Targeting endogenous analgesia systems for endometriosis treatment

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Abstract

Endometriosis is a chronic inflammatory disease that affects 1 in 10 women of childbearing age. It is characterized by the growth of endometrium in extrauterine locations and is associated with chronic pelvic pain and infertility. This persistent pain is linked to emotional distress and loss of working ability that negatively impact the quality of life of the patients. Current clinical management provides unsatisfactory outcomes and produces unwanted side effects. Thus, the development of more effective therapeutic strategies is still an unmet clinical need, and their development relies on the establishment of animal models that recapitulate the features of clinical endometriosis. The present Thesis has characterized a surgical model of endometriosis that shows nociceptive, affective-like behaviors and impaired cognition, reproducing the symptoms observed in endometriosis patients. In this model of endometriosis, natural cannabinoids alleviate nociceptive behaviors, restore cognitive function and inhibit the development of endometriotic growths. A kappa opioid receptor agonist also shows strong pain-relieving properties in this model, although affective and cognitive disturbances persist regardless of the complete alleviation of pain. A minimally invasive model of endometriosis that also mimics the symptoms of human endometriosis was used to explore the neuroinflammatory changes induced by the presence of ectopic endometrial cells. In this model, minimal endometriosis leads to neuroinflammation in brain areas related to pain, emotion and cognitive processes.

Resumen

La endometriosis es una enfermedad inflamatoria crónica que afecta a 1 de cada 10 mujeres en edad fértil. Se caracteriza por el crecimiento de tejido endometrial fuera del útero y se asocia con dolor pélvico crónico e infertilidad. Este dolor persistente está relacionado con alteraciones emocionales y disminución de la capacidad de trabajo que impactan negativamente en la calidad de vida. Los tratamientos actuales proporcionan resultados insatisfactorios y producen efectos secundarios. Por lo tanto, se necesitan estrategias terapéuticas más eficaces y su desarrollo depende de la disponibilidad de modelos animales que recapitulen las características de la endometriosis clínica. La presente Tesis ha caracterizado un modelo quirúrgico de endometriosis que muestra alteraciones nociceptivas, afectivas y cognitivas, reproduciendo los síntomas observados en pacientes con endometriosis. En este modelo, el tratamiento con cannabinoides naturales proporciona alivio de las manifestaciones nociceptivas, restaura la función cognitiva e inhibe el desarrollo del tejido endometrial ectópico. El tratamiento con un agonista del receptor opioide kappa también proporciona alivio de las manifestaciones nociceptivas observadas en este modelo, aunque las alteraciones afectivas y cognitivas persisten pese al alivio del dolor. Se ha utilizado un modelo mínimamente invasivo, que también reproduce los síntomas de la endometriosis humana, para explorar los cambios neuroinflamatorios inducidos por la presencia de células endometriales ectópicas. En este modelo, la endometriosis mínima-leve provoca neuroinflamación en áreas del cerebro relacionadas con el dolor, las emociones y los procesos cognitivos.

Abbreviations

2-AG 2-arachidonoylglycerol

AC Adenylyl cyclase

AEA Anandamide

ATP Adenosine triphosphate

BDNF Brain-derived neurotrophic factor

cAMP Cyclic adenosine monophosphate

CB1R Cannabinoid receptor 1

CB2R Cannabinoid receptor 2

CBD Cannabidiol

CGRP Calcitonin gene related peptide

CNS Central nervous system

COCs Combined oral contraceptives

DOR Delta opioid receptor

DRG Dorsal root ganglia

ERK Extracellular-regulated kinase

FAAH Fatty acid amide hydrolase

GABA Gamma-aminobutyric acid

GnRH Gonadotropin-releasing hormone

GPCR G protein-coupled receptor

IASP International association for the study of pain

IL1β Interleukin 1 beta

JNK c-Jun N-terminal kinase

$K_{ir}3$ Inwardly rectifying K^+ channels

KOR Kappa opioid receptor

MAGL Monoacylglycerol lipase

MAPK Mitogen-associated kinase

MMPs Metalloproteinases

MOR Mu opioid receptor

NF-κB Nuclear factor kappa B

NGF Nerve growth factor

NOR Nociceptin or orphanin opioid receptor

NSAIDs Nonsteroidal anti-inflammatory drugs

PAG Periaqueductal gray

PDYN Prodynorphin

PEA N-palmitoyl ethanolamine

PENK Preproenkephalin

PI3K Phosphatidyl inositol 3

PKA Protein kinase A

PNOC Pronociceptin

POMC Proopiomelanocortin

PPARS Peroxisome-proliferator-activating receptors

RMV Rostral ventromedial medulla

ROS Reactive oxygen species

SP Substance P

THC Δ9-tetrahydrocannabinol

TNF-α Tumor necrosis factor alpha

TRVP1 Transient receptor potential vanilloid 1

VEGF Vascular endothelial growth factor

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INTRODUCTION

1 Pain

Thomas Lewis wrote in the preface to his monograph entitled Pain: “Reflection tells me that I am so far from being able to define pain that the attempt could serve no useful purpose” (Lewis, 1942). Defining the concept of pain in a concise and precise manner represents, indeed, a challenge. In 2020, the International Association for the Study of Pain (IASP) published a definition adopted the following definition of pain: “an **unpleasant sensory and emotional experience** associated with, or resembling that associated with, actual or potential tissue damage” (Raja *et al.*, 2020). This new definition does not rely upon the ability to describe the experience of pain, and therefore includes populations such as neonates and infants, demented people, unconscious individuals, and nonhuman animals that can not verbally communicate their pain.

Both sensorial and emotional elements compose the experience of pain (Baños *et al.*, 2006). The sensorial or nociceptive element is the painful sensation consequence of the transmission of a noxious stimulus from peripheral sensory nerves to the central nervous system. The emotional component comprises the unpleasant character of pain perception, and it is influenced to varying degrees by the previous experience and by several psychological and social factors.

1.1 Classification of pain

Pain has been classified according to several criteria, including anatomical localization, intensity, etiology, duration and pathophysiological mechanisms, among others.

1.1.1 According to duration

Acute and chronic pain differ in the physiological and pathophysiological mechanisms involved, as well as in their temporal duration (Aliaga *et al.*, 2002). **Acute pain** is an immediate, short-lasting event caused by a noxious stimulus. It serves a biological function as a warning mechanism, and it resolves with healing of the injured tissue. In contrast, **chronic pain** lasts or recurs for more than 3 months, and may persist beyond the injury, remaining after tissue healing and losing its biological purpose. Chronic pain is, indeed, considered a disease of its own (Treede *et al.*, 2019), which is usually associated with significant emotional distress and functional disability (Nicholas *et al.*, 2019). Chronic pain may progress from acute pain as a consequence of repeated nerve stimulation, resulting in functional and structural changes at peripheral and central levels (Feizerfan and Sheh, 2015; Kuner and Flor, 2016).

1.1.2 According to the mechanisms involved

Nociceptive pain is described as pain that arises from actual or threatened damage to non-neural tissue occurring with a normally functioning somatosensory nervous system. Nociceptive pain is caused by the activation of specialized high-threshold sensory fibers and continues as long as the noxious stimulus is present (Treede, 2018). **Inflammatory pain** results from activation of nociceptors by inflammatory mediators released in response to damage and inflammation. Acute inflammatory pain is necessary for protective reflexes and wound healing; on the other hand, persistent inflammation may lead to peripheral and central sensitization (Woolf,

2011). **Neuropathic pain** is caused by a lesion or disease of the somatosensory nervous system, including peripheral fibers and central neurons (Colloca *et al.*, 2017). Cerebrovascular diseases, spinal lesions, diabetes, infections, chemotherapy or inflammatory disorders are examples of conditions that may cause neuropathic pain. This type of pain is maladaptive, since it lengthens beyond the injury and remains once the lesion disappears (Costigan *et al.*, 2009). **Nociplastic pain** arises from altered nociception despite no clear evidence of actual or threatened tissue damage or evidence of a possible cause of lesion or disease of the somatosensory nervous system. This term was recently proposed to describe pain states characterized by clinical and psychophysical findings that suggest altered nociception despite the absence of clear evidence of actual or threatened damage (Kosek *et al.*, 2016).

1.2 The route of pain transmission

1.2.1 Detection of noxious stimuli in the periphery

Nociceptors are high-threshold sensory receptors of the peripheral somatosensory nervous system that detect and transduce noxious thermal, mechanical and chemical stimuli. Nociceptors are the peripheral terminals of primary afferent neurons that have the cellular body in dorsal root ganglia (DRG) or the trigeminal ganglion. The peripheral axonal branch of these neurons innervates the target tissue or organ, and the central axon synapses with second-order neurons in the dorsal horn of the spinal cord or the trigeminal nucleus caudalis (Basbaum *et al.*, 2009; Dubin and Patapoutian, 2010).

There are two main types of nerve fibers conveying pain signals. The first includes medium diameter (1 – 5 µm) thinly-myelinated **Aδ fibers** that conduct well-localized first and fast (5-30 m/s) pain signals (Basbaum *et al.*, 2009). They can be divided into two categories: type I, which mediate the first pain response to intense mechanical stimuli, and type II, which have a low heat threshold and mediate fast responses to noxious heat. **C fibers** are small diameter (0.2 – 1.5 µm) unmyelinated fibers that conduct slow (2 m/s), diffuse and longer-lasting pain. Most C fibers are polymodal, responsive to mechanical, thermal and chemical stimuli (Dubin and Patapoutian, 2010), although there is a group of C fibers (the so-called "silent nociceptors") that are only heat responsive but develop mechanical sensitivity after injury. C fibers can be divided into peptidergic or non-peptidergic. Both types express the transient receptor potential vanilloid 1 (TRPV1), which responds to heat and capsaicin, but only peptidergic C fibers express the neuropeptides substance P and calcitonin gene related peptide (CGRP) (Usoskin *et al.*, 2015). Peptidergic C fibers mainly mediate thermal pain transmission, whereas non-peptidergic C fibers transmit noxious thermal, mechanical and chemical stimuli (Basbaum *et al.*, 2009).

Under normal conditions, Aδ fibers immediately conduct localized pain signals, which are followed by diffuse pain conducted by C fibers. However, in the setting of an injury, damaged tissue, nociceptors and immune cells release mediators including peptides, neurotransmitters and cytokines that lead to the development of **peripheral sensitization** (Scholz and Woolf, 2002; Schaible, 2007; Gold and Gebhart, 2010).

Under these sensitizing circumstances, Aδ fibers and C fibers become activated by low-threshold stimuli, provoking exaggerated stimulus-evoked painful sensations like allodynia (pain induced by non-noxious stimuli) and hyperalgesia (increased response to noxious stimuli) (Schaible, 2007).

1.2.2 Ascending spinal pain pathways and supraspinal integration

The central terminals of primary afferent fibers transmitting nociceptive information enter the **spinal cord** through the dorsal roots and project to different layers of spinal grey matter, the laminae. Aδ and C fibers contact to second order neurons in laminae I, II and V (D'Mello and Dickenson, 2008). The majority of primary afferents synapsing in the dorsal horn of the spinal cord use glutamate as neurotransmitter. Glutamate exerts an excitatory effect via the post-synaptic α-amino-3-hydroxy 5-methyl-4-isoxazeloproprionic acid (AMPA) receptor, N-methyl-d-aspartate (NMDA) receptors, kainate, and G-protein coupled metabotropic (mGluR) receptors (D'Mello and Dickenson, 2008). Substance P, CGRP, brain-derived neurotrophic factor (BDNF) and purines (ATP) also contribute to pain transmission in the spinal cord (Willis, 1985; Julius and Basbaum, 2001; Basbaum *et al.*, 2009). These neuromodulators are overexpressed in the setting of persistent inflammation or nerve injury, contributing to the development of **central sensitization** (Latremoliere and Woolf, 2009). Central sensitization refers to a state of hyperexcitability established in the central nervous system that leads to enhanced processing of nociceptive signals, generating allodynia and hyperalgesia. Numerous

mechanisms are implicated in central sensitization, including the participation of Aβ fibers, which normally respond to innocuous mechanical stimulation, in the transmission of nociceptive responses (Basbaum *et al.*, 2009).

Besides neuronal cells, resident **microglia and astrocytes** also modulate pain transmission. Microglia are macrophage-related cells that conduct a constant immune surveillance in the central nervous system. Upon activation by an injury or an infection, microglia release proinflammatory factors that contribute to central sensitization (Tsuda *et al.*, 2005). Astrocytes become activated with a slower onset and for longer time than microglia in several models of chronic pain (Ji *et al.*, 2019). Astrocytes are involved in the regulation of almost all aspects of neuronal functioning in the central nervous system, such as extracellular ion homeostasis, neurotransmitter reuptake and release, and metabolic control (Gosselin *et al.*, 2010). In the setting of an injury, astrocytes are activated and release mediators that modulate excitatory and inhibitory synaptic transmission, further contributing to central sensitization and transition from acute to chronic pain (Ji *et al.*, 2013).

In pathological conditions, other cells of the innate immune system including mast cells, macrophages, basophils, eosinophils and neutrophils, interact with neuronal cells to modulate pain transmission (Baral *et al.*, 2019). Cells of the adaptative immune system, namely T and B cells, which are hardly detectable in the nervous system of naïve animals, also contribute to the regulation of pain transmission has

demonstrated in recent studies in animal models of chronic pain (Sorge *et al.*, 2015; Cabañero *et al.*, 2020).

From the spinal cord, nociceptive signaling ascends to supraspinal areas through one of the tracts that constitute the anterolateral system (Figure 1). Axons of second order neurons localized in the spinal cord decussate and ascend via the spinothalamic tract to synapse directly in the contralateral thalamus via the spinoreticulothalamic tract, synapsing indirectly in the reticular formation, or as spinomesencephalic, spinotectal or spinohypothalamic fibers that synapse in brainstem nuclei (Patestas and Gartner, 2016).

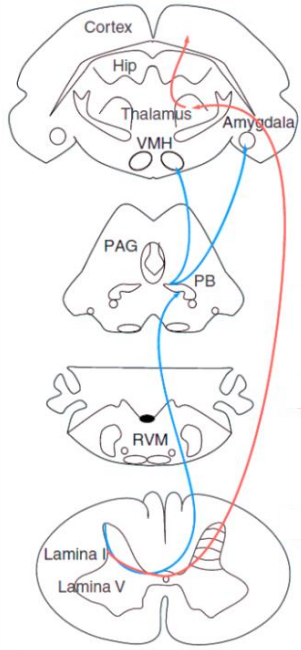


Figure 1. Main ascending pain pathways. Nociceptive signals travel from the spinal dorsal horn to the thalamus, which distributes the information to areas of the cortex concerned with both discrimination and affect (blue line), or directly to regions involved in the cognitive and affective aspects of pain (red line). RVM, rostroventral medial medulla; PB, Parabranchial area; PAG, periaqueductal gray; VMH, ventral medial nucleus of the hypothalamus; Hipp, hippocampus. Adapted from (Bee and Dickenson, 2007).

The **thalamus** acts as a key relay station for the transmission of nociceptive information to other supraspinal areas (Ab Aziz and Ahmad, 2006). Thalamic projections conduct tactile, proprioceptive and nociceptive signals to the **somatosensory cortex**, which mediates the sensory discriminative aspects of pain such as quality, location and intensity. The thalamus also projects to limbic structures including the **prefrontal**, **anterior cingulate and insular cortices** and the **amygdala**, contributing to the cognitive and affective-motivational components of pain (Groh *et al.*, 2017). The spinoreticulothalamic tract conducts nociceptive signals to the thalamus by forming multiple synapses in the **reticular formation**, a region involved in arousal and wakefulness that alerts the organism of an injury (Martins and Tavares, 2017).

Spinomesencephalic fibers project to the parabrachial nucleus, which sends fibers to the **amygdala**. The amygdala receives nociceptive inputs also from the thalamus and the cortex, contributing to the emotional processing of nociceptive information and to certain cognitive aspects such as pain-related decision-making (Ji *et al.*, 2010). Spinotectal fibers conduct the nociceptive signal mainly to the superior colliculi, and spinohypothalamic fibers ascend to the hypothalamus, structures that are associated with the autonomic responses to nociception (Patestas and Gartner, 2016).

1.2.3 Descending pain modulation

The descending modulatory circuit modulates the complex experience of pain (Figure 2) through changes in pain thresholds as a response to

attention, emotion, context, expectations and internal states (Millan, 2002; Chen and Heinricher, 2019).

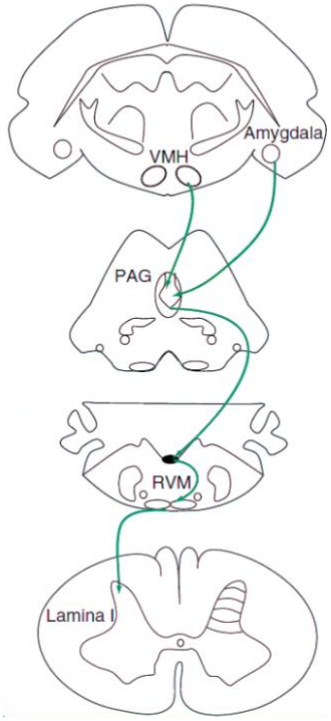


Figure 2. Descending pain pathway. The amygdala and the hypothalamus project to the periaqueductal grey, where descending information is distributed to lower brainstem to regulate nociceptive and autonomic responses that follow noxious stimulation. RVM, rostroventral medial medulla; PAG, periaqueductal gray; VMH, ventral medial nucleus of the hypothalamus. Adapted from (Bee and Dickenson, 2007).

The **periaqueductal gray** (PAG) receives inputs from higher brain regions such as the ventral tegmental area (VTA), the prefrontal cortex, the hypothalamus and the amygdala. Neurons in the PAG project to the **rostral ventromedial medulla** (RVM), which also receives nociceptive information from the thalamus, the parabrachial area, the locus coeruleus and the parabrachial tract. From the RVM, projections to the **spinal dorsal horns** and the **trigeminal nucleus caudalis** exert a

bidirectional modulatory effect. OFF-and ON-cells from the RVM send outputs to the spinal cord or the trigeminal nucleus caudalis, inhibiting or facilitating pain perception, respectively (Chen and Heinricher, 2019). The PAG-RVM circuit integrates information from higher brain centers involved in the emotional and cognitive aspects of pain. Thus, the descending pathway modulates pain thresholds as a response to attention, mood, context and expectations, allowing environmental, contextual and cognitive factors to influence the pain experience (Ossipov *et al.*, 2010).

2 Endometriosis

Endometriosis is defined as the presence of endometrial cells outside the uterine cavity, mainly, but not exclusively, in the pelvic compartment (Zondervan *et al.*, 2020). It is associated with pelvic pain and infertility; however, the same symptoms can be associated to other etiologies, and endometriosis can be present in the absence of obvious lesions (Johnson *et al.*, 2017). From a clinical perspective, endometriosis could be better defined as a chronic, estrogen-dependent, inflammatory and systemic disease that commonly presents as pelvic pain and infertility (Agarwal *et al.*, 2019).

2.1 Epidemiology

Endometriosis is estimated to affect 10% of females in reproductive age, which translates to 190 million people worldwide (Zondervan *et al.*, 2020). The number of affected people is probably underestimated, since the definitive diagnosis of endometriosis requires direct visualization of lesions (Agarwal *et al.*, 2019). The prevalence ranges from 2 to 11% in adult asymptomatic females, from 5 to 50% in females with fertility problems, and 5 to 21% in females hospitalized for pelvic pain (Shafrir *et al.*, 2018). Among symptomatic adolescents, the prevalence of endometriosis ranges from 49% of those with chronic pelvic pain to 75% of those with pain that is unresponsive to medical treatment (Shafrir *et al.*, 2018).

Endometriosis has a heterogeneous **presentation** (Figure 3). Superficial peritoneal and serosal lesions of different colors, cysts in the ovaries (endometriomas) or infiltrating nodules of more than 5 mm in depth

(deep endometriosis) that are often accompanied by fibrosis and adhesions, and many other forms of extra-pelvic lesions can be found in endometriosis patients (Zondervan *et al.*, 2018). The American Fertility Society (AFS) and the American Society of Reproductive Medicine (ASRM) classify endometriosis according to a point system that takes into account the location, extent and depth of lesions (Revised American Society for Reproductive Medicine classification of endometriosis: 1996., 1997). Stage I or minimal comprises a low number of superficial endometriotic spots or adhesions. Stage II or mild can be a few, deep peritoneal lesions solely or in combination with superficial lesions and filmy adhesions. Stage III or moderate often includes an endometrioma by itself or in combination with superficial or deep endometriosis and/or dense adhesions. Stage IV or severe is characterized by all of the above as well as bilateral ovarian endometrioma and/or dense adhesions that can lead to an obliteration of the pelvic cavity (Revised American Society for Reproductive Medicine classification of endometriosis: 1996., 1997). Notably, the severity of endometriosis according to this system does not correlate with the severity and location of the symptoms, the treatment response or the prognosis of the disease (Schliep *et al.*, 2015; Johnson *et al.*, 2017).

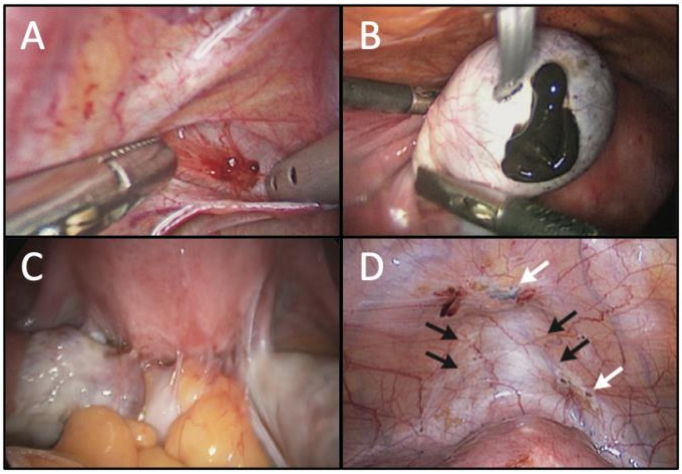


Figure 3. Different presentations of endometriosis. (A) Superficial red peritoneal endometriotic lesion and hyperemia. (B) Chocolate cyst in the ovary. (C) Endometriosis with bowel adhesions to the uterus. (D) Deep nodules (black arrows) and red, brown, and black peritoneal endometriotic lesions (white arrows). Adapted from (Zondervan *et al.*, 2020).

Symptoms are also variable among patients of endometriosis. The most frequent manifestations of endometriosis are dysmenorrhea (pelvic pain during menstruation), dyspareunia (pelvic pain during or after sexual intercourse) and cyclical and non-cyclical abdominopelvic pain (Zondervan *et al.*, 2020). Dyschezia (painful defecation), as well as other effects on bowel habits such as diarrhea or constipation, are common in cases of deep infiltrating endometriosis (Fauconnier *et al.*, 2002), while painful urination or cyclical presence of blood in the urine are associated to endometriosis affecting the bladder (Chung *et al.*, 2002). Some endometriosis patients also experience regional hyperalgesia and allodynia (Stratton *et al.*, 2015), as well as chronic fatigue (Sinaii *et al.*, 2002). These manifestations overlap with symptoms of interstitial cystitis and irritable bowel disease, conditions that often co-exist with endometriosis (Surrey *et al.*, 2018).

2.1.1 Impact on quality of life

Patients of endometriosis usually present **psychiatric comorbidities** that exert a negative effect on their quality of life. Endometriosis pain is accompanied by anxiety in 28 to 87% of the cases and by depression in 14 to 86% of the patients (Sepulcri and do Amaral, 2009; Friedl *et al.*, 2015). Dyspareunia and chronic pelvic pain associated to endometriosis usually impact sexual life, compromising overall sexual activity, self-esteem and sexual satisfaction (Montanari *et al.*, 2013). Furthermore, patients report that endometriosis affects their social, family and work relationships, principally due to the perceived lack of understanding and support (Seear, 2009; Rush and Misajon, 2018).

The symptoms of endometriosis can also affect **work productivity** and professional development (Sperschneider *et al.*, 2019). Patients included in a cross-sectional study reported a substantial impact of symptoms in work productivity and daily life activities. They also reported a 13% of average loss in work time and disturbances in 65% of their work (Fourquet *et al.*, 2011). Benign chronic pain has been associated to impairments in several cognitive outputs, including attention, learning, memory and decision-making (Dick *et al.*, 2002; Apkarian *et al.*, 2004; Muñoz and Esteve, 2005). However, the effects of endometriosis pain on specific cognitive functions have not been investigated yet.

The causal relationship between pain and emotional and cognitive alterations is complex, since chronic pain leads to a negative affective and cognitive state and, in turn, this negative state contributes to pain perception (Bushnell *et al.*, 2013). Indeed, women with anxiety

disorders show higher pain sensitivity (Keogh and Mansoor, 2001; Defrin *et al.*, 2008), although both pain-attenuating and exacerbating effects of depressive disorders have been described (Bär *et al.*, 2005; Chiu *et al.*, 2005; Schwier *et al.*, 2010). On the other hand, social support has been associated with lower pain intensity (Montoya *et al.*, 2004).

2.2 Pathogenesis and pathophysiology

The **retrograde menstruation hypothesis**, proposed by Sampson in the 1920s, is the most accepted hypothesis for the pathogenesis of endometriosis. It states that menstrual debris containing viable endometrial glands and stroma reach the peritoneal cavity through the fallopian tubes during menstruation (Sampson, 1927). The association of a higher risk of endometriosis with shorter menstrual cycles and obstructed menstrual flow supports this theory (Missmer *et al.*, 2004; Shafrir *et al.*, 2018). Retrograde neonatal uterine bleeding containing stem or progenitor endometrial cells could also explain premenarchal endometriosis and severe endometriosis in adolescents (Gargett *et al.*, 2014). Another postulated origin of endometriosis is the coelomic metaplasia hypothesis, which arises from the transformation of mesothelium into endometrial-like tissue. This hypothesis has been suggested as an origin of extra-pelvic endometriosis (Davis and Goldberg, 2017) and in patients with Müllerian duct defects (Troncon *et al.*, 2014). Alternative explanations include the lymphatic and vascular metastasis hypothesis, which implies the transport of endometrial cells from the uterus to distant ectopic sites through

lymphatic and blood vessels, and could explain endometriosis occurring outside the pelvis (Jerman and Hey-Cunningham, 2015).

The **altered hormonal milieu** plays a key role in the proliferation of ectopic endometrial cells. In ectopic endometrium, overexpression of the steroidogenic factor 1 (SF1) and aromatase increases estradiol synthesis, while decreased expression of hydroxysteroid 17β dehydrogenase 2 (HSD17B2) reduces estradiol degradation (Bulun *et al.*, 2004; Xue *et al.*, 2011). High local concentrations of estradiol, together with an upregulation of the estrogen receptor β (ERβ), induce endometriotic cell mitogenesis (Pellegrini *et al.*, 2012) and diminish estradiol induction of the progesterone receptor (PR) (Burney *et al.*, 2007). Alteration of these signaling pathways causes progesterone resistance, which is associated to increased adhesive properties of the tissues, activity of matrix metalloproteinases (MMPs) and angiogenic response in the lesion microenvironment (Al-Sabbagh *et al.*, 2012). In this setting, the **epithelial–mesenchymal transition** (EMT) occurs in endometriotic cells, which acquire an invasive mesenchymal phenotype and trigger fibroblast-to-myofibroblast trans-differentiation and collagen production, further contributing to formation of adhesions and fibrosis (Vigano *et al.*, 2018).

An **altered immune surveillance** also sustains the growth and maintenance of ectopic endometrium (Shigesi *et al.*, 2019). Patients with endometriosis have defective natural killer (NK) cells (Kang et al., 2014) and macrophages with decreased phagocytic capacity (Symons *et al.*, 2018) that may contribute to immune evasion of endometrial cells. Aberrant responses of cells of the adaptative immune system may

also contribute to the development of endometriosis, since T lymphocytes from endometriosis patients show a lower response to autologous endometrial cells (Dmowski *et al.*, 1981; Helvacioglu *et al.*, 1997).

Ectopic endometrial tissue develops their own neural and vascular supplies through **neuroangiogenesis** processes. Indeed, the peritoneal fluid of endometriosis patients presents increased amounts of neurotrophic and angiogenic factors such as BDNF, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), neurotrophins 4 and 5 (NT4/5) or nerve injury-induced protein 1 (Ninj1) (Shifren *et al.*, 1996; Barcena de Arellano *et al.*, 2013; Miyashita *et al.*, 2019).

2.3 Physiopathology of endometriosis pain

2.3.1 Pain originated from endometriotic lesions

Endometriosis pain may originate directly from endometriotic lesions, which are innervated by nociceptive, sympathetic and parasympathetic fibers. A high density of unmyelinated C-fiber sensory afferents and an upregulation of TRPV1 have been found in endometriotic lesions (Tokushige *et al.*, 2006b; Wang *et al.*, 2009b; Bohonyi *et al.*, 2017), suggesting that fibers iinervating the lesions are involved in the generation of pain associated to endometriosis. In fact, patients whose endometriotic lesions are highly innervated report more severe pain symptoms (Mechsner *et al.*, 2009; McKinnon *et al.*, 2012; Kajitani *et al.*, 2013).

Sensory fibers innervating endometriotic lesions may converge into the same spinal pathways that the peripheral fibers they originally

sprouted from (Malykhina, 2007). Therefore, pelvic organs and endometriotic lesions may converge in the same central terminals within the spinal cord (Costa *et al.*, 2004). This cross-sensitization might be a partial explanation of widespread visceral hypersensitivity (Hsu *et al.*, 2011) and high comorbidity rates between endometriosis and other chronic pain diseases, such as irritable bowel syndrome or interstitial cystitis (Surrey *et al.*, 2018).

2.3.2 Inflammatory pain mechanisms in endometriosis

When located in ectopic sites, endometrial cells produce CC chemokines that attract monocytes, T cells and eosinophils, and CXC chemokines that attract monocytes and neutrophils (Reis *et al.*, 2013). Macrophages also invade endometriotic lesions and overexpress proinflammatory cytokines such as the tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 1 (IL-1) (Symons *et al.*, 2018). The production of cytokines is also promoted by overactivation of the nuclear factor kappa B (NF-κB) pathway, the production of reactive oxygen species (ROS), and the activation of mitogen-associated kinase (MAPK) signaling pathways (Beste *et al.*, 2014).

Similar to eutopic endometrium, endometriotic lesions suffer cyclical bleeding in response to hormonal variations (Halme *et al.*, 1984; Burney and Lathi, 2009). Cell death during endometrial breakdown exacerbates the already ongoing inflammatory response caused by the presence of endometrial cells in ectopic sites (Figure 4). Degenerating endometriotic tissue releases numerous damage-associated molecular patterns (DAMPs), including calgranulins and soluble extracellular

matrix components (Gilabert-Estelles *et al.*, 2005; Ferrero *et al.*, 2008) and enriches lesion environment with ROS, prostaglandins (PGE2), iron and protons (Kobayashi *et al.*, 2009; Sacco *et al.*, 2012) that directly stimulate nociceptors in close proximity to endometriotic lesions. These products also activate macrophages and mast cells through the NF-κB pathway (Lousse *et al.*, 2008; Morgan and Liu, 2011), provoking further release of NGF, interleukins 8 and 1 beta (IL-8 and IL-1β) and TNF-α. In patients with endometriosis, elevated levels of these proinflammatory and pronociceptive mediators are associated with a higher severity of pain symptoms (Scholl *et al.*, 2009; McKinnon *et al.*, 2015).

Nerve fibers themselves also participate in the generation of inflammatory endometriosis-associated pain through the release of inflammatory neuropeptides, causing neurogenic inflammation. These neuropeptides include CGRP and substance P (SP), which have been detected in sensory nerve fibers close to endometriotic lesions (Tokushige *et al.*, 2006b; Wang *et al.*, 2009a). CGRP and SP induce mast cell degranulation, edema and activation of macrophages that release more proinflammatory cytokines (Ottosson and Edvinsson, 1997; Brain and Grant, 2004). Re-activation of sensory nerve fibers by these cytokines completes a vicious cycle that sustains the inflammatory milieu and contributes to the generation of pain in endometriosis patients.

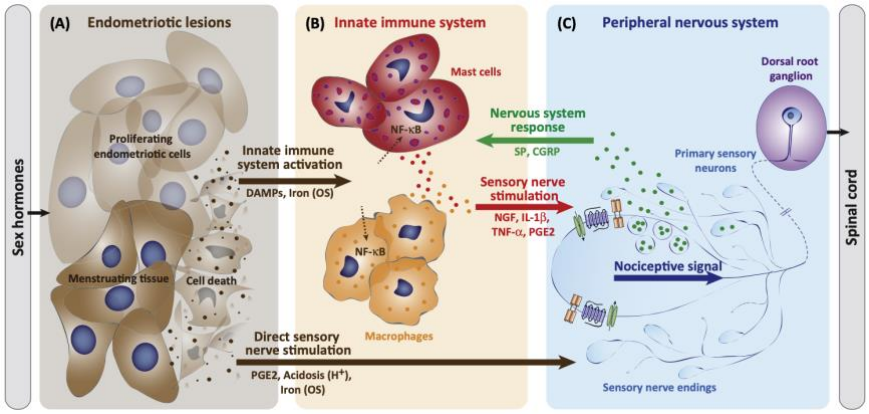


Figure 4. Main pathways involved in the pathogenesis of inflammatory pain in endometriosis. Endometriotic lesions (A), the innate immune system (B), and the peripheral nervous system (C) contribute to inflammatory pain. Endometriotic lesions are under the control of sex hormones, thus, proliferation and death of endometriotic cells occur cyclically. Mediators released during this process directly stimulate sensory nerve endings to generate nociceptive signals. In addition, products released from tissue degeneration activate the innate immune system. Proinflammatory and pronociceptive mediators are then released by activated mast cells and macrophages, which are also able to stimulate nociceptors. In response to stimulation, sensory nerves further increase and maintain inflammation by secreting proinflammatory mediators. PGE2, prostaglandin; OS, oxidative stress; DAMPs, damage-associated molecular patterns; NF-κB, nuclear factor kappa B; NGF, nerve growth factor; IL-1β, interleukin 1 beta; TNF- α, tumor necrosis factor alpha; SP, substance P; CGRP, calcitonin gene related peptide. Adapted from (Laux-Biehlmann *et al.*, 2015).

2.3.3 Endometriosis-induced sensitization of pain pathways

Persistent inflammation leads to sensitization of nociceptive fibers (Ren and Dubner, 2010). Inflammatory and neurotrophic mediators released by endometriotic and immune cells can activate TRPV1-positive fibers and induce TRPV1 expression, potentiating nociceptor sensitization (Wang, 2008). Indeed, elevated expression of TRPV1 correlates with higher pelvic pain intensity in patients with deep infiltrating endometriosis (Bohonyi *et al.*, 2017), suggesting that

TRPV1-dependent peripheral sensitization mechanisms may promote pain.

There is rising evidence for the role of central sensitization in chronic pelvic pain associated to endometriosis. The fact that endometriosis patients often experience persistent pain even after surgical removal of endometrial lesions (Shakiba *et al.*, 2008) suggests the presence of central mechanisms. Moreover, some endometriosis patients present regional hyperalgesia and allodynia (Stratton *et al.*, 2015), further supporting the existence of central sensitization. In addition, decreases in regional gray matter volume in brain regions associated with pain processing, such as the insula or the thalamus, have been found in endometriosis patients (As-Sanie *et al.*, 2012), as well as increased levels of excitatory neurotransmitters in the insula of patients with chronic pelvic pain associated to endometriosis, but not in asymptomatic individuals (As-Sanie *et al.*, 2016).

2.4 Treatment strategies for endometriosis pain

There is currently no cure for endometriosis. Therefore, the primary goal of pharmacological and surgical treatments and their complementary approaches is managing endometriosis symptoms. The choice of treatment depends on many factors such as the extent of the disease, preferences and tolerability of the patient, desire for conception, long-term safety and costs (Figure 5) (Dunselman *et al.*, 2014).

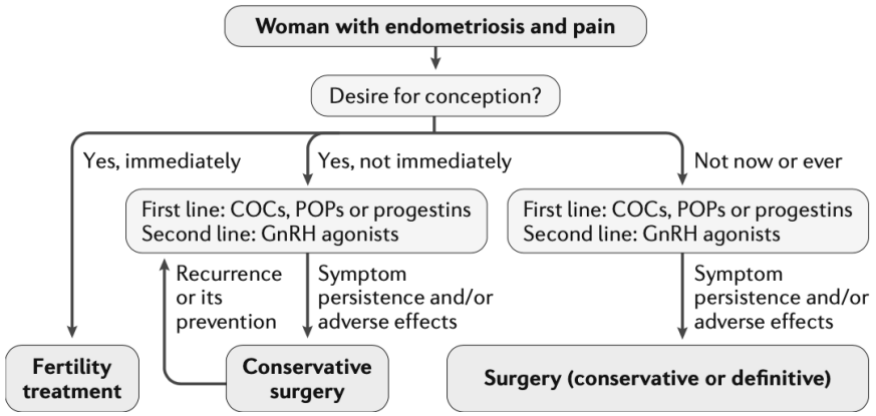


Figure 5. Algorithm for management of endometriosis-associated pain. COCs, combined oral contraceptives; POPs, progestin-only pills; GnRH, gonadotropin-releasing hormone. Adapted from (Zondervan *et al.*, 2018).

2.4.1 Pharmacological treatment of endometriosis pain

Since endometriosis is an estrogen-dependent disease, pharmacological treatments are focused on estrogen suppression. These treatments are symptomatic but not cytoreductive, and symptom recurrence after treatment cessation is common (Becker *et al.*, 2017).

Combined oral contraceptives (COCs) taken in a continuous or a cyclic regimen are the first-line treatment for endometriosis associated pain (Dunselman *et al.*, 2014). COCs induce central inhibition of gonadotropin secretion, suppress ovulation and reduce secretion of ovarian estrogen. This results in the establishment of a hyper-progestogenic milieu that provokes the decidualization and subsequent atrophy of both eutopic and ectopic endometrium (Olive, 2003).

Progestins, administered through oral, sub-cutaneous, intramuscular, intrauterine or vaginal route (Vercellini *et al.*, 2009), also induce atrophy of endometrial tissues (Olive, 2003) and lack some of the more concerning side effects of estrogen, such its associated risk of thromboembolism (Lidegaard *et al.*, 2011). The levonorgestrel-releasing intrauterine system (LNG-IUS) has also proved effectivity reducing dysmenorrhea in endometriosis patients (Abou-Setta *et al.*, 2013). COCs and progestins show overall safety and efficacy, however, they are associated with side effects including irregular bleeding, breast tenderness and psychological disturbances (Robakis *et al.*, 2019).

Gonadotropin-releasing hormone (GnRH) agonists are second-line treatments (Dunselman *et al.*, 2014). GnRH agonists downregulate pituitary GnRH receptors inhibiting the hypothalamic pituitary ovarian axis and therefore blocking ovarian secretion of estrogen. This eventually leads to hypoestrogenism, amenorrhea and regression of the endometriotic implants (Olive, 2008). GnRH agonist therapy is associated with menopause-like side effects, including bone loss, vasomotor symptoms (hot flashes), insomnia, and urogenital atrophy. Many of these adverse effects can be mitigated with the use of high-doses of progestins or low-doses of estrogen–progestin therapies. These add-back treatments increase sex steroid levels without providing sufficient estrogen for the growth and maintenance of endometriosis. Elagolix is the first GnRH antagonist approved in North America for the treatment of endometriosis-associated pelvic pain

(Taylor *et al.*, 2017). Other GnRH antagonists (linzagolix and relugolix) are under evaluation in phase 3 clinical trials.

Off-label use of **aromatase inhibitors** for the treatment of endometriosis pain is based on the overexpression of aromatase and subsequent estrogen production in endometriotic lesions. Used in combination with COCs, progestins or GnRH agonists, aromatase inhibitors have proven useful in patients with pain refractory to other medical or surgical treatments (Nawathe *et al.*, 2008; Ferrero *et al.*, 2011). However, long-term use of aromatase inhibitors is associated to bone-density loss, joint pain and increased multiple-pregnancy rates (Verma and Konje, 2009; Alborzi *et al.*, 2011).

Danazol, a derivative of 17 alpha-ethinyl–testosterone, **and gestrinones** suppress gonadotropin secretion and inhibit steroid synthesis, inducing an hyperandrogenic and hypoestrogenic environment that hinders the proliferation of endometrial tissues and produces amenorrhea (Olive, 2003). Although treatments with danazol and gestrinones have proven effective in controlling endometriosis-associated pain (Fedele *et al.*, 1989; Selak *et al.*, 2007), its use has fallen over the years due to their multiple adverse effects that include acne, hypertrichosis, weight gain or muscle cramps (Fedele *et al.*, 1989).

The previously mentioned treatments are often accompanied by **analgesics**. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for endometriosis-associated pain (Dunselman *et al.*, 2014) since they are useful for the treatment of primary dysmenorrhea (Marjoribanks *et al.*, 2015). However, the effectiveness of NSAIDs in treating endometriosis associated dysmenorrhea is not

well established (Brown *et al.*, 2017), and frequent use of NSAIDs is associated with gastrointestinal complications (Massó González *et al.*, 2010). Most of the guidelines for the treatment of endometriosis (Dunselman *et al.*, 2014; Practice Committee of the American Society for Reproductive Medicine, 2014) do not include recommendations for the use of strong analgesics. However, other guidelines suggest that physicians could prescribe opioids or non-standard analgesics such as gabapentin or pregabalin if necessary (Ministerio de Sanidad, 2013). In this line, a recent report describes a frequent use of prescription opioids by endometriosis patients with unmanageable pain (Lamvu *et al.*, 2019).

Available pharmacological approaches for the treatment of endometriosis-associated pain produce, therefore, a large number of side effects and show variable efficacy. Furthermore, hormonal treatments are not an option for patients with endometriosis who wish to conceive. Novel approaches with pain-relieving properties but also disease-modifying effects are needed for endometriosis. With these objectives, several clinical trials are currently testing different nonhormonal treatments, including dopamine agonists, polyphenols or cannabinoids (Table 1).

Table 1. Active clinical trials of non-hormonal treatments for endometriosis. IL-1, interleukin 1; IRAK-4 interleukin-1 receptor associated kinase 4; TLR, Toll-like receptor; TBD, to be defined; PEA, Palmitoylethanolamide, N/A, not available; ATP, adenosine triphosphate; THC, Δ9-tetrahydrocannabinol; CBD, cannabidiol. Adapted from (Zondervan *et al.*, 2020).

Drug	Drug class	Assumed mechanism	Application	Phase
Anakira	IL-1 antagonist	Anti- inflammatory	Subcutaneous	1
Botulinum toxin	Neurotoxic protein from *Clostridium botulinum*	Neurotoxic protein from *Clostridium botulinum*	Intramuscular	1/2
Cabergoline	Dopamine agonist	Anti-angiogenic	Oral	2
DLBS1442	Bioactive fraction of Phaleria macrocarpa	Apoptotic Anti-inflammatory Anti-angiogenic	Oral	2 /3
Epigallocatechin Gallate	Polyphenol (green tea)	Anti-angiogenic	Oral	P 2
IRAK-4	Inhibition of TLR/IL-1 receptor complex	Reduction in expression of inflammatory genes in immune cells	TBD	1
Melatonin	Neurohormone	Analgesic Anti-oxidant Anti-inflammatory	Oral	2
Micronized PEA-Transpolydatin	Endogenous fatty acid amide	Anti- inflammatory by inhibition of mast cell degranulation	Oral/sublingual	N/A
MT-2990	Antibody	Anti- inflammatory	Intravenous	2
P2X3 Antagonist (BAY 181708)	ATP-gated channel antagonist	Direct inhibition efferent nerve signaling	Oral	1/2
P2X3 Antagonist (Gefaxipant)	ATP-gated channel antagonist	Direct inhibition efferent nerve signaling	Oral	2
P2X4 Antagonist	ATP-gated channel antagonist	Immune cell-mediated inhibition of nerve signaling	TBD	1
Quinagolide	Dopamine 2-receptor agonist	Anti-angiogenic and Anti- inflammatory	Vaginal ring	2
THC/CBD	Cannabinoid	Cannabinoid receptors	Inhaler	2

2.4.2 Surgical treatment of endometriosis pain

Surgery should be considered in endometriosis patients with pain refractory to the pharmacological treatment. Another indication for surgery could be temporary pain relief in patients with desire of a future pregnancy (Berlanda *et al.*, 2013). Surgical treatment of endometriosis-associated pain aims to eliminate endometriotic lesions and remove adhesions to restore the normal anatomy, or to interrupt nerve pathways innervating the affected region, usually trough laparoscopy. The effect of surgery on pain may be temporarily satisfactory, however, additional surgical and pharmacological treatments are commonly required due to symptom recurrence (Nirgianakis *et al.*, 2020).

Elimination of endometriotic lesions may be achieved by excision, drainage, coagulation or vaporization. Conservative excision of ovarian endometriomas has been associated with greater pain alleviation and lower recurrence than vaporization or coagulation (Hart *et al.*, 2005), although a combination of these different techniques may be a better approach to limit damage to the gonadal reserve (Donnez *et al.*, 2010; Tsolakidis *et al.*, 2010). On the other hand, elimination of peritoneal lesions through coagulation and excision provide similar pain relief (Wright *et al.*, 2005; Healey *et al.*, 2010). Deep endometriotic lesions are usually excised effectively, although the procedure is associated with a high risk of complications and post-surgical pain (Donnez and Squifflet, 2010).

Hysterectomy, with or without oophorectomy, and removal of all visible lesions should be considered only in patients that have already

completed childbearing or have no desire to retain reproductive function and failed to respond to conservative treatments (Dunselman *et al.*, 2014). Since hysterectomy is effective in many, but not all cases of endometriosis (Martin, 2006), it is important to inform the patient that this procedure does not necessarily cure endometriosis or its symptoms. Furthermore, surgical menopause induced by hysterectomy is associated to a variety of long-term health outcomes including cardiovascular disease, fracture risk, pelvic floor dysfunction and alterations in neurologic functions (Stewart *et al.*, 2012).

Pelvic nerve transection procedures may be carried out during the course of conservative surgery to interrupt pelvic nerve pathways in endometriosis patients. Laparoscopic uterosacral nerve ablation (LUNA) is one of the most common nerve transection procedures for the treatment of chronic pelvic pain, however, studies show that it is not effective reducing endometriosis-associated pain, nor offers any additional benefit over excision surgery alone (Vercellini *et al.*, 2003). Presacral neurectomy (PSN) involves the excision or incision of the uterus innervation. Although it may be beneficial for the treatment of dysmenorrhea as an adjunct to conservative surgery (Proctor *et al.*, 2005), it is associated with adverse effects such as bleeding, constipation and urinary urgency (Zullo *et al.*, 2003).

2.4.3 Complementary strategies for endometriosis pain

Current treatments for endometriosis may be insufficient or have undesirable effects for many patients. For this reason, they often use self-care or lifestyle interventions to manage their symptoms and the adverse effects of medications (Table 2).

Table 2. Self-management strategies used for endometriosis, listed from most to least common, and perceived pain relief. Adapted from (Armour *et al.*, 2019; Sinclair *et al.*, 2020).

Self-management modality	Users		Reported pain relief (0-10 scale)	
	N	%	Mean	Standard deviation
Heat	259	70	7.6	2.0
Rest	252	68	6.5	1.7
Meditation/Breathing	175	47	6.4	2.4
Dietary choices	163	44	6.3	3.0
Exercise	158	42	6.3	1.6
Stretching	148	40	5.5	2.7
Yoga/Pilates	131	35	5.5	2.1
Massage	118	32	5.3	2.1
Herbal medicines	61	16	4.9	2.4
Alcohol	51	14	4.8	2.5
Cannabis	48	13	4.7	2.3
Acupressure	29	8	4.6	2.1
Cold	18	5	4.6	2.1
Hemp oil/CBD oil	12	3	4.5	2.0
Taichi/Qigong	8	2	4.0	1.7

The most common forms of self-management among endometriosis patients are heat, rest, meditation and breathing exercises (Armour *et al.*, 2019; Schwartz *et al.*, 2019). Although there are no studies on the effectivity of local **heat** relieving endometriosis related pain, studies suggest that it can reduce primary dysmenorrhea (Akin *et al.*, 2004). On the other hand, **mindfulness meditation** has proved effective reducing pain associated to endometriosis in a 6-year follow-up study (Hansen *et al.*, 2017). **Dietary choices**, such as gluten-free, vegan or low monosaccharides and polyols (known as FODMAP) diets are also

commonly adopted by endometriosis patients. Although there is a complex relationship between diet and endometriosis, gluten-free and FODMAP diets have shown positive effects reducing pain (Marziali *et al.*, 2012) and gastrointestinal symptoms (Moore *et al.*, 2017), respectively. Some endometriosis patients also use **physiotherapy** to reduce pain symptoms, although the evidence of its efficacy is limited (Fuentes-Márquez *et al.*, 2019). **Cannabis** and hemp or cannabidiol (CBD) oil use is popular among endometriosis patients (Armour *et al.*, 2019), which self-report a high effectiveness of cannabis improving pain, sleep and nausea and vomiting, allowing them to even reduce pharmaceutical medications (Sinclair *et al.*, 2020).

2.5 Animal models of endometriosis

Endometriosis occurs spontaneously in human and **nonhuman primates** (D'Hooghe *et al.*, 2009). A high prevalence of endometriosis has been observed in colonies of ageing rhesus macaques (Zondervan *et al.*, 2004), and endometriosis has also been experimentally induced in baboons by intrapelvic injection of autologous menstrual affluent (D'Hooghe *et al.*, 1995; Fazleabas, 2010). Although endometriosis in these animals closely resembles human condition, the use of nonhuman primates is limited by economical and ethical issues (D'Hooghe *et al.*, 2009). Rodents do not develop endometriosis spontaneously, but they are cost-effective and widely available (Grümmer, 2006). Furthermore, there is a vast variety of genetically modified mice that can be applied to study the role of specific genes on endometriosis.

Heterologous rodent models have been developed through xenotransplantation or injection of human endometrial tissue (ectopic, eutopic, menstrual or cultured stromal and epithelial cells) into immunodeficient animals (Greaves *et al.*, 2017b). These models can be very useful to evaluate potential therapies on endometriotic lesions that maintain human histological characteristics (Hull *et al.*, 2008), as well as to investigate species-specific factors involved in lesion formation. However, the lack of a normal immunological response represents the major limitation of these models.

Homologous rodent models involve surgical transplantation or injection of endometrial tissue from the same animal or a syngeneic donor into the pelvic or abdominal cavities of immunocompetent animals. On the one hand, **autologous models** consist in the surgical transplantation of uterine fragments into the arterial cascades of the small intestine, the peritoneal wall or the ovaries of the same animal (Vernon and Wilson, 1985; Berkley *et al.*, 2001). On the other hand, **syngeneic models** do not involve the resection of the uterus from the same animal, and allow the study of the contribution of host and donor cells on endometriosis (Zhao *et al.*, 2016). Among syngeneic models, the ones involving suturing of uterine fragments to the peritoneal lining have the advantage of easy localization and analysis of the lesions. Endometriosis can also be induced by injection of minced uterine, endometrial or "menstrual" (endometrial tissue obtained after a menses-like event produced by hormonal manipulation) tissue into the peritoneum of a syngeneic animal. These models are less invasive that the ones involving surgery, but evaluation of effectivity of disease-

modifying drug is complicated due to the homogeneity of the lesions and the difficulty to identify them unless reporter mice are used as donors (Fortin *et al.*, 2003).

Most of the published rodent models of endometriosis use **ovariectomized** animals supplemented with estradiol, avoiding the natural variations in hormone levels across the estrous cycle (Greaves *et al.*, 2017b). This manipulation promotes lesion establishment and growth; however, it also involves an additional surgery to remove the ovaries and an alteration in the hormonal status that may affect animal behavior.

3 The endocannabinoid system

The *Cannabis sativa* plant has been used for therapeutic and recreational purposes for millennia (Russo and Guy, 2006). Yet, it took until the second half of the XIX century that its mechanism of action began to be unraveled. The identification of its major active components, the cannabinoids Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) in the 1960s (Mechoulam and Shvo, 1963; Gaoni and Mechoulam, 1964), led to the discovery of the cannabinoid receptors (Matsuda *et al.*, 1990; Munro *et al.*, 1993) and their endogenous ligands (Devane *et al.*, 1992; Mechoulam *et al.*, 1995) in the early 1990s. Since then, the endocannabinoid system has been assigned a wealth of roles related to the maintenance of homeostasis in physiological and pathological conditions (Di Marzo *et al.*, 2004).

3.1 Components of the endocannabinoid system

3.1.1 Cannabinoid receptors

The two canonical receptors of the endocannabinoid system are the cannabinoid receptor 1 (CB1R) and 2 (CB2R), which are G protein-coupled receptors (GPCRs) with seven transmembrane domains (Figure 6**Error! Reference source not found.**) (Childers and Deadwyler, 1996). Nevertheless, other GPCRs, such as GPR55, GPR18 and GPR110 (Irving *et al.*, 2017), TRPV1 (De Petrocellis *et al.*, 2017) and peroxisome-proliferator-activating receptors (PPARs) (O'Sullivan, 2007) can also be modulated by cannabinoids.

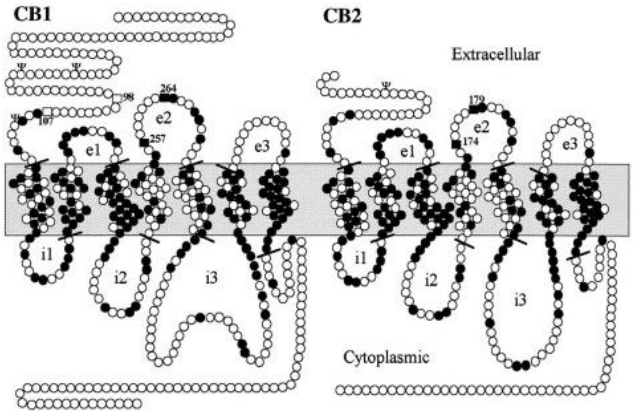


Figure 6. Schematic representation of the human CB1 and CB2 receptors. Black circles represent amino acids common to the two receptors, and white circles represent different amino acids. CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2. Adapted from (Shire *et al.*, 1996).

The **CB1R** is highly expressed in the central nervous system (Figure 7), and its distribution has been well characterized in rodents (Tsou *et al.*, 1998) and humans (Westlake *et al.*, 1994). The high expression of CB1R in brain areas such as the cortex, hippocampus, amygdala, basal ganglia and cerebellum (Freund *et al.*, 2003) underlies the major psychotropic effects of THC and synthetic CB1R agonists. The CB1R is also present in pain-processing areas such as the hypothalamus, thalamus, PAG, RVM, and the dorsal horn of the spinal cord (Freund *et al.*, 2003). Besides being expressed in neurons, CB1R is also expressed in other cells of the central nervous system (CNS) such as astrocytes (Han *et al.*, 2012). Furthermore, CB1R is expressed in peripheral tissues including male and female reproductive organs (Das *et al.*, 1995; Gye *et al.*, 2005), urinary bladder (Walczak *et al.*, 2009), liver (Tam *et al.*, 2010) and peripheral sensory nerves (Ahluwalia *et al.*, 2000), among others. CB1Rs are primarily expressed in presynaptic terminals (Busquets-Garcia *et al.*, 2018) inhibiting the release of glutamate and gamma-

aminobutyric acid (GABA), but also acetylcholine, noradrenaline, dopamine, serotonin, and cholecystokinin, among other neurotransmitters (Szabo and Schlicker, 2005). In addition, postsynaptic actions (Maroso *et al.*, 2016) and presence of CB1Rs in mitochondria (Bénard *et al.*, 2012) have also been described recently.

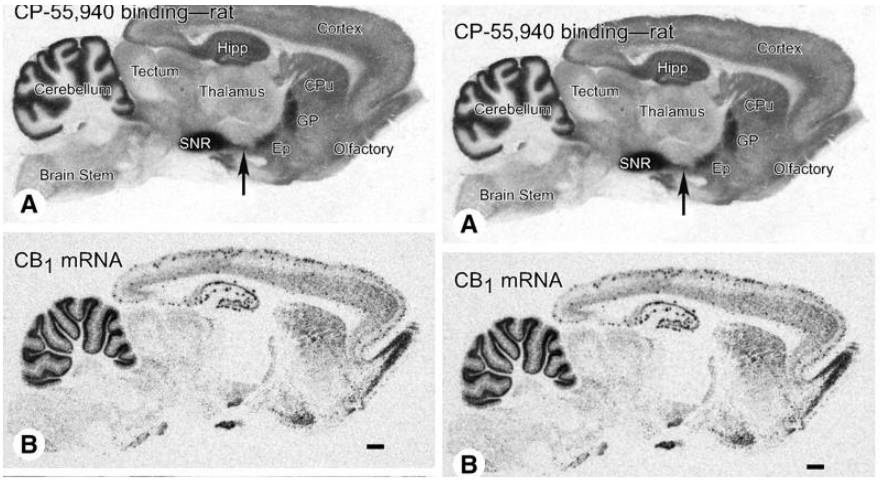


Figure 7. Distribution of CB1R in the brain. (**A**) CB1R localization in the rat brain marked by the tritiated ligand CP-55,940 and (**B**) CB1R gene transcript hybridized with a CB1R-specific oligonucleotide probe. (**C**) CB1R localization in the human brain marked by the tritiated ligand CP-55,940. In both rat and human brains, high levels CB1R are found in basal ganglia, cerebellum, hippocampus, cortex, and caudate putamen. Adapted from (Freund *et al.*, 2003).

The **CB2R** is mainly expressed in the periphery, particularly in immune cells like macrophages, B and T lymphocytes, neutrophils and monocytes (Galiègue *et al.*, 1995). Examples of other peripheral tissues where CB2R are also present are the liver (Julien *et al.*, 2005), pancreas (Bermúdez-Silva *et al.*, 2008), ovaries (El-Talatini *et al.*, 2009) and sensory neurons (Anand *et al.*, 2008). It was long accepted that the expression of the CB2R was restricted to the periphery, however, the presence of this receptor has been demonstrated in the CNS in

microglial cells (Sánchez *et al.*, 2001), astrocytes (Walter *et al.*, 2003) and neurons of the spinal cord and the brain (Van Sickle *et al.*, 2005). Although expression levels of CB2R in rat and mouse brains is much lower than those of CB1R (Gong *et al.*, 2006; Onaivi *et al.*, 2006), this receptor seems to be involved in several central responses, including emotional and rewarding processes (Onaivi *et al.*, 2012) and pain attenuation (Shang and Tang, 2017).

Stimulation of CB1R and CB2R leads to the activation of multiple **signaling pathways**. As members of the GPCR superfamily, cannabinoid receptors mediate their effects by activating heterotrimeric $G_{i/o}$ proteins (McAllister and Glass, 2002) (Figure 8**Error! Reference source not found.Error! Reference source not found.**). Activation of Gi/o proteins is coupled to the inhibition of the adenylyl cyclase activity (AC), which causes a decrease in cyclic adenosine monophosphate (cAMP) production and the corresponding attenuation of protein kinase A (PKA) activity. Coupling to heterotrimeric $G_{i/o}$ proteins also leads to stimulation of enzymes of the MAPK family such as extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinase (JNK) (Bosier *et al.*, 2010). In addition, activation of complex protein cascades such as those involving the phosphoinositide-3-kinase (PI3K) have also been proposed (Piomelli, 2003). These intracellular events lead to downstream regulation of gene transcription. Stimulation of coupled $G_{i/o}$ proteins is also associated to inhibition of voltage activated Ca^{2+} channels and stimulation of inwardly rectifying K^+ channels ($K_{ir}3$) in neurons to inhibit neurotransmitter release (McAllister and Glass, 2002).

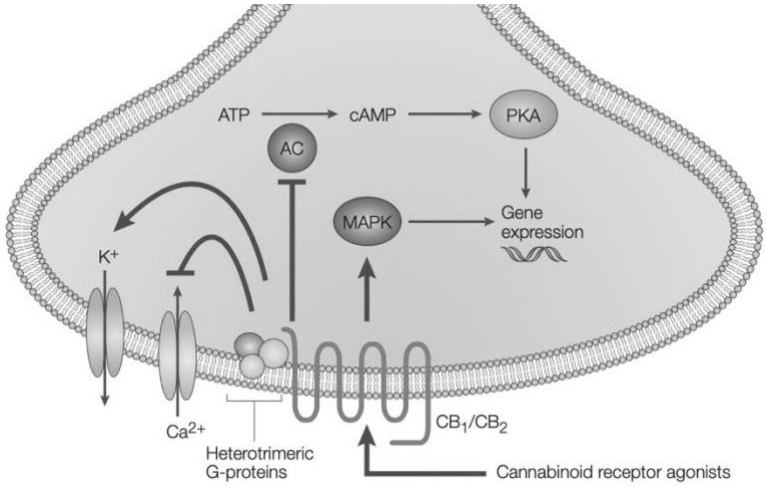
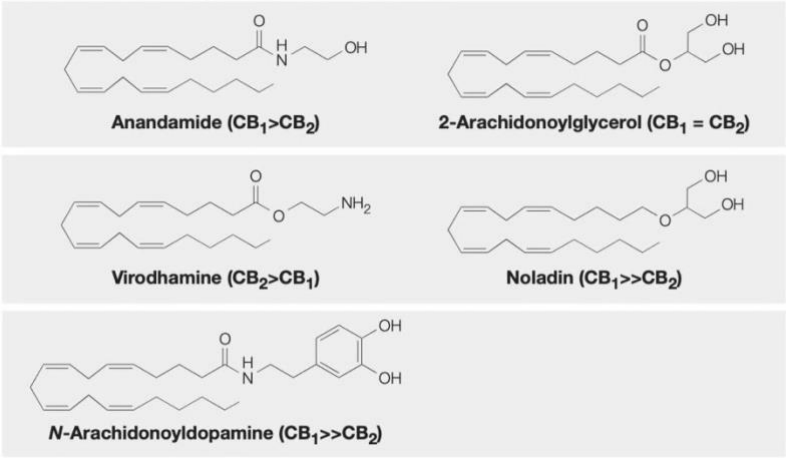


Figure 8. Major signaling pathways of cannabinoids. CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; AC, adenylyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; MAPK, mitogen-activated protein kinase. Adapted from (Di Marzo *et al.*, 2004).

