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EP₂ receptor antagonism reduces peripheral and central hyperalgesia in a preclinical mouse model of endometriosis

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Endometriosis is an incurable gynecological disorder characterized by debilitating pain and the establishment of innervated endometriosis lesions outside the uterus. In a preclinical mouse model of endometriosis we demonstrated overexpression of the PGE₂-signaling pathway (including COX-2, EP₂, EP₄) in endometriosis lesions, dorsal root ganglia (DRG), spinal cord, thalamus and forebrain. TRPV1, a PGE₂-regulated channel in nociceptive neurons was also increased in the DRG. These findings support the concept that an amplification process occurs along the pain neuroaxis in endometriosis. We then tested TRPV1, EP₂, and EP₄ receptor antagonists: The EP₂ antagonist was the most efficient analgesic, reducing primary hyperalgesia by 80% and secondary hyperalgesia by 40%. In this study we demonstrate reversible peripheral and central hyperalgesia in mice with induced endometriosis.

Endometriosis is a chronic gynecological disorder affecting 176 million women worldwide¹ associated with chronic pain and infertility. Current therapies include invasive surgery, drugs that suppress endogenous hormones² and non-steroidal anti-inflammatory drugs (NSAIDs) all of which have unwanted side effects. Better treatments for endometriosis-associated pain are needed but their development has been hampered by the lack of a robust preclinical model that fully reproduces the altered pain perception experienced by women with endometriosis.

Endometriosis is caused by the presence of endometrial-like tissue (endometriosis lesions) outside of the uterine cavity³. An association between small nerve fiber infiltration of endometriosis lesions and increased central and peripheral pain has been described (reviewed in ref. 4). Alterations in pain perception in women are thought to involve release of inflammatory mediators and neuropeptides by efferent peripheral nerve endings⁵, an increase in the sensitivity of nociceptive neurons⁶, and peripheral hyperalgesia. Continuous input from peripheral afferents can also trigger spinal hyper-excitability (central sensitization)⁷ resulting in increased pain perception, secondary hyperalgesia and allodynia⁸. Prostaglandin E₂ (PGE₂) is a well-established mediator of inflammation and nociception in inflammatory^{9,10} and neuropathic pain conditions¹¹ and synthesis of prostaglandins within endometriosis lesions has previously been reported^{12–15}. As the association of PGE₂ signaling and endometriosis is well established we chose to validate this pathway as a target for pain attenuation in our mouse model.

A rat model of endometriosis exhibits vaginal hyperalgesia and increased abdominal muscle activity^{16–18}. Recently, a study utilizing the same rat model demonstrated increased hyperalgesia in response to von Frey filaments, although an increase was also observed in the sham group¹⁹. Another model using autologous transplantation of endometrial tissue onto the gastrocnemius muscle²⁰ has proved useful for the identification of pronociceptive molecules that may be relevant in endometriosis²¹. Our murine model of endometriosis uses decidualized endometrial tissue injected (rather than sutured) into the peritoneum of recipient mice²². Resultant lesions phenocopy those in women; they are vascularized, innervated, infiltrated by macrophages and also exhibit oestrogen-dependent regulation of vascular-nerve and macrophage-nerve interactions^{23,24}. The aim of our study

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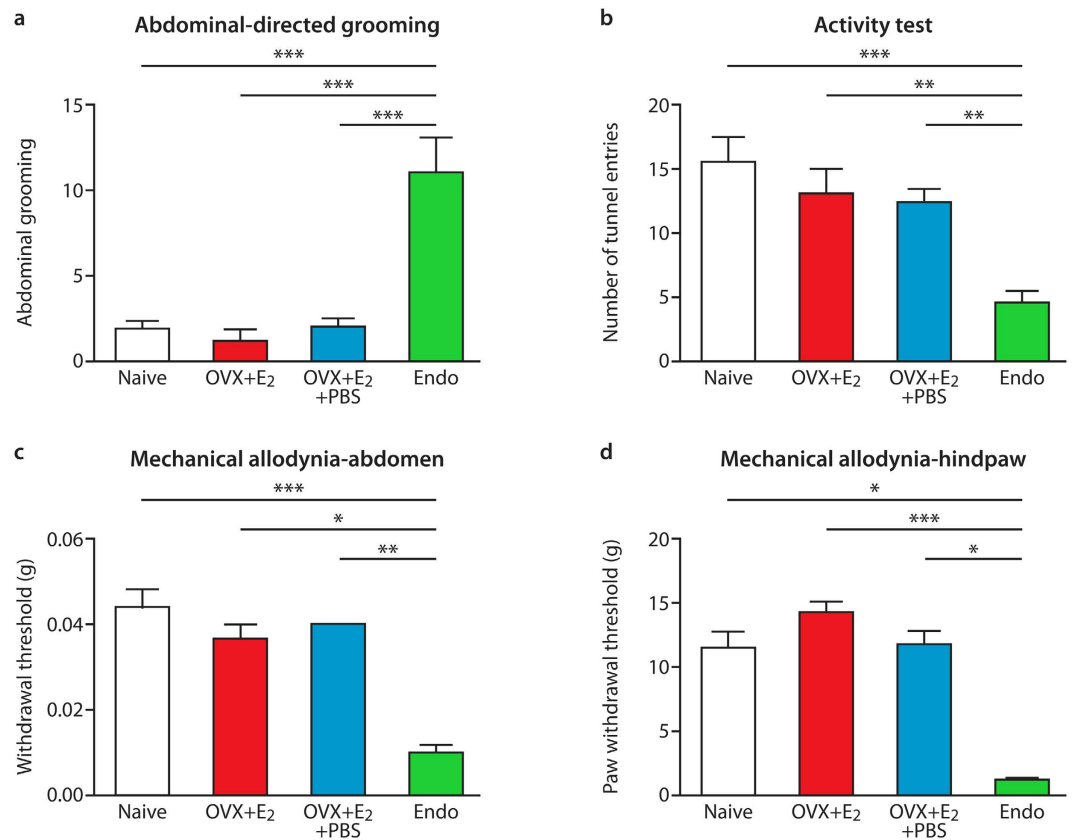


Figure 1. Behavior testing in control and endometriosis mice. Endometriosis mice, together with ovariectomised, estradiol-treated sham recipients, with or without i.p injection of PBS, and naïve controls were scored for general behaviors indicative of discomfort that might be associated with pelvic pain. (a) Shows abdominally directed licking (average number of grooming events recorded by two observers over two 5 min periods) was significantly increased in endometriosis mice ($n = 5$) compared to estradiol-treated ($OVX + E_2$; $n = 3$, $OVX + E_2 + PBS$; $n = 6$) and naïve controls ($n = 7$). (b) Shows that exploratory activity (average number of open-field tunnel entries recorded by two observers) was significantly reduced in endometriosis mice ($n = 9$) compared to estradiol-treated ($OVX + E_2$; $n = 6$, $OVX + E_2 + PBS$; $n = 6$) and naïve controls ($n = 11$). Mechanical withdrawal threshold, shown by von Frey filament testing, was also measured on the lower abdomen and plantar hind-paw of endometriosis mice ($n = 6$), $OVX + E_2$, $OVX + E_2 + PBS$ ($n = 6$) and naïve controls ($n = 7$). For quantitative sensory testing, von Frey filaments were applied to caudal regions of the abdomen; (c and d) show that withdrawal thresholds (g = grams) for both abdomen and paw testing were significantly decreased in endometriosis mice compared to the other groups. Statistical analysis was performed using a one-way ANOVA and Newman-Keuls post-hoc test (a and b) or a Kruskal-Wallis test and Dunn's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

was to determine if mice with induced endometriosis exhibit peripheral (abdominal) and central (secondary/referred) hyperalgesia and to test compounds targeting prostaglandin receptors to validate this model as a platform for preclinical testing of compounds to treat pain.

