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1 **Title: Ethosuximide improves chronic pain-induced anxiety- and depression-**  
2 **like behaviors**

3

4

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14

15 **Short title:** Ethosuximide to treat chronic pain-induced anxiety and depression

16

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21 **ABSTRACT**

22

23 Chronic pain is a heavy burden disease. Current treatments are generally weakly effective or  
24 associated with adverse effects. New therapeutic approaches are therefore needed. Recent  
25 studies have suggested T-type calcium channels as an attractive target for the treatment of  
26 chronic pain. In this perspective, it was decided to perform a preclinical evaluation of the  
27 efficacy of ethosuximide, a T-type channel blocker used clinically as an antiepileptic, as a  
28 novel pharmacological treatment for chronic pain. Assessment of the effect of ethosuximide  
29 was thus made in both nociception and pain-related comorbidities as anxiety and depression  
30 are frequently encountered in chronic pain patients. Our results show that such symptoms  
31 occurred in three animal models of chronic pain designed to reflect traumatic neuropathic,  
32 chemotherapy-induced neuropathic and inflammatory pain conditions. Administration of  
33 ethosuximide reduced both chronic pain and comorbidities with a marked intensity ranging  
34 from partial reduction to a complete suppression of symptoms. These results make  
35 ethosuximide, and more broadly the inhibition of T-type calcium channels, a new strategy  
36 for the management of uncontrolled chronic pain, likely to improve not only pain but also  
37 the accompanying anxiety and depression.

38

39

40 **ABBREVIATIONS**

41 CFA, complete Freund's adjuvant; EPM, elevated plus maze; ETX, ethosuximide; FST, forced  
42 swimming test; NSF, novelty suppressed feeding; OIPN, oxaliplatin-induced peripheral  
43 neuropathy; PWR, paw withdrawal response; SNI, spared nerve injury; TST, tail suspension  
44 test; Veh, vehicle.

45

46

47 **KEYWORDS**

48 Chronic pain; Neuropathy; Inflammation; T-type calcium channels; Ethosuximide;  
49 Anxiety/Depression.

## 50 **1. Introduction**

51 With a worldwide prevalence estimated at 20% (Goldberg and McGee, 2011), chronic pain is  
52 a heavy burden for individuals and society. For a large proportion of patients, chronic pain is  
53 accompanied by various comorbidities such as anxiety and depression which contribute to  
54 the deterioration of their quality of life (Conrad et al., 2013; World Health Organization  
55 technical report series, 2003). The prevalence of anxiety disorders in patients with chronic  
56 pain is up to 26% as against 7% to 18% in the general population (Global Burden of Disease  
57 Study 2013 Collaborators, 2015; Twillman, 2007), and signs of depression are estimated to  
58 occur in 50% of painful patients (Dworkin and Gitlin, 1991; Oliveira et al., 2018) who, in  
59 addition, are two to three times more likely to develop anxiety or depression (Demyttenaere  
60 et al., 2007; McWilliams et al., 2004; Price, 2000; Wilson et al., 2002). In parallel, patients  
61 with anxiety disorders have a higher risk of developing chronic pain (Sareen et al., 2005).  
62 Evidence also shows that anxiety and depression are amplifiers of pain perception  
63 (Klauenberg et al., 2008; Ploghaus et al., 2001).

64 These comorbidities have been widely documented in patients suffering from traumatic  
65 (Bailey et al., 2009; Gustorff et al., 2008; Radat et al., 2013) or iatrogenic (Bao et al., 2016;  
66 Thornton et al., 2008; Tofthagen et al., 2013; Ventzel et al., 2016) painful neuropathies. They  
67 have also been commonly reported in patients suffering from other etiologies of chronic  
68 pain, such as rheumatoid arthritis (Edwards et al., 2011; Goldenberg, 2010; Isik et al., 2007;  
69 Kojima et al., 2009; McWilliams et al., 2008; Sheehy et al., 2006). Unfortunately, the  
70 analgesic therapies for these chronic pain conditions and their associated comorbidities have  
71 limited efficacy and benefit-risk ratio. First line treatments for neuropathic pain  
72 (antidepressants and antiepileptics) are of limited efficacy (Finnerup et al., 2015).  
73 Chemotherapy-induced neuropathy have no treatment (Hershman et al., 2014; Poupon et  
74 al., 2015). Current drugs for the management of rheumatoid arthritis (Smolen et al., 2017)  
75 have also a limited effect on pain and entail serious adverse effects (Boyman et al., 2014;  
76 Laine et al., 2003; Solomon et al., 2017). Clearly, innovative treatments are needed to reduce  
77 pain and comorbidities in chronic pain patients and thereby to improve their quality of life.

78 Several studies have shown the interest of T-type calcium channels in different pain  
79 condition (Choi et al., 2007; Francois et al., 2013; Snutch and Zamponi, 2018), especially  
80 neuropathic (Bourinet et al., 2016) and inflammatory pain (Kerckhove et al., 2014).  
81 Ethosuximide, a non-specific blocker of T-type channels, has an analgesic effect on

82 neuropathic (Dogrul et al., 2003; Flatters and Bennett, 2004; Hamidi et al., 2012; Kawashiri  
83 et al., 2012; Okubo et al., 2011) and inflammatory pain (Barton et al., 2005; Cheng et al.,  
84 2007; Munro et al., 2007; Shannon et al., 2005), but no study has yet assessed its effect, and  
85 more broadly that of T-type calcium channel inhibition, on anxiety and depression resulting  
86 from chronic pain.

87 The aim of the present study was to evaluate the effect of ethosuximide on the nociceptive  
88 and emotional (anxiety and depression) manifestations of neuropathic and inflammatory  
89 chronic pain. For this purpose, we used different well-established models of chronic pain:  
90 the spared nerve injury (SNI) (Shields et al., 2003) and oxaliplatin-induced peripheral  
91 neuropathy (OIPN) (Poupon et al., 2018) models of neuropathic pain and the complete  
92 Freund's adjuvant (CFA) model (Kerckhove et al., 2014) of monoarthritic pain. Five tests were  
93 performed assessing anxiety (elevated plus maze), depression (tail suspension test and  
94 forced swimming test) or both (novelty suppressed feeding test), and two tests assessing  
95 pain (von Frey and thermal place preference tests). The results presented here show that  
96 ethosuximide reduced nociception and improved anxiety- and depression-like behaviors  
97 observed in the three murine models of chronic pain.