3.1.2 Endocannabinoids

All identified endocannabinoids are long-chain arachidonic acid derivatives (Figure 9**Error! Reference source not found.**). The different endocannabinoids exhibit varying selectivity for CB1R and CB2R, as well as for other targets (McAllister and Glass, 2002). The most relevant endocannabinoids are N-arachidonoylethanolamine (anandamide or AEA) and 2-arachidonoylglycerol (2-AG) (Devane *et al.*, 1992; Mechoulam *et al.*, 1995). **AEA** is a partial agonist of CB1R and CB2R, and also has affinity for TRPV1 (Cristino *et al.*, 2008). **2-AG** is a full agonist of CB1R and CB2R, and is present at higher concentrations than AEA in the brain (Sugiura *et al.*, 2006). Other putative endocannabinoids with unknown physiological function are 2-arachidonoylglycerol ether (noladin ether) (Sugiura *et al.*, 1995), N-

arachidonoyldopamine, (Huang *et al.*, 2002) and O-arachidonoylethanolamine (virodhamine) (Porter *et al.*, 2002) (Figure



9).

Figure 9. Endocannabinoids and putative endocannabinoids and their rank of affinity for cannabinoid receptors. Chemical structures of the two best-studied endocannabinoids, anandamide and 2-arachidonoylglycerol and three proposed endogenous ligands of cannabinoid receptors. CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2. Adapted from (Di Marzo *et al.*, 2004).

Structurally-related fatty acid amines such as N-acylethanolamines (NAEs), N-oleoyl ethanolamine (OEA) and N-palmitoyl ethanolamine (PEA) are also widely distributed in the CNS and the periphery, although their affinity for CB1R and CB2R is very weak. It has been proposed that these compounds may induce indirect activation of the principal cannabinoid receptors through the entourage effect increasing the activity of endogenous cannabinoids, and through the stimulation of TRVP1, PPAR or GPR55 (Petrosino and Di Marzo, 2017).

Unlike other neurotransmitters, endocannabinoids are not pre-stored in secretory vesicles, but are biosynthesized on demand from cell membrane lipids, responding to an increase in intracellular calcium

concentration. Endocannabinoids are then released from the postsynaptic terminal and travel across the synapse to act as **retrograde messengers** (Freund *et al.*, 2003). As mentioned above, activation of presynaptic CB1R leads to the suppression of neurotransmitter release on excitatory or inhibitory synapses. Endocannabinoid activation of CB1R for a few seconds results in short-term plasticity characterized by transient depolarization-induced suppression of inhibition or excitation, depending on the nature of the presynaptic terminal. This mechanism involves direct G protein-dependent inhibition of presynaptic Ca^{2+} influx (Castillo *et al.*, 2012). Endocannabinoids also mediate long-term depression at both excitatory and inhibitory synapses through the inhibition of AC and downregulation of the cAMP/PKA pathway in active synapses (Heifets and Castillo, 2009).

3.1.3 Enzymes involved in the biosynthesis and degradation of endocannabinoids

Endocannabinoid levels are the result of a balance between their enzymatic synthesis and degradation (Figure 10). AEA **synthesis** involves two enzymatic reactions: glycerophospholipids and phosphatidylethanolamine are converted into N-arachidonoyl phosphatidylethanolamide (NArPE) by a calcium-dependent N-acyltransferase (NAT), then, NArPE is hydrolyzed to AEA by the phospholipase D (NAPE-PLD) (Di Marzo *et al.*, 1994). 2-AG is also synthetized in a two-step process: phospholipase C (PLC) produces sn-1-Acyl-2-arachidonyl-diacylglycerol (DAG) from glycerophospholipids,

which is then hydrolyzed by diacylglycerol lipases (DAGL) into 2-AG (Murataeva *et al.*, 2014).

Once AEA and 2-AG activate their target receptors, AEA **degradation** is carried out by fatty-acid amide hydrolase (FAAH) (Di Marzo *et al.*, 1994), whereas 2-AG is primarily metabolized by monoacylglycerol lipase (MAGL) (Dinh *et al.*, 2002), both on pre- and postsynaptic neurons (Chicca *et al.*, 2017). Nevertheless, alternative metabolic pathways for the degradation of these endocannabinoids have also been described (Jhaveri *et al.*, 2007).

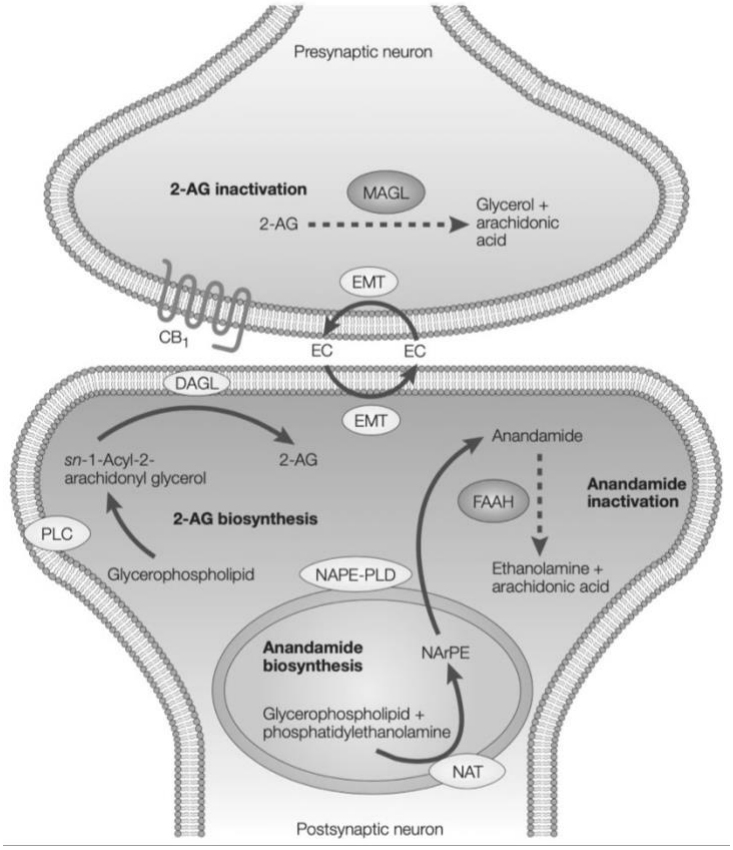


Figure 10. Synthesis and degradation of endocannabinoids. CB_1, cannabinoid receptor 1; EC, endocannabinoid; 2-AG, 2-arachidonoylglycerol; PLC, phospholipases C; DAGL, diacylglycerol lipase; MAGL, monoacylglycerol lipase;

NAT, N-acyltransferase; NAPE-PLD, N-acylphosphatidyl-ethanolamine-specific phospholipase D; FAAH, fatty acid amide hydrolase; EMT, endocannabinoid membrane transporter; NArPE, N-arachidonoyl-phosphatidyl-ethanolamine. Adapted from (Di Marzo *et al.*, 2004).

3.1.4 Exogenous cannabinoid receptor ligands

About 100 **phytocannabinoids** have been isolated from the *Cannabis sativa* plant during the last century. The first cannabinoids to be discovered, and also the most studied ones, were Δ9-tetrahydrocannabinol (THC), the main psychoactive component of the plant, and cannabidiol (CBD), a major bioactive compound that lacks important psychotropic effects. Examples of other phytocannabinoids are Δ9-tetrahydrocannabivarin, cannabinol, cannabidivarin, cannabigerol and cannabichromene (Pertwee, 2006) (Figure 11).

The structures of these phytocannabinoids have been used as models to develop several **synthetic compounds** with cannabimimetic properties (Figure 11). Non-selective cannabinoid agonists include HU-210, CP55940 and WIN55212. Examples of CB1R and CB2R selective agonists are arachidonyl-2'-chloroethylamide (ACEA), and JWH-133, respectively. Cannabinoid receptor antagonists have also been synthetized, and include SR141716A (rimonabant) and AM251 which act on CB1R, and AM630, which acts on CB2R (Pertwee *et al.*, 2010).

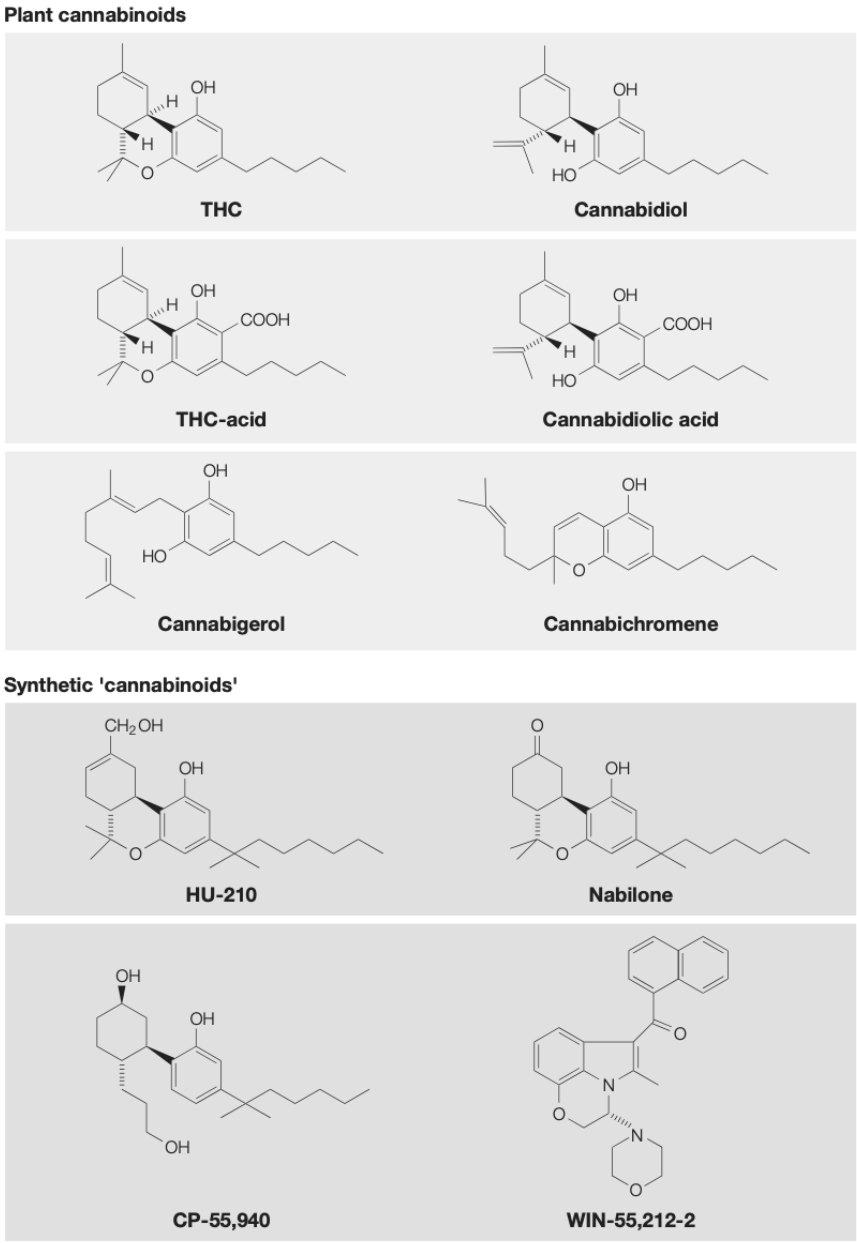


Figure 11. Chemical structures of some plant and synthetic cannabinoids. Only Δ9-tetrahydrocannabinol binds to cannabinoid receptors with high affinity. None of the synthetic cannabinoids is selective for one cannabinoid receptor over the other. THC, Δ9-tetrahydrocannabinol. Adapted from (Di Marzo *et al.*, 2004).

3.2 Physiological functions of the endocannabinoid system

The endocannabinoid system participates in physiological functions and pathological states since it is involved in the regulation of cell, organ and organism homeostasis, contributing to the regulation of reproductive, cardiovascular, respiratory and gastrointestinal functions, metabolism and energy storage, and immune responses, among others (Grotenhermen, 2004). At central levels, endocannabinoid signaling is implicated in neural functions, including movement and motor coordination (de Fonseca *et al.*, 2005), learning and memory (Marsicano and Lafenêtre, 2009), emotion (Lutz *et al.*, 2015), reward and motivation (Manzanares *et al.*, 2018) and pain modulation (Woodhams *et al.*, 2017).

3.2.1 Cannabinoid effects in pain

The endocannabinoid system plays an important role in the modulation of nociception in physiological and pathological conditions. Multiple lines of evidence suggest that endocannabinoids naturally suppress pain. Indeed, physiological pain stimuli elevate the endocannabinoid tone in a rapid and transient manner, while pathological pain conditions produce slow but persistent changes in endocannabinoid levels (Zogopoulos *et al.*, 2013). The contribution of endocannabinoid signaling to pain transmission occurs at both peripheral and central levels. Stimulation of cannabinoid receptors in rodent DRG and human nociceptors decreases nociceptive transmission in electrophysiology studies (Millns *et al.*, 2001; Anand *et al.*, 2008). At the spinal level, CB1R mediates long-term depression (LTD) in synapses involving Aδ nociceptors, whereas, it prevents the induction of long-term

potentiation (LTP) in C-fiber synapses, indicating participation of CB1R in synaptic plasticity of nociceptors (Kato *et al.*, 2012). In the cortex, suppression of GABAergic inhibition by CB2R but not CB1R agonism has also been evidenced using electrophysiological approaches (Morgan *et al.*, 2009).

Studies performed in rodent models of **acute pain** have shown that the elevation of the endocannabinoid tone by exogenous administration of endocannabinoids or by inhibition of endocannabinoid degradation or uptake produces antinociception (reviewed by Guindon and Hohmann, 2009). Most of the studies attribute these antinociceptive effects to CB1R stimulation (Mason *et al.*, 1999; Hohmann *et al.*, 2005; Suplita *et al.*, 2005; Hasanein and Javanmardi, 2008), although others suggest that antinociception is mediated through a CB1R-independent mechanism (Adams *et al.*, 1998; Wiley *et al.*, 2006). Exogenous cannabinoids have also proven effective against thermal (Khanna *et al.*, 2011), mechanical (Bloom *et al.*, 1977) and chemical stimuli (Sofia *et al.*, 1973) in models of acute pain. Transgenic mice lacking CB1R in nociceptive (Nav1.8) sensory neurons show increased sensitivity to heat and mechanical stimuli (Agarwal *et al.*, 2007), implying that peripheral CB1R participate in the modulation of the nociceptive responses. CB1R stimulation inhibits pain transmission also on supraspinal areas such as the thalamus (Martin *et al.*, 1999) and modulates neuronal activity in frontal and limbic structures to influence nociceptive responses to heat and chemical stimuli (Manning *et al.*, 2003). Microinjection of cannabinoid agonists into areas involved in the descending inhibitory pathway, including the PAG (Martin *et al.*,

1999) and RVM (Martin *et al.*, 1998), also results in CB1R-dependent analgesia. Endocannabinoids released in these areas have been suggested to mediate stress-induced analgesia via CB1R (Hohmann *et al.*, 2005). Studies using CB2R knockout mice show that this receptor does not play a relevant role in mechanical or heat sensitivity in physiological conditions (Racz *et al.*, 2008; Cabañero *et al.*, 2020). Nevertheless, others have described that baseline responses to heat stimuli increase in mice lacking CB2R (Ibrahim *et al.*, 2006), and that besides neuronal cells, immune cells and keratinocytes also participate in peripheral CB2R analgesia (Ibrahim *et al.*, 2005).

Inflammatory pain models such as the carrageenin, the capsaicin and the complete Freund's adjuvant models have been useful to reveal the antinociceptive effects of cannabinoid agonists, endocannabinoids, and their modulators in inflammatory pain (reviewed by Guindon and Hohmann, 2009). Both CB1R and CB2R have been implicated in the antinociceptive effects of FAAH inhibition in the carrageenin model (Jayamanne *et al.*, 2006; Ahn *et al.*, 2011). Furthermore, it has been described that spinal CB1R expressed on nociceptive fibers and local interneurons inhibits the release of neurotransmitters involved in pain (Drew et al., 2000) and that peripheral CB1R mediates AEA antinociceptive and anti-inflammatory effects in this model (Clapper *et al.*, 2010). On the other hand, CB2R plays an important role in peripheral inflammation, nociception and central sensitization in osteoarthritic rats (Burston *et al.*, 2013). Indeed, mechanical allodynia is enhanced in osteoarthritic mice lacking CB2R, and attenuated in mice overexpressing this cannabinoid receptor (La Porta *et al.*, 2013).

Pharmacological increase of endocannabinoid levels also reduces nociception in rodent models of **neuropathic pain** (reviewed by Guindon and Hohmann, 2009). Although the upregulation of spinal CB1R promotes enhanced antinociceptive effects of cannabinoids in nerve-injured rats (Lim *et al.*, 2003), the constitutive lack of CB1R does not alter neuropathic pain manifestations in mice (Castañé *et al.*, 2006). On the contrary, absence of CB1R in peripheral nociceptors enhances nociception and reduces the analgesic effects of cannabinoid administration, suggesting that the participation of peripheral CB1Rs is particularly important in neuropathic pain (Agarwal *et al.*, 2007). Indeed, peripheral CB1R mediates AEA antinociceptive and anti-inflammatory effects in nerve-injured mice (Clapper et al., 2010). On the other hand, spinal CB2R expression is also increased during neuropathic pain (Zhang *et al.*, 2003), and constitutive and neuronal lack of CB2R exacerbate behavioral manifestations of neuropathic pain in mice, while lymphocyte CB2R depletion inhibits antinociception provided by a selective CB2R agonist (Cabañero *et al.*, 2020). Furthermore, exacerbated neuropathic pain behaviors in mice constitutively lacking CB2R match with microglia and astrocyte activation in the spinal cord (Racz *et al.*, 2008). In supraspinal areas, endocannabinoid levels also increase during neuropathic pain (Petrosino *et al.*, 2007), although it remains to be investigated whether enhancement or blocking of endocannabinoid signaling in these brain regions could affect neuropathic pain manifestations.

Increases in circulating AEA concentrations have been found in **patients** with chronic pain syndromes, such as fibromyalgia (Kaufmann *et al.*, 2008) and complex regional pain syndrome (Kaufmann *et al.*, 2009). Furthermore, individuals with a single nucleotide polymorphism in the gene for FAAH that results in elevated circulating concentrations of AEA is associated with lower sensitivity to cold pain (Cajanus *et al.*, 2016), suggesting that AEA reduces pain perception. Increased amounts of circulating 2-AG concentrations have also been reported in patients of neuromyelitis optica. In this individuals, 2-AG levels are negatively correlated with mechanical pain thresholds, suggesting an analgesic role of this endocannabinoid (Pellkofer *et al.*, 2013). On the contrary, elevated concentrations of 2-AG correlate with knee pain in osteoarthritis patients, indicating that it may also contribute to pain perception (La Porta *et al.*, 2015). On the other hand, studies suggest that cannabinoids are not effective for acute pain in humans (Beaulieu, 2006; Holdcroft *et al.*, 2006; Kraft *et al.*, 2008). However, medicinal cannabis and cannabinoids are associated with chronic pain relief and good tolerability in cancer and non-cancer pain patients (Lynch, 2016; Vučković *et al.*, 2018). Nevertheless, there is still insufficient evidence to support their long-term use for the management of chronic pain.

3.2.2 Cannabinoid effects in emotional responses

The endocannabinoid system is extensively distributed in the CNS and plays an important and complex role in the regulation of emotional homeostasis. This is illustrated by the fact that THC can cause euphoria, relaxation and stress-relieving effects, or dysphoria and anxiety

depending on the dose and on the basal status of the individual (Maldonado *et al*., 2020; Mechoulam and Parker, 2013).

The same complex picture applies to animal studies evaluating the effects of exogenous cannabinoids in **anxiety-like behavior** (Micale *et al.*, 2013). Cannabinoid agonists elicit anxiolytic responses at low doses, and anxiogenic effects at higher doses (Moreira and Wotjak, 2010), although genetic background or environmental context can modify these effects. This bimodality has been attributed to differences in the baseline stress levels of the animals, recruitment of non-canonical cannabinoid receptors, or differential effects of CB1R activation on distinct neuronal populations (Häring *et al.*, 2012). Elevation of endocannabinoid tone by administration of AEA and 2-AG or blocking of FAAH and MAGL induces anxiolytic-like responses in rodents (Rubino *et al.*, 2008; Mechoulam and Parker, 2013). Thus, CB1R seems to mediate the anxiolytic-like effects produced by increased AEA levels, while CB2R seems to contribute mainly to the effects of elevated 2-AG (Busquets-Garcia *et al.*, 2011). Studies performed with transgenic mice show that the lack of either CB1R or CB2R is associated to increased anxiety-like behaviors (La Porta *et al.*, 2015), evidencing a direct involvement of both cannabinoid receptors in emotional responses.

Modulation of the endocannabinoid system also has an impact on **depressive-like behavior** in rodents. Different cannabinoid agonists reduce depressive-like behavior via CB1R mechanisms (Patel and Hillard, 2009). Results with CB1R and CB2R antagonists are, however, inconsistent. While some studies show no effect on depressive-like behavior (Bambico *et al.*, 2007; Gobshtis *et al.*, 2007; Onaivi *et al.*,

2008), others conclude that cannabinoid receptor antagonism reduces this behavior (Steiner *et al.*, 2008a). Studies using knockout animals are inconclusive, since they have shown that mice lacking CB1R or CB2R display similar (Jardinaud *et al.*, 2005) or higher (Steiner *et al.*, 2008b; Ortega-Alvaro *et al.*, 2011) depressive-like behavior than wild type mice, depending on the strain and the experimental conditions.

Epidemiological studies have demonstrated that cannabis use for anxiety is highly prevalent (Kosiba *et al.*, 2019). However, **clinical trials** testing the effects of acute consumption of isolated THC concluded that this cannabinoid is associated to anxiogenic responses (reviewed by Sharpe *et al.*, 2020). On the contrary, CBD has anxiolytic effects in individuals with social anxiety disorders (Bergamaschi *et al.*, 2011) and reduces the anxiety induced by acute THC consumption (Zuardi *et al.*, 1982).

3.2.3 Cannabinoid effects in cognition

The components of the endocannabinoid system are present in brain areas such as the medial prefrontal cortex or the hippocampus, where participate in learing and memory processes.

CB1R agonists impair **emotional and non-emotional memory** in naïve rodents (Mechoulam and Parker, 2013), while CB2R agonism has shown a beneficial or neutral effect on cognition (García-Gutiérrez *et al.*, 2013). Blockade of cannabinoid receptors produce a variety of effects on cognitive processes. CB1R antagonism and genetic deletion of CB1R in mice improve olfactory memory, working memory and object recognition memory, although they impair the extinction of aversive memories (Terranova *et al.*, 1996; Hampson and Deadwyler,

1998; Maccarrone *et al.*, 2002). On the contrary, pharmacological and genetic inactivation of CB2R produces cognitive impairments in mice (García-Gutiérrez *et al.*, 2013). Nevertheless, others have shown no cognitive effects when cannabinoid receptors are blocked or absent (Lichtman *et al.*, 2002; La Porta *et al.*, 2016). Some studies show that increases in AEA, but not 2-AG, interfere with emotional and non-emotional memory (Busquets-Garcia *et al.*, 2011), while others have reported improvements in memory associated to the elevation of both AEA and 2-AG (Varvel *et al.*, 2007; Pan *et al.*, 2011; Mechoulam and Parker, 2013). Altogether, these data reveals that the relationship between cannabinoid signaling and memory and cognition is complex.

In **humans**, cannabis use affects several aspects of cognitive performance, including attention, working memory, verbal learning, mental flexibility and consolidation of memories (Mechoulam and Parker, 2013). These effects are dose-dependent, and although they are stronger in the first six hours after acute consumption, impairments in cognitive function may be present even after three weeks of abstinence and beyond (Crean *et al.*, 2011)

3.3 The endocannabinoid system in endometriosis

The endocannabinoid system components coexist in the human endometrium. Cannabinoid receptors, endocannabinoids and enzymes involved in their biosynthesis and degradation are distinctly expressed during the different phases of the menstrual cycle (Scotchie *et al.*, 2015), suggesting a role of the endocannabinoid system on the proper functioning of healthy female reproductive tissues. Thus, the involvement of this system in specific mechanisms critical to

endometrial growth and maintenance suggest a role of this system in endometriosis.

Cannabinoids modulate **cell growth and survival** through activation of pathways that correlate with the abnormal cell growth occurring in endometriosis (**Error! Reference source not found.**). One of those is the PI3K/protein kinase B pathway, primary involved in the regulation of cell cycle through apoptosis mediators such as Bax, Bad and caspase 9. This signaling pathway can be negatively regulated by the phosphatase and tensin homolog (PTEN) (Myers and Tonks, 1997). PTEN is downregulated in eutopic and ectopic endometrial tissue from endometriosis patients, suggesting that these cells may be resistant to apoptosis due to a downstream decrease in the pro-apoptotic factor Bax (Meresman et al., 2000).

Cannabinoids reduce levels of the MMP2, a mediator involved in **cell migration**, in cells from murine gliomas (Blázquez *et al.*, 2008). During endometriosis, an increased synthesis of MMPs in both eutopic and ectopic endometrium (Sanchez *et al.*, 2012) is associated to cell migration, as it occurs in cancerous cells. On the other hand, endocannabinoids can stimulate cell migration through the activation of PI3K/Akt and ERK1/2 pathways. Indeed, this activation is suggested to be specifically induced by the CB1R agonist metanandamide, which promotes the migration of endometrial stromal cells *in vitro* (Gentilini *et al.*, 2010). Thus, cannabinoid stimulation could both inhibit or promote endometrial cell migration in endometriosis.

Exogenous and endogenous cannabinoids are emerging as potential suppressors of **angiogenesis**, especially in cancer studies (Bifulco *et al.*,

2007). The selective CB2R agonist JWH-133 reduces vascular irrigation in a mouse model of glioma (Blázquez *et al.*, 2003) and decreases the expression of proangiogenic factors in human glioma cells (Blázquez *et al.*, 2004). In contrast, AEA seems to stimulate the fibroblast growth factor (FGF) through CB1R, inducing proliferation of human umbilical vein endothelial cells (HUVECs) (Pisanti *et al.*, 2011). As it occurs in tumors, endometriotic cells require their own vascular supply to survive and grow, and the endocannabinoid system may play a role in the regulation of angiogenesis during endometriosis.

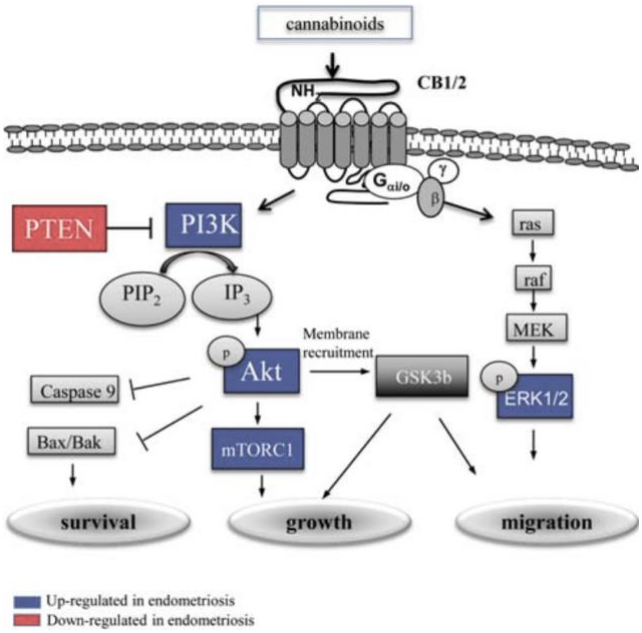


Figure 12. Schematic diagram of cannabinoid receptor signaling pathways potentially altered in endometriosis. Endometrial cell survival, growth and migration could be modulated by cannabinoids in endometriosis. PKB, protein kinase B; Bax, pro-apoptotic protein; Bak, anti-apoptotic protein; CB1/2, cannabinoid 1/2 receptor; GPCR, G protein-coupled receptor; ERK, extracellular-regulated kinase; TKR, tyrosin kinase receptor; PI3K, phosphatidyl inositol 3 kinase; PTEN, phosphatase and tensin homolog; GSK3b, glycogen synthase kinase 3 b; mTOR, mammalian target of rapamycin complex 1. Adapted from (Sanchez *et al.*, 2012).

Cannabinoid receptors are expressed in almost every type of **immune cells** in humans and mice (Parolaro, 1999). In agreement, their ligands have demonstrated *in vivo* and *in vitro* effects on the production and function of inflammatory mediators. For example, THC stimulates CB2R to inhibit the chemotactic response of murine peritoneal macrophages to the RANTES/chemokine C-C motif ligand 5 (CCL5) (Raborn *et al.*, 2008). On the other hand, CB1R antagonism reduces plasma levels of monocyte chemoattractant protein-1 (MCP-1/CCL2) and of interleukin 12 (IL-12) in a model of atherosclerotic lesion development (Dol-Gleizes *et al.*, 2009). In isolated endometrial stromal cells, CB1R expression is disrupted after exposure to the inflammatory 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCCD) (Resuehr *et al.*, 2012), suggesting an inverse relationship between inflammation and endometrial CB1R signaling. On the other hand, activation of CB2R has been associated with the release of nitric oxide in endometrial inflammation, suggesting that this receptor participates in inflammatory processes in endometrial tissues (Iuvone *et al.*, 2008). Furthermore, TRPV1 is upregulated in deep-infiltrating endometriosis cells (Bohonyi *et al.*, 2017), where it may play a role in pain and inflammation.

Clinical data on the effects of cannabinoid drugs in endometriosis is scarce. Nevertheless, a small open-label study has shown the efficacy of ultramicronized PEA and co-micronized PEA/polydatin reducing chronic pelvic pain, dysmenorrhea, dyspareunia and dyschezia associated to endometriosis, as well as improving overall quality of life (Stochino Loi *et al.*, 2019). Hence, the endocannabinoid system could be a potential target for the treatment of endometriosis.

4 The endogenous opioid system

Similarly to the *Cannabis sativa* plant, the opium poppy has been used for millennia for ritual, recreative and medicinal purposes (Booth, 2013). Morphine was isolated from opium in the early XIX century (Krishnamurti and Rao, 2016), although it took until the 1970s to became apparent that opiate compounds exerted their actions at specific receptors in the CNS (McClane and Martin, 1967; Goldstein *et al.*, 1971). Soon, endorphins enkephalins and dynorphins were isolated and identified as the endogenous compounds that bind to opioid receptors (Hughes *et al.*, 1975; Cox *et al.*, 1976; Goldstein *et al.*, 1979). The discovery of the opioid receptors and their endogenous opioid ligands was the first step to characterize the endogenous opioid system.

4.1 Components of the endogenous opioid system

4.1.1 Opioid receptors

Mu (MOR), delta (DOR) and kappa (KOR) opioid receptors are considered the three principal opioid receptors (Kieffer, 1999), although the nociceptin or orphanin receptor (NOR or opioid receptor-like 1, ORL-1) has also been accepted as part of the opioid receptor family (Bunzow *et al.*, 1994; Mollereau *et al.*, 1994). Opioid receptors are seven-transmembrane domain proteins that couple to G proteins sensitive to the pertussis toxin ($G_{\alpha i}$ and $G_{\alpha o}$), and display different conformations on their inactive and active states **Error! Reference source not found.**) (Granier *et al.*, 2012; Manglik *et al.*, 2012; Thompson *et al.*, 2012; Wu *et al.*, 2012).

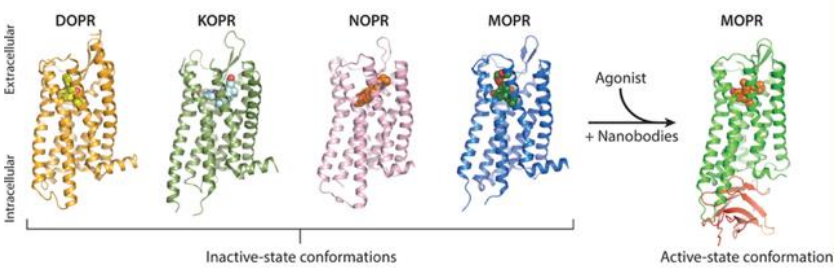


Figure 13. Structures of the four opioid receptors. Crystal structures of the inactive state of all four opioid receptors. When an opioid agonist enters the binding pocket of the receptor, a conformational change in the transmembrane domains allows for intracellular effector molecules to bind and activate downstream signalling cascades. Stabilizing nanobodies were used to elucidate the active state of MOR. DORP, delta opioid receptor, KORP, kappa opioid receptor, NOPR, nociceptin opioid receptor, MOPR, mu opioid receptor. Adapted from (Corder *et al.*, 2018).

Opioid receptors are widely distributed in the peripheral and central nervous systems (Mansour *et al.*, 1988; Stein, 1993). In areas of the brain such as the cortex, the limbic circuitry and the brain stem, opioid receptors are abundant (Mansour *et al.*, 1994; Neal *et al.*, 1999). Although the three classical opioid receptors coincide in most structures, some regions show higher expression of one particular receptor (**Error! Reference source not found.**). Thus, MOR is the most expressed opioid receptor in the amygdala, DOR is the most abundant in the olfactory tract, the cortex and the striatum and KOR is highly expressed in the basal anterior forebrain (Le Merrer *et al.*, 2009; Lutz and Kieffer, 2013). In the spinal cord dorsal horn and DRG, MOR is mainly present in C-fibers (Scherrer *et al.*, 2009), whereas DOR is highly expressed in myelinated Aβ primary afferents (Bardoni *et al.*, 2014; François and Scherrer, 2018). MOR and DOR can also be co-expressed in a subset of polymodal Aδ fibers (H.-B. Wang *et al.*, 2010; François and Scherrer, 2018). Expression of KOR has been described in low-

thereshold mechanoreceptor C- fibers and Aδ and Aβ fibers (Snyder *et al.*, 2018).

Opioid receptor expression (mRNA) generally matches the distribution of binding sites (protein), implying that opioid receptor synthesis is carried out by local neurons. However, the absence of binding sites in places where there is mRNA expression suggest that receptors synthesized in some brain regions are transported to projection areas where they are localized presynaptically (Le Merrer *et al.*, 2009).

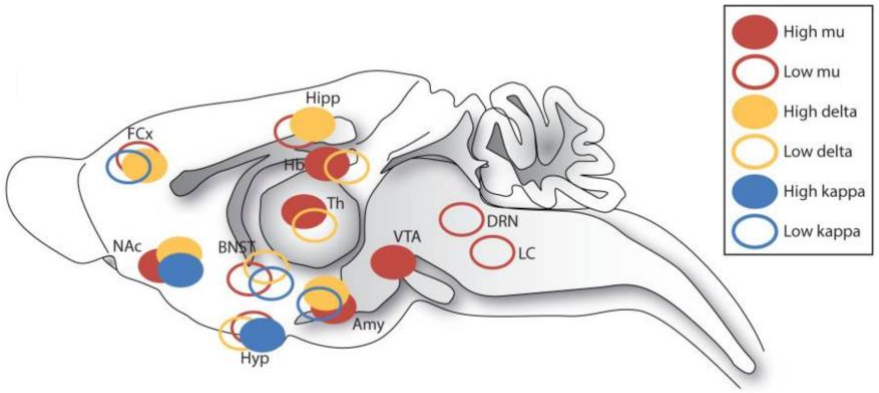


Figure 14. Distribution of the classical opioid receptors in the rodent brain. Opioid receptors overlap their localization throughout rodent brain. FCx, prefrontal cortex; NAc, nucleus accumbens; BNST, bed nucleus of the stria terminalis; Hyp, hypothalamus; Hipp, hippocampus; Hb, habenula; Th, thalamus; Amy, amygdala; VTA, ventral tegmental area; DRN, dorsal raphe nucleus; LC, locus coeruleus. Adapted from (Lutz and Kieffer, 2013).

Opioid receptors have also been found in sensory and sympathetic fibers innervating peripheral tissues such as the gastrointestinal tract, dermis and epidermis, bones or joint tissue (Bigliardi and Bigliardi-Qi, 2014). Besides their presence in neuronal cells, opioid receptors can be found in immune cells and in other peripheral tissues (Hedner and Cassuto, 1987).

When opioid receptors are activated, G proteins dissociate into Gα and Gβγ subunits, which subsequently act on **intracellular cascades** that typically depress neural functions (**Error! Reference source not found.**). The Gα subunit positively interacts with Kir3 (Wickman and Clapham, 1995; Torrecilla *et al.*, 2002), while the Gβγ subunit inhibits N-, P/Q- and L-type voltage-gated calcium channels (Zamponi and Snutch, 1998). The Gα subunit also inhibits AC activity and reduces cAMP formation (Law *et al.*, 2000). These processes lead to a strong neuronal hyperpolarization and inhibition of neuronal excitability. With opioid receptor activation, GPCRs are phosphorylated and can recruit β-arrestins, which stimulate components of the MAPK cascade (Al-Hasani and Bruchas, 2011) including ERK1/2, c-Jun JNK1–3 and p38 to modulate the activity of transcription factors, ion channels and neurotransmitter transporters among others (Raman *et al.*, 2007). β-arrestins also regulate desensitization and internalization of the opioid receptors (Corder *et al.*, 2018). Although it was initially accepted that internalized receptors were inactive (Bohn *et al.*, 1999), latter studies have shown that opioid receptors still signal from endosomal compartments (Irannejad *et al.*, 2013; Eichel *et al.*, 2016).

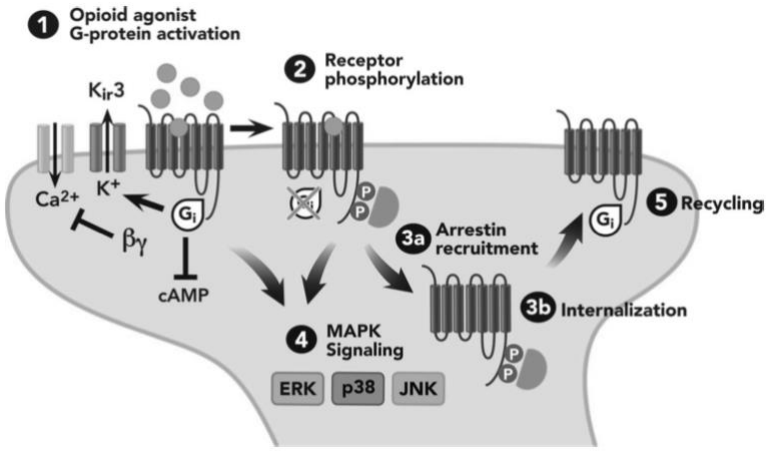


Figure 15. Opioid receptor signaling pathways. (1) Activated G protein subunits (1) interact with K^+ and Ca^{2+} channels and (2) are phosphorylated causing (3a) arrestin recruitment and subsequent (3b) receptor internalization. (4) Dissociation of G protein subunits stimulate components of the MAPK cascade. (5) Opioid receptors can be recycled to the cell Surface. Bγ, G protein B-γ subunit; $K_{ir}3$, inwardly rectigying potassium channels; cAMP, cyclic adenosine monophosphate; ERK, extracellular signal- regulated kinase; JNK c-jun N-terminal kinase; MAPK, mitogen-activated protein kinases; P, phosphorylation. Adapted from (Al-Hasani and Bruchas, 2011).

4.1.2 Endogenous opioids

There are four major families of endogenous opioid ligands, **β-endorphins, enkephalins, dynorphins, and nociceptin/orphaninFQ**, (**Error! Reference source not found.**), which exhibit different affinities for each opioid receptor but share a common N-terminal sequence (Tyr-Gly-Gly-Phe-Met-Leu) (Akil *et al.*, 1997). β-endorphin acts on both MOR and DOR with similar affinity; enkephalins bind to DOR and MOR, with higher affinity for DOR; dynorphins can activate KOR, MOR and DOR with a greater affinity for KOR; and noiceptin/orphanin FQ binds to NOR (Kieffer, 1995; Meunier *et al.*, 1995).

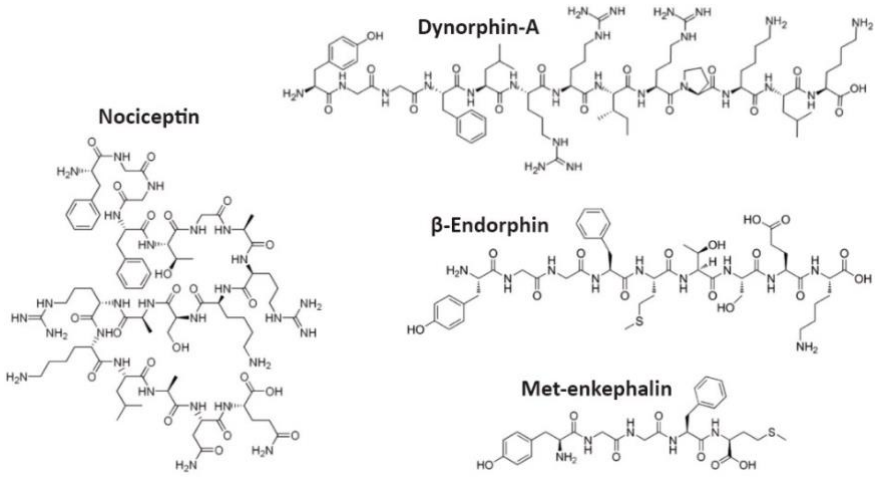


Figure 16. Chemical structure of endogenous opioid peptides. Adapted from (Corder *et al.*, 2018).

Endogenous opioids are released into synaptic and extra-synaptic spaces (Duggan, 2000; Banghart and Sabatini, 2012; Chavkin, 2013). Activation of opioid receptors can occur hundreds of microns away from the site where opioid peptides were released (Svingos *et al.*, 1996; Glass *et al.*, 2009), implying that opioid synapses include a much broader area than typical fast synapses.

4.1.3 Enzymes involved in the biosynthesis and degradation of endogenous peptides

β-endorphins, enkephalins, dynorphins, and nociceptin/orphanin FQ result from **proteolytic cleavage** of precursor proteins proopiomelanocortin (POMC), preproenkephalin (PENK), prodynorphin (PDYN) and pronociceptin (PNOC), respectively (Corder *et al.*, 2018). Opioid precursors are stored in dense core vesicles in the neuron soma and are cleaved during their transport to axon terminals (Corder et al, 2018). PENK are abundant and widely expressed in the

brain, while POMC cell bodies have been only detected in the hypothalamus, the nucleus tractus solitarius, and the pituitary gland. PDYN cell bodies are also widespread, but particularly abundant in the hypothalamus matching with high KOR density (Le Merrer *et al.*, 2009). Dynorphins are also synthetized from PDYN in primary afferents and immune cells, which also synthetize and secrete enkephalins (Labuz *et al.*, 2016; Maldonado *et al.*, 2018).

Once released into the synaptic cleft, enkephalins are **cleaved** by the two zinc metallopeptidases endopeptidase neprilysin and aminopeptidase N, which catalyze the cleavage of peptide bonds on the N-terminal side of Tyr-Gly-Gly and Tyr residues, respectively (Roques *et al.*, 2012). Mass spectrometry studies of the metabolism products of dynorphins and β-endorphins suggest that these endogenous opioids have their own metabolizing enzymes (Reed *et al.*, 2003, 2008).

4.1.4 Exogenous opioid receptor ligands

The term opioid is used broadly to describe all compounds that act on opioid receptors. **Natural** opioids include morphine and codeine, which display higher affinity for MOR. Nonetheless, other natural opioids with selectivity for specific receptors have been identified, like the KOR-selective agonist salvinorin A (Roth *et al.*, 2002). A broad spectrum of **semi-synthetic and synthetic** opioid receptor agonist and antagonist ligands has been developed. Examples of MOR agonists include oxycodone and oxymorphone, tramadol, DAMGO, and the partial agonist buprenorphine, which acts also as a KOR antagonist. Naloxone and naltrexone are opioid antagonists with preferential affinity for

MOR (Jordan *et al.*, 2000). DOR agonists include SNC-80, AZD2327 or BU-48, among others; DOR antagonists include naltrindole and ICI174,864 (Peppin and Raffa, 2015). U50,488H, U689593 and GR89,696, are examples of KOR agonists, and nor-binaltorphimine and JDTic are some of the available KOR antagonists (Beck *et al.*, 2019). NOP can be activated by agonists such as NNC 63-0532, and blocked by the antagonists JTC081 and J113397 (Gear *et al.*, 2014).

In addition to these ligands, several peripherally-restricted agonists have been synthetized to study the contribution of peripheral and central mechanisms in opioid signaling and to avoid the centrally-mediated adverse effects of opioids (Iwaszkiewicz *et al.*, 2013). Furthermore, biased agonists that elicit a preferential signaling pathway downstream the receptor, activating G-protein or β-arrestin cascades, have been developed recently (Faouzi *et al.*, 2020).

4.2 Physiological functions of the opioid system

The endogenous opioid system participates in a wide range of functions related to behavior, including pain, reward and addiction, stress, learning and memory, mood or sociability. This system is also involved in the regulation of gastrointestinal transit, and respiratory, cardiovascular and immunological functions, among others (Bodnar, 2017). The role of MOR and DOR in the control of pain has been widely investigated, although more recent investigations have centered the attention in opioid signaling through KOR. For the aim of this Thesis, we will focus on the involvement of KOR in pain, emotions and cognition.

4.2.1 Role of the kappa opioid receptor in pain

The endogenous opioid system plays a crucial role in modulating pain transmission at the periphery and the CNS (Corder *et al.*, 2018). At the peripheral level, immune cells release opioid peptides that inhibit pain transmission locally (Rittner *et al.*, 2008). In the DRG, each opioid receptor preferentially controls distinct types of pain and somatosensory modalities, with KOR mainly controlling visceral pain (Kivell and Prisinzano, 2010; Vanderah, 2010; Naser and Kuner, 2018). At the spinal level, opioid signaling modulates nociceptive responses by inhibiting activity in synapses between primary afferent nociceptive neurons and second-order neurons. Indeed, dynorphins and enkephalins are expressed by interneurons in the dorsal horn (Boyle *et al.*, 2017; François *et al.*, 2017) and are upregulated following peripheral injury (Xu *et al.*, 2004; Lai *et al.*, 2008; Podvin *et al.*, 2016). KOR is expressed in neurons, interneurons and astrocytes at this level of the pain transmission route (Eckert and Light, 2002; Xu *et al.*, 2007). Supraspinally, KOR mediates descending antinociception particularly in the dorsal raphe nucleus (Zhao *et al.*, 2007; Land *et al.*, 2009). The KOR-dynorphin system also gates affective information from the basolateral amygdala and the locus coeruleus (Crowley *et al.*, 2016; McCall *et al.*, 2017), and mediates pain-induced negative affect by acting on the nucleus accumbens circuitry (Liu *et al.*, 2019; Massaly *et al.*, 2019).

KOR agonists are able to attenuate **acute pain** in different animal models. Administration of KOR agonists reduce acute pain in response to thermal stimuli via serotonergic (Vonvoigtlander *et al.*, 1984) and GABAergic pathways (Nemmani and Mogil, 2003). KOR agonists also

inhibit pain responses to chemical stimulus in rodents (Craft *et al.*, 1995; McCurdy *et al.*, 2006; Labuz *et al.*, 2007). However, mechanical stimuli elicit similar responses in mice lacking either KOR or PDNY and wild type mice (Negrete *et al.*, 2017). The antinociceptive effects of KOR on **inflammatory pain** have also been investigated. Exogenous KOR ligands reduce pain behaviors in carrageenin (Amarante *et al.*, 2004), formalin (Clemente *et al.*, 2004), capsaicin (Lomas *et al.*, 2007) and complete Freund's adjuvant (Auh and Ro, 2012) inflammation models. Studies using knockout mice show that the absence of either KOR or PDYN is associated to exacerbated mechanical allodynia and increased spinal microglial activation in a model of osteoarthritis (Negrete *et al.*, 2017).

KOR is also involved in chronic **neuropathic pain**. Administration of peripherally-selective KOR agonists reduces pain behaviors in a rat model of nerve injury (Catheline *et al.*, 1998; Walker *et al.*, 1999), indicating that activation of peripheral KOR induces antinociception in neuropathic pain conditions. The KOR antagonists norbinaltorphimine and 5'-guanidinonaltrindole (GNTI) enhance mechanical and thermal in rodent models of neuropathic pain (Obara *et al.*, 2003), providing further evidence of the role of KOR in pain associated to nerve injuries. Nevertheless, rodents subjected to different models of neuropathic pain show enhanced expression and pronociceptive actions of dynorphin at spinal levels (Xu *et al.*, 2004; Lai *et al.*, 2008; Podvin *et al.*, 2016), suggesting that dynorphin can also promote pain in certain conditions.

Clinical and preclinical studies suggest a **sexual dimorphism** in the effects of KOR agonists on pain. Some studies report that KOR agonists exert higher antinociceptive effects in male rodents (Barrett *et al.*, 2002; Terner *et al.*, 2003; Sternberg *et al.*, 2004). However, others show greater antinociception in females (Bartok and Craft, 1997; Lawson *et al.*, 2010). In rodent models of inflammatory pain, KOR agonists are more efficient reducing pain behaviors in males (Lomas *et al.*, 2007; Rasakham and Liu-Chen, 2011; Auh and Ro, 2012), or females (Bereiter, 2001; Clemente *et al.*, 2004; Lawson *et al.*, 2010), depending on the model and the experimental conditions. Estrogen-dependence of KOR-mediated antinociceptive effects could, at least partially, explain these differences (Lawson *et al.*, 2010).

In **humans**, mixed KOR/MOR agonists including pentazocine, nalbuphine and butorphanol produce greater analgesia for postoperative pain following molar extraction in women than in man (Gear *et al.*, 1996a, 1996b, 1999). Furthemore, the analgesic effect of pentazocine against thermal and ischemic pain is also more robust in women (Mogil *et al.*, 2003), although no sex differences on its effect against heat, ischemic and pressure pain have also been reported (Fillingim *et al.*, 2004). Moreover, butorphanol provides better analgesia in men in the cold-water stimulus pain assay (Zacny and Beckman, 2004), but is equaly effective in both sexes for heat an preassure pain (Sibille *et al.*, 2011).

Substantial evidence shows that KOR stimulation produces strong analgesic effects. Furthermore, KOR agonists are free from the abuse potential and some of the adverse effects of MOR agonists. However,

KOR agonists produce as well severe undesirable effects such as diuresis, salivation, emesis and sedation (Y. Wang *et al.*, 2010), which limit their use in the clinic.

4.2.2 Role of the kappa opioid receptor in emotional responses

The abundance of components of the opioid system in the cortex, limbic areas and other brain regions related to mood control indicates that this system plays a crucial role in emotional processing. Indeed, increases in the levels of enkephalins and endorphins by exogenous administration or by inhibition of catabolic enzymes is associated to reduced anxiety and depressive-like behaviors (Kastin *et al.*, 1978; Tejedor-Real *et al.*, 1993; Nieto *et al.*, 2005; Jutkiewicz *et al.*, 2006; Peppin and Raffa, 2015).

The role of KOR in **anxiety and depression**-like behaviors has been investigated using pharmacological and genetic approaches. Systemic KOR agonists induce anxiogenic and pro-depressant effects in rodents (Knoll and Carlezon, 2010; Chartoff and Mavrikaki, 2015; Chavkin and Koob, 2016). It has been proposed that KOR stimulation may reduce extracellular dopamine levels in the nucleus accumbens (Carlezon *et al.*, 2006), an area playing a crucial role in depression (Nestler and Carlezon, 2006). It has also been hypothesized that the activation of KOR in the locus coeruleus diminishes neural discharge evoked by engaging either glutamate or corticotropin-releasing factor inputs. In turn, noradrenergic signaling in forebrain areas decreases, potentially contributing to the pro-depressive effect of KOR agonists (Kreibich *et al.*, 2008).

Opposite effects are observed when KOR is blocked with selective antagonists, producing antidepressant and anxiolytic-like behavioral effects (Shirayama *et al.*, 2004; Beardsley *et al.*, 2005; Knoll *et al.*, 2007; Zhang *et al.*, 2007). It has been proposed that KOR antagonists attenuate the behavioral effects of elevated cAMP response element-binding (CREB) expression within the nucleus accumbens by disinhibiting KOR-mediated neurotransmitter release from mesolimbic dopaminergic neurons, thus contributing to an antidepressant-like effect (Pliakas *et al.*, 2001). Furthermore, ablation of KOR from dopamine neurons (Van't Veer and Carlezon, 2013) or from glutamatergic neurons that project from the basolateral amygdala to the medial prefrontal cortex (Tejeda *et al.*, 2015) results in an anxiolytic phenotype. Others have shown that the lack of KOR or PDYN does not alter anxiety and depression-like behaviors in basal conditions. However, the absence of these genes is associated to exacerbated depressive-like behavior and slightly reduced anxiety-like behavior in animals subjected to a model of osteoarthritis pain (Negrete *et al.*, 2017).

In **humans**, KOR agonism is associated with dysphoria, anxiety, depression and psychotomimetic effects (Pfeiffer *et al.*, 1986; Chappell *et al.*, 1993; González *et al.*, 2006). On the other hand, the MOR agonist/KOR antagonist buprenorphine has shown antidepressant efficacy in major depressive disorder studies (Nyhuis *et al.*, 2008; Karp *et al.*, 2014).

4.2.3 Role of the kappa opioid receptor in cognition

KOR and its endogenous ligand dynorphin are present in the hippocampus and amygdala, areas closely related to learning and memory processes. It has been described that dynorphins modulate signal transmission between the dentate gyrus and the CA3 region of the hippocampus by decreasing excitatory glutamatergic signaling and therefore diminishing hippocampal activity (Bilkei-Gorzo *et al.*, 2014).

Most of the behavioral studies in rodents show that KOR agonists cause **cognitive impairments**. Several KOR agonists produce attention deficits and learning disruptions, which can also be blocked by pretreatment with selective KOR antagonists (Shannon *et al.*, 2007; Nemeth *et al.*, 2010; Abraham *et al.*, 2018b). In this line, KOR agonists also cause prepulse inhibition deficits that can be prevented by KOR antagonists (Bortolato *et al.*, 2005), although others report no changes on this behavior after KOR stimulation (Tejeda *et al.*, 2010). Pharmacological KOR activation also produces aversive emotional behaviors that contribute to stress-induced learning and social memory dysfunctions in mice (A. N. Carey *et al.*, 2009; Bertran-Gonzalez *et al.*, 2013).

Similar effects of KOR agonists have been found in **humans**. A clinical trial in healthy adults found that salvavorin A induces impairments in recall and recognition memory in a dose-related manner (MacLean *et al.*, 2013). Interestingly, a genetic study revealed that a rare gene polymorphism associated with reduced PDYN expression is associated with better episodic memory in humans (Kölsch et al, 2009).

4.3 The endogenous opioid system and endometriosis

The endogenous opioid system plays a role in the regulation of reproductive physiology at multiple sites (Rosen *et al.*, 1990). Opioid peptides inhibit the secretion of GnRH at hypothalamic level and also the tonic release of luteinizing hormone (Mehmanesh *et al.*, 1988; Yilmaz and Gilmore, 1999; Kumru *et al.*, 2001). Furthermore, there is a growing body of evidence indicating that the opioid system participates in the regulation of reproductive function through a direct local action in healthy reproductive tissues (Jin *et al.*, 1988) which, by extension, may also occur in endometriotic growths.

Opioid receptors and peptides are present in human (Wahlström *et al.*, 1985; Petraglia *et al.*, 1986; Makrigiannakis *et al.*, 1992; Chatzaki *et al.*, 2000) and rodent (Zhu *et al.*, 1998) endometrial cells. This denotes a role of this system in the functioning of the endometrium. It has been described that dynorphin participates in **apoptotic processes** of endometrial tissue remodeling, such as menstruation, via the Fas/FasL pathway (Chatzaki *et al.*, 2001). Like the cells of the eutopic endometrium, ectopic endometrial cells proliferate and suffer apoptosis cyclically, and the opioid system could be involved in their survival and growth.

Opioid signaling may be involved in the establishment of ectopic endometrial lesions, since it has been described that MMPs drive cell **migration** in endometriosis (Sanchez et al., 2012). In this line, morphine can decrease MMP secretion from fibrosarcoma cells (Shariftabrizi *et al.*, 2006), although agonists targeting the different opioid receptors

have also been described to induce MMP secretion in bladder cancer cells, increasing **migration**, adhesion, spreading and invasion through a rapid modification of the MAPK signaling cascade and the cytoskeleton distribution (Vassou *et al.*, 2011). Thus, exogenous opioid receptor agonists could both inhibit and enhance migration of endometrial cells in endometriosis.

Angiogenesis, another crucial event in the development and maintenance of endometriotic lesions, can be modulated by opioids. Pro-angiogenic effects of morphine have been described in breast cancer xenograft models (Bimonte *et al.*, 2015) and allograft tumor models with mammary carcinoma and adenocarcinoma cells (Farooqui *et al.*, 2007; Ustun *et al.*, 2011). In an spontaneous breast cancer model, morphine-promoted angiogenesis correlated with enhanced degranulation of mast cells and elevated levels of tryptase (Nguyen *et al.*, 2014). The number of mast cells increases in the vicinity of endometriotic lesions, where tryptase may contribute to fibrosis and inflammation (Kempuraj *et al.*, 2004). On the other hand, there are studies reporting anti-angiogenic effects of morphine in lung carcinoma (Koodie *et al.*, 2014) and melanoma (Yamamizu *et al.*, 2013) mouse models. It has been proposed that morphine could inhibit VEGF synthesis via the MAPK cascade in cancerous cells, inhibiting angiogenesis (Koodie *et al.*, 2010). Another possibility is an angiogenic effect of morphine associated to a reduction of tumor-infiltrating neutrophils and monocyte and macrophage cells (Koodie *et al.*, 2014), which can act as VEGF donors (Chanmee *et al.*, 2014). Interestingly, it has been reported that administration of KOR agonists also reduce

vascularization of B16 melanomas in mice by inducing VEGF receptor down-regulation (Yamamizu *et al.*, 2013). Since KOR agonism had no effect on angiogenesis in mice lacking KOR, it is likely that this process requires KOR activity. Thus, VEGF receptors could be co-internalized and degraded with stimulated KORs (Yamamizu *et al.*, 2013), or, their expression may be down-regulated through protein kinase C (PKC) activity, which initiates VEGF receptor internalization and degradation (Singh *et al.*, 2005).

The opioid system is involved in **inflammatory** processes. Systemic MOR and KOR agonists, as well as peripherally restricted KOR agonists, reduce joint swelling and damage in models of chronic inflammation associated to adjuvant arthritis, while KOR antagonism exacerbates inflammation (Binder and Walker, 1998). MOR, DOR and KOR agonists have also proven effective reducing visceral inflammation in several models, including peritonitis and gut inflammation models (reviewed by Pol and Puig, 2004). Moreover, pharmacological or genetic depletion of MOR increases inflammation in the mouse colon (Philippe *et al.*, 2003). All these data suggest the presence of tonic endogenous opioid anti-inflammatory actions that could also be occurring in endometriosis. Furthermore, since KOR agonists are effective relieving inflammatory and visceral pain in females, targeting KOR could be a potential strategy for the treatment of endometriosis.

OBJECTIVES

Objective 1

To determine the effects of the phytocannabinoids Δ9-tetrahydrocannabinol and cannabidiol on the development of endometriosis and its symptoms.

Article #1

Disease-modifying effects of natural Δ9-tetrahydrocannabinol in endometriosis-associated pain

Alejandra Escudero-Lara, Josep Argerich, David Cabañero*, Rafael Maldonado*

***eLife* 2020;9:e50356 DOI: 10.7554/eLife.50356**

Supplementary results I

The effects of Δ9-tetrahydrocannabinol on cortical neuroinflammatory markers are different in mice with ectopic endometrium

Alejandra Escudero-Lara, David Cabañero*, Rafael Maldonado*

Supplementary results II

Δ9-tetrahydrocannabinol and cannabidiol, alone or in combination, inhibit endometriosis manifestations and development

Alejandra Escudero-Lara, David Cabañero*, Rafael Maldonado*

Article #2

Surgical induction of endometriosis to female mice

Alejandra Escudero-Lara, David Cabañero*, Rafael Maldonado*

***Bio-protocol* 2020;10(18): e3763. DOI: 10.21769/BioProtoc.3763**

Objective 2

To study the effects of kappa opioid receptor stimulation in the behavioral and histopathological alterations associated to endometriosis.

Article #3

Kappa opioid modulation of endometriosis pain in mice

Alejandra Escudero-Lara, David Cabañero*, Rafael Maldonado*

Submitted to *Neuropharmacology* (2021)

Objective 3

To explore the behavioral al neuroinflammatory changes associated to minimal endometriosis.

Article #4

Behavioral and neuroinflammatory changes induced by minimal endometriosis in mice

Alejandra Escudero-Lara, David Cabañero*, Rafael Maldonado*

(In preparation)

RESULTS

Article #1

Disease-modifying effects of natural Δ9-tetrahydrocannabinol in endometriosis-associated pain

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SHORT REPORT

Disease-modifying effects of natural Δ9-tetrahydrocannabinol in endometriosis-associated pain

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Abstract Endometriosis is a chronic painful disease highly prevalent in women that is defined by growth of endometrial tissue outside the uterine cavity and lacks adequate treatment. Medical use of cannabis derivatives is a current hot topic and it is unknown whether phytocannabinoids may modify endometriosis symptoms and development. Here we evaluate the effects of repeated exposure to Δ9-tetrahydrocannabinol (THC) in a mouse model of surgically-induced endometriosis. In this model, female mice develop mechanical hypersensitivity in the caudal abdomen, mild anxiety-like behavior and substantial memory deficits associated with the presence of extrauterine endometrial cysts. Interestingly, daily treatments with THC (2 mg/kg) alleviate mechanical hypersensitivity and pain unpleasantness, modify uterine innervation and restore cognitive function without altering the anxiogenic phenotype. Strikingly, THC also inhibits the development of endometrial cysts. These data highlight the interest of scheduled clinical trials designed to investigate possible benefits of THC for women with endometriosis.

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Introduction

Endometriosis is a chronic inflammatory disease that affects 1 in 10 women of childbearing age (***Zondervan et al., 2019***). It is characterized by the growth of endometrium in extrauterine locations, chronic pain in the pelvis and the lower abdomen, infertility, emotional distress and loss of working ability (***Fourquet et al., 2011***; ***Márki et al., 2017***; ***Zondervan et al., 2019***). Current clinical management provides unsatisfactory outcomes. On the one hand, hormonal therapy has unwanted effects including contraception and emotional disturbances (***Ross and Kaiser, 2017***; ***Skovlund et al., 2016***), whereas surgical excision of the growths is associated with high-recurrence rates and post-surgical pain (***Garry, 2004***). Hence, clinical treatments are limited and women often unsatisfactorily self-manage their pain (***Armour et al., 2019***). In this context, marijuana legalization for medical purposes in American and European states has led to increased availability of phytocannabinoids (***Abuhasira et al., 2018***). While cannabis may provide pain relief in certain conditions (***Campbell et al., 2001***), it is unclear whether it may modify endometriosis symptoms or development.