Results

Induction of endometriosis lesions resulted in altered pain-associated behaviors. We confirmed lesions in 90% of endometriosis mice (Endo) and those that we did not recover lesions from were excluded from our analysis. The average number of lesions recovered was 1.9 (Supplementary Table 1). Endo mice had significantly higher levels of abdominally-directed licking (Fig. 1a) and decreased exploratory activity (Fig. 1b) compared to controls ($p < 0.001$). Endo mice had significantly lower mechanical withdrawal thresholds for von Frey filaments, not only on the abdomen (Fig. 1c) but also on the hind-paws (Fig. 1d; $p < 0.05$ and $p < 0.01$, respectively). We found no correlation between mechanical allodynia (hypersensitivity) and number of lesions in mice with endometriosis (Supplementary Fig. 1a,b).

Over-representation of the PGE₂ signaling pathway and nociceptive ion channels in endometriosis lesions and in the nervous system of mice with endometriosis. Consistent with our previous studies^{22–24} endometriosis-like lesions were recovered from the walls of the parietal peritoneum and the visceral peritoneum covering the uterus, gut, and intestines; mesentery associated with the gut and intestines; adipose tissue associated with the kidney; and underneath the kidneys. EP_2 , EP_4 , cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) were significantly increased in endometriosis lesions compared to

the peritoneum of naive mice (NP) or mice with endometriosis (EP; $p < 0.01$; Supplementary Fig. 2a–d); EP₂, EP₄, COX-1 and COX-2 proteins were immunolocalised to glandular and stromal cells in lesions and mesothelial cells in the peritoneum (Supplementary Fig. 2). PGE₂ concentrations in the peritoneal fluid of Endo mice were significantly increased ($p < 0.05$; Supplementary Fig. 2E). mRNA concentrations of EP₁ and EP₃ were unchanged (Supplementary Fig. 2f,g). EP₂, *Cox-1*, *Scn11a* and *Trpv1* mRNA concentrations were significantly increased in dorsal root ganglia (DRG; clusters of cell bodies of afferent sensory neurons that transmit noxious stimuli from the periphery to the spinal cord) from endometriosis mice ($p < 0.05$; Fig. 2a–d); EP₄ and *Cox-2* were unchanged (Supplementary Fig. 3c,d). *Trpv1* mRNA was also increased in the DRGs from OVX + E₂ mice. Dual immunofluorescence showed that in small DRG cells expressing peripherin (a marker for small unmyelinated sensory neurons; C-fibre nociceptors), the proportion that were immunopositive for TRPV1 was significantly increased in Endo mice compared to naive and OVX + E₂-treated controls ($p < 0.05$; Fig. 2e,f). The proportion of TRPV1-immunopositive neurons in DRG that express EP₂ was also increased in these animals (Supplementary Fig. 3e,f). Mice with endometriosis had significantly increased concentrations of COX-2 protein in the spinal dorsal horn ($p < 0.001$), the thalamus ($p < 0.001$) and the anterior cingulate cortex of the brain ($p < 0.001$; Fig. 2f,i). All of these changes would be expected to contribute to pain hypersensitivity.

Pre-clinical testing and stratification of potential therapies identified EP₂ as a key target.

Figure 3 indicates that injection of the TRPV1 inhibitor JNJ 17203212 (Fig. 3a,b) or the EP₄ antagonist L-161982 (Fig. 3c,d), did not reverse abdominal or paw hyperalgesia to a statistically significant extent. Administration of the EP₂ antagonist TG6-10-1 resulted in a statistically significant reversal of mechanical allodynia as tested on the abdomen ($p < 0.05$) at 45 mins post administration but this did not reach significance for the hind paw (Fig. 3e,f). Time-course graphs are shown in Supplementary Fig. 4. Results were extended using injection (Fig. 3g,h) or oral administration (Fig. 4a,b) of a second EP₂ antagonist (PF-04418948)²⁵ and this reduced allodynia in both abdomen and hind-paw tests ($p < 0.001$ using either route). Oral administration resulted in striking, time dependent, and significant impacts (Fig. 4a,b, $p < 0.001$).

Discussion

The re-purposing of drugs and the development of novel treatments for endometriosis-associated pelvic pain has been limited by the paucity of accessible pre-clinical models. In this study we tested pain responses in a mouse model of endometriosis that phenocopies interactions between endometriosis lesions and peritoneal tissue^{22,24} identified in humans. Endometriosis (Endo) mice exhibited increased levels of abdominally-directed grooming, a reduction in normal exploratory behavior and reduced mechanical withdrawal thresholds on both the abdomen and plantar hind-paw. The rodent models reported by Berkley, and by Levine and Giudice, both report evidence of hyperalgesia^{16,20,26,27}. These models involve the artificial implantation of uterine tissue (full thickness i.e. endometrium plus myometrium) onto the mesenteric arteries of the small intestine and the gastrocnemius muscle, respectively. Therefore the microenvironment of the endometriosis lesion created in these models may not closely mirror that of the peripheral lesions in women.

PGE₂ is increased in the peritoneal fluid of patients with endometriosis²⁸, this up-regulation results from induced expression of cyclooxygenase-2 (COX-2) in endometriotic tissue. PGE₂ is thought to be a key player in the pathophysiology of endometriosis and studies have shown that inhibition of COX-2 decreases survival, migration and invasion of endometriotic cells¹⁵. The same authors demonstrated that inhibition of EP₂ and EP₄ can inhibit the epithelial and stromal cell invasion via suppression of matrix metalloproteinases²⁹. Attenuation of PGE₂ signaling via lipoxin A₄ can also modulate disease progression by attenuation of pro-inflammatory and angiogenic mediators³⁰. A recent study using xenografted endometriotic cell lines in a nude mouse model of endometriosis demonstrated that dual inhibition of EP₂ and EP₄ could attenuate mechanical hyperalgesia of the pelvic floor via suppression of pro-inflammatory mediators in dorsal root ganglia³¹, however the authors did not test secondary hyperalgesia or analyze changes in the central nervous system.

