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

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1 **EP<sub>2</sub> receptor antagonism reduces peripheral and central hyperalgesia in a preclinical mouse**  
2 **model of endometriosis**

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

17 **The authors have no competing financial interests**

18

19

20 **Abstract**

21 Endometriosis is an incurable gynecological disorder characterized by debilitating pain and the  
22 establishment of innervated endometriosis lesions outside the uterus. In a preclinical mouse model of  
23 endometriosis we demonstrated overexpression of the PGE<sub>2</sub>-signaling pathway (including COX-2, EP<sub>2</sub>,  
24 EP<sub>4</sub>) in endometriosis lesions, dorsal root ganglia (DRG), spinal cord, thalamus and forebrain. TRPV1,  
25 a PGE<sub>2</sub>-regulated channel in nociceptive neurons was also increased in the DRG. These findings support  
26 the concept that an amplification process occurs along the pain neuroaxis in endometriosis. We then  
27 tested TRPV1, EP<sub>2</sub>, and EP<sub>4</sub> receptor antagonists: EP<sub>2</sub> antagonist was the most efficient analgesic,  
28 reducing primary hyperalgesia by 80% and secondary hyperalgesia by 40%. In this study we  
29 demonstrate reversible peripheral and central hyperalgesia in mice with induced endometriosis.

30

## Preclinical evaluation of pain in endometriosis

31 Endometriosis is a chronic gynecological disorder affecting 176 million women worldwide<sup>1</sup>  
32 associated with chronic pain and infertility. Current therapies include invasive surgery, drugs that  
33 suppress endogenous hormones<sup>2</sup> and non-steroidal anti-inflammatory drugs (NSAIDs) all of which have  
34 unwanted side effects. Better treatments for endometriosis-associated pain are needed but their  
35 development has been hampered by the lack of a robust preclinical model that fully reproduces the  
36 altered pain perception experienced by women with endometriosis.

37 Endometriosis is caused by the presence of endometrial-like tissue (endometriosis lesions) outside  
38 of the uterine cavity<sup>3</sup>. An association between small nerve fiber infiltration of endometriosis lesions and  
39 increased central and peripheral pain has been described (reviewed in<sup>4</sup>). Alterations in pain perception  
40 in women are thought to involve release of inflammatory mediators and neuropeptides by efferent  
41 peripheral nerve endings,<sup>5</sup> an increase in the sensitivity of nociceptive neurons,<sup>6</sup> and peripheral  
42 hyperalgesia. Continuous input from peripheral afferents can also trigger spinal hyper-excitability  
43 (central sensitization)<sup>7</sup> resulting in increased pain perception, secondary hyperalgesia and allodynia<sup>8</sup>.  
44 Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a well-established mediator of inflammation and nociception in  
45 inflammatory<sup>9,10</sup> and neuropathic pain conditions<sup>11</sup> and synthesis of prostaglandins within endometriosis  
46 lesions has previously been reported<sup>12, 13, 14, 15</sup>. As the association of PGE<sub>2</sub> signaling and endometriosis  
47 is well established we chose to validate this pathway as a target for pain attenuation in our mouse model.

48 A rat model of endometriosis exhibits vaginal hyperalgesia and increased abdominal muscle  
49 activity<sup>16, 17, 18</sup>. Recently, a study utilizing the same rat model demonstrated increased hyperalgesia in  
50 response to von Frey filaments, although an increase was also observed in the sham group<sup>19</sup>. Another  
51 model using autologous transplantation of endometrial tissue onto the gastrocnemius muscle<sup>20</sup> has  
52 proved useful for the identification of pronociceptive molecules that may be relevant in endometriosis<sup>21</sup>.  
53 Our murine model of endometriosis uses decidualized endometrial tissue injected (rather than sutured)  
54 into the peritoneum of recipient mice<sup>22</sup>. Resultant lesions phenocopy those in women; they are  
55 vascularized, innervated, infiltrated by macrophages and also exhibit oestrogen-dependent regulation of  
56 vascular-nerve and macrophage-nerve interactions<sup>23, 24</sup>. The aim of our study was to determine if mice  
57 with induced endometriosis exhibit peripheral (abdominal) and central (secondary/referred)

58 hyperalgesia and to test compounds targeting prostaglandin receptors to validate this model as a platform  
59 for preclinical testing of compounds to treat pain.

## 60 **Results**

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

## 70 **Over-representation of the PGE<sub>2</sub> signaling pathway and nociceptive ion channels in endometriosis 71 lesions and in the nervous system of mice with endometriosis**