Δ9-tetrahydrocannabinol (THC) is the main psychoactive constituent of the *Cannabis sativa* plant, and multiple animal and clinical studies suggest its efficacy relieving chronic pain (***De Vry et al., 2004***; ***Harris et al., 2016***; ***King et al., 2017***; ***Ueberall et al., 2019***; ***Williams et al., 2008***), although controversial results have been obtained in human clinical trials (***Stockings et al., 2018***). However, THC has important side effects including cognitive deficits and anxiety (***Célérier et al., 2006***; ***Kasten et al., 2017***; ***Puighermanal et al., 2013***). This work investigates the effects of natural THC in a mouse model of endometriosis that reproduces the ectopic endometrial growths and some of the

eLife digest Endometriosis is a common disease in women caused by tissue that lines the uterus growing outside the uterine cavity on to other organs in the pelvis. This can cause a variety of symptoms including chronic pelvic pain, infertility, and pain during menstruation or sexual intercourse. These symptoms may contribute to anxiety, depression, loss of working ability and a reduced quality of life.

Currently available treatments for endometriosis, including hormonal therapy and surgery, have a limited effect and can produce unwanted side effects. For example, women who undergo surgery to remove the growths may experience post-surgical pain or a recurrence. As a result, women with endometriosis often rely on self-management strategies like dietary changes or exercise. Although cannabis consumption has a large number of potential side effects and can lead to substance abuse, it has been shown to provide pain relief in some conditions. But it is unknown whether it could be useful for treating endometriosis.

Now, Escudero-Lara et al. have created a mouse model that mimics some of the conditions of human endometriosis: pelvic pain, anxiety and memory impairments. The mice were treated with moderate doses of Δ9-tetrahydrocannabinol (THC), which is the main pain-relieving component of cannabis. The THC reduced pelvic pain and cognitive impairments in the mice with the endometriosis-like condition, but it had no effect on their anxious behavior. Escudero-Lara et al. also noticed that endometrial growths were also smaller in the treated mice indicating that THC may also inhibit endometriosis development.

These experiments suggest that THC may be a useful treatment for patients with endometriosis. Clinical trials are already ongoing to test whether these findings translate to patients with the condition. Although THC and cannabis are readily available in some areas, Escudero-Lara et al. discourage using unregulated cannabis products due to the potential risks.

behavioral alterations of clinical endometriosis. Our data show that THC is effective inhibiting hypersensitivity in the caudal abdominal area without inducing tolerance, as well as reducing the pain unpleasantness associated with endometriosis. Notably, THC also prevents the cognitive impairment observed in mice with ectopic endometrium without modifying anxiety-like behavior at this particular dose. Interestingly, THC shows efficacy limiting the development of ectopic endometrium, revealing disease-modifying effects of this natural cannabinoid.

Results and discussion

Ectopic endometrium leads to pain sensitivity in the caudal abdomen, anxiety-like behavior and memory impairment

Our first aim was to characterize a novel experimental procedure to evaluate at the same time nociceptive, cognitive and emotional manifestations of endometriosis pain in female mice. Mice were subjected to a surgical implantation of endometrial tissue in the peritoneal wall of the abdominal compartment or to a sham procedure. Mice receiving ectopic endometrial implants developed persistent mechanical hypersensitivity in the caudal abdominal area, whereas sham mice recovered their baseline sensitivity and showed significant differences in comparison to endometriosis mice since the second week of implantation (***Figure 1a*** and ***Figure 1—figure supplement 1***). To test whether mechanical hypersensitivity of endometriosis mice was specific to this abdominal region, nociceptive responses were also measured in the hind paw. In this distant area, mechanical sensitivity remained unaltered, indicating that pain sensitization did not generalize to other sites (***Figure 1b*** and ***Figure 1—figure supplement 2***). To discern whether increased nociception was accompanied by a component of negative affect, a measure of pain unpleasantness was taken on day 14 after the surgeries (***Figure 1c***). Endometriosis mice showed increased nocifensive behaviors to mechanical stimuli when compared with sham mice. Similarly, endometriosis mice exhibited enhanced anxiety-like behavior reflected in lower percentages of time and entries to the open arms of the elevated plus maze (***Figure 1d***). Total arm entries were similar in both groups (***Figure 1d***). In line with these findings, previous rodent models of endometriosis found increased mechanosensitivity in the lower

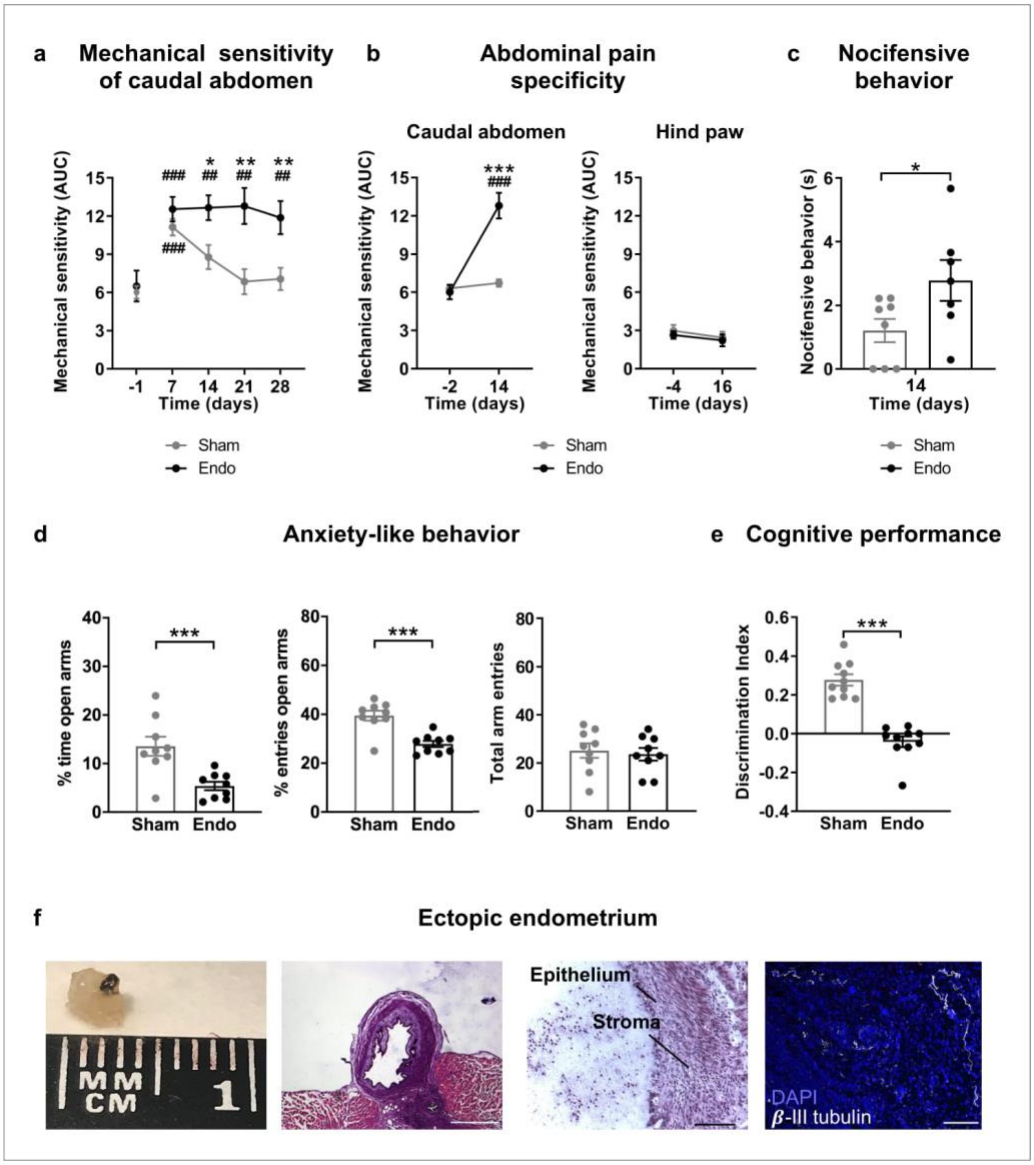

Figure 1. Behavioral and histological alterations in female mice with ectopic endometrial implants. Endometriosis mice showed (**a**) persistent mechanical abdominal hypersensitivity that (**b**) was localized in the caudal abdominal area but not detectable in distant areas (hind paw). Mechanical sensitivity is represented by the area under the curve of frequency of response to von Frey filaments. Higher values mean higher mechanical pain. Mice receiving endometrial implants also showed (**c**) increased nocifensive behavior, (**d**) anxiety-like behavior in the elevated plus maze test and (**e**) cognitive

Figure 1 continued on next page

Figure 1 continued

impairment in the novel object recognition task. (**f**) From left to right: cysts were recovered from endometriosis mice, were filled with fluid (scale bar = 1 mm), contained endometrial epithelium and stroma (scale bar = 100 µm) and were innervated by beta-III tubulin-labeled fibers (scale bar = 100 µm, blue is DAPI and white is β-III tubulin). Error bars are mean ± SEM. One-way repeated measures ANOVA + Bonferroni (**a and b**) and Student t-test (**c, d and e**). *p<0.05, **p<0.01, ***p<0.001 vs sham. ##p<0.01, ###p<0.001 vs baseline. Endo, endometriosis, AUC, area under the curve.
The online version of this article includes the following source data and figure supplement(s) for figure 1:

Source data 1. Effects of ectopic endometrium.
Figure supplement 1. Nociceptive responses to abdominal mechanical stimulation with von Frey filaments.
Figure supplement 2. Nociceptive responses to abdominal and paw mechanical stimulation with von Frey filaments.
Figure supplement 3. Density of beta-III tubulin-labeled fibers in uteri of endometriosis and sham mice.

abdomen (***Arosh et al., 2015***; ***Greaves et al., 2017***) and affective-like disturbances (***Filho et al., 2019***; ***Li et al., 2018***). Previous works associate nociceptive and emotional distress in chronic pain settings with cognitive decline (***Bushnell et al., 2015***; ***La Porta et al., 2015***; ***You et al., 2018***), although this cognitive impairment has not yet been revealed in rodent models of endometriosis. We found in our model a dramatic impairment of long-term memory in endometriosis mice (***Figure 1e***). While mnemonic effects of this pathology have not been thoroughly evaluated, a cognitive impairment may contribute to the loss of working ability consistently reported in women with endometriosis (***Hansen et al., 2013***; ***Sperschneider et al., 2019***). Hence, mice with ectopic endometrium recapitulate in our model some of the symptomatology observed in the clinics, although manifestations of spontaneous pain could not be evaluated in this work.

Mice receiving endometrial implants developed 3 to 5 endometrial cysts in the peritoneal wall of the abdominal compartment. Cysts were of 2.59 ± 0.34 mm diameter, filled with fluid, with glandular epithelium and stroma and innervated by beta-III tubulin positive fibers (***Figure 1f***), as shown in women (***Tokushige et al., 2006***; ***Wang et al., 2009***) and other rodent models (***Arosh et al., 2015***; ***Berkley et al., 2004***). Interestingly, we also found increased expression of the neuronal marker beta-III tubulin in the uteri of endometriosis mice (***Figure 1—figure supplement 3***), mimicking not only some of the symptoms but also the histological phenotype observed in women with endometriosis (***Miller and Fraser, 2015***; ***Tokushige et al., 2006***).

Δ9-tetrahydrocannabinol alleviates pain in the caudal abdomen, restores cognitive function and limits the growth of ectopic endometrium

Our second objective was to assess the effects of THC exposure on the endometriosis model to select an appropriate dose for a chronic treatment. Acute doses of THC were first tested in endometriosis and sham mice at a time point in which endometriotic lesions and hypersensitivity in the caudal abdomen were fully developed. Acute THC administration produced a dose-dependent reduction of abdominal mechanical hypersensitivity (***Figure 2***). The acute ED50 of THC 1.916 mg/kg (≈2 mg/kg) was chosen for the repeated administration.

Repeated exposure to THC 2 mg/kg, once daily for 28 days, provided a sustained alleviation of mechanical hypersensitivity during the whole treatment period (***Figure 3a*** and ***Figure 3—figure supplement 1***). Repeated THC starting on day 1 could have exerted a preventive effect at endometriosis stages in which pain sensitivity may have not been fully developed. To discern whether the absence in loss of efficacy was due to an inhibition of endometriosis development or to an actual lack of tolerance, we assessed the persistence of THC efficacy once pain was already present. THC given for the first time on day 14 was as effective as THC given on the same day after a daily treatment starting on day 8 (7 days long, ***Figure 3b*** and ***Figure 3—figure supplement 2***). Therefore, THC did not lose its efficacy when repeated administration started once painful symptomatology was established. The absence of tolerance to THC-induced antinociception is in contrast with the tolerance described at higher THC doses in other pain models (***Greene et al., 2018***; ***LaFleur et al., 2018***; ***Wakley et al., 2014***). As expected, no effects of endometriosis or THC treatments were found in mechanical sensitivity of distant areas (Hind paw, ***Figure 3—figure supplement 3***). Endometriosis mice treated with vehicle showed an increase in nocifensive behaviors compared with sham mice (***Figure 3c***). Interestingly, the 7 day treatment with THC inhibited this component of negative affect, while the effects of an acute administration of THC were highly variable. This variable

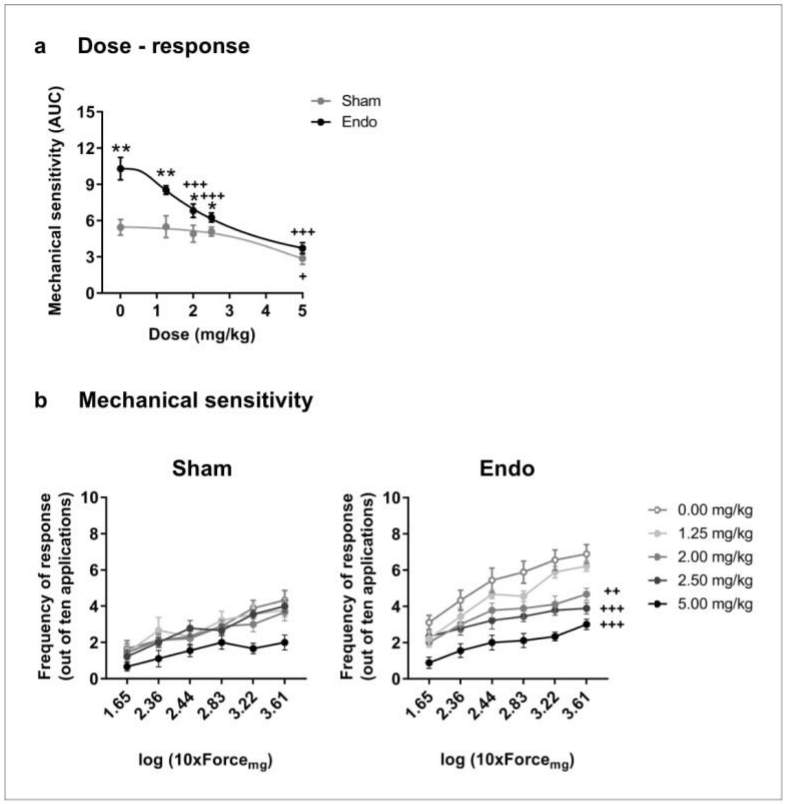

Figure 2. Effect of acute THC administration on the nociceptive responses to mechanical stimulation. (**a**) Acute THC produced a dose-dependent reduction of mechanical hypersensitivity in the caudal abdominal area. Mechanical sensitivity is represented by the area under the curve of frequency of response to von Frey filaments. Higher values mean higher mechanical pain. (**b**) Administration of 2, 2.5 and 5 mg/kg of THC decreased the frequency of response to von Frey filaments in endometriosis mice. Error bars are mean ± SEM. One-way repeated measures ANOVA + Bonferroni. *p<0.05, **p<0.01 vs sham; +p<0.05, ++p<0.01, +++p<0.001 vs vehicle. Endo, endometriosis; THC, Δ9-tetrahydrocannabinol, AUC, area under the curve.
The online version of this article includes the following source data for figure 2:

Source data 1. Acute THC effects.

response could be associated to aversive effects associated with a first exposure to THC, an event described in humans (***MacCallum and Russo, 2018***) and mice (***Kubilius et al., 2018***).

Additional experiments were conducted to assess the effects of THC on the anxiety-like behavior induced by endometriosis pain (***Figure 3d***). As in previous experiments, endometriosis mice showed a lower percentage of time in the open arms of the elevated plus maze (***Figure 3d***), revealing increased anxiety-like behavior. However, the percentage of entries to open arms was similar in endometriosis and sham mice. Therefore, the anxiogenic-like effect of ectopic endometrium in these experimental conditions was mild and the present model was not optimal to reveal the emotional component of this painful situation. No significant effects of repeated THC 2 mg/kg were observed on the percentages of time and entries, although THC-treated mice showed a subtle increase in anxiety-like behavior (***Figure 3d***, percentage of time in open arms). Previous studies described

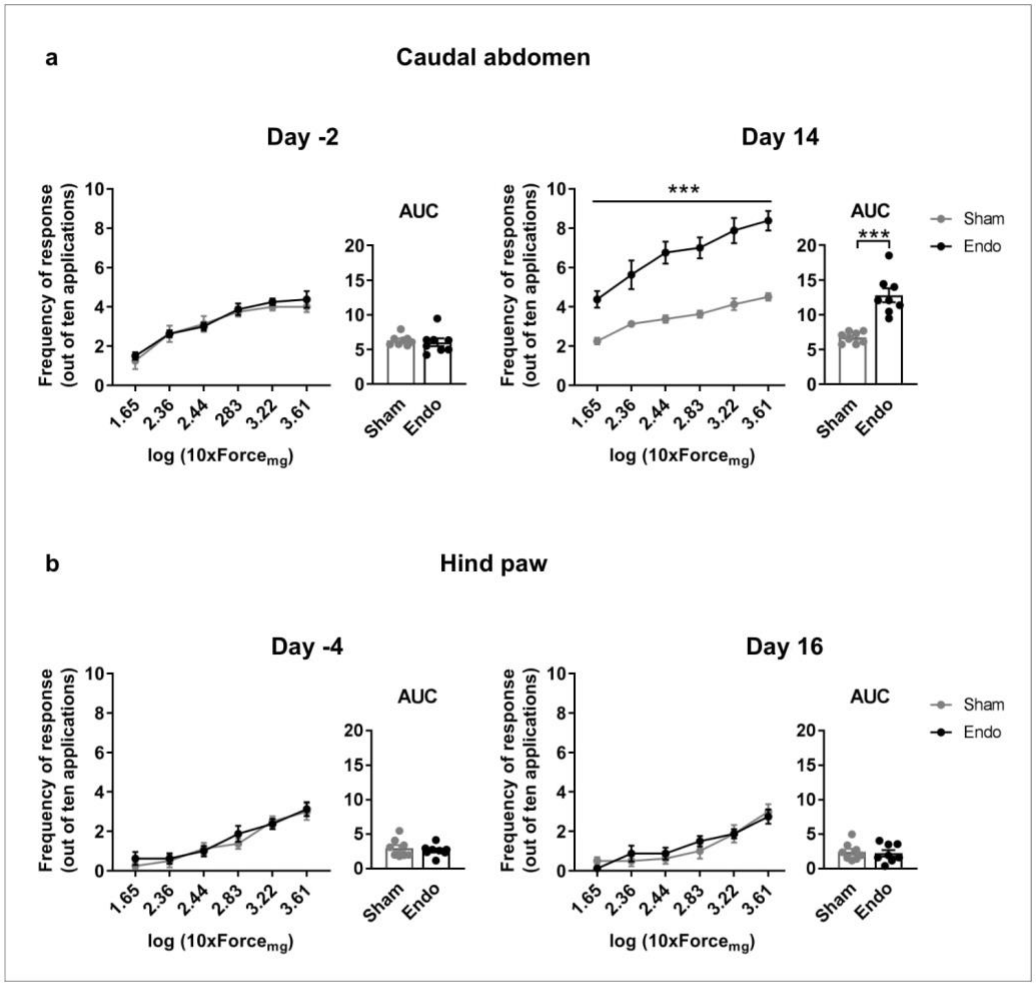

Figure 1—figure supplement 2. Nociceptive responses to abdominal and paw mechanical stimulation with von Frey filaments. (**a**) Frequency of responses to von Frey filaments applied against the caudal abdominal area and corresponding AUCs on day 14 after surgery were significantly higher in endometriosis mice when compared to sham mice. (**b**) Frequency of responses to von Frey filaments applied against the hind paw and corresponding AUCs were similar before and after surgery in endometriosis and sham mice. For each day, left panel is frequency of response to each von Frey filament and right panel is the corresponding mechanical sensitivity represented by the area under the curve of frequency of response to von Frey filaments. Higher values mean higher mechanical pain. Error bars are mean ± SEM. One-way repeated measures ANOVA (left panels) and Student t-test (right panels). ***p<0.001 vs sham. Endo, endometriosis; AUC, area under the curve.

anxiogenic-like effects of slightly higher doses (3 mg/kg) in naïve male mice (**Viñals et al., 2015**), and anxiolytic-like effects when using lower doses (0.3 mg/kg, **Puighermanal et al., 2013**; **Viñals et al., 2015**). Thus, possible effects of THC alleviating pain-related anxiety-like behavior in endometriosis mice could be hindered by intrinsic anxiogenic effects of this THC dose. Therefore, doses with less pain-relieving efficacy could potentially be effective promoting anxiolytic-like effects considering the intrinsic effects of THC on emotional-like behavior. Alternatively, the absence of clear effects of THC on anxiety-like behavior may be associated to the evaluation time point, which was 6 hr after administration to study the impact of pain relief on anxiety-like behavior, rather than to assess direct drug effects. Total arm entries were similar among groups (**Figure 3d**). Memory performance was also assessed the third week after starting the THC treatment. As expected, mice exposed to the chronic nociceptive manifestations of endometriosis showed a pronounced cognitive impairment, as well as sham mice exposed to THC, in accordance with previous reports in naïve males (**Kasten et al., 2017**; **Puighermanal et al., 2013**). Surprisingly, endometriosis mice repeatedly treated with natural THC showed intact discrimination indices (**Figure 3e**) suggesting protective effects of THC in this chronic inflammatory condition. In agreement, recent studies have shown cognitive improvements after THC exposure in old male and female mice (**Bilkei-Gorzo et al., 2017**; **Sarne et al., 2018**).

Exogenous and endogenous cannabinoids have shown modulatory effects on the female reproductive system (**Walker et al., 2019**). Thus, we analyzed the effects of THC on the ectopic and eutopic endometrium and on ovarian follicle maturation. Interestingly, endometriosis mice receiving THC 2 mg/kg for 32 days showed an evident inhibition of the development of endometrial cysts (cyst diameter and area of endometrial tissue, **Figure 4a**) without significant effects on cyst innervation (**Figure 4—figure supplement 1a**). In agreement, a previous study showed antiproliferative effects of WIN 55212–2, a synthetic cannabinoid agonist, on endometrial cell cultures and in ectopic endometrium implanted in immunodepressed mice (**Leconte et al., 2010**). The assessment of the uterine diameter and the area of eutopic endometrium (**Figure 4—figure supplement 1b**) showed no effects of the THC treatment, suggesting that the antiproliferative activity of THC on endometrial cells is restricted to ectopic sites. However, possible effects of THC on established endometriosis lesions were not evaluated. Repeated THC increased the expression of neuronal markers in the uteri of sham mice, similar to the increase provoked by the ectopic endometrium (**Figure 4b**). Interestingly, THC prevented this increase in endometriosis mice (**Figure 4b**) indicating again that THC exposure may have different consequences under chronic inflammatory conditions. In agreement, recent studies showed differential effects of THC on the nervous system of rodents with and without chronic inflammation (**Bilkei-Gorzo et al., 2017**; **Sarne et al., 2018**). To investigate a possible estrogenic influence on these histological findings, we analyzed 17 β-estradiol plasma levels. As expected, 17 β-estradiol plasma levels depended on the phase of the estrous cycle: mice in proestrus had the highest concentration followed by mice in diestrus, and mice in estrus showed the lowest levels (**Figure 4c**, left graph). We found that 17 β-estradiol was similar in all experimental groups (**Figure 4c**, right graph), although the levels of this estrogen were positively correlated with cyst diameter (**Figure 4d**, left), proving the estrogenic influence on ectopic endometrial lesions. 17 β-estradiol levels were not correlated with endometrial area of the cysts (**Figure 4d**, middle), or uterine innervation (**Figure 4d**, right), suggesting independent THC effects on these histological changes.

We also assessed possible effects of THC on ovarian functioning, since previous works have suggested inhibitory effects of THC on folliculogenesis and ovulation (**Adashi et al., 1983**; **El-Talatini et al., 2009**). Numbers of preantral follicles, antral follicles and corpora lutea were similar in all groups in our experimental conditions (**Figure 4—figure supplement 1c**). These data suggest that endometriosis and THC were void of overt effects on ovarian follicle maturation and luteinization, however, other effects of endometriosis or THC on fertility cannot be excluded in our model. Similarly, the presence of prominent symptoms of endometriosis such as dysmenorrhea or dyspareunia could not be evaluated.

Conclusions

Here we show for the first time that chronic administration of a moderate dose of the phytocannabinoid THC relieves mechanical hypersensitivity of caudal abdominal area, pain unpleasantness and cognitive impairment associated with the presence of ectopic endometrial cysts. These behavioral manifestations correlate with a decrease in the size of ectopic endometrium in THC-exposed mice.

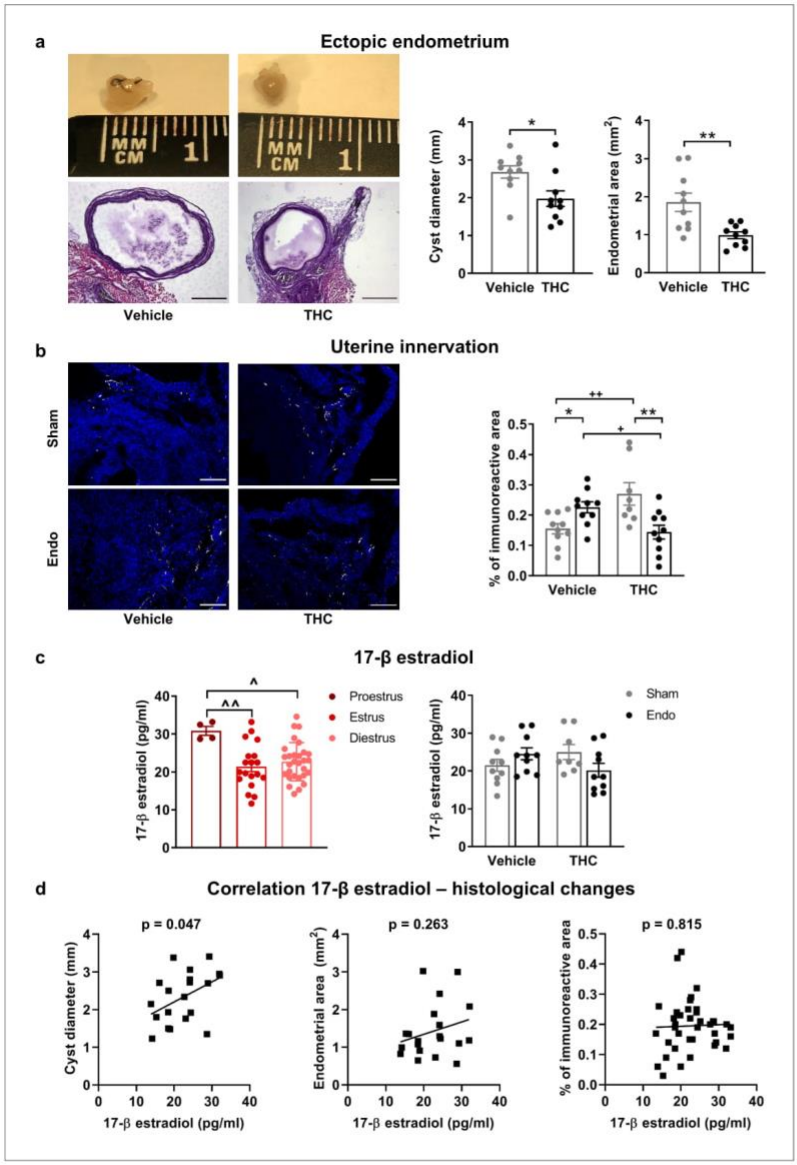

Figure 4. Effects of THC on the histological changes observed in mice with ectopic endometrium. (**a**) Ectopic endometrial growths of mice treated with THC were smaller (left graph) and had less endometrial tissue (right graph) than those of mice receiving vehicle. Scale bar = 1 mm. (**b**) THC increased innervation in sham mice but prevented uterine hyperinnervation in endometriosis mice. Blue is DAPI and white is β-III tubulin. Scale bar = 100 µm. (**c**) As expected, 17-β estradiol levels were higher in mice in proestrus (left). Estrogen levels were similar in all experimental conditions (right). (**d**) There was

Figure 4 continued on next page

Figure 4 continued

a positive correlation between cyst diameter and plasma levels of 17-β estradiol (left, r = 0.450). Absence of correlation of estrogen levels with cyst endometrial area (middle, r = 0.263) and uterine innervation (right, r = 0.039). THC dose: 2 mg/kg/day. Error bars are mean ± SEM. Student t-test (a, left graph), Mann Whitney U (a, right graph), two-way ANOVA + Bonferroni (**b**), mixed model + Bonferroni (c, left); Two-way ANOVA (c, right) and Pearson correlation (**d**). *p<0.05, **p<0.01 vs sham. +p<0.05, ++p<0.01 vs vehicle. ¯p<0.05, ¯p<0.01 vs proestrus. Endo, endometriosis; THC, Δ9-tetrahydrocannabinol.

The online version of this article includes the following source data and figure supplement(s) for figure 4:

Source data 1. Effects of repeated THC on histopathological features.
Figure supplement 1. Histological features of reproductive tissues after chronic THC treatment.

However, the pain-relieving effects of this particular dose of THC were not accompanied by a modification of anxiety-like behavior associated with endometriosis and effects on spontaneous pain were not evaluated in this work. Interestingly, THC produced opposite cognitive effects in sham and endometriosis mice. THC also induced an increase in markers of uterine innervation in sham animals, but prevented such changes in endometriosis mice, suggesting again different effects of THC under chronic inflammatory conditions. Importantly, THC also inhibited the growth of ectopic endometrium without apparent consequences on the eutopic endometrium and ovarian tissues. Altogether, the present data obtained in a preclinical model of endometriosis underline the interest in conducting clinical research to assess the effects of moderate doses of THC on endometriosis patients. Based on our results, we (clinicaltrials.gov, #NCT03875261) and others (gynica.com) have planned the initiation of clinical trials to provide evidence on the translatability of these results to women with endometriosis. These novel clinical trials will evaluate this new possible endometriosis treatment under pathological human conditions. However, cannabis has a large number of potential side effects, as well as a high potential for abuse liability (***Curran et al., 2016***), that have to be considered by physicians and patients. Therefore, the use of cannabis in unregulated scenarios should be discouraged taking into account these serious side effects.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Strain, strain background (*Mus musculus*, female)	C57Bl/6J	Charles Rivers, Lyon, France	C57Bl/6J	Female
Chemical compound, drug	THC (Tetrahydrocannabinol)	THC-Pharm-GmbH		Natural THC
Chemical compound, drug	Cremophor EL	Sigma-Aldrich	C5135; Kolliphor EL	
Chemical compound, drug	0.9%, NaCl physiological saline	Laboratorios Ern	Vitulia	
Chemical compound, drug	Ethanol	Scharlab	ET00051000	
Chemical compound, drug	Vaporised Isoflurane in oxygen	Virbac	Vetflurane	4% V/V for induction; 2.5% V/V for maintenance
Chemical compound, drug	Optimal cutting temperature compound	Sakura finetek	25608–930	Item code 4583
Biological sample (*Equus asinus*)	Normal donkey serum	Sigma-Aldrich	D9663-10ML	3% in PBS with 0.3% Triton X-100
Biological sample (*Capra aegagrus hircus*)	Normal goat serum	Vector lab	S-1000	
Antibody	Rabbit polyclonal anti-beta-III tubulin antibody	Abcam	ab18207	(1:2000)

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Continued

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Antibody	Donkey polyuclonal anti-rabbit Alexa Fluor A488 antibody	Thermo Fisher Scientific	A21206	(1:1000)
Antibody	Goat polyclonal anti-rabbit Alexa Fluor A555 antibody	Abcam	ab150078	(1:1000)
Chemical compound, drug	DAPI Fluoromount-G mounting media	SouthernBiotech	0100–20	
Chemical compound, drug	Calcium EDTA (Sodium calcium edetate)	Sigma-Aldrich	Sodium calcium edetate	
Commercial assay or kit	Enzyme-linked immunosorbent assay; ELISA	Calbiotech	ES180S-100	
Software, algorithm	NIH Image J software	Wayne Rasband		
Software, algorithm	GraphPad Prism 8	GraphPad Software, Inc		
Software, algorithm	IBM SPSS 23 software	IBM Corporation		
Software, algorithm	Smart 3.0 videotracking software	Panlab		

Animals

Female C57Bl/6J mice (Charles Rivers, Lyon, France) were used in all the experiments. Mice were 8 weeks old at the beginning of the experiments and were housed in cages of 4 to 5 mice with ad libitum access to water and food. The housing conditions were maintained at 21 ± 1˚C and 55 ± 10% relative humidity in controlled light/dark cycle (light on between 8 AM and 8 PM). Animals were habituated to housing conditions and handled for 1 week before the start of the experiments. All animal procedures were conducted in accordance with standard ethical guidelines (European Communities Directive 2010/63/EU and NIH Guide for Care and Use of Laboratory Animals, 8th Edition) and approved by autonomic (Generalitat de Catalunya, Departament de Territori i Sostenibilitat) and local (Comitè Ètic d'Experimentació Animal, CEEA-PRBB) ethical committees. Mice were randomly assigned to treatment groups and all experiments were performed blinded for pharmacological and surgical conditions.

Drugs

THC was purchased from THC-Pharm-GmbH (Frankfurt, Germany) as natural THC with 98.8% purity. This source of natural THC has been widely used in multiple research studies (***Busquets-Garcia et al., 2018***; ***Busquets-Garcia et al., 2011***; ***Cutando et al., 2013***; ***Flores et al., 2014***; ***Forsberg, 1970***; ***Gunasekaran et al., 2009***; ***Lopez-Rodriguez et al., 2014***; ***Morrison et al., 2011***; ***Puighermanal et al., 2013***). To corroborate the purity of the THC samples, High Performance Liquid Cromatography – Ultraviolet (HPLC-UV) was used for cannabinoid analysis and Gas Chromatography and Flame Ionization Detection (GC-FID) for terpenes (Canna Foundation, Paterna, Spain). These analyses revealed no detectable amounts of other cannabinoids or terpenes (Source Data Files 2, 3 and 4). THC was diluted in a vehicle composed of 2.5% ethanol, 5% Cremophor EL (C5135, Sigma-Aldrich St. Louis, MO, USA), and 92.5% saline, and was administered subcutaneously in a volume of 5 ml/kg.

Estrous cycle determination

The phase of the estrous cycle was assessed by histological examination of cells extracted by vaginal lavage (***Byers et al., 2012***) the day of the surgeries and the day of euthanasia. Briefly, mice were gently restrained and 20 µl of saline were flushed 5 times into the vagina. The resulting fluid was

placed onto gelatinized slides, stained with methylene blue and observed at 40X magnification under a light microscope (DM6000 B, Leica Biosystems, Nussloch, Germany).

Surgical induction of endometriosis

Endometriotic lesions were surgically-induced as previously described (***Somigliana et al., 1999***), with some modifications. Briefly, uterine horns from donor mice at diestrus were excised, opened longitudinally and biopsied into four pieces (2 × 2 mm). Recipient mice were anesthetized with vaporized isoflurane in oxygen (4% V/V for induction; 2.5% V/V for maintenance) and a midline incision of 1 cm was made to expose the abdominal compartment. Endometriosis mice had four uterine fragments sutured to the parietal peritoneum, whereas sham-operated mice received four similar-sized fragments of abdominal fat. Transplanted tissues and abdominal muscle and skin were stitched using 6–0 black silk (8065195601, Alcon Cusi S.A., Barcelona, Spain).

Experimental protocols

The nociceptive, affective and cognitive manifestations associated with the presence of ectopic endometrium were determined in a first experiment. After the measurement of baseline mechanical sensitivity (day −1), endometriosis or sham surgery was performed (day 0), and nociceptive responses were assessed again 7, 14, 21 and 28 days after surgery. Anxiety-like behavior and cognitive performance were evaluated on days 23 and 27, respectively. At the end of the experimental sequence (day 32), mice were euthanized by cervical dislocation for sample collection.

A second experiment was conducted to investigate the presence of generalized nociceptive sensitization. Nociceptive responses to hind paw mechanical stimulation were assessed before (day −4) and 16 days after surgery. In parallel, mechanical sensitivity of the caudal abdominal area was evaluated on days −2 and 14 after surgery. An additional evaluation of nocifensive behaviors to abdominal mechanical stimulation was performed on day 14.

A third experiment was conducted to obtain the ED50 of acute THC administration for the alleviation of mechanical hypersensitivity. Endometriosis and sham mice were tested in the von Frey assay after administration of different doses of THC (1.25, 2, 2.5 and 5 mg/kg) or vehicle. Measurements were done 45 min after subcutaneous administration of THC or vehicle at time points in which endometriotic lesions and hypersensitivity in the caudal abdomen were fully developed (days 33–41).

The effects of chronic THC or vehicle were evaluated in endometriosis and sham mice in a fourth experiment. Chronic treatment with THC (2 mg/kg) or vehicle administered once a day (9 AM) started on day 1 after surgery and lasted until day 32. Behavioral measures were conducted as in the first experiment. Mice were tested on the nociceptive paradigm 45 min after drug or vehicle administration and on the anxiety-like and memory tests 6 hr after administration. Mice were euthanized on day 32 by cervical dislocation for sample collection.

A fifth experiment with 4 sets of mice was conducted to investigate THC tolerance development once the pain symptomatology was established. One of the groups underwent a sham surgery and the other three received endometrial implants. The sham group and one of the endometriosis groups received vehicle from day 1 to 16; one of the endometriosis groups received vehicle for 13 days and on day 15, and acute doses of THC (2 mg/kg) on days 14 and 16; the last endometriosis group received a repeated treatment with a daily administration of THC (2 mg/kg) from day 7 to 16. All mice were tested for mechanical sensitivity in the caudal abdominal area and the hind paw 45 min after drug or vehicle administration on days −2, 7 and 14 (caudal abdomen), and −4 and 16 (hind paw), respectively. The effects of THC on nocifensive behavior were measured on day 14.

Nociceptive behavior

Mechanical sensitivity was quantified by measuring the responses to von Frey filament stimulation of the caudal abdominal area or the right hind paw. Von Frey filaments (1.65, 2.36, 2.44, 2.83, 3.22 and 3.61 corresponding to 0.008, 0.02, 0.04, 0.07, 0.16 and 0.4 g; Bioseb, Pinellas Park, FL, USA) were applied in increasing order of force, 10 times each, for 1–2 s, with an inter-stimulus interval of 5–10 s. Abrupt retraction of abdomen, immediate licking, jumping and scratching of the site of application were considered positive responses in the evaluation of abdominal mechanical sensitivity. Paw withdrawal, shaking or licking was considered a positive response in the evaluation of paw mechanical sensitivity. The area under the curve (AUC) was calculated by applying the linear trapezoidal rule to

the plots representing the frequency of response versus the numbers of von Frey filaments, which represent the logarithm of the filament force expressed in mg x 10.

Nocifensive behavior

Unpleasantness of pain in response to a mechanical stimulus was measured as previously described (***Corder et al., 2019***; ***Corder et al., 2017***) with minor modifications. Briefly, this parameter was evaluated using a single application of the von Frey filament 4.08 (corresponding to 1 g) against the caudal abdominal area shown in ***Figure 1—figure supplement 1b***. The time spent protecting the area by guarding or seeking escape during the following 30 s was considered nocifensive behavior.

Anxiety-like behavior

The elevated plus maze test was used to evaluate anxiety-like behavior in a black Plexiglas apparatus consisting of 4 arms (29 cm long x 5 cm wide), 2 open and 2 closed, set in cross from a neutral central square (5 × 5 cm) elevated 40 cm above the floor. Light intensity in the open and closed arms was 45 and 5 lux, respectively. Mice were placed in the central square facing one of the open arms and tested for 5 min. The percentages of time and entries to the open arms were determined as 100 x (time or entries to open arms) / (time or entries to open arms + time or entries to closed arms) as a measure of anxiety-like behavior.

Cognitive behavior

The novel object recognition task was assayed in a V-shaped maze to measure cognitive performance (***Puighermanal et al., 2009***). On the first day, mice were habituated for 9 min to the maze. On the second day, mice were placed again in the maze for 9 min and two identical objects were presented at the ends of the arms of the maze. Twenty-four h later, one of the familiar objects was replaced with a novel one and mice were placed back in the maze for 9 min. The time spent exploring each object (novel and familiar) was recorded and a discrimination index (DI) was calculated as the difference between the time spent exploring the novel and the familiar object, divided by the total time exploring the two objects. A threshold of 10 s of total interaction with the objects was set to discard low levels of general activity.

Sample harvesting and tissue preparation

Endometriotic lesions, uterine horns and ovaries were harvested from each mouse and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 4 hr and cryoprotected in 30% sucrose with 0.1% sodium azide for 6 days at 4˚C. Samples were then embedded in molds filled with optimal cutting temperature compound (4583, Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and stored at −80˚C until use.

Histology and immunostaining

Endometriotic lesions and uteri were serially sectioned at 20 µm with a cryostat (CM3050, Leica Biosystems, Nussloch, Germany), mounted onto gelatinized slides and stored at −20˚C until use. Sections of endometriotic lesions and uteri were stained with hematoxylin and eosin and observed under a Macro Zoom Fluorescence Microscope (MVX10, Olympus, Tokyo, Japan) for assessment of diameter and histological features.

Cyst sections were blocked and permeabilized with 3% normal donkey serum in PBS with 0.3% Triton X-100 for 2 hr and incubated overnight with rabbit anti-beta-III tubulin antibody (ab18207, 1:2000, Abcam, Cambridge, United Kingdom) in 3% normal donkey serum in PBS with 0.3% Triton X-100 at 4˚C. After washing with PBS, sections were incubated for 1 hr at room temperature with anti-rabbit Alexa Fluor A488 antibody (A21206, 1:1000, Thermo Fisher Scientific, Waltham, MA, USA). Slides were washed with PBS and coverslipped with DAPI Fluoromount-G (0100–20, Southern-Biotech, Birmingham, AL, USA) mounting media.

Uterine sections were blocked and permeabilized with 5% normal goat serum in PBS with 0.3% Triton X-100 for 2 hr and incubated overnight with rabbit anti-beta-III tubulin antibody (ab18207, 1:2000, Abcam) in 5% normal goat serum in PBS with 0.3% Triton X-100 at 4˚C. After washing with PBS, sections were incubated for 1 hr at room temperature with anti-rabbit Alexa Fluor A555

antibody (ab150078, 1:1000, Abcam, Cambridge, United Kingdom). Slides were washed with PBS and coverslipped with DAPI Fluoromount-G (0100–20, SouthernBiotech).

Image analysis

Images of immunostained sections of cysts and uteri were captured with the X2 objective of a Macro Zoom Fluorescence Microscope (MVX10, Olympus, Shinjuku, Tokyo, Japan) and processed and quantified using the NIH Image J software. An observer who was blinded to treatment group assignment converted from 4 to 8 images per animal into negative black-and-white images and the threshold was manually adjusted. Images were then dilated, skeletonized and the mean percentage of immunoreactive area was obtained by running the 'Analyze particles' function.

Determination of 17 β-estradiol plasma levels

Plasma samples were collected the day of euthanasia in tubes containing calcium EDTA. 17β-estradiol levels were determined with an enzyme-linked immunosorbent assay - ELISA (ES180S-100, Calbiotech, El Cajon, CA, USA) according to manufacturer instructions.

Ovarian follicle counting

Sections of ovaries were stained with hematoxylin and eosin and observed under an upright microscope (DM6000 B, Leica Biosystems). The number of pre-antral and antral follicles was determined in every nine sections. Only follicles containing an oocyte were counted and the total number of follicles was estimated by multiplying the raw counts by nine according to published criteria (***Myers et al., 2004***). The number of corpora lutea was determined by direct counting of every 18 sections according to the average corpus luteum diameter (***Numazawa and Kawashima, 1982***).

Statistical analysis

Data obtained with the nociception model were analyzed using one-way repeated measures ANOVA (surgery as between-subject factor), two-way repeated measures ANOVA (surgery and treatment as between-subject factors) or mixed models (surgery and treatment as between-subject factors and time as within-subject factor) whenever appropriate. Dose-response curve was fitted and ED50 determined using GraphPad Prism 8 (San Diego, CA, USA). Data obtained with the elevated plus maze test, novel object recognition task, histology, immunostaining and ovarian follicle counting were analyzed using a Student t-test (surgery) or a two-way ANOVA (surgery and treatment). Post hoc Bonferroni analysis was performed after ANOVA when appropriate. The nonparametric Kruskal-Wallis test was used whenever data did not have a normal distribution or equal variances, followed by Mann Whitney U when appropriate. Correlation between variables was determined using the Pearson correlation coefficient. Data are expressed as individual data points and mean ± SEM, and statistical analyses were performed using IBM SPSS 23 software (Chicago, IL, USA). The differences were considered statistically significant when the p value was below 0.05.

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Author contributions

Alejandra Escudero-Lara, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing - original draft, Conducted the behavioral and molecular experiments, Performed immunohistochemistry and microscopy, Wrote the manuscript; Josep Argerich, Formal analysis, Investigation, Methodology, Performed immunohistochemistry and microscopy; David Cabañero, Conceptualization, Supervision, Investigation, Visualization, Methodology, Writing - review and editing, Conceptualized and supervised the project, Participated in the experimental design, Wrote the manuscript; Rafael Maldonado, Conceptualization, Resources, Supervision, Funding acquisition, Investigation, Project administration, Writing - review and editing, Conceptualized, supervised and funded the project, Participated in the experimental design, Wrote the manuscript

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Ethics

Animal experimentation: All animal procedures were conducted in accordance with standard ethical guidelines (European Communities Directive 2010/63/EU and NIH Guide for Care and Use of Laboratory Animals, 8th Edition) and approved by autonomic (Generalitat de Catalunya, Departament de Territori i Sostenibilitat) and local (Comitè Ètic d'Experimentació Animal, CEEA-PRBB) ethical committees.

Decision letter and Author response

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Additional files

Supplementary files

- Transparent reporting form

Data availability

All data supporting the findings of this study are available within the manuscript and its source data files. Source data files have been provided for Figures 1, 2, 3 and 4 and their figure supplements.

References

Abuhasira R, Shbiro L, Landschaft Y. 2018. Medical use of Cannabis and cannabinoids containing products - Regulations in Europe and north america. *European Journal of Internal Medicine* **49**:2–6. DOI: https://doi.org/10.1016/j.ejim.2018.01.001, PMID: 29329891

Adashi EY, Jones PB, Hsueh AJ. 1983. Direct antigonadal activity of cannabinoids: suppression of rat granulosa cell functions. *American Journal of Physiology-Endocrinology and Metabolism* **244**:E177–E185. DOI: https://doi.org/10.1152/ajpendo.1983.244.2.E177

Armour M, Sinclair J, Chalmers KJ, Smith CA. 2019. Self-management strategies amongst Australian women with endometriosis: a national online survey. *BMC Complementary and Alternative Medicine* **19**:17. DOI: https://doi.org/10.1186/s12906-019-2431-x, PMID: 30646891

Arosh JA, Lee J, Balasubbramanian D, Stanley JA, Long CR, Meagher MW, Osteen KG, Bruner-Tran KL, Burghardt RC, Starzinski-Powitz A, Banu SK. 2015. Molecular and preclinical basis to inhibit PGE2 receptors EP2 and EP4 as a novel nonsteroidal therapy for endometriosis. *PNAS* **112**:9716–9721. DOI: https://doi.org/10.1073/pnas.1507931112, PMID: 26199416

Berkley KJ, Dmitrieva N, Curtis KS, Papka RE. 2004. Innervation of ectopic endometrium in a rat model of endometriosis. *PNAS* **101**:11094–11098. DOI: https://doi.org/10.1073/pnas.0403663101, PMID: 15256593

Bilkei-Gorzo A, Albayram O, Draffehn A, Michel K, Piyanova A, Oppenheimer H, Dvir-Ginzberg M, Rácz I, Ulas T, Imbeault S, Bab I, Schultze JL, Zimmer A. 2017. A chronic low dose of Δ9-tetrahydrocannabinol (THC) restores cognitive function in old mice. *Nature Medicine* **23**:782–787. DOI: https://doi.org/10.1038/nm.4311, PMID: 28481360

Bushnell MC, Case LK, Ceko M, Cotton VA, Gracely JL, Low LA, Pitcher MH, Villemure C. 2015. Effect of environment on the long-term consequences of chronic pain. *Pain* **156**:S42–S49. DOI: https://doi.org/10.1097/01.j.pain.0000460347.77341.bd, PMID: 25789436

Busquets-Garcia A, Puighermanal E, Pastor A, de la Torre R, Maldonado R, Ozaita A. 2011. Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses. *Biological Psychiatry* **70**:479–486. DOI: https://doi.org/10.1016/j.biopsych.2011.04.022, PMID: 21684528

Busquets-Garcia A, Gomis-González M, Salgado-Mendialdúa V, Galera-López L, Puighermanal E, Martín-García E, Maldonado R, Ozaita A. 2018. Hippocampal protein kinase C signaling mediates the Short-Term memory impairment induced by Delta9-Tetrahydrocannabinol. *Neuropsychopharmacology* **43**:1021–1031. DOI: https://doi.org/10.1038/npp.2017.175, PMID: 28816239

Byers SL, Wiles MV, Dunn SL, Taft RA. 2012. Mouse estrous cycle identification tool and images. *PLOS ONE* **7**:e35538. DOI: https://doi.org/10.1371/journal.pone.0035538, PMID: 22514749

Campbell FA, Tramèr MR, Carroll D, Reynolds DJ, Moore RA, McQuay HJ. 2001. Are cannabinoids an effective and safe treatment option in the management of pain? A qualitative systematic review. *BMJ* **323**:13. DOI: https://doi.org/10.1136/bmj.323.7303.13, PMID: 11440935

Célérier E, Ahdepil T, Wikander H, Berrendero F, Nyberg F, Maldonado R. 2006. Influence of the anabolic-androgenic steroid nandrolone on cannabinoid dependence. *Neuropharmacology* **50**:788–806. DOI: https://doi.org/10.1016/j.neuropharm.2005.11.017

Corder G, Tawfik VL, Wang D, Sypek EI, Low SA, Dickinson JR, Sotoudeh C, Clark JD, Barres BA, Bohlen CJ, Scherrer G. 2017. Loss of μ opioid receptor signaling in nociceptors, but not microglia, abrogates morphine tolerance without disrupting analgesia. *Nature Medicine* **23**:164–173. DOI: https://doi.org/10.1038/nm.4262, PMID: 28092666

Corder G, Ahanonu B, Grewe BF, Wang D, Schnitzer MJ, Scherrer G. 2019. An amygdalar neural ensemble that encodes the unpleasantness of pain. *Science* **363**:276–281. DOI: https://doi.org/10.1126/science.aap8586, PMID: 30655440

Curran HV, Freeman TP, Mokrysz C, Lewis DA, Morgan CJ, Parsons LH. 2016. Keep off the grass? Cannabis, cognition and addiction. *Nature Reviews Neuroscience* **17**:293–306. DOI: https://doi.org/10.1038/nrn.2016.28, PMID: 27052382

Cutando L, Busquets-Garcia A, Puighermanal E, Gomis-González M, Delgado-García JM, Gruart A, Maldonado R, Ozaita A. 2013. Microglial activation underlies cerebellar deficits produced by repeated Cannabis exposure. *Journal of Clinical Investigation* **123**:2816–2831. DOI: https://doi.org/10.1172/JCI67569, PMID: 23934130

De Vry J, Kuhl E, Franken-Kunkel P, Eckel G. 2004. Pharmacological characterization of the chronic constriction injury model of neuropathic pain. *European Journal of Pharmacology* **491**:137–148. DOI: https://doi.org/10.1016/j.ejphar.2004.03.051, PMID: 15140630

El-Talatini MR, Taylor AH, Elson JC, Brown L, Davidson AC, Konje JC. 2009. Localisation and function of the endocannabinoid system in the human ovary. *PLOS ONE* **4**:e4579. DOI: https://doi.org/10.1371/journal.pone.0004579, PMID: 19238202

Filho P, Chaves Filho AJM, Vieira CFX, Oliveira TQ, Soares MVR, Jucá PM, Quevedo J, Barichello T, Macedo D, das Chagas Medeiros F. 2019. Peritoneal endometriosis induces time-related depressive- and anxiety-like alterations in female rats: involvement of hippocampal pro-oxidative and BDNF alterations. *Metabolic Brain Disease* **34**:909–925. DOI: https://doi.org/10.1007/s11011-019-00397-1, PMID: 30798429

Flores Á, Maldonado R, Berrendero F. 2014. The hypocretin/orexin receptor-1 as a novel target to modulate cannabinoid reward. *Biological Psychiatry* **75**:499–507. DOI: https://doi.org/10.1016/j.biopsych.2013.06.012, PMID: 23896204

Forsberg JG. 1970. An estradiol mitotic rate inhibiting effect in the müllerian epithelium in neonatal mice. *Journal of Experimental Zoology* **175**:369–374. DOI: https://doi.org/10.1002/jez.1401750310, PMID: 5529519

Fourquet J, Báez L, Figueroa M, Iriarte RI, Flores I. 2011. Quantification of the impact of endometriosis symptoms on health-related quality of life and work productivity. *Fertility and Sterility* **96**:107–112. DOI: https://doi.org/10.1016/j.fertnstert.2011.04.095, PMID: 21621771

Garry R. 2004. The effectiveness of laparoscopic excision of endometriosis. *Current Opinion in Obstetrics and Gynecology* **16**:299–303. DOI: https://doi.org/10.1097/01.gco.0000136496.95075.79, PMID: 15232483

Greaves E, Horne AW, Jerina H, Mikolajczak M, Hilferty L, Mitchell R, Fleetwood-Walker SM, Saunders PTK. 2017. EP2 receptor antagonism reduces peripheral and central hyperalgesia in a preclinical mouse model of endometriosis. *Scientific Reports* **7**:1–10. DOI: https://doi.org/10.1038/srep44169

Greene NZ, Wiley JL, Yu Z, Clowers BH, Craft RM. 2018. Cannabidiol modulation of antinociceptive tolerance to Δ^9-tetrahydrocannabinol. *Psychopharmacology* **235**:3289–3302. DOI: https://doi.org/10.1007/s00213-018-5036-z, PMID: 30238130

Gunasekaran N, Long LE, Dawson BL, Hansen GH, Richardson DP, Li KM, Arnold JC, McGregor IS. 2009. Reintoxication: the release of fat-stored Delta(9)-tetrahydrocannabinol (THC) into blood is enhanced by food deprivation or ACTH exposure. *British Journal of Pharmacology* **158**:1330–1337. DOI: https://doi.org/10.1111/j.1476-5381.2009.00399.x, PMID: 19681888

Hansen KE, Kesmodel US, Baldursson EB, Schultz R, Forman A. 2013. The influence of endometriosis-related symptoms on work life and work ability: a study of danish endometriosis patients in employment. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **169**:331–339. DOI: https://doi.org/10.1016/j.ejogrb.2013.03.008, PMID: 23537616

Harris HM, Sufka KJ, Gul W, ElSohly MA. 2016. Effects of Delta-9-Tetrahydrocannabinol and cannabidiol on Cisplatin-Induced neuropathy in mice. *Planta Medica* **82**:1169–1172. DOI: https://doi.org/10.1055/s-0042-106303, PMID: 27214593

Kasten CR, Zhang Y, Boehm SL. 2017. Acute and long-term effects of Δ9-tetrahydrocannabinol on object recognition and anxiety-like activity are age- and strain-dependent in mice. *Pharmacology Biochemistry and Behavior* **163**:9–19. DOI: https://doi.org/10.1016/j.pbb.2017.10.012, PMID: 29107728

King KM, Myers AM, Soroka-Monzo AJ, Tuma RF, Tallarida RJ, Walker EA, Ward SJ. 2017. Single and combined effects of Δ^9 -tetrahydrocannabinol and cannabidiol in a mouse model of chemotherapy-induced neuropathic pain. *British Journal of Pharmacology* **174**:2832–2841. DOI: https://doi.org/10.1111/bph.13887, PMID: 28548225

Kubilius RA, Kaplick PM, Wotjak CT. 2018. Highway to hell or magic smoke? The dose-dependence of Δ^9-THC in place conditioning paradigms. *Learning & Memory* **25**:446–454. DOI: https://doi.org/10.1101/lm.046870.117, PMID: 30115766

La Porta C, Bura SA, Llorente-Onaindia J, Pastor A, Navarrete F, García-Gutiérrez MS, De la Torre R, Manzanares J, Monfort J, Maldonado R. 2015. Role of the endocannabinoid system in the emotional manifestations of osteoarthritis pain. *Pain* **156**:2001–2012. DOI: https://doi.org/10.1097/j.pain.0000000000000260

LaFleur RA, Wilson RP, Morgan DJ, Henderson-Redmond AN. 2018. Sex differences in antinociceptive response to Δ-9-tetrahydrocannabinol and CP 55,940 in the mouse Formalin test. *NeuroReport* **29**:447–452. DOI: https://doi.org/10.1097/WNR.0000000000000993, PMID: 29461336

Leconte M, Nicco C, Ngô C, Arkwright S, Chéreau C, Guibourdenche J, Weill B, Chapron C, Dousset B, Batteux F. 2010. Antiproliferative effects of cannabinoid agonists on deep infiltrating endometriosis. *The American Journal of Pathology* **177**:2963–2970. DOI: https://doi.org/10.2353/ajpath.2010.100375, PMID: 21057002

Li T, Mamillapalli R, Ding S, Chang H, Liu ZW, Gao XB, Taylor HS. 2018. Endometriosis alters brain electrophysiology, gene expression and increases pain sensitization, anxiety, and depression in female mice. *Biology of Reproduction* **99**:349–359. DOI: https://doi.org/10.1093/biolre/ioy035, PMID: 29425272

Lopez-Rodriguez AB, Llorente-Berzal A, Garcia-Segura LM, Viveros MP. 2014. Sex-dependent long-term effects of adolescent exposure to THC and/or MDMA on neuroinflammation and serotoninergic and cannabinoid systems in rats. *British Journal of Pharmacology* **171**:1435–1447. DOI: https://doi.org/10.1111/bph.12519, PMID: 24236988

MacCallum CA, Russo EB. 2018. Practical considerations in medical Cannabis administration and dosing. *European Journal of Internal Medicine* **49**:12–19. DOI: https://doi.org/10.1016/j.ejim.2018.01.004, PMID: 29307505

Márki G, Bokor A, Rigó J, Rigó A. 2017. Physical pain and emotion regulation as the main predictive factors of health-related quality of life in women living with endometriosis. *Human Reproduction* **32**:1432–1438. DOI: https://doi.org/10.1093/humrep/dex091, PMID: 28482063

Miller EJ, Fraser IS. 2015. The importance of pelvic nerve fibers in endometriosis. *Women's Health* **11**:611–618. DOI: https://doi.org/10.2217/whe.15.47, PMID: 26314611

Morrison PD, Nottage J, Stone JM, Bhattacharyya S, Tunstall N, Brenneisen R, Holt D, Wilson D, Sumich A, McGuire P, Murray RM, Kapur S, Ffytche DH. 2011. Disruption of frontal θ coherence by Δ9-tetrahydrocannabinol is associated with positive psychotic symptoms. *Neuropsychopharmacology* **36**:827–836. DOI: https://doi.org/10.1038/npp.2010.222, PMID: 21150914

Myers M, Britt KL, Wreford NG, Ebling FJ, Kerr JB. 2004. Methods for quantifying follicular numbers within the mouse ovary. *Reproduction* **127**:569–580. DOI: https://doi.org/10.1530/rep.1.00095, PMID: 15129012

Numazawa A, Kawashima S. 1982. Morphometric studies on ovarian follicles and corpora lutea during the oestrous cycle in the mouse. *Reproduction* **64**:275–283. DOI: https://doi.org/10.1530/jrf.0.0640275

Puighermanal E, Marsicano G, Busquets-Garcia A, Lutz B, Maldonado R, Ozaita A. 2009. Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. *Nature Neuroscience* **12**:1152–1158. DOI: https://doi.org/10.1038/nn.2369

Puighermanal E, Busquets-Garcia A, Gomis-González M, Marsicano G, Maldonado R, Ozaita A. 2013. Dissociation of the pharmacological effects of THC by mTOR blockade. *Neuropsychopharmacology* **38**:1334–1343. DOI: https://doi.org/10.1038/npp.2013.31, PMID: 23358238

Ross RA, Kaiser UB. 2017. The emotional cost of contraception. *Nature Reviews Endocrinology* **13**:7–9. DOI: https://doi.org/10.1038/nrendo.2016.194

Sarne Y, Toledano R, Rachmany L, Sasson E, Doron R. 2018. Reversal of age-related cognitive impairments in mice by an extremely low dose of tetrahydrocannabinol. *Neurobiology of Aging* **61**:177–186. DOI: https://doi.org/10.1016/j.neurobiolaging.2017.09.025, PMID: 29107185

Skovlund CW, Mørch LS, Kessing LV, Lidegaard Ø. 2016. Association of hormonal contraception with depression. *JAMA Psychiatry* **73**:1154–1162. DOI: https://doi.org/10.1001/jamapsychiatry.2016.2387, PMID: 27680324

Somigliana E, Viganò P, Rossi G, Carinelli S, Vignali M, Panina-Bordignon P. 1999. Endometrial ability to implant in ectopic sites can be prevented by interleukin-12 in a murine model of endometriosis. *Human Reproduction* **14**:2944–2950. DOI: https://doi.org/10.1093/humrep/14.12.2944, PMID: 10601076

Sperschneider ML, Hengartner MP, Kohl-Schwartz A, Geraedts K, Rauchfuss M, Woelfler MM, Haeberlin F, von Orelli S, Eberhard M, Maurer F, Imthurn B, Imesch P, Leeners B. 2019. Does endometriosis affect professional life? A matched case-control study in Switzerland, Germany and Austria. *BMJ Open* **9**:e019570. DOI: https://doi.org/10.1136/bmjopen-2017-019570, PMID: 30782670

Stockings E, Campbell G, Hall WD, Nielsen S, Zagic D, Rahman R, Murnion B, Farrell M, Weier M, Degenhardt L. 2018. Cannabis and cannabinoids for the treatment of people with chronic noncancer pain conditions: a systematic review and meta-analysis of controlled and observational studies. *Pain* **159**:1932–1954. DOI: https://doi.org/10.1097/j.pain.0000000000001293, PMID: 29847469

Tokushige N, Markham R, Russell P, Fraser IS. 2006. High density of small nerve fibres in the functional layer of the endometrium in women with endometriosis. *Human Reproduction* **21**:782–787. DOI: https://doi.org/10.1093/humrep/dei368, PMID: 16253968

Ueberall M, Essner U, Mueller-Schwefe GHH. 2019. Effectiveness and tolerability of THC:cbd oromucosal spray as add-on measure in patients with severe chronic pain: analysis of 12-week open-label real-world data provided by the german pain e-Registry]]>. *Journal of Pain Research* **12**:1577–1604. DOI: https://doi.org/10.2147/JPR.S192174

Viñals X, Moreno E, Lanfumey L, Codomí A, Pastor A, La TRD, Gasperini P, Navarro G, Howell LA, Pardo L, Lluís C, Canela EI, McCormick PJ, Maldonado R, Robledo P. 2015. Cognitive impairment induced by Delta9-tetrahydrocannabinol occurs through heteromers between cannabinoid CB 1 and serotonin 5-HT 2A receptors 1. *PLOS Biology* **40**:e1002194. DOI: https://doi.org/10.1371/journal.pbio.1002194

Wakley AA, Wiley JL, Craft RM. 2014. Sex differences in antinociceptive tolerance to delta-9-tetrahydrocannabinol in the rat. *Drug and Alcohol Dependence* **143**:22–28. DOI: https://doi.org/10.1016/j.drugalcdep.2014.07.029

Walker OS, Holloway AC, Raha S. 2019. The role of the endocannabinoid system in female reproductive tissues. *Journal of Ovarian Research* **12**:3. DOI: https://doi.org/10.1186/s13048-018-0478-9, PMID: 30646937

Wang G, Tokushige N, Markham R, Fraser IS. 2009. Rich innervation of deep infiltrating endometriosis. *Human Reproduction* **24**:827–834. DOI: https://doi.org/10.1093/humrep/den464, PMID: 19151028

Williams J, Haller VL, Stevens DL, Welch SP. 2008. Decreased basal endogenous opioid levels in diabetic rodents: effects on morphine and delta-9-tetrahydrocannabinoid-induced antinociception. *European Journal of Pharmacology* **584**:78–86. DOI: https://doi.org/10.1016/j.ejphar.2007.12.035

You Z, Zhang S, Shen S, Yang J, Ding W, Yang L, Lim G, Doheny JT, Tate S, Chen L, Mao J. 2018. Cognitive impairment in a rat model of neuropathic pain: role of hippocampal microtubule stability. *Pain* **159**:1518–1528. DOI: https://doi.org/10.1097/j.pain.0000000000001233, PMID: 29613911

Zondervan KT, Becker CM, Koga K, Missmer SA, Taylor RN, Viganò P. 2019. Endometriosis. *Medical Radiology* **4**:9. DOI: https://doi.org/10.1038/s41572-018-0008-5

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SHORT REPORT

Figures and figure supplements

Disease-modifying effects of natural Δ9-tetrahydrocannabinol in endometriosis-associated pain

Alejandra Escudero-Lara *et al*

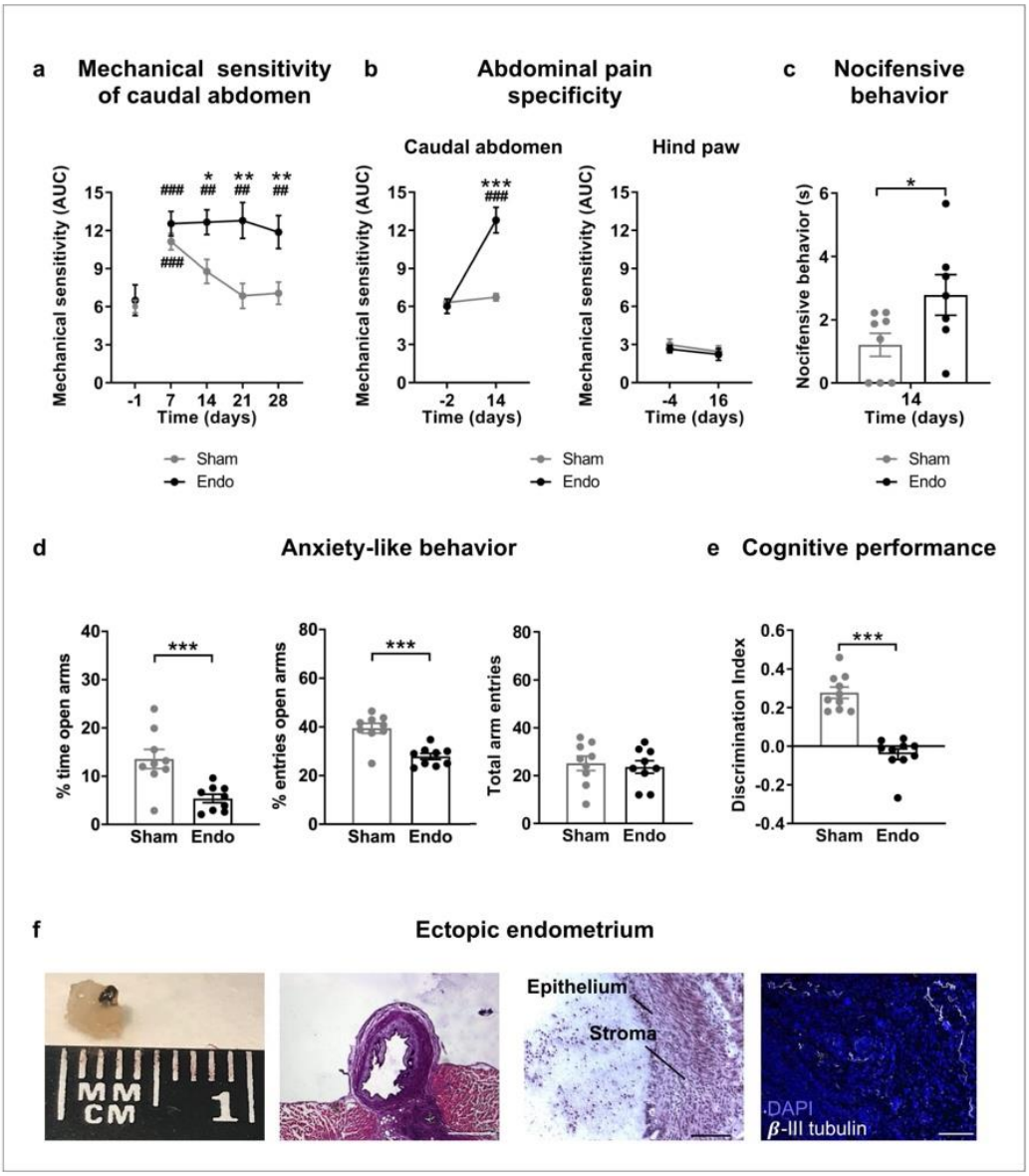


Figure 1. Behavioral and histological alterations in female mice with ectopic endometrial implants. Endometriosis mice showed (a) persistent mechanical abdominal hypersensitivity that (b) was localized in the caudal abdominal area but not detectable in distant areas (hind paw). Mechanical sensitivity is represented by the area under the curve of frequency of response to von Frey filaments. Higher values mean higher mechanical pain. Mice

Figure 1 continued on next page

Figure 1 continued

receiving endometrial implants also showed (**c**) increased nocifensive behavior, (**d**) anxiety-like behavior in the elevated plus maze test and (**e**) cognitive impairment in the novel object recognition task. (**f**) From left to right: cysts were recovered from endometriosis mice, were filled with fluid (scale bar = 1 mm), contained endometrial epithelium and stroma (scale bar = 100 µm) and were innervated by beta-III tubulin-labeled fibers (scale bar = 100 µm, blue is DAPI and white is β-III tubulin). Error bars are mean ± SEM. One-way repeated measures ANOVA + Bonferroni (**a and b**) and Student t-test (**c, d and e**). *p<0.05, **p<0.01, ***p<0.001 vs sham. ##p<0.01, ###p<0.001 vs baseline. Endo, endometriosis, AUC, area under the curve.