In our mouse model we confirmed that the prostaglandin E signaling pathway was over-expressed in the pelvic cavity of our Endo mice and concentrations of PGE₂ were increased in their peritoneal fluid. Release of PGE₂ at sites of peripheral inflammation can contribute to pain hypersensitivity by lowering the threshold and enhancing the excitability of nociceptor sensory fibers³². This occurs at least in part via EP receptor-mediated activation of intracellular kinases in the nociceptor terminal that causes phosphorylation of the nociceptive ion channel TRPV1^{33,34} and an up-regulation of Nav1.9 voltage-gated sodium channel (SCN11A)³⁵, which is then transported to peripheral nerve terminals to contribute to increased excitability⁷. We detected a significant increase in expression of EP₂, COX-1 and both TRPV1 and SCN11A ion channels in the DRGs of mice with endometriosis. This parallels observations of increased COX-1 and COX-2 in TRPV1-positive DRG cells in other models of inflammatory hyperalgesia³⁶. TRPV1-immunoreactivity was increased in small, peripherin-positive, nociceptive neurons in DRG of mice with endometriosis and EP₂ expression was further increased in these cells. All of these findings are consistent with the development of sensory neuron hyperexcitability in our mice. The possibility of some additional role of prostaglandin signaling in non-neuronal cells, as seen in some other pain models³⁷ cannot be excluded. Elevated TRPV1 expression in small DRG cells innervating pelvic regions in mice with endometriosis is consistent with our previous findings that TRPV1 mRNA is elevated in peritoneal lesions from women with endometriosis³⁸.

In this study, assessment of COX-2 expression in CNS regions within the pain-processing pathway revealed striking increases in expression at spinal, thalamic and cortical levels. COX-2 expression in the CNS is established as a sensitive and responsive biomarker of centralized inflammatory pain^{39–41} and is an important finding consistent with the inflammatory pain and widespread central sensitization as experienced by women with endometriosis. The phenomenon of central sensitization⁴² has been postulated as a key contributor to the co-morbid pain syndromes experienced by women with the condition⁴. Central sensitization is described as a maladaptation

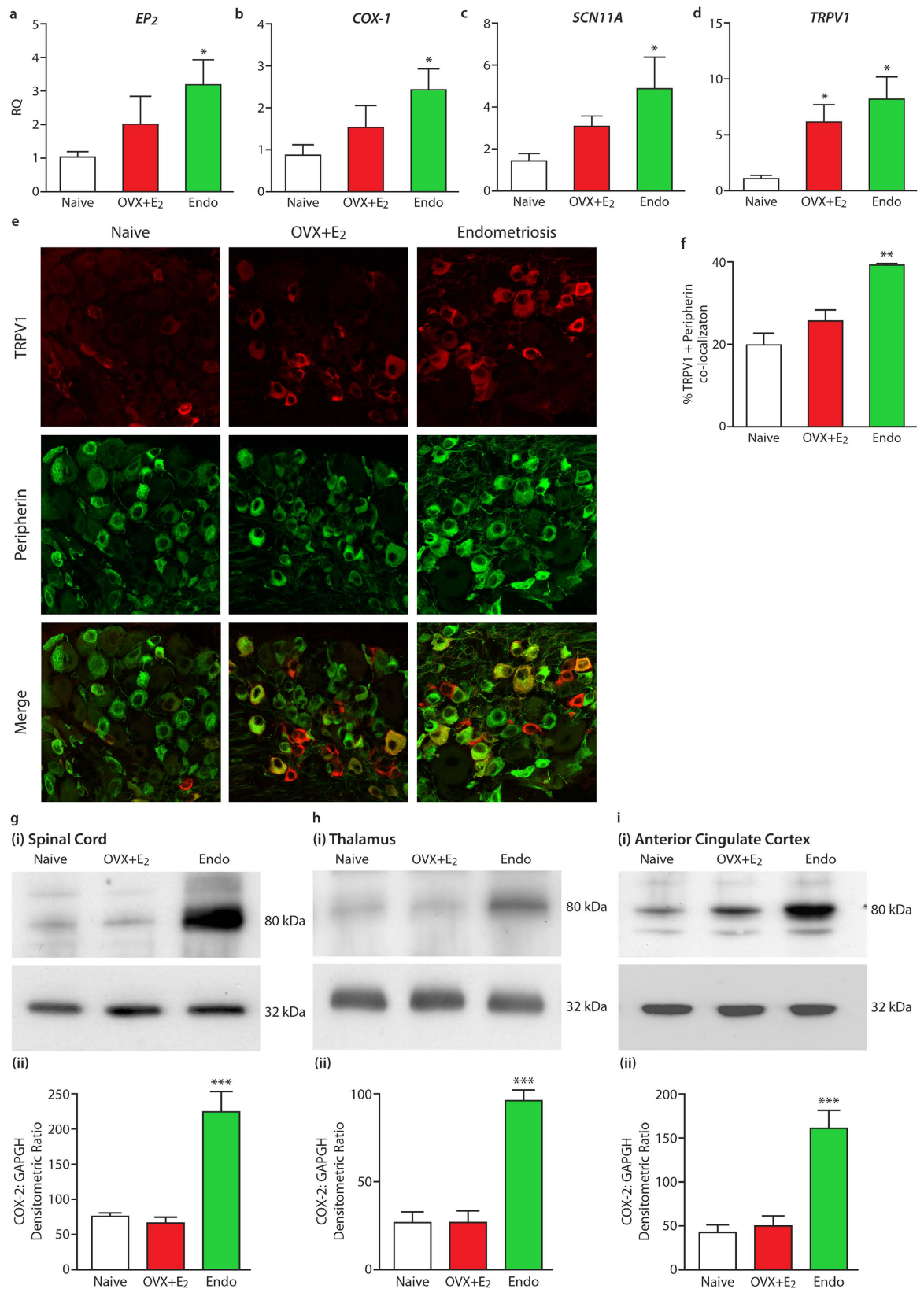


Figure 2. Molecular changes in the nervous system of mice with endometriosis. Expression of the PGE₂ pathway and nociceptive ion channels is elevated in the DRGs, spinal cord and brain of mice with endometriosis. (a–d) QPCR analysis of the prostaglandin E receptor 2(a) *EP2*, (b) *COX-1* and nociceptive ion channels (c) *SCN11A* and (d) *TRPV1* in L5–L6 DRGs from mice with endometriosis (n = 9) compared to naïve (n = 7) and OVX + E₂ control mice (n = 6). RQ: Relative quantification. Values were normalized to a single naïve DRG sample given the arbitrary value of one. (e,f) Dual label immunofluorescence was carried out to

identify TRPV1 expression (red) in L5-6 DRG cells co-expressing peripherin (green). (e) Shows typical confocal images for TRPV1 and peripherin from naïve, OVX + E₂-treated sham-recipient and endometriosis mice (field of view, 160 × 160 μm). Total cells counted were 330, 320, and 543, accumulated from three different naïve, OVX + E₂-treated and endometriosis mice in each case. (f) Shows a bar chart that summarizes % expression of TRPV1 in peripherin-positive cells and indicates that the number of TRPV1 + peripherin + small DRG cells is significantly increased in mice with endometriosis. (g–i) Images (i) show representative examples of COX-2 (top panel; 80 kDa) and GAPDH (bottom panel; 36 kDa) expression as analyzed using Western blot in (g) spinal cord, (h) thalamus, (i) and anterior cingulate cortex of endometriosis mice, OVX + E₂-treated sham-recipients and naïve controls, n = 5–6 for all groups. (ii) Graphs showing COX-2: GAPDH densitometric ratio derived from quantitative densitometry of films. Both images and bar charts indicate marked elevation of COX-2 expression in each region. No changes were observed in OVX + E₂-treated and naïve controls. Statistical analysis was performed using a one-way ANOVA and Tukey's post comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.

