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Authors: Erin Greaves[1*], Andrew W Horne[1], Helen Jerina[2], Marta Mikolajczak[2], Lisa Hilferty[1], Rory Mitchell[2], Sue M Fleetwood-Walker[2], Philippa TK Saunders[3]

Affiliations:

1. MRC Centre for Reproductive Health, The University of Edinburgh, Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ
2. Centre for Integrative Physiology, The University of Edinburgh, Hugh Robson Building, 15 George Square, Edinburgh EH8 9XD
3. MRC Centre for Inflammation Research, The University of Edinburgh, Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ

***** To whom correspondence should be addressed:

Dr Erin Greaves; egreaves@ed.ac.uk

Tel: 0131-242-6642

One Sentence Summary: A pre-clinical mouse model of endometriosis-associated pain allows testing of potential therapies for attenuation of peripheral and secondary hyperalgesia.

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Abstract

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 PGE_2-signaling pathway (including COX-2, EP_2, EP_4) in endometriosis lesions, dorsal root ganglia (DRG), spinal cord, thalamus and forebrain. TRPV1, a PGE_2-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, EP_2, and EP_4 receptor antagonists: EP_2 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 worldwide[1] associated with chronic pain and infertility. Current therapies include invasive surgery, drugs that suppress endogenous hormones[2] 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 cavity[3]. An association between small nerve fiber infiltration of endometriosis lesions and increased central and peripheral pain has been described (reviewed in[4]). Alterations in pain perception in women are thought to involve release of inflammatory mediators and neuropeptides by efferent peripheral nerve endings,[5] an increase in the sensitivity of nociceptive neurons,[6] 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 allodynia[8]. Prostaglandin E_2 (PGE_2) is a well-established mediator of inflammation and nociception in inflammatory[9, 10] and neuropathic pain conditions[11] and synthesis of prostaglandins within endometriosis lesions has previously been reported [12, 13, 14, 15]. As the association of PGE_2 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, 17, 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 group[19]. Another model using autologous transplantation of endometrial tissue onto the gastrocnemius muscle[20] has proved useful for the identification of pronociceptive molecules that may be relevant in endometriosis[21]. Our murine model of endometriosis uses decidualized endometrial tissue injected (rather than sutured) into the peritoneum of recipient mice[22]. 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 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 PGE_2 signaling pathway and nociceptive ion channels in endometriosis lesions and in the nervous system of mice with endometriosis

Consistent with our previous studies[22, 23, 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 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); EP_2, EP_4, COX-1 and COX-2 proteins were immunolocalised to glandular and stromal cells in lesions and mesothelial cells in the peritoneum (Supplementary Fig 2). PGE_2 concentrations in the peritoneal fluid of Endo mice were significantly increased ($p<0.05$; Supplementary Fig.2E). mRNA concentrations of *EP_1* and *EP3* were unchanged (Supplementary Fig.2f-g)**.** *EP_2*, *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); *EP_4* and *Cox-2* were unchanged (Supplementary Fig.3c-d). *Trpv1* mRNA was also increased in the DRGs from OVX+E_2 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 naïve and OVX+E_2-treated controls ($p<0.05$; Fig.2e, f). The proportion of TRPV1-immunopositive neurons in DRG that express EP_2 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 EP_2 as a key target

Figure 3 indicates that injection of the TRPV1 inhibitor JNJ 17203212 (Fig3a, b) or the EP_4 antagonist L-161982 (Fig.3c, d), did not reverse abdominal or paw hyperalgesia to a statistically significant extent. Administration of the EP_2 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 EP_2 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 host tissue[22, 24] identified in human tissues. 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, 26, 27] [20]. 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.