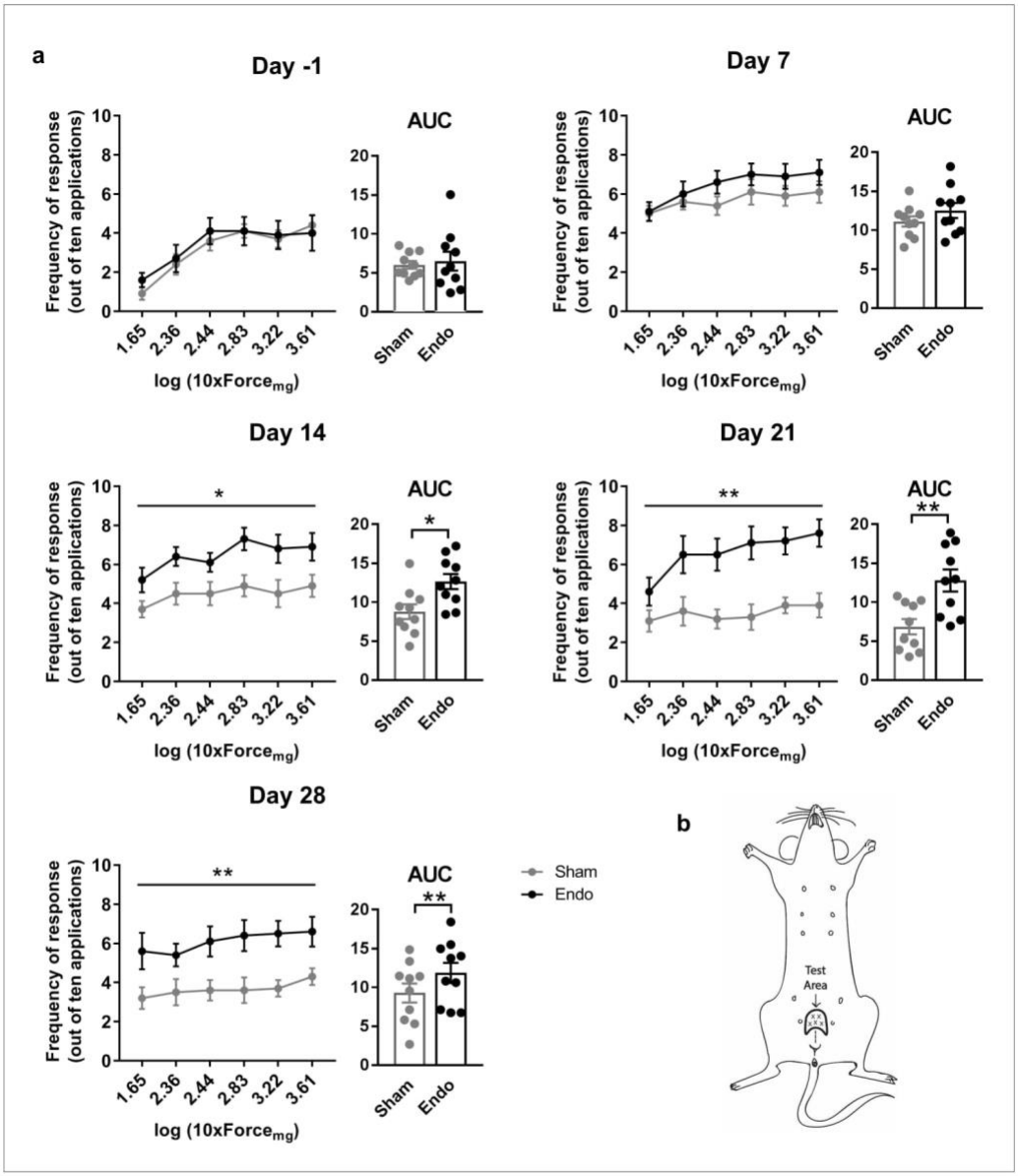


Figure 1—figure supplement 1. Nociceptive responses to abdominal mechanical stimulation with von Frey filaments. (a) Significantly higher frequency of responses and AUC was observed in endometriosis mice when compared to sham mice on days 14, 21 and 28 after the surgery. For each day, left

Figure 1—figure supplement 1 continued on next page

Figure 1—figure supplement 1 continued

panel is frequency of response to each von Frey filament and right panel is the corresponding mechanical sensitivity represented by the area under the curve of frequency of response to von Frey filaments. Higher values mean higher mechanical pain. (**b**) Site of application of the von Frey filaments (Test area). Error bars are mean ± SEM. For each day, one-way repeated measures ANOVA (left panels) and Student t-test (right panels). *p<0.05, **p<0.01 vs sham. Endo, endometriosis; AUC, area under the curve.

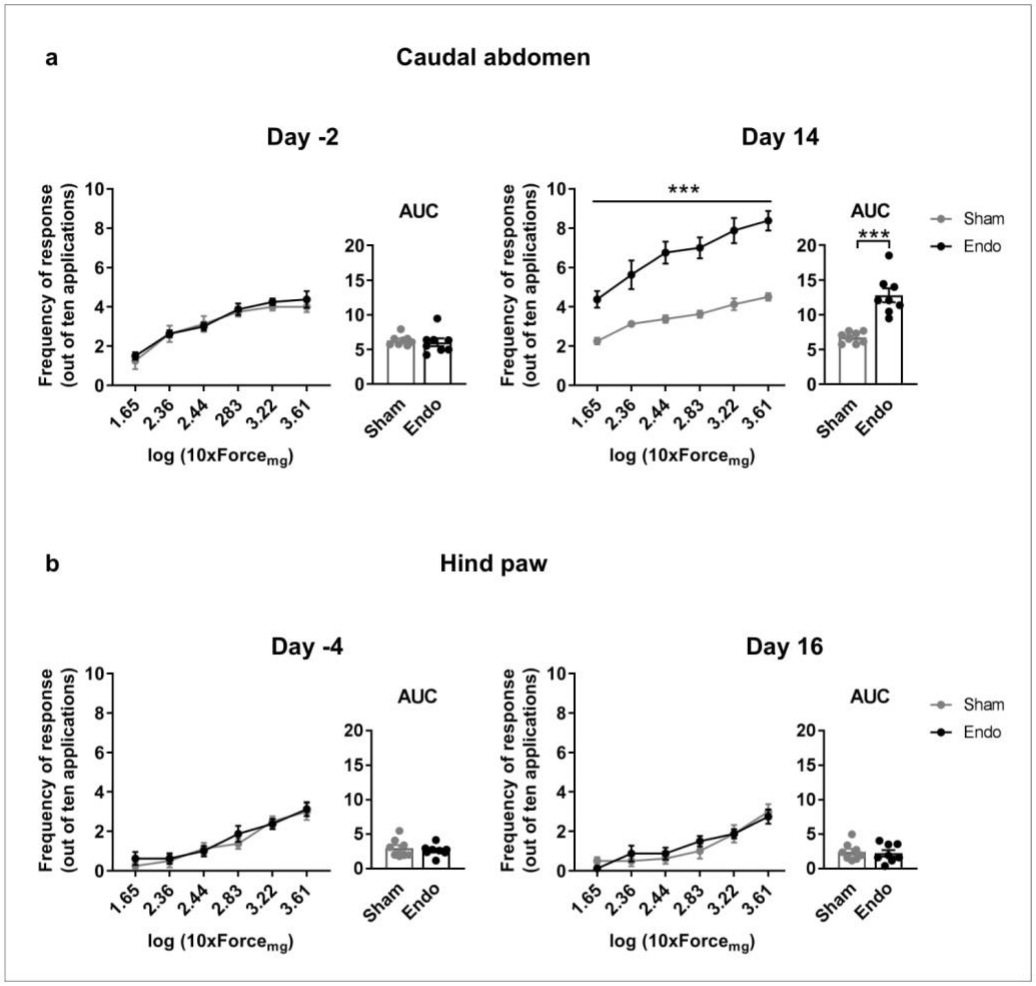


Figure 1—figure supplement 2. Nociceptive responses to abdominal and paw mechanical stimulation with von Frey filaments. (**a**) Frequency of responses to von Frey filaments applied against the caudal abdominal area and corresponding AUCs on day 14 after surgery were significantly higher in endometriosis mice when compared to sham mice. (**b**) Frequency of responses to von Frey filaments applied against the hind paw and corresponding AUCs were similar before and after surgery in endometriosis and sham mice. For each day, left panel is frequency of response to each von Frey filament and right panel is the corresponding mechanical sensitivity represented by the area under the curve of frequency of response to von Frey filaments. Higher values mean higher mechanical pain. Error bars are mean ± SEM. One-way repeated measures ANOVA (left panels) and Student t-test (right panels). ***p<0.001 vs sham. Endo, endometriosis; AUC, area under the curve.

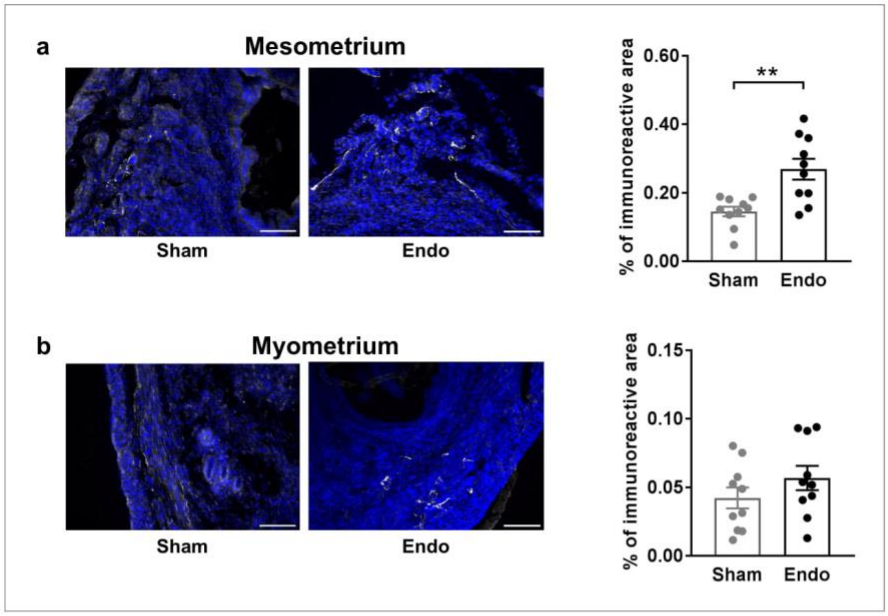


Figure 1—figure supplement 3. Density of beta-III tubulin-labeled fibers in uteri of endometriosis and sham mice. (**a**) The percentage of immunoreactive area of the mesometrial aspect of the uterus was higher in endometriosis mice. (**b**) The percentage of immunoreactive area of myometrium did not differ between groups. Blue is DAPI and white is β-III tubulin. Scale bar = 100 μm. Error bars are mean ± SEM. Student t-test. **p<0.01 vs sham. Endo, endometriosis.

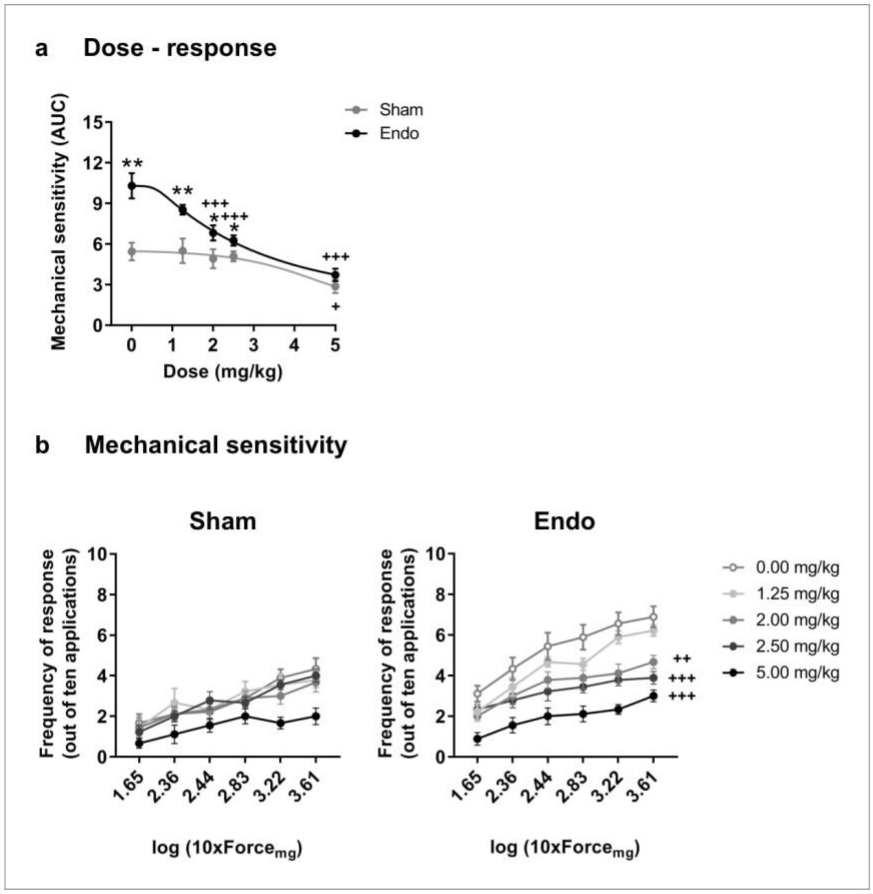


Figure 2. Effect of acute THC administration on the nociceptive responses to mechanical stimulation. (**a**) Acute THC produced a dose-dependent reduction of mechanical hypersensitivity in the caudal abdominal area. Mechanical sensitivity is represented by the area under the curve of frequency of response to von Frey filaments. Higher values mean higher mechanical pain. (**b**) Administration of 2, 2.5 and 5 mg/kg of THC decreased the frequency of response to von Frey filaments in endometriosis mice. Error bars are mean ± SEM. One-way repeated measures ANOVA + Bonferroni. *p<0.05, **p<0.01 vs sham; +p<0.05, ++p<0.01, +++p<0.001 vs vehicle. Endo, endometriosis; THC, Δ9-tetrahydrocannabinol, AUC, area under the curve.

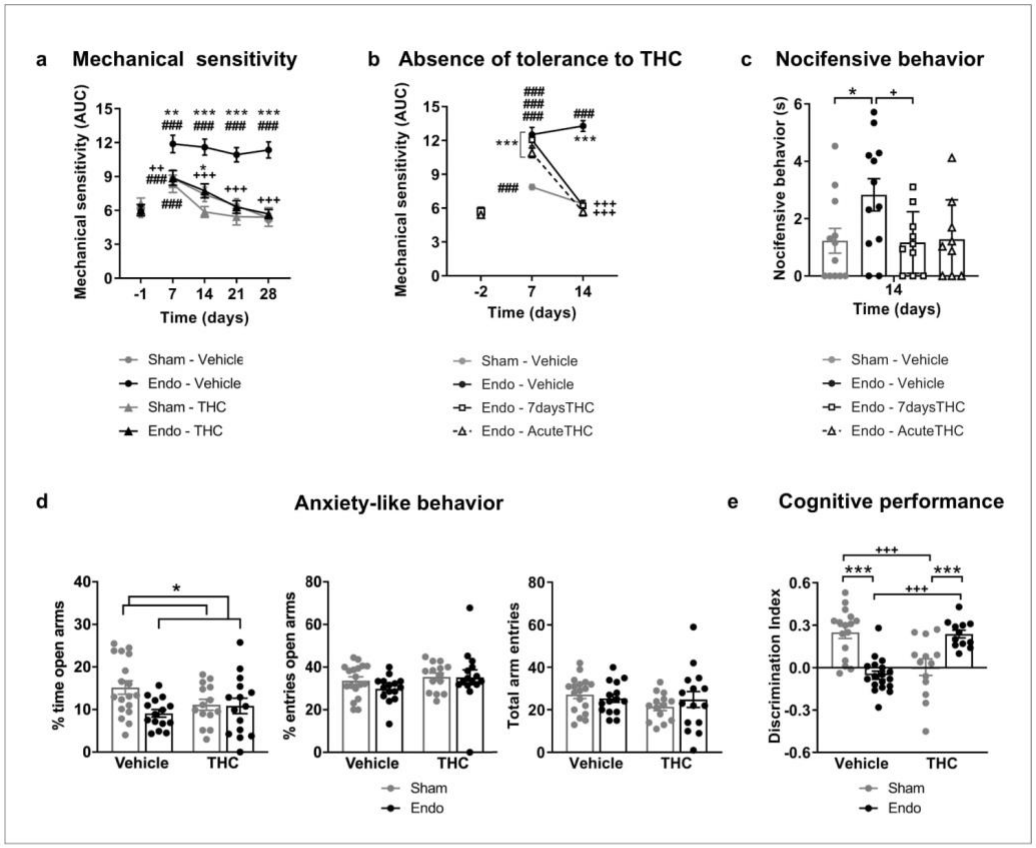


Figure 3. Effects of THC on the behavioral changes observed in mice with ectopic endometrium. (**a**) Repeated THC (28 days) alleviated mechanical hypersensitivity in the caudal abdominal area of endometriosis mice in the von Frey test. (**b**) THC administered on day 14 after a 6 day treatment (Endo – 7daysTHC) was as effective as an acute dose given on day 14 (Endo – AcuteTHC). Mechanical sensitivity is represented by the area under the curve of frequency of response to von Frey filaments. Higher values mean higher mechanical pain. (**c**) Nocifensive behaviors were abolished in endometriosis mice after a 7 day treatment with THC (Endo – 7daysTHC). (**d**) Endometriosis-associated anxiety-like behavior was unaltered after THC in the elevated plus maze test. (**e**) THC impaired object recognition memory in sham mice and prevented memory deficits of endometriosis mice in the novel object recognition test. THC dose: 2 mg/kg/day. Error bars are mean ± SEM. Two-way repeated measures ANOVA + Bonferroni (**a**), Mixed model + Bonferroni (**b**), Kruskal-Wallis + Mann Whitney U (**c**) and Two-way ANOVA + Bonferroni (**d and e**). ###p<0.001 vs baseline. *p<0.05, **p<0.01, ***p<0.001 vs sham. ++p<0.01, +++p<0.001 vs vehicle,. Endo, endometriosis; THC, Δ9-tetrahydrocannabinol; AUC, area under the curve.

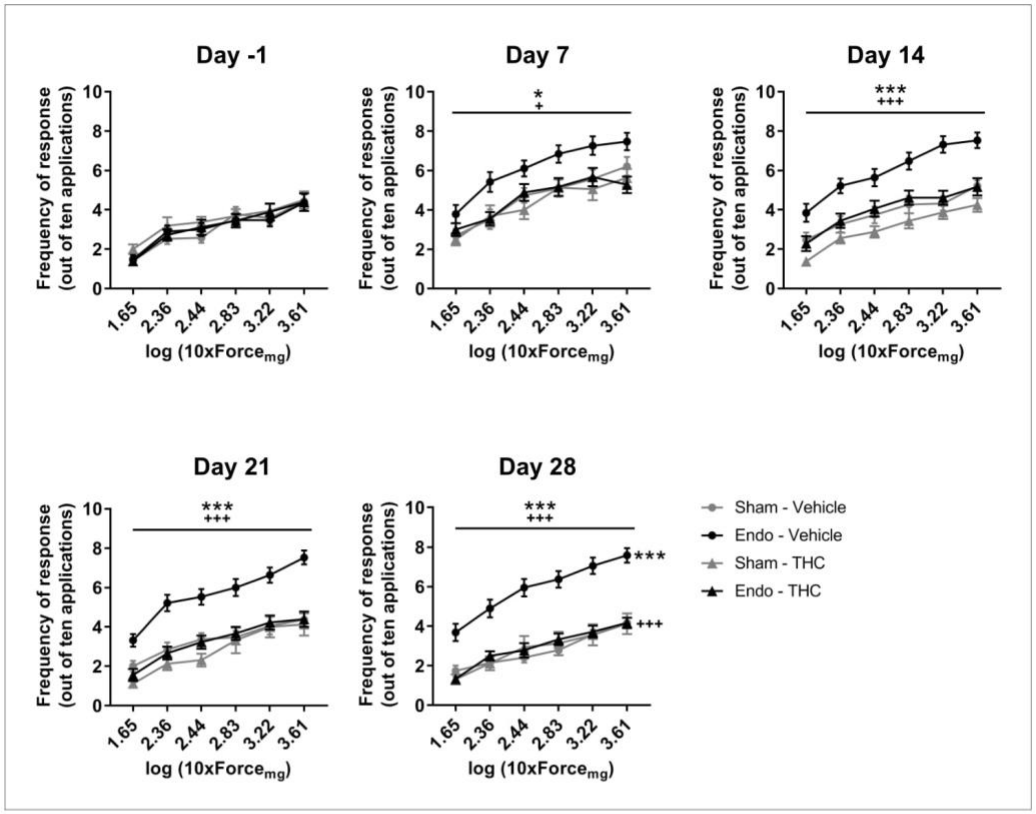


Figure 3—figure supplement 1. Effect of chronic THC treatment on nociceptive responses to abdominal mechanical stimulation with von Frey filaments. Endometriosis mice treated with vehicle showed higher frequency of response than endometriosis mice treated with THC and sham mice treated with vehicle. THC dose: 2 mg/kg/day. Error bars are mean ± SEM. Two-way repeated measures ANOVA + Bonferroni. *p<0.05, ***p<0.001 vs sham. +p<0.05, +++p<0.001 vs vehicle. Endo, endometriosis; THC, Δ9-tetrahydrocannabinol.

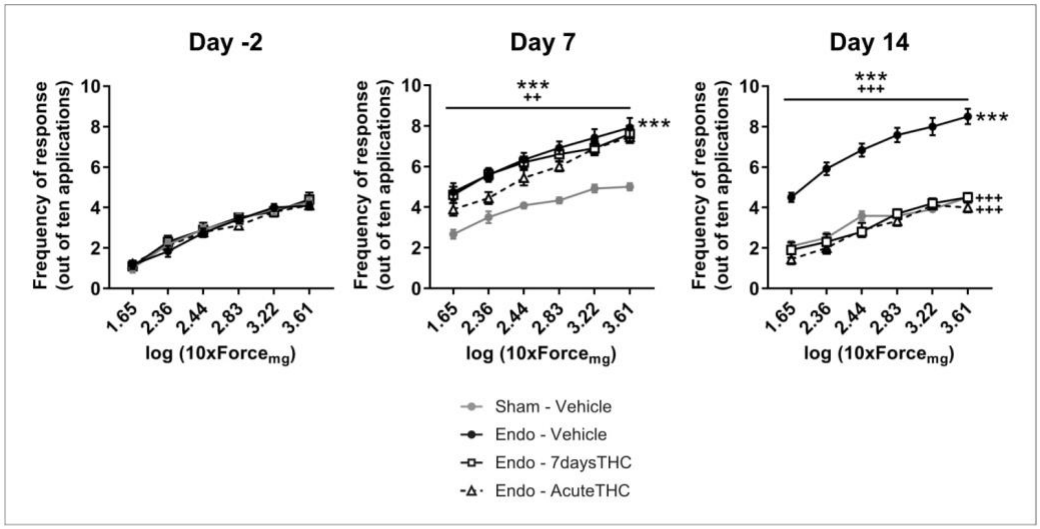


Figure 3—figure supplement 2. Effect of a repeated THC treatment starting on day 8 after surgeries on nociceptive responses to abdominal mechanical stimulation with von Frey filaments. Endometriosis mice treated with THC for 7 days (Endo – 7daysTHC) and endometriosis mice treated acutely with THC (Endo – AcuteTHC) showed a reduction in the frequency of response to von Frey filaments on day 14. THC dose: 2 mg/kg/day. Error bars are mean ± SEM. Mixed model + Bonferroni. ***p<0.001 vs sham; +++p<0.001 vs vehicle. Endo, endometriosis; THC, Δ9-tetrahydrocannabinol.

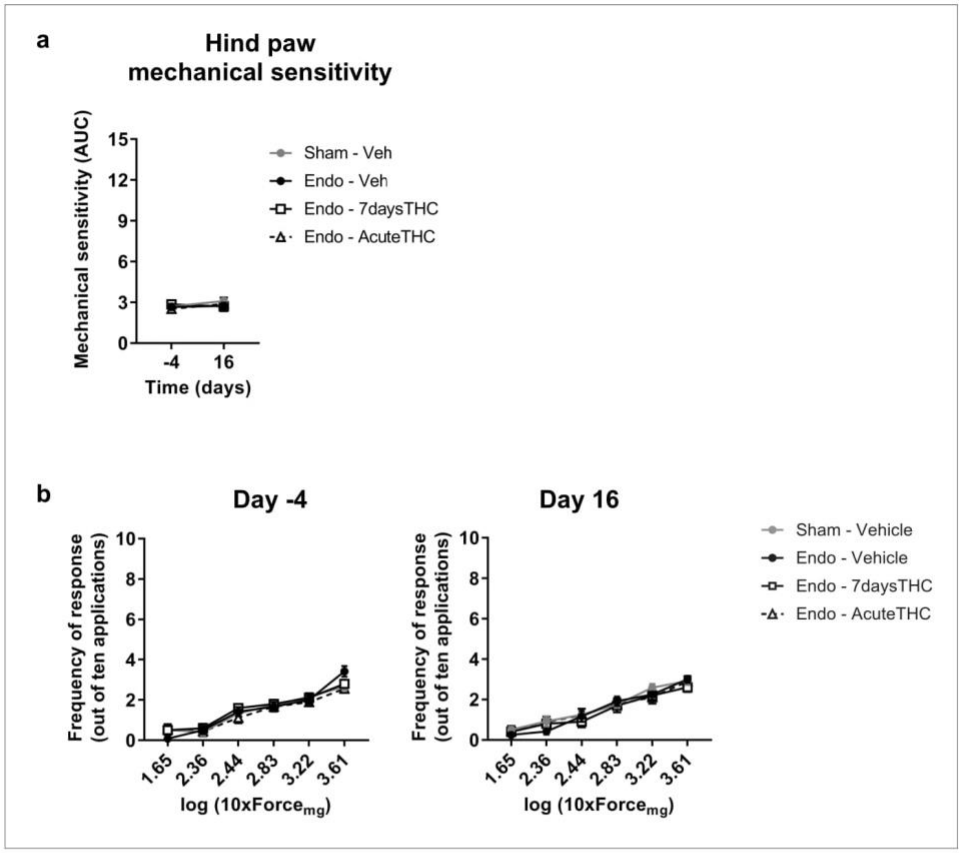


Figure 3—figure supplement 3. Effect of a repeated THC treatment starting on day eight after surgeries on nociceptive responses to hind paw mechanical stimulation with von Frey filaments. (**a**) Mechanical sensitivity in the hind paw remained stable after endometriosis surgery or THC treatment. Mechanical sensitivity is represented by the area under the curve of frequency of response to von Frey filaments. Higher values mean higher mechanical pain. (**b**) Similar frequency of responses was observed in all groups of mice before and after the surgery. THC dose: 2 mg/kg/day. Error bars are mean ± SEM. Mixed model + Bonferroni. Endo, endometriosis; THC, Δ9-tetrahydrocannabinol, AUC, area under the curve.

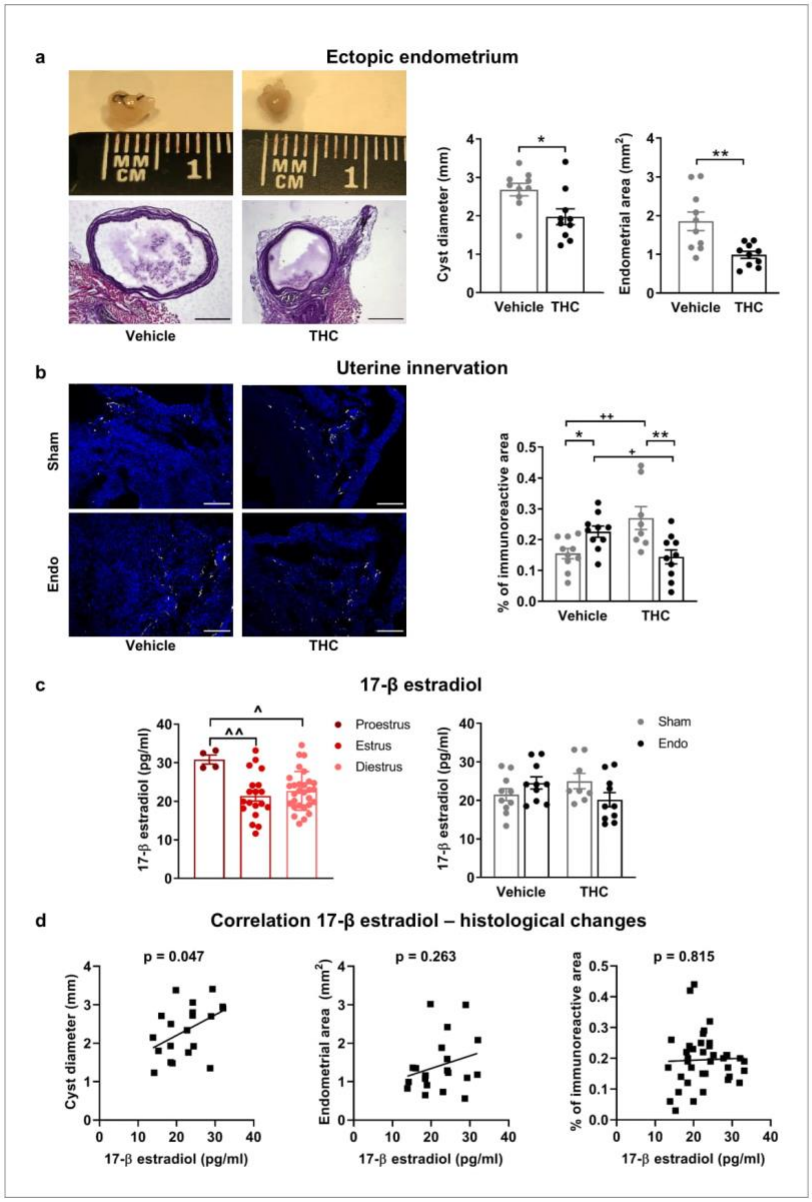


Figure 4. Effects of THC on the histological changes observed in mice with ectopic endometrium. (**a**) Ectopic endometrial growths of mice treated with THC were smaller (left graph) and had less endometrial tissue (right graph) than those of mice receiving vehicle. Scale bar = 1 mm. (**b**) THC increased

Figure 4 continued on next page

Figure 4 continued

innervation in sham mice but prevented uterine hyperinnervation in endometriosis mice. Blue is DAPI and white is β-III tubulin. Scale bar = 100 μm. (**c**) As expected, 17-β estradiol levels were higher in mice in proestrus (left). Estrogen levels were similar in all experimental conditions (right). (**d**) There was a positive correlation between cyst diameter and plasma levels of 17-β estradiol (left, r = 0.450). Absence of correlation of estrogen levels with cyst endometrial area (middle, r = 0.263) and uterine innervation (right, r = 0.039). THC dose: 2 mg/kg/day. Error bars are mean ± SEM. Student t-test (a, left graph), Mann Whitney U (a, right graph), two-way ANOVA + Bonferroni (**b**), mixed model + Bonferroni (c, left); Two-way ANOVA (c, right) and Pearson correlation (**d**). *p<0.05, **p<0.01 vs sham. +p<0.05, ++p<0.01 vs vehicle. ¯p<0.05, ¯¯p<0.01 vs proestrus. Endo, endometriosis; THC, Δ9-tetrahydrocannabinol.

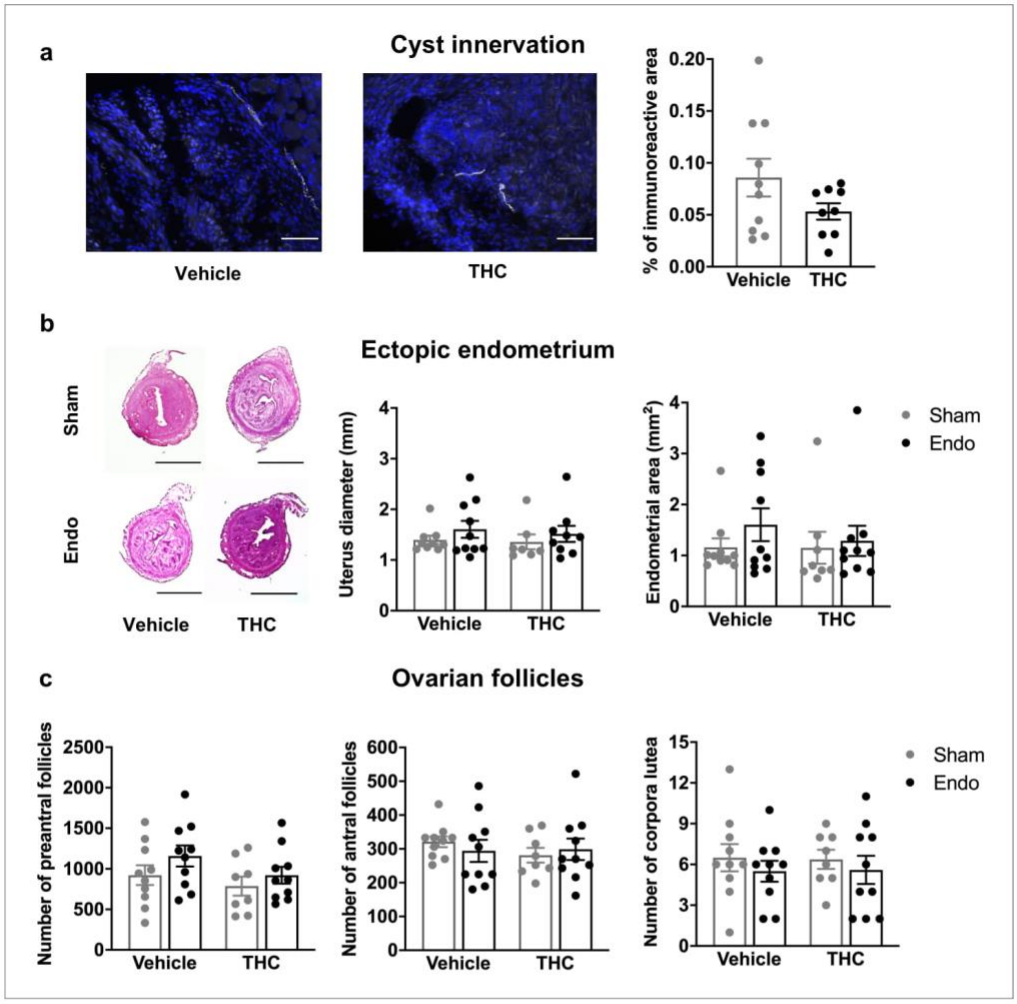


Figure 4—figure supplement 1. Histological features of reproductive tissues after chronic THC treatment. (**a**) Cyst innervation was unaffected by THC. Blue is DAPI and white is β-III tubulin. Scale bar = 100 μm. (**b**) Uterine diameter and area of endometrial tissue were similar among the groups. Scale bar = 1 mm. (**c**) Number of preantral follicles, antral follicles and corpora lutea were unchanged after endometriosis or THC treatment. Error bars are mean ± SEM. Student t-test (**a**) and two-way ANOVA (**b and c**). Endo, endometriosis; THC, Δ9-tetrahydrocannabinol.

Supplementary results I

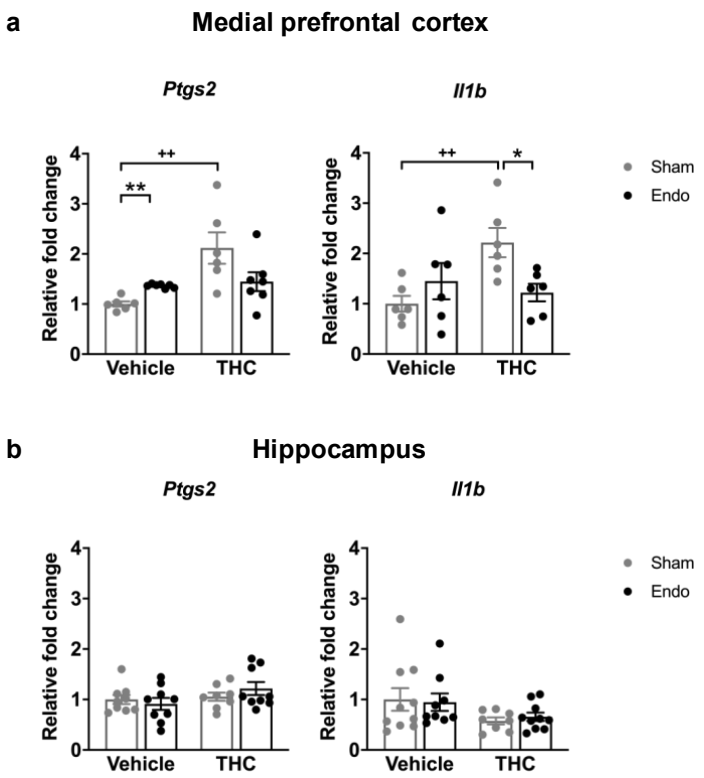
Pro-inflammatory effects of Δ9-tetrahydrocannabinol are absent in endometriosis mice

Alejandra Escudero-Lara, David Cabañero*, Rafael Maldonado*

Δ9-tetrahydrocannabinol increases mRNA levels of neuroinflammatory markers in medial prefrontal cortices of sham animals, but not in endometriosis mice

We found that Δ9-tetrahydrocannabinol (THC) produced amnesic effects in control animals and prevented these cognitive deficits in mice with ectopic endometrium. Since chronic pain is associated to local inflammatory events brain areas involved in memory processing such as the medial prefrontal cortex and the hippocampus (Del Rey *et al.*, 2011; Ong *et al.*, 2019), we investigated in these brain areas expression levels of genes related with inflammation, including the genes coding for cyclooxygenase 2 (*Ptgs2*) and interleukin 1 beta (*Il1b*).

Endometriosis mice treated with vehicle showed increased expression levels of *Ptg2* and a trend for increased levels of *Il1b* in the medial prefrontal cortex (Supplementary figure IA). As expected, sham mice receiving THC also showed significant overexpression of both markers (**Error! Reference source not found.**a). Interestingly, these effects of THC were absent in endometriosis mice, since levels of *Ptg2* and *Il1b* were unaltered after the THC treatment in these animals (Supplementary figure IA). These neuroinflammatory changes were not observed in the hippocampus (Supplementary figure IB). Hence, the presence of ectopic endometrium was associated with a pro-cognitive effect of THC exposure and with a decrease on the mRNA levels of cortical neuroinflammatory markers.



Supplementary figure I. Effects of THC on gene expression levels of *Ptgs2* and *Il1b* in the medial prefrontal cortex and the hippocampus of mice with ectopic endometrium. (**a**) Cortical expression levels of the genes coding for interleukin 1 beta (*Il1b*) and cyclooxygenase 2 (*Ptgs2*) increased after THC in sham mice but not in endometriosis mice. (**b**) Hippocampal expression levels of these genes remained unaltered. THC dose: 2 mg/kg/day for 32 days. Error bars are mean ± SEM. (a) Kruskal-Wallis + U Mann Whitney, (b) Kruskal-Wallis. *p<0.05, **p<0.01 vs sham. ++p<0.01 vs vehicle. Endo, endometriosis; THC, Δ9-tetrahydrocannabinol; Ptgs2, prostaglandin-endoperoxide synthase 2 (cyclooxygenase 2, COX2); Il1b, interleukin 1 beta.

Supplementary results II

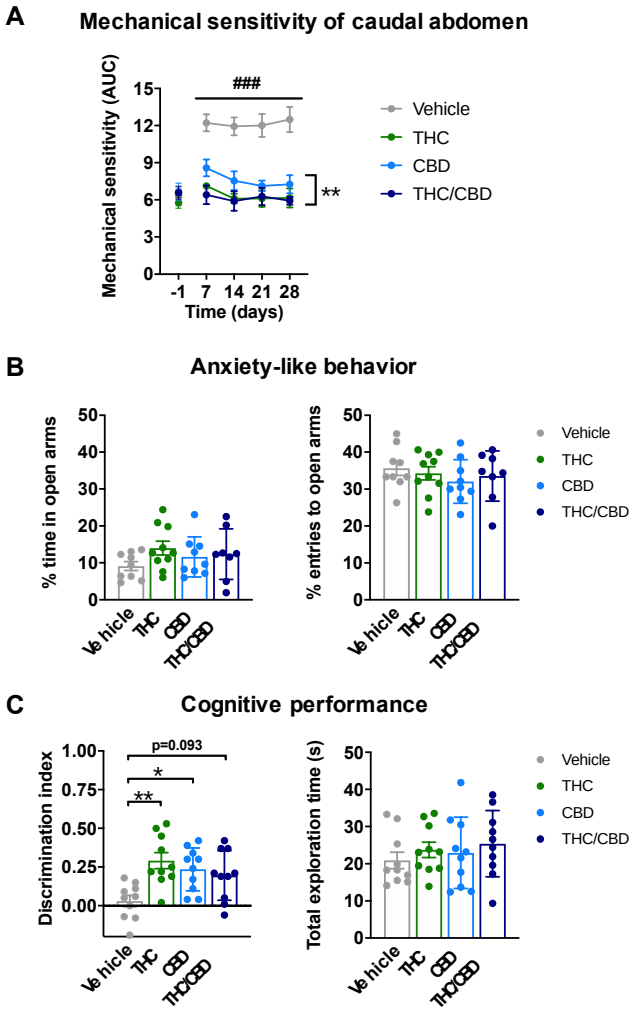
Δ9-tetrahydrocannabinol and cannabidiol inhibit endometriosis development and its manifestations

Alejandra Escudero-Lara, David Cabañero*, Rafael Maldonado*

Δ9-tetrahydrocannabinol and cannabidiol, alone and in combination, alleviate hypersensitivity in the caudal abdomen and prevent cognitive deficits associated to endometriosis

Female mice receiving implants of endometrial tissue (endometriosis mice) were treated with vehicle or daily doses of Δ9-tetrahydrocannabinol (THC, 2 mg·kg^{-1}), cannabidiol (CBD, 2 mg·kg^{-1}) or the combination of both (THC/CBD, 2 mg·kg^{-1}/2 mg·kg^{-1}) for 32 days, starting the first day after surgery. Mechanical sensitivity in the caudal abdomen, anxiety-like behavior and cognitive performance were evaluated to assess the effects of these phytocannabinoids on the behavioral manifestations of endometriosis.

Mice receiving vehicle developed a persistent punctate mechanical hypersensitivity in the caudal abdominal area that was measured through repeated application of von Frey filaments (Supplementary figure IIA). THC, CBD and THC/CBD treatments showed similar efficacy alleviating such mechanical hypersensitivity during the whole experiment (Supplementary figure IIA). Previous experiments comparing endometriosis and sham-operated mice treated with vehicle revealed that mechanical hypersensitivity in the caudal abdomen associated with ectopic endometrium was accompanied by increased anxiety-like behavior. THC, CBD or THC/CBD did not modify the percentage of time and entries to the open arms displayed by endometriosis mice in the elevated plus maze test (Supplementary figure IIB) indicating that none of the treatments affected the anxiety-like behavior associated to endometriotic growths.

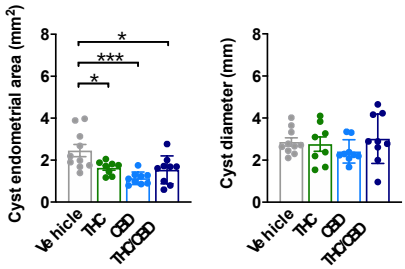


Supplementary figure II. Effects of THC, CBD and THC/CBD on the behavior of mice with ectopic endometrium. (**A**) Chronic THC, CBD and THC/CBD treatments alleviated mechanical hypersensitivity in the caudal abdominal area of endometriosis mice in the von Frey test. (**B**) Endometriosis-associated anxiety-like behavior was unaltered in the elevated plus maze test after 23 days of repeated administration of the three diferent cannabinoid treatments. (**C**) THC, CBD and THC/CBD improved cognitive performance in endometriosis mice in the novel object recognition test. Doses: THC (2 mg·kg^{-1}), CBD (2 mg·kg^{-1}),THC/CBD (2 mg·kg^{-1}/2 mgkg^{-1}). Error bars are mean ± SEM. (A) mixed model analysis + Bonferroni, (B, left and right panels) one-way ANOVA, (C, left panel) one-way ANOVA + Bonferroni, (C, right panel) Kruskal-Wallis. ###p<0.001 vs baseline. *p<0.05, **p<0.01 vs vehicle. THC, Δ9-tetrahydrocannabinol; CBD, cannabidiol; AUC, area under the curve.

Previous experiments showed that endometriosis mice treated with vehicle have discrimination indices close to 0 in the novel object recognition test. As previously described, THC prevented the cognitive decline associated with the presence of ectopic endometrium (Supplementary figure IIC). Interestingly, CBD also prevented this manifestation and the combination of THC/CBD induced similar effect, suggesting that these phytocannabinoids protect against cognitive impairments in mice with ectopic endometrial implants. Nevertheless, the THC/CBD combination did not induce significant improvement when compared to vehicle treated mice, possibly due to a ceiling effect obtained at these doses of THC and CBD.

Chronic exposure to Δ9-tetrahydrocannabinol and cannabidiol inhibits the growth of ectopic endometrial tissue

Surgically-implanted endometrium developed into cysts with glandular epithelium and stroma. Mice receiving THC, CBD and THC/CBD showed reduced endometrial area of their cystic lesions when compared to vehicle-treated mice, although the diameter of the cysts was similar regardless of the treatment (Supplementary figure III). Therefore, cannabinoid treatments inhibited the growth of ectopic endometrium.



Supplementary figure III. Effects of THC, CBD and THC/CBD on the behavior of mice with ectopic endometrium. Effects of THC, CBD and THC/CBD on ectopic endometrium. Ectopic endometrial growths of mice receiving cannabinoid treatments had less endometrial tissue than those of mice receiving vehicle (left graph), although cyst diameters were similar among groups (right graph). Doses: THC (2 mg·kg^{-1}), CBD (2 mg·kg^{-1}), THC/CBD (2 mg·kg^{-1}/2 mgkg^{-1}). Error bars are mean ± SEM. (left panel) Kruskal-Wallis + Mann Whitney U; (right panel) one-way ANOVA. *p<0.05, ***p<0.001 vs vehicle. THC, Δ9-tetrahydrocannabinol; CBD, cannabidiol.

Article #2

Surgical induction of endometriosis in female mice

Alejandra Escudero-Lara, David Cabañero*, Rafael Maldonado*

Bio-protocol **(2020)**

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Surgical Induction of Endometriosis in Female Mice

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#Contributed equally to this work

[Abstract] Endometriosis is a common gynecological disease characterized by the presence of endometrial tissue outside the uterine cavity. It is frequently associated with pain, infertility and a reduced quality of life, and it lacks adequate treatment. Several rodent models of endometriosis have been developed through heterologous and homologous transplantation of endometrial tissue into the abdominal compartment. Here we describe a surgical procedure to generate a syngeneic model of endometriosis in immunocompetent mice with intact uterine and ovarian tissues. In this model, four uterine fragments from a donor mouse at diestrus are sutured to the abdominal wall of a recipient mouse. One month after surgeries, endometrial implants develop into cysts with glandular epithelium and stroma, mimicking the endometriotic lesions observed in women with endometriosis. Therefore, this mouse model provides a valuable tool to study the pathophysiology of endometriosis and the efficacy of potential treatments.

Keywords: Endometriosis, Female, Mouse, Model, Surgery, Syngeneic

[Background] Endometriosis is a chronic gynecological condition defined by the growth of endometrial tissue outside the uterus, mainly in pelvic and abdominal surfaces (Zondervan *et al.*, 2018). It affects 10% of women in reproductive age and it is associated with pain, infertility and reduced quality of life (Fourquet *et al.*, 2011; Márki *et al.*, 2017; Zondervan *et al.*, 2018). Available pharmacological and surgical therapies for endometriosis have undesired effects and fail providing long-term alleviation of the symptoms (Falcone and Flyckt, 2018). Hence, new therapeutic strategies are needed, and their development relies on the establishment of animal models that recapitulate the pathophysiological and behavioral features of clinical endometriosis.

Endometriosis occurs spontaneously in human and nonhuman primates (D'Hooghe *et al.*, 1992; Hadfield *et al.*, 1997). In baboons, endometriosis has also been experimentally induced by injection of menstrual effluent into the pelvis (D'Hooghe *et al.*, 1995), resulting in a model that closely mimics endometriosis in women. However, the use of nonhuman primates is limited by economical and ethical issues (D'Hooghe *et al.*, 2009). In this scenario, rodents are cost-effective and widely available, representing a very useful tool for endometriosis research (Grümmer, 2006). Different heterologous and homologous rodent models have been developed to study the etiology of endometriosis and the efficacy

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of compounds on its development and symptoms. On the one hand, heterologous models involve xenotransplantation of human endometrial tissue–ectopic, eutopic or cultured stromal and epithelial cells–into immunodeficient mice (Greaves *et al.*, 2017). These models allow the evaluation of potential therapies on endometriotic lesions that maintain human histological characteristics (Hull *et al.*, 2008), however, the lack of a normal immunological response represents an important limitation for the study of this chronic inflammatory condition. On the other hand, homologous models have been developed through surgical transplantation or injection of endometrial tissue from the same animal or a syngeneic donor into the peritoneal cavity of immunocompetent animals (Vernon and Wilson, 1985; Somigliana *et al.*, 1999; Fattori *et al.*, 2020). Syngeneic transplantation of endometrial tissue avoids complications related to the resection of one uterine tissue from the same animal and allows studying the contribution of host and donor cells on endometriosis (Zhao *et al.*, 2016). Among syngeneic models, the one presented here involves suturing of uterine fragments and has the advantage of high reproducibility of the lesions, allowing easy identification and size comparison when testing disease-modifying drugs.

Here we describe the implementation of a syngeneic surgical model of endometriosis in immunocompetent mice with intact uterine tissue and ovarian function. This model reproduces the ectopic endometrial growths observed in clinical endometriosis as well as some of its symptoms, including persistent pain hypersensitivity in the lower abdomen, anxiety-related behavior and potential cognitive deficits (Escudero-Lara *et al.*, 2020). Furthermore, we have used this model to show the efficacy of Δ9-tetrahydrocannabinol limiting the development of endometriosis and its pain-related manifestations (Escudero-Lara *et al.*, 2020). Since the present approach allows the use of immunocompetent mice with an unaltered estrous cycle, we consider this resource a model with high construct and face validity, valuable for identification and assessment of novel therapeutic strategies for endometriosis.

Materials and Reagents

1. 24 x 60 mm coverslips (Deltalab, Euroturbo®, catalog number: D102460)
2. 200 µl pipette tips (Daslab, catalog number: 162006)
3. Microscope slides (Avantor, VWR™, catalog number: 631-1553)
4. Rolled cotton 100% (Acofarma, Acofar®, catalog number: 4957051)
5. Iodine-povidone (Meda Pharma SAU, Betadine®, catalog number: 716720.4)
6. No. 15 Sterile Disposable Scalpels (Swann Morton, catalog number: 0505)
7. Petri dishes (BD, Falcon™, catalog number: 353001)
8. 0.7 METRIC, 45 cm (6-0 18") silk sutures (Alcon, catalog number: 184801)
9. 26 G needles (BD, Microlance™, catalog number: 300300)
10. 1 ml syringes (BD, Plastipak™, catalog number: 303172)
11. Reaction tubes, 1.5 ml (Sarstedt, catalog number: 72.690.001)
12. Isoflurane-absorbing charcoal filter (Bickford, OMNICON f/air, catalog number: 80120)
13. 8-week-old female C57Bl/6J mice (Charles Rivers Laboratories, JAX™, strain code: 632)

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Note: Housed in cages of four to five mice with water and food available ad libitum. Housing conditions are maintained at 21 ± 1 °C and 55 ± 10% relative humidity in a 12 h light/dark cycle (light on from 8 AM to 8 PM).

14. Saline solution 0.9% sodium chloride (ERN, Vitulia, catalog number: 999791.5)
15. Distilled water (dH_2O)
16. Medical Oxygen (Abelló Linde, Conoxia®, catalog number: 652547.0)
17. Isoflurane (Virbac, Vetflurane®, catalog number: 575837-4)
18. Ophthalmic ointment (Nicox, Xilin, catalog number: 171324)
19. Optimal cutting temperature compound (O.C.T) (Sakura Finetek, Tissue-Tek® O.C.T.™, catalog number: 4583)
20. Cryomolds (Sakura Finetek Tissue-Tek® Cryomold®, catalog number: 4565)
21. Papanicolaou's solution 1a Harris' hematoxylin solution (Merck Millipore, catalog number: 104302)
22. Eosin G or Y 0.5% alcoholic (Diapath, catalog number: C0353)
23. Xylene Cyanol FF (Sigma-Aldrich, catalog number: X4126)
24. Mounting medium for microscopy (Deltalab, VITROCLUD®, catalog number: A20250)
25. Gelatin (Sigma-Aldrich, catalog number: G9391)
26. $KCr(SO_4)_2 \cdot 12H_2O$ (Merck Millipore, EMSURE®, catalog number: 101036)
27. $NaH_2PO_4 \cdot 2H_2O$ (Sigma-Aldrich, catalog number: 71505)
28. $Na_2HPO_4 \cdot 12H_2O$ (Sigma-Aldrich, catalog number: 71649)
29. NaCl (Sigma-Aldrich, catalog number: S7653)
30. PFA (Merck Millipore, catalog number: 104005)
31. NaOH (Merck Millipore, catalog number: 109137)
32. Sodium azide (Sigma-Aldrich, catalog number: S2002)
33. Sucrose (Merck Millipore, catalog number: 107687)
34. 100% ethanol (Merck Millipore, EMSURE®, catalog number: 1009831)
35. Gentamicin (Laboratorios Normon, Genta-Gobens®, catalog number: 999037.7)
36. Meloxicam (Boehringer Ingelheim, Metacam®, catalog number: 210028)
37. Methylene blue (Scharlau, EsssentQ®, catalog number: AZ02030025)
38. Gelatinized microscope slides (see Recipes)
39. 0.3 mg/ml gentamicin (see Recipes)
40. 0.2 mg/ml meloxicam (see Recipes)
41. 0.01 M phosphate buffered saline (PBS) (see Recipes)
42. 4% paraformaldehyde (PFA) (see Recipes)
43. Cryoprotectant solution: 30% sucrose with 0.1% sodium azide (see Recipes)
44. 96% and 70% ethanol (see Recipes)
45. 0.01% methylene blue in saline solution (see Recipes)

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Equipment

1. P20 pipette (Gilson, model: PIPETMAN® P20, catalog number: F123600)
2. Plastic staining jars and baskets (Brand, Brand®, catalog number: 471800)
3. Light microscope (Leica Microsystems, model: DM6000 B) with camera (Leica Microsystems, model: DFC300 FX)
4. Anesthesia circuit including isoflurane vaporizer (Midmark, Isoflurane VIP 3000®-Well-Fill, catalog number: 91305430), tubing, regulating valves, nose cones and induction chamber (Midmark, catalog number: 93805108) (Figure 1)
5. Cold light source (Leica Microsystems, model: CLS 50 X)
6. Electric hair-clipper (BBraun, Aesculap®, model: ISIS, catalog number: GT421)
7. Heating pads (Daga, catalog number: N2P 220-230)
8. Small serrated-semi curved tip forceps (Allgaier Instrumente, A-line endoscopy, catalog number: 08-515-005)
9. Haemostatic forceps (Allgaier Instrumente, A-line endoscopy, catalog number:13-028-120)
10. Rounded tip scissors (Medicon, catalog number: 02.50.62)
11. Dissection scissors (Medicon, catalog number: 02.70.14)
12. Microdissection scissors (Dimeda, catalog number: 09.102.11)
13. Cryostat (Leica Biosystems, model: CM3050)
14. Macro Zoom Microscope (Olympus, model: MVX10) with camera (Olympus, model: DP71)
15. -80 °C freezer (Thermo Fisher, -86 C ULT Freezer, model: 917)

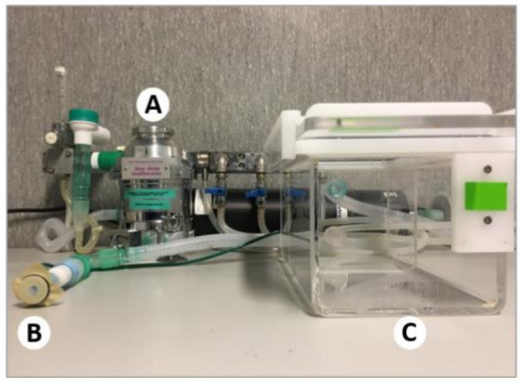

Figure 1. Anesthesia circuit. A. Isoflurane vaporizer. B. Nose cone. C. Induction chamber.