of the CNS resulting from continued or repetitive input from nociceptors, in endometriosis it is likely that this input is provided by afferent nerve fibers innervating endometriosis lesions. One of the first steps in this process of central sensitization is an increase in expression of genes encoding neurotrophins, neuropeptides and ion channels critical in sensing and detecting noxious stimuli⁸. In the model of endometriosis generated by Berkley et al, rats exhibit vaginal hyperalgesia²⁶ and it is argued by the authors that this finding suggests central sensitization as an underlying factor because spinal segments associated with the induced endometriosis cysts are distant from spinal segments receiving input from the vagina^{43,44}. Using this same model a decrease in μ-opioid and NMDA receptor immunoreactivity in the periaqueductal gray area of the brain was detected in rats with endometriosis compared to controls. Torres-Reverón et al suggested that a decrease in NMDA receptor expression could be an attempt to homeostatically regulate pain perception, whilst a decrease in μ-opioid receptor expression suggests decreased modulatory activity of opioid receptors that could contribute to hyperalgesia in the condition⁴⁵. We have also documented molecular alterations in the CNS of mice with endometriosis and we believe this as an important step change in our understanding of endometriosis-associated pain.

Having demonstrated amplified pain behaviors, we tested therapeutic strategies for reversal of mechanical allodynia. The effects of TRPV1 inhibition were modest, whilst antagonism of EP₂, particularly following oral administration of the highly selective antagonist PF-04418948 had pronounced effects on peripheral and secondary hyperalgesia. This is consistent with reports that EP₂ null mice do not develop spinal hyperalgesia following induced peripheral inflammation⁴⁶. In a recent study, the effect of a mixture of EP₂/EP₄ antagonists had a modest impact on pelvic floor hyperalgesia in mice which may either reflect the limited specificity of the antagonists tested or use of human endometriotic cell lines (not intact tissue fragments) in recipient mice lacking a full complement of immune cells³¹. In our hands, the selective EP₄ antagonist L-161982, did not reverse the sensitivity to mechanical stimulation.

In summary, we show that induction of endometriosis in mice with an intact immune system is associated with maladaptation of the CNS, consistent with central sensitization⁴. We have demonstrated striking reversal of both peripheral and secondary hyperalgesia via EP₂ antagonism. In conclusion, we present evidence that a murine model of endometriosis displaying local and central sensitization can be used for pre-clinical testing of therapeutics for endometriosis-associated pain.

Methods

Mouse model of endometriosis. Experiments were performed in accordance with the Guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Experiments were performed under licensed approval from the UK Home Office (London). C57BL/6 mice (Harlan Laboratories; Derby, UK) were given access to food and water *ad libitum*, ambient temperature and humidity were 21 °C and 50% respectively. Endometriosis was induced in the mice as previously described²². In brief, the endometrium of syngeneic donor mice underwent hormonal manipulation and induction of decidualization using an in-house protocol to model endometrial differentiation, breakdown and repair⁴⁷: detailed analysis of tissue samples has shown that progesterone withdrawal (removal of P4 pellet) results in rapid induction of hypoxia, tissue breakdown and induction of angiogenic genes^{47,48}. Endometrial tissue (~6 hours post-progesterone withdrawal) was recovered by opening the horn and scraping with a scalpel. Approximately 40 mg tissue (equivalent to one decidualized horn) was suspended in 0.2 ml PBS and injected into the peritoneal cavity of ovariectomised recipient mice that were supplemented with 500 ng Estradiol Valerate (EV). This supplementation was maintained by subcutaneous injection of 500 ng EV every 3 days (modification of previously published model). After allowing lesions to form over 21 days behavioral assessments were performed.

Behavioral assessments. All behavioral tests were performed starting 21 days after endometriosis induction on 2 consecutive days (day 21 and 22). Mechanical allodynia was measured using calibrated Semmes-Weinstein von Frey filaments (Stoelting, Wood Dale, IL), according to the manufacturer's instructions. Von Frey filaments were applied to the skin perpendicular to the plantar surface of the hindpaws or to the lower abdomen, as in refs 49–51. Filaments were applied to the abdomen or hind-paw ten times, force in grams (g) of the filament evoking a withdrawal response in 50% of cases was recorded. Initial testing of the abdomen in naïve mice indicated lower thresholds in more caudal regions, in agreement with a previous report⁵⁰. For the following spontaneous behavior tests mice were placed in observation boxes for two 5 min periods and manually observed by two independent investigators (one blinded to experimental group). Periods of spontaneous