72 Consistent with our previous studies<sup>22, 23, 24</sup> endometriosis-like lesions were recovered from the walls  
73 of the parietal peritoneum and the visceral peritoneum covering the uterus, gut, and intestines;  
74 mesentery associated with the gut and intestines; adipose associated with the kidney; and underneath  
75 the kidneys. *EP<sub>2</sub>*, *EP<sub>4</sub>*, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) were significantly  
76 increased in endometriosis lesions compared to the peritoneum of naive mice (NP) or mice with  
77 endometriosis (EP;  $p<0.01$ ; Supplementary Fig.2a-d); *EP<sub>2</sub>*, *EP<sub>4</sub>*, COX-1 and COX-2 proteins were  
78 immunolocalised to glandular and stromal cells in lesions and mesothelial cells in the peritoneum  
79 (Supplementary Fig 2). PGE<sub>2</sub> concentrations in the peritoneal fluid of Endo mice were significantly  
80 increased ( $p<0.05$ ; Supplementary Fig.2E). mRNA concentrations of *EP<sub>1</sub>* and *EP<sub>3</sub>* were unchanged  
81 (Supplementary Fig.2f-g). *EP<sub>2</sub>*, *Cox-1*, *Scn11a* and *Trpv1* mRNA concentrations were significantly  
82 increased in dorsal root ganglia (DRG; clusters of cell bodies of afferent sensory neurons that transmit  
83 noxious stimuli from the periphery to the spinal cord) from endometriosis mice ( $p<0.05$ ; Fig.2a-d);  
84 *EP<sub>4</sub>* and *Cox-2* were unchanged (Supplementary Fig.3c-d). *Trpv1* mRNA was also increased in the  
85 DRGs from OVX+E<sub>2</sub> mice. Dual immunofluorescence showed that in small DRG cells expressing

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86 peripherin (a marker for small unmyelinated sensory neurons; C-fibre nociceptors), the proportion that  
87 were immunopositive for TRPV1 was significantly increased in Endo mice compared to naïve and  
88 OVX+E<sub>2</sub>-treated controls (p<0.05; Fig.2e, f). The proportion of TRPV1-immunopositive neurons in  
89 DRG that express EP<sub>2</sub> was also increased in these animals (Supplementary Fig.3e-f). Mice with  
90 endometriosis had significantly increased concentrations of COX-2 protein in the spinal dorsal horn  
91 (p<0.001), the thalamus (p<0.001) and the anterior cingulate cortex of the brain (p<0.001; Fig.2f, i).  
92 All of these changes would be expected to contribute to pain hypersensitivity.

### 93 **Pre-clinical testing and stratification of potential therapies identified EP<sub>2</sub> as a key target**

94 Figure 3 indicates that injection of the TRPV1 inhibitor JNJ 17203212 (Fig3a, b) or the EP<sub>4</sub> antagonist  
95 L-161982 (Fig.3c, d), did not reverse abdominal or paw hyperalgesia to a statistically significant extent.  
96 Administration of the EP<sub>2</sub> antagonist TG6-10-1 resulted in a statistically significant reversal of  
97 mechanical allodynia as tested on the abdomen (p<0.05) at 45 mins post administration but this did not  
98 reach significance for the hind paw (Fig.3e, f). Time-course graphs are shown in Supplementary Fig.4.  
99 Results were extended using injection (Fig 3g, h) or oral administration (Fig 4a, b) of a second EP<sub>2</sub>  
100 antagonist (PF-04418948)<sup>25</sup> and this reduced allodynia in both abdomen and hind-paw tests (p<0.001  
101 using either route). Oral administration resulted in striking, time dependent, and significant impacts  
102 (Fig.4a, b, p<0.001).

### 103 **Discussion**

104 The re-purposing of drugs and the development of novel treatments for endometriosis-  
105 associated pelvic pain has been limited by the paucity of accessible pre-clinical models. In this study we  
106 tested pain responses in a mouse model of endometriosis that phenocopies interactions between  
107 endometriosis lesions and host tissue<sup>22, 24</sup> identified in human tissues. Endometriosis (Endo) mice  
108 exhibited increased levels of abdominally-directed grooming, a reduction in normal exploratory  
109 behavior and reduced mechanical withdrawal thresholds on both the abdomen and plantar hind-paw.  
110 The rodent models reported by Berkley, and by Levine and Giudice, both report evidence of  
111 hyperalgesia<sup>16, 26, 27 20</sup>. These models involve the artificial implantation of uterine tissue (full thickness  
112 i.e. endometrium plus myometrium) onto the mesenteric arteries of the small intestine and the

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113 gastrocnemius muscle, respectively. Therefore the microenvironment of the endometriosis lesion  
114 created in these models may not closely mirror that of the peripheral lesions in women.

115 PGE<sub>2</sub> is increased in the peritoneal fluid of patients with endometriosis<sup>28</sup>, this up-regulation  
116 results from induced expression of cyclooxygenase-2 (COX-2) in endometriotic tissue. PGE<sub>2</sub> is thought  
117 to be a key player in the pathophysiology of endometriosis and studies have shown that inhibition of  
118 COX-2 decreases survival, migration and invasion of endometriotic cells<sup>13</sup>. The same authors  
119 demonstrated that inhibition of EP<sub>2</sub> and EP<sub>4</sub> can inhibit the epithelial and stromal cell invasion via  
120 suppression of matrix metalloproteinases<sup>29</sup>. Attenuation of PGE<sub>2</sub> signaling via lipoxin A<sub>4</sub> can also  
121 modulate disease progression by attenuation of pro-inflammatory and angiogenic mediators<sup>30</sup>. A recent  
122 study using xenografted endometriotic cell lines in a nude mouse model of endometriosis demonstrated  
123 that dual inhibition of EP<sub>2</sub> and EP<sub>4</sub> could attenuate mechanical hyperalgesia of the pelvic floor via  
124 suppression of pro-inflammatory mediators in dorsal root ganglia<sup>31</sup>, however the authors did not test  
125 secondary hyperalgesia or analyze changes in the central nervous system.