Software

1. Leica Application Suite software v4.0 (Leica Microsystems, www.leica-microsystems.com)

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2. Cell^D Life Science documentation software (Matrix Optics, www.matrixoptics.com)

Procedure

A. Selection of donors in diestrus: estrous cycle determination
   1. Place the mouse on the cage grid, and gently pull the tail backwards to induce the mouse gripping the grid and pulling forward. With the other hand, grasp the mouse by the scruff of the neck and pin the tail between the palm and the fourth finger of the hand holding the scruff.
   2. Place the pipette tip at the opening of the vaginal canal and flush the vagina 5 times with 20 µl of saline solution in and out.
   3. Place the resulting fluid onto a gelatinized slide.
   4. Let the drop air-dry.
   5. Stain with 0.01% methylene blue for 2 min.
   6. Wash twice in distilled water for 2 min.
   7. Let air-dry.
   8. Take images of stained cells using the 40x objective of a light microscope to determine the stage of the estrous cycle (Figure 2):
      a. In proestrus, most cells are nucleated.
      b. In estrus, mostly cornified epithelial cells are present.
      c. In metestrus, cornified epithelial cells and leukocytes are found.
      d. In diestrus, leukocytes are predominant, and some nucleated and cornified epithelial cells are present.

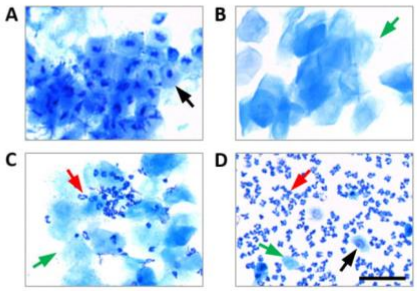

Figure 2. Vaginal cytology from mice in each stage of the estrous cycle. A. Proestrus. B. Estrus. C. Metestrus. D. Diestrus. Nucleated epithelial cells, cornified epithelial cells, and leukocytes are indicated by black, green and red arrows, respectively. Scale bar = 50 µm.

B. Obtention of endometrial and control tissues: excision of uterine fragments and abdominal fat
   1. Euthanize the donor mouse in diestrus by cervical dislocation.

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2. Make a midline incision on the abdomen using a scalpel and excise the uterus (Figures 3A and 3B).
3. Place the uterus in a Petri dish containing cold saline solution (4 ºC, on ice) (Figure 3C).
4. Place abdominal fat of a size similar to the uterus in another Petri dish containing cold saline solution (Figure 3D).
5. Strip one excised uterine horn and open it longitudinally with microdissection scissors, exposing the endometrium (Figures 3E and 3F).
   Note: Keep the other uterine horn unopened and in cold saline solution until used to induce endometriosis on a second recipient mouse.
6. Use a new scalpel to cut the uterine horn or the fat in four squares with each side measuring 2 x 2 mm (Figures 3G and 3H).
7. Keep the uterine fragments in cold saline solution until needed.

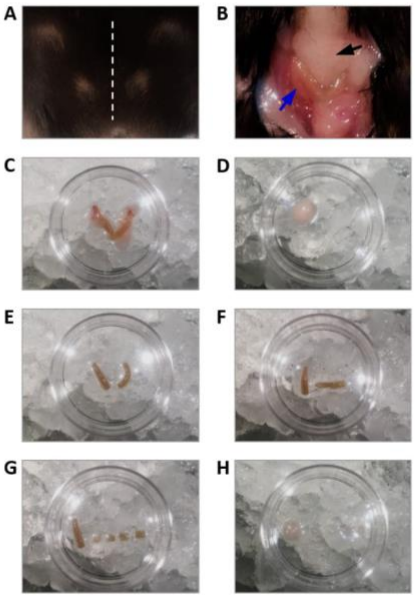

Figure 3. Excision of uterine fragments and abdominal fat. A. A midline incision (dashed line) is made on the abdomen of the euthanized donor mouse. B. The uterus (blue arrow) and the abdominal fat (black arrow) are exposed through the incision. C. The uterus is excised and placed in a Petri dish containing cold saline solution. D. Abdominal fat tissue is also excised and placed in another Petri dish containing cold saline solution. E. The uterine horns are stripped. F. One uterine horn is opened longitudinally, exposing the endometrium. G. The opened uterine horn is cut in four fragments of 2 x 2 mm that are used for the endometriosis surgery. H.

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Abdominal fat is also cut in similar-sized fragments that are used for the control surgery.

C. Surgical induction of endometriosis: peritoneal implantation of ectopic endometrium
1. Anesthetize the recipient mouse with 4% V/V isoflurane in medical oxygen (2 L/min) in the induction chamber.
2. Once anesthetized, place the mouse on top of a heating pad and maintain anesthesia with 2.5% V/V isoflurane in medical oxygen (2 L/min) using a nose cone.
3. Apply ophthalmic ointment to avoid drying of the eyes.
4. Shave the abdomen of the mouse using an electric hair-clipper.
5. Clean the skin with a piece of cotton soaked in iodine-povidone solution (Figure 4A).
6. Make a 1 cm midline incision on the skin using a scalpel, ending approximately 0.5 cm cranial to the vaginal ending.
7. Insert closed rounded tip scissors between the skin and the abdominal wall and open them to detach the skin from the muscle.
8. Make a 1 cm incision on the abdominal wall using the small scissors and use blunt forceps to expose its inner surface (Figure 4B).
9. Suture the uterine fragments into the abdominal wall (two on each side of the incision) with loose square knots of 6-0 black silk. Uterine fragments must be sutured with the endometrial surface facing the peritoneal cavity (Figures 4C and 4D).
10. Close the muscular abdominal wall with three loose square knots of 6-0 black silk (Figure 4E).
11. Close the skin with three square knots of 6-0 black silk.
12. Clean the skin with a piece of cotton soaked in povidone-iodine solution (Figure 4F).
13. Leave the animal lying over one side in a clean cage partially placed over a heating pad until it recovers.

Notes:

a. *Whenever the use of analgesics and antibiotics does not interfere with the purpose of the experiment, give meloxicam (2 mg/kg, subcutaneously, injection volume 10 ml/kg) and gentamicin (1 mg/kg, intraperitoneally, injection volume 3 ml/kg) right after surgery and 24 h later.*

b. *To induce endometriosis to a second recipient mouse, repeat the procedure from Step B4 using the remaining uterine horn excised from the same donor mouse.*

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Figure 4. Surgical induction of endometriosis. A. The abdomen of an anesthetized recipient mouse is shaved and cleaned with iodine-povidone solution. B. The inner surface of the abdominal wall is exposed through a 1 cm incision. C and D. Two fragments of uterine tissue or fat from a donor mouse are sutured on each side of the incision. E. The muscular abdominal wall is closed with three loose square knots. F. The skin is closed with three square knots and cleaned with iodine-povidone solution.

D. Control mice: peritoneal implantation of abdominal fat

To obtain control mice repeat the procedure from Section C, substituting the Step C9 by suturing the fat fragments into the abdominal wall (two on each side of the incision) with loose square knots of 6-0 black silk.

E. Confirmation of endometriosis: histology of endometriotic lesions

Note: Establish the time of necropsy according to the purpose of the experiment. Control mice should not develop endometriotic lesions.

1. Perform a vaginal lavage as described in Section A if the stage of the estrous cycle at the time of sample collection needs to be considered.
2. Euthanize the mouse by cervical dislocation.
3. Use dissection scissors to make a dorsal incision in the back of the mouse at the level of the pelvis and peel the skin (Figure 5A).
4. Remove the spine from S1 to L1 by making a first cut at the level of the most caudal rib and a second cut 1 cm caudal to the first one. Then, cut both sides along the spinal column (Figure 5A).

   Note: Spinal cord and dorsal root ganglia can be isolated from the extracted fragment of spinal column and used for biochemical and immunohistochemical analyses.

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5. Carefully pull out the intestines to allow the examination of the abdominal wall (Figure 5B).
6. Excise the cystic endometriotic lesions and remove the surrounding fat tissue using dissection scissors and forceps. Special care should be taken to avoid lancing the fluid-filled cysts (Figure 5C).
7. Place the endometriotic lesions in reaction tubes with paraformaldehyde 4% and allow tissue fixation for 4 h at 4 °C.
8. Wash 3 x 5 min with 0.01 M PBS at room temperature.
9. Keep samples in sucrose 30% with 0.1% sodium azide for 6 days to cryoprotect the tissue.
10. Take the samples out of the sucrose solution, remove sucrose excess with filter paper, embed in cryomolds filled with O.C.T. and store at -80 °C.
11. Section samples at 20 µm with a cryostat and mount onto gelatinized slides. Store at -20 °C.
12. Stain with hematoxylin and eosin:
    a. Leave the slides in staining baskets and allow them to thaw.
    b. Sink in 100% ethanol for 5 min.
    c. Sink in 96% ethanol for 5 min.
    d. Sink in 70% ethanol for 5 min.
    e. Wash with distilled water.
    f. Immerse in Papanicolaou's solution 1a Harris' hematoxylin solution for 5 min.
    g. Wash in distilled water for 2 min.
    h. Immerse in Eosin G or Y 0.5% alcoholic for 30 s.
    i. Sink in 70% ethanol for 5 min.
    j. Sink in 96% ethanol for 5 min.
    k. Sink in 100% ethanol for 5 min.
    l. Clear with Xylene Cyanol FF for 5 min.
    m. Mount coverslip with mounting medium for microscopy.
    n. Allow to dry horizontally for 3 days.
13. Capture images of stained sections using a macro zoom microscope for assessment of diameter and histological features (Figure 5D).

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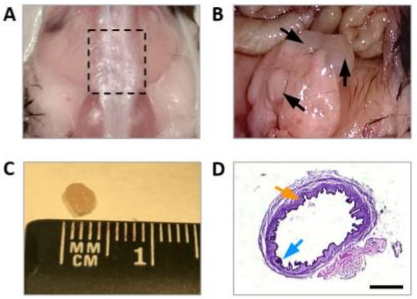

Figure 5. Endometriotic lesions recovered 32 days after surgical induction of endometriosis. A. Removal of the spine from S1 to L1 (dashed lines). B. Uterine fragments implanted into the abdominal wall develop into cysts. C. Endometriotic cyst of approximately 2.5 mm diameter. D. Hematoxylin and eosin stained section of an endometriotic lesion showing the presence of stroma (orange arrow) and glandular epithelium (blue arrow). Scale bar = 1 mm.

Notes

1. Use different scalpels and silk sutures for uterine fragments or fat, and for muscle and skin.
2. Mice should be monitored daily for at least 3 days after surgeries. If a mouse removes the skin sutures, briefly anesthetize with isoflurane (4% V/V for induction, 2.5% V/V for maintenance), wash the incision site with saline solution and close the skin with 6-0 black silk square knots. Afterwards, disinfect with iodine-povidone solution.
3. Mice should be supervised following an adaptation of the Morton and Griffiths guidelines on the recognition of pain, distress and discomfort (Morton and Griffiths, 1985). This adapted protocol considers four variables, and a score is assigned for each variable:
   a. Weight loss:
      0 Normal. There is no weight loss or the animal grows normally.
      1 Weight loss less than 10%.
      2 Weight loss between 10 and 20%. Alteration in the appearance or amount of stool.
      3 Weight loss greater than 20%. The animal does not consume water or food.
   b. Coat appearance:
      0 Normal.
      1 Hair in poor condition.
      2 Hair in poor condition and ocular or nasal secretions.
      3 Piloerection.
   c. Movement/posture/behavior of the animal:
      0 Normal.
      1 Small changes.

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2 Moderate changes.

3 Inactivity, aggressiveness, self-mutilation or vocalizations.

d. Aspect of the incision site:

0 Normal.

1 Slight redness.

2 Local edema.

3 Infection, darkening (signs of necrosis).

The final score is calculated as the sum of the scores obtained for each variable, and it determines the application of corrective actions:

a. 0 to 3. The mouse does not require any corrective action.

b. 4 to 7. The mouse requires the application of corrective actions.

c. Equal to 8. The mouse should be euthanized.

*Euthanasia will be practiced if the score for any of the variables is 3.

Corrective measures include cleaning the wound with disinfectant and applying topical antibiotic, placing the cage on top of a heating pad and supplying nutrient-enriched hydrating gel or food pellets soaked in water to facilitate feeding.

Recipes

1. Gelatinized microscope slides
   a. Prepare 0.5% gelatin-coating solution
      0.5 g gelatin
      0.5 g $KCr(SO_4)_2 \cdot 12H_2O$
      dH_2O up to 400 ml
      stir at 50 °C until dissolved
   b. Gelatinize slides
      Place the slides in staining baskets
      Soak the baskets 3 x 30 s in 0.5% gelatin-coating solution, waiting 30 s between immersions
      Blot excess solution onto filter paper and cover the baskets containing the slides with aluminum foil
      Dry in an incubator at 37 °C for 24 h
2. 0.3 mg/ml gentamicin
   31.25 µl gentamicin
   5 ml saline solution 0.9% sodium chloride
3. 0.2 mg/ml meloxicam
   0.2 ml meloxicam
   4.8 ml saline solution 0.9% sodium chloride
4. 0.01 M phosphate buffered saline (PBS)
   a. Prepare Solution A: 0.2 M sodium phosphate monobasic

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3.12 g $NaH_2PO_4 \cdot 2H_2O$
dH_2O up to 100 ml

b. Prepare Solution B: 0.2 M sodium phosphate dibasic
28.65 g $Na_2HPO_4 \cdot 12H_2O$
dH_2O up to 400 ml

c. Prepare 0.1 M phosphate buffer (PB), pH 7.4
100 ml Solution A
400 ml Solution B
500 ml dH_2O

d. Prepare 0.1 M PBS
9 g NaCl
1,000 ml 0.1 M PB

e. Prepare 0.01 M PBS
100 ml 0.1 M PBS
1,000 ml dH_2O

5. 4% paraformaldehyde (PFA)
10 g PFA
1 ml 1 M NaOH
0.1 M PB (see Recipe 3) up to 250 ml

6. Cryoprotectant solution: 30% sucrose with 0.1% sodium azide
a. Prepare 0.01 M PBS (see Recipe 3)
b. Prepare 20% sodium azide solution
2 g sodium azide
dH_2O up to 10 ml
c. Prepare cryoprotectant solution
30 g sucrose
0.5 ml 20% sodium azide
0.01 M PBS up to 100 ml

7. 96% and 70% ethanol
a. 96% ethanol
480 ml 100% ethanol
20 ml dH_2O
b. 70% ethanol
350 ml 100% ethanol
150 ml dH_2O

8. 0.01% methylene blue in saline solution
2 mg methylene blue
20 ml saline solution 0.9% sodium chloride

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This protocol was adapted from an established published procedure to induce endometriosis to rats (Somigliana *et al.*, 1999), and has already been used by our group (Escudero-Lara *et al.*, 2020).

Competing interests

The authors declare no conflicts of interest.

Ethics

Animal experimentation: All animal procedures were conducted in accordance with standard ethical guidelines (European Communities Directive 2010/63/EU and NIH Guide for Care and Use of Laboratory Animals, 8th Edition) and approved by autonomic (Generalitat de Catalunya, Departament de Territori i Sostenibilitat) and local (Comitè Ètic d'Experimentació Animal, CEEA-PRBB) ethical committees.

References

1. D'Hooghe, T. M., Bambra, C. S., Isahakia, M. and Koninckx, P. R. (1992). Evolution of spontaneous endometriosis in the baboon (*Papio anubis*, *Papio cynocephalus*) over a 12-month period. *Fertil Steril* 58(2): 409-412.
2. D'Hooghe, T. M., Bambra, C. S., Raeymaekers, B. M., De Jonge, I., Lauweryns, J. M. and Koninckx, P. R. (1995). Intrapelvic injection of menstrual endometrium causes endometriosis in baboons (*Papio cynocephalus* and *Papio anubis*). *Am J Obstet Gynecol* 173(1): 125-134.
3. D'Hooghe, T. M., Kyama, C. M., Chai, D., Fassbender, A., Vodolazkaia, A., Bokor, A. and Mwenda, J. M. (2009). Nonhuman primate models for translational research in endometriosis. *Reprod Sci* 16(2): 152-161.
4. Escudero-Lara, A., Argerich, J., Cabanero, D. and Maldonado, R. (2020). Disease-modifying effects of natural Delta9-tetrahydrocannabinol in endometriosis-associated pain. *Elife* 9: e50356.
5. Falcone, T. and Flyckt, R. (2018). Clinical Management of Endometriosis. *Obstet Gynecol* 131(3): 557-571.
6. Fattori, V., Franklin, N. S., Gonzalez-Cano, R., Peterse, D., Ghalali, A., Madrian, E., Verri, W. A., Jr., Andrews, N., Woolf, C. J. and Rogers, M. S. (2020). Nonsurgical mouse model of

bio-protocol www.bio-protocol.org/e3763 Bio-protocol 10(18): e3763. DOI:10.21769/BioProtoc.3763

endometriosis-associated pain that responds to clinically active drugs. *Pain* 161(6): 1321-1331.

7. Fourquet, J., Baez, L., Figueroa, M., Iriarte, R. I. and Flores, I. (2011). Quantification of the impact of endometriosis symptoms on health-related quality of life and work productivity. *Fertil Steril* 96(1): 107-112.
8. Greaves, E., Critchley, H. O. D., Horne, A. W. and Saunders, P. T. K. (2017). Relevant human tissue resources and laboratory models for use in endometriosis research. *Acta Obstet Gynecol Scand* 96(6): 644-658.
9. Grümmer, R. (2006). Animal models in endometriosis research. *Hum Reprod Update* 12(5): 641-649.
10. Hadfield, R. M., Yudkin, P. L., Coe, C. L., Scheffler, J., Uno, H., Barlow, D. H., Kemnitz, J. W. and Kennedy, S. H. (1997). Risk factors for endometriosis in the rhesus monkey (*Macaca mulatta*): a case-control study. *Hum Reprod Update* 3(2): 109-115.
11. Hull, M. L., Escareno, C. R., Godsland, J. M., Doig, J. R., Johnson, C. M., Phillips, S. C., Smith, S. K., Tavare, S., Print, C. G. and Charnock-Jones, D. S. (2008). Endometrial-peritoneal interactions during endometriotic lesion establishment. *Am J Pathol* 173(3): 700-715.
12. Márki, G., Bokor, A., Rigo, J. and Rigo, A. (2017). Physical pain and emotion regulation as the main predictive factors of health-related quality of life in women living with endometriosis. *Hum Reprod* 32(7): 1432-1438.
13. Morton, D. B. and Griffiths, P. H. (1985). Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *Vet Rec* 116(16): 431-436.
14. Somigliana, E., Vigano, P., Rossi, G., Carinelli, S., Vignali, M. and Panina-Bordignon, P. (1999). Endometrial ability to implant in ectopic sites can be prevented by interleukin-12 in a murine model of endometriosis. *Hum Reprod* 14(12): 2944-2950.
15. Vernon, M. W. and Wilson, E. A. (1985). Studies on the surgical induction of endometriosis in the rat. *Fertil Steril* 44(5): 684-694.
16. Zhao, Y., Chen, Y., Kuang, Y., Bagchi, M. K., Taylor, R. N., Katzenellenbogen, J. A. and Katzenellenbogen, B. S. (2016). Multiple Beneficial Roles of Repressor of Estrogen Receptor Activity (REA) in Suppressing the Progression of Endometriosis. *Endocrinology* 157(2): 900-912.
17. Zondervan, K. T., Becker, C. M., Koga, K., Missmer, S. A., Taylor, R. N. and Vigano, P. (2018). Endometriosis. *Nat Rev Dis Primers* 4(1): 9.

Article #3

Kappa opioid modulation of endometriosis pain in mice

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Kappa opioid modulation of endometriosis pain in mice

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Abstract

Endometriosis is an estrogen-dependent disease that affects 1 in 10 women in reproductive age and is characterized by the presence of endometrial tissue in extrauterine locations. Its main symptom is chronic pelvic pain that may lead to emotional and cognitive disorders and the neurobiological substrate of these manifestations remain unclear. The kappa opioid receptor is a constituent of the endogenous opioid analgesia system widely expressed in somatosensory nervous pathways and also in endometrial tissues. This work investigates the possible involvement of kappa opioid receptor on the nociceptive, behavioral and histopathological manifestations of endometriosis in a murine model. Female mice receiving endometrial implants develop a persistent mechanical hypersensitivity in the pelvic area that is stronger during the estrus phase of the estrous cycle. The kappa opioid receptor agonist U50,488H produces a dose-dependent relief of this mechanical hypersensitivity, regardless of the cycle phase. Repeated exposure to a low dose of U50,488H (1 mg/kg/day s.c. for one month) provides sustained relief of mechanical hypersensitivity, without tolerance development or sedative side effects. Interestingly, this treatment also inhibits a decreased rearing behavior associated with spontaneous pain or discomfort in endometriosis mice. This KOR-mediated pain relief does not prevent the anxiety-like behavior or the cognitive impairment exhibited by endometriosis mice, and the growth of endometriotic cysts is also unaltered. These data provide evidence of strong pain-relieving properties of kappa opioid receptor stimulation in female mice with endometriosis pain. The persistence of affective

and cognitive manifestations suggests that these comorbidities are independent of pelvic pain and simultaneous treatment of these co-morbidities may be necessary for successful management of endometriosis.

Highlights

- Endometriosis mice have increased pelvic mechanical sensitivity during estrus
- Kappa opioid stimulation relieves evoked and spontaneous endometriosis pain
- Endometriosis and kappa opioid agonism disrupt long-term memory in female mice
- Kappa opioid pain relief does not avoid anxiety-like behavior of endometriosis mice
- Low doses of the kappa opioid agonist U50,488H lack sedative effects in female mice
- Exogenous kappa opioid receptor stimulation does not modify endometriosis lesions

Keywords

Endometriosis, chronic pelvic pain, kappa opioid receptor, U50,488H, female

1. Introduction

Endometriosis is a chronic disease defined by the presence of endometrial tissue outside the uterine cavity. It affects 10% of women in reproductive age and is associated with infertility and chronic pelvic pain (Zondervan et al., 2020). This persistent pain is often associated with emotional and cognitive disorders (Fourquet et al., 2011). Current endometriosis treatments involve hormonal and surgical treatments that produce important unwanted side effects such as psychiatric alterations or persistent post-surgical pain (Garry, 2004; Ross and Kaiser, 2017). 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 endometriosis pain and its associated co-morbidities. Recent murine models of endometriosis reproduce the behavioral and histopathological features of endometriosis in women (Arosh et al., 2015; Escudero-Lara et al., 2020a, 2020b) and represent a novel approach for the elucidation of endometriosis mechanisms and the possible development of therapeutic alternatives.

The endogenous opioid system plays a fundamental role in the limitation of pain (Nadal et al., 2013; Ossipov et al., 2010; Przewłocki and Przewłocka, 2001) and is implicated in the physiological control of emotional (Lutz and Kieffer, 2013) and cognitive responses (Izquierdo et al., 1980). It is integrated by the endogenous opioid peptides and the opioid receptors mu (MOR), delta (DOR), and kappa (KOR) (Kieffer, 1995). Among the opioid receptors, KOR play a crucial role in visceral and inflammatory pain (Kivell and Prisinzano, 2010; Vanderah, 2010)

and their stimulation produces greater analgesia in women than in men (Gear et al., 1996b). KOR agonists reduce abdominal pain thresholds in patients of non-ulcer dyspepsia (Fraitag et al., 1994) and irritable bowel syndrome (Dapoigny et al., 1995; Delvaux et al., 1999) and KOR activation reduces visceral nociception in multiple rodent models of visceral and inflammatory pain (Kamp et al., 2003; Rivière et al., 1993; Sengupta et al., 1999; Su et al., 1997; Tao et al., 2008). Moreover, KOR expression has been described in endometrial tissues of female rodents and women (Chatzaki et al., 2000; Makrigiannakis et al., 1992; Petraglia et al., 1986; Wahlström et al., 1985; Zhu et al., 1998). Despite all the evidence on the role of KOR in pain processing specially in females, the possible involvement of this opioid receptor in endometriosis is still unknown.

The aim of this study was to evaluate the efficacy of KOR activation in modulating the nociceptive, behavioral and histopathological manifestations of endometriosis using a murine model of endometriosis that mimics the alterations found in the clinical settings (Escudero-Lara et al., 2020a, 2020b). The selective KOR agonist U50,488H was used to assess its effects on pelvic sensitivity, anxiety-like behavior and cognitive impairment associated with endometriosis. Locomotor activity and motor coordination were also evaluated to identify possible sedative effects and decreases in rearing behavior were used as a surrogate measure of spontaneous abdominal pain or discomfort. The possible implication of KOR in the development of endometriosis lesions was also evaluated by measuring the sizes of ectopic endometrial growths.

2.Materials and methods

2.1.Animals

C57BL/6J female mice (Charles Rivers, Lyon, France), 8-week-old at the beginning of the experiment, were used. Mice were housed in cages of 4 to 5 mice with ad libitum access to water and food. Housing conditions were maintained at 21 ± 1°C and 55 ± 10% relative humidity in a controlled light/dark cycle (light on between 07:30 AM and 07:30 PM). Animals were habituated to housing conditions and handling for 1 week before the start of the experiments. All experimental procedures and animal husbandry were conducted following the ARRIVE guidelines, in accordance with standard ethical guidelines (European Communities Directive 2010/63/EU and NIH Guide for Care and Use of Laboratory Animals, 8th Edition) and approved by regional (Generalitat de Catalunya, Departament de Territori i Sostenibilitat) and local (Comitè Ètic d'Experimentació Animal, CEEA-PRBB) ethical committees. Sample size was based on previous studies in our laboratory using comparable behavioral approaches (Carcolé et al., 2019; Escudero-Lara et al., 2020a; La Porta et al., 2016). Whenever possible, experiments were performed blinded for surgical and pharmacological conditions, and treatments were randomized between groups.

2.2. Drugs

The KOR agonist U50,488H (trans-(1S,2S)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl] benzeneacetamide hydrochloride hydrate;

U111, Sigma Aldrich, St. Louis, MO, USA) was diluted in saline and was administered subcutaneously in a volume of 10 ml/kg.

2.3. Estrous cycle determination

The phase of the estrous cycle was determined by histological examination of vaginal smears, as previously described (Escudero-Lara et al., 2020b). Briefly, mice were gently restrained and 20 µl of saline were flushed into the vagina. The resulting smear was placed onto gelatinized slides, dried, stained with methylene blue and observed at 40X magnification under a light microscope (DM6000 B, Leica Biosystems, Nussloch, Germany).

2.4. Surgical induction of endometriosis

Endometriotic lesions were surgically induced as previously described (Escudero-Lara et al., 2020b). Briefly, uterine horns from donor mice at diestrus were excised, opened longitudinally and biopsied into 4 pieces (2 x 2 mm). Recipient mice were anesthetized with vaporized isoflurane in oxygen (4% V/V for induction; 2.5% V/V for maintenance) and a midline incision of 1 cm was made to expose the pelvic compartment. Endometriosis mice had 4 uterine fragments sutured to the peritoneal wall, whereas sham-operated mice received 4 similar-sized fragments of abdominal fat. Transplanted tissues and abdominal muscle and skin were stitched using 6-0 black silk (8065195601, Alcon® Cusi S.A., Barcelona, Spain).

2.5. Experimental protocols

Expression levels of the genes coding for the opioid receptors were measured by RT-PCR in endometriotic cysts obtained from untreated mice 32 days after induction of endometriosis. To evaluate the effect of acute U50,488H on pelvic mechanical sensitivity, endometriosis and sham mice were tested in the von Frey assay after administration of vehicle or different doses of U50,488H (0.5, 1, 1.5, 2 and 2.5 mg/kg). Measurements were done 60 min after subcutaneous administration of U50,488H or vehicle at time points in which endometriotic lesions and pelvic hypersensitivity were fully developed (from day 37 to 47).

To assess the effect of sustained kappa opioid receptor stimulation, chronic U50,488H (1 mg/kg) or vehicle were administered once a day (9 AM) starting on day 1 after surgery until day 38. After the measurement of baseline mechanical sensitivity (day -1), endometriosis or sham surgery was performed (day 0), and nociceptive responses were assessed again 7, 14, 21 and 28 days after surgery. The phase of the estrous cycle was also determined on day 28. Motor coordination was assessed on day 18 after surgeries. Anxiety-like behavior and cognitive performance were evaluated on days 23 and 27, respectively. Locomotor activity was evaluated on day 30. At the end of the experimental sequence (day 39), mice were euthanized by cervical dislocation for sample collection.

2.6. Gene expression analysis by RT-PCR

Ectopic endometrial growths from untreated mice were fresh-frozen on dry ice immediately after euthanasia (day 32 after induction of

endometriosis) and stored at -80°C until use. Total RNA was isolated from frozen endometrial cysts using Trizol® (15596018, Invitrogen, Waltham, MA, USA) and subsequently reverse-transcribed to cDNA with a High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Foster City, CA, USA) according to the manufacturer instructions. RT-PCR was carried out in triplicate with a QuantStudio 12K Flex Real-Time PCR System (4471134, Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix (04707516001, Roche, Basel, Switzerland). The following specific primers were used: 5'-TCTTCACCCTCTGCACCATG-3' (Oprm1 forward); 5'-TCTATGGACCCCTGCCTGTA-3' (Oprm1 reverse), 5'-TTTGGCATCGTCCGGTACAC-3' (Oprd1 forward); 5'-AGAGCACAGCCTTGCACAGC -3' (Oprd1 reverse); 5'-ATCTGTGTCTTCGTCTTTGCCT-3' (Oprk1 forward); 5'-GATTTCGGTCCTTCTCTCGGG-3' (Oprk1 reverse); 5'-ATGACTCCACTCACGGCAAAT-3' (Gapdh forward); 5'-GGGTCTCGCTCCTGGAAGAT-3' (Gapdh reverse). Data for each target gene were analyzed by the 2-ΔΔCt method (Livak and Schmittgen, 2001) after normalization to the endogenous control Gapdh.

2.7. Nociceptive behavior

Mechanical sensitivity was quantified by measuring the responses to von Frey filament stimulation of the pelvic area 60 min after drug or vehicle administration. Von Frey filaments (1.65, 2.36, 2.44, 2.83, 3.22 and 3.61 corresponding to 0.008, 0.02, 0.04, 0.07, 0.16 and 0.4 g; Bioseb, Pinellas Park, FL, USA) were applied in increasing order of force, 10 times each, for 1-2 sec, with an inter-stimulus interval of 5-10 sec.

Abrupt retraction of abdomen, jumping and immediate licking of the site of application were considered positive responses in the evaluation of pelvic mechanical sensitivity. The area under the curve (AUC) was calculated by applying the linear trapezoidal rule to the plots representing the frequency of response versus the number of von Frey filaments, which represent the logarithm of the filament force expressed in mg x 10. The percentage of the maximum possible effect was calculated as the percentage difference between the measured response for each dose and the measured response after vehicle administration, divided by the difference between the maximum response (response of sham mice treated with vehicle) and the measured response after vehicle administration (response of endometriosis mice treated with vehicle).

2.8. Motor coordination

The accelerating rotarod test was used to evaluate motor coordination. The apparatus consists of a 5 cm-diameter black-striated rod with 5 crossing compartments each 5 cm-wide. Animals were trained using a low-speed rotation of 4 rpm. If a mouse fell before a 90-s time limit, it was returned to the rod until it was able to stay on it at least 90 s for 2 consecutive trials. Each mouse carried out a maximum of 15 trials with a 2-min rest period between them. Mice that did not accomplish the training were excluded from the experiment. The test was performed 24 h later, 60 min after drug or vehicle administration. The rotarod accelerated from 4 to 20 rpm over 50 s and was maintained at 20 rpm thereafter. Mice performed a total of 5 trials, with a 2-min period between them. In each trial, the latency to fall was recorded

automatically, with a cut-off time established at 90 s. The mean fall latency of the 5 trials was used for statistical analyses.

2.9. Anxiety-like behavior

The elevated plus maze test was used to evaluate anxiety-like behavior 6 h after drug or vehicle administration in order to discard a possible intrinsic effect of the acute drug exposure. A black Plexiglas apparatus consisting of 4 arms (29 cm long x 5 cm wide), 2 open and 2 closed, set in cross from a neutral central square (5 x 5 cm) elevated 40 cm above the floor was used. Light intensity in the open and closed arms was 45 and 5 lux, respectively. Mice were placed in the central square facing one of the open arms and tested for 5 min. The percentage time spent in the open and closed arms of the maze was determined as a measure of anxiety-like behavior. Mice that fell from the maze were excluded from the experiment.

2.10. Cognitive behavior

The novel object recognition task was assayed in a V-shaped maze to measure cognitive performance (Puighermanal et al., 2009) 6 h after drug or vehicle administration in order to discard a possible intrinsic effect of the acute drug exposure. On the first day, mice were habituated for 9 min to the empty maze. On the second day, mice were placed again in the maze for 9 min and 2 identical objects were presented at the ends of the arms of the maze. The day of the test, 24 h later, one of the familiar objects was replaced with a novel one and mice were placed back in the maze for 9 min. The time spent exploring each object (novel and familiar) was recorded and a discrimination

index was calculated as the difference between the time spent exploring the novel and the familiar object, divided by the total time exploring the 2 objects. A threshold of 10 s of total interaction with the objects was set to discard low levels of general activity.

2.11. Locomotion

Locomotor activity was evaluated by using individual locomotor activity boxes of 10.8 x 20.3 x 18.6 cm (Imetronic, Pessac, France) equipped with infrared sensors to detect horizontal activity and an infrared plane to detect rearings. Mice were placed in the boxes right after U50,488H or vehicle administration. Horizontal activity (number of beam breaks) and rearings were recorded for 90 min.

2.12. Histology of endometriotic lesions

Endometriotic lesions from mice treated with U50,488H or vehicle were harvested from each mouse on day 39 after surgeries. Samples were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 4 h and cryoprotected in 30% sucrose with 0.1% sodium azide for 6 days. Then, samples were embedded in molds filled with optimal cutting temperature compound (4583, Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and stored at -80°C until use. Endometriotic lesions were serially sectioned at 50 µm with a cryostat (CM3050, Leica Biosystems, Nussloch, Germany), mounted onto gelatinized slides and stored at -20°C until use. Sections of endometriotic lesions were stained with hematoxylin and eosin and images were taken under a Macro Zoom Microscope (MVX10, Olympus, Tokyo, Japan) for assessment of diameter and area of

endometrial tissue. The mean diameter and area of all endometrial cysts from each mouse was used for statistical analyses.

2.13. Statistical analysis

The adequate statistical test was applied according to the data distribution defined by the Shapiro-Wilk normality test. Mixed model analyses (surgery and treatment as between-subject factors, dose or day as within-subject factors) were used for repeated measures. Kruskal-Wallis followed by U Mann-Whitney and 2-way ANOVA followed by Bonferroni post hoc analysis were used for multiple group comparisons. Comparisons between two groups were analyzed with U Mann-Whitney test. Dose-response curve was fitted and ED50 was determined using GraphPad Prism 8 (San Diego, CA, USA). Data are expressed as individual data points and mean ± SEM. Values that felt outside of the interval determined as the mean ± 2 times the standard deviation were excluded from the analyses. All statistical analyses were performed using IBM SPSS 24 software (Chicago, IL, USA). Differences were considered statistically significant when the p value was below 0.05. Statistical results are available in supplementary tables.

3. Results

3.1. Acute stimulation of KOR produced dose-dependent alleviation of pelvic mechanical hypersensitivity in endometriosis mice

In order to investigate the presence of opioid receptors in ectopic endometrial tissues, expression levels of the genes coding for MOR (Oprm1), DOR (Oprd1) and KOR (Oprk1) were measured in endometriotic cysts obtained from mice 1 month after induction of endometriosis (Fig. 1A). The expression level of the gene coding for KOR was an average of 10-fold higher than the expression levels of the genes coding for MOR ($p<0.001$) or DOR ($p<0.01$). The high expression of KOR in ectopic endometrium prompted us to investigate the effects of KOR stimulation in the endometriosis model.

Vehicle or increasing doses of U50,488H (0, 0.5, 1, 1.5, 2 and 2.5 mg/kg s.c.) were administered to endometriosis and sham mice after 4 weeks of the surgical induction of endometriosis or the sham procedure, when the behavioral phenotype of endometriosis mice is fully developed (Escudero-Lara et al., 2020a). Mechanical sensitivity in the pelvic area was evaluated 60 min after drug or vehicle administration by measuring the responses to von Frey filament stimulation. Endometriosis mice treated with vehicle or U50,488H at 0.5 mg/kg showed a pronounced mechanical hypersensitivity in the pelvic area when compared to sham mice ($p<0.05$ vs. sham; Fig. 2B and 2C). However, U50,488H doses of 1, 1.5, 2 and 2.5 mg/kg induced significant inhibition of pelvic hypersensitivity (NS vs. sham), with the thresholds obtained after the acute doses of 2 and 2.5 mg/kg revealing complete

pain relief and significant differences with the thresholds obtained after vehicle treatment ($p<0.05$ vs. vehicle; Fig. 2B and 2C). U50,488H did not induce significant modification of mechanical thresholds in sham mice. Thus, acute U50,488H produced a dose-dependent reduction of pelvic mechanical hypersensitivity in endometriosis mice with an ED50 of 0.7386 mg/kg (Fig. 2D). Therefore, the alleviation of pelvic hypersensitivity through activation of KOR suggests usefulness of KOR stimulation for the acute relief of pelvic mechanical sensitivity in endometriosis mice.

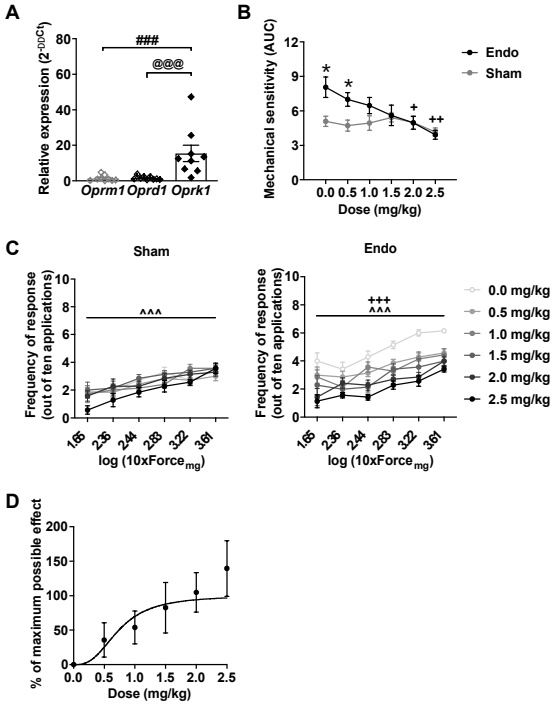

Figure 1. Kappa opioid receptor was highly expressed in endometriotic cysts and its acute stimulation alleviated pelvic mechanical hypersensitivity in a dose-dependent manner. (A) Expression levels of the gene coding for KOR (*Oprk1*) were ten-fold higher than expression levels of the genes coding for mu (μ) (*Oprm1*) and delta (∂) (*Oprd1*) opioid receptors in endometriotic cysts. (**B**) Acute administration of U50,488H 1, 1.5, 2 and 2.5 mg/kg alleviated mechanical sensitivity in endometriosis mice without affecting sham mice. (**C**) Acute administration of

increasing doses of U50,488H decreased the frequency of response to von Frey filaments in endometriosis mice without affecting sham mice. (**D**)The ED50 for the acute alleviation of mechanical hypersensitivity was 0.7386 mg/kg. Error bars are mean ± SEM. (A) Kruskal-Wallis + U Mann Whitney; (B) Mixed model analysis + Bonferroni, n=7 mice/group; (C) Mixed model analysis, n=7 mice/group; (D) n=7 mice. ###p<0.001 vs. *Oprm1*; @@@p<0.01 vs. *Oprd1*; *p<0.05 vs. sham; +p<0.05, ++p<0.01, +++p<0.001 vs. vehicle; ^^^p<0.001 effect of filament. Endo, endometriosis; AUC, area under the curve. Detailed statistical analyses are shown in Supplementary Table S1.

3.2. Chronic KOR activation provided sustained alleviation of pelvic mechanical hypersensitivity in endometriosis mice

We next evaluated the effects of chronic U50,488H exposure on pelvic sensitivity of endometriosis mice. The dose of 1 mg/kg was chosen to study the effects of repeated administration based on the dose-response curve (Fig. 1B and 1C). Thus, vehicle or U50,488H (1 mg/kg) were administered once a day for 38 days to sham and endometriosis mice, starting the day after the surgeries (Fig. 2). Baseline pelvic mechanical sensitivity was similar among groups. On day 7 after surgeries, endometriosis and sham mice treated with vehicle showed heightened pelvic sensitivity (p<0.001 vs. baseline; Fig. 3A and 3B). At this time point, the KOR agonist alleviated pelvic hypersensitivity of both endometriosis and sham groups (p<0.05 vs. vehicle; Fig. 3A and 3B). The heightened pelvic sensitivity of endometriosis mice treated with vehicle persisted until the end of the experiment (p<0.001 vs. sham-vehicle; Fig. 3A). Conversely, endometriosis mice receiving U50,488H showed complete recovery of their baseline sensitivity since day 14 after the beginning of the treatment (p<0.001 vs. endo-vehicle; Fig. 3A). Similarly, sham mice treated with vehicle recovered their

baseline sensitivity on day 14 (Fig. 3A and 3B), whereas sham mice treated with U50,488H showed unaltered mechanical thresholds during the entire experiment (Fig. 3A and 3B). Thus, chronic KOR stimulation provided sustained alleviation of pelvic mechanical hypersensitivity in endometriosis mice and antinociceptive tolerance was not developed.

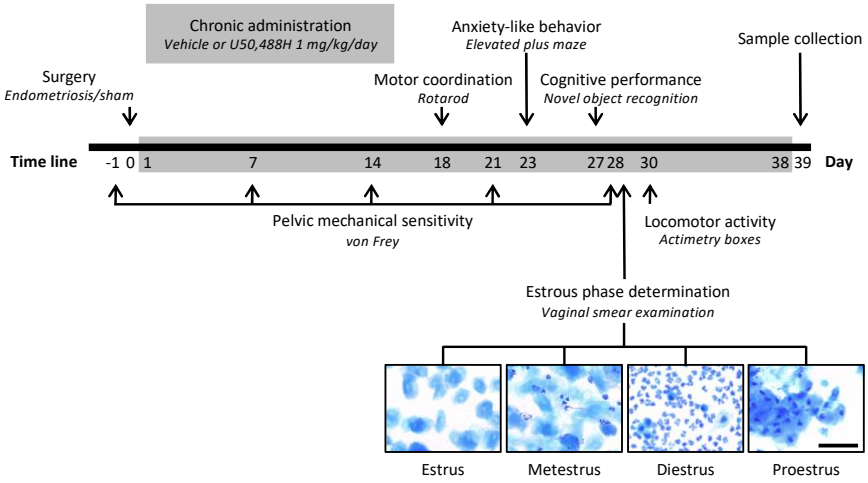

Figure 2. Experimental sequence to assess the effects of sustained kappa opioid receptor stimulation in mice subjected to a model of endometriosis. After the measurement of baseline mechanical sensitivity (day -1), endometriosis or sham surgery was performed (day 0). Chronic U50,488H (1 mg/kg) or vehicle were administered once a day from day 1 to day 38 after surgery. Nociceptive responses were assessed again 7, 14, 21 and 28 days after surgery, and the phase of the estrous cycle was also determined on day 28 by histological examination of vaginal smears. Motor coordination was assessed on day 18 after surgeries. Anxiety-like behavior, cognitive performance and locomotor activity were evaluated on days 23, 27 and 30, respectively. On day 39, mice were euthanized for sample collection. Scale bar=50 µm.

Endometriosis pain in women is usually worse during menstruation, but an effect of the estrous cycle on endometriosis-related pain in mice has not been yet investigated. The stage of the estrous cycle was

determined in the mouse model of endometriosis on the last day of evaluation of mechanical thresholds (day 28) to study its possible influence on pelvic mechanical sensitivity. Mice of the different groups were subdivided into those that were in stages of low estrogen levels (estrus) and those in stages of high estrogen levels (proestrus, metestrus and diestrus), as previously described (Nilsson et al., 2015; Zenclussen et al., 2014). Pelvic mechanical sensitivity was significantly increased in endometriosis mice treated with vehicle that were in the estrus phase ($p<0.001$ vs. high-estrogen phases; Fig. 3C and 3D). On the other hand, sham mice showed similar mechanical sensitivity regardless of stage of the cycle or the pharmacological treatment, although a trend for increased sensitivity was observed in vehicle-treated females in estrus (Fig. 3C). Indeed, significant hypersensitivity was detected in these females when the data were analyzed separately ($p<0.05$, Fig. 3D). Treatment with U50,488H reduced the mechanical sensitivity of endometriosis mice both in low and high-estrogen groups ($p<0.05$ vs endo-vehicle, Fig. 3C and 3D). Therefore, endometriosis mice showed greater mechanical sensitivity during estrus, which was associated with low estrogen levels, and KOR stimulation was effective alleviating pelvic hypersensitivity independently of the estrous cycle phase.

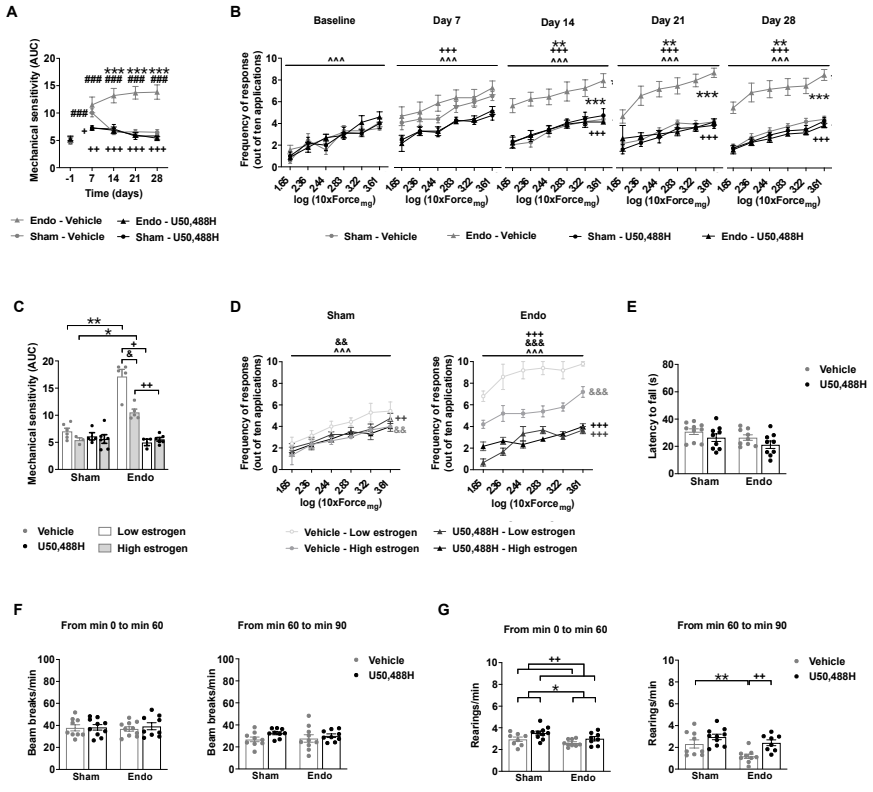

Figure 3. Chronic KOR activation relieved pelvic hypersensitivity in mice with ectopic endometrium. (**A**) Daily administration of U50,488H 1 mg/kg alleviated mechanical hypersensitivity in endometriosis mice without inducing tolerance. (**B**) Endometriosis mice treated with vehicle showed higher frequencies of response to von Frey filaments than endometriosis mice treated with U50,488H and sham mice treated with vehicle. U50,488H did not affect sham mice. (**C**) On day 28 after surgeries, pelvic hypersensitivity of endometriosis mice in estrous phases associated with low estrogen levels (estrus) was greater than the pelvic sensitivity of endometriosis mice in estrous phases associated with higher estrogen levels (proestrus, metestrus and diestrus). U50,488H abolished pelvic mechanical sensitivity in both groups. (**D**) Frequency of response to von Frey filaments was significantly higher in vehicle-treated sham mice that were in the low estrogen phase. Although frequency of response was higher in endometriosis mice in phases of low estrogen levels than in endometriosis mice in phases of high estrogen levels, U50,488H reduced the frequency of response to mechanical stimulation in both groups. (**E**) No significant differences among groups were found in the latency to fall from the rotarod. (**F**) Horizontal locomotor activity (number of beam breaks/min) was similar among groups during the first 60 min

following U50,488H or vehicle administration and also during the time period of evaluation of mechanical sensitivity (60 to 90 min). (**G**) The presence of ectopic endometrium reduced rearing behavior while U50,488H increased it during the first 60 min after the treatment (left panel). During the following 30 min (60 to 90, right panel), endometriosis mice treated with vehicle performed a lower number of rearings compared to vehicle-treated sham mice, but U50,488H prevented this alteration in endometriosis mice. (A) Mixed model analysis + Bonferroni, n= 9-10 mice/group; (B) Mixed model analysis + Bonferroni, n=9-10 mice/group; (C) Kruskal-Wallis + U Mann Whitney; (D) Mixed model analysis + Bonferroni, n=3-7 mice/group; (E) 2-way ANOVA; (F, left and right panels) 2-way ANOVA; (G, left panel) 2-way ANOVA + Bonferroni; (G, right panel) Kruskal-Wallis + U Mann Whitney. Error bars are mean ± SEM. ###p<0.001 vs. baseline; *p<0.05, **p<0.01, ***p<0.001 vs. sham; +p<0.05, ++p<0.01, +++p<0.001 vs. vehicle; &p<0.05, &&p<0.01, &&&p<0.001 vs low estrogen; ^^^p<0.001 effect of filament. Endo, endometriosis; AUC, area under the curve. Detailed statistical analyses are shown in Supplementary Table S2.

KOR agonists can induce sedation in rodents (Vonvoigtlander et al., 1983) and humans (Rimoy et al., 1994). To investigate possible motor effects that could interfere with the observed nociceptive responses, motor coordination and locomotor activity were investigated in endometriosis and sham mice chronically treated with the low dose of U50,488H. First, an accelerating rotarod was used on day 18 of the experiment, 60 min after drug or vehicle administration. No significant differences among groups were found in the latency to fall from the rotarod (NS, Fig. 3E). Spontaneous locomotor activity of endometriosis and sham mice receiving either U50,488H or vehicle for 30 days was also evaluated. Mice were placed in actimetry boxes immediately after the injection and their activity was recorded for 90 min. Horizontal activity was unaffected by endometriosis or the pharmacological treatment during the first 60 min after drug or vehicle administration (NS, Fig. 3F), and similar results were obtained at the time period of evaluation of mechanical sensitivity (60-90 min, NS, Fig. 3F). Since

motor coordination and horizontal locomotion were not affected by endometriosis or U50,488H treatments, we discarded any possible bias of disrupted locomotion on the responses to mechanical stimulation.

Reduced rearing behavior is associated to visceral pain in rodents, and can be interpreted as a measure of spontaneous pain or discomfort (Lucarini et al., 2020; Salameh et al., 2019; Schwartz et al., 2013). Endometriosis mice showed reduced number of rearings/min from min 0 to 60 after drug or vehicle administration ($p<0.05$ vs Sham; Fig. 3G), and U50,488H induced significant reinstatement of this behavior both in sham-operated and endometriosis mice (from min 0 to min 60; $p<0.01$ vs vehicle; Fig. 3G). During the following 30 min (60-90 min), endometriosis mice treated with vehicle showed further decrease of rearings when compared to vehicle-treated sham mice (from min 60 to min 90; $p<0.01$ vs. sham-vehicle, Fig. 3G). Interestingly, the treatment with U50,488H normalized rearing behavior of endometriosis mice at this time point ($p<0.01$ vs. endo-vehicle, Fig. 3G), suggesting acute efficacy of this U50,488H dose inhibiting spontaneous pain or discomfort.

3.3. KOR-mediated pain relief did not mitigate the anxiety-like behavior or the memory impairment of endometriosis mice

Chronic pelvic pain is often accompanied by affective disorders in women with endometriosis (Fourquet et al., 2011). To assess the effect of KOR-mediated pain relief on the anxiety-like behavior revealed in endometriosis mice, the elevated plus maze was used 23 days after the surgeries. Endometriosis mice spent significantly less time in the open arms of the maze ($p<0.05$ vs sham, Fig. 4A). This increased anxiety-like

behavior was present regardless of the treatment with U50,488H or its vehicle, suggesting a lack of effect of the KOR agonist on this affective behavior.

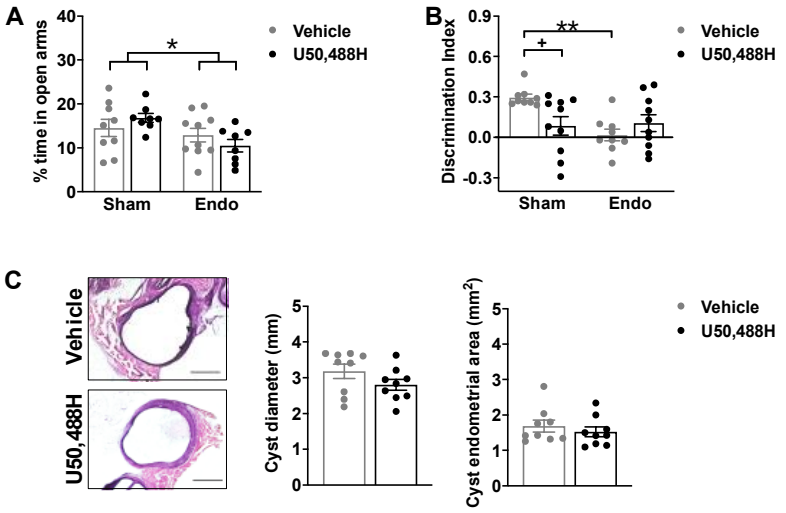

Figure 4. Sustained KOR stimulation does not modify anxiety-like behavior, cognitive impairment or endometriotic cysts in endometriosis mice. (A) Endometriosis mice showed an enhanced anxiety-like behavior reflected in lower percentages of time spent in the open arms of the elevated plus maze, but the treatment with U50,488H did not modify this behavior. (**B**) Both endometriosis and chronic U50,488H impair long-term memory in the novel object recognition test. (**C**) The diameter and endometrial area of endometriotic cysts from endometriosis mice were similar regardless of the treatment with U50,488H. Scale bar = 1 mm. (A) 2-way ANOVA; (B) Kruskal-Wallis + U Mann Whitney; (C) U Mann Whitney. Error bars are mean ± SEM. *p<0.05, **p<0.01 vs. sham; +p<0.05 vs. vehicle. Endo, endometriosis. Detailed statistical analyses are shown in Supplementary Table S3.

We previously reported that the presence of ectopic endometrium in mice was associated with cognitive impairment (Escudero-Lara et al., 2020a). The effect of chronic U50,488H was evaluated 27 days after the surgeries in the novel object recognition task to investigate the impact of KOR-mediated pain relief on the memory deficits observed in this

model. Endometriosis mice treated with vehicle or U50,488H presented a significant decrease in the discrimination index when compared to vehicle-treated sham mice ($p<0.01$ vs. sham-vehicle; Fig. 4B). Similarly, sham mice chronically treated with U50,488H exhibited impairment in object recognition memory ($p<0.05$ vs. sham-vehicle; Fig.4B). Therefore, KOR stimulation did not prevent the cognitive impairment of endometriosis mice and showed primary effects disrupting long-term recognition memory.

3.4. KOR stimulation did not modify the growth of ectopic endometrial tissue

Mice were euthanized after the behavioral characterization and their pelvic compartment was carefully explored. Only mice receiving endometrial implants developed endometrial cysts that were filled of fluid and contained glandular epithelium and stroma (Fig. 4C), as we previously reported (Escudero-Lara et al., 2020a, 2020b). Endometriosis mice treated with vehicle or U50,488H developed endometrial cysts of similar diameter and endometrial area (NS; Fig. 4C). Therefore, exogenous KOR stimulation did not produce significant effects on the growth of the ectopic endometrium.

4. Discussion

This study revealed a strong efficacy of kappa opioid receptor (KOR) stimulation in the attenuation of evoked and spontaneous pain in a murine model of endometriosis that closely mimics this clinical condition. Chronic KOR stimulation with a low dose of U50,488H (1 mg/kg) induced sustained alleviation of pelvic hypersensitivity in female mice subjected to the model, and this pain inhibition was more efficient during the estrus phase of the estrous cycle, which was associated with higher pain sensitivity. Kappa activation did not modify anxiety-like behavior nor the cognitive impairment exhibited by mice with ectopic endometrium, suggesting that these co-morbidities occur through independent mechanisms and anxiety or memory deficits may develop in the absence of pain.

The gene coding for KOR was highly expressed in endometrial cysts obtained from mice subjected to the endometriosis model. The expression of endogenous opioids and their receptors has been previously described in mouse (Zhu et al., 1998), swine (Dziekonski et al., 2018) and human (Chatzaki et al., 2000; Makrigiannakis et al., 1992; Petraglia et al., 1986; Wahlström et al., 1985) endometrial cells. In line with our findings, KOR has been reported as the most abundant opioid receptor in human endometrial cells (Hatzoglou et al., 1995). KOR is also widely expressed in peripheral nerve fibers (Stein, 2016) and cells of the immune system (Sharp, 2006). Since endometrial cysts are innervated and contain immune cells (Escudero-Lara et al., 2020a, 2020b), the presence of KOR in these tissues suggests that this opioid

receptor may be implicated in pain transmission and inflammation in endometriosis.

Acute administration of the KOR agonist U50,488H alleviated in a dose-dependent manner pelvic mechanical hypersensitivity of endometriosis mice. Doses in the range of 1 to 2.5 mg/kg alleviated pelvic hypersensitivity in these mice without modifying nociceptive sensitivity in sham animals. Interestingly, repeated exposure of a low dose of U50,488H (1 mg/kg) provided efficient alleviation of mechanical hypersensitivity during the complete pharmacological treatment. This is in contrast with the tolerance observed after administration of higher doses of U50,488H (3-100 mg/kg) to male mice (Bhalla et al., 2010; McLaughlin et al., 2004; Narita et al., 2003; Vonvoigtlander et al., 1983). To the best of our knowledge, this is the first work showing sustained pain-relieving efficacy of a KOR agonist in female mice subjected to chronic pain and no signs of antinociceptive tolerance were observed. Together with the higher analgesic efficacy of KOR agonists in women (Gear et al., 1999, 1996b, 1996a), these results suggest that modulation of KOR could represent an effective approach for the chronic treatment of endometriosis pain.

Female mice implanted with ectopic endometrium showed higher mechanical sensitivity during estrus, the phase of the estrous cycle in which estrogen levels decrease. In agreement, low estrogen levels have been associated to higher sensitivity in rats subjected to the tail-flick test (Kayser et al., 1996; Martínez-Gómez et al., 1994; Stoffel et al., 2003). Furthermore, the pain symptoms associated to endometriosis in women, are exacerbated during menstruation (Laux-Biehlmann et al.,

2015) the phase of the menstrual cycle equivalent to the estrus in mice. Thus, the mouse model replicates the increased pain sensitivity observed during menstruation in women with endometriosis (Laux-Biehlmann et al., 2015) and is consistent with the reduction of pain obtained with contraceptive treatments that prevent menstruation in women (Vercellini et al., 2009). However, the role of estrogens in animal models of nociception seems complex, since increased responses to organ distension have also been described when estrogen levels are high (Cason et al., 2003; Holdcroft et al., 2000; Sapsed-Byrne et al., 1996).

KOR activation reduced pelvic hypersensitivity in mice with ectopic endometrium in all the phases of the estrous cycle, in agreement with the lack of effect of estradiol administration in the antinociceptive efficacy of U50,488H in a rat model of visceral pain (Sandner-Kiesling and Eisenach, 2002). However, the antinociceptive effects of KOR activation were higher in endometriosis mice during estrus. Previous works also describe higher KOR-mediated antinociception in naïve mice in estrus (Abraham et al., 2018b).

Endometriosis mice showed a significant reduction in rearing, an exploratory behavior that involves stretching of the abdominal area, which has been related to manifestation of spontaneous pain. In accordance, decreased number of rearings has been previously observed in multiple models of visceral pain in rodents (Lucarini et al., 2020; Salameh et al., 2019; Schwartz et al., 2013). KOR stimulation normalized this rearing behavior in mice with ectopic endometrium. Acute U50,488H has been reported to both increased (Kuzmin et al.,

2000) or decreased rearing behaviors in male rodents (Kuzmin et al., 2000; Milman et al., 2006) depending on the dose. Since the number of rearings performed by sham mice chronically exposed to this low dose of U50,488H was unaffected, our data suggest that normalization of the rearing behavior was due to the alleviation of spontaneous pain or discomfort. Thus, restoration of rearing behavior provides further evidence of the potential analgesic efficacy of KOR agonists in endometriosis.