PGE_2 is increased in the peritoneal fluid of patients with endometriosis[28], this up-regulation results from induced expression of cyclooxygenase-2 (COX-2) in endometriotic tissue. PGE_2 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 cells[13]. The same authors demonstrated that inhibition of EP_2 and EP_4 can inhibit the epithelial and stromal cell invasion via suppression of matrix metalloproteinases[29]. Attenuation of PGE_2 signaling via lipoxin A_4 can also modulate disease progression by attenuation of pro-inflammatory and angiogenic mediators[30]. A recent study using xenografted endometriotic cell lines in a nude mouse model of endometriosis demonstrated that dual inhibition of EP_2 and EP_4 could attenuate mechanical hyperlagesia of the pelvic floor via suppression of pro-inflammatory mediators in dorsal root ganglia[31], 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 PGE_2 were increased in their peritoneal fluid. Release of PGE_2 at sites of peripheral inflammation can contribute to pain hypersensitivity by lowering the threshold and enhancing the excitability of nociceptor sensory fibers[32]. 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 excitability[7]. We detected a significant increase in expression of EP_2, 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 hyperalgesia[36]. TRPV1-immunoreactivity was increased in small, peripherin-positive, nociceptive neurons in DRG of mice with endometriosis and EP_2 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 models[37] 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 endometriosis[38].

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, 40, 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 sensitization[42] has been postulated as a key contributor to the co-morbid pain syndromes experienced by women with the condition[4]. Central sensitization is described as a maladaptation 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 stimuli[8]. In the model of endometriosis generated by Berkley et al, rats exhibit vaginal hyperalgesia[26] 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 condition[45]. 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 EP_2, 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 EP_2 null mice do not develop spinal hyperalgesia following induced peripheral inflammation[46]. In a recent

study, the effect of a mixture of EP_2/EP_4 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 cells[31]. In our hands, the selective EP_4 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 sensitization[4]. We have demonstrated striking reversal of both peripheral and secondary hyperalgesia via EP_2 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 described[22]. 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 repair[47]: 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 40mg 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 500ng Estradiol Valerate (EV). This supplementation was maintained by subcutaneous injection of 500ng EV every 3 days (modification of previously published model). After allowing lesions to form over 21 days behavioral assessments were performed.

198 **Behavioral assessments.** All behavioral tests were performed starting 21 days after endometriosis
199 induction on 2 consecutive days (day 21 and 22). Mechanical allodynia was measured using calibrated
200 Semmes-Weinstein von Frey filaments (Stoelting, Wood Vale, IL), according to the manufacturer's
201 instructions. Von Frey filaments were applied to the skin perpendicular to the plantar surface of the
202 hindpaws or to the lower abdomen, as in [49, 50, 51]. Filaments were applied to the abdomen or hind-paw
203 ten times force in grams (g) of the filament evoking a withdrawal response in 50% of cases was recorded.
204 Initial testing of the abdomen in naïve mice indicated lower thresholds in more caudal regions, in
205 agreement with a previous report[50]. For the following spontaneous behavior tests mice were placed in
206 observation boxes for two 5 min periods and manually observed by two independent investigators (one
207 blinded to experimental group). Periods of spontaneous abdominally directed licking (an element of
208 normal grooming behavior[52]) were recorded and an average generated. Excessive abdominally-directed
209 licking has been reported to represent a useful biomarker of abdominal visceral pain[46]. Each time a
210 mouse exhibited abdominal grooming was recorded as an event. The matrix of brain regions activated
211 during pain includes areas impacting on affective behaviors, corresponding to the anxiety- and
212 depression-like signs associated with chronic pain states[53]. Paradigms of altered affective sate in rodent
213 pain models include reduced exploratory behavior[54]. Exploratory activity here was recorded in a
214 modification of the open-field setting[55, 56], with mice retained within their home box with a cardboard
215 tunnel in the centre of the enclosure; open-field tunnel entries were manually recorded by two
216 independent investigators (one blinded to experimental group).
217 **Experimental groups and sample collection.** Four groups of mice were analysed; (i) naïve controls
218 (no surgical procedures; n=9), (ii) OVX+E_2 controls (n=9), for which, mice were ovariectomised and
219 given E_2 valerate s.c. 500 ng in sesame oil every 2 days to mirror the surgical and hormonal status on
220 the Endo mice; (Sigma, UK), (iii) OVX+E_2+PBS controls (n=6); as in group ii plus i.p injection of
221 PBS, to mirror injection of tissue as in Endo mice (iv) endometriosis mice (Endo mice; as group iii,
222 with 'menstrual' donor material in PBS injected i.p (n=18)). On day 23 mice were culled and the
223 following samples recovered: peritoneal fluid (PF, recovered as in [57] by injecting 3 ml ice cold PBS
224 into the peritoneal cavity followed by gentle massage and recovery (approximately 2 ml was
225 recovered from the injected 3ml). 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 and frozen (Applied Biosystems, Warrington, UK), 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% ddH2O and injected in a volume of 100 µl/25g using 30 mg/kg JNJ 17203212 (TRPV1 antagonist), 10 mg/kg TG6-10-1 (EP_2 antagonist; Calbiochem)[58], 10 mg/kg PF-04418948[25] (EP_2 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[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 ΔΔCt 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.