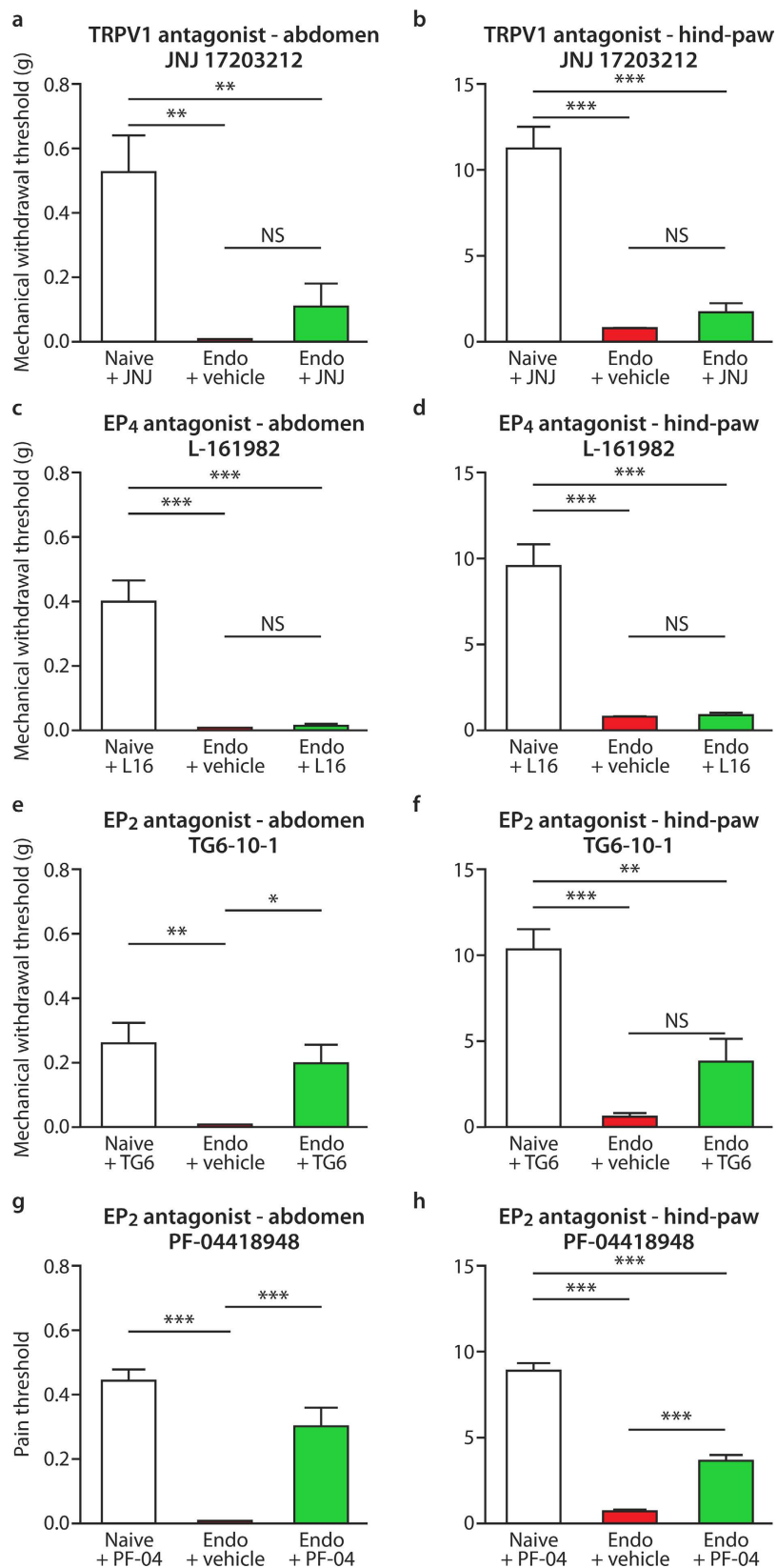


Figure 3. Pre-clinical testing of potential therapeutics in a mouse model of endometriosis. Graphs depict 50% mechanical withdrawal thresholds for von Frey filaments (g = grams) applied to abdomen or hindpaw of mice with endometriosis or naïve controls, $n = 5$ all groups. The time-point showing maximal reversal of pain is shown for each antagonist. **(a)** Effects of the TRPV1 inhibitor JNJ 17203212 (30 mg/kg ip), 30 mins post-injection on abdominal withdrawal responses in mice with endometriosis compared to naïve mice. **(b)** Shows corresponding results from hindpaw. Concurrently measured naïve values were 0.55 ± 0.17 g and

11.25 ± 1.44 g respectively for abdomen and hindpaw. In both tests, withdrawal thresholds were significantly lower in mice with endometriosis (Endo + Vehicle) than in naïve controls or in naïve animals treated with the drug (Naïve + JNJ; $p < 0.01$ and $p < 0.001$). This difference was modestly but not significantly attenuated by JNJ 17203212, which had no discernible effect on the responses of naïve animals. (c) Effects of the selective EP₄ antagonist L-161982 (10 mg/kg ip) 30 mins post i.p injection on abdominal and (d) hindpaw mechanical withdrawal thresholds. Naïve values were 0.39 ± 0.07 g and 9.43 ± 1.43 g. In both tests, thresholds were significantly lower in mice with endometriosis than in naïve animals treated with the drug ($p < 0.001$). L-161982 had no discernible effect on hypersensitivity in mice with endometriosis or in naïve animals. (e) Effect of the EP₂ antagonist TG6-10-1 (10 mg/kg, ip), on abdominal and (f) hindpaw mechanical withdrawal thresholds at 45 mins post i.p injection. Naïve values were 0.31 ± 0.11 g and 9.69 ± 1.62 g. In both tests, thresholds were significantly lower in mice with endometriosis compared to naïve controls or in naïve animals treated with the drug ($p < 0.01$ and $p < 0.001$). This difference was significantly reversed by TG6-10-1 in abdominal tests ($p < 0.05$). TG6-10-1 also induced a modest but not statistically significant pain reversal in paw tests. The highly selective EP₂ antagonist PF-04418948 (10 mg/kg, i.p) significantly attenuated both (g) abdominal and (h) hindpaw mechanical hypersensitivity in mice with endometriosis ($p < 0.001$) 45 mins post injection. There was no discernible effect of PF-04418948 in naïve animals. Naïve values were 0.48 ± 0.05 g and 8.77 ± 0.51 g. Statistical analysis was performed using a one-way ANOVA and Tukey's post comparison test. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$.

abdominally directed licking (an element of normal grooming behavior⁵²) were recorded and an average generated. Excessive abdominally-directed licking has been reported to represent a useful biomarker of abdominal visceral pain⁴⁶. Each time a mouse exhibited abdominal grooming was recorded as an event. The matrix of brain regions activated during pain includes areas impacting on affective behaviors, corresponding to the anxiety- and depression-like signs associated with chronic pain states⁵³. Paradigms of altered affective state in rodent pain models include reduced exploratory behavior⁵⁴. Exploratory activity here was recorded in a modification of the open-field setting^{55,56}, with mice retained within their home box with a cardboard tunnel in the centre of the enclosure; open-field tunnel entries were manually recorded by two independent investigators (one blinded to experimental group).

Experimental groups and sample collection. Four groups of mice were analysed; (i) naïve controls (no surgical procedures; $n = 9$), (ii) OVX + E₂ controls ($n = 9$), for which, mice were ovariectomised and given E₂ valerate (Sigma, UK), s.c. 500 ng in sesame oil every 2 days to mirror the surgical and hormonal status of the Endo mice; (iii) OVX + E₂ + PBS controls ($n = 6$); as in group ii plus i.p injection of PBS, to mirror injection of tissue as in Endo mice (iv) endometriosis mice (Endo mice; as group iii, with 'menstrual' donor material in PBS injected i.p ($n = 18$)). On day 23 mice were culled and the following samples recovered: peritoneal fluid (PF, recovered as in ref. 57 by injecting 3 ml ice cold PBS into the peritoneal cavity followed by gentle massage and recovery (approximately 2 ml was recovered from the injected 3 ml). PF was then centrifuged and frozen), peritoneal biopsy, endometriotic lesions, L5-L6 DRGs, lumbar spinal cord, thalamus and anterior cingulate cortex. Samples were collected into RNAlater (Applied Biosystems, Warrington, UK) and frozen, neutral-buffered formalin prior to paraffin embedding for immunohistochemical analysis (uterus, peritoneum, and endometriosis lesions) or frozen on dry ice prior to sectioning and immunofluorescence staining (DRG) or protein extraction for Western blot analysis (spinal cord and brain). Endometriotic lesions were recognized as red, brown or white tissue deposits on the visceral or parietal peritoneum and were carefully dissected away from any surrounding fat or peritoneum. The presence of glands plus stroma in suspected lesions were confirmed by haematoxylin/eosin staining. Biopsies that did not contain both glands and stroma were not included in further analysis.