Sedation is an important side effect of opioids and other analgesic prescription drugs (Caplan et al., 2007). The endometriosis model showed that repeated exposure to U50,488H (1 mg/kg) did not affect horizontal activity or motor coordination of female mice. The sedative effects of higher doses (5-30 mg/kg) of U50,488H have been widely described in male rodents (Dunn et al., 2018; J. J. Liu et al., 2019; Vonvoigtlander et al., 1983), but lower doses (3 mg/kg) have been reported to be void of sedative effects (Dunn et al., 2020). In agreement with our results, previous studies have described that 1 mg/kg of U50,488H does not affect horizontal locomotion in female mice (Liu et al., 2019). Thus, our data suggest that analgesic efficacy can be achieved with doses that lack significant effects on motor performance, an advantage against other available treatments for chronic pain treatment.

The anxiety-like behavior of endometriosis mice was unaffected by the sustained pain relief achieved through KOR stimulation. Our previous work investigating the antinociceptive efficacy of delta-9-tetrahydrocannabinol in the same endometriosis model also showed

persistence of anxiety-like behavior in spite of a complete relief of pelvic hypersensitivity (Escudero-Lara et al., 2020a). On the contrary, other models of chronic pain have shown reinstatement of normal anxiety-like behavior after chronic treatment with antinociceptive drugs (Cabañero et al., 2020; La Porta et al., 2016, 2015). Hence, the present data suggest that the affective impairment observed in endometriosis mice may be independent of the persistent pain sensitization. In this line, a primary effect of endometriosis on affective behavior has been suggested after the detection of depressive symptomatology in women with endometriosis that did not suffer from chronic pain (Lorençatto et al., 2006). Therefore, a primary effect of endometriosis on the promotion of anxiety and depression could also have an impact favoring the development of chronic pain (Boakye et al., 2016). On the other hand, anxiogenic (Gillett et al., 2013; Privette and Terrian, 1995; Smith et al., 2012; Tejeda et al., 2012), anxiolytic (Privette and Terrian, 1995) and no effects (Gillett et al., 2013; Privette and Terrian, 1995; Robles et al., 2014) of KOR agonists have been described in the literature, depending on the dose, the test and the animal model. However, a study addressing this issue in naïve female mice shows an absence of effects of U50,488H (2.5-10 mg/kg) on anxiety-like behavior (Robles et al., 2014), in accordance with our results. Hence, the pain-relieving effects of KOR stimulation had not significant effects in the emotional impact of endometriosis and simultaneous treatment of pain and affective symptoms may be an appropriate approach for endometriosis management. KOR stimulation did not modify the cognitive impairment of mice with ectopic endometrium and impaired cognitive performance in sham

mice. KOR agonists have been reported to impair novel object recognition (Carey et al., 2009; Paris et al., 2011), spatial mnemonic performance (Daumas et al., 2007) and emotional memory (Castellano et al., 1988; Daumas et al., 2007) in male mice. In female mice, U50,488H impairs the differential reinforcement of the low-response rate task (Abraham et al., 2018a), an operational test of cognition that involves timing and behavioral inhibition. Therefore, the memory deficits observed in sham mice and the lack of memory improvement in mice subjected to endometriosis were expected after U50,488H treatment. Although the observed KOR-mediated cognitive impairment is in agreement with the literature, it could interfere with a possible improvement of memory associated to the alleviation of pain.

Mice showed ectopic endometrial growths of similar size after 38 days of treatment with U50,488H or vehicle. It has been reported that KOR stimulation increases the apoptotic rate of human endometrial cells in vitro (Chatzaki et al., 2001), but the effects of KOR stimulation on ectopic endometrial cells has not been studied in vivo. Our data indicate that exogenous activation of KOR does not induce significant changes in the development of ectopic endometrial growths in spite of the high levels of KOR expression in endometriotic cysts in our model. This fact implies that the pain-relieving effects of the KOR agonist are not due to an inhibition of the development of endometriosis lesions, but to an actual alleviation of pain associated to the presence of ectopic endometrium.

In summary, our study revealed that KOR stimulation can alleviate and prevent chronic pelvic mechanical sensitivity and discomfort in female mice subjected to endometriosis. This KOR-mediated pain relief was void of antinociceptive tolerance and was highly effective during estrus, the phase of the estrous cycle in which mice became more sensitive. Such an increased sensitivity resembles the intense perimenstrual pain observed in endometriosis patients. Interestingly, KOR-mediated pain relief did not modify the anxiety-like behavior or the memory impairment of mice with ectopic endometrial growths, suggesting that these may represent primary emotional and cognitive alterations independent of pain triggered by endometriosis. Thus, a multidisciplinary approach simultaneously targeting nociceptive, emotional and cognitive alterations of endometriosis may be necessary for successful management of the disease in women.

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References

Abraham AD, Fontaine HM, Song AJ, Andrews MM, Baird MA, Kieffer BL, Land BB, Chavkin C. 2018a. κ-Opioid receptor activation in dopamine neurons disrupts behavioral inhibition. Neuropsychopharmacology 43:362–372. doi:10.1038/npp.2017.133

Abraham AD, Schattauer SS, Reichard KL, Cohen JH, Fontaine HM, Song AJ, Johnson SD, Land BB, Chavkin C. 2018b. Estrogen regulation of GRK2 inactivates Kappa opioid receptor signaling mediating analgesia, but not aversion. J Neurosci 38:8031–8043. doi:10.1523/JNEUROSCI.0653-18.2018

Arosh JA, Lee J, Balasubbramanian D, Stanley JA, Long CR, Meagher MW, Osteen KG, Bruner-Tran KL, Burghardt RC, Starzinski-Powitz A, Banu SK. 2015. Molecular and preclinical basis to inhibit PGE2 receptors EP2 and EP4 as a novel nonsteroidal therapy for endometriosis. Proc Natl Acad Sci U S A 112:9716–9721. doi:10.1073/pnas.1507931112

Bhalla S, Zhang Z, Patterson N, Gulati A. 2010. Effect of endothelin-A receptor antagonist on mu, delta and kappa opioid receptor-mediated antinociception in mice. Eur J Pharmacol 635:62–71. doi:10.1016/j.ejphar.2010.03.003

Boakye PA, Olechowski C, Rashiq S, Verrier MJ, Kerr B, Witmans M, Baker G, Joyce A, Dick BD. 2016. A Critical Review of Neurobiological Factors Involved in the Interactions Between Chronic Pain, Depression, and Sleep Disruption. Clin J Pain 32:327–336. doi:10.1097/AJP.0000000000000260

Burnett M, Lemyre M. 2017. No. 345-Primary Dysmenorrhea Consensus Guideline. J Obstet Gynaecol Canada 39:585–595. doi:https://doi.org/10.1016/j.jogc.2016.12.023

Cabañero D, Ramírez-López A, Drews E, Schmöle A, Otte DM, Wawrzczak-Bargiela A, Huerga Encabo H, Kummer S, Ferrer-Montiel A, Przewlocki R, Zimmer A, Maldonado R. 2020. Protective role of neuronal and lymphoid cannabinoid CB2 receptors in neuropathic pain. Elife 9:e55582. doi:10.7554/eLife.55582

Caplan JP, Epstein LA, Quinn DK, Stevens JR, Stern TA. 2007. Neuropsychiatric effects of prescription drug abuse. Neuropsychol Rev 17:363–380. doi:10.1007/s11065-007-9037-7

Carcolé M, Zamanillo D, Merlos M, Fernández-Pastor B, Cabañero D, Maldonado R. 2019. Blockade of the Sigma-1 Receptor Relieves Cognitive and Emotional Impairments Associated to Chronic Osteoarthritis Pain . Front Pharmacol .

Carey AN, Lyons AM, Shay CF, Dunton O, McLaughlin JP. 2009. Endogenous κ opioid activation mediates stress-induced deficits in learning and memory. J Neurosci 29:4293–4300. doi:10.1523/JNEUROSCI.6146-08.2009

Cason AM, Samuelsen CL, Berkley KJ. 2003. Estrous changes in vaginal nociception in a rat model of endometriosis. Horm Behav 44:123–131. doi:10.1016/S0018-506X(03)00121-1

Castellano C, Libri V, Ammassari-Teule M. 1988. The amygdala mediates the impairing effect of the selective kappa-opioid receptor

agonist U-50,488 on memory in CD1 mice. Behav Brain Res 30:259–263. doi:10.1016/0166-4328(88)90168-4

Chatzaki E, Makrigiannakis A, Margioris AN, Kouimtzoglou E, Gravanis A. 2001. The Fas/FasL apoptotic pathway is involved in κ-opioidinduced apoptosis of human endometrial stromal cells. Mol Hum Reprod 7:867–874. doi:10.1093/molehr/7.9.867

Chatzaki E, Margioris AN, Makrigiannakis A, Castanas E, Georgoulias V, Gravanis A. 2000. Kappa opioids and TGFbeta1 interact in human endometrial cells. Mol Hum Reprod 6:602–609. doi:10.1093/molehr/6.7.602

Dapoigny M, Abitbol JL, Fraitag B. 1995. Efficacy of peripheral kappa agonist fedotozine versus placebo in treatment of irritable bowel syndrome. A multicenter dose-response study. Dig Dis Sci 40:2244–2249. doi:10.1007/BF02209014

Daumas S, Betourne A, Halley H, Wolfer DP, Lipp H-P, Lassalle J-M, Francés B. 2007. Transient activation of the CA3 Kappa opioid system in the dorsal hippocampus modulates complex memory processing in mice. Neurobiol Learn Mem 88:94–103. doi:10.1016/j.nlm.2007.02.001

Delvaux M, Louvel D, Lagier E, Scherrer B, Abitbol JL, Frexinos J. 1999. The kappa agonist fedotozine relieves hypersensitivity to colonic distention in patients with irritable bowel syndrome. Gastroenterology 116:38–45. doi:10.1016/s0016-5085(99)70226-x

Dunn A, Windisch K, Ben-Ezra A, Pikus P, Morochnik M, Erazo J, Reed B, Kreek MJ. 2020. Modulation of cocaine-related behaviors by low doses of the potent KOR agonist nalfurafine in male C57BL6 mice. Psychopharmacology (Berl) 237:2405–2418. doi:10.1007/s00213-020-05543-7

Dunn AD, Reed B, Guariglia C, Dunn AM, Hillman JM, Kreek MJ. 2018. Structurally related kappa opioid receptor agonists with substantial differential signaling bias: Neuroendocrine and behavioral effects in C57BL6 Mice. Int J Neuropsychopharmacol 21:847–857. doi:10.1093/ijnp/pyy034

Dziekonski M, Zmijewska A, Czelejewska W, Wojtacha P, Okrasa S. 2018. The effect of selective agonists of opioid receptors on in vitro secretion of steroid hormones by porcine endometrium during the estrous cycle and early pregnancy. J Physiol Pharmacol 69:727–735. doi:10.26402/jpp.2018.5.07

Escudero-Lara A, Argerich J, Cabañero D, Maldonado R. 2020a. Disease-modifying effects of natural Δ9-tetrahydrocannabinol in endometriosis-associated pain. Elife 9. doi:10.7554/eLife.50356

Escudero-Lara A, Cabañero D, Maldonado R. 2020b. Surgical Induction of Endometriosis in Female Mice. Bio-Protocol 10:1–14. doi:10.21769/bioprotoc.3763

Fourquet J, Báez L, Figueroa M, Iriarte RI, Flores I. 2011. Quantification of the impact of endometriosis symptoms on health-related quality of life and work productivity. Fertil Steril. doi:10.1016/j.fertnstert.2011.04.095

Fraitag B, Homerin M, Hecketsweiler P. 1994. Double-blind dose-response multicenter comparison of fedotozine and placebo in treatment of nonulcer dyspepsia. Dig Dis Sci 39:1072–1077. doi:10.1007/BF02087560

Gambadauro P, Carli V, Hadlaczky G. 2018. Depressive symptoms among women with endometriosis: a systematic review and meta-analysis. Am J Obstet Gynecol. doi:10.1016/j.ajog.2018.11.123

Garry R. 2004. The effectiveness of laparoscopic excision of endometriosis. Curr Opin Obstet Gynecol 16:299–303. doi:10.1097/01.gco.0000136496.95075.79

Gear RW, Gordon NC, Heller PH, Paul S, Miaskowski C, Levine JD. 1996a. Gender difference in analgesic response to the kappa-opioid pentazocine. Neurosci Lett 205:207–209. doi:https://doi.org/10.1016/0304-3940(96)12402-2

Gear RW, Miaskowski C, Gordon NC, Paul SM, Heller PH, Levine JD. 1999. The kappa opioid nalbuphine produces gender- and dose-dependent analgesia and antianalgesia in patients with postoperative pain. Pain 83:339–345. doi:10.1016/s0304-3959(99)00119-0

Gear RW, Miaskowski C, Gordon NC, Paul SM, Heller PH, Levine JD. 1996b. Kappa-opioids produce significantly greater analgesia in women than in men. Nat Med 2:1248–1250. doi:10.1038/nm1196-1248

Gillett K, Harshberger E, Valdez GR. 2013. Protracted withdrawal from ethanol and enhanced responsiveness stress: Regulation via the dynorphin/kappa opioid receptor system. Alcohol 47:359–365. doi:10.1016/j.alcohol.2013.05.001

Hatzoglou A, Gravanis A, Margioris AN, Zoumakis E, Castanas E. 1995. Identification and characterization of opioid-binding sites present in the Ishikawa human endometrial adenocarcinoma cell line. J Clin Endocrinol Metab 80:418–423. doi:10.1210/jcem.80.2.7852499

Holdcroft A, Sapsed-Byrne S, Ma D, Hammal D, Forsling ML. 2000. Sex and oestrous cycle differences in visceromotor responses and vasopressin release in response to colonic distension in male and female rats anaesthetized with halothane. Br J Anaesth 85:907–910. doi:10.1093/bja/85.6.907

Izquierdo I, Dias RD, Souza DO, Carrasco MA, Elisabetsky E, Perry ML. 1980. The role of opioid peptides in memory and learning. Behav Brain Res 1:451–468. doi:10.1016/0166-4328(80)90001-7

Kamp EH, Jones RCW, Tillman SR, Gebhart GF. 2003. Quantitative assessment and characterization of visceral nociception and hyperalgesia in mice. Am J Physiol - Gastrointest Liver Physiol 284:434–444. doi:10.1152/ajpgi.00324.2002

Kayser V, Berkley KJ, Keita H, Gautron M, Guilbaud G. 1996. Estrous and sex variations in vocalization thresholds to hindpaw and tail pressure stimulation in the rat. Brain Res 742:352–354. doi:10.1016/s0006-8993(96)01108-0

Kieffer BL. 1995. Recent advances in molecular recognition and signal transduction of active peptides: Receptors for opioid peptides. Cell Mol Neurobiol 15:615–635. doi:10.1007/BF02071128

Kivell B, Prisinzano TE. 2010. Kappa opioids and the modulation of pain. Psychopharmacology (Berl) 210:109–19. doi:10.1007/s00213-010-1819-6

Kuzmin A, Sandin J, Terenius L, Ögren SO. 2000. Dose- and time-dependent bimodal effects of κ-opioid agonists on locomotor activity in mice. J Pharmacol Exp Ther 295:1031–1042.

La Porta C, Bura SA, Llorente-Onaindia J, Pastor A, Navarrete F, García-Gutiérrez MS, De la Torre R, Manzanares J, Monfort J, Maldonado R. 2015. Role of the endocannabinoid system in the emotional manifestations of osteoarthritis pain. Pain 156:2001–12. doi:10.1097/j.pain.0000000000000260

La Porta C, Lara-Mayorga IM, Negrete R, Maldonado R. 2016. Effects of pregabalin on the nociceptive, emotional and cognitive manifestations of neuropathic pain in mice. Eur J Pain (United Kingdom). doi:10.1002/ejp.868

Laux-Biehlmann A, D'hooghe T, Zollner TM. 2015. Menstruation pulls the trigger for inflammation and pain in endometriosis. Trends Pharmacol Sci. doi:10.1016/j.tips.2015.03.004

Liu JJ, Chiu YT, DiMattio KM, Chen C, Huang P, Gentile TA, Muschamp JW, Cowan A, Mann M, Liu-Chen LY. 2019. Phosphoproteomic approach for agonist-specific signaling in mouse brains: mTOR pathway is involved in κ opioid aversion. Neuropsychopharmacology 44:939–949. doi:10.1038/s41386-018-0155-0

Liu SS, Pickens S, Burma NE, Ibarra-Lecue I, Yang H, Xue L, Cook C, Hakimian JK, Severino AL, Lueptow L, Komarek K, Taylor AMW,

Olmstead MC, Carroll FI, Bass CE, Andrews AM, Walwyn W, Trang T, Evans CJ, Leslie FM, Cahill CM. 2019. Kappa opioid receptors drive a tonic aversive component of chronic pain. J Neurosci 39:4162–4178. doi:10.1523/JNEUROSCI.0274-19.2019

Livak KJ, Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using RealTime Quantitative PCR and the 2–ΔΔCt Method. Methods 25:402–408. doi:10.1006/meth.2001.1262

Lorençatto C, Petta CA, Navarro MJ, Bahamondes L, Matos A. 2006. Depression in women with endometriosis with and without chronic pelvic pain. Acta Obstet Gynecol Scand 85:88–92. doi:10.1080/00016340500456118

Lucarini E, Parisio C, Branca JJV, Segnani C, Ippolito C, Pellegrini C, Antonioli L, Fornai M, Micheli L, Pacini A, Bernardini N, Blandizzi C, Ghelardini C, Di Cesare Mannelli L. 2020. Deepening the Mechanisms of Visceral Pain Persistence: An Evaluation of the Gut-Spinal Cord Relationship. Cells 9. doi:10.3390/cells9081772

Lutz P-E, Kieffer BL. 2013. Opioid receptors: distinct roles in mood disorders. Trends Neurosci 36:195–206. doi:10.1016/j.tins.2012.11.002

Makrigiannakis A, Margioris A, Markogiannakis E, Stournaras C, Gravanis A. 1992. Steroid hormones regulate the release of immunoreactive beta-endorphin from the Ishikawa human endometrial cell line. J Clin Endocrinol Metab 75:584–589. doi:10.1210/jcem.75.2.1639959

Martínez-Gómez M, Cruz Y, Salas M, Hudson R, Pacheco P. 1994. Assessing pain threshold in the rat: Changes with estrus and time of day. Physiol Behav 55:651–657. doi:10.1016/0031-9384(94)90040-X

McLaughlin JP, Myers LC, Zarek PE, Caron MG, Lefkowitz RJ, Czyzyk TA, Pintar JE, Chavkin C. 2004. Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. J Biol Chem 279:1810–1818. doi:10.1074/jbc.M305796200

Milman A, Weizman R, Rigai T, Rice KC, Pick CG. 2006. Behavioral effects of opioid subtypes compared with benzodiazepines in the staircase paradigm. Behav Brain Res 170:141–147. doi:10.1016/j.bbr.2006.02.017

Nadal X, La Porta C, Andreea Bura S, Maldonado R. 2013. Involvement of the opioid and cannabinoid systems in pain control: new insights from knockout studies. Eur J Pharmacol 716:142–157. doi:10.1016/j.ejphar.2013.01.077

Narita M, Khotib J, Suzuki M, Ozaki S, Yajima Y, Suzuki T. 2003. Heterologous μ-opioid receptor adaptation by repeated stimulation of κ-opioid receptor: Up-regulation of G-protein activation and antinociception. J Neurochem 85:1171–1179. doi:10.1046/j.1471-4159.2003.01754.x

Nilsson ME, Vandenput L, Tivesten Å, Norlén AK, Lagerquist MK, Windahl SH, Börjesson AE, Farman HH, Poutanen M, Benrick A, Maliqueo M, Stener-Victorin E, Ryberg H, Ohlsson C. 2015. Measurement of a comprehensive sex steroid profile in rodent serum

by high-sensitive gas chromatography-tandem mass spectrometry. Endocrinology 156:2492–2502. doi:10.1210/en.2014-1890

Ossipov MH, Dussor GO, Porreca F. 2010. Central modulation of pain. J Clin Invest 120:3779–3787. doi:10.1172/JCI43766

Paris JJ, Reilley KJ, McLaughlin JP. 2011. Kappa Opioid Receptor-Mediated Disruption of Novel Object Recognition: Relevance for Psychostimulant Treatment. J Addict Res Ther S4. doi:10.4172/2155-6105.S4-007

Petraglia F, Facchinetti F, M'Futa K, Ruspa M, Bonavera JJ, Gandolfi F, Genazzani AR. 1986. Endogenous opioid peptides in uterine fluid. Fertil Steril 46:247–251. doi:10.1016/s0015-0282(16)49520-8

Privette TH, Terrian DM. 1995. Kappa opioid agonists produce anxiolytic-like behavior on the elevated plus-maze. Psychopharmacology (Berl) 118:444–450. doi:10.1007/BF02245945

Przewłocki R, Przewłocka B. 2001. Opioids in chronic pain. Eur J Pharmacol 429:79–91. doi:10.1016/s0014-2999(01)01308-5

Puighermanal E, Marsicano G, Busquets-Garcia A, Lutz B, Maldonado R, Ozaita A. 2009. Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. Nat Neurosci. doi:10.1038/nn.2369

Rimoy GH, Wright DM, Bhaskar NK, Rubin PC. 1994. The cardiovascular and central nervous system effects in the human of U-62066E. A selective opioid receptor agonist. Eur J Clin Pharmacol 46:203–207. doi:10.1007/BF00192549

Rivière PJM, Pascaud X, Chevalier E, Le Gallou B, Junien JL. 1993. Fedotozine reverses ileus induced by surgery or peritonitis: Action at peripheral κ-Opioid receptors. Gastroenterology 104:724–731. doi:10.1016/0016-5085(93)91007-5

Robles CF, McMackin MZ, Campi KL, Doig IE, Takahashi EY, Pride MC, Trainor BC. 2014. Effects of kappa opioid receptors on conditioned place aversion and social interaction in males and females. Behav Brain Res 262:84–93. doi:10.1016/j.bbr.2014.01.003

Ross RA, Kaiser UB. 2017. The emotional cost of contraception. Nat Rev Endocrinol 13:7–9. doi:10.1038/nrendo.2016.194

Salameh E, Meleine M, Gourcerol G, Do Rego JC, Do Rego JL, Legrand R, Breton J, Aziz M, Guérin C, Coëffier M, Savoye G, Marion-Letellier R. 2019. Chronic colitis-induced visceral pain is associated with increased anxiety during quiescent phase. Am J Physiol - Gastrointest Liver Physiol 316:G692–G700. doi:10.1152/ajpgi.00248.2018

Sandner-Kiesling A, Eisenach JC. 2002. Estrogen reduces efficacy of μ- but not κ-opioid agonist inhibition in response to uterine cervical distension. Anesthesiology 96:375–380. doi:10.1097/00000542-200202000-00024

Sapsed-Byrne S, Ma D, Ridout D, Holdcroft A. 1996. Estrous cycle phase variations in visceromotor and cardiovascular responses to colonic distension in the anesthetized rat. Brain Res 742:10–16. doi:10.1016/S0006-8993(96)00989-4

Schwartz ES, La JH, Scheff NN, Davis BM, Albers KM, Gebhart GF. 2013. TRPV1 and TRPA1 antagonists prevent the transition of acute to chronic

inflammation and pain in chronic pancreatitis. J Neurosci 33:5603–5611. doi:10.1523/JNEUROSCI.1806-12.2013

Sengupta JN, Snider A, Su X, Gebhart GF. 1999. Effects of kappa opioids in the inflamed rat colon. Pain 79:175–185. doi:10.1016/S0304-3959(98)00175-4

Sharp BM. 2006. Multiple opioid receptors on immune cells modulate intracellular signaling. Brain Behav Immun 20:9–14. doi:10.1016/j.bbi.2005.02.002

Smith JS, Schindler AG, Martinelli E, Gustin RM, Bruchas MR, Chavkin C. 2012. Stress-induced activation of the dynorphin/κ-opioid receptor system in the amygdala potentiates nicotine conditioned place preference. J Neurosci 32:1488–1495. doi:10.1523/JNEUROSCI.2980-11.2012

Stein C. 2016. Opioid Receptors. Annu Rev Med 67:433–451. doi:10.1146/annurev-med-062613-093100

Stoffel EC, Ulibarri CM, Craft RM. 2003. Gonadal steroid hormone modulation of nociception, morphine antinociception and reproductive indices in male and female rats. Pain 103:285–302. doi:10.1016/s0304-3959(02)00457-8

Su X, Sengupta JN, Gebhart GF. 1997. Effects of opioids on mechanosensitive pelvic nerve afferent fibers innervating the urinary bladder of the rat. J Neurophysiol 77:1566–1580. doi:10.1152/jn.1997.77.3.1566

Tao YM, Li QL, Zhang CF, Xu XJ, Chen J, Ju YW, Chi ZQ, Long YQ, Liu JG. 2008. LPK-26, a novel κ-opioid receptor agonist with potent

antinociceptive effects and low dependence potential. Eur J Pharmacol 584:306–311. doi:10.1016/j.ejphar.2008.02.028

Tejeda HA, Natividad LA, Orfila JE, Torres O V, O'Dell LE. 2012. Dysregulation of kappa-opioid receptor systems by chronic nicotine modulate the nicotine withdrawal syndrome in an age-dependent manner. Psychopharmacology (Berl) 224:289–301. doi:10.1007/s00213-012-2752-7

Vanderah TW. 2010. Delta and kappa opioid receptors as suitable drug targets for pain. Clin J Pain 26 Suppl 1:S10-5. doi:10.1097/AJP.0b013e3181c49e3a

Vercellini P, Somigliana E, Viganò P, Abbiati A, Barbara G, Crosignani PG. 2009. Endometriosis: current therapies and new pharmacological developments. Drugs 69:649–675. doi:10.2165/00003495-200969060-00002

Vonvoigtlander PF, Lahti RA, Ludens JH. 1983. U-50,488: a selective and structurally novel non-Mu (kappa) opioid agonist. J Pharmacol Exp Ther 224:7–12.

Wahlström T, Laatikainen T, Salminen K, Leppäluoto J. 1985. Immunoreactive beta-endorphin is demonstrable in the secretory but not in the proliferative endometrium. Life Sci 36:987–990. doi:10.1016/0024-3205(85)90395-9

Zenclussen ML, Casalis PA, Jensen F, Woidacki K, Zenclussen AC. 2014. Hormonal fluctuations during the estrous cycle modulate heme oxygenase-1 expression in the uterus. Front Endocrinol (Lausanne) 5:1–6. doi:10.3389/fendo.2014.00032

Zhu Y, Hsu MS, Pintar JE. 1998. Developmental expression of the mu, kappa, and delta opioid receptor mRNAs in mouse. J Neurosci 18:2538–2549. doi:10.1523/JNEUROSCI.18-07-02538.1998

Zondervan KT, Becker CM, Missmer SA. 2020. Endometriosis. N Engl J Med 382:1244–1256. doi:10.1056/NEJMra1810764

Supplementary material

Supplementary table S1. Statistical results for Figure 1.

	Statistical analysis		Result
(A) Gene expression fold change	Kruskal-Wallis Test		H(2)=15.881; p=0.0004
(B) Dose-response curve of U50,488H for pelvic mechanical sensitivity	Mixed Model Analysis	Endometriosis	F(1,64.428)=10.239; p=0.0021
		Dose	F(5,26.950)=6.076; p=0.0007
		Endometriosis*dose	F(1,26.950)=2.846; p=0.0344
(Sham) Mechanical sensitivity after administration of different doses of U50,488H (frequency of response)	Mixed Model Analysis	Filament	F(5, 78.177)=20.247; p=0.0000
		Dose	F(5,1.912)=3.970; p=0.0942
		Filament*dose	F(25, 78.177)=0.799; p=0.7313
(Endo) Mechanical sensitivity after administration of different doses of U50,488H (frequency of response)	Mixed Model Analysis	Filament	F(5,69.872)=20.183; p=0.0000
		Dose	F(5,189.179)=15.570; p=0.0000
		Filament*dose	F(25,69.872)=0.568; p=0.9424

Supplementary table S2. Statistical results for Figure 3.

	Statistical analysis		Result
(A) Pelvic mechanical sensitivity through time (AUC)	Mixed Model Analysis	Endometriosis	F(1,153.604)=43.772; p=0.0000
		Treatment	F(1,153.604)=76.339; p=0.0000
		Day	F(4,62.567)=24.843; p=0.0000
		Endometriosis*treatment	F(1,153.604)=44.595; p=0.0000
		Endometriosis*day	F(4,62.567)=5.519; p=0.0007
		Treatment*day	F(4,62.567)=9.747; p=0.0000
		Endometriosis*treatment*day	F(4,62.567)=7.451; p=0.0001
(B) (Day -1, baseline) Effect of chronic U50,488H on pelvic mechanical sensitivity (frequency of response)	Mixed Model Analysis	Endometriosis	F(1,198.289)=2.715; p=0.1010
		Treatment	F(1,198.289)=0.242; p=0.6232
		Filament	F(5, 66.027)=34.713; p=0.0000
		Endometriosis*treatment	F(1,198.289)=0.011; p=0.9149
		Endometriosis*filament	F(5, 66.027)=1.139; p=0.3487
		Treatment*filament	F(5, 66.027)=0.600; p=0.7001
		Endometriosis*treatment*filament	F(5, 66.027)=1.062; p=0.3895
(B) (Day 7) Effect of chronic U50,488H on pelvic mechanical sensitivity (frequency of response)	Mixed Model Analysis	Endometriosis	F(1,184.408)=3.392; p=0.0671
		Treatment	F(1,184.408)=63.352; p=0.0000
		Filament	F(5, 63.121)=11.156; p=0.0000
		Endometriosis*treatment	F(1,184.408)=2.551; p=0.1119
		Endometriosis*filament	F(5, 63.121)=0.174; p=0.9712
		Treatment*filament	F(5, 63.121)=0.120; p=0.9876
		Endometriosis*treatment*filament	F(5, 63.121)=0.182; p=0.9684
(B) (Day 14) Effect of chronic U50,488H on pelvic mechanical sensitivity (frequency of response)	Mixed Model Analysis	Endometriosis	F(1,207.173)=56.904; p=0.0000
		Treatment	F(1,207.173)=47.222; p=0.0000
		Filament	F(5,64.733)=11.011; p=0.0000
		Endometriosis*treatment	F(1,207.173)=69.197; p=0.0000
		Endometriosis*filament	F(5,64.733)=0.200; p=0.9614
		Treatment*filament	F(5,64.733)=0.74; p=0.9959
		Endometriosis*treatment*filament	F(5,64.733)=0.149; p=0.9796
(B) (Day 21) Effect of chronic U50,488H on pelvic mechanical sensitivity (frequency of response)	Mixed Model Analysis	Endometriosis	F(1,192.292)=106.759; p=0.0000
		Treatment	F(1,192.292)=141.677; p=0.0000
		Filament	F(5,76.989)=24.656; p=0.0000
		Endometriosis*treatment	F(1,192.292)=94.134; p=0.0000
		Endometriosis*filament	F(5,76.989)=1.379; p=0.2413
		Treatment*filament	F(5,76.989)=0.288; p=0.9186
		Endometriosis*treatment*filament	F(5,76.989)=0.448; p=0.8132
(B) (Day 28) Effect of chronic U50,488H on pelvic mechanical sensitivity (frequency of response)	Mixed Model Analysis	Endometriosis	F(1,192.594)=90.741; p=0.0000
		Treatment	F(1,192.594)=159.458; p=0.0000
		Filament	F(5,61.985)=20.168; p=0.0000
		Endometriosis*treatment	F(1,192.594)=118.518; p=0.0000
		Endometriosis*filament	F(5,61.985)=0.131; p=0.9846
		Treatment*filament	F(5,61.985)=0.270; p=0.9278
		Endometriosis*treatment*filament	F(5,61.985)=0.151; p=0.9791
(C) Pelvic mechanical sensitivity on day 28, in different stages of the estrous cycle (AUC)	Kruskal-Wallis Test		H(7)=25.166; p=0.0007

(continues)

(D) (Sham) Effects of U50,488H on pelvic mechanical sensitivity (frequency of response)	Mixed Model Analysis	Filament	F(5,27.377)=9.255; p=0.0000
		Estrous phase	F(1,78.951)=10.578; p=0.0017
		Treatment	F(1,78.951)=2.400; p=0.1253
		Filament*Estrous phase	F(5,27.377)=0.162; p=0.9743
		Filament*Treatment	F(5,27.377)=0.165; p=0.9734
		Estrous phase*Treatment	F(1,78.951)=4.013; p=0.0486
		Filament*Estrous phase*Treatment	F(5,27.377)=0.179; p=0.9683
(D) (Endo) Effects of U50,488H on pelvic mechanical sensitivity (frequency of response)	Mixed Model Analysis	Filament	F(5,34.150)=19.068; p=0.0000
		Estrous phase	F(1,68.174)=50.150; p=0.0000
		Treatment	F(1,68.174)=399.220; p=0.0000
		Filament*Estrous phase	F(5,34.150)=2.585; p=0.0436
		Filament*Treatment	F(5,34.150)=0314; p=0.9009
		Estrous phase*Treatment	F(1,68.174)=65.502; p=0.0000
		Filament*Estrous phase*Treatment	F(1,68.174)=0.351; p=0.8779
(E) Motor coordination (latency to fall from the rotarod)	2-way ANOVA	Endometriosis	F(1,33)=3.861; p=0.0579
		Treatment	F(1,33)=3.970; p=0.0546
		Endometriosis*treatment	F(1,33)=0.029; p=0.8662
(F, left panel) Horizontal activity (beam breaks/min) from min 0 to min 60	2-way ANOVA	Endometriosis	F(1,34)=0.000; p=0.9905
		Treatment	F(1,34)=0.283; p=0.5980
		Endometriosis*treatment	F(1,34)=0.147; p=0.7034
(F, right panel) Horizontal activity (beam breaks/min) from min 60 to min 90	2-way ANOVA	Endometriosis	F(1,33)=0.093; p=0.7624
		Treatment	F(1,33)=2.992; p=0.0930
		Endometriosis*treatment	F(1,33)=0.477; p=0.4946
(G, left panel) Rearings/min from min 0 to min 60	2-way ANOVA	Endometriosis	F(1,31)=5.956; p=0.0206
		Treatment	F(1,31)=7.9876; p=0.0082
		Endometriosis*treatment	F(1,31)=0.283; p=0.5985
(G, right panel) Rearings/min from min 60 to min 90	Kruskal-Wallis Test		H(3)=16.041; p=0.0011

Supplementary table S3. Statistical results for Figure 4.

	Statistical analysis		Result
(A) Anxiety-like behavior (% of time in open arms)	2-way ANOVA	Endometriosis	F(1,30)=5.524; p=0.0255
		Treatment	F(1,30)=0.025; p=0.8745
		Endometriosis*treatment	F(1,30)=2.5; p=0.1244
(B) Cognitive performance (discrimination index)	Kruskal-Wallis Test		H(3)=10.539; p=0.0145
(C, left panel) Cyst diameter	U Mann Whitney		u=39, z=-0.832, p=0.436
(C, right panel) Cyst endometrial area	U Mann Whitney		u=33, z=-0.663, p=0.546

Article #4

Behavioral and neuroinflammatory changes induced by minimal endometriosis in mice

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(In preparation)

Behavioral and neuroinflammatory changes induced by minimal endometriosis in mice

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Declarations of interest: none

Abstract

Endometriosis is defined by the growth of endometrial cells in extrauterine locations and is associated with pain and infertility. However, the extent of the ectopic endometrial growths does not correlate with the severity of the symptoms. Although altered immune function has been associated to this disease, the neuroinflammatory changes associated to endometriosis have not been yet investigated. Here, we show that minimal endometriosis in immunocompetent mice leads to abdominopelvic hypersensitivity, anxiety and depression-like behaviors and cognitive deficits that correlate with neuroinflammatory changes in the periaqueductal gray, the medial prefrontal cortex and the hippocampus. Mice subjected to this model of endometriosis show increased expression levels of the inflammatory markers COX2 and Il1β, the astrocyte marker GFAP, and of the T lymphocyte markers CD4 and CD2 in these brain areas. The presence of endometrial cells in the peritoneum of mice subjected to this model was confirmed by flow cytometry of the peritoneal lavage fluid from mice that received GFP+ endometrial cells. This model, which recapitulates the symptoms of human endometriosis, may be a useful tool to study the immune mechanisms involved in pain and psychological alterations associated to minimal endometriosis.

Keywords

Endometriosis, female, chronic pelvic pain, lymphocytes

Introduction

Endometriosis is a chronic inflammatory disease that affects 1 in 10 women of reproductive age. It is defined by the presence of endometrial cells outside the uterus and is diagnosed by laparoscopy (Agarwal et al., 2019). However, the absence of obvious lesions does not eliminate the possibility of endometriosis, and there is no correlation between extent of the disease and symptom severity (Johnson et al., 2017). The main symptoms of endometriosis are pelvic pain and infertility, which are often accompanied by anxiety, depression and reduced work productivity (Fourquet et al., 2011).

Endometriosis has been hypothesized to develop from retrograde menstruation and is associated with dysregulation of the immune system (Ahn et al., 2015). On the other hand, chronic pelvic pain caused by endometriosis has been related to changes in regional gray matter volume in patients (As-Sanie et al., 2012). Chronic pain in experimental models leads to inflammatory events in brain areas related to pain processing, emotional responses and learning and memory (Del Rey et al., 2011; Zhuang et al., 2016; Ong et al., 2019). Nevertheless, whether endometriosis induces inflammation in the central immune system has not been investigated yet.

This work characterizes the behavioral manifestations showed by mice subjected to a model of minimal endometriosis provoked through the injection of syngeneic endometrial cells into the peritoneal cavity of immunocompetent female mice. Furthermore, it describes the neuroinflammatory changes associated to these behavioral alterations.

Methods

Animals

C57Bl/6J 8-week-old female mice (Charles Rivers) were used in behavioral experiments. Female transgenic mice ubiquitously expressing enhanced GFP (C57BL/6 background, GPF+ mice) and their wild type (WT) littermates were used in the experiment involving detection of injected endometrial cells by flow cytometry, and were a generous gift from Dr.Pura Muñoz-Cánoves laboratory. Mice were housed in cages of 4-5 mice with ad libitum access to water and food. Housing conditions were 21 ± 1°C and 55 ± 10% relative humidity in a controlled light/dark cycle. Mice were habituated to housing and handling for 1 week prior to the experiments. All procedures were conducted in accordance with standard ethical guidelines (European Communities Directive 2010/63/EU and NIH Guide for Care and Use of Laboratory Animals, 8th Edition) and approved by ethical committees. Treatment groups were randomly assigned, and experiments were performed under blind conditions.

Endometriosis model

Endometriosis was induced through the injection of syngeneic endometrial fragments as previously described (Somigliana et al., 1999), with some modifications. Briefly, animals were injected subcutaneously with β -estradiol 3 μg/mouse (E8875, Sigma) in corn oil 7 days before induction of endometriosis. The phase of the estrous cycle on the day of endometriosis induction was assessed by histological examination of cells extracted by vaginal lavage as

previously described (Escudero-Lara et al., 2020b), and donor mice at diestrus were euthanized by cervical dislocation. Their uterine horns were excised and placed on a petri dish with 100µl gentamicin 0.3 mg/ml solution in saline 0.9% and opened longitudinally. The endometrium was separated from the myometrium by scraping with two spatulas and suspended in gentamicin 0.3 mg/ml solution up to a volume of 0.4 ml. Endometrial cells obtained from a single uterine horn were injected into the lower middle abdomen of each recipient mice (endometriosis mice) with an 18G syringe. Control mice received 0.4 ml of gentamicin solution.

Study design

A first experiment evaluated the nociceptive, affective and cognitive behaviors of endometriosis mice. Baseline mechanical sensitivity was evaluated on day -1, and endometriosis or control injections were performed on day 0. Nociceptive responses were measured again on days 7, 14, 21 and 28. Anxiety-like, cognitive and depression-like behaviors were assessed on days 16, 20, and 33, respectively. On day 35, mice were euthanized for examination of the peritoneum.

In a second experiment, endometrium from GFP+ mice was injected to wild-type littermates to determine whether endometrial cells could be found in the peritoneum 35 days later. The presence of GFP+ cells in the fluid obtained by peritoneal lavage was assessed by flow cytometry.

Behavioral evaluation

Mechanical sensitivity was quantified by measuring the responses to von Frey filament stimulation of abdominopelvic area, anxiety-like behavior was evaluated with the elevated plus maze test, and cognitive performance was assayed with the novel object recognition task as previously reported (Escudero-Lara et al., 2020a).

The forced swimming test was performed to evaluate depression-like behavior. Mice were placed in a cylinder with water (23–25°C), being forced to swim for 6 min. Duration of immobility was quantified over the last 4 min.

Gene expression analysis

Periaqueductal gray, medial prefrontal cortex and hippocampus were obtained from untreated mice on day 35. RNA was isolated using Trizol® (Invitrogen, cat. 15596018) and reverse-transcribed with a High-Capacity cDNA Reverse-Transcription Kit (Applied Biosystems, cat.4368814). RT-PCR was performed with a QuantStudio 12KFlex Real-Time PCR System (Applied Biosystems, cat.4471134) using the SYBR Green PCR MasterMix (Roche, cat.04707516001). Primers are listed in supplementary table 1. Data for each gene were analyzed by the $2^{-\Delta\Delta Ct}$ method after normalization to *Actb*.

Flow cytometry analysis of peritoneal cells

Peritoneal lavages were performed on day 35 to wild-type mice that received GFP+ endometrial cells. Samples from wild-type and GFP+ mice were obtained to establish gate conditions. Immunofluorescence was measured using a BD™ LSR II flow cytometer (BD biosciences), and

data were analyzed with the FACSDiva^{TM}v6.2 software (BD biosciences).

Statistics

All statistical analyses were performed using IBM SPSS 24 software (Chicago, IL, USA). The adequate statistical test was applied according to the data distribution defined by the Shapiro-Wilk normality test. Mixed model analyses followed by Bonferroni test were used for repeated measures. Comparisons between two groups were analyzed with U Mann-Whitney or Student T tests. Data are expressed as individual data points and mean ± SEM. Values that felt outside of the interval determined as the mean ± 2 times the standard deviation were excluded from the analyses. Differences were considered statistically significant when the p value was below 0.05.

Results and discussion

The first aim was to investigate the nociceptive, affective and cognitive behaviors of female mice receiving endometrial cells (endometriosis mice) or control solution. Endometriosis mice showed persistent mechanical hypersensitivity in the abdominopelvic area, whereas nociceptive behavior of control mice remained unaltered (Fig.1A). Endometriosis mice also showed exacerbated anxiety-like behavior reflected in lower percentages of entries to the open arms of the elevated plus maze (Fig.1B), and increased depression-like behavior revealed by longer immobility times in the forced swimming test (Fig.1C). Accordingly, previous studies in other models of endometriosis described abdominopelvic hypersensitivity (Escudero-Lara et al., 2020a; Fattori et al., 2020) and affective-like disturbances (Escudero-Lara et al., 2020a; Filho et al., 2019; Li et al., 2018). These alterations could be a direct consequence of abdominopelvic hypersensitivity; however, recent data obtained in a chronic neuropathic pain model revealed that a high-anxiety phenotype can also contribute to exacerbated nociception (Martínez-Navarro et al., 2019). Interestingly, higher anxiety, psychoticism and introversion scores have been reported in women with endometriosis compared to women with other painful gynecological conditions (Low et al., 1993). Furthermore, endometriosis mice showed decreased discrimination indices in the novel object recognition test (Fig.1D), in agreement with our previous study with a surgical endometriosis model (Escudero-Lara et al., 2020a). Hence, this model mimics the symptomatology observed in clinical endometriosis.

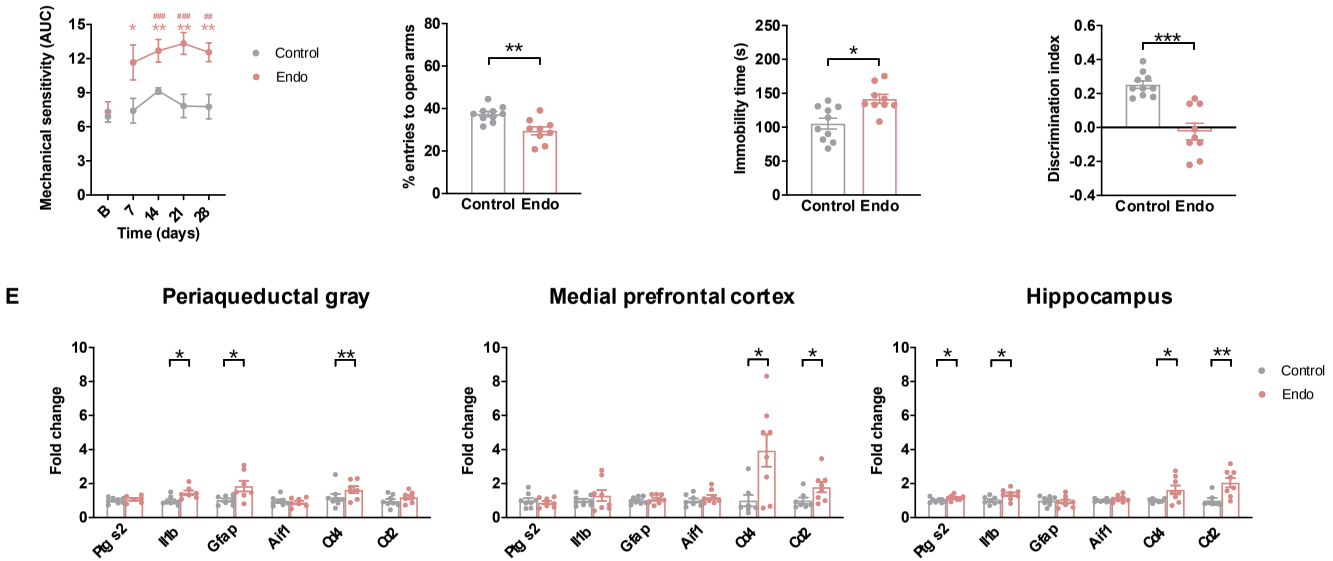

Figure 1. Behavioral and neuroinflammatory alterations associated to the presence of ectopic endometrial cells. Endometriosis mice showed (**A**) persistent mechanical hypersensitivity in the abdominopelvic area after stimulation with von Frey filaments, (**B**) increased anxiety-like behavior in the elevated plus maze test, (**C**) increased depression-like behavior in the forced swimming test and (**D**) cognitive impairment in the novel object recognition task. (**E**, left panel) Endometriosis mice showed increased expression of the genes coding for interleukin 1 beta (*Il1b*), the astrocyte marker GFAP (*Gfap*) and the T lymphocyte marker CD4 (*Cd4*) in the periaqueductal gray, (central panel) the genes coding for the T lymphocyte markers CD4 (*Cd4*) and CD2 (*Cd2*) in the medial prefrontal cortex and, (right panel) the genes coding for cyclooxygenase 2 (*Ptgs2*), interleukin 1 beta (*Il1b*), CD4 (*Cd4*) and CD2 (*Cd2*) in the hippocampus. (A) Mixed model analysis, (B, C, and D) Student t-test. (D) Student t-test or Mann Whitney U. ##p<0.01, ###p<0.001 vs baseline; *p<0.05, **p<0.01, ***p<0.001 vs control. Endo, endometriosis; AUC, area under the curve. Ptgs2, prostaglandin-endoperoxide synthase 2; Il1b, interleukin 1 beta; Gfap, glial fibrillary acidic protein; Aif1, allograft inflammatory factor 1; CD4, cluster of differentiation 4; CD2, cluster of differentiation 2.

The presence of macroscopic endometriotic growths in mice receiving endometrial cells could not be confirmed. Nonetheless, flow cytometry analysis of the peritoneal lavage fluid of wild-type mice receiving GPF+ endometrial cells revealed the presence of GFP+ cells (0.095±0.042%) 35 days after injection (Fig.2). This indicates that mice subjected to this model could develop microscopic lesions that led to behavioral alterations. In endometriosis patients, the extent of endometriotic lesions is not correlated with symptom severity, with minimal endometriosis being able to cause high degrees of pain (Johnson et al., 2017).

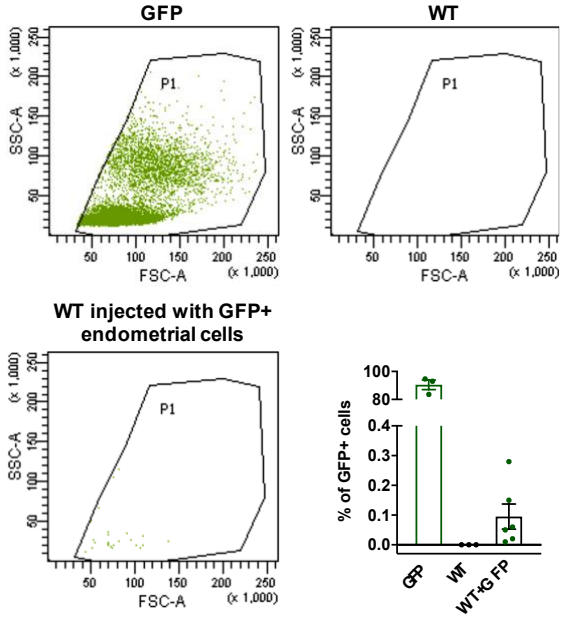

Figure 2. Flow cytometry analysis of peritoneal fluid from wild type mice receiving GFP+ endometrial cells. A percentage 0.095±0.042 GFP+ cells was identified in the peritoneum of WT mice 35 days after the injection. GFP, green fluorescent protein; WT, wild type.

References

Agarwal, S.K., Chapron, C., Giudice, L.C., Laufer, M.R., Leyland, N., Missmer, S.A., Singh, S.S., Taylor, H.S., 2019. Clinical diagnosis of endometriosis: a call to action. Am. J. Obstet. Gynecol. 220, 354.e1-354.e12. https://doi.org/10.1016/j.ajog.2018.12.039

As-Sanie, S., Harris, R.E., Napadow, V., Kim, J., Neshewat, G., Kairys, A., Williams, D., Clauw, D.J., Schmidt-Wilcke, T., 2012. Changes in regional gray matter volume in women with chronic pelvic pain: a voxel-based morphometry study. Pain 153, 1006–1014. https://doi.org/10.1016/j.pain.2012.01.032

Brynskikh, A., Warren, T., Zhu, J., Kipnis, J., 2008. Adaptive immunity affects learning behavior in mice. Brain. Behav. Immun. 22, 861–869. https://doi.org/10.1016/j.bbi.2007.12.008

Chen, Z., Xie, F., Bao, M., Li, X., Chao, Y., Lin, C., Guo, R., Zhang, C., Wu, A., Yue, Y., Guan, Y., Wang, Y., 2015. Activation of p38 MAPK in the rostral ventromedial medulla by visceral noxious inputs transmitted via the dorsal columns may contribute to pelvic organ cross-sensitization in rats with endometriosis. Neuroscience 291, 272–278. https://doi.org/10.1016/j.neuroscience.2015.02.021

Clark, S.M., Soroka, J.A., Song, C., Li, X., Tonelli, L.H., 2016. CD4(+) T cells confer anxiolytic and antidepressant-like effects, but enhance fear memory processes in Rag2(-/-) mice. Stress 19, 303–311. https://doi.org/10.1080/10253890.2016.1191466

Dodds, K.N., Beckett, E.A.H., Evans, S.F., Hutchinson, M.R., 2019. Spinal Glial Adaptations Occur in a Minimally Invasive Mouse Model of

Endometriosis: Potential Implications for Lesion Etiology and Persistent Pelvic Pain. Reprod. Sci. 26, 357–369. https://doi.org/10.1177/1933719118773405

Escudero-Lara, A., Argerich, J., Cabañero, D., Maldonado, R., 2020a. Disease-modifying effects of natural Δ9-tetrahydrocannabinol in endometriosis-associated pain. Elife 9. https://doi.org/10.7554/eLife.50356

Escudero-Lara, A., Cabañero, D., Maldonado, R., 2020b. Surgical Induction of Endometriosis in Female Mice. Bio-Protocol 10, 1–14. https://doi.org/10.21769/bioprotoc.3763

Fan, K., Li, Yi-yuan, Wang, H., Mao, X., Guo, J., Wang, F., Huang, L., Li, Yi-ning, Ma, X., Gao, Z., Chen, W., Qian, D., Xue, W., Cao, Q., Zhang, Lei, Shen, L., Zhang, Long, Tong, C., Zhong, J., Lu, W., Lu, L., Ren, K., Zhong, G., Wang, Y., Tang, M., Feng, X.-H., Chai, R., Jin, J., 2019. Stress-Induced Metabolic Disorder in Peripheral CD4+ T Cells Leads to Anxiety-like Behavior. Cell 179, 864-879.e19. https://doi.org/10.1016/j.cell.2019.10.001

Fattori, V., Franklin, N.S., Gonzalez-Cano, R., Peterse, D., Ghalali, A., Madrian, E., Verri, W.A.J., Andrews, N., Woolf, C.J., Rogers, M.S., 2020. Nonsurgical mouse model of endometriosis-associated pain that responds to clinically active drugs. Pain 161, 1321–1331. https://doi.org/10.1097/j.pain.0000000000001832

Filho, P.W.L.L., Chaves Filho, A.J.M., Vieira, C.F.X., Oliveira, T. de Q., Soares, M.V.R., Jucá, P.M., Quevedo, J., Barichello, T., Macedo, D., das Chagas Medeiros, F., 2019. Peritoneal endometriosis induces time-

related depressive- and anxiety-like alterations in female rats: involvement of hippocampal pro-oxidative and BDNF alterations. Metab. Brain Dis. https://doi.org/10.1007/s11011-019-00397-1

Fourquet, J., Báez, L., Figueroa, M., Iriarte, R.I., Flores, I., 2011. Quantification of the impact of endometriosis symptoms on health-related quality of life and work productivity. Fertil. Steril. 96, 107–112. https://doi.org/10.1016/j.fertnstert.2011.04.095

Gogacz, M., Winkler, I., Bojarska-Junak, A., Tabarkiewicz, J., Semczuk, A., Rechberger, T., Adamiak, A., 2016. Increased percentage of Th17 cells in peritoneal fluid is associated with severity of endometriosis. J. Reprod. Immunol. 117, 39–44. https://doi.org/https://doi.org/10.1016/j.jri.2016.04.289

Greaves, E., Horne, A.W., Jerina, H., Mikolajczak, M., Hilferty, L., Mitchell, R., Fleetwood-Walker, S.M., Saunders, P.T.K., 2017. EP2 receptor antagonism reduces peripheral and central hyperalgesia in a preclinical mouse model of endometriosis. Sci. Rep. 7. https://doi.org/10.1038/srep44169

Johnson, N.P., Hummelshoj, L., Adamson, G.D., Keckstein, J., Taylor, H.S., Abrao, M.S., Bush, D., Kiesel, L., Tamimi, R., Sharpe-Timms, K.L., Rombauts, L., Giudice, L.C., 2017. World endometriosis society consensus on the classification of endometriosis. Hum. Reprod. 32, 315–324. https://doi.org/10.1093/humrep/dew293

Kim, S.-J., Lee, H., Lee, G., Oh, S.-J., Shin, M.-K., Shim, I., Bae, H., 2012. CD4+CD25+ regulatory T cell depletion modulates anxiety and

depression-like behaviors in mice. PLoS One 7, e42054–e42054. https://doi.org/10.1371/journal.pone.0042054

Kobayashi, Y., Kiguchi, N., Fukazawa, Y., Saika, F., Maeda, T., Kishioka, S., 2015. Macrophage-T cell interactions mediate neuropathic pain through the glucocorticoid-induced tumor necrosis factor ligand system. J. Biol. Chem. 290, 12603–12613. https://doi.org/10.1074/jbc.M115.636506

Labuz, D., Schmidt, Y., Schreiter, A., Rittner, H.L., Mousa, S.A., Machelska, H., 2009. Immune cell-derived opioids protect against neuropathic pain in mice. J. Clin. Invest. 119, 278–286. https://doi.org/10.1172/JCI36246

Laumet, G., Edralin, J.D., Dantzer, R., Heijnen, C.J., Kavelaars, A., 2019. Cisplatin educates CD8+ T cells to prevent and resolve chemotherapy-induced peripheral neuropathy in mice. Pain 160, 1459–1468. https://doi.org/10.1097/j.pain.0000000000001512

Li, T., Mamillapalli, R., Ding, S., Chang, H., Liu, Z.W., Gao, X.B., Taylor, H.S., 2018. Endometriosis alters brain electrophysiology, gene expression and increases pain sensitization, anxiety, and depression in female mice. Biol. Reprod. 99, 349–359. https://doi.org/10.1093/biolre/ioy035

Liu, Z., Chen, S., Qiu, C., Sun, Y., Li, W., Jiang, J., Zhang, J.-M., 2018. Fractalkine/CX3CR1 Contributes to Endometriosis-Induced Neuropathic Pain and Mechanical Hypersensitivity in Rats. Front. Cell. Neurosci. 12, 495. https://doi.org/10.3389/fncel.2018.00495

Pasciuto, E., Burton, O.T., Roca, C.P., Lagou, V., Rajan, W.D., Theys, T., Mancuso, R., Tito, R.Y., Kouser, L., Callaerts-Vegh, Z., de la Fuente, A.G., Prezzemolo, T., Mascali, L.G., Brajic, A., Whyte, C.E., Yshii, L., Martinez-Muriana, A., Naughton, M., Young, A., Moudra, A., Lemaitre, P., Poovathingal, S., Raes, J., De Strooper, B., Fitzgerald, D.C., Dooley, J., Liston, A., 2020. Microglia Require CD4 T Cells to Complete the Fetal-to-Adult Transition. Cell 182, 625-640.e24. https://doi.org/10.1016/j.cell.2020.06.026

Petrović, J., Silva, J.R., Bannerman, C.A., Segal, J.P., Marshall, A.S., Haird, C.M., Gilron, I., Ghasemlou, N., 2019. γδ T Cells Modulate Myeloid Cell Recruitment but Not Pain During Peripheral Inflammation. Front. Immunol. 10, 473. https://doi.org/10.3389/fimmu.2019.00473

Ron-Harel, N., Segev, Y., Lewitus, G.M., Cardon, M., Ziv, Y., Netanely, D., Jacob-Hirsch, J., Amariglio, N., Rechavi, G., Domany, E., Schwartz, M., 2008. Age-Dependent Spatial Memory Loss Can Be Partially Restored by Immune Activation. Rejuvenation Res. 11, 903–913. https://doi.org/10.1089/rej.2008.0755

Rosen, S.F., Ham, B., Drouin, S., Boachie, N., Chabot-Dore, A.-J., Austin, J.-S., Diatchenko, L., Mogil, J.S., 2017. T-Cell Mediation of Pregnancy Analgesia Affecting Chronic Pain in Mice. J. Neurosci. 37, 9819–9827. https://doi.org/10.1523/JNEUROSCI.2053-17.2017

Rosen, S.F., Ham, B., Haichin, M., Walters, I.C., Tohyama, S., Sotocinal, S.G., Mogil, J.S., 2019. Increased pain sensitivity and decreased opioid analgesia in T-cell-deficient mice and implications for sex differences. Pain 160.

Sorge, R.E., Mapplebeck, J.C.S., Rosen, S., Beggs, S., Taves, S., Alexander, J.K., Martin, L.J., Austin, J.S., Sotocinal, S.G., Chen, D., Yang, M., Shi, X.Q., Huang, H., Pillon, N.J., Bilan, P.J., Tu, Y., Klip, A., Ji, R.R., Zhang, J., Salter, M.W., Mogil, J.S., 2015. Different immune cells mediate mechanical pain hypersensitivity in male and female mice. Nat. Neurosci. 18, 1081–1083. https://doi.org/10.1038/nn.4053

Verma–Gandhu, M., Bercik, P., Motomura, Y., Verdu, E.F., Khan, W.I., Blennerhassett, P.A., Wang, L., El–Sharkawy, R.T., Collins, S.M., 2006. CD4+ T-Cell Modulation of Visceral Nociception in Mice. Gastroenterology 130, 1721–1728. https://doi.org/10.1053/j.gastro.2006.01.045

Supplementary Table 1. Sequences of the primers used in gene expression analyses.

	Forward	Reverse
Ptgs2	5'-CAGACAACATAAACTGCGCCTT-3'	5'-GATACACCTCTCCACCAATGACC-3'
Il1b	5'-GAAGTTGACGGACCCCAAAA-3'	5'-TGATGTGCTGCTGCGAGATT -3'
Gfap	5'-CAGAGGAGTGGTATCGGTCTAAGTT-3'	5'-CGATAGTCGTTAGCTTCGTGCTT-3'
Aif1	5'-CCCCCAGCCAAGAAAGCTAT-3'	5'-GCCCCACCGTGTGACATC-3'
Cd4	5'- CCCAGGTCTCGCTTCAGTTT -3'	5'-GGGAGAGGTAGGTCCCATCA-3'
Cd2	5'- ACCAACCTGAACGCACCATT -3'	5'- CCAAGAGCACCAAGAGGAGT -3'
Actb	5'-CGTGAAAAGATGACCCAGATCA-3'	5'-CACAGCCTGGATGGCTACGT-3'

DISCUSSION

The overall purpose of this Doctoral Thesis was to explore the possibility of targeting endogenous analgesia systems for the treatment of endometriosis, as well as to investigate the role of the adaptative immune system on endometriosis pain and its associated behavioral alterations.

A mouse model of endometriosis to test new therapeutic approaches

Available pharmacological and surgical therapies for endometriosis have important adverse effects and fail providing long-term alleviation of the symptoms (Falcone and Flyckt, 2018). Hence, new therapeutic strategies are needed, and their development relies on the establishment of animal models that recapitulate the features of clinical endometriosis. In this Thesis, two different models of endometriosis have been developed to evaluate at the same time nociceptive, cognitive and emotional manifestations of endometriosis in naturally-cycling immunocompetent mice. The first model consisted of a surgical implantation of endometrial tissue into the peritoneal wall of the abdominal compartment of syngeneic recipient mice. The second model was induced through the injection of endometrial tissue into the peritoneum of syngeneic recipient mice, and will be discussed in the last section of the Discussion.