## 98 **2. Materials and methods**

99

### 100 **2.1. Animals**

101 Male mice C57BL6/J (20-25 g, Janvier, France) were acclimatized for a week before testing.  
102 They were housed under controlled environmental conditions (21-22°C; 55% humidity, 12h  
103 light/dark cycles, food and water *ad libitum*). All experiments were approved by the local  
104 ethics committees and performed according to European legislation (Directive 2010/63/EU)  
105 on the protection of animals used for scientific purposes, and complied with the  
106 recommendations of the International Association for the Study of Pain.

107

### 108 **2.2. Products**

109 Ethosuximide (Ref E7138, Sigma-Aldrich, France), administered intraperitoneally (i.p.) 20 min  
110 before behavior tests at a concentration of 200 mg/kg, is reconstituted in an aqueous saline  
111 solution (0.9% NaCl). Complete Freund's adjuvant (CFA) administered by periarticular  
112 injection, consists of *Mycobacterium butyricum* (Ref DF0640-33-7, Difco Laboratories,  
113 Detroit, USA) dissolved in paraffin oil and aqueous saline solution (0.9% NaCl). The solution is  
114 autoclaved 20 min at 120°C. Oxaliplatin (Ref O9512, Sigma-Aldrich, France), administered i.p.  
115 at a concentration of 3 mg/kg, is dissolved in glucose 5%.

116

### 117 **2.3. Animal models**

118

#### 119 *2.3.1. Spared Nerve Injury (SNI) Model*

120 Mice were anesthetized with i.p. injection of xylazine hydrochloride (10 mg/kg) and  
121 ketamine (100 mg/kg). The tibial and peroneal nerves were ligatured with non-resorbable  
122 suture. One centimeter of nerve was cut distal of the ligature (Shields et al., 2003). The sural  
123 nerve was kept intact. The behaviors tests were performed before and 21 days after surgery.

124

#### 125 *2.3.2. Oxaliplatin-Induced Peripheral Neuropathic (OIPN) Pain Model*

126 This model was modified from that of Poupon *et al.* (2018). The neuropathic pain model was  
127 induced by 6 i.p. injections (day 0, day 3, day 7, day 10, day 14 and day 17) of oxaliplatin at 3  
128 mg/kg. The behaviors tests were performed before any injection and 5 days after the last  
129 injection.

130

### 131 *2.3.3. Monoarthritic Model*

132 A persistent inflammatory pain model was produced by injection under brief anesthesia  
133 (2.5% isoflurane inhalation) of 5  $\mu$ l of CFA on either side of the left ankle joint of mice  
134 (Kerckhove et al., 2014). The behaviors tests were performed before and 14 days after CFA  
135 injection.

136

### 137 *2.3.4. Restraint Stress-Induced Depression Model*

138 Depression was produced by repeated restraint stress (Agnihotri et al., 2019; Choi et al.,  
139 2017). Briefly, mice were placed head first into a modified 50 ml falcon tube with ventilation  
140 holes. Mice were restrained for 2h each day, for 7 days. The behaviors tests occurred 24h  
141 following the 7 days restraint.

142

## 143 **2.4. Behavioral tests**

144

### 145 *2.4.1. von Frey Test*

146 Mice were habituated to the testing environment before baseline testing. The experimenter  
147 was blinded to the mice treatments. On the behavior testing day, the mice were placed  
148 individually in Plexiglas compartments 8 cm (L) x 3.5 cm (W) x 8 cm (D), on an elevated wire  
149 mesh platform to allow access to the ventral surface of the hindpaws and allowed to  
150 acclimatize for one hour before testing. Von Frey filaments ranging from 0.02 to 1.4 g were  
151 applied perpendicularly to the plantar surface of the paw. Paw withdrawal or licking was  
152 considered as a positive response. 50% paw withdrawal threshold (PWT) was determined  
153 using an adaptation of the Dixon up–down method, as described previously (Chaplan et al.,  
154 1994).

155

### 156 *2.4.2. Thermal Place Preference*

157 Mice were placed in an arena containing two identical platforms placed side by side such  
158 that the adjacent thermal surfaces at different temperatures were enclosed in a single  
159 chamber (32 x 16 cm, Bioseb, France) (Pereira et al., 2014). The temperature of one platform  
160 was maintained at 25°C and the other at 23°C. The movement of the mice between the two

161 plates was recorded with a video tracking system over 3 min and the parameter evaluated  
162 was the time spent on each platform. The percentage of time spent at 25°C was recorded.

163

#### 164 *2.4.3. Elevated Plus Maze (EPM) Test*

165 The elevated plus maze test is the common method for assessing anxiety-like behavior in  
166 mice (Rodgers and Johnson, 1995). The apparatus is placed 50 cm from the floor. It consists  
167 of two open arms (without a sidewall anxiety zone) (38 x 6 cm) and two closed arms (side  
168 walls 17 cm in height, no anxiety zone) connected by a central platform (6 x 6 cm) The test  
169 was performed in an evenly lighted room. Light intensity was fixed at 60 lux. A decrease in  
170 the time spent in open arms (compared to the baseline) is characteristic of anxious behavior.  
171 The animal was placed alone in the center of the maze with its head toward the closed arms  
172 for 5 min. A camera recorded the movements of the animal. The time spent in each arm and  
173 the frequency entries in open arms were determined by videotracking with ViewPoint®  
174 software.

175

#### 176 *2.4.4. Tail Suspension Test (TST)*

177 Tail suspension test was performed as previously described (Steru et al., 1985). Briefly, each  
178 mouse was suspended by its tail and secured by adhesive tape to the suspension bar leaving  
179 a gap of 20-25 cm between the animal's nose and the apparatus floor. Adhesive tape was  
180 applied 2-3 mm from the end of the tail. Total immobility time was recorded over a period of  
181 5 min directly by an experimenter. The mouse was considered immobile only when it hung  
182 passively and completely motionless. A long period of total immobility is a sign of depressive  
183 behavior.

184

#### 185 *2.4.5. Forced Swimming Test (FST)*

186 The animals were placed individually into glass cylinders (height, 25 cm; diameter, 16 cm)  
187 filled with 10 cm of warm water (22-25°C) for 6 min. The time when the mice remained  
188 floating passively or immobile in the water was recorded directly by an experimenter  
189 (Porsolt et al., 1977). A long period of total immobility is a sign of depressive behavior.