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 established[62]. 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 E_2 (PGE_2) ELISA. Approximately 2 ml of the PF was collected into tubes containing indomethacin (10μM) to prevent ex vivo PGE_2 metabolism. PGE_2 levels were analyzed using DetectX® prostaglandin E_2 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 *Sure*Lock™ Minicell gel electrophoresis system (Invitrogen) with approximately 12μg 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

1. Rogers PA, *et al.* Priorities for endometriosis research: recommendations from an international consensus workshop. *Reprod Sci* **16**, 335-346 (2009).

2. Dunselman GA, *et al.* ESHRE guideline: management of women with endometriosis. *Hum Reprod* **29**, 400-412 (2014).

3. Giudice LC, Kao LC. Endometriosis. *Lancet* **364**, 1789-1799 (2004).

4. Stratton P, Berkley KJ. Chronic pelvic pain and endometriosis: translational evidence of the relationship and implications. *Hum Reprod Update* **17**, 327-346 (2011).

5. Holzer P. Neurogenic vasodilatation and plasma leakage in the skin. *Gen Pharmacol* **30**, 5-11 (1998).

6. Gebhart GF. Peripheral contributions to visceral hyperalgesia. *Can J Gastroenterol* **13 Suppl A**, 37A-41A (1999).

7. Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. *Science* **288**, 1765-1769 (2000).

8. Woolf CJ. Central sensitization: implications for the diagnosis and treatment of pain. *Pain* **152**, S2-15 (2011).

9. Schaible HG, Ebersberger A, Von Banchet GS. Mechanisms of pain in arthritis. *Ann N Y Acad Sci* **966**, 343-354 (2002).

10. Schaible HG, *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 RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. *Fertil Steril* **98**, 511-519 (2012).

13. Banu SK, Lee J, Speights VO, Jr., Starzinski-Powitz A, Arosh JA. 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 LS, *et al.* Prostaglandin E2 stimulates aromatase expression in endometriosis-derived stromal cells. *J Clin Endocrinol Metab* **82**, 600-606 (1997).

16. Berkley KJ, Dmitrieva N, Curtis KS, Papka RE. Innervation of ectopic endometrium in a rat model of endometriosis. *Proc Natl Acad Sci U S A* **101**, 11094-11098 (2004).

17. Cason AM, Samuelsen CL, Berkley KJ. Estrous changes in vaginal nociception in a rat model of endometriosis. *Hormones and behavior* **44**, 123-131 (2003).

18. Nagabukuro H, Berkley KJ. 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 ML, Torres-Reveron A, Appleyard CB. Impact of physical activity on pain perception in an animal model of endometriosis. **7**, 100.

20. Alvarez P, *et al.* Ectopic uterine tissue as a chronic pain generator. *Neuroscience* **225**, 269-282 (2012).

21. Alvarez P, Levine JD. 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 AW, Saunders PT. 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 AW, Saunders PT. Estradiol Is a Critical Mediator of Macrophage-Nerve Cross Talk in Peritoneal Endometriosis. *Am J Pathol*, (2015).

25. af Forselles KJ, *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 KJ, Cason A, Jacobs H, Bradshaw H, Wood E. Vaginal hyperalgesia in a rat model of endometriosis. *Neurosci Lett* **306**, 185-188 (2001).

27. Berkley KJ, McAllister SL, Accius BE, Winnard KP. Endometriosis-induced vaginal hyperalgesia in the rat: effect of estropause, ovariectomy, and estradiol replacement. *Pain* **132 Suppl 1**, S150-159 (2007).

28. De Leon FD, Vijayakumar R, Brown M, Rao CV, Yussman MA, 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 SK, Subbarao T, Starzinski-Powitz A, Arosh JA. 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 JA, *et al.* 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*, (2015).

32. Basbaum AI, Bautista DM, 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 AM, Waxman SG. 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 U S A* **110**, 3603-3608 (2013).