126 In our mouse model we confirmed that the prostaglandin E signaling pathway was over-  
127 expressed in the pelvic cavity of our Endo mice and concentrations of PGE<sub>2</sub> were increased in their  
128 peritoneal fluid. Release of PGE<sub>2</sub> at sites of peripheral inflammation can contribute to pain  
129 hypersensitivity by lowering the threshold and enhancing the excitability of nociceptor sensory fibers<sup>32</sup>.  
130 This occurs at least in part via EP receptor-mediated activation of intracellular kinases in the nociceptor  
131 terminal that causes phosphorylation of the nociceptive ion channel TRPV1<sup>33,34</sup> and an up-regulation of  
132 Nav1.9 voltage-gated sodium channel (SCN11A)<sup>35</sup>, which is then transported to peripheral nerve  
133 terminals to contribute to increased excitability<sup>7</sup>. We detected a significant increase in expression of  
134 EP<sub>2</sub>, COX-1 and both TRPV1 and SCN11A ion channels in the DRGs of mice with endometriosis. This  
135 parallels observations of increased COX-1 and COX-2 in TRPV1-positive DRG cells in other models  
136 of inflammatory hyperalgesia<sup>36</sup>. TRPV1-immunoreactivity was increased in small, peripherin-positive,  
137 nociceptive neurons in DRG of mice with endometriosis and EP<sub>2</sub> expression was further increased in  
138 these cells. All of these findings are consistent with the development of sensory neuron hyperexcitability  
139 in our mice. The possibility of some additional role of prostaglandin signaling in non-neuronal cells, as  
140 seen in some other pain models<sup>37</sup> cannot be excluded. Elevated TRPV1 expression in small DRG cells

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141 innervating pelvic regions in mice with endometriosis is consistent with our previous findings that  
142 TRPV1 mRNA is elevated in peritoneal lesions from women with endometriosis<sup>38</sup>.

143 In this study, assessment of COX-2 expression in CNS regions within the pain-processing  
144 pathway revealed striking increases in expression at spinal, thalamic and cortical levels. COX-2  
145 expression in the CNS is established as a sensitive and responsive biomarker of centralized  
146 inflammatory pain<sup>39, 40, 41</sup> and is an important finding consistent with the inflammatory pain and  
147 widespread central sensitization as experienced by women with endometriosis. The phenomenon of  
148 central sensitization<sup>42</sup> has been postulated as a key contributor to the co-morbid pain syndromes  
149 experienced by women with the condition<sup>4</sup>. Central sensitization is described as a maladaptation of the  
150 CNS resulting from continued or repetitive input from nociceptors, in endometriosis it is likely that this  
151 input is provided by afferent nerve fibers innervating endometriosis lesions. One of the first steps in this  
152 process of central sensitization is an increase in expression of genes encoding neurotrophins,  
153 neuropeptides and ion channels critical in sensing and detecting noxious stimuli<sup>8</sup>. In the model of  
154 endometriosis generated by Berkley et al, rats exhibit vaginal hyperalgesia<sup>26</sup> and it is argued by the  
155 authors that this finding suggests central sensitization as an underlying factor because spinal segments  
156 associated with the induced endometriosis cysts are distant from spinal segments receiving input from  
157 the vagina<sup>43, 44</sup>. Using this same model a decrease in  $\mu$ -opioid and NMDA receptor immunoreactivity in  
158 the periaqueductal gray area of the brain was detected in rats with endometriosis compared to controls.  
159 Torres-Reverón et al suggested that a decrease in NMDA receptor expression could be an attempt to  
160 homeostatically regulate pain perception, whilst a decrease in  $\mu$ -opioid receptor expression suggests  
161 decreased modulatory activity of opioid receptors that could contribute to hyperalgesia in the  
162 condition<sup>45</sup>. We have also documented molecular alterations in the CNS of mice with endometriosis and  
163 we believe this as an important step change in our understanding of endometriosis-associated pain.

164 Having demonstrated amplified pain behaviors, we tested therapeutic strategies for reversal of  
165 mechanical allodynia. The effects of TRPV1 inhibition were modest, whilst antagonism of EP<sub>2</sub>,  
166 particularly following oral administration of the highly selective antagonist PF-04418948 had  
167 pronounced effects on peripheral and secondary hyperalgesia. This is consistent with reports that EP<sub>2</sub>  
168 null mice do not develop spinal hyperalgesia following induced peripheral inflammation<sup>46</sup>. In a recent



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169 study, the effect of a mixture of EP<sub>2</sub>/EP<sub>4</sub> antagonists had a modest impact on pelvic floor hyperalgesia  
170 in mice which may either reflect the limited specificity of the antagonists tested or use of human  
171 endometriotic cell lines (not intact tissue fragments) in recipient mice lacking a full complement of  
172 immune cells<sup>31</sup>. In our hands, the selective EP<sub>4</sub> antagonist L-161982, did not reverse the sensitivity to  
173 mechanical stimulation..

174 In summary, we show that induction of endometriosis in mice with an intact immune system is  
175 associated with maladaptation of the CNS, consistent with central sensitization<sup>4</sup>. We have demonstrated  
176 striking reversal of both peripheral and secondary hyperalgesia via EP<sub>2</sub> antagonism. In conclusion, we  
177 present evidence that a murine model of endometriosis displaying local and central sensitization can be  
178 used for pre-clinical testing of therapeutics for endometriosis-associated pain.