Mice subjected to the surgically-induced model of endometriosis developed persistent mechanical hypersensitivity in the caudal abdominal area, but not in the hind paw, indicating that pain sensitization did not generalize to other sites in this model. Endometriosis mice also exhibited increased nocifensive behaviors to mechanical stimuli, revealing a component of negative affect

associated to abdominal hypersensitivity. In agreement, studies in other rodent models of endometriosis have found increased sensitivity to mechanical stimuli in the lower abdomen (Arosh *et al.*, 2015; Greaves *et al.*, 2017a). Mice subjected to this model of endometriosis also displayed a reduced number of rearings, a behavior that involves stretching of the abdomen and that can be interpreted as a measure of abdominal pain or discomfort. Indeed, reduced rearing behavior has been described in multiple models of visceral pain (Schwartz *et al.*, 2013; Salameh *et al.*, 2019; Lucarini *et al.*, 2020). Furthermore, mice receiving ectopic endometrial implants showed enhanced anxiety-like behavior, as previously reported in different models of endometriosis (Li *et al.*, 2018; Filho *et al.*, 2019). In patients, pelvic pain associated to endometriosis is commonly accompanied by anxiety (Laganà *et al.*, 2017). Notably, endometriosis patients report higher psychoticism and anxiety scores than individuals with pelvic pain of other origins (Low *et al.*, 1993). Thus, the exacerbated anxiety-like behavior found in mice subjected to the endometriosis model could be consequence of persistent hypersensitivity, and, in turn, the chronification of this hypersensitivity could also be favored by the increased anxiety-like behavior associated to the presence of ectopic endometrium.

Mice receiving ectopic endometrial implants also developed a pronounced impairment in long-term memory. In line with this finding, cognitive deficits have been reported in rodent models of inflammatory and neuropathic chronic pain (La Porta *et al.*, 2015; You *et al.*, 2018). It has not been investigated whether endometriosis could affect cognitive function in humans, however, endometriosis patients often

report loss of working ability and productivity (Fourquet et al., 2011; Sperschneider et al., 2019).

Ectopic endometrial implants grew into cysts filled with fluid and immune cells and with a wall consisting of glandular epithelium and stroma. The surface of these endometrial cysts was innervated by neurites, in agreement with previous studies in rodents (Berkley *et al.*, 2004; Arosh *et al.*, 2015) and endometriosis patients (Tokushige *et al.*, 2006a; Wang *et al.*, 2009b). Mice receiving endometrial implants also developed increased uterine innervation, suggesting that the presence of ectopic endometrial lesions facilitated nerve sprouting in the eutopic endometrium. Accordingly, women with endometriosis show high density of uterine nerve fibers, which may contribute to chronic pelvic pain (Tokushige *et al.*, 2006a; Miller EJ, 2015). Therefore, this mouse model of surgically-induced endometriosis recapitulates the histological and behavioral features of human endometriosis, providing a valuable tool to study the efficacy of potential treatments in the development of endometriosis and its symptoms.

Cannabinoids as potential treatments for endometriosis

Cannabis consumption is one of the self-management strategies that many endometriosis patients adopt to complement their medical treatments (Armour *et al.*, 2019). However, it is unknown how cannabis and cannabinoids like THC, the main psychoactive component of the plant, and CBD, which lacks important psychotropic properties, can affect endometriosis and its symptoms. With the objective of determining the effects of these cannabinoids on endometriosis, they

were chronically administered to mice subjected to the above-described surgical model of endometriosis.

On the one hand, acute THC reduced abdominal mechanical hypersensitivity in a dose-dependent manner in mice with fully developed endometriotic cysts. On the other hand, repeated administration of a moderate dose THC (2 mg/kg) starting right after induction of endometriosis or when the mechanical hypersensitivity was already developed, provided sustained alleviation of abdominal hypersensitivity. Interestingly, female mice subjected to this model did not become tolerant to the antinociceptive effects of this dose of THC, in contrast to the studies describing the development of tolerance to higher THC doses in other pain models (Greene *et al.*, 2018; Lafleur *et al.*, 2018). Subchronic THC treatment also inhibited nocifensive behaviors in mice with ectopic endometrium, although acute THC administration produced variable effects in this measure of pain unpleasantness. A separate experiment evaluated the effects of the non-psychoactive cannabinoid CBD (2 mg/kg), alone or in combination with THC (2 mg/kg), in the mechanical hypersensitivity associated to the presence of ectopic endometrium. CBD and CBD/THC treatments were as effective as THC alone relieving abdominal hypersensitivity. Although synergetic interactions between THC and CBD have been described in models of chronic pain (Casey *et al.*, 2017), no additive effects of the two cannabinoids were observed, since they already provided complete alleviation of mechanical hypersensitivity when administered alone.

Repeated exposure to THC, CBD or their combination did not modify the increased anxiety-like behavior of mice receiving endometrial implants, suggesting that pain relief was not sufficient to prevent this affective-like alteration. However, since THC shows anxiogenic and anxiolytic-like effects at slightly higher and lower doses (Viñals *et al.*, 2015), possible relief of pain-related anxiety-like behavior provided by THC could have been hindered by its intrinsic effects on this behavior. On the other hand, although anxiolytic-like effects of CBD have been widely described in the literature (reviewed by Blessing *et al.*, 2015), absence of effects of this cannabinoid alone or in combination with THC have also been reported in female mice (Kasten *et al.*, 2019), in accordance with this result.

Interestingly, chronic treatment with THC reverted the cognitive impairment shown by mice with ectopic endometrial implants, while it caused the expected memory deficits in sham animals, suggesting protective effects of THC in endometriosis. In agreement, recent studies have shown cognitive improvements after THC exposure in old male and female mice (Bilkei-Gorzo *et al.*, 2017; Sarne *et al.*, 2018) associated to THC effects in brain areas related to cognitive function, such as the prefrontal cortex and the hippocampus. The cognitive deficits of endometriosis mice treated with vehicle and sham mice receiving THC were associated with increased expression COX2 and IL1β in the medial prefrontal cortex, but not in the hippocampus. Remarkably, THC did not produce such effects in mice with ectopic endometrial implants. Both aging and chronic pain are associated to local inflammatory events in these brain areas (Di Benedetto *et al.*,

2017; Ong *et al.*, 2019). These data suggest that the effects of THC on cognitive function and neuroinflammation are different in the presence of an inflammatory condition. Similarly to the THC treatment, the repeated administration of CBD and the combination of both cannabinoids increased discrimination indices in mice with ectopic endometrial implants, although the improvement provided by THC/CBD did not reach statistical significance possibly due to a ceiling effect at these doses. Furthermore, a recent study has shown that repeated administration of the combination THC/CBD to naive female mice causes a stronger cognitive impairment than the treatment with each cannabinoid alone (Kasten *et al.*, 2017). This effect could also explain the slightly lower protective effect of THC/CBD on cognitive function of mice subjected to the model of endometriosis.

The studied cannabinoids also affected the growth of the endometrial implants in this model. A one-month treatment with THC inhibited the development of endometriotic lesions, without effects on the size of the eutopic endometrium, suggesting that this antiproliferative effect in endometrial cells was restricted to ectopic locations. Furthermore, estrogen levels in plasma were positively correlated with cyst diameter, showing an influence of this hormone on ectopic endometrial lesions. Nevertheless, estrogen levels did not correlate with endometrial area of the cysts, suggesting independent THC effects on this change. The treatments with CBD and the THC/CBD combination also inhibited the growth of the ectopic endometrium. This is in agreement with previous studies showing antiproliferative effects of WIN 55212-2 in endometrial cells *in vitro* and in an endometriosis model in

immunodepressed mice (Leconte *et al.*, 2010). However, THC did not produce any change in the innervation of the cysts. Since a previous study described that ACPA, a specific CB1 receptor agonist, enhanced nerve sprouting of cysts in a rat model of endometriosis (Han *et al.*, 2017), this finding suggests that other cannabinoid receptors may modulate the effect of cannabinoids on cyst innervation. On the other hand, repeated exposure to THC also modified the altered uterine innervation observed in this model. While THC increased the expression of neuronal markers in the uteri of sham mice, THC prevented this increase in mice with ectopic endometrium. This result suggests again that THC exposure may have different effects under this chronic condition. Since THC has been suggested to inhibit folliculogenesis and ovulation (Adashi *et al.*, 1983; El-Talatini *et al.*, 2009), the numbers of ovarian preantral follicles, antral follicles and corpora lutea were determined. These numbers were similar in all groups, suggesting that the implantation of ectopic endometrial tissue and the THC treatment lacked overt effects on ovarian follicle maturation and luteinization.

In summary, the phytocannabinoids THC, CBD and the THC/CBD combinations relieved mechanical pain sensitivity in a surgical model of endometriosis without inducing tolerance to its antinociceptive effects. THC also inhibited the negative affect associated to abdominal hypersensitivity. In addition, the three cannabinoid treatments prevented the cognitive impairment associated to the presence of ectopic endometrial cysts, with THC inducing cortical expression of neuroinflammatory markers in sham animals but reducing such

neuroinflammatory changes in mice subjected to endometriosis. However, the increased anxiety-like behavior was not modified by any of the treatments regardless of the complete alleviation of abdominal hypersensitivity, suggesting that anxiety and pain are independent events. Interestingly, THC, CBD and the combination of THC/CBD treatments inhibited the growth of ectopic endometrial cysts. The observed pain-relieving and disease modifying effects of cannabinoids in this model underline the possibility of targeting the endocannabinoid system for the treatment of endometriosis. Indeed, the effects of cannabinoids in endometriosis are already being evaluated in clinical trials (#NCT03875261 and gynica.com) that will provide evidence on the translatability of these results to the clinic.

Targeting the kappa opioid receptor for endometriosis pain

The surgical model of endometriosis was also used to study the effects of kappa opioid receptor (KOR) stimulation in the behavioral and histopathological alterations associated to endometriosis. It is known that KOR plays a crucial role in visceral and inflammatory pain (Kivell and Prisinzano, 2010; Vanderah, 2010), however, the involvement of this receptor in endometriosis had not been previously investigated.

This model allowed to study the expression of the three classical opioid receptors in ectopic endometrial growths. KOR was highly expressed in endometriotic cysts, in line with the studies reporting that it is the most abundant opioid receptor in endometrial cells (Hatzoglou *et al.*, 1995). On the other hand, since endometrial cysts are innervated and filled with fluid with immune cells, KOR could be involved in pain

transmission and inflammation in endometriosis. Hence, KOR could be a druggable target for endometriosis treatment.

Administration of the KOR agonist U50,488H provided acute dose-dependent alleviation of pelvic mechanical hypersensitivity in mice with ectopic endometrial implants. Repeated exposure to a low dose of U50,488H (1 mg/kg) also provided sustained alleviation of mechanical hypersensitivity during the entire experiment. The use of such a low dose of U50,488H could explain the lack of tolerance development to the antinociceptive effects of this KOR agonist, which is in contrast with the tolerance described for male rodents after administration of higher doses (McLaughlin *et al.*, 2004; Bhalla *et al.*, 2010). Notably, mice subjected to this model of endometriosis showed higher mechanical sensitivity during estrus, the phase of the estrous cycle associated to the lowest estrogen levels. The role of estrogens in animal models of nociception is complex, since both increased (Martínez-Gómez *et al.*, 1994; Kayser *et al.*, 1996) and decreased (Holdcroft *et al.*, 2000; Cason *et al.*, 2003) pain sensitivities have been reported in rodents in phases with low estrogen levels. Nevertheless, this model of endometriosis replicates the increased pain observed in women during menstruation (Laux-Biehlmann *et al.*, 2015), the phase of the menstrual cycle in which estrogen levels are at their lowest. Interestingly, KOR activation reduced pelvic hypersensitivity in mice with ectopic endometrium independently of the phase of the estrous cycle. Therefore, U50,488H produced the greatest alleviation of hypersensitivity during estrus, in agreement with the higher KOR-mediated antinociception described in naïve mice in this phase of the

estrous cycle (Abraham *et al.*, 2018a). Administration of U50,488H also normalized the decreased rearing behavior in mice with ectopic endometrium, suggesting that KOR stimulation induced alleviation of spontaneous pain or discomfort. Notably, the low dose of U50,488H used in this study did not affect horizontal activity or motor coordination in female mice, indicating that analgesic efficacy can be achieved with low doses that lack significant effects on motor performance.

The increased anxiety-like behavior of mice subjected to the model of endometriosis was not affected by the U50,488H treatment. In agreement, it has been reported that this KOR agonist lacks effects on anxiety-like behavior in female mice (Robles *et al.*, 2014). Therefore, although the chronic treatment with U50,488H provided relief of mechanical and spontaneous pain behaviors in endometriosis mice, the increased anxiety-like behavior was not prevented. This result was similar to the observed with chronic THC, suggesting again that the affective disturbances shown by mice with ectopic endometrium may be independent of the increased pain-like behaviors. In this line, depressive symptomatology has been observed in a significant percentage of women with endometriosis without chronic pain (Lorençatto *et al.*, 2006) indicating that endometriosis could have a primary effect on emotional disturbances.

The treatment with the KOR agonist did not modify the cognitive impairment of mice with ectopic endometrium. Since the U50,488H produced cognitive deficits in sham mice, the lack of memory improvement in mice subjected to the model of endometriosis was

expected. Accordingly, KOR agonists have been widely reported to disrupt cognitive functions in female and male rodents (Daumas *et al.*, 2007; Amanda N. Carey *et al.*, 2009; Paris *et al.*, 2011; Abraham *et al.*, 2018b). Therefore, the observed amnesic effect of U50,488H is in agreement with the literature, but, unfortunately, it could have interfered with a possible improvement of memory associated to the alleviation of the nociceptive manifestations of endometriosis.

The chronic treatment with the KOR agonist did not affect the size of ectopic endometrial growths. It has been reported that activation of KOR stimulates apoptosis in cultured human endometrial cells (Chatzaki *et al.*, 2001), although the effects of KOR agonism *in vivo* had not been studied before. On the one hand, this result indicates that KOR stimulation in endometriotic cysts did not affect their growth. On the other hand, it indicates that the observed alleviation of pain-behaviors was due to the antinociceptive effects of U50,488H and not to an inhibition of the development of endometriosis.

In summary, KOR stimulation alleviated chronic hypersensitivity in mice subjected to a surgical model of endometriosis without inducing tolerance to its antinociceptive effects. KOR-mediated analgesia was particularly effective in estrus, the phase of the estrous cycle associated to the lowest levels of estrogen and in which endometriosis mice showed higher mechanical sensitivity. Exogenous KOR activation also normalized rearing behavior in mice with ectopic endometrium without altering motor coordination or horizontal activity, suggesting an alleviation of spontaneous abdominal pain or discomfort. Interestingly, KOR-mediated pain relief did not modify the anxiety-like

behavior and the cognitive deficits of endometriosis mice, suggesting, together with the results obtained in the study with THC, that pain, anxiety and cognitive alterations are independent processes in endometriosis. Therefore, a successful strategy for the management of endometriosis should be multidisciplinary and target simultaneously the different symptomatology.

Neuroinflammatory changes associated to minimal endometriosis

The second model of endometriosis used in this Thesis consisted of an injection of endometrial tissue into the peritoneum of syngeneic recipient mice. Female mice subjected to this model also showed sustained mechanical hypersensitivity in the abdominopelvic area and increased anxiety- and depression-like behaviors. Similarly to anxiety, exacerbated depression-like behaviors have also been previously reported in rodent models of endometriosis (Li *et al.*, 2018; Filho *et al.*, 2019). As in the surgical model, mice receiving ectopic endometrial cells through an intraperitoneal injection also displayed impairments in long-term memory, indicating that experimental endometriosis is strongly associated to cognitive deficits.

Interestingly, mice subjected to this model of endometriosis showed neuroinflammatory changes in brain areas related to pain, emotion, and cognitive processes. On the one hand, ectopic endometrial cells induced increases in the expression of IL1β in the periaqueductal gray and the hippocampus, where expression of COX2 was also increased. In agreement, endometriosis has been found to increase COX2 expression in the spinal cord, the thalamus and the cortex of mice that displayed pain-like behaviors (Greaves *et al.*, 2017a). On the other

hand, mice subjected to this model of endometriosis showed overexpression of the astrocyte marker GFAP in the periaqueductal gray, in line with the spinal astrocytosis previously described in a different model of endometriosis (Dodds *et al.*, 2019). On the contrary, ectopic endometrial cells did not induce changes in the expression of the microglia marker IBA1, although others have found microgliosis in the spinal cord (Liu *et al.*, 2018) and the rostromedial medulla (Chen *et al.*, 2015) associated to endometriosis. Therefore, changes in microglia activity in other areas besides the studied ones could not be discarded. Surprisingly, ectopic endometrial cells produced an elevated expression of the T lymphocyte markers CD4 and CD2 in the periaqueductal gray, the medial prefrontal cortex and the hippocampus. This result suggests that cells of the adaptative immune system may have infiltrated the brain of mice receiving ectopic endometrial cells. Hence, this model of endometriosis revealed neuroinflammatory changes in response to ectopic endometrial cells, and therefore, it could be a suitable model to study the contribution of the immune system to the behavioral manifestations of endometriosis.

Injection models of endometriosis are less invasive that the ones involving surgery, however, identification of endometriotic lesions is often difficult unless reporter mice are used as donors (Fortin *et al.*, 2003). In fact, mice subjected to this second model of endometriosis did not show macroscopic endometriotic growths one month after induction of endometriosis, although the presence of microscopic lesions could not be excluded. Indeed, when GFP+ endometrial cells were injected into the peritoneal cavity of wild type mice, the presence

of GFP+ cells one month after injection was detected by flow cytometry in the peritoneal lavage fluid of recipient mice. Hence, mice subjected to this model could develop microscopic endometriotic lesions able to induce nociceptive, affective-like and cognitive alterations. Since very small endometriotic growths are able to cause high degrees of pain in patients (Johnson *et al.*, 2017), this mouse model could be useful to investigate the behavioral manifestations of minimal endometriosis.

In conclusion, the present Thesis has characterized two models of endometriosis and has described the effects of cannabinoid and kappa opioid receptor stimulation on the behavioral and histopathological features of experimental endometriosis. On the one hand, the data presented in this Thesis have revealed that natural cannabinoids could have beneficial effects in the context of endometriosis, limiting its development and symptoms. On the other hand, the results obtained in the study with the kappa opioid agonist suggest that the different manifestations of endometriosis may constitute separate events, and therefore a multidisciplinary approach targeting the different symptoms could be necessary for an effective management of endometriosis.

CONCLUSIONS

The main conclusions of the work presented in this Thesis can be summarized as follows:

1. The presence of ectopic endometrium leads to persistent abdominopelvic pain sensitivity and associated negative affect, spontaneous abdominal pain, enhanced anxiogenic behavior and severe impairments in long-term memory.
2. Acute administration of THC produces a dose-dependent reduction of abdominopelvic mechanical hypersensitivity.
3. Repeated exposure to moderate doses of THC staring the day after induction of endometriosis provides sustained alleviation of abdominopelvic hypersensitivity and the associated negative affect without inducing tolerance.
4. THC is equally effective reducing pain-related behaviors when repeated administration starts once the painful symptomatology is established.
5. Chronic treatment with THC does not alter the enhanced anxiety-like behavior of endometriosis mice, suggesting that pain and affective disorders in endometriosis may constitute independent processes.
6. Chronic THC induces memory loss associated to cortical inflammatory changes, but it restores cognitive function in endometriosis mice without provoking the inflammatory alterations. This suggests that the presence of chronic inflammation modifies the consequences of THC exposure.
7. THC shows a striking effect limiting the growth of ectopic endometrium, without inducing apparent effects on the eutopic

endometrium or the ovarian follicle maturation of endometriosis mice.

8. Chronic treatments with THC, CBD and THC/CBD, show similar efficacy alleviating abdominopelvic mechanical hypersensitivity and restoring cognitive function in endometriosis mice.
9. The exacerbated anxiety-like behavior displayed by endometriosis mice is not modified by THC, CBD or THC/CBD treatments.
10. THC, CBD and the combination of both inhibit the growth of ectopic endometrium.
11. Repeated KOR stimulation with a low dose of the specific agonist U50,488H alleviates pelvic mechanical hypersensitivity and spontaneous pain associated to endometriosis without inducing antinociceptive tolerance.
12. This KOR-mediated pain relief is particularly effective during estrus, the phase of the estrous cycle in which mice are more sensitive to mechanical stimuli.
13. Sustained KOR-mediated pain relief does not modify the anxiety-like behavior or the memory impairment of mice with ectopic endometrial growths.
14. KOR is highly expressed in endometriotic cysts in this model, however, exogenous activation of KOR does not modify the development of ectopic endometrial growths.
15. Minimal endometriosis induces nociceptive, affective-like and cognitive manifestations that correlate with neuroinflammatory changes in the periaqueductal gray, the medial prefrontal cortex and the hippocampus.

REFERENCES

Ab Aziz, C. B. and Ahmad, A. H. (2006) The role of the thalamus in modulating pain, *Malaysian Journal of Medical Sciences*. School of Medical Sciences, Universiti Sains Malaysia, 13(2), pp. 11–18.

Abou-Setta, A. M., Houston, B., Al-Inany, H. G. and Farquhar, C. (2013) Levonorgestrel-releasing intrauterine device (LNG-IUD) for symptomatic endometriosis following surgery., *The Cochrane database of systematic reviews*. England, (1), p. CD005072.

Abraham, A. D. *et al.* (2018a) Estrogen regulation of GRK2 inactivates Kappa opioid receptor signaling mediating analgesia, but not aversion, *Journal of Neuroscience*, 38(37), pp. 8031–8043.

Abraham, A. D. *et al.* (2018b) κ-Opioid receptor activation in dopamine neurons disrupts behavioral inhibition, *Neuropsychopharmacology*. Nature Publishing Group, 43(2), pp. 362–372.

Adams, I. B., Compton, D. R. and Martin, B. R. (1998) Assessment of anandamide interaction with the cannabinoid brain receptor: SR 141716A antagonism studies in mice and autoradiographic analysis of receptor binding in rat brain., *The Journal of pharmacology and experimental therapeutics*. United States, 284(3), pp. 1209–1217.

Adashi, E. Y., Jones, P. B. C. and Hsueh, A. J. W. (1983) Direct antigonadal activity of cannabinoids: Suppression of rat granulosa cell functions, *American Journal of Physiology - Endocrinology and Metabolism*, 7(2), pp. E177-85.

Agarwal, N. *et al.* (2007) Cannabinoids mediate analgesia largely via

peripheral type 1 cannabinoid receptors in nociceptors., *Nature neuroscience*, 10(7), pp. 870–879.

Agarwal, S. K. *et al.* (2019) Clinical diagnosis of endometriosis: a call to action, *American Journal of Obstetrics and Gynecology*. Elsevier Inc., 220(4), pp. 354.e1-354.e12.

Ahluwalia, J. *et al.* (2000) Cannabinoid 1 receptors are expressed in nociceptive primary sensory neurons., *Neuroscience*. United States, 100(4), pp. 685–688.

Ahn, K. *et al.* (2011) Mechanistic and pharmacological characterization of PF-04457845: a highly potent and selective fatty acid amide hydrolase inhibitor that reduces inflammatory and noninflammatory pain., *The Journal of pharmacology and experimental therapeutics*, 338(1), pp. 114–124.

Akil, H., Meng, F., Devine, D. P. and Watson, S. J. (1997) Molecular and Neuroanatomical Properties of the Endogenous Opioid System: Implications for Treatment of Opiate Addiction, *Seminars in Neuroscience*, 9(3), pp. 70–83.

Akin, M. *et al.* (2004) Continuous, low-level, topical heat wrap therapy as compared to acetaminophen for primary dysmenorrhea., *The Journal of reproductive medicine*. United States, 49(9), pp. 739–745.

Al-Hasani, R. and Bruchas, M. R. (2011) Molecular mechanisms of opioid receptor-dependent signaling and behavior, *Anesthesiology*, 115(6), pp. 1363–1381.

Al-Sabbagh, M., Lam, E. W.-F. and Brosens, J. J. (2012) Mechanisms of endometrial progesterone resistance., *Molecular and cellular endocrinology*. Ireland, 358(2), pp. 208–215.

Alborzi, Saeed *et al.* (2011) A comparison of the effect of short-term aromatase inhibitor (letrozole) and GnRH agonist (triptorelin) versus case control on pregnancy rate and symptom and sign recurrence after laparoscopic treatment of endometriosis., *Archives of gynecology and obstetrics*. Germany, 284(1), pp. 105–110.

Aliaga, L. *et al.* (2002) *Tratamiento del dolor : teoría y práctica*. 2nd. ed. Barcelona: Publicaciones Permanyer.

Amarante, L. H., Alves, D. P. and Duarte, I. D. G. (2004) Study of the involvement of K+ channels in the peripheral antinociception of the κ-opioid receptor agonist bremazocine, *European Journal of Pharmacology*, 494(2), pp. 155–160.

Anand, U. *et al.* (2008) Cannabinoid receptor CB2 localisation and agonist-mediated inhibition of capsaicin responses in human sensory neurons., *Pain*. United States, 138(3), pp. 667–680.

Apkarian, A. V. *et al.* (2004) Chronic pain patients are impaired on an emotional decision-making task, *Pain*, 108(1–2), pp. 129–136.

Armour, M., Sinclair, J., Chalmers, K. J. and Smith, C. A. (2019) Self-management strategies amongst Australian women with endometriosis: a national online survey, *BMC Complementary and Alternative Medicine*. BMC Complementary and Alternative Medicine, 19(1), p. 17.

Arosh, J. A. *et al.* (2015) Molecular and preclinical basis to inhibit PGE2 receptors EP2 and EP4 as a novel nonsteroidal therapy for endometriosis, *Proceedings of the National Academy of Sciences of the United States of America*, 112(31), pp. 9716–9721.

As-Sanie, S. *et al.* (2012) Changes in regional gray matter volume in women with chronic pelvic pain: a voxel-based morphometry study, *Pain*. 2012/03/02, 153(5), pp. 1006–1014.

As-Sanie, S. *et al.* (2016) Functional connectivity is associated with altered brain chemistry in women with endometriosis-associated chronic pelvic pain, *J pain*, 17(1), pp. 1–13.

Auh, Q.-S. and Ro, J. Y. (2012) Effects of peripheral κ opioid receptor activation on inflammatory mechanical hyperalgesia in male and female rats, *Neuroscience letters*. 2012/07/20, 524(2), pp. 111–115.

Bambico, F. R., Katz, N., Debonnel, G. and Gobbi, G. (2007) Cannabinoids elicit antidepressant-like behavior and activate serotonergic neurons through the medial prefrontal cortex., *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(43), pp. 11700–11711.

Banghart, M. R. and Sabatini, B. L. (2012) Photoactivatable neuropeptides for spatiotemporally precise delivery of opioids in neural tissue., *Neuron*. NIH Public Access, 73(2), pp. 249–59.

Baños, J. E., Bosch, F. and Farré, M. (2006) *Historia de la terapéutica analgésica.*

Bär, K. J. *et al.* (2005) Pain perception in major depression depends

on pain modality, *Pain*, 117(1–2), pp. 97–103.

Baral, P., Udit, S. and Chiu, I. M. (2019) Pain and immunity: implications for host defence, *Nature Reviews Immunology*, 19(7), pp. 433–447.

Barcena de Arellano, M. L. *et al.* (2013) Evidence of neurotrophic events due to peritoneal endometriotic lesions, *Cytokine*, 62(2), pp. 253–261.

Bardoni, R. *et al.* (2014) Delta opioid receptors presynaptically regulate cutaneous mechanosensory neuron input to the spinal cord dorsal horn, *Neuron*, 81(6), pp. 1312–1327.

Barrett, A. C., Smith, E. S. and Picker, M. J. (2002) Sex-related differences in mechanical nociception and antinociception produced by μ- and κ-opioid receptor agonists in rats, *European Journal of Pharmacology*, 452(2), pp. 163–173.

Bartok, R. E. and Craft, R. M. (1997) Sex differences in opioid antinociception, *Journal of Pharmacology and Experimental Therapeutics*, 282(2), pp. 769–778.

Basbaum, A. I., Bautista, D. M., Scherrer, G. and Julius, D. (2009) Cellular and Molecular Mechanisms of Pain, *Cell*, 139(2), pp. 267–284.

Beardsley, P. M., Howard, J. L., Shelton, K. L. and Carroll, F. I. (2005) Differential effects of the novel kappa opioid receptor antagonist, JDTic, on reinstatement of cocaine-seeking induced by footshock stressors vs cocaine primes and its antidepressant-like effects in

rats, *Psychopharmacology*, 183(1), pp. 118–126.

Beaulieu, P. (2006) Effects of nabilone, a synthetic cannabinoid, on postoperative pain., *Canadian journal of anaesthesia = Journal canadien d'anesthesie*. United States, 53(8), pp. 769–775.

Beck, T. C., Hapstack, M. A., Beck, K. R. and Dix, T. A. (2019) Therapeutic Potential of Kappa Opioid Agonists, *Pharmaceuticals (Basel, Switzerland)*. MDPI, 12(2), p. 95.

Becker, C. M., Gattrell, W. T., Gude, K. and Singh, S. S. (2017) Reevaluating response and failure of medical treatment of endometriosis: a systematic review, *Fertility and Sterility*. Elsevier Inc., 108(1), pp. 125–136.

Bee, L. A. and Dickenson, A. H. (2007) Neuropathic pain: Multiple mechanisms at multiple sites, *Future Neurology*, 2(6), pp. 661–671.

Bénard, G. *et al.* (2012) Mitochondrial CB1 receptors regulate neuronal energy metabolism, *Nature Neuroscience*, 15(4), pp. 558–564.

Di Benedetto, S. *et al.* (2017) Contribution of neuroinflammation and immunity to brain aging and the mitigating effects of physical and cognitive interventions, *Neuroscience and Biobehavioral Reviews*. Elsevier Ltd, 75, pp. 114–128.

Bereiter, D. A. (2001) Sex Differences in Brainstem Neural Activation after Injury to the TMJ Region, *Cells Tissues Organs*, 169(3), pp. 226–237.

Bergamaschi, M. M. *et al.* (2011) Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naïve social phobia patients., *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 36(6), pp. 1219–1226.

Berkley, K. J. *et al.* (2001) Vaginal hyperalgesia in a rat model of endometriosis., *Neuroscience letters*. Ireland, 306(3), pp. 185–188.

Berkley, K. J., Dmitrieva, N., Curtis, K. S. and Papka, R. E. (2004) Innervation of ectopic endometrium in a rat model of endometriosis, *Proceedings of the National Academy of Sciences of the United States of America*, 101(30), pp. 11094–11098.

Berlanda, N. *et al.* (2013) Role of surgery in endometriosis-associated subfertility., *Seminars in reproductive medicine*. United States, 31(2), pp. 133–143.

Bermúdez-Silva, F. J. *et al.* (2008) Presence of functional cannabinoid receptors in human endocrine pancreas, *Diabetologia*, 51(3), pp. 476–487.

Bertran-Gonzalez, J. *et al.* (2013) Learning-related translocation of δ-Opioid receptors on ventral striatal cholinergic interneurons mediates choice between goal-directed actions, *Journal of Neuroscience*. Society for Neuroscience, 33(41), pp. 16060–16071.

Beste, M. T. *et al.* (2014) Molecular network analysis of endometriosis reveals a role for c-Jun-regulated macrophage activation., *Science translational medicine*, 6(222), p. 222ra16.

Bhalla, S., Zhang, Z., Patterson, N. and Gulati, A. (2010) Effect of endothelin-A receptor antagonist on mu, delta and kappa opioid receptor-mediated antinociception in mice, *European Journal of Pharmacology*. Elsevier B.V., 635(1–3), pp. 62–71.

Bifulco, M., Laezza, C., Gazzerro, P. and Pentimalli, F. (2007) Endocannabinoids as emerging suppressors of angiogenesis and tumor invasion (review)., *Oncology reports*. Greece, 17(4), pp. 813–816.

Bigliardi, P. L. and Bigliardi-Qi, M. (2014) *Peripheral Opioids*, *Itch: Mechanisms and Treatment*.

Bilkei-Gorzo, A., Mauer, D., Michel, K. and Zimmer, A. (2014) Dynorphins regulate the strength of social memory, *Neuropharmacology*, 77, pp. 406–413.

Bilkei-Gorzo, A. *et al.* (2017) A chronic low dose of Δ9-tetrahydrocannabinol (THC) restores cognitive function in old mice, *Nature Medicine*, 23(6), pp. 782–787.

Bimonte, S. *et al.* (2015) Morphine Promotes Tumor Angiogenesis and Increases Breast Cancer Progression., *BioMed research international*, 2015, p. 161508.

Binder, W. and Walker, J. S. (1998) Effect of the peripherally selective κ-opioid agonist, asimadoline, on adjuvant arthritis, *British Journal of Pharmacology*. John Wiley & Sons, Ltd, 124(4), pp. 647–654.

Blázquez, C. *et al.* (2003) Inhibition of tumor angiogenesis by

cannabinoids, *The FASEB Journal*. John Wiley & Sons, Ltd, 17(3), pp. 1–16.

Blázquez, C. *et al.* (2004) Cannabinoids inhibit the vascular endothelial growth factor pathway in gliomas., *Cancer research*. United States, 64(16), pp. 5617–5623.

Blázquez, C. *et al.* (2008) Cannabinoids Inhibit Glioma Cell Invasion by Down-regulating Matrix Metalloproteinase-2 Expression, *Cancer Research*, 68(6), pp. 1945 LP – 1952.

Blessing, E. M., Steenkamp, M. M., Manzanares, J. and Marmar, C. R. (2015) Cannabidiol as a Potential Treatment for Anxiety Disorders, *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics*. Springer US, 12(4), pp. 825–836.

Bloom, A. S., Dewey, W. L., Harris, L. S. and Brosius, K. K. (1977) 9-nor-9beta-hydroxyhexahydrocannabinol, a cannabinoid with potent antinociceptive activity: comparisons with morphine., *The Journal of pharmacology and experimental therapeutics*. United States, 200(2), pp. 263–270.

Bodnar, R. J. (2017) Endogenous Opiates and Behavior: 2015, *Peptides*, 88, pp. 126–188.

Bohn, L. M. *et al.* (1999) Enhanced morphine analgesia in mice lacking beta-arrestin 2., *Science (New York, N.Y.)*, 286(5449), pp. 2495–8.

Bohonyi, N. *et al.* (2017) Local upregulation of transient receptor

potential ankyrin I and transient receptor potential vanilloid I ion channels in rectosigmoid deep infiltrating endometriosis, *Molecular Pain*, 13, pp. 1–13.

Booth, M. (2013) *Opium: A history*. St. Martin's Griffin.

Bortolato, M. *et al.* (2005) Kappa opioid receptor activation disrupts prepulse inhibition of the acoustic startle in rats., *Biological psychiatry*. United States, 57(12), pp. 1550–1558.

Bosier, B., Muccioli, G. G., Hermans, E. and Lambert, D. M. (2010) Functionally selective cannabinoid receptor signalling: therapeutic implications and opportunities., *Biochemical pharmacology*. England, 80(1), pp. 1–12.

Boyle, K. A. *et al.* (2017) A quantitative study of neurochemically defined populations of inhibitory interneurons in the superficial dorsal horn of the mouse spinal cord, *Neuroscience*, 363, pp. 120–133.

Brain, S. D. and Grant, A. D. (2004) Vascular actions of calcitonin gene-related peptide and adrenomedullin., *Physiological reviews*. United States, 84(3), pp. 903–934.

Brown, J. *et al.* (2017) Nonsteroidal anti-inflammatory drugs for pain in women with endometriosis., *The Cochrane database of systematic reviews*, 1(1), p. CD004753.

Bulun, S. E. *et al.* (2004) Aromatase and endometriosis., *Seminars in reproductive medicine*. United States, 22(1), pp. 45–50.

Bunzow, J. R. *et al.* (1994) Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a μ, δ or κ opioid receptor type, *FEBS Letters*, 347(2–3), pp. 284–288.

Burney, R. O. *et al.* (2007) Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis., *Endocrinology*. United States, 148(8), pp. 3814–3826.

Burney, R. O. and Lathi, R. B. (2009) Menstrual bleeding from an endometriotic lesion., *Fertility and sterility*. United States, 91(5), pp. 1926–1927.

Burston, J. J. *et al.* (2013) Cannabinoid CB2 receptors regulate central sensitization and pain responses associated with osteoarthritis of the knee joint, *PloS one*. Public Library of Science, 8(11), pp. e80440–e80440.

Bushnell, M. C., Čeko, M. and Low, L. A. (2013) Cognitive and emotional control of pain and its disruption in chronic pain, *Nature Reviews Neuroscience*, 14(7), pp. 502–511.

Busquets-Garcia, A. *et al.* (2011) Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses, *Biological Psychiatry*. Elsevier Inc., 70(5), pp. 479–486.

Busquets-Garcia, A., Bains, J. and Marsicano, G. (2018) CB1 Receptor Signaling in the Brain: Extracting Specificity from Ubiquity, *Neuropsychopharmacology*, 43(1), pp. 4–20.

Cabañero, D. *et al.* (2020) Protective role of neuronal and lymphoid cannabinoid CB2 receptors in neuropathic pain, *eLife*. Edited by C. Büchel and A. Basbaum. eLife Sciences Publications, Ltd, 9, p. e55582.

Cajanus, K. *et al.* (2016) Effect of endocannabinoid degradation on pain: role of FAAH polymorphisms in experimental and postoperative pain in women treated for breast cancer., *Pain*. United States, 157(2), pp. 361–369.

Carey, A. N. *et al.* (2009) Endogenous Opioid Activation Mediates Stress-Induced Deficits in Learning and Memory, *Journal of Neuroscience*, 29(13), pp. 4293–4300.

Carey, Amanda N. *et al.* (2009) Endogenous κ opioid activation mediates stress-induced deficits in learning and memory, *Journal of Neuroscience*, 29(13), pp. 4293–4300.

Carlezon, W. A. *et al.* (2006) Depressive-like effects of the κ-opioid receptor agonist salvinorin A on behavior and neurochemistry in rats, *Journal of Pharmacology and Experimental Therapeutics*, 316(1), pp. 440–447.

Casey, S. L., Atwal, N. and Vaughan, C. W. (2017) *Cannabis constituent synergy in a mouse neuropathic pain model*, *Pain*.

Cason, A. M., Samuelsen, C. L. and Berkley, K. J. (2003) Estrous changes in vaginal nociception in a rat model of endometriosis, *Hormones and Behavior*, 44(2), pp. 123–131.

Castañé, A. *et al.* (2006) Development and expression of

neuropathic pain in CB1 knockout mice., *Neuropharmacology*. England, 50(1), pp. 111–122.

Castillo, P. E., Younts, T. J., Chávez, A. E. and Hashimotodani, Y. (2012) Endocannabinoid Signaling and Synaptic Function, *Neuron*, 76(1), pp. 70–81.

Catheline, G., Guilbaud, G. and Kayser, V. (1998) Peripheral component in the enhanced antinociceptive effect of systemic U-69,593, a kappa-opioid receptor agonist in mononeuropathic rats., *European journal of pharmacology*, 357(2–3), pp. 171–8.

Chanmee, T., Ontong, P., Konno, K. and Itano, N. (2014) Tumor-associated macrophages as major players in the tumor microenvironment., *Cancers*, 6(3), pp. 1670–1690.

Chappell, P. B. *et al.* (1993) Neuroendocrine and behavioral effects of the selective kappa agonist spiradoline in Tourette's syndrome: a pilot study., *Psychiatry research*. Ireland, 47(3), pp. 267–280.

Chartoff, E. H. and Mavrikaki, M. (2015) Sex Differences in Kappa Opioid Receptor Function and Their Potential Impact on Addiction., *Frontiers in neuroscience*, 9, p. 466.

Chatzaki, E. *et al.* (2000) Kappa opioids and TGFbeta1 interact in human endometrial cells., *Molecular human reproduction*. England, 6(7), pp. 602–609.

Chatzaki, E. *et al.* (2001) The Fas/FasL apoptotic pathway is involved in κ-opioidinduced apoptosis of human endometrial stromal cells, *Molecular Human Reproduction*, 7(9), pp. 867–874.

Chavkin, C. (2013) Dynorphin--still an extraordinarily potent opioid peptide., *Molecular pharmacology*. American Society for Pharmacology and Experimental Therapeutics, 83(4), pp. 729–736.

Chavkin, C. and Koob, G. F. (2016) Dynorphin, Dysphoria, and Dependence: the Stress of Addiction., *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 41(1), pp. 373–374.

Chen, Q. L. and Heinricher, M. M. (2019) Descending Control Mechanisms and Chronic Pain, *Current Rheumatology Reports*, 21(5), p. 13.

Chen, Z. *et al.* (2015) Activation of p38 MAPK in the rostral ventromedial medulla by visceral noxious inputs transmitted via the dorsal columns may contribute to pelvic organ cross-sensitization in rats with endometriosis., *Neuroscience*. United States, 291, pp. 272–278.

Chicca, A. *et al.* (2017) Chemical probes to potently and selectively inhibit endocannabinoid cellular reuptake., *Proceedings of the National Academy of Sciences of the United States of America*, 114(25), pp. E5006–E5015.

Childers, S. R. and Deadwyler, S. A. (1996) Role of cyclic AMP in the actions of cannabinoid receptors., *Biochemical pharmacology*. England, 52(6), pp. 819–827.

Chiu, Y. H. *et al.* (2005) Poor sleep and depression are independently associated with a reduced pain threshold. Results of a population

based study, *Pain*, 115(3), pp. 316–321.

Chung, M. K., Chung, R. R., Gordon, D. and Jennings, C. (2002) The evil twins of chronic pelvic pain syndrome: endometriosis and interstitial cystitis., *JSLS : Journal of the Society of Laparoendoscopic Surgeons*, 6(4), pp. 311–314.

Clapper, J. R. *et al.* (2010) Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism, *Nature neuroscience*. 2010/09/19, 13(10), pp. 1265–1270.

Clemente, J. T. *et al.* (2004) Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint., *Neuroscience letters*. Ireland, 372(3), pp. 250–255.

Colloca, L. *et al.* (2017) Neuropathic pain, *Nature reviews. Disease primers*, 3, p. 17002.

Corder, G., Castro, D. C., Bruchas, M. R. and Scherrer, G. (2018) Endogenous and exogenous opioids in pain, *Annual Review of Neuroscience*, 41(1), pp. 453–473.

Costa, M., Brookes, S. H. J. and Zagorodnyuk, V. (2004) How many kinds of visceral afferents?, *Gut*, 53(SUPPL. 2), pp. 1–4.

Costigan, M., Scholz, J. and Woolf, C. J. (2009) Neuropathic pain: A maladaptive response of the nervous system to damage, *Annual Review of Neuroscience*, 32(1), pp. 1–32.

Cox, B. M., Goldstein, A. and Hi, C. H. (1976) Opioid activity of a

peptide, beta-lipotropin-(61-91), derived from beta-lipotropin., *Proceedings of the National Academy of Sciences of the United States of America*, 73(6), pp. 1821–1823.

Craft, R. M. *et al.* (1995) Opioid antinociception in a rat model of visceral pain: systemic versus local drug administration., *The Journal of pharmacology and experimental therapeutics*. United States, 275(3), pp. 1535–1542.

Crean, R. D., Crane, N. A. and Mason, B. J. (2011) An evidence based review of acute and long-term effects of cannabis use on executive cognitive functions, *Journal of addiction medicine*, 5(1), pp. 1–8.

Cristino, L. *et al.* (2008) Immunohistochemical localization of anabolic and catabolic enzymes for anandamide and other putative endovanilloids in the hippocampus and cerebellar cortex of the mouse brain., *Neuroscience*. United States, 151(4), pp. 955–968.

Crowley, N. A. *et al.* (2016) Dynorphin Controls the Gain of an Amygdalar Anxiety Circuit, *Cell reports*. 2016/03/17, 14(12), pp. 2774–2783.

D'Hooghe, T. M. *et al.* (1995) Intrapelvic injection of menstrual endometrium causes endometriosis in baboons (Papio cynocephalus and Papio anubis)., *American journal of obstetrics and gynecology*. United States, 173(1), pp. 125–134.

D'Hooghe, T. M. *et al.* (2009) Nonhuman primate models for translational research in endometriosis., *Reproductive sciences (Thousand Oaks, Calif.)*. United States, 16(2), pp. 152–161.

D'Mello, R. and Dickenson, A. H. (2008) Spinal cord mechanisms of pain, *British Journal of Anaesthesia*, 101(1), pp. 8–16.

Das, S. K. *et al.* (1995) Cannabinoid ligand-receptor signaling in the mouse uterus, *Pharmacology*, 92(May), pp. 4332–4336.

Daumas, S. *et al.* (2007) Transient activation of the CA3 Kappa opioid system in the dorsal hippocampus modulates complex memory processing in mice., *Neurobiology of learning and memory*. United States, 88(1), pp. 94–103.

Davis, A. C. and Goldberg, J. M. (2017) Extrapelvic Endometriosis., *Seminars in reproductive medicine*. United States, 35(1), pp. 98–101.

Defrin, R. *et al.* (2008) Quantitative testing of pain perception in subjects with PTSD - Implications for the mechanism of the coexistence between PTSD and chronic pain, *Pain*, 138(2), pp. 450–459.

Devane, W. A. *et al.* (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor., *Science (New York, N.Y.)*. United States, 258(5090), pp. 1946–1949.

Dick, B., Eccleston, C. and Crombez, G. (2002) Attentional functioning in fibromyalgia, rheumatoid arthritis, and musculoskeletal pain patients, *Arthritis Care and Research*, 47(6), pp. 639–644.

Dinh, T. P., Freund, T. F. and Piomelli, D. (2002) A role for monoglyceride lipase in 2-arachidonoylglycerol inactivation., *Chemistry and physics of lipids*. Ireland, 121(1–2), pp. 149–158.

Dmowski, W. P., Steele, R. W. and Baker, G. F. (1981) Deficient cellular immunity in endometriosis., *American journal of obstetrics and gynecology*. United States, 141(4), pp. 377–383.

Dodds, K. N., Beckett, E. A. H., Evans, S. F. and Hutchinson, M. R. (2019) Spinal Glial Adaptations Occur in a Minimally Invasive Mouse Model of Endometriosis: Potential Implications for Lesion Etiology and Persistent Pelvic Pain., *Reproductive sciences (Thousand Oaks, Calif.)*. United States, 26(3), pp. 357–369.

Dol-Gleizes, F. *et al.* (2009) Rimonabant, a selective cannabinoid CB1 receptor antagonist, inhibits atherosclerosis in LDL receptor-deficient mice., *Arteriosclerosis, thrombosis, and vascular biology*. United States, 29(1), pp. 12–18.

Donnez, J. and Squifflet, J. (2010) Complications, pregnancy and recurrence in a prospective series of 500 patients operated on by the shaving technique for deep rectovaginal endometriotic nodules, *Human Reproduction*, 25(8), pp. 1949–1958.

Donnez, J. *et al.* (2010) Laparoscopic management of endometriomas using a combined technique of excisional (cystectomy) and ablative surgery., *Fertility and sterility*. United States, 94(1), pp. 28–32.

Dubin, A. E. and Patapoutian, A. (2010) Nociceptors: The sensors of the pain pathway, *Journal of Clinical Investigation*. American Society for Clinical Investigation, 120(11), pp. 3760–3772.

Duggan, A. W. (2000) Neuropeptide spread in the brain and spinal

cord, in *Progress in Brain Research*, pp. 369–380.

Dunselman, G. A. J. *et al.* (2014) ESHRE guideline: management of women with endometriosis †, *Human Reproduction*, 29(3), pp. 400–412.

Eckert, W. A. and Light, A. R. (2002) Hyperpolarization of substantia gelatinosa neurons evoked by μ-, κ-, δ1-, and δ2-selective opioids, *Journal of Pain*, 3(2), pp. 115–125.

Eichel, K., Jullié, D. and Von Zastrow, M. (2016) β-Arrestin drives MAP kinase signalling from clathrin-coated structures after GPCR dissociation, *Nature Cell Biology*. NIH Public Access, 18(3), pp. 303–310.

El-Talatini, M. R. *et al.* (2009) Localisation and function of the endocannabinoid system in the human ovary, *PLoS ONE*, 4(2), p. e4579.

Falcone, T. and Flyckt, R. (2018) Clinical Management of Endometriosis., *Obstetrics and gynecology*. United States, 131(3), pp. 557–571.

Faouzi, A., Varga, B. R. and Majumdar, S. (2020) Biased Opioid Ligands, *Molecules (Basel, Switzerland)*. MDPI, 25(18), p. 4257.

Farooqui, M. *et al.* (2007) COX-2 inhibitor celecoxib prevents chronic morphine-induced promotion of angiogenesis, tumour growth, metastasis and mortality, without compromising analgesia., *British journal of cancer*, 97(11), pp. 1523–1531.

Fauconnier, A. *et al.* (2002) Relation between pain symptoms and the anatomic location of deep infiltrating endometriosis., *Fertility and sterility*. United States, 78(4), pp. 719–726.

Fazleabas, A. T. (2010) Progesterone resistance in a baboon model of endometriosis., *Seminars in reproductive medicine*. United States, 28(1), pp. 75–80.

Fedele, L. *et al.* (1989) Gestrinone versus danazol in the treatment of endometriosis., *Fertility and sterility*. United States, 51(5), pp. 781–785.

Feizerfan, A. and Sheh, G. (2015) Transition from acute to chronic pain, *Continuing Education in Anaesthesia Critical Care & Pain*, 15(2), pp. 98–102.

Ferrero, S. *et al.* (2008) Peritoneal fluid proteome in women with different ASRM stages of endometriosis., *Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology*. England, 24(8), pp. 433–441.

Ferrero, S., Gillott, D. J., Venturini, P. L. and Remorgida, V. (2011) Use of aromatase inhibitors to treat endometriosis-related pain symptoms: a systematic review, *Reproductive biology and endocrinology : RB&E*. BioMed Central, 9, p. 89.

Filho, P. W. L. L. *et al.* (2019) Peritoneal endometriosis induces time-related depressive- and anxiety-like alterations in female rats: involvement of hippocampal pro-oxidative and BDNF alterations, *Metabolic Brain Disease*. Metabolic Brain Disease.

Fillingim, R. B. *et al.* (2004) Experimental pain models reveal no sex differences in pentazocine analgesia in humans., *Anesthesiology*. United States, 100(5), pp. 1263–1270.

de Fonseca, F. R. *et al.* (2005) The endocannabinoid system: Physiology and pharmacology, *Alcohol and Alcoholism*, 40(1), pp. 2–14.

Fortin, M. *et al.* (2003) An improved mouse model for endometriosis allows noninvasive assessment of lesion implantation and development, *Fertility and Sterility*, 80(SUPPL. 2), pp. 832–838.

Fourquet, J. *et al.* (2011) Quantification of the impact of endometriosis symptoms on health-related quality of life and work productivity, *Fertility and Sterility*, 96(1), pp. 107–112.

François, A. *et al.* (2017) A Brainstem-Spinal Cord Inhibitory Circuit for Mechanical Pain Modulation by GABA and Enkephalins., *Neuron*, 93(4), pp. 822-839.e6.

François, A. and Scherrer, G. (2018) Delta opioid receptor expression and function in primary afferent somatosensory neurons, *Handbook of Experimental Pharmacology*, 247, pp. 87–114.

Freund, T. F., Katona, I. and Piomelli, D. (2003) Role of endogenous cannabinoids in synaptic signaling., *Physiological reviews*. United States, 83(3), pp. 1017–1066.

Friedl, F. *et al.* (2015) Impact of endometriosis on quality of life, anxiety, and depression: an Austrian perspective, *Archives of Gynecology and Obstetrics*, 292(6), pp. 1393–1399.

Fuentes-Márquez, P., Cabrera-Martos, I. and Valenza, M. C. (2019) Physiotherapy interventions for patients with chronic pelvic pain: A systematic review of the literature., *Physiotherapy theory and practice*. England, 35(12), pp. 1131–1138.

Galiègue, S. *et al.* (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations., *European journal of biochemistry*. England, 232(1), pp. 54–61.

Gaoni, Y. and Mechoulam, R. (1964) Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish, *Journal of the American Chemical Society*. American Chemical Society, 86(8), pp. 1646–1647.

García-Gutiérrez, M. S. *et al.* (2013) Synaptic plasticity alterations associated with memory impairment induced by deletion of CB2 cannabinoid receptors., *Neuropharmacology*. England, 73, pp. 388–396.

Gargett, C. E. *et al.* (2014) Potential role of endometrial stem/progenitor cells in the pathogenesis of early-onset endometriosis., *Molecular human reproduction*. England, 20(7), pp. 591–598.

Gear, R. W. *et al.* (1996a) Gender difference in analgesic response to the kappa-opioid pentazocine, *Neuroscience Letters*, 205(3), pp. 207–209.

Gear, R. W. *et al.* (1996b) Kappa-opioids produce significantly

greater analgesia in women than in men., *Nature medicine*. United States, 2(11), pp. 1248–1250.

Gear, R. W. *et al.* (1999) The kappa opioid nalbuphine produces gender- and dose-dependent analgesia and antianalgesia in patients with postoperative pain., *Pain*. United States, 83(2), pp. 339–345.

Gear, R. W. *et al.* (2014) NOP receptor mediates anti-analgesia induced by agonist-antagonist opioids, *Neuroscience*. 2013/11/01, 257, pp. 139–148.

Gentilini, D. *et al.* (2010) Endocannabinoid system regulates migration of endometrial stromal cells via cannabinoid receptor 1 through the activation of PI3K and ERK1/2 pathways, *Fertility and Sterility*, 93(8), pp. 2588–2593.

Gilabert-Estelles, J. *et al.* (2005) Plasminogen activators and plasminogen activator inhibitors in endometriosis., *Frontiers in bioscience : a journal and virtual library*. United States, 10, pp. 1162–1176.

Glass, M. J., Vanyo, L., Quimson, L. and Pickel, V. M. (2009) Ultrastructural relationship between N-methyl-d-aspartate-NR1 receptor subunit and mu-opioid receptor in the mouse central nucleus of the amygdala, *Neuroscience*. NIH Public Access, 163(3), pp. 857–867.

Gobshtis, N., Ben-Shabat, S. and Fride, E. (2007) Antidepressant-induced undesirable weight gain: prevention with rimonabant without interference with behavioral effectiveness., *European*

journal of pharmacology. Netherlands, 554(2–3), pp. 155–163.

Gold, M. S. and Gebhart, G. F. (2010) Nociceptor sensitization in pain pathogenesis, *Nature Medicine*. NIH Public Access, 16(11), pp. 1248–1257.

Goldstein, A., Lowney, L. I. and Pal, B. K. (1971) Stereospecific and nonspecific interactions of the morphine congener levorphanol in subcellular fractions of mouse brain., *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 68(8), pp. 1742–1747.

Goldstein, A. *et al.* (1979) Dynorphin-(1-13), an extraordinarily potent opioid peptide, *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 76(12), pp. 6666–6670.

Gong, J.-P. *et al.* (2006) Cannabinoid CB2 receptors: immunohistochemical localization in rat brain, *Brain research*, 1071(1), p. 10—23.

González, D. *et al.* (2006) Pattern of use and subjective effects of Salvia divinorum among recreational users, *Drug and Alcohol Dependence*, 85(2), pp. 157–162.

Gosselin, R.-D., Suter, M. R., Ji, R.-R. and Decosterd, I. (2010) Glial cells and chronic pain, *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry*. 2010/06/25, 16(5), pp. 519–531.

Granier, S. *et al.* (2012) Structure of the δ-opioid receptor bound to

naltrindole, *Nature*. NIH Public Access, 485(7398), pp. 400–404.

Greaves, E. *et al.* (2017a) EP2 receptor antagonism reduces peripheral and central hyperalgesia in a preclinical mouse model of endometriosis, *Scientific Reports*, 7.

Greaves, E., Critchley, H. O. D., Horne, A. W. and Saunders, P. T. K. (2017b) Relevant human tissue resources and laboratory models for use in endometriosis research, *Acta Obstetricia et Gynecologica Scandinavica*, pp. 644–658.

Greene, N. Z. *et al.* (2018) Cannabidiol modulation of antinociceptive tolerance to Δ9-tetrahydrocannabinol, *Psychopharmacology*. Psychopharmacology, 235(11), pp. 3289–3302.

Groh, A., Mease, R. and Krieger, P. (2017) Pain processing in the thalamocortical system, *e-Neuroforum*, 23(3), pp. 117–122.

Grotenhermen, F. (2004) Pharmacology of cannabinoids., *Neuro endocrinology letters*. Sweden, 25(1–2), pp. 14–23.

Grümmer, R. (2006) Animal models in endometriosis research, *Human Reproduction Update*, pp. 641–649.

Guindon, J. and Hohmann, A. G. (2009) The endocannabinoid system and pain, *CNS & neurological disorders drug targets*, 8(6), pp. 403–421.

Gye, M. C., Kang, H. H. and Kang, H. J. (2005) Expression of cannabinoid receptor 1 in mouse testes., *Archives of andrology*.

England, 51(3), pp. 247–255.

Halme, J., Becker, S. and Wing, R. (1984) Accentuated cyclic activation of peritoneal macrophages in patients with endometriosis., *American journal of obstetrics and gynecology*. United States, 148(1), pp. 85–90.

Hampson, R. E. and Deadwyler, S. A. (1998) Role of Cannabinoid Receptors in Memory Storage, *Neurobiology of Disease*, 5(6), pp. 474–482.

Han, H. *et al.* (2017) Cannabinoid receptor 1 contributes to sprouted innervation in endometrial ectopic growth through mitogen-activated protein kinase activation, *Brain Research*, 1663, pp. 132–140.

Han, J. *et al.* (2012) Acute cannabinoids impair working memory through astroglial CB1 receptor modulation of hippocampal LTD., *Cell*. United States, 148(5), pp. 1039–1050.

Hansen, K. E., Kesmodel, U. S., Kold, M. and Forman, A. (2017) Long-term effects of mindfulness-based psychological intervention for coping with pain in endometriosis: A six-year follow-up on a pilot study, *Nordic Psychology*. Routledge, 69(2), pp. 100–109.

Häring, M., Guggenhuber, S. and Lutz, B. (2012) Neuronal populations mediating the effects of endocannabinoids on stress and emotionality., *Neuroscience*. United States, 204, pp. 145–158.

Hart, R. *et al.* (2005) Excisional surgery versus ablative surgery for ovarian endometriomata: a Cochrane Review., *Human reproduction*

(Oxford, England). England, 20(11), pp. 3000–3007.

Hasanein, P. and Javanmardi, K. (2008) A potent and selective inhibitor of endocannabinoid uptake, UCM707, potentiates antinociception induced by cholestasis., *Fundamental & clinical pharmacology*. England, 22(5), pp. 517–522.

Hatzoglou, A. *et al.* (1995) Identification and characterization of opioid-binding sites present in the Ishikawa human endometrial adenocarcinoma cell line., *The Journal of clinical endocrinology and metabolism*. United States, 80(2), pp. 418–423.

Healey, M., Ang, W. C. and Cheng, C. (2010) Surgical treatment of endometriosis: a prospective randomized double-blinded trial comparing excision and ablation., *Fertility and sterility*. United States, 94(7), pp. 2536–2540.

Hedner, T. and Cassuto, J. (1987) Opioids and opioid receptors in peripheral tissues., *Scandinavian journal of gastroenterology. Supplement*. England, 130, pp. 27–46.

Heifets, B. D. and Castillo, P. E. (2009) Endocannabinoid signaling and long-term synaptic plasticity, *Annual review of physiology*, 71, pp. 283–306.

Helvacioglu, A., Aksel, S. and Peterson, R. D. (1997) Endometriosis and autologous lymphocyte activation by endometrial cells. Are lymphocytes or endometrial cell defects responsible?, *The Journal of reproductive medicine*. United States, 42(2), pp. 71–75.

Hohmann, A. G. *et al.* (2005) An endocannabinoid mechanism for

stress-induced analgesia., *Nature*. England, 435(7045), pp. 1108–1112.

Holdcroft, A. *et al.* (2000) Sex and oestrous cycle differences in visceromotor responses and vasopressin release in response to colonic distension in male and female rats anaesthetized with halothane, *British Journal of Anaesthesia*, 85(6), pp. 907–910.

Holdcroft, A. *et al.* (2006) A multicenter dose-escalation study of the analgesic and adverse effects of an oral cannabis extract (Cannador) for postoperative pain management., *Anesthesiology*. United States, 104(5), pp. 1040–1046.

Hsu, A. L. *et al.* (2011) Relating pelvic pain location to surgical findings of endometriosis, *Obstetrics and gynecology*, 118(2 Pt 1), pp. 223–230.

Huang, S. M. *et al.* (2002) An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors., *Proceedings of the National Academy of Sciences of the United States of America*, 99(12), pp. 8400–8405.

Hughes, J. *et al.* (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity, *Nature*, 258(5536), pp. 577–579.

Hull, M. L. *et al.* (2008) Endometrial-peritoneal interactions during endometriotic lesion establishment., *The American journal of pathology*, 173(3), pp. 700–715.

Ibrahim, M. M. *et al.* (2005) CB2 cannabinoid receptor activation

produces antinociception by stimulating peripheral release of endogenous opioids, *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 102(8), pp. 3093–3098.

Ibrahim, M. M. *et al.* (2006) CB2 cannabinoid receptor mediation of antinociception., *Pain*. United States, 122(1–2), pp. 36–42.

Irannejad, R. *et al.* (2013) Conformational biosensors reveal GPCR signalling from endosomes, *Nature*. NIH Public Access, 495(7442), pp. 534–538.

Irving, A. *et al.* (2017) Cannabinoid Receptor-Related Orphan G Protein-Coupled Receptors., *Advances in pharmacology (San Diego, Calif.)*. United States, 80, pp. 223–247.

Iuvone, T. *et al.* (2008) Selective CB2 up-regulation in women affected by endometrial inflammation., *Journal of cellular and molecular medicine*, 12(2), pp. 661–670.

Iwaszkiewicz, K., Schneider, J. and Hua, S. (2013) Targeting peripheral opioid receptors to promote analgesic and anti-inflammatory actions , *Frontiers in Pharmacology* , p. 132.

Jardinaud, F. *et al.* (2005) CB1 receptor knockout mice show similar behavioral modifications to wild-type mice when enkephalin catabolism is inhibited., *Brain research*. Netherlands, 1063(1), pp. 77–83.

Jayamanne, A. *et al.* (2006) Actions of the FAAH inhibitor URB597 in neuropathic and inflammatory chronic pain models., *British journal*

of pharmacology, 147(3), pp. 281–288.

Jerman, L. F. and Hey-Cunningham, A. J. (2015) The role of the lymphatic system in endometriosis: a comprehensive review of the literature., *Biology of reproduction*. United States, 92(3), p. 64.

Jhaveri, M. D., Richardson, D. and Chapman, V. (2007) Endocannabinoid metabolism and uptake: novel targets for neuropathic and inflammatory pain., *British journal of pharmacology*, 152(5), pp. 624–632.

Ji, G. *et al.* (2010) Cognitive impairment in pain through amygdala-driven prefrontal cortical deactivation, *Journal of Neuroscience*, 30(15), pp. 5451–5464.

Ji, R.-R., Berta, T. and Nedergaard, M. (2013) Glia and pain: is chronic pain a gliopathy?, *Pain*. 2013/06/20, 154 Suppl(0 1), pp. S10–S28.