37. Kras JV, Dong L, Winkelstein BA. 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 AW, Saunders PT. Elevated peritoneal expression and estrogen regulation of nociceptive ion channels in endometriosis. *J Clin Endocrinol Metab*, jc20142282 (2014).

39. Samad TA, *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 KJ, Ebersberger A, Klein B, Vanegas H, Schaible HG. 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 CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain* **10**, 895-926 (2009).

43. Berkley KJ, Rapkin AJ, Papka RE. The pains of endometriosis. *Science* **308**, 1587-1589 (2005).

44. McAllister SL, McGinty KA, Resuehr D, Berkley KJ. 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 FL, Murray A, Esnal A, Gibson DA, Critchley HO, Saunders PT. 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 FL, Murray AA, Scanlon JP, Saunders PT. Hypoxyprobe 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 EM, Moss A, Rosie R, Delaney A, Mitchell R, Fleetwood-Walker SM. 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 JM, 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 MC, Klaus K, Miller AL, Scotto di Perrotolo M, Sotocinal SG, Flecknell PA. The assessment of post-vasectomy pain in mice using behaviour and the Mouse Grimace Scale. *PLoS One* **7**, e35656 (2012).

53. Usdin TB, Dimitrov EL. 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 HL, Brunton PJ, Russell JA, 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 YT, Shao YW, Yen CT, Shaw FZ. Acid-induced hyperalgesia and anxio-depressive comorbidity in rats. *Physiology & behavior* **131**, 105-110 (2014).

57. Wieser F, Wu J, Shen Z, Taylor RN, 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 WA, Dudek FE, Dingledine R. Inhibition of the prostaglandin receptor EP2 following status epilepticus reduces delayed mortality and brain inflammation. *Proc Natl Acad Sci U S A* **110**, 3591-3596 (2013).

59. Greaves E, Collins F, Esnal-Zufiaurre A, Giakoumelou S, Horne AW, Saunders PT. 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 AW, Saunders PT. 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 MF, Seguela P, Bourque CW. 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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Data and materials availability: N/A