### 179 **Methods**

180 **Mouse model of endometriosis.** Experiments were performed in accordance with the Guidelines of the  
181 Committee for Research and Ethical Issues of the International Association for the Study of Pain.  
182 Experiments were performed under licensed approval from the UK Home Office (London). C57BL/6  
183 mice (Harlan Laboratories; Derby, UK) were given access to food and water *ad libitum*, ambient  
184 temperature and humidity were 21°C and 50% respectively. Endometriosis was induced in the mice as  
185 previously described<sup>22</sup>. In brief, the endometrium of syngeneic donor mice underwent hormonal  
186 manipulation and induction of decidualization using an in-house protocol to model endometrial  
187 differentiation, breakdown and repair<sup>47</sup>: detailed analysis of tissue samples has shown that progesterone  
188 withdrawal (removal of P4 pellet) results in rapid induction of hypoxia, tissue breakdown and induction  
189 of angiogenic genes<sup>47, 48</sup>. Endometrial tissue (~6 hours post-progesterone withdrawal) was recovered by  
190 opening the horn and scraping with a scalpel. Approximately 40mg tissue (equivalent to one  
191 decidualized horn) was suspended in 0.2 ml PBS and injected into the peritoneal cavity of  
192 ovariectomised recipient mice that were supplemented with 500ng Estradiol Valerate (EV). This  
193 supplementation was maintained by subcutaneous injection of 500ng EV every 3 days (modification of  
194 previously published model). After allowing lesions to form over 21 days behavioral assessments were  
195 performed.

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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 Dale, 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 <sup>49, 50, 51</sup>. 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<sup>50</sup>. 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<sup>52</sup>) were recorded and an average generated. Excessive abdominally-directed  
209 licking has been reported to represent a useful biomarker of abdominal visceral pain<sup>46</sup>. 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<sup>53</sup>. Paradigms of altered affective state in rodent  
213 pain models include reduced exploratory behavior<sup>54</sup>. Exploratory activity here was recorded in a  
214 modification of the open-field setting<sup>55, 56</sup>, 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<sub>2</sub> controls (n=9), for which, mice were ovariectomised and  
219 given E<sub>2</sub> 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<sub>2</sub>+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 <sup>57</sup> 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,

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226 endometriotic lesions, L5-L6 DRGs, lumbar spinal cord, thalamus and anterior cingulate cortex.

227 Samples were collected into RNAlater and frozen (Applied Biosystems, Warrington, UK), neutral-

228 buffered formalin prior to paraffin embedding for immunohistochemical analysis (uterus, peritoneum,

229 and endometriosis lesions) or frozen on dry ice prior to sectioning and immunofluorescence staining

230 (DRG) or protein extraction for Western blot analysis (spinal cord and brain). Endometriotic lesions

231 were recognized as red, brown or white tissue deposits on the visceral or parietal peritoneum and were

232 carefully dissected away from any surrounding fat or peritoneum. The presence of glands plus stroma

233 in suspected lesions were confirmed by haematoxylin/eosin staining. Biopsies that did not contain

234 both glands and stroma were not included in further analysis.

235 **Antagonists.** Agents for i.p. injections were dissolved in 10% dimethylsulphoxide, 50% PEG-400, 40%

236 ddH<sub>2</sub>O and injected in a volume of 100  $\mu$ l/25g using 30 mg/kg JNJ 17203212 (TRPV1 antagonist), 10

237 mg/kg TG6-10-1 (EP<sub>2</sub> antagonist; Calbiochem)<sup>58</sup>, 10 mg/kg PF-04418948<sup>25</sup> (EP<sub>2</sub> antagonist; Abcam), 10

238 mg/kg L-161982 (EP<sub>4</sub> antagonist; Abcam). Von Frey testing was performed at 30, 45 and 60 minutes

239 post injection: for PF-0418948, testing was also carried out at 75 minutes). PF-04418948 (10 mg/kg in

240 0.5% w/v methylcellulose + 0.1% V/V Tween-20 in purified water) was also administered as an oral

241 gavage and the von Frey test performed every 15 mins starting at 30 mins.

242 **Quantitative real time PCR.** RNA was extracted from control uterine biopsies recovered from naïve

243 mice, peritoneal biopsies from naïve and endometriosis mice, endometriosis lesions and dorsal root

244 ganglia using an RNeasy kit (QIAGEN) according to the manufacturers instructions. RNA was

245 quantified using a NanoDrop ND 1000. Quantitative PCR was performed as detailed in<sup>59, 60</sup>; briefly,

246 cDNA was synthesized using SuperScript VILO enzyme (Invitrogen) with 100ng starting template.

247 PCRs were performed using Roche Universal Probe Library (Roche Applied Science) using primer

248 sequences detailed in Supplementary Table 2. 18S was used as a reference gene. Thermal cycling was

249 performed on a 7900 Fast real-time PCR machine. Data was analysed with RQ manager software

250 (Applied Biosystems) using the  $\Delta\Delta$ Ct method; samples were normalised to a uterine control sample.

251 **Immunodetection.** Single antigen immunohistochemistry was performed according to standard

252 protocols<sup>47, 61</sup> with citrate antigen retrieval.