190

#### 191 *2.4.6. Novelty Suppressed Feeding (NSF) Test*

192 Tests based on hyponeophagia phenomena were initially designed to study the efficacy of  
193 anxiolytic treatments but have been increasingly used to study chronic and subchronic  
194 antidepressant treatments in rodent models (Blasco-Serra et al., 2017). The novelty  
195 suppressed feeding test induces a situation of conflicting motivations in animals between  
196 the feeding and the fear of venturing into the center of the brightly lit arena (anxiety zone).  
197 The animal, deprived of food for 24 h, is placed at the beginning of the experiment in a  
198 corner of the arena 46 x 46 cm face against the wall for a period of 5 min. At the center of  
199 this arena, is placed a ramekin filled with food (standard pellets) that is illuminated by a  
200 strong light. The time taken by the animal to go to the center of the arena (parameter  
201 assessing anxiety) and to eat (parameter assessing depression) are measured (Bodnoff et al.,  
202 1989).

203

## 204 **2.5. Experimental Protocol**

205 The design, analysis and reporting of the research were carried out in accordance with the  
206 ARRIVE guidelines (Kilkenny et al., 2010) and those of the European Community guiding for  
207 the care and use of animals (Directive, 2010/63/EU). Animals were randomly divided into 10  
208 mice *per* group. Treatments were administered according to the method of equal blocks, in  
209 order to assess the effect of the different treatments over the same time interval thereby  
210 avoiding unverifiable and time-variable environmental influences. All behavioral tests were  
211 performed in a quiet room by the same blinded experimenter. To ensure the methodological  
212 quality of the study, we followed the recommendations of Rice *et al.* (2008).

213

## 214 **2.6. Statistical Analysis**

215 Results are expressed as mean  $\pm$  SD and were recorded with Prism 7 (GraphPad Software  
216 Inc., San Diego, CA). Data were tested for normality (Shapiro-Wilk test) and for equal  
217 variance (Fisher test). Statistical analysis for mean comparison was performed with *t*-test for  
218 parametric data and Mann-Whitney test for non-parametric data. Multiple measurements  
219 were compared two-way ANOVA. The post hoc comparisons were performed by the Sidak  
220 method. Values of  $p < 0.05$  were considered statistically significant. The area under the time-  
221 course (0–120 min) curves (AUC) of 50% mechanical threshold variations (individual values)  
222 were calculated by the trapezoidal rule and expressed as mean  $\pm$  SD (in g x min). The  
223 percentages given in Table 1 were calculated as follows:  $100 - [(mean\ of\ ethosuximide\ group$

224 after model induction - mean of ethosuximide group before model induction) \* 100 / (mean  
225 of vehicle group after model induction - mean of vehicle group before model induction)].

## 226 **3. Results**

227

### 228 **3.1. Ethosuximide reduced hypersensitivity in two different neuropathic pain models**

229

230 In the SNI model, mechanical hypersensitivity, symptom commonly found in patients  
231 suffering from traumatic neuropathy, was assessed by the von Frey test. Twenty-one days  
232 after surgery, the mechanical paw withdrawal threshold (PWT) decreased from  $0.64 \pm 0.17$  g  
233 to  $0.07 \pm 0.02$  g for the vehicle group and from  $0.58 \pm 0.15$  g to  $0.09 \pm 0.06$  g for the  
234 ethosuximide group (**Fig. 1A**). Ethosuximide administration significantly reduced the  
235 mechanical hypersensitivity from 20 min to 60 min after the injection ( $p < 0.0001$ ; 2-way  
236 ANOVA; **Fig. 1A**) with a maximum effect at 40 min (PWT of  $0.40 \pm 0.10$  g vs  $0.07 \pm 0.02$  g,  
237 vehicle vs ethosuximide,  $p < 0.0001$ ; Sidak *post-hoc*; **Fig. 1A**).

238 In the OIPN model, five days after the last injection of oxaliplatin the PWT of mice  
239 was lower compared to baseline, from  $0.55 \pm 0.25$  g to  $0.10 \pm 0.05$  g for vehicle group and  
240 from  $0.47 \pm 0.17$  g to  $0.13 \pm 0.08$  g for ethosuximide group (**Fig. 1B**). Mice that received  
241 ethosuximide have a significantly higher mechanical threshold at 20 and 40 min after the  
242 injection ( $p < 0.01$ ; 2-way ANOVA; **Fig. 1B**) with a maximum effect at 40 min (PWT of  $0.14 \pm$   
243  $0.02$  g vs  $0.38 \pm 0.12$  g, vehicle vs ethosuximide,  $p < 0.0001$ ; Sidak *post-hoc*; **Fig. 1B**).  
244 Moreover, we used the thermal place preference to assess cold thermal hypersensitivity, a  
245 neuropathic symptom frequently expressed by patients treated with oxaliplatin (Attal et al.,  
246 2009; Forstenpointner et al., 2018; Lehky et al., 2004). After oxaliplatin treatment, mice  
247 spent more time on the 25°C plate ( $72.3 \pm 10.8\%$  of the time) than before injection ( $59.2 \pm$   
248  $7.0\%$  of the time,  $p < 0.01$ ; *t*-test; **Fig. 1C**), reflecting the development of thermal allodynia.  
249 Administration of ethosuximide reduced time spent on the plate at 25°C ( $44.9 \pm 13.0\%$  of the  
250 time) reflecting the suppression of cold allodynia (**Fig. 1C**).

251

### 252 **3.2. Ethosuximide reduced the anxiety- and depression-like behavior resulting from** 253 **chronic neuropathic pain**

254

255 We first investigated the effect of ethosuximide on the anxiety-like behavior by  
256 measuring the time spent and the number of entries in open arms in the elevated plus maze

257 (EPM) test and the time needed to reach the center of the arena in the novelty suppressed  
258 feeding (NSF) test were measured.

259 In the EPM test, the induction of neuropathic pain significantly decreased the time  
260 spent in open arms from  $30.9 \pm 25.8$  s to  $4.3 \pm 9.6$  s in SNI ( $p < 0.01$ ; Mann-Whitney; **Fig. 2A**  
261 **left**) and from  $9.0 \pm 9.3$  s to  $1.4 \pm 2.7$  s in OIPN ( $p < 0.05$ ; Mann-Whitney; **Fig. 2C left**)  
262 compared to baseline. Mirrored results were observed with the number of entries in the  
263 open arm (**Fig. 2A right and 2C right**). Likewise, in the NSF test, the time needed to reach the  
264 center was longer in the two models (SNI:  $72.4 \pm 26.7$  s,  $p < 0.001$ ; OIPN:  $137.2 \pm 111.6$  s,  $p <$   
265  $0.01$ ; Mann-Whitney; **Fig. 2B and Fig. 2D**) compared to their respective baseline ( $25.8 \pm 12.5$   
266 s and  $24.6 \pm 7.5$  s).