Figures

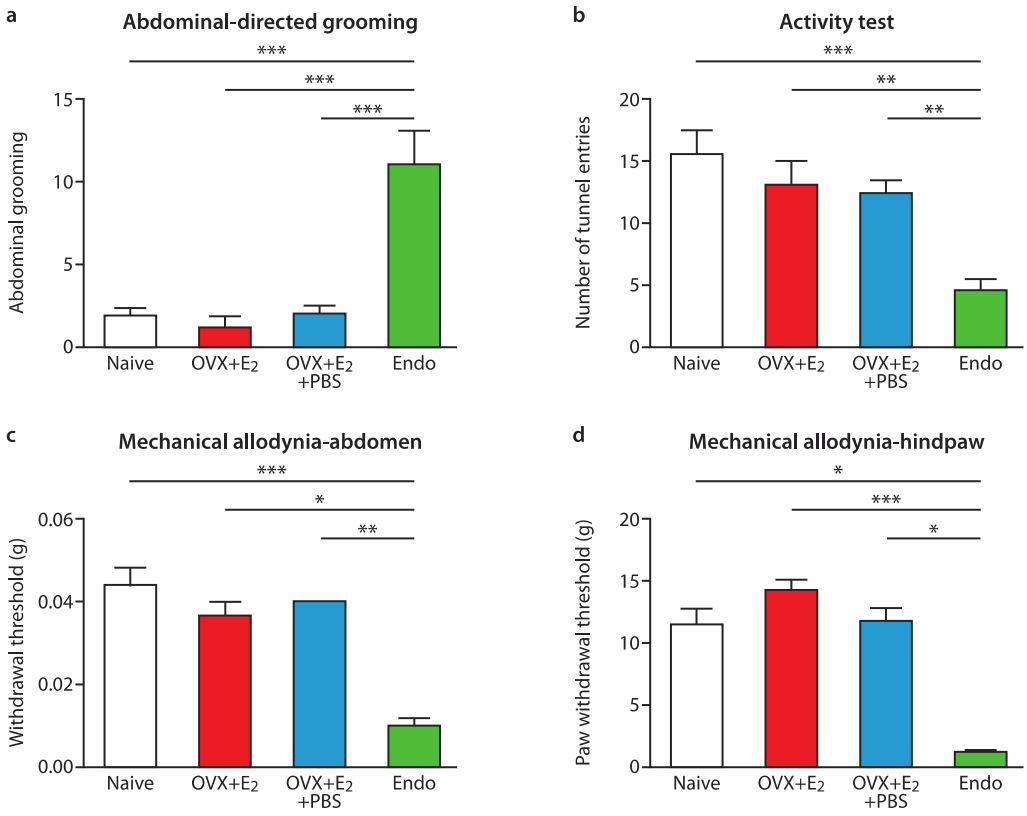


Fig.1 | Behavior testing in control and endometriosis mice. Endometriosis mice, together withovariectomised, estradiol-treated sham recipients, with or without i.p injection of PBS, and naïve controls were scored for general behaviors indicative of discomfort that might be associated with pelvic pain. **(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 Kruskall-Wallis test and Dunn's multiple comparison test. *$p<0.05$. **$p<0.01$, ***$p<0.001$.