Ji, R.-R., Donnelly, C. R. and Nedergaard, M. (2019) Astrocytes in chronic pain and itch, *Nature Reviews Neuroscience*, 20(11), pp. 667–685.

Jin, D. F., Muffly, K. E., Okulicz, W. C. and Kilpatrick, D. L. (1988) Estrous Cycle- and Pregnancy-Related Differences in Expression of the Proenkephalin and Proopiomelanocortin Genes in the Ovary and Uterus*, *Endocrinology*, 122(4), pp. 1466–1471.

Johnson, N. P. *et al.* (2017) World endometriosis society consensus on the classification of endometriosis, *Human Reproduction*, 32(2), pp. 315–324.

Jordan, B. A., Cvejic, S. and Devi, L. A. (2000) Opioids and Their Complicated Receptor Complexes, *Neuropsychopharmacology*, 23(1), pp. S5–S18.

Julien, B. *et al.* (2005) Antifibrogenic role of the cannabinoid receptor CB2 in the liver., *Gastroenterology*. United States, 128(3), pp. 742–755.

Julius, D. and Basbaum, A. I. (2001) Molecular mechanisms of nociception, *Nature*, 413(6852), pp. 203–210.

Jutkiewicz, E. M. *et al.* (2006) Behavioral and neurobiological effects of the enkephalinase inhibitor RB101 relative to its antidepressant effects, *European Journal of Pharmacology*, 531(1–3), pp. 151–159.

Kajitani, T. *et al.* (2013) Possible involvement of nerve growth factor in dysmenorrhea and dyspareunia associated with endometriosis., *Endocrine journal*. Japan, 60(10), pp. 1155–1164.

Karp, J. F. *et al.* (2014) Safety, tolerability, and clinical effect of low-dose buprenorphine for treatment-resistant depression in midlife and older adults., *The Journal of clinical psychiatry*, 75(8), pp. e785-93.

Kasten, C. R., Zhang, Y. and Boehm, S. L. (2017) Acute and long-term effects of Δ9-tetrahydrocannabinol on object recognition and anxiety-like activity are age- and strain-dependent in mice, *Pharmacology Biochemistry and Behavior*, 163, pp. 9–19.

Kasten, C. R., Zhang, Y. and Boehm 2nd, S. L. (2019) Acute Cannabinoids Produce Robust Anxiety-Like and Locomotor Effects in

Mice, but Long-Term Consequences Are Age- and Sex-Dependent, *Frontiers in behavioral neuroscience*. Frontiers Media S.A., 13, p. 32.

Kastin, A. J. *et al.* (1978) Enkephalin and other peptides reduce passiveness., *Pharmacology, biochemistry, and behavior*, 9(4), pp. 515–9.

Kato, A. *et al.* (2012) Endocannabinoid-dependent plasticity at spinal nociceptor synapses, *The Journal of physiology*. 2012/07/23. Blackwell Science Inc, 590(19), pp. 4717–4733.

Kaufmann, I. *et al.* (2008) Anandamide and neutrophil function in patients with fibromyalgia., *Psychoneuroendocrinology*. England, 33(5), pp. 676–685.

Kaufmann, I. *et al.* (2009) Enhanced anandamide plasma levels in patients with complex regional pain syndrome following traumatic injury: a preliminary report., *European surgical research. Europaische chirurgische Forschung. Recherches chirurgicales europeennes*. Switzerland, 43(4), pp. 325–329.

Kayser, V. *et al.* (1996) Estrous and sex variations in vocalization thresholds to hindpaw and tail pressure stimulation in the rat., *Brain research*. Netherlands, 742(1–2), pp. 352–354.

Kempuraj, D. *et al.* (2004) Increased numbers of activated mast cells in endometriosis lesions positive for corticotropin-releasing hormone and urocortin., *American journal of reproductive immunology (New York, N.Y. : 1989)*. Denmark, 52(4), pp. 267–275.

Keogh, E. and Mansoor, L. (2001) Investigating the effects of anxiety

sensitivity and coping on the perception of cold pressor pain in healthy women, *European Journal of Pain*, 5(1), pp. 11–22.

Khanna, N. *et al.* (2011) Interaction of morphine and potassium channel openers on experimental models of pain in mice., *Fundamental & clinical pharmacology*. England, 25(4), pp. 479–484.

Kieffer, B. L. (1995) Recent advances in molecular recognition and signal transduction of active peptides: Receptors for opioid peptides, *Cellular and Molecular Neurobiology*, 15(6), pp. 615–635.

Kieffer, B. L. (1999) Opioids: First lessons from knockout mice, *Trends in Pharmacological Sciences*, 20(1), pp. 19–26.

Kivell, B. and Prisinzano, T. E. (2010) Kappa opioids and the modulation of pain, *Psychopharmacology*, 210(2), pp. 109–119.

Knoll, A. T. *et al.* (2007) Anxiolytic-like effects of κ-opioid receptor antagonists in models of unlearned and learned fear in rats, *Journal of Pharmacology and Experimental Therapeutics*, 323(3), pp. 838–845.

Knoll, A. T. and Carlezon, W. A. (2010) Dynorphin, stress, and depression, *Brain Research*. Elsevier, 1314, pp. 56–73.

Kobayashi, H. *et al.* (2009) The role of iron in the pathogenesis of endometriosis, *Gynecological Endocrinology*. Taylor & Francis, 25(1), pp. 39–52.

Koodie, L., Ramakrishnan, S. and Roy, S. (2010) Morphine suppresses tumor angiogenesis through a HIF-1alpha/p38MAPK

pathway., *The American journal of pathology*, 177(2), pp. 984–997.

Koodie, L. *et al.* (2014) Morphine inhibits migration of tumor-infiltrating leukocytes and suppresses angiogenesis associated with tumor growth in mice., *The American journal of pathology*, 184(4), pp. 1073–1084.

Kosek, E. *et al.* (2016) Do we need a third mechanistic descriptor for chronic pain states?, *PAIN*, 157(7).

Kosiba, J. D., Maisto, S. A. and Ditre, J. W. (2019) Patient-reported use of medical cannabis for pain, anxiety, and depression symptoms: Systematic review and meta-analysis., *Social science & medicine (1982)*. England, 233, pp. 181–192.

Kraft, B. *et al.* (2008) Lack of analgesia by oral standardized cannabis extract on acute inflammatory pain and hyperalgesia in volunteers., *Anesthesiology*. United States, 109(1), pp. 101–110.

Kreibich, A. *et al.* (2008) Presynaptic inhibition of diverse afferents to the locus ceruleus by kappa-opiate receptors: a novel mechanism for regulating the central norepinephrine system., *The Journal of neuroscience : the official journal of the Society for Neuroscience*. NIH Public Access, 28(25), pp. 6516–25.

Krishnamurti, C. and Rao, S. C. (2016) The isolation of morphine by Serturner, *Indian journal of anaesthesia*. Medknow Publications & Media Pvt Ltd, 60(11), pp. 861–862.

Kumru, S. *et al.* (2001) Differential regulation of preovulatory luteinizing hormone and follicle-stimulating hormone release by

opioids in the proestrous rat., *Physiological research*. Czech Republic, 50(4), pp. 397–403.

Kuner, R. and Flor, H. (2016) Structural plasticity and reorganisation in chronic pain., *Nature reviews. Neuroscience*. England, 18(1), pp. 20–30.

Labuz, D. *et al.* (2007) Relative contribution of peripheral versus central opioid receptors to antinociception., *Brain research*. Netherlands, 1160, pp. 30–38.

Labuz, D., Celik, M. O., Zimmer, A. and Machelska, H. (2016) Distinct roles of exogenous opioid agonists and endogenous opioid peptides in the peripheral control of neuropathy-triggered heat pain, *Scientific Reports*, 6(1), p. 32799.

Lafleur, R. A., Wilson, R. P., Morgan, D. J. and Henderson-Redmond, A. N. (2018) Sex differences in antinociceptive response to Δ-9-tetrahydrocannabinol and CP 55,940 in the mouse formalin test, *NeuroReport*, 29(6), pp. 447–452.

Laganà, A. S. *et al.* (2017) Anxiety and depression in patients with endometriosis: impact and management challenges, *International journal of women's health*. Dove Medical Press, 9, pp. 323–330.

Lai, J., Luo, M. chyi, Chen, Q. and Porreca, F. (2008) Pronociceptive actions of dynorphin via bradykinin receptors, *Neuroscience Letters*, 437(3), pp. 175–179.

Lamvu, G. *et al.* (2019) Patterns of Prescription Opioid Use in Women With Endometriosis: Evaluating Prolonged Use, Daily Dose,

and Concomitant Use With Benzodiazepines, *Obstetrics and Gynecology*, 133(6), pp. 1120–1130.

Land, B. B. *et al.* (2009) Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking, *Proceedings of the National Academy of Sciences of the United States of America*, 106(45), pp. 19168–19173.

Latremoliere, A. and Woolf, C. J. (2009) Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity, *Journal of Pain*, 10(9), pp. 895–926.

Laux-Biehlmann, A., D'hooghe, T. and Zollner, T. M. (2015) Menstruation pulls the trigger for inflammation and pain in endometriosis, *Trends in Pharmacological Sciences*, 36(5), pp. 270–276.

Law, P. Y., Wong, Y. H. and Loh, H. H. (2000) Molecular mechanisms and regulation of opioid receptor signaling, *Annual Review of Pharmacology and Toxicology*, 40, pp. 389–430.

Lawson, K. P., Nag, S., Thompson, A. D. and Mokha, S. S. (2010) Sex-specificity and estrogen-dependence of kappa opioid receptor-mediated antinociception and antihyperalgesia, *PAIN®*, 151(3), pp. 806–815.

Leconte, M. *et al.* (2010) Antiproliferative effects of cannabinoid agonists on deep infiltrating endometriosis, *American Journal of Pathology*, 177(6), pp. 2963–2970.

Lewis, T. (1942) *Pain.*, *New York: Macmillan*. Routledge.

Li, T. *et al.* (2018) Endometriosis alters brain electrophysiology, gene expression and increases pain sensitization, anxiety, and depression in female mice, *Biology of Reproduction*, 99(2), pp. 349–359.

Lichtman, A. H., Varvel, S. A. and Martin, B. R. (2002) Endocannabinoids in cognition and dependence., *Prostaglandins, leukotrienes, and essential fatty acids*. Scotland, 66(2–3), pp. 269–285.

Lidegaard, Ø. *et al.* (2011) Risk of venous thromboembolism from use of oral contraceptives containing different progestogens and oestrogen doses: Danish cohort study, 2001-9., *BMJ (Clinical research ed.)*, 343, p. d6423.

Lim, G., Sung, B., Ji, R.-R. and Mao, J. (2003) Upregulation of spinal cannabinoid-1-receptors following nerve injury enhances the effects of Win 55,212-2 on neuropathic pain behaviors in rats., *Pain*. United States, 105(1–2), pp. 275–283.

Liu, S. S. *et al.* (2019) Kappa opioid receptors drive a tonic aversive component of chronic pain, *Journal of Neuroscience*, 39(21), pp. 4162–4178.

Liu, Z. *et al.* (2018) Fractalkine/CX3CR1 Contributes to Endometriosis-Induced Neuropathic Pain and Mechanical Hypersensitivity in Rats., *Frontiers in cellular neuroscience*, 12, p. 495.

Lomas, L. M. *et al.* (2007) Sex differences in the potency of kappa opioids and mixed-action opioids administered systemically and at

the site of inflammation against capsaicin-induced hyperalgesia in rats., *Psychopharmacology*. Germany, 191(2), pp. 273–285.

Lorençatto, C. *et al.* (2006) Depression in women with endometriosis with and without chronic pelvic pain, *Acta Obstetricia et Gynecologica Scandinavica*, 85(1), pp. 88–92.

Lousse, J.-C. *et al.* (2008) Increased activation of nuclear factor-kappa B (NF-kappaB) in isolated peritoneal macrophages of patients with endometriosis., *Fertility and sterility*. United States, 90(1), pp. 217–220.

Low, W. Y., Edelmann, R. J. and Suttonf, C. (1993) Psychological Profile of Endometriosis Patientes in Comparasion To Patientes With Pain of Other Origins, 37(2), pp. 111–116.

Lucarini, E. *et al.* (2020) Deepening the Mechanisms of Visceral Pain Persistence: An Evaluation of the Gut-Spinal Cord Relationship, *Cells*, 9(8).

Lutz, B., Marsicano, G., Maldonado, R. and Hillard, C. J. (2015) The endocannabinoid system in guarding against fear, anxiety and stress, *Nature Reviews Neuroscience*. Nature Publishing Group, 16(12), pp. 705–718.

Lutz, P.-E. and Kieffer, B. L. (2013) Opioid receptors: distinct roles in mood disorders., *Trends in neurosciences*, 36(3), pp. 195–206.

Lynch, M. E. (2016) Cannabinoids in the management of chronic pain: A front line clinical perspective, *Journal of Basic and Clinical Physiology and Pharmacology*, 27(3), pp. 189–191.

Maccarrone, M. *et al.* (2002) Age-related changes of anandamide metabolism in CB1 cannabinoid receptor knockout mice: correlation with behaviour., *The European journal of neuroscience*. France, 15(7), pp. 1178–1186.

MacLean, K. A. *et al.* (2013) Dose-related effects of salvinorin A in humans: dissociative, hallucinogenic, and memory effects, *Psychopharmacology*, 226(2), pp. 381–392.

Makrigiannakis, A. *et al.* (1992) Steroid hormones regulate the release of immunoreactive beta-endorphin from the Ishikawa human endometrial cell line., *The Journal of clinical endocrinology and metabolism*. United States, 75(2), pp. 584–589.

Maldonado, R., Baños, J. E. and Cabañero, D. (2018) Usefulness of knockout mice to clarify the role of the opioid system in chronic pain, *British Journal of Pharmacology*, 175(14), pp. 2791–2808.

Malykhina, A. P. (2007) Neural mechanisms of pelvic organ cross-sensitization, *Neuroscience*, 149(3), pp. 660–672.

Manglik, A. *et al.* (2012) Crystal structure of the μ-opioid receptor bound to a morphinan antagonist., *Nature*. NIH Public Access, 485(7398), pp. 321–6.

Manning, B. H., Martin, W. J. and Meng, I. D. (2003) The rodent amygdala contributes to the production of cannabinoid-induced antinociception, *Neuroscience*, 120(4), pp. 1157–1170.

Mansour, A. *et al.* (1988) Anatomy of CNS opioid receptors., *Trends in neurosciences*, 11(7), pp. 308–14.

Mansour, A. *et al.* (1994) Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study., *The Journal of comparative neurology*, 350(3), pp. 412–38.

Manzanares, J. *et al.* (2018) Role of the endocannabinoid system in drug addiction., *Biochemical pharmacology*. England, 157, pp. 108–121.

Marjoribanks, J., Ayeleke, R. O., Farquhar, C. and Proctor, M. (2015) Nonsteroidal anti-inflammatory drugs for dysmenorrhoea., *The Cochrane database of systematic reviews*, 2015(7), p. CD001751.

Maroso, M. *et al.* (2016) Cannabinoid Control of Learning and Memory through HCN Channels, *Neuron*, 89(5), pp. 1059–1073.

Marsicano, G. and Lafenêtre, P. (2009) Roles of the endocannabinoid system in learning and memory., *Current topics in behavioral neurosciences*. Germany, 1, pp. 201–230.

Martin, D. C. (2006) Hysterectomy for treatment of pain associated with endometriosis, *Journal of Minimally Invasive Gynecology*, 13(6), pp. 566–572.

Martin, W. J., Tsou, K. and Walker, J. M. (1998) Cannabinoid receptor-mediated inhibition of the rat tail-flick reflex after microinjection into the rostral ventromedial medulla., *Neuroscience letters*. Ireland, 242(1), pp. 33–36.

Martin, W. J. *et al.* (1999) Anatomical basis for cannabinoid-induced antinociception as revealed by intracerebral microinjections, *Brain Research*, 822(1–2), pp. 237–242.

Martínez-Gómez, M. *et al.* (1994) Assessing pain threshold in the rat: Changes with estrus and time of day, *Physiology and Behavior*, 55(4), pp. 651–657.

Martins, I. and Tavares, I. (2017) Reticular Formation and Pain: The Past and the Future, *Frontiers in neuroanatomy*. Frontiers Media S.A., 11, p. 51.

Marziali, M. *et al.* (2012) Gluten-free diet: a new strategy for management of painful endometriosis related symptoms?, *Minerva chirurgica*. Italy, 67(6), pp. 499–504.

Di Marzo, V. *et al.* (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons., *Nature*. England, 372(6507), pp. 686–691.

Di Marzo, V., Bifulco, M. and De Petrocellis, L. (2004) The endocannabinoid system and its therapeutic exploitation, *Nature Reviews Drug Discovery*, pp. 771–784.

Mason, D. J. J., Lowe, J. and Welch, S. P. (1999) Cannabinoid modulation of dynorphin A: correlation to cannabinoid-induced antinociception., *European journal of pharmacology*. Netherlands, 378(3), pp. 237–248.

Massaly, N. *et al.* (2019) Pain-Induced Negative Affect Is Mediated via Recruitment of The Nucleus Accumbens Kappa Opioid System, *Neuron*. 2019/03/13, 102(3), pp. 564-573.e6.

Massó González, E. L., Patrignani, P., Tacconelli, S. and García Rodríguez, L. A. (2010) Variability among nonsteroidal

antiinflammatory drugs in risk of upper gastrointestinal bleeding., *Arthritis and rheumatism*. United States, 62(6), pp. 1592–1601.

Matsuda, L. A. *et al.* (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA., *Nature*. England, 346(6284), pp. 561–564.

McAllister, S. D. and Glass, M. (2002) CB(1) and CB(2) receptor-mediated signalling: a focus on endocannabinoids., *Prostaglandins, leukotrienes, and essential fatty acids*. Scotland, 66(2–3), pp. 161–171.

McCall, J. G. *et al.* (2017) Locus coeruleus to basolateral amygdala noradrenergic projections promote anxiety-like behavior., *eLife*, 6.

McClane, T. K. and Martin, W. R. (1967) Effects of morphine, nalorphine, cyclazocine, and naloxone on the flexor reflex, *Neuropharmacology*, 6(2), pp. 89–98.

McCurdy, C. R. *et al.* (2006) Antinociceptive profile of salvinorin A, a structurally unique kappa opioid receptor agonist, *Pharmacology Biochemistry and Behavior*, 83(1), pp. 109–113.

McKinnon, B., Bersinger, N. A., Wotzkow, C. and Mueller, M. D. (2012) Endometriosis-associated nerve fibers, peritoneal fluid cytokine concentrations, and pain in endometriotic lesions from different locations., *Fertility and sterility*. United States, 97(2), pp. 373–380.

McKinnon, B. D., Bertschi, D., Bersinger, N. A. and Mueller, M. D. (2015) Inflammation and nerve fiber interaction in endometriotic

pain, *Trends in Endocrinology & Metabolism*, 26(1), pp. 1–10.

McLaughlin, J. P. *et al.* (2004) Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance., *The Journal of biological chemistry*, 279(3), pp. 1810–1818.

Mechoulam, R. and Shvo, Y. (1963) Hashish. I. The structure of cannabidiol., *Tetrahedron*. England, 19(12), pp. 2073–2078.

Mechoulam, R. *et al.* (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors., *Biochemical pharmacology*. England, 50(1), pp. 83–90.

Mechoulam, R. and Parker, L. A. (2013) The endocannabinoid system and the brain., *Annual review of psychology*. United States, 64, pp. 21–47.

Mechsner, S. *et al.* (2009) A pilot study to evaluate the clinical relevance of endometriosis-associated nerve fibers in peritoneal endometriotic lesions, *Fertility and Sterility*. Elsevier Ltd, 92(6), pp. 1856–1861.

Mehmanesh, H., Almeida, O. F. X., Nikolarakis, K. E. and Herz, A. (1988) Hypothalamic LH-RH release after acute and chronic treatment with morphine studied in a combined in vivo/in vitro model, *Brain Research*, 451(1), pp. 69–76.

Le Merrer, J., Becker, J. A. J., Befort, K. and Kieffer, B. L. (2009) Reward processing by the opioid system in the brain, *Physiological Reviews*, 89(4), pp. 1379–1412.

Meunier, J. C. *et al.* (1995) Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor, *Nature*, 377(6549), pp. 532–535.

Micale, V. *et al.* (2013) Endocannabinoid system and mood disorders: Priming a target for new therapies, *Pharmacology and Therapeutics*, 138(1), pp. 18–37.

Millan, M. J. (2002) Descending control of pain, *Progress in Neurobiology*, 66(6), pp. 355–474.

Miller EJ, F. I. (2015) The importance of pelvic nerve fibers in endometriosis, *Women's Health*, 11(5), pp. 611–618.

Millns, P. J., Chapman, V. and Kendall, D. A. (2001) Cannabinoid inhibition of the capsaicin-induced calcium response in rat dorsal root ganglion neurones., *British journal of pharmacology*, 132(5), pp. 969–971.

Ministerio de Sanidad, S. sociales e igualdad (2013) Guía de atención a las mujeres con endomtriosis en el Sistema Nacional de Salud (SNS).

Missmer, S. A. *et al.* (2004) Reproductive history and endometriosis among premenopausal women., *Obstetrics and gynecology*. United States, 104(5 Pt 1), pp. 965–974.

Miyashita, M. *et al.* (2019) Expression of Nerve Injury-Induced Protein1 (Ninj1) in Endometriosis., *Reproductive sciences (Thousand Oaks, Calif.)*. United States, 26(8), pp. 1105–1110.

Mogil, J. S. *et al.* (2003) The melanocortin-1 receptor gene mediates female-specific mechanisms of analgesia in mice and humans, *Proceedings of the National Academy of Sciences*, 100(8), pp. 4867 LP – 4872.

Mollereau, C. *et al.* (1994) ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization, *FEBS Letters*, 341(1), pp. 33–38.

Montanari, G. *et al.* (2013) Women with deep infiltrating endometriosis: sexual satisfaction, desire, orgasm, and pelvic problem interference with sex., *The journal of sexual medicine*. Netherlands, 10(6), pp. 1559–1566.

Montoya, P. *et al.* (2004) Influence of social support and emotional context on pain processing and magnetic brain responses in fibromyalgia, *Arthritis and Rheumatism*, 50(12), pp. 4035–4044.

Moore, J. S., Gibson, P. R., Perry, R. E. and Burgell, R. E. (2017) Endometriosis in patients with irritable bowel syndrome: Specific symptomatic and demographic profile, and response to the low FODMAP diet., *The Australian & New Zealand journal of obstetrics & gynaecology*. Australia, 57(2), pp. 201–205.

Moreira, F. A. and Wotjak, C. T. (2010) Cannabinoids and anxiety., *Current topics in behavioral neurosciences*. Germany, 2, pp. 429–450.

Morgan, M. J. and Liu, Z. G. (2011) Crosstalk of reactive oxygen species and NF-κB signaling, *Cell Research*. Nature Publishing Group,

21(1), pp. 103–115.

Morgan, N. H., Stanford, I. M. and Woodhall, G. L. (2009) Functional CB2 type cannabinoid receptors at CNS synapses, *Neuropharmacology*, 57(4), pp. 356–368.

Muñoz, M. and Esteve, R. (2005) Reports of memory functioning by patients with chronic pain, *Clinical Journal of Pain*, 21(4), pp. 287–291.

Munro, S., Thomas, K. L. and Abu-Shaar, M. (1993) Molecular characterization of a peripheral receptor for cannabinoids, *Nature*, 365(6441), pp. 61–65.

Murataeva, N., Straiker, A. and Mackie, K. (2014) Parsing the players: 2-arachidonoylglycerol synthesis and degradation in the CNS, *British journal of pharmacology*. Blackwell Publishing Ltd, 171(6), pp. 1379–1391.

Naser, P. V and Kuner, R. (2018) Peripheral Kappa Opioid Receptor Signaling Takes on a Central Role, *Neuron*, 99(6), pp. 1102–1104.

Nawathe, A. *et al.* (2008) Systematic review of the effects of aromatase inhibitors on pain associated with endometriosis., *BJOG : an international journal of obstetrics and gynaecology*. England, 115(7), pp. 818–822.

Neal, C. R. *et al.* (1999) Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: comparison of ORL1 receptor mRNA expression with (125)I-[(14)Tyr]-orphanin FQ binding., *The Journal of comparative neurology*, 412(4), pp. 563–

605.

Negrete, R., García Gutiérrez, M. S., Manzanares, J. and Maldonado, R. (2017) Involvement of the dynorphin/KOR system on the nociceptive, emotional and cognitive manifestations of joint pain in mice, *Neuropharmacology*, 116, pp. 315–327.

Nemeth, C. L. *et al.* (2010) Role of kappa-opioid receptors in the effects of salvinorin A and ketamine on attention in rats., *Psychopharmacology*, 210(2), pp. 263–274.

Nemmani, K. V. S. and Mogil, J. S. (2003) Serotonin–GABA interactions in the modulation of mu- and kappa-opioid analgesia, *Neuropharmacology*, 44(3), pp. 304–310.

Nestler, E. J. and Carlezon, W. A. (2006) The Mesolimbic Dopamine Reward Circuit in Depression, *Biological Psychiatry*, 59(12), pp. 1151–1159.

Nguyen, J. *et al.* (2014) Morphine stimulates cancer progression and mast cell activation and impairs survival in transgenic mice with breast cancer., *British journal of anaesthesia*, 113 Suppl(Suppl 1), pp. i4-13.

Nicholas, M. *et al.* (2019) The IASP classification of chronic pain for ICD-11: chronic primary pain, *PAIN*, 160(1).

Nieto, M. M. *et al.* (2005) Physiological control of emotion-related behaviors by endogenous enkephalins involves essentially the delta opioid receptors, *Neuroscience*, 135(2), pp. 305–313.

Nirgianakis, K., Ma, L., McKinnon, B. and Mueller, M. D. (2020) Recurrence Patterns after Surgery in Patients with Different Endometriosis Subtypes: A Long-Term Hospital-Based Cohort Study, *Journal of Clinical Medicine*, 9(2), p. 496.

Nyhuis, P. W., Gastpar, M. and Scherbaum, N. (2008) Opiate treatment in depression refractory to antidepressants and electroconvulsive therapy., *Journal of clinical psychopharmacology*. United States, pp. 593–595.

O'Sullivan, S. E. (2007) Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors., *British journal of pharmacology*, 152(5), pp. 576–582.

Obara, I., Mika, J., Schafer, M. K.-H. and Przewlocka, B. (2003) Antagonists of the kappa-opioid receptor enhance allodynia in rats and mice after sciatic nerve ligation., *British journal of pharmacology*, 140(3), pp. 538–46.

Olive, D. L. (2003) Medical therapy of endometriosis., *Seminars in reproductive medicine*. United States, 21(2), pp. 209–222.

Olive, D. L. (2008) Gonadotropin-releasing hormone agonists for endometriosis., *The New England journal of medicine*. United States, 359(11), pp. 1136–1142.

Onaivi, E. S. *et al.* (2006) Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain., *Annals of the New York Academy of Sciences*. United States, 1074, pp. 514–536.

Onaivi, E. S. *et al.* (2008) Brain neuronal CB2 cannabinoid receptors

in drug abuse and depression: from mice to human subjects., *PloS one*, 3(2), p. e1640.

Onaivi, E. S., Ishiguro, H., Gu, S. and Liu, Q.-R. (2012) CNS effects of CB2 cannabinoid receptors: beyond neuro-immuno-cannabinoid activity, *Journal of psychopharmacology (Oxford, England)*. 2011/03/29, 26(1), pp. 92–103.

Ong, W. Y., Stohler, C. S. and Herr, D. R. (2019) Role of the Prefrontal Cortex in Pain Processing, *Molecular Neurobiology*. Molecular Neurobiology, 56(2), pp. 1137–1166.

Ortega-Alvaro, A. *et al.* (2011) Deletion of CB2 cannabinoid receptor induces schizophrenia-related behaviors in mice, *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2011/03/23. Nature Publishing Group, 36(7), pp. 1489–1504.

Ossipov, M. H., Dussor, G. O. and Porreca, F. (2010) Central modulation of pain., *The Journal of clinical investigation*, 120(11), pp. 3779–3787.

Ottosson, A. and Edvinsson, L. (1997) Release of histamine from dural mast cells by substance P and calcitonin gene-related peptide., *Cephalalgia : an international journal of headache*. England, 17(3), pp. 166–174.

Pan, B. *et al.* (2011) Alterations of endocannabinoid signaling, synaptic plasticity, learning, and memory in monoacylglycerol lipase knock-out mice., *The Journal of neuroscience : the official journal of*

the Society for Neuroscience, 31(38), pp. 13420–13430.

Paris, J. J., Reilley, K. J. and McLaughlin, J. P. (2011) Kappa Opioid Receptor-Mediated Disruption of Novel Object Recognition: Relevance for Psychostimulant Treatment., *Journal of addiction research & therapy*, S4.

Parolaro, D. (1999) Presence and functional regulation of cannabinoid receptors in immune cells., *Life sciences*. Netherlands, 65(6–7), pp. 637–644.

Patel, S. and Hillard, C. J. (2009) Role of endocannabinoid signaling in anxiety and depression, *Current topics in behavioral neurosciences*, 1, pp. 347–371.

Patestas, M. A. and Gartner, L. P. (2016) *A textbook of neuroanatomy*, *Wiley-Blackwell*.

Pellegrini, C. *et al.* (2012) The expression of estrogen receptors as well as GREB1, c-MYC, and cyclin D1, estrogen-regulated genes implicated in proliferation, is increased in peritoneal endometriosis., *Fertility and sterility*. United States, 98(5), pp. 1200–1208.

Pellkofer, H. L. *et al.* (2013) The major brain endocannabinoid 2-AG controls neuropathic pain and mechanical hyperalgesia in patients with neuromyelitis optica., *PloS one*, 8(8), p. e71500.

Peppin, J. F. and Raffa, R. B. (2015) Delta opioid agonists: A concise update on potential therapeutic applications, *Journal of Clinical Pharmacy and Therapeutics*, 40(2), pp. 155–166.

Pertwee, R. G. (2006) Cannabinoid pharmacology: the first 66 years., *British journal of pharmacology*, 147 Suppl(Suppl 1), pp. S163-71.

Pertwee, R. G. *et al.* (2010) International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB_1 and CB_2, *Pharmacological reviews*. The American Society for Pharmacology and Experimental Therapeutics, 62(4), pp. 588–631.

Petraglia, F. *et al.* (1986) Endogenous opioid peptides in uterine fluid., *Fertility and sterility*. United States, 46(2), pp. 247–251.

De Petrocellis, L., Nabissi, M., Santoni, G. and Ligresti, A. (2017) Actions and Regulation of Ionotropic Cannabinoid Receptors., *Advances in pharmacology (San Diego, Calif.)*. United States, 80, pp. 249–289.

Petrosino, S. *et al.* (2007) Changes in spinal and supraspinal endocannabinoid levels in neuropathic rats., *Neuropharmacology*. England, 52(2), pp. 415–422.

Petrosino, S. and Di Marzo, V. (2017) The pharmacology of palmitoylethanolamide and first data on the therapeutic efficacy of some of its new formulations, *British journal of pharmacology*. 2016/09/29. John Wiley and Sons Inc., 174(11), pp. 1349–1365.

Pfeiffer, A., Brantl, V., Herz, A. and Emrich, H. M. (1986) Psychotomimesis mediated by κ opiate receptors, *Science*, 233(4765), pp. 774–776.

Philippe, D. *et al.* (2003) Anti-inflammatory properties of the mu

opioid receptor support its use in the treatment of colon inflammation., *The Journal of clinical investigation*, 111(9), pp. 1329–1338.

Piomelli, D. (2003) The molecular logic of endocannabinoid signalling, *Nature Reviews Neuroscience*, 4(11), pp. 873–884.

Pisanti, S. *et al.* (2011) Genetic and pharmacologic inactivation of cannabinoid CB1 receptor inhibits angiogenesis., *Blood*. United States, 117(20), pp. 5541–5550.

Pliakas, A. M. *et al.* (2001) Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens., *The Journal of neuroscience : the official journal of the Society for Neuroscience*. NIH Public Access, 21(18), pp. 7397–403.

Podvin, S., Yaksh, T. and Hook, V. (2016) The Emerging Role of Spinal Dynorphin in Chronic Pain: A Therapeutic Perspective, *Annual Review of Pharmacology and Toxicology*. NIH Public Access, 56, pp. 511–533.

Pol, O. and Puig, M. M. (2004) Expression of Opioid Receptors During Peripheral Inflammation, *Current Topics in Medicinal Chemistry*, pp. 51–61.

La Porta, C. *et al.* (2013) Role of CB1 and CB2 cannabinoid receptors in the development of joint pain induced by monosodium iodoacetate, *Pain*, 154(1), pp. 160–174.

La Porta, C. *et al.* (2015) Role of the endocannabinoid system in the emotional manifestations of osteoarthritis pain., *Pain*, 156(10), pp. 2001–12.

La Porta, C., Lara-Mayorga, I. M., Negrete, R. and Maldonado, R. (2016) Effects of pregabalin on the nociceptive, emotional and cognitive manifestations of neuropathic pain in mice, *European Journal of Pain (United Kingdom)*, 20(9), pp. 1454–1466.

Porter, A. C. *et al.* (2002) Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor., *The Journal of pharmacology and experimental therapeutics*. United States, 301(3), pp. 1020–1024.

Practice Committee of the American Society for Reproductive Medicine (2014) Treatment of pelvic pain associated with endometriosis: a committee opinion, *Fertility and Sterility*. Elsevier, 101(4), pp. 927–935.

Proctor, M. L. *et al.* (2005) Surgical interruption of pelvic nerve pathways for primary and secondary dysmenorrhoea., *The Cochrane database of systematic reviews*. England, (4), p. CD001896.

Raborn, E. S. *et al.* (2008) The cannabinoid delta-9-tetrahydrocannabinol mediates inhibition of macrophage chemotaxis to RANTES/CCL5: linkage to the CB2 receptor, *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology*. 2007/07/11, 3(2), pp. 117–129.

Racz, I. *et al.* (2008) Crucial role of CB(2) cannabinoid receptor in the regulation of central immune responses during neuropathic pain, *The Journal of neuroscience : the official journal of the Society for Neuroscience*. Society for Neuroscience, 28(46), pp. 12125–12135.

Raja, S. N. *et al.* (2020) The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises., *Pain*, 161(9), pp. 1976–1982.

Raman, M., Chen, W. and Cobb, M. H. (2007) Differential regulation and properties of MAPKs, *Oncogene*, 26(22), pp. 3100–3112.

Rasakham, K. and Liu-Chen, L.-Y. (2011) Sex differences in kappa opioid pharmacology, *Life Sciences*, 88(1), pp. 2–16.

Reed, B., Zhang, Y., Chait, B. T. and Kreek, M. J. (2003) Dynorphin A(1–17) biotransformation in striatum of freely moving rats using microdialysis and matrix-assisted laser desorption/ionization mass spectrometry, *Journal of Neurochemistry*. John Wiley & Sons, Ltd, 86(4), pp. 815–823.

Reed, B., Bidlack, J. M., Chait, B. T. and Kreek, M. J. (2008) Extracellular biotransformation of beta-endorphin in rat striatum and cerebrospinal fluid., *Journal of neuroendocrinology*, 20(5), pp. 606–616.

Reis, F. M., Petraglia, F. and Taylor, R. N. (2013) Endometriosis: hormone regulation and clinical consequences of chemotaxis and apoptosis., *Human reproduction update*, 19(4), pp. 406–418.

Ren, K. and Dubner, R. (2010) Interactions between the immune and

nervous systems in pain, *Nature Medicine*. NIH Public Access, 16(11), pp. 1267–1276.

Resuehr, D. *et al.* (2012) Progesterone-dependent regulation of endometrial cannabinoid receptor type 1 (CB1-R) expression is disrupted in women with endometriosis and in isolated stromal cells exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), *Fertility and Sterility*, 98(4).

Revised American Society for Reproductive Medicine classification of endometriosis: 1996. (1997) *Fertility and sterility*. United States, 67(5), pp. 817–821.

Del Rey, A. *et al.* (2011) Chronic neuropathic pain-like behavior correlates with IL-1β expression and disrupts cytokine interactions in the hippocampus, *Pain*, 152(12), pp. 2827–2835.

Rittner, H. L., Brack, A. and Stein, C. (2008) Pain and the immune system, *British Journal of Anaesthesia*. IASP Press, Seattle, 101(1), pp. 40–44.

Robakis, T., Williams, K. E., Nutkiewicz, L. and Rasgon, N. L. (2019) Hormonal Contraceptives and Mood: Review of the Literature and Implications for Future Research, *Current Psychiatry Reports*. Current Psychiatry Reports, 21(7).

Robles, C. F. *et al.* (2014) Effects of kappa opioid receptors on conditioned place aversion and social interaction in males and females, *Behavioural Brain Research*, 262, pp. 84–93.

Roques, B. P., Fournié-Zaluski, M. C. and Wurm, M. (2012) Inhibiting

the breakdown of endogenous opioids and cannabinoids to alleviate pain, *Nature Reviews Drug Discovery*, 11(4), pp. 292–310.

Rosen, H., Itin, A., Schiff, R. and Keshet, E. (1990) Local Regulation within the Female Reproductive System and upon Embryonic Implantation: Identification of Cells Expressing Proenkephalin A, *Molecular Endocrinology*, 4(1), pp. 146–154.

Roth, B. L. *et al.* (2002) Salvinorin A: a potent naturally occurring nonnitrogenous kappa opioid selective agonist., *Proceedings of the National Academy of Sciences of the United States of America*, 99(18), pp. 11934–11939.

Rubino, T. *et al.* (2008) Role in Anxiety Behavior of the Endocannabinoid System in the Prefrontal Cortex, *Cerebral Cortex*, 18(6), pp. 1292–1301.

Rush, G. and Misajon, R. (2018) Examining subjective wellbeing and health-related quality of life in women with endometriosis., *Health care for women international*. England, 39(3), pp. 303–321.

Russo, E. and Guy, G. W. (2006) A tale of two cannabinoids: the therapeutic rationale for combining tetrahydrocannabinol and cannabidiol., *Medical hypotheses*. United States, 66(2), pp. 234–246.

Sacco, K. *et al.* (2012) The role of prostaglandin E2 in endometriosis., *Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology*. England, 28(2), pp. 134–138.

Salameh, E. *et al.* (2019) Chronic colitis-induced visceral pain is associated with increased anxiety during quiescent phase, *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 316(6), pp. G692–G700.

Sampson, J. A. (1927) Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity, *American Journal of Obstetrics & Gynecology*. Elsevier, 14(4), pp. 422–469.

Sanchez, A. M. *et al.* (2012) The molecular connections between the cannabinoid system and endometriosis, *Molecular Human Reproduction*, pp. 563–571.

Sánchez, C. *et al.* (2001) The CB(1) cannabinoid receptor of astrocytes is coupled to sphingomyelin hydrolysis through the adaptor protein fan., *Molecular pharmacology*. United States, 59(5), pp. 955–959.

Sarne, Y. *et al.* (2018) Reversal of age-related cognitive impairments in mice by an extremely low dose of tetrahydrocannabinol, *Neurobiology of Aging*. Elsevier Inc., 61, pp. 177–186.

Schaible, H. G. (2007) Peripheral and central mechanisms of pain generation, *Handbook of Experimental Pharmacology*, 177(177), pp. 3–28.

Scherrer, G. *et al.* (2009) Dissociation of the Opioid Receptor Mechanisms that Control Mechanical and Heat Pain, *Cell*, 137(6), pp. 1148–1159.

Schliep, K. C. *et al.* (2015) Pain typology and incident endometriosis, *Human Reproduction*, 30(10), pp. 2427–2438.

Scholl, B., Bersinger, N. A., Kuhn, A. and Mueller, M. D. (2009) Correlation between symptoms of pain and peritoneal fluid inflammatory cytokine concentrations in endometriosis., *Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology*. England, 25(11), pp. 701–706.

Scholz, J. and Woolf, C. J. (2002) Can we conquer pain?, *Nature Neuroscience*, 5(11s), pp. 1062–1067.

Schwartz, A. S. K. *et al.* (2019) The use of home remedies and complementary health approaches in endometriosis, *Reproductive BioMedicine Online*, 38(2), pp. 260–271.

Schwartz, E. S. *et al.* (2013) TRPV1 and TRPA1 antagonists prevent the transition of acute to chronic inflammation and pain in chronic pancreatitis, *Journal of Neuroscience*, 33(13), pp. 5603–5611.

Schwier, C., Kliem, A., Boettger, M. K. and Bär, K. J. (2010) Increased Cold-Pain Thresholds in Major Depression, *Journal of Pain*, 11(3), pp. 287–290.

Scotchie, J. G., Savaris, R. F., Martin, C. E. and Young, S. L. (2015) Endocannabinoid regulation in human endometrium across the menstrual cycle, *Reproductive sciences (Thousand Oaks, Calif.)*. 2014/05/12. SAGE Publications, 22(1), pp. 113–123.

Seear, K. (2009) The etiquette of endometriosis: stigmatisation,

menstrual concealment and the diagnostic delay., *Social science & medicine (1982)*. England, 69(8), pp. 1220–1227.

Selak, V., Farquhar, C., Prentice, A. and Singla, A. (2007) Danazol for pelvic pain associated with endometriosis., *The Cochrane database of systematic reviews*. England, (4), p. CD000068.

Sepulcri, R. de P. and do Amaral, V. F. (2009) Depressive symptoms, anxiety, and quality of life in women with pelvic endometriosis., *European journal of obstetrics, gynecology, and reproductive biology*. Ireland, 142(1), pp. 53–56.

Shafrir, A. L. *et al.* (2018) Risk for and consequences of endometriosis: A critical epidemiologic review., *Best practice & research. Clinical obstetrics & gynaecology*. Netherlands, 51, pp. 1–15.

Shakiba, K. *et al.* (2008) Surgical treatment of endometriosis: a 7-year follow-up on the requirement for further surgery., *Obstetrics and gynecology*. United States, 111(6), pp. 1285–1292.

Shang, Y. and Tang, Y. (2017) The central cannabinoid receptor type-2 (CB2) and chronic pain., *The International journal of neuroscience*. England, 127(9), pp. 812–823.

Shannon, H. E. *et al.* (2007) Effects of kappa opioid receptor agonists on attention as assessed by a 5-choice serial reaction time task in rats., *Neuropharmacology*. England, 53(8), pp. 930–941.

Shariftabrizi, A. *et al.* (2006) Matrix metalloproteinase 2 secretion in WEHI 164 fibrosarcoma cells is nitric oxide-related and modified by

morphine., *European journal of pharmacology*. Netherlands, 530(1–2), pp. 33–39.

Sharpe, L. *et al.* (2020) Cannabis, a cause for anxiety? A critical appraisal of the anxiogenic and anxiolytic properties, *Journal of translational medicine*. BioMed Central, 18(1), p. 374.

Shifren, J. L. *et al.* (1996) Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis., *The Journal of clinical endocrinology and metabolism*. United States, 81(8), pp. 3112–3118.

Shigesi, N. *et al.* (2019) The association between endometriosis and autoimmune diseases: a systematic review and meta-analysis., *Human reproduction update*, 25(4), pp. 486–503.

Shirayama, Y. *et al.* (2004) Stress increases dynorphin immunoreactivity in limbic brain regions and dynorphin antagonism produces antidepressant-like effects, *Journal of Neurochemistry*, 90(5), pp. 1258–1268.

Shire, D. *et al.* (1996) Structural Features of the Central Cannabinoid CB1 Receptor Involved in the Binding of the Specific CB1 Antagonist SR 141716A (∗), *Journal of Biological Chemistry*. Elsevier, 271(12), pp. 6941–6946.

Sibille, K. T. *et al.* (2011) Individual differences in morphine and butorphanol analgesia: a laboratory pain study, *Pain medicine (Malden, Mass.)*. 2011/06/13, 12(7), pp. 1076–1085.

Van Sickle, M. D. *et al.* (2005) Identification and functional characterization of brainstem cannabinoid CB2 receptors., *Science (New York, N.Y.)*. United States, 310(5746), pp. 329–332.

Sinaii, N. *et al.* (2002) High rates of autoimmune and endocrine disorders, fibromyalgia, chronic fatigue syndrome and atopic diseases among women with endometriosis: a survey analysis., *Human reproduction (Oxford, England)*. England, 17(10), pp. 2715–2724.

Sinclair, J. *et al.* (2020) Cannabis Use, a Self-Management Strategy Among Australian Women With Endometriosis: Results From a National Online Survey., *Journal of obstetrics and gynaecology Canada : JOGC = Journal d'obstetrique et gynecologie du Canada : JOGC*. Netherlands, 42(3), pp. 256–261.

Singh, A. J., Meyer, R. D., Band, H. and Rahimi, N. (2005) The carboxyl terminus of VEGFR-2 is required for PKC-mediated down-regulation., *Molecular biology of the cell*, 16(4), pp. 2106–2118.

Snyder, L. M. *et al.* (2018) Kappa Opioid Receptor Distribution and Function in Primary Afferents, *Neuron*, 99(6), pp. 1274-1288.e6.

Sofia, R. D., Nalepa, S. D., Harakal, J. J. and Vassar, H. B. (1973) Anti-edema and analgesic properties of delta9-tetrahydrocannabinol (THC)., *The Journal of pharmacology and experimental therapeutics*. United States, 186(3), pp. 646–655.

Sorge, R. E. *et al.* (2015) Different immune cells mediate mechanical pain hypersensitivity in male and female mice, *Nature Neuroscience*,

18(8), pp. 1081–1083.

Sperschneider, M. L. *et al.* (2019) Does endometriosis affect professional life? A matched case-control study in Switzerland, Germany and Austria., *BMJ open*, 9(1), p. e019570.

Stein, C. (1993) Peripheral mechanisms of opioid analgesia, *Anesthesia and Analgesia*, 76(1), pp. 182–191.

Steiner, M. *et al.* (2008a) Antidepressant-like behavioral effects of impaired cannabinoid receptor type 1 signaling coincide with exaggerated corticosterone secretion in mice., *Psychoneuroendocrinology*, 33(1), pp. 54–67.

Steiner, M. *et al.* (2008b) Impaired cannabinoid receptor type 1 signaling interferes with stress-coping behavior in mice., *The pharmacogenomics journal*. United States, 8(3), pp. 196–208.

Sternberg, W. F., Chesler, E. J., Wilson, S. G. and Mogil, J. S. (2004) Acute progesterone can recruit sex-specific neurochemical mechanisms mediating swim stress-induced and κ-opioid analgesia in mice, *Hormones and Behavior*, 46(4), pp. 467–473.

Stewart, E. A., Shuster, L. T. and Rocca, W. A. (2012) Reassessing hysterectomy., *Minnesota medicine*, 95(3), pp. 36–39.

Stochino Loi, E. *et al.* (2019) Effect of ultramicronized-palmitoylethanolamide and co-micronized palmitoylethanolamide/polydatin on chronic pelvic pain and quality of life in endometriosis patients: An open-label pilot study, *International journal of women's health*. Dove, 11, pp. 443–449.

Stratton, P. *et al.* (2015) Association of chronic pelvic pain and endometriosis with signs of sensitization and myofascial pain., *Obstetrics and gynecology*, 125(3), pp. 719–728.

Sugiura, T. *et al.* (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain., *Biochemical and biophysical research communications*. United States, 215(1), pp. 89–97.

Sugiura, T., Kishimoto, S., Oka, S. and Gokoh, M. (2006) Biochemistry, pharmacology and physiology of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand., *Progress in lipid research*. England, 45(5), pp. 405–446.

Suplita, R. L. 2nd, Farthing, J. N., Gutierrez, T. and Hohmann, A. G. (2005) Inhibition of fatty-acid amide hydrolase enhances cannabinoid stress-induced analgesia: sites of action in the dorsolateral periaqueductal gray and rostral ventromedial medulla., *Neuropharmacology*. England, 49(8), pp. 1201–1209.

Surrey, E. S. *et al.* (2018) Risk of Developing Comorbidities Among Women with Endometriosis: A Retrospective Matched Cohort Study., *Journal of women's health (2002)*. United States, 27(9), pp. 1114–1123.

Svingos, A. L. *et al.* (1996) Ultrastructural immunocytochemical localization of mu-opioid receptors in rat nucleus accumbens: extrasynaptic plasmalemmal distribution and association with Leu5-enkephalin., *The Journal of neuroscience : the official journal of the*

Society for Neuroscience, 16(13), pp. 4162–73.

Symons, L. K. *et al.* (2018) The Immunopathophysiology of Endometriosis., *Trends in molecular medicine*. England, 24(9), pp. 748–762.

Szabo, B. and Schlicker, E. (2005) Effects of cannabinoids on neurotransmission., *Handbook of experimental pharmacology*. Germany, (168), pp. 327–365.

Tam, J. *et al.* (2010) Peripheral CB1 cannabinoid receptor blockade improves cardiometabolic risk in mouse models of obesity., *The Journal of clinical investigation*, 120(8), pp. 2953–2966.

Taylor, H. S. *et al.* (2017) Treatment of Endometriosis-Associated Pain with Elagolix, an Oral GnRH Antagonist, *New England Journal of Medicine*, 377(1), pp. 28–40.

Tejeda, H. A., Chefer, V. I., Zapata, A. and Shippenberg, T. S. (2010) The effects of kappa-opioid receptor ligands on prepulse inhibition and CRF-induced prepulse inhibition deficits in the rat, *Psychopharmacology*. 2010/03/16, 210(2), pp. 231–240.

Tejeda, H. A. *et al.* (2015) Prefrontal Cortical Kappa Opioid Receptors Attenuate Responses to Amygdala Inputs, *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2015/05/14. Nature Publishing Group, 40(13), pp. 2856–2864.

Tejedor-Real, P. *et al.* (1993) Effect of mixed (RB 38A) and selective (RB 38B) inhibitors of enkephalin degrading enzymes on a model of

depression in the rat, *Biological Psychiatry*, 34(1–2), pp. 100–107.

Terner, J. M., Barrett, A. C., Cook, C. D. and Picker, M. J. (2003) Sex differences in (−)-pentazocine antinociception: Comparison to morphine and spiradoline in four rat strains using a thermal nociceptive assay, *Behavioural Pharmacology*, 14(1).

Terranova, J. P. *et al.* (1996) Improvement of memory in rodents by the selective CB1 cannabinoid receptor antagonist, SR 141716., *Psychopharmacology*. Germany, 126(2), pp. 165–172.

Thompson, A. A. *et al.* (2012) Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic, *Nature*. NIH Public Access, 485(7398), pp. 395–399.

Tokushige, N., Markham, R., Russell, P. and Fraser, I. S. (2006a) High density of small nerve fibres in the functional layer of the endometrium in women with endometriosis, *Human Reproduction*, 21(3), pp. 782–787.

Tokushige, N., Markham, R., Russell, P. and Fraser, I. S. (2006b) Nerve fibres in peritoneal endometriosis., *Human reproduction (Oxford, England)*. England, 21(11), pp. 3001–3007.

Torrecilla, M. *et al.* (2002) G-Protein-Gated Potassium Channels Containing Kir3.2 and Kir3.3 Subunits Mediate the Acute Inhibitory Effects of Opioids on Locus Ceruleus Neurons, *Journal of Neuroscience*, 22(11), pp. 4328–4334.

Treede, R.-D. (2018) The International Association for the Study of Pain definition of pain: as valid in 2018 as in 1979, but in need of

regularly updated footnotes, *Pain reports*. Wolters Kluwer, 3(2), pp. e643–e643.

Treede, R.-D. *et al.* (2019) Chronic pain as a symptom or a disease: the IASP Classification of Chronic Pain for the International Classification of Diseases (ICD-11), *PAIN*, 160(1).

Troncon, J. K. *et al.* (2014) Endometriosis in a patient with mayer-rokitansky-küster-hauser syndrome., *Case reports in obstetrics and gynecology*, 2014, p. 376231.

Tsolakidis, D. *et al.* (2010) The impact on ovarian reserve after laparoscopic ovarian cystectomy versus three-stage management in patients with endometriomas: a prospective randomized study., *Fertility and sterility*. United States, 94(1), pp. 71–77.

Tsou, K. *et al.* (1998) Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system, *Neuroscience*, 83(2), pp. 393–411.

Tsuda, M., Inoue, K. and Salter, M. W. (2005) Neuropathic pain and spinal microglia: a big problem from molecules in ‘small’ glia, *Trends in Neurosciences*. Elsevier, 28(2), pp. 101–107.

Usoskin, D. *et al.* (2015) Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing, *Nature Neuroscience*. Nature Publishing Group, 18(1), pp. 145–153.

Ustun, F. *et al.* (2011) Evaluation of morphine effect on tumour angiogenesis in mouse breast tumour model, EATC., *Medical*

oncology (Northwood, London, England). United States, 28(4), pp. 1264–1272.

Van't Veer, A. and Carlezon, W. A. J. (2013) Role of kappa-opioid receptors in stress and anxiety-related behavior., *Psychopharmacology*, 229(3), pp. 435–452.

Vanderah, T. W. (2010) Delta and kappa opioid receptors as suitable drug targets for pain., *The Clinical journal of pain*, 26 Suppl 1(Supplement 10), pp. S10-5.

Varvel, S. A. *et al.* (2007) Inhibition of fatty-acid amide hydrolase accelerates acquisition and extinction rates in a spatial memory task., *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. England, 32(5), pp. 1032–1041.

Vassou, D. *et al.* (2011) Opioids increase bladder cancer cell migration via bradykinin B2 receptors., *International journal of oncology*. Greece, 39(3), pp. 697–707.

Vercellini, P. *et al.* (2003) Laparoscopic uterosacral ligament resection for dysmenorrhea associated with endometriosis: Results of a randomized, controlled trial, *Fertility and Sterility*, 80(2), pp. 310–319.

Vercellini, P. *et al.* (2009) Endometriosis: current therapies and new pharmacological developments., *Drugs*. New Zealand, 69(6), pp. 649–675.

Verma, A. and Konje, J. C. (2009) Successful treatment of refractory

endometriosis-related chronic pelvic pain with aromatase inhibitors in premenopausal patients, *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 143(2), pp. 112–115.

Vernon, M. W. and Wilson, E. A. (1985) Studies on the surgical induction of endometriosis in the rat**Presented at the Thirtieth Annual Meeting of the Society for Gynecological Investigation, March 17 to 20, 1983, Washington, D.C.††Supported in part by National Institutes of Health (NIH) grant, *Fertility and Sterility*. Elsevier Masson SAS, 44(5), pp. 684–694.

Vigano, P. *et al.* (2018) Time to redefine endometriosis including its pro-fibrotic nature., *Human reproduction (Oxford, England)*. England, 33(3), pp. 347–352.

Viñals, X. *et al.* (2015) Cognitive Impairment Induced by Delta9-tetrahydrocannabinol Occurs through Heteromers between Cannabinoid CB1 and Serotonin 5-HT2A Receptors., *PLoS biology*, 13(7), p. e1002194.

Vonvoigtlander, P. F., Lewis, R. A. and Neff, G. L. (1984) Kappa opioid analgesia is dependent on serotonergic mechanisms., *The Journal of pharmacology and experimental therapeutics*. United States, 231(2), pp. 270–274.

Vučković, S. *et al.* (2018) Cannabinoids and Pain: New Insights From Old Molecules, *Frontiers in pharmacology*. Frontiers Media S.A., 9, p. 1259.

Wahlström, T., Laatikainen, T., Salminen, K. and Leppäluoto, J.

(1985) Immunoreactive beta-endorphin is demonstrable in the secretory but not in the proliferative endometrium., *Life sciences*. Netherlands, 36(10), pp. 987–990.

Walczak, J. S., Price, T. J. and Cervero, F. (2009) Cannabinoid CB1 receptors are expressed in the mouse urinary bladder and their activation modulates afferent bladder activity., *Neuroscience*. United States, 159(3), pp. 1154–1163.

Walker, J., Catheline, G., Guilbaud, G. and Kayser, V. (1999) Lack of cross-tolerance between the antinociceptive effects of systemic morphine and asimadoline, a peripherally-selective kappa-opioid agonist, in CCI-neuropathic rats., *Pain*, 83(3), pp. 509–16.

Walter, L. *et al.* (2003) Nonpsychotropic cannabinoid receptors regulate microglial cell migration., *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 23(4), pp. 1398–1405.

Wang, G. *et al.* (2009a) Hyperinnervation in Intestinal Deep Infiltrating Endometriosis, *Journal of Minimally Invasive Gynecology*, 16(6), pp. 713–719.

Wang, G., Tokushige, N., Markham, R. and Fraser, I. S. (2009b) Rich innervation of deep infiltrating endometriosis, *Human Reproduction*, 24(4), pp. 827–834.

Wang, H.-B. *et al.* (2010) Coexpression of delta- and mu-opioid receptors in nociceptive sensory neurons., *Proceedings of the National Academy of Sciences of the United States of America*.

National Academy of Sciences, 107(29), pp. 13117–22.

Wang, Y. (2008) The functional regulation of TRPV1 and its role in pain sensitization, *Neurochemical Research*, 33(10), pp. 2008–2012.

Wang, Y. *et al.* (2010) The role of kappa-opioid receptor activation in mediating antinociception and addiction., *Acta pharmacologica Sinica*, 31(9), pp. 1065–1070.

Westlake, T. M. *et al.* (1994) Cannabinoid receptor binding and messenger RNA expression in human brain: an in vitro receptor autoradiography and in situ hybridization histochemistry study of normal aged and Alzheimer's brains., *Neuroscience*. United States, 63(3), pp. 637–652.

Wickman, K. and Clapham, D. E. (1995) Ion channel regulation by G proteins., *Physiological reviews*, 75(4), pp. 865–85.

Wiley, J. L., Razdan, R. K. and Martin, B. R. (2006) Evaluation of the role of the arachidonic acid cascade in anandamide's in vivo effects in mice., *Life sciences*. Netherlands, 80(1), pp. 24–35.

Willis, W. D. (1985) Central nervous system mechanisms for pain modulation, in McMahon, S. and Koltzenburg, M. (eds) *Applied Neurophysiology*. Edinburgh: Churchill Livingstone, pp. 153–165.

Woodhams, S. G. *et al.* (2017) The cannabinoid system and pain, *Neuropharmacology*, pp. 105–120.

Woolf, C. J. (2011) Central sensitization: Implications for the diagnosis and treatment of pain, *Pain*, 152(SUPPL.3), pp. S2–S15.

Wright, J., Lotfallah, H., Jones, K. and Lovell, D. (2005) A randomized trial of excision versus ablation for mild endometriosis., *Fertility and sterility*. United States, 83(6), pp. 1830–1836.

Wu, H. *et al.* (2012) Structure of the human κ-opioid receptor in complex with JDTic, *Nature*. NIH Public Access, 485(7398), pp. 327–332.

Xu, M. *et al.* (2004) Neuropathic pain activates the endogenous κ opioid system in mouse spinal cord and induces opioid receptor tolerance, *Journal of Neuroscience*. NIH Public Access, 24(19), pp. 4576–4584.

Xu, M. *et al.* (2007) Sciatic nerve ligation-induced proliferation of spinal cord astrocytes is mediated by κ opioid activation of p38 mitogen-activated protein kinase, *Journal of Neuroscience*, 27(10), pp. 2570–2581.

Xue, Q., Zhou, Y. F., Zhu, S. N. and Bulun, S. E. (2011) Hypermethylation of the CpG island spanning from exon II to intron III is associated with steroidogenic factor 1 expression in stromal cells of endometriosis, *Reproductive Sciences*, 18(11), pp. 1080–1084.

Yamamizu, K. *et al.* (2013) κ Opioids inhibit tumor angiogenesis by suppressing VEGF signaling., *Scientific reports*, 3, p. 3213.

Yilmaz, B. and Gilmore, D. P. (1999) Opioid modulation of hypothalamic catecholaminergic neurotransmission and the pre-ovulatory LH surge in the rat., *Neuro endocrinology letters*. Sweden,

20(1–2), pp. 115–121.

You, Z. *et al.* (2018) Cognitive impairment in a rat model of neuropathic pain: role of hippocampal microtubule stability, *Pain*, 159(8), pp. 1518–1528.

Zacny, J. P. and Beckman, N. J. (2004) The effects of a cold-water stimulus on butorphanol effects in males and females., *Pharmacology, biochemistry, and behavior*. United States, 78(4), pp. 653–659.

Zamponi, G. W. and Snutch, T. P. (1998) Modulation of voltage-dependent calcium channels by G proteins, *Current Opinion in Neurobiology*, 8(3), pp. 351–356.

Zhang, H. *et al.* (2007) Central kappa-opioid receptor-mediated antidepressant-like effects of nor-Binaltorphimine: behavioral and BDNF mRNA expression studies., *European journal of pharmacology*. NIH Public Access, 570(1–3), pp. 89–96.

Zhang, J. *et al.* (2003) Induction of CB2 receptor expression in the rat spinal cord of neuropathic but not inflammatory chronic pain models., *The European journal of neuroscience*. France, 17(12), pp. 2750–2754.

Zhao, Y. *et al.* (2016) Multiple Beneficial Roles of Repressor of Estrogen Receptor Activity (REA) in Suppressing the Progression of Endometriosis., *Endocrinology*, 157(2), pp. 900–912.

Zhao, Z.-Q. *et al.* (2007) Central serotonergic neurons are differentially required for opioid analgesia but not for morphine

tolerance or morphine reward, *Proceedings of the National Academy of Sciences*, 104(36), pp. 14519–14524.

Zhu, Y., Hsu, M. S. and Pintar, J. E. (1998) Developmental expression of the mu, kappa, and delta opioid receptor mRNAs in mouse., *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 18(7), pp. 2538–2549.

Zogopoulos, P., Vasileiou, I., Patsouris, E. and Theocharis, S. E. (2013) The role of endocannabinoids in pain modulation., *Fundamental & clinical pharmacology*. England, 27(1), pp. 64–80.

Zondervan, K. T. *et al.* (2004) Familial aggregation of endometriosis in a large pedigree of rhesus macaques., *Human reproduction (Oxford, England)*. England, 19(2), pp. 448–455.

Zondervan, K. T. *et al.* (2018) Endometriosis., *Nature reviews. Disease primers*. England, 4(1), p. 9.

Zondervan, K. T., Becker, C. M. and Missmer, S. A. (2020) Endometriosis, *The new england journal of medicine Review*, 384, pp. 1244–1256.

Zuardi, A. W., Shirakawa, I., Finkelfarb, E. and Karniol, I. G. (1982) Action of cannabidiol on the anxiety and other effects produced by delta 9-THC in normal subjects., *Psychopharmacology*. Germany, 76(3), pp. 245–250.

Zullo, F. *et al.* (2003) Effectiveness of presacral neurectomy in women with severe dysmenorrhea caused by endometriosis who were treated with laparoscopic conservative surgery: A 1-year

prospective randomized double-blind controlled trial, *American Journal of Obstetrics and Gynecology*, 189(1), pp. 5–10.