267 After ethosuximide treatment, the time spent in open arms in the EPM test was  
268 significantly increased in SNI ( $12.4 \pm 11.9$  s,  $p < 0.05$ ; Mann-Whitney; **Fig. 2A left**) and in OIPN  
269 ( $14.2 \pm 17.3$  s,  $p < 0.05$ ; Mann-Whitney; **Fig. 2C left**) compared to vehicle (SNI:  $4.3 \pm 9.5$  s and  
270 OIPN:  $1.4 \pm 2.7$  s), corresponding to a correction of 13.2% and a complete suppression of the  
271 disorder, respectively. The number of entries in the open arm was significantly increased by  
272 ethosuximide in the SNI model ( $p < 0.01$ ; Mann-Whitney; **Fig. 2A right**). In the OIPN model,  
273 the increase observed did not reach the statistical significance ( $p = 0.07$ ; Mann-Whitney; **Fig.**  
274 **2A right**). Ethosuximide also reduced the time to go to the center in the NSF test in the SNI  
275 ( $38.3 \pm 49.0$  s) and the OIPN ( $16.3 \pm 8.2$  s); these scores are not statistically different from  
276 those of the corresponding baselines (**Fig. 2B and Fig. 2D**) and are evidence of the ability of  
277 ethosuximide in this test to reverse anxiety-like symptoms.

278

279 In the SNI and OIPN models, we next evaluated the depression-like behavior and how  
280 it was affected by ethosuximide. To this end, we measured the latency to eat in the NSF test  
281 and determined the total immobility time in the tail suspension test (TST) and in the forced  
282 swimming test (FST).

283 Compared to baseline, mice submitted to SNI surgery or treated with oxaliplatin took  
284 longer to eat in the NSF test (SNI:  $87.4 \pm 34.5$  s vs  $143.7 \pm 41.7$  s,  $p < 0.01$ , baseline vs post-  
285 SNI; OIPN:  $186.3 \pm 67.4$  s vs  $300.0 \pm 0.0$  s,  $p < 0.0001$ ; Mann-Whitney; baseline vs post-OIPN;  
286 **Fig. 3A and Fig. 3B**). In addition, we observed an increased immobility time in the TST (SNI:  
287  $123.5 \pm 21.4$  s vs  $164.0 \pm 21.1$  s,  $p < 0.01$ ; *t*-test; baseline vs post-SNI, **Fig. 3C**; OIPN:  $147.8 \pm$   
288  $26.4$  s vs  $188.0 \pm 17.7$  s,  $p < 0.001$ ; *t*-test; baseline vs post-OIPN; **Fig. 3D**) and in the FST (SNI:

289 132.4 ± 22.9 s vs 193.3 ± 22.9 s,  $p < 0.0001$ ; *t*-test; baseline vs post-SNI, **Fig. 3E**; OIPN: 177.6 ±  
290 21.1 s vs 225.8 ± 27.3 s,  $p < 0.001$ ; *t*-test; baseline vs post-OIPN, **Fig. 3F**). These results  
291 indicated depression-like behavior in both SNI and OIPN models.

292 Compared to vehicle, ethosuximide treatment decreased the depressive-like  
293 behaviors in the SNI model suppressing the behavioral modifications observed in the NFS  
294 test (76.1 ± 52.7 s,  $p < 0.01$ ; **Fig. 3A**), in the TST (137.0 ± 23.4 s,  $p < 0.05$ ; *t*-test; **Fig. 3C**) and in  
295 the FST (152.5 ± 37.3 s,  $p < 0.01$ ; *t*-test; **Fig. 3E**). The same effect of ethosuximide was  
296 observed in the OIPN model submitted to the NSF test (251.3 ± 36.0 s,  $p < 0.001$ ; Mann-  
297 Whitney; **Fig. 3B**), the TST (116.5 ± 45.7 s,  $p < 0.0001$ ; Mann-Whitney; **Fig. 3D**) and the FST  
298 (146.4 ± 45.8 s,  $p < 0.001$ ; *t*-test; **Fig. 3F**).

299 Interestingly, in naïve mice, compared to the vehicle, ethosuximide reduced anxiety-  
300 and depression-like behaviors, in the EPM test (time in open arms: 6.7 ± 8.0 s vs 30.7 ± 20.9  
301 s,  $p < 0.01$ ; Mann-Whitney; entries number: 3.3 ± 2.8 vs 9.3 ± 3.6,  $p < 0.001$ ; *t*-test), in the  
302 NFS test (54.8 ± 20.9 s vs 35.6 ± 13.2 s to go to the center,  $p < 0.05$ ; *t*-test; 188.6 ± 34.5 s vs  
303 165 ± 18.4 s to eat,  $p = 0.078$ ; *t*-test), in the TST (151.8 ± 16.0 s vs 60.9 ± 43.5 s,  $p < 0.0001$ ;  
304 Mann-Whitney) and in the FST (243.0 ± 34.3 s vs 50.7 ± 47.2 s,  $p < 0.0001$ ; *t*-test) (**Suppl. Fig.**  
305 **1**).

306 Finally, the anti-depressive effect of ethosuximide was investigated in a murine  
307 model of depression (**Suppl. Fig. 2**). Compared to baseline, chronic restrain stress induced a  
308 statistical significant increase of immobility time (135.6 ± 22.7 s vs 176.1 ± 18.2,  $p < 0.001$ ; *t*-  
309 test; 167.6 ± 26.9 s vs 212.0 ± 36.4 s,  $p < 0.01$ ; *t*-test) in the TST (**Suppl. Fig. 2A**) and FST  
310 (**Suppl. Fig. 2B**), respectively. After stress, mice treated with ethosuximide showed a  
311 significant reduction of immobility time in both tests compared to vehicle (**Suppl. Fig. 2**).

312

### 313 **3.3. Ethosuximide reduced hypersensitivity, anxiety and depression-like disorders in the** 314 **context of chronic inflammatory pain**

315

316 Paw withdrawal threshold was decreased 14 days after CFA injection from 0.55 ±  
317 0.18 g to 0.17 ± 0.09 g for vehicle group and from 0.59 ± 0.15 g to 0.13 ± 0.09 g for  
318 ethosuximide group (**Fig. 4**). Ethosuximide administration after CFA significantly increased  
319 the PWT from 20 min to 60 min after the injection ( $p < 0.01$ ; 2-way ANOVA; **Fig. 4**) with a

320 maximum effect at 40 min (PWT of  $0.16 \pm 0.07$  g vs  $0.40 \pm 0.15$  g, vehicle vs ethosuximide,  $p$   
321  $< 0.001$ ; Sidak *post-hoc*; **Fig. 4**).