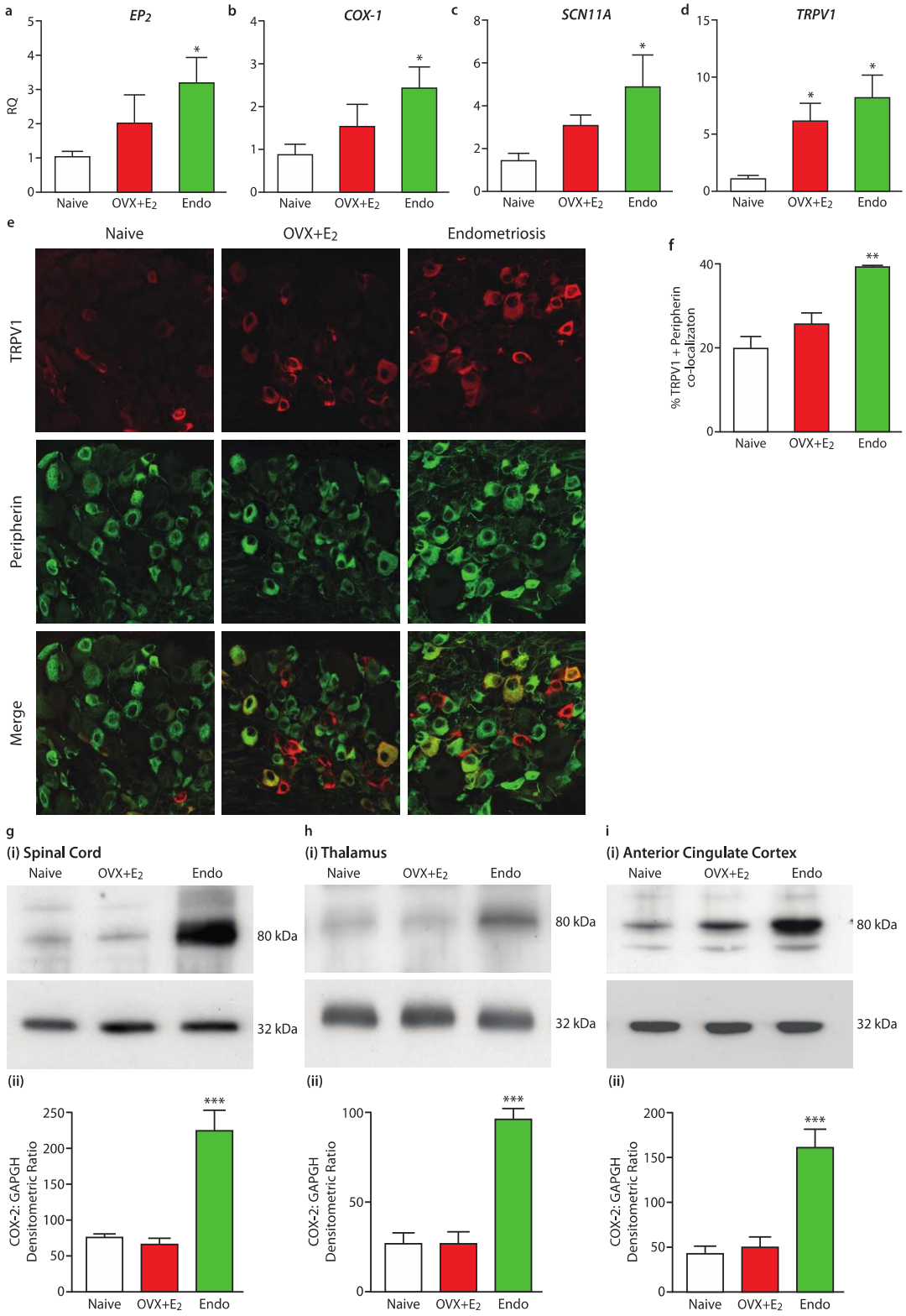
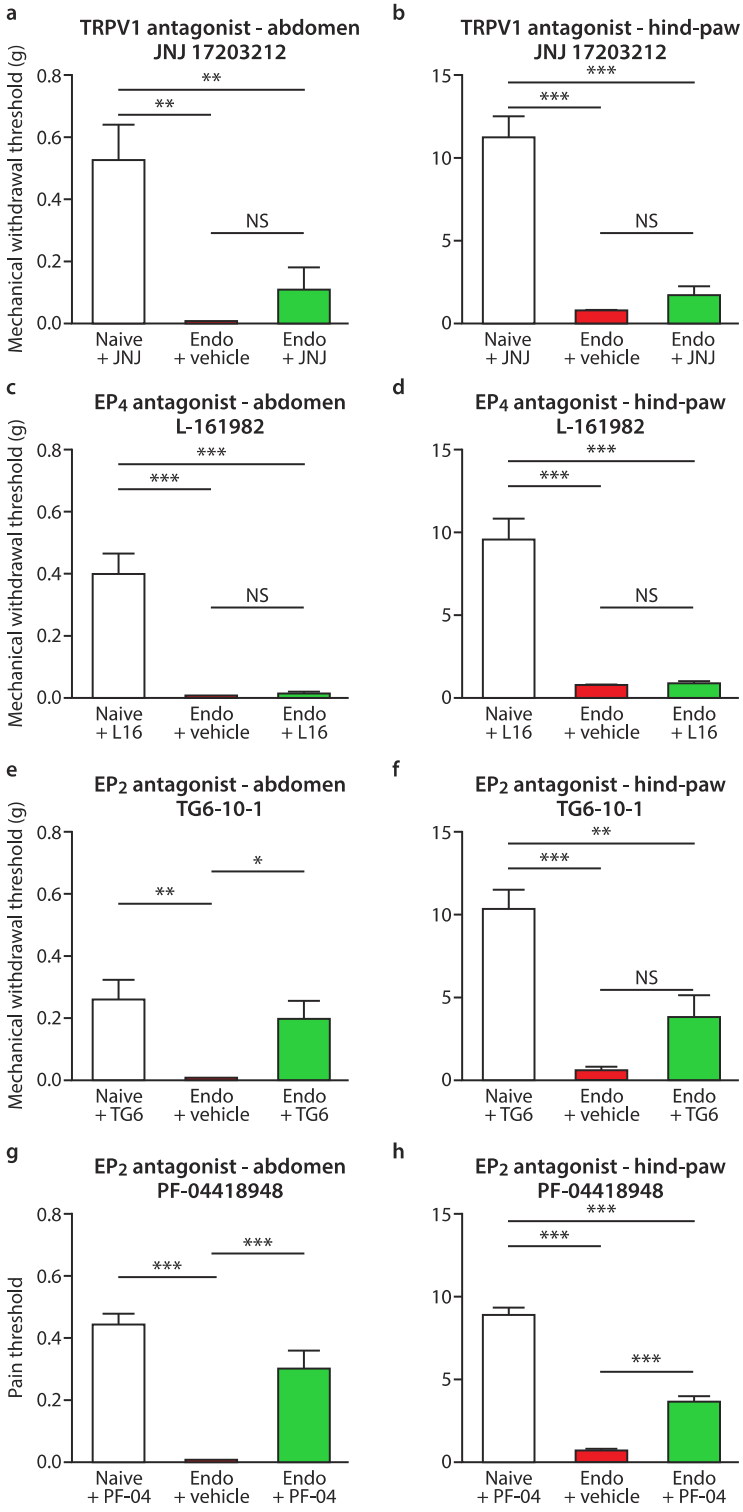