253 *Dual immunofluorescence*

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254 Dorsal root ganglia (DRGs) were embedded in OCT (CellPath) and frozen on dry ice. Sixteen  $\mu\text{m}$   
255 cryostat sections were blocked for 1 hour at room temperature then incubated with primary antibodies  
256 (supplementary Table 3). Specificity of the TRPV1 antibody has been previously established<sup>62</sup>.  
257 Secondary antibodies, from Molecular Probes or Sigma-Aldrich, goat anti-chicken Alexafluor 488  
258 (1:1000), goat anti-guinea pig AlexaFluor 568 (1:1000) or goat anti-guinea-pig CF405A (1:1000) were  
259 applied for 1 hour. Sections were mounted in ProLong® Gold Antifade (Life Technologies). Confocal  
260 images were acquired at x20 magnification using a Nikon A1R microscope and ImageJ software was  
261 used to quantify co-staining. Standard controls omitting primary antibodies were immunonegative.

262 **Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) ELISA.** Approximately 2 ml of the PF was collected into tubes containing  
263 indomethacin (10 $\mu\text{M}$ ) to prevent ex vivo PGE<sub>2</sub> metabolism. PGE<sub>2</sub> levels were analyzed using DetectX®  
264 prostaglandin E<sub>2</sub> enzyme immunoassay kit (Arbor Assays, MI, USA).

### 265 **Western blotting**

266 Tissue samples were collected into sealable tubes and frozen on dry ice, and then subsequently  
267 homogenized in Laemmli buffer, heated to 80°C for 5 min and centrifuged. Aliquots of lysate  
268 supernatant were analysed using the NuPage XCell SureLock™ Minicell gel electrophoresis system  
269 (Invitrogen) with approximately 12 $\mu\text{g}$  protein loaded per lane. Membranes were incubated overnight at  
270 4°C in 2% non-fat dried milk in 0.1 M PBS with 0.1% Tween-20, containing anti-COX-2 antibody  
271 (Supplementary Table 3)<sup>63</sup>. Membranes were washed and incubated for 50 min at room temperature  
272 with peroxidase-conjugated donkey anti-rabbit antibody (Chemicon, 1:20,000) and detected by  
273 peroxidase-linked enhanced chemiluminescence. Membranes were re-probed with mouse monoclonal  
274 anti-GAPDH (Supplementary Table 2). Films were scanned and band intensities were quantified by  
275 densitometry using ImageJ.

276  
277 **Statistical analysis.** Statistical analysis used a one-way ANOVA with a Newman Keuls or Tukey's test,  
278 or a Kruskal Wallis with a Dunn's multiple comparison test. A p value of less than 0.05 was considered  
279 significant. \*:p<0.05, \*\*:p<0.01, \*\*\*:p<0.001.

280  
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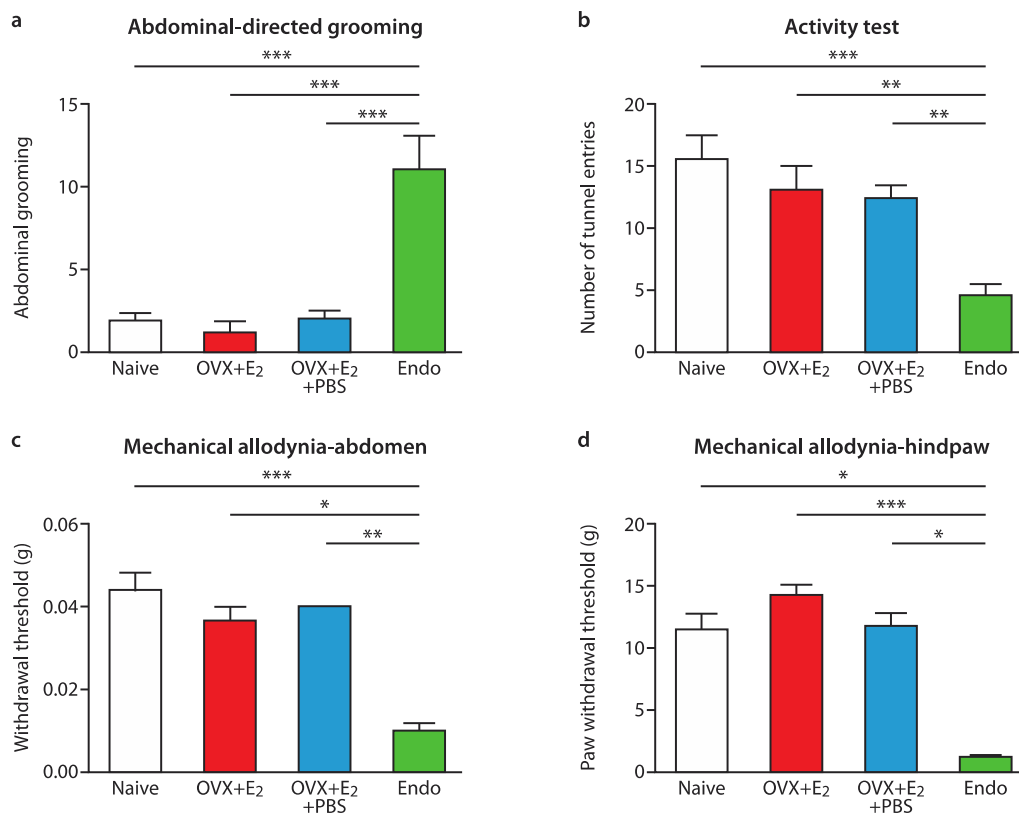
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511 **Figures**