322 Related comorbidities were assessed with the same tests previously performed (EPM  
323 and time to go to center of NSF test for anxiety component and time to go to eat of NSF test,  
324 TST and FST for depressive component).

325 Firstly, CFA injection induced a development of anxiety-like behavior as evidenced by  
326 shorter time spent in the open arms in the EPM test ( $16.3 \pm 12.4$  s vs  $0.8 \pm 1.2$  s,  $p < 0.001$ ;  
327 Mann-Whitney; baseline vs post-CFA; **Fig. 5A**), reduction of entries in the open arms in the  
328 EPM test ( $8.9 \pm 3.8$  vs  $0.8 \pm 1.0$ ,  $p < 0.001$ ; Mann-Whitney; baseline vs post-CFA; **Fig. 5B**) and  
329 longer time to go to center in the NSF test ( $18.5 \pm 12.4$  s vs  $126.4 \pm 31.9$  s,  $p < 0.0001$ ; Mann-  
330 Whitney; baseline vs post-CFA; **Fig. 5C**). This anxiety-like behavior was reduced after  
331 injection of ethosuximide (EPM:  $7.3 \pm 4.6$  s in the open arms,  $p < 0.01$ ; Mann-Whitney;  $4.1 \pm$   
332  $2.5$  entries in the open arms,  $p < 0.05$ ; Mann-Whitney; NSF:  $11.0 \pm 5.3$  s to go to the center,  $p$   
333  $< 0.0001$ ; Mann-Whitney) compared to vehicle (**Fig. 5A, Fig 5B and Fig. 5C**).

334 Secondly, CFA injection increased the latency to eat (NFS:  $175.9 \pm 58.3$  s; **Fig. 5D**) and  
335 the immobility time (TST:  $198.6 \pm 25.6$  s; FST:  $230.4 \pm 22.9$  s; **Fig. 5E and Fig. 5F**) compared to  
336 baseline values. Such modifications indicated the development of depressive-like behavior in  
337 the CFA model. The treatment with ethosuximide reversed depressive-like behaviors,  
338 resulting in a decreased latency to eat (NFS:  $84.4 \pm 36.0$  s,  $p < 0.001$ ; Mann-Whitney; **Fig 5D**)  
339 and total immobilization time (TST:  $156.9 \pm 48.0$  s,  $p < 0.05$ ; *t*-test; and FST:  $174.5 \pm 75.5$  s,  $p$   
340  $= 0.07$ ; Mann-Whitney; **Fig. 5E and Fig. 5F**), compared to vehicle.

341 **DISCUSSION**

342

343 This work shows that ethosuximide, a non-specific T-type calcium channel blockers used in  
344 humans as an anticonvulsant (Coulter et al., 1990; Leresche et al., 1998; Todorovic and  
345 Lingle, 1998), associates an analgesic action with an effect on anxiety- and depression-like  
346 symptoms in different chronic pain conditions. These effects were observed with multiple  
347 chronic pain models of a different nature (neuropathic and inflammatory) and etiology  
348 (post-traumatic and post-chemotherapy neuropathy) and using multiple assessment  
349 modalities for both pain and comorbidities. This strategy for exploring animal emotional  
350 symptoms similar to those found in chronic pain patients with affective disorders  
351 (Demyttenaere et al., 2007; Dworkin and Gitlin, 1991; Global Burden of Disease Study 2013  
352 Collaborators, 2015; McWilliams et al., 2004; Price, 2000) has been used by other authors  
353 (For reviews, see Leite-Almeida et al., 2015; Yalcin et al., 2014) and recently in neuropathy  
354 induced by oxaliplatin (Hache et al., 2015) or paclitaxel (Toma et al., 2017), and makes  
355 studies clinically more relevant.

356

357 In our experimental conditions, ethosuximide reduced both chronic pain and comorbidities  
358 with a marked intensity ranging from partial reduction to complete suppression (**Table 1**). Its  
359 effect on the painful component recalls that previously obtained in animal models of  
360 neuropathic (Dogrul et al., 2003; Duggett and Flatters, 2017; Flatters and Bennett, 2004;  
361 Hamidi et al., 2012; Kawashiri et al., 2012; Okubo et al., 2011) and inflammatory (Barton et  
362 al., 2005; Cheng et al., 2007; Munro et al., 2007; Shannon et al., 2005) pain. In contrast, its  
363 beneficial effect of reducing emotional manifestations in a chronic pain context had not, to  
364 our knowledge, been previously documented. This effect is in line with results obtained with  
365 other anticonvulsants such as lamotrigine and valproate and pregabalin, which are approved  
366 in several countries for generalized anxiety disorder and which were found to have  
367 beneficial effects on depressive symptoms independently of their anticonvulsant efficacy  
368 (Edwards et al., 2001; Ettinger et al., 2007; Miller et al., 2008).

369

370 Thus, this broad spectrum of action of ethosuximide makes it a potential potentially  
371 interesting in the treatment of patients with chronic pain, whose management requires a  
372 holistic approach that in particular integrates comorbidities. The relationship between

373 depression and chronic pain is frequent and bi-directional in nature (Zhuo, 2016). Symptoms  
374 of depression or anxiety can exacerbate the severity of pain (Klauenberg et al., 2008;  
375 Ploghaus et al., 2001; Wilson et al., 2002), contribute to the persistence of chronic pain  
376 (Boogaard et al., 2011) and have an impact on response to treatments (Hider et al., 2009;  
377 Jamison et al., 2013; Wasan et al., 2015). Chronic pain therapy is thus not limited in clinical  
378 practice to the prescription of analgesics and treatments targeted at emotional  
379 manifestations are often necessary even if pain reduction by analgesics can, by itself, help to  
380 reduce emotional manifestations. In this respect, the decrease in nociceptive  
381 hypersensitivity achieved by ethosuximide in this study could have resulted from both the  
382 intrinsic effect of ethosuximide on nociception and the inhibition of comorbidities: the two  
383 mechanisms are not exclusive. We demonstrated that ethosuximide presented an anxiolytic-  
384 and an antidepressant-like effects in naïve mice and an antidepressant-like effect in a murine  
385 model of depression devoided of pain which plaid for an intrinsic action. However, this  
386 question remains to be more investigated.