Antagonists. Agents for i.p. injections were dissolved in 10% dimethylsulphoxide, 50% PEG-400, 40% de-ionised H₂O and injected in a volume of 100 µl/25 g using 30 mg/kg JNJ 17203212 (TRPV1 antagonist), 10 mg/kg TG6-10-1 (EP₂ antagonist; Calbiochem)⁵⁸, 10 mg/kg PF-04418948²⁵ (EP₂ antagonist; Abcam), 10 mg/kg L-161982 (EP₄ antagonist; Abcam). Von Frey testing was performed at 30, 45 and 60 minutes post injection: for PF-0418948, testing was also carried out at 75 minutes). PF-04418948 (10 mg/kg in 0.5% w/v methylcellulose + 0.1% V/V Tween-20 in purified water) was also administered as an oral gavage and the von Frey test performed every 15 mins starting at 30 mins.

Quantitative real time PCR. RNA was extracted from control uterine biopsies recovered from naïve mice, peritoneal biopsies from naïve and endometriosis mice, endometriosis lesions and dorsal root ganglia using an RNeasy kit (QIAGEN) according to the manufacturers instructions. RNA was quantified using a NanoDrop ND 1000. Quantitative PCR was performed as detailed in refs 59,60; briefly, cDNA was synthesized using SuperScript VILO enzyme (Invitrogen) with 100ng starting template. PCRs were performed using Roche Universal Probe Library (Roche Applied Science) using primer sequences detailed in Supplementary Table 2. 18S was used as a reference gene. Thermal cycling was performed on a 7900 Fast real-time PCR machine. Data was analysed with RQ manager software (Applied Biosystems) using the $\Delta\Delta C_t$ method; samples were normalised to a uterine control sample.

Immunodetection. Single antigen immunohistochemistry was performed according to standard protocols^{47,61} with citrate antigen retrieval.

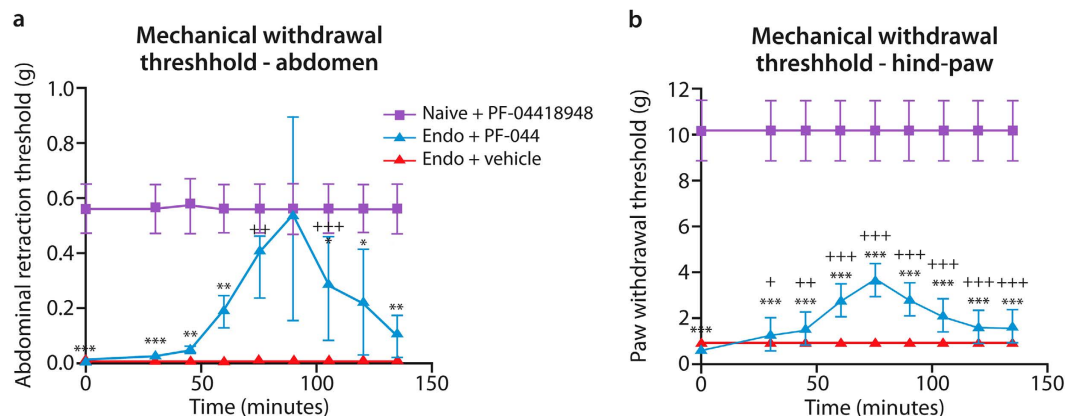


Figure 4. Oral administration of the selective EP₂ antagonist PF-04418948. To test the efficacy of oral administration of PF-04418948, the drug was administered at a dose of 10 mg/kg by oral gavage and testing was initiated after 30 min. The graphs show the time-course of effects of PF-04418948 on mechanical withdrawal responses in mice with endometriosis (blue and pink lines) or naïve controls (purple lines). In both (a) the abdomen and (b) the hindpaw, withdrawal thresholds were significantly lower in endometriosis mice than in naïve controls ($p < 0.001$). The pre-drug withdrawal thresholds in the naïve, drug-treated animals, were not discernibly different from those in naïve, untreated animals (0.55 ± 0.06 g for abdomen and 9.00 ± 0.67 g for hindpaw). In each case the hypersensitivity due to endometriosis was substantially and significantly attenuated by PF-04418948 ($p < 0.001$), $n = 5$ all groups. Statistical analysis was performed using a Two-Way ANOVA and Dunnett's multiple comparison test. * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.001$ compared to naïve mice + pharmacological agent. + $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$ compared to pre-administration baseline.

Dual immunofluorescence. Dorsal root ganglia (DRGs) were embedded in OCT (CellPath) and frozen on dry ice. Sixteen μm cryostat sections were blocked for 1 hour at room temperature then incubated with primary antibodies (Supplementary Table 3). Specificity of the TRPV1 antibody has been previously established⁶². Secondary antibodies, from Molecular Probes or Sigma-Aldrich, goat anti-chicken Alexafluor 488 (1:1000), goat anti-guinea pig AlexaFluor 568 (1:1000) or goat anti-guinea-pig CF405A (1:1000) were applied for 1 hour. Sections were mounted in ProLong[®] Gold Antifade (Life Technologies). Confocal images were acquired at x20 magnification using a Nikon A1R microscope and ImageJ software was used to quantify co-staining. Standard controls omitting primary antibodies were immunonegative.

Prostaglandin E₂ (PGE₂) ELISA. Approximately 2 ml of the PF was collected into tubes containing indomethacin (10 μM) to prevent *ex vivo* PGE₂ metabolism. PGE₂ levels were analyzed using DetectX[®] prostaglandin E₂ enzyme immunoassay kit (Arbor Assays, MI, USA).

Western blotting. Tissue samples were collected into sealable tubes and frozen on dry ice, and then subsequently homogenized in Laemmli buffer, heated to 80 °C for 5 min and centrifuged. Aliquots of lysate supernatant were analysed using the NuPage XCell SureLock[™] Minicell gel electrophoresis system (Invitrogen) with approximately 12 μg protein loaded per lane. Membranes were incubated overnight at 4 °C in 2% non-fat dried milk in 0.1 M PBS with 0.1% Tween-20, containing anti-COX-2 antibody (Supplementary Table 3)⁶³. Membranes were washed and incubated for 50 min at room temperature with peroxidase-conjugated donkey anti-rabbit antibody (Chemicon, 1:20,000) and detected by peroxidase-linked enhanced chemiluminescence. Membranes were re-probed with mouse monoclonal anti-GAPDH (Supplementary Table 2). Films were scanned and band intensities were quantified by densitometry using ImageJ.