512

513 **Fig.1 | Behavior testing in control and endometriosis mice.** Endometriosis mice, together  
 514 ovariectomised, estradiol-treated sham recipients, with or without i.p injection of PBS, and naïve  
 515 controls were scored for general behaviors indicative of discomfort that might be associated with pelvic  
 516 pain. **(a)** Shows abdominally directed licking (average number of grooming events recorded by two  
 517 observers over two 5 min periods) was significantly increased in endometriosis mice (n=5) compared to  
 518 estradiol-treated (OVX+E<sub>2</sub>; n=3, OVX+E<sub>2</sub>+PBS; n=6) and naïve controls (n=7). **(b)** Shows that  
 519 exploratory activity (average number of open-field tunnel entries recorded by two observers was  
 520 significantly reduced in endometriosis mice (n=9) compared to estradiol-treated (OVX+E<sub>2</sub>; n=6,  
 521 OVX+E<sub>2</sub>+PBS; n=6) and naïve controls (n=11). Mechanical withdrawal threshold, shown by von Frey  
 522 filament testing, was also measured on the lower abdomen and plantar hind-paw of endometriosis mice  
 523 (n=6), OVX+E<sub>2</sub>, OVX+E<sub>2</sub>+PBS (n=6) and naïve controls (n=7). For quantitative sensory testing, von  
 524 Frey filaments were applied to caudal regions of the abdomen; **(c)** and **(d)** show that withdrawal

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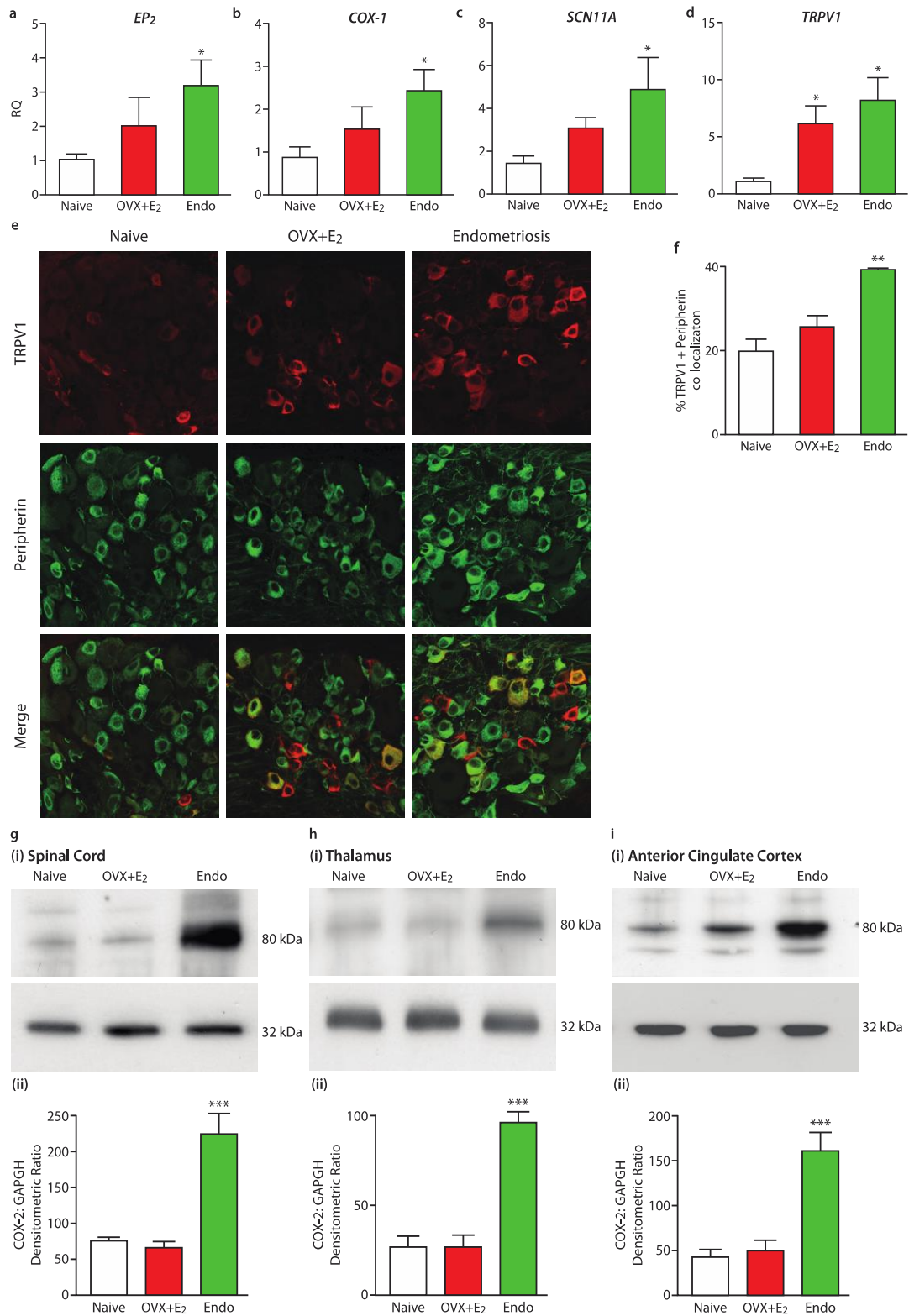
525 thresholds (g = grams) for both abdomen and paw testing were significantly decreased in endometriosis  
526 mice compared to the other groups. Statistical analysis was performed using a one-way ANOVA and  
527 Newman-Keuls post-hoc test (a and b) or a Kruskal-Wallis test and Dunn's multiple comparison test.  
528 \*p<0.05. \*\*p<0.01, \*\*\*p<0.001.