387

388 The effects of ethosuximide observed in our study can be explained by its mobilization of  
389 low voltage activated, also called T-type, calcium channels. The location of these channels on  
390 nociceptive message transmission pathways or in message transfer and modulation  
391 structures (For reviews, see Bourinet et al., 2016; Snutch and Zamponi, 2018) could explain  
392 the analgesia induced by their inhibition. These channels are also largely expressed in the  
393 brain structures involved in anxiety and depression (Talley et al., 1999). However, few  
394 studies have explored the involvement of T-type channels in anxiety and the results are  
395 conflicting (Choi et al., 2007; Gangarossa et al., 2014). Among ionic channels, ethosuximide  
396 inhibits Cav3.1/3.2/3.3 channels, sodium and Ca<sup>2+</sup>-activated K<sup>+</sup> channels (Leresche et al.,  
397 1998; Todorovic and Lingle, 1998). The Cav3.2 isoform could be involved because Cav3.2  
398 channels play a role in pain (Okubo et al., 2011; Sekiguchi and Kawabata, 2013), anxiety  
399 (Gangarossa et al., 2014) and depression (Llinás et al., 1999). However, certain questions  
400 remain concerning the mechanism of the anxiolytic effect of ethosuximide following the  
401 publication of Gangarossa *et al.* which showed that Cav3.2 knock-out mice exhibited an  
402 anxious phenotype (Gangarossa et al., 2014).

403

404 Whatever the molecular mechanism in question, this study could allow a rapid clinical proof  
405 of concept by performing a study in patients suffering from chronic pain and related  
406 comorbidities. Given its adverse effects and the great sensitivity of chronic pain patients to  
407 certain drug therapies as recently reported for amitriptyline (Maarrawi et al., 2018), doses of  
408 ethosuximide should be accordingly adapted. This risk was observed in our recent clinical  
409 study, EDONOT (Kerckhove et al., 2017) evaluating ethosuximide in patients with peripheral  
410 neuropathic pain in which the high number of adverse events resulted in the discontinuation  
411 of the study during the interim analysis (59% of drop-outs rate). This major limitation made  
412 it impossible to evidence a significant difference from the control treatment despite that,  
413 with a per-protocol analysis, ethosuximide reduced significantly pain intensity (up to 20%  
414 after 4 weeks of treatment). Indeed, our post-hoc analyses of the EDONOT study  
415 (unpublished data), suggested a better tolerance and an analgesic action of low doses of  
416 ethosuximide (< 10 ml/day). This promising result and the results obtained here justify  
417 conducting a clinical study with reduced doses in order to validate the analgesic and anti-  
418 comorbidity effects of ethosuximide. In addition, for the longer term, the use of more  
419 specific Cav3.2 channel inhibitors could be an interesting therapeutic route.

420

421 Thus, our results make ethosuximide, and more broadly the inhibition of T-type calcium  
422 channels, a promising strategy for the management not only of uncontrolled chronic pain  
423 but also of the accompanying anxiety and depression.

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431

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433 The authors have no conflict of interest to declare.

434

435 **Author Contributions:**

436 Conceived and designed the experiments (AE, CM, NK, LD, JB, LB, GO), Performed the  
437 experiments (NK, JB, LB, GO), analyzed the data (CM, NK, GO, JB, LB), wrote the paper (AE,  
438 CM, NK, LD, LB, GO).

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717

718 **LEGENDS FOR FIGURES**

719

720 **Figure 1: Ethosuximide reduced neuropathic pain induced in SNI and OIPN models.** To  
721 assess the impact of ethosuximide (ETX, 200 mg/kg, i.p.) on mechanical hypersensitivity in  
722 neuropathic pain induced by the spared nerve injury (SNI) model (**A**) and on oxaliplatin-  
723 induced peripheral neuropathic pain (OIPN) model (**B**), von Frey test were performed. This  
724 test was assessed before the surgery (baseline) and after vehicle (Veh) or ETX treatments  
725 realized 21 days after the surgery. 50% paw withdrawal threshold (PWT) was determined  
726 using an adaptation of the Dixon up-down method. Area under the time-course (0-120 min)  
727 of PWT variations are represented in the right side. To assess the impact of ethosuximide on  
728 cold allodynia in OIPN model, the thermal place preference test was performed (**C**). This test  
729 was assessed before surgery (baseline) and after vehicle (Veh) or ETX treatments realized  
730 five days after the last injection of oxaliplatin. Mice were placed for 3 min in an arena  
731 containing two identical platforms, one at 25°C and one at 23°C, and the percentage of time  
732 spent at 25°C was measured. Data are shown as mean  $\pm$  SD ( $n = 10$  per group). **A and B:**  $***p < 0.001$ ,  $****p < 0.0001$ ; two way ANOVA followed by Sidak's *post hoc* test; *t*-test or Mann-  
733 Whithney for AUC mean comparing. **C :**  $***p < 0.001$ , compared with the baseline / vehicle  
734 group,  $####p < 0.001$ , compared with the OIPN / vehicle group; *t*-test.

736

737 **Figure 2: Ethosuximide reduced anxiety-like behavior in SNI and OIPN models.** Two tests  
738 were performed, the elevated plus maze test (**A and C**) in which the total time spent in the  
739 anxious area (open arms (**left**)) and the and the number of entries in the open arms (**right**)  
740 were recorded over 5 min; and (**B and D**) the novelty suppress feeding test (anxious  
741 modality) in which the time to reach the center of the arena (anxious zone) was measured,  
742 to assess the impact of ethosuximide (ETX, 200 mg/kg, i.p.) on anxiety-like behavior  
743 developed in (**A and B**) spared nerve injury (SNI) and (**C and D**) oxaliplatin-induced peripheral  
744 neuropathy (OIPN) models. In the SNI model, tests were performed before the surgery  
745 (baseline) and after vehicle (Veh) or ETX treatments realized 21 days after the surgery. In the  
746 OIPN model, tests were performed before the first oxaliplatin injection (baseline) and after  
747 vehicle (Veh) or ETX treatments realized five days after the last injection of oxaliplatin. Data  
748 are shown as mean  $\pm$  SD ( $n = 10$  per group).  $*p < 0.05$ ,  $**p < 0.01$ ,  $****p < 0.0001$ , compared

749 with the baseline / vehicle group, # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  compared with the SNI or  
750 OIPN / vehicle group; Mann-Whitney test.