Statistical analysis. Statistical analysis used a one-way ANOVA with a Newman Keuls or Tukey's test, or a Kruskal Wallis with a Dunn's multiple comparison test. A p value of less than 0.05 was considered significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

References

- Rogers, P. A. *et al.* Priorities for endometriosis research: recommendations from an international consensus workshop. *Reprod Sci* **16**, 335–346 (2009).
- Dunselman, G. A. *et al.* ESHRE guideline: management of women with endometriosis. *Hum Reprod* **29**, 400–412 (2014).
- Giudice, L. C. & Kao, L. C. Endometriosis. *Lancet* **364**, 1789–1799 (2004).
- Stratton, P. & Berkley, K. J. Chronic pelvic pain and endometriosis: translational evidence of the relationship and implications. *Hum Reprod Update* **17**, 327–346 (2011).
- Holzer, P. Neurogenic vasodilatation and plasma leakage in the skin. *Gen Pharmacol* **30**, 5–11 (1998).
- Gebhart, G. F. Peripheral contributions to visceral hyperalgesia. *Can J Gastroenterol* **13** Suppl A, 37A–41A (1999).
- Wolf, C. J. & Salter, M. W. Neuronal plasticity: increasing the gain in pain. *Science* **288**, 1765–1769 (2000).
- Wolf, C. J. Central sensitization: implications for the diagnosis and treatment of pain. *Pain* **152**, S2–15 (2011).
- Schaible, H. G., Ebersberger, A. & Von Banchet, G. S. Mechanisms of pain in arthritis. *Ann N Y Acad Sci* **966**, 343–354 (2002).

10. Schaible, H. G. *et al.* Joint pain. *Exp Brain Res* **196**, 153–162 (2009).
11. Ma, W. & Quirion, R. Does COX2-dependent PGE2 play a role in neuropathic pain? *Neurosci Lett* **437**, 165–169 (2008).
12. Burney, R. O. & Giudice, L. C. Pathogenesis and pathophysiology of endometriosis. *Fertil Steril* **98**, 511–519 (2012).
13. Banu, S. K., Lee, J., Speights, V. O. Jr., Starzinski-Powitz, A. & Arosh, J. A. Cyclooxygenase-2 regulates survival, migration, and invasion of human endometriotic cells through multiple mechanisms. *Endocrinology* **149**, 1180–1189 (2008).
14. Chishima, F. *et al.* Increased expression of cyclooxygenase-2 in local lesions of endometriosis patients. *American journal of reproductive immunology (New York, NY: 1989)* **48**, 50–56 (2002).
15. Noble, L. S. *et al.* Prostaglandin E2 stimulates aromatase expression in endometriosis-derived stromal cells. *J Clin Endocrinol Metab* **82**, 600–606 (1997).
16. Berkley, K. J., Dmitrieva, N., Curtis, K. S. & Papka, R. E. Innervation of ectopic endometrium in a rat model of endometriosis. *Proc Natl Acad Sci USA* **101**, 11094–11098 (2004).
17. Cason, A. M., Samuelsen, C. L. & Berkley, K. J. Estrous changes in vaginal nociception in a rat model of endometriosis. *Hormones and behavior* **44**, 123–131 (2003).
18. Nagabukuro, H. & Berkley, K. J. Influence of endometriosis on visceromotor and cardiovascular responses induced by vaginal distention in the rat. *Pain* **132** Suppl 1, S96–103 (2007).
19. Hernandez, S., Cruz, M. L., Torres-Reveron, A. & Appleyard, C. B. Impact of physical activity on pain perception in an animal model of endometriosis. *7*, 100 (2015).
20. Alvarez, P. *et al.* Ectopic uterine tissue as a chronic pain generator. *Neuroscience* **225**, 269–282 (2012).
21. Alvarez, P. & Levine, J. D. Screening the role of pronociceptive molecules in a rodent model of endometriosis pain. *J Pain* **15**, 726–733 (2014).
22. Greaves, E. *et al.* A novel mouse model of endometriosis mimics human phenotype and reveals insights into the inflammatory contribution of shed endometrium. *Am J Pathol* **184**, 1930–1939 (2014).
23. Greaves, E., Collins, F., Esnal, A., Giakoumelou, S., Horne, A. W. & Saunders, P. T. Estrogen receptor (ER) agonists differentially regulate neuroangiogenesis in peritoneal endometriosis via the repellent factor SLIT3. *Endocrinology*, en20141086 (2014).
24. Greaves, E., Temp, J., Esnal-Zufiurre, A., Mechsner, S., Horne, A. W. & Saunders, P. T. Estradiol Is a Critical Mediator of Macrophage-Nerve Cross Talk in Peritoneal Endometriosis. *Am J Pathol* (2015).
25. Forselles, K. J. *et al.* *In vitro* and *in vivo* characterization of PF-04418948, a novel, potent and selective prostaglandin EP(2) receptor antagonist. *British journal of pharmacology* **164**, 1847–1856 (2011).
26. Berkley, K. J., Cason, A., Jacobs, H., Bradshaw, H. & Wood, E. Vaginal hyperalgesia in a rat model of endometriosis. *Neurosci Lett* **306**, 185–188 (2001).
27. Berkley, K. J., McAllister, S. L., Accius, B. E. & Winnard, K. P. Endometriosis-induced vaginal hyperalgesia in the rat: effect of ovariectomy, ovariectomy, and estradiol replacement. *Pain* **132** Suppl 1, S150–159 (2007).
28. De Leon, F. D., Vijayakumar, R., Brown, M., Rao, C. V., Yussman, M. A. & Schultz, G. Peritoneal fluid volume, estrogen, progesterone, prostaglandin, and epidermal growth factor concentrations in patients with and without endometriosis. *Obstetrics and gynecology* **68**, 189–194 (1986).
29. Lee, J., Banu, S. K., Subbarao, T., Starzinski-Powitz, A. & Arosh, J. A. Selective inhibition of prostaglandin E2 receptors EP2 and EP4 inhibits invasion of human immortalized endometriotic epithelial and stromal cells through suppression of metalloproteinases. *Mol Cell Endocrinol* **332**, 306–313 (2011).
30. Kumar, R. *et al.* Lipoxin A(4) prevents the progression of *de novo* and established endometriosis in a mouse model by attenuating prostaglandin E(2) production and estrogen signaling. *PLoS One* **9**, e89742 (2014).
31. Arosh, J. A. *et al.* Molecular and preclinical basis to inhibit PGE2 receptors EP2 and EP4 as a novel nonsteroidal therapy for endometriosis. *Proc Natl Acad Sci USA* (2015).
32. Basbaum, A. I., Bautista, D. M., Scherrer, G. & Julius, D. Cellular and molecular mechanisms of pain. *Cell* **139**, 267–284 (2009).
33. Moriyama, T. *et al.* Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. *Mol Pain* **1**, 3 (2005).
34. Schnizler, K. *et al.* Protein kinase A anchoring via AKAP150 is essential for TRPV1 modulation by forskolin and prostaglandin E2 in mouse sensory neurons. *J Neurosci* **28**, 4904–4917 (2008).
35. Rush, A. M. & Waxman, S. G. PGE2 increases the tetrodotoxin-resistant Nav1.9 sodium current in mouse DRG neurons via G-proteins. *Brain Res* **1023**, 264–271 (2004).
36. Araldi, D. *et al.* Peripheral inflammatory hyperalgesia depends on the COX increase in the dorsal root ganglion. *Proc Natl Acad Sci USA* **110**, 3603–3608 (2013).
37. Kras, J. V., Dong, L. & Winkelstein, B. A. The prostaglandin E2 receptor, EP2, is upregulated in the dorsal root ganglion after painful cervical facet joint injury in the rat. *Spine* **38**, 217–222 (2013).
38. Greaves, E., Grieve, K., Horne, A. W. & Saunders, P. T. Elevated peritoneal expression and estrogen regulation of nociceptive ion channels in endometriosis. *J Clin Endocrinol Metab*, jc20142282 (2014).
39. Samad, T. A. *et al.* Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* **410**, 471–475 (2001).
40. Vardeh, D. *et al.* COX2 in CNS neural cells mediates mechanical inflammatory pain hypersensitivity in mice. *J Clin Invest* **119**, 287–294 (2009).
41. Vasquez, E., Bar, K. J., Ebersberger, A., Klein, B., Vanegas, H. & Schaible, H. G. Spinal prostaglandins are involved in the development but not the maintenance of inflammation-induced spinal hyperexcitability. *J Neurosci* **21**, 9001–9008 (2001).
42. Latremoliere, A. & Woolf, C. J. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain* **10**, 895–926 (2009).
43. Berkley, K. J., Rapkin, A. J. & Papka, R. E. The pains of endometriosis. *Science* **308**, 1587–1589 (2005).
44. McAllister, S. L., McGinty, K. A., Resuehr, D. & Berkley, K. J. Endometriosis-induced vaginal hyperalgesia in the rat: role of the ectopic growths and their innervation. *Pain* **147**, 255–264 (2009).
45. Torres-Reveron, A. *et al.* Endometriosis Is Associated With a Shift in MU Opioid and NMDA Receptor Expression in the Brain Periaqueductal Gray. *Reprod Sci* **23**, 1158–1167 (2016).
46. Reinold, H. *et al.* Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. *J Clin Invest* **115**, 673–679 (2005).
47. Cousins, F. L., Murray, A., Esnal, A., Gibson, D. A., Critchley, H. O. & Saunders, P. T. Evidence from a Mouse Model That Epithelial Cell Migration and Mesenchymal-Epithelial Transition Contribute to Rapid Restoration of Uterine Tissue Integrity during Menstruation. *PLoS One* **9**, e86378 (2014).
48. Cousins, F. L., Murray, A. A., Scanlon, J. P. & Saunders, P. T. Hypoxyprome reveals dynamic spatial and temporal changes in hypoxia in a mouse model of endometrial breakdown and repair. *BMC research notes* **9**, 30 (2016).
49. Garry, E. M., Moss, A., Rosie, R., Delaney, A., Mitchell, R. & Fleetwood-Walker, S. M. Specific involvement in neuropathic pain of AMPA receptors and adapter proteins for the GluR2 subunit. *Molecular and cellular neurosciences* **24**, 10–22 (2003).
50. Laird, J. M., Martinez-Caro, L., Garcia-Nicas, E. & Cervero, F. A new model of visceral pain and referred hyperalgesia in the mouse. *Pain* **92**, 335–342 (2001).
51. Moss, A. *et al.* A role of the ubiquitin-proteasome system in neuropathic pain. *J Neurosci* **22**, 1363–1372 (2002).