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## Preclinical evaluation of pain in endometriosis



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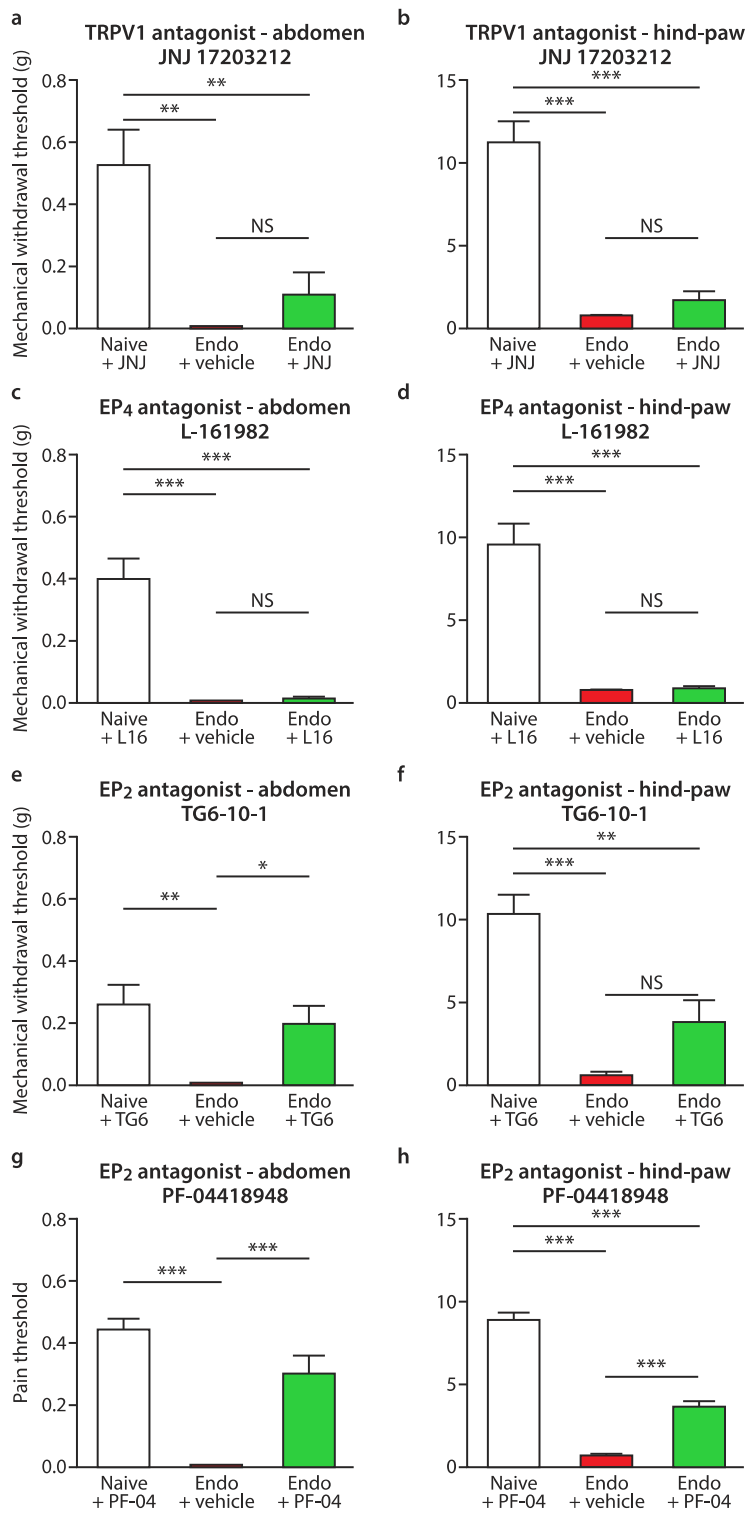
533 **Fig.2 | Molecular changes in the nervous system of mice with endometriosis.** Expression of the PGE<sub>2</sub>

534 pathway and nociceptive ion channels is elevated in the DRGs, spinal cord and brain of mice with

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535 endometriosis. **(a-d)** QPCR analysis of the prostaglandin E receptor **(a)** *EP<sub>2</sub>*, **(b)** *COX-1* and nociceptive  
536 ion channels **(c)** *Scn11a* and **(d)** *Trpv1* in L5-L6 DRGs from mice with endometriosis (n=9) compared  
537 to naïve (n=7) and OVX+E<sub>2</sub> control mice (n=6). RQ: Relative quantification. Values were normalized  
538 to a single naïve DRG sample given the arbitrary value of one. **(e-f)** Dual label immunofluorescence  
539 was carried out to identify TRPV1 expression (red) in L5-6 DRG cells co-expressing peripherin (green).  
540 **(e)** Shows typical confocal images for TRPV1 and peripherin from naïve, OVX+E<sub>2</sub>-treated sham-  
541 recipient and endometriosis mice (field of view, 160x 160µm). Total cells counted were 330, 320, and  
542 543, accumulated from three different naïve, OVX+E<sub>2</sub>-treated and endometriosis mice in each case. **(f)**  
543 Shows a bar chart that summarizes % expression of TRPV1 in peripherin-positive cells and indicates  
544 that the number of TRPV1+peripherin+ small DRG cells is significantly increased in mice with  
545 endometriosis. **(g-i)** Images (i) show representative examples of COX-2 (top panel; 80 kDa) and  
546 GAPDH (bottom panel; 36 kDa) expression as analyzed using Western blot in **(g)** spinal cord, **(h)**  
547 thalamus, **(i)** and anterior cingulate cortex of endometriosis mice, OVX+E<sub>2</sub>-treated sham-recipients and  
548 naïve controls, n=5-6 for all groups. (ii) Graphs showing COX-2: GAPDH densitometric ratio derived  
549 from quantitative densitometry of films. Both images and bar charts indicate marked elevation of COX-  
550 2 expression in each region. No changes were observed in OVX+E<sub>2</sub>-treated and naïve controls.  
551 Statistical analysis was performed using a one-way ANOVA and Tukey's post comparison test.  
552 \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