751

752 **Figure 3: Ethosuximide reduced the depressive-like behavior in SNI and OIPN mice models.**

753 To assess the impact of ethosuximide (ETX, 200 mg/kg, i.p.) on depressive-like behaviors in  
754 (A, C and E) spared nerve injury (SNI) and in (B, D, and F) oxaliplatin-induced peripheral  
755 neuropathy (OIPN) models, various tests were performed: (A and B) the novelty suppressed  
756 feeding test (latency to feed, depressive modality), (C and D) the tail suspension test (time of  
757 total immobility) and (E and F) the forced swimming test (time of total immobility). In the  
758 SNI model, tests were performed before the surgery (baseline) and after vehicle (Veh) or ETX  
759 treatments realized 21 days after the surgery. In the OIPN model, tests were performed  
760 before the first oxaliplatin injection (baseline) and after vehicle (Veh) or ETX treatments  
761 realized five days after the last injection of oxaliplatin. Data are shown as mean  $\pm$  SD (n = 10  
762 per group). \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  compared with the baseline / vehicle  
763 group, # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$ , compared with the SNI or OIPN / vehicle group;  
764 Mann-Whitney or *t*-test.

765

766 **Figure 4: Ethosuximide relieved nociceptive behavior of mice in a chronic inflammatory**  
767 **context induced by CFA.** The effect of ethosuximide (ETX, 200 mg/kg, i.p.) on chronic

768 inflammatory pain (monoarthritic model, 14 days after periarticular injection of CFA) was  
769 evaluated by the von Frey test to evidence the symptoms of mechanical hypersensitivity (A).  
770 This test was assessed before CFA injection (baseline) and after vehicle (Veh) or ETX  
771 treatments realized 14 days after CFA injection. 50% paw withdrawal threshold (PWT) was  
772 determined using an adaptation of the Dixon up-down method. Area under the time-course  
773 (0-120 min) of PWT variations are represented in the right side. Data are shown as mean  $\pm$   
774 SD (n = 10 per group). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; two way ANOVA followed by  
775 Sidak's *post hoc* test and *t*-test for AUC mean comparing.

776

777 **Figure 5: Ethosuximide abolished the anxiety and depressive-like behavior resulting from**  
778 **chronic inflammatory pain induced by CFA.** To assess the impact of ethosuximide (ETX, 200

779 mg/kg, i.p.) on anxiety-like behavior developed during chronic inflammatory pain  
780 (monoarthritic model, 14 days after periarticular injection of CFA), two tests were

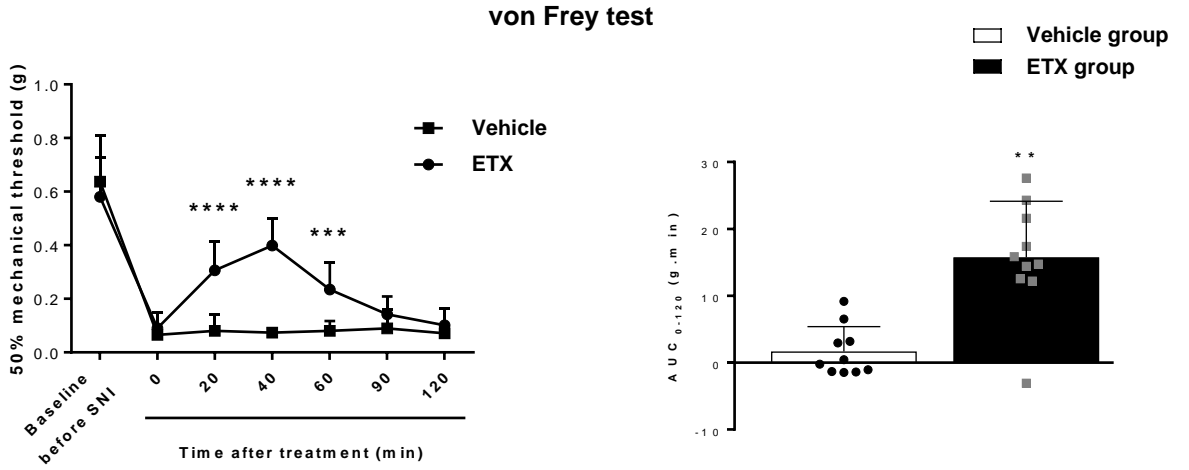
781 performed: the elevated plus maze test in which the total time spent in the anxious area  
782 (open arms) (A) and the and the number of entries in the open arms (B) were recorded over  
783 5 min; and (C) the novelty suppress feeding test (anxious modality) in which the time to go  
784 to the center of arena was measured. To assess the effect of ethosuximide (ETX, 200 mg/kg,  
785 i.p.) on depressive-like suppress feeding test (latency to feed, depressive modality), (E) the  
786 tail suspension test (time behaviors, (D) the novelty of total immobility) and (F) the forced  
787 swimming test (time of total immobility) were performed before (baseline) and 14 days after  
788 CFA injection. All tests were assessed before CFA injection (baseline) and after vehicle (Veh)  
789 or ETX treatments realized 14 days after CFA injection. Data are shown as mean  $\pm$  SD (n = 10  
790 *per group*). \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001, compared with the baseline / vehicle group, # $p$  <  
791 0.05, ## $p$  < 0.01, ### $p$  < 0.001, #### $p$  < 0.0001 compared with the CFA / vehicle group; Mann-  
792 Whitney or *t*-test.

793

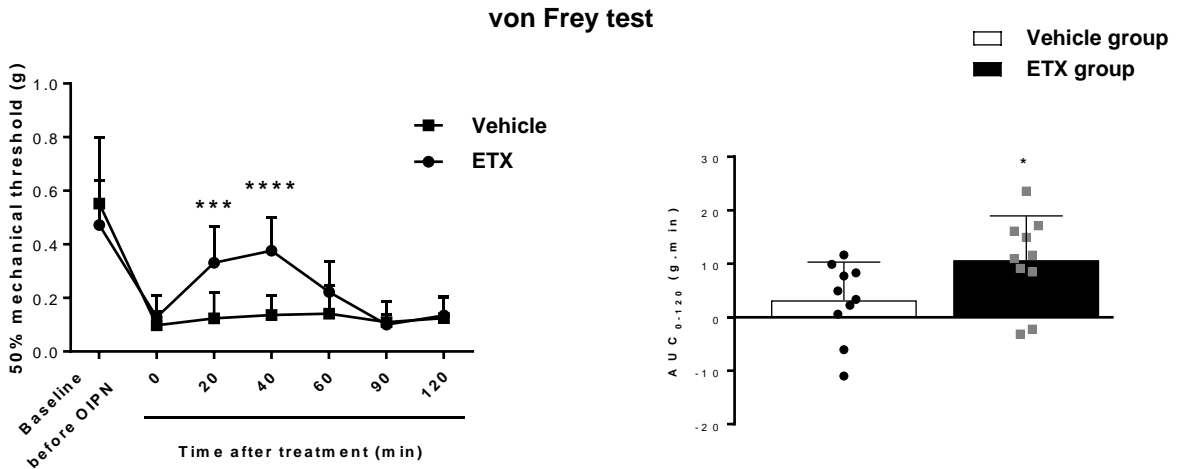
794 **Table 1: Ethosuximide reduced hypersensitivity, anxiety and depressive-like behaviors**  
795 **resulting from neuropathic pain and chronic inflammatory pain.** This table summarizes the  
796 effect of ethosuximide on nociception, anxiety and depressive-like behaviors in two models  
797 of neuropathic pain (SNI and OIPN) and one model of chronic inflammatory pain (CFA),  
798 expressed as percent of reduction. Nociception was assessed by the von Frey test or the  
799 thermal place preference test. Anxiety-like behavior was assessed by the elevated plus maze  
800 test and the novelty suppressed feeding test (time to reach the center). Depressive-like  
801 behavior was evaluated with the novelty suppressed feeding test (time to eat), the tail  
802 suspension test and the forced swimming test.

# Figure 1

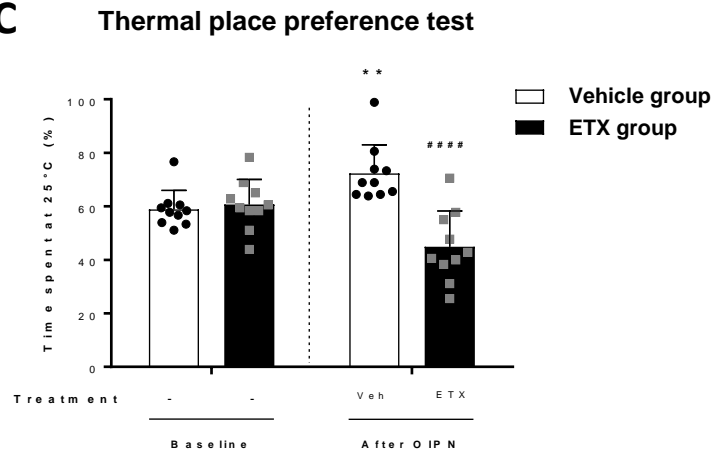
**A**



**B**



**C**



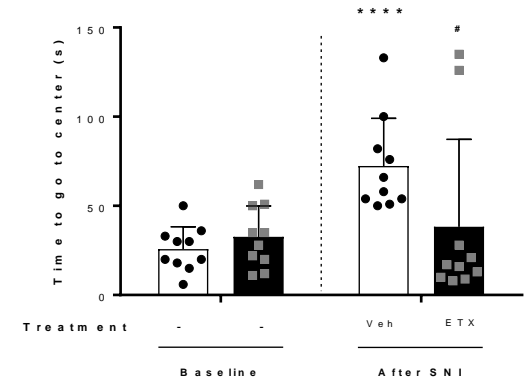
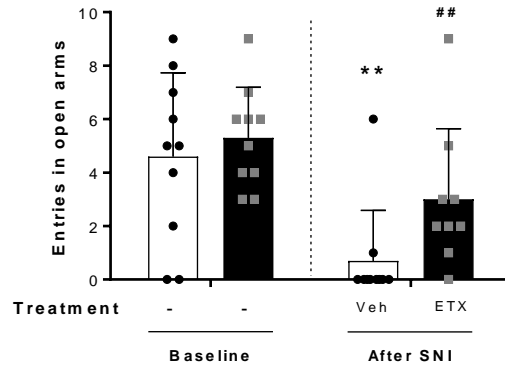
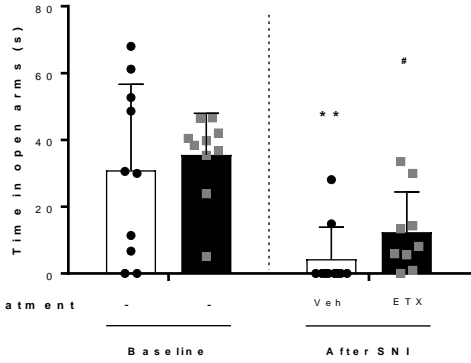
# Figure 2

## Elevated plus maze test

## Novelty suppressed feeding test

**A**

**B**

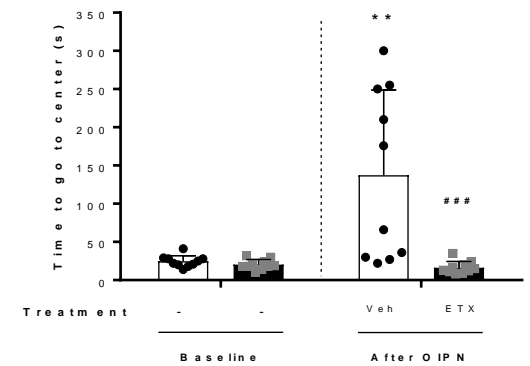
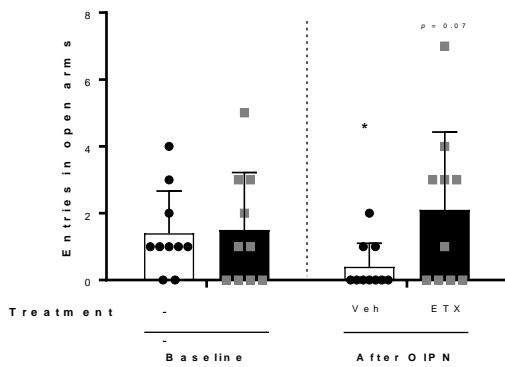
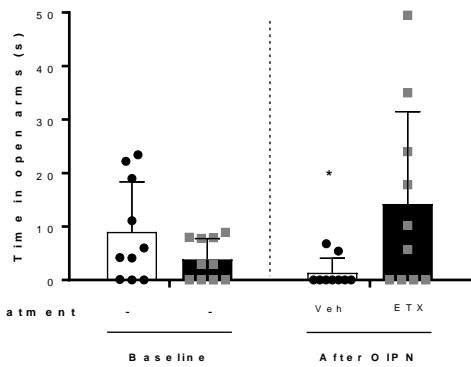


## Elevated plus maze test

## Novelty suppressed feeding test