52. Leach, M. C., Klaus, K., Miller, A. L., Scotto di Perrotolo, M., Sotocinal, S. G. & Flecknell, P. A. The assessment of post-vasectomy pain in mice using behaviour and the Mouse Grimace Scale. *PLoS One* **7**, e35656 (2012).
53. Usdin, T. B. & Dimitrov, E. L. The Effects of Extended Pain on Behavior: Recent Progress. *The Neuroscientist: a review journal bringing neurobiology, neurology and psychiatry* **22**, 521–533 (2016).
54. Leite-Almeida, H., Pinto-Ribeiro, F. & Almeida, A. Animal Models for the Study of Comorbid Pain and Psychiatric Disorders. *Modern trends in pharmacopsychiatry* **30**, 1–21 (2015).
55. Sun, L., Gooding, H. L., Brunton, P. J., Russell, J. A., Mitchell, R. & Fleetwood-Walker, S. Phospholipase D-mediated hypersensitivity at central synapses is associated with abnormal behaviours and pain sensitivity in rats exposed to prenatal stress. *The international journal of biochemistry & cell biology* **45**, 2706–2712 (2013).
56. Liu, Y. T., Shao, Y. W., Yen, C. T. & Shaw, F. Z. Acid-induced hyperalgesia and anxio-depressive comorbidity in rats. *Physiology & behavior* **131**, 105–110 (2014).
57. Wieser, F., Wu, J., Shen, Z., Taylor, R. N. & Sidell, N. Retinoic acid suppresses growth of lesions, inhibits peritoneal cytokine secretion, and promotes macrophage differentiation in an immunocompetent mouse model of endometriosis. *Fertil Steril* **97**, 1430–1437 (2012).
58. Jiang, J., Quan, Y., Ganesh, T., Pouliot, W. A., Dudek, F. E. & Dingledine, R. Inhibition of the prostaglandin receptor EP2 following status epilepticus reduces delayed mortality and brain inflammation. *Proc Natl Acad Sci USA* **110**, 3591–3596 (2013).
59. Greaves, E., Collins, F., Esnal-Zufiaurre, A., Giakoumelou, S., Horne, A. W. & Saunders, P. T. Estrogen receptor (ER) agonists differentially regulate neuroangiogenesis in peritoneal endometriosis via the repellent factor SLIT3. *Endocrinology* **155**, 4015–4026 (2014).
60. Greaves, E., Grieve, K., Horne, A. W. & Saunders, P. T. Elevated peritoneal expression and estrogen regulation of nociceptive ion channels in endometriosis. *The Journal of clinical endocrinology and metabolism* **99**, E1738–1743 (2014).
61. Collins, F. *et al.* Expression of oestrogen receptors, ERalpha, ERbeta, and ERbeta variants, in endometrial cancers and evidence that prostaglandin F may play a role in regulating expression of ERalpha. *BMC Cancer* **9**, 330 (2009).
62. Sharif Naeini, R., Witty, M. F., Seguela, P. & Bourque, C. W. An N-terminal variant of Trpv1 channel is required for osmosensory transduction. *Nat Neurosci* **9**, 93–98 (2006).
63. Rahmouni, S. *et al.* Cyclo-oxygenase type 2-dependent prostaglandin E2 secretion is involved in retrovirus-induced T-cell dysfunction in mice. *The Biochemical journal* **384**, 469–476 (2004).

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Author Contributions

E.G. and S.F.W. conceived manuscript, performed experimental work, analysed results and wrote manuscript. H.J., M.M. and L.H. performed experimental work. R.M. performed experimental work and contributed critical feedback on manuscript. P.T.K.S. and A.H. conceived and wrote manuscript.

Additional Information

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