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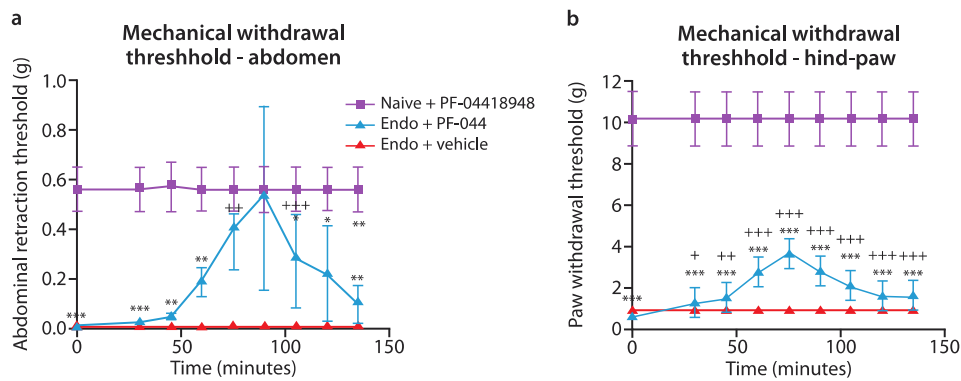
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554 **Fig.3| Pre-clinical testing of potential therapeutics in a mouse model of endometriosis.** Graphs  
 555 depict 50% mechanical withdrawal thresholds for von Frey filaments (g=grams) applied to abdomen or  
 556 hindpaw of mice with endometriosis or naïve controls, n=5 all groups. The time-point showing maximal

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557 reversal of pain is shown for each antagonist. **(a)** Effects of the TRPV1 inhibitor JNJ 17203212  
558 (30mg/kg ip), 30 mins post-injection on abdominal withdrawal responses in mice with endometriosis  
559 compared to naïve mice. **(b)** Shows corresponding results from hindpaw. Concurrently measured naïve  
560 values were  $0.55 \pm 0.17$ g and  $11.25 \pm 1.44$ g respectively for abdomen and hindpaw. In both tests,  
561 withdrawal thresholds were significantly lower in mice with endometriosis (Endo+Vehicle) than in  
562 naïve controls or in naïve animals treated with the drug (Naïve+JNJ;  $p < 0.01$  and  $p < 0.001$ ). This  
563 difference was modestly but not significantly attenuated by JNJ 17203212, which had no discernible  
564 effect on the responses of naïve animals. **(c)** Effects of the selective EP<sub>4</sub> antagonist L-161982 (10mg/kg  
565 ip) 30 mins post i.p injection on abdominal and **(d)** hindpaw mechanical withdrawal thresholds. Naïve  
566 values were  $0.39 \pm 0.07$ g and  $9.43 \pm 1.43$ g. In both tests, thresholds were significantly lower in mice  
567 with endometriosis than in naïve animals treated with the drug ( $p < 0.001$ ). L-161982 had no discernible  
568 effect on hypersensitivity in mice with endometriosis or in naïve animals. **(e)** Effect of the EP<sub>2</sub> antagonist  
569 TG6-10-1 (10mg/kg, ip), on abdominal and **(f)** hindpaw mechanical withdrawal thresholds at 45 mins  
570 post i.p injection. Naïve values were  $0.31 \pm 0.11$ g and  $9.69 \pm 1.62$ g. In both tests, thresholds were  
571 significantly lower in mice with endometriosis compared to naïve controls or in naïve animals treated  
572 with the drug ( $p < 0.01$  and  $p < 0.001$ ). This difference was significantly reversed by TG6-10-1 in  
573 abdominal tests ( $p < 0.05$ ). TG6-10-1 also induced a modest but not statistically significant pain reversal  
574 in paw tests. The highly selective EP<sub>2</sub> antagonist PF-04418948 (10 mg/kg, i.p) significantly attenuated  
575 *both* **(g)** abdominal and **(h)** hindpaw mechanical hypersensitivity in mice with endometriosis ( $p < 0.001$ )  
576 45 mins post injection. There was no discernible effect of PF-04418948 in naïve animals. Naïve values  
577 were  $0.48 \pm 0.05$ g and  $8.77 \pm 0.51$ g. Statistical analysis was performed using a one-way ANOVA and  
578 Tukey's post comparison test. \* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.001$ .

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579

580 **Fig.4| Oral administration of the selective EP<sub>2</sub> 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 ( $0.55 \pm 0.06g$  for abdomen and  $9.00 \pm 0.67g$  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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