Fig.2 | Molecular changes in the nervous system of mice with endometriosis. Expression of the PGE_2 pathway and nociceptive ion channels is elevated in the DRGs, spinal cord and brain of mice with

endometriosis. **(a-d)** QPCR analysis of the prostaglandin E receptor **(a)** *EP_2*, **(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+E_2 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+E_2-treated sham-recipient and endometriosis mice (field of view, 160x 160μm). Total cells counted were 330, 320, and 543, accumulated from three different naïve, OVX+E_2-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+E_2-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+E_2-treated and naïve controls. Statistical analysis was performed using a one-way ANOVA and Tukey's post comparison test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.



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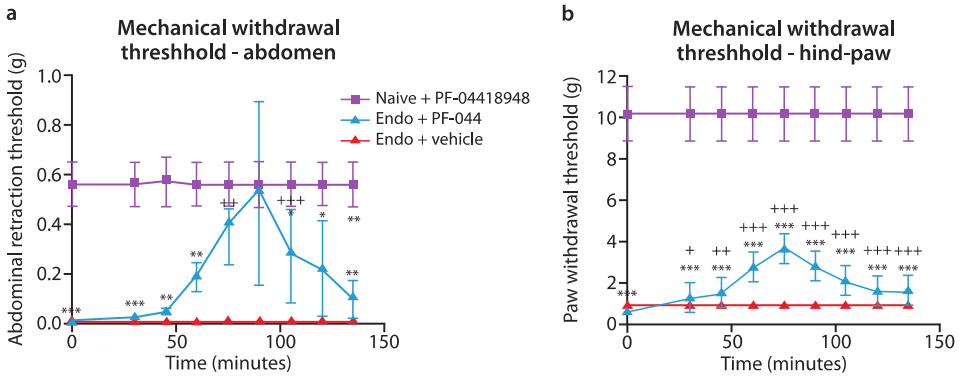
554 **Fig.3| Pre-clinical testing of potential therapeutics in a mouse model of endometriosis.** Graphs

555 depict 50% mechanical withdrawal thresholds for von Frey filaments (g=grams) applied to abdomen or

556 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 (30mg/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±0.17g and 11.25± 1.44g 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 EP_4 antagonist L-161982 (10mg/kg ip) 30 mins post i.p injection on abdominal and **(d)** hindpaw mechanical withdrawal thresholds. Naïve values were 0.39 ± 0.07g and 9.43 ± 1.43g. 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 EP_2 antagonist TG6-10-1 (10mg/kg, ip), on abdominal and **(f)** hindpaw mechanical withdrawal thresholds at 45 mins post i.p injection. Naïve values were 0.31± 0.11g and 9.69 ± 1.62g. 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 EP_2 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 ± 0.05g and 8.77 ± 0.51g. Statistical analysis was performed using a one-way ANOVA and Tukey's post comparison test. *$p<0.05$, **$p<0.001$, ***$p<0.001$.



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580 **Fig.4| Oral administration of the selective EP_2 antagonist PF-04418948.** To test the efficacy of oral
581 administration of PF-04418948, the drug was administered at a dose of 10mg/kg by oral gavage and
582 testing was initiated after 30 min. The graphs show the time-course of effects of PF-04418948 on
583 mechanical withdrawal responses in mice with endometriosis (blue and pink lines) or naïve controls
584 (purple lines). In both **(a)** the abdomen and **(b)** the hindpaw, withdrawal thresholds were significantly
585 lower in endometriosis mice than in naïve controls ($p<0.001$). The pre-drug withdrawal thresholds in
586 the naïve, drug-treated animals, were not discernibly different from those in naïve, untreated animals
587 (0.55 ±0.06g for abdomen and 9.00 ± 0.67g for hindpaw). In each case the hypersensitivity due to
588 endometriosis was substantially and significantly attenuated by PF-04418948 ($p<0.001$), n=5 all groups.
589 Statistical analysis was performed using a Two-Way ANOVA and Dunnett's multiple comparison test.
590 *$p<0.05$, **$p<0.001$ and ***$p<0.001$ compared to naïve mice + pharmacological agent. +$p<0.05$,
591 ++$p<0.01$ and +++$p<0.001$ compared to pre-administration baseline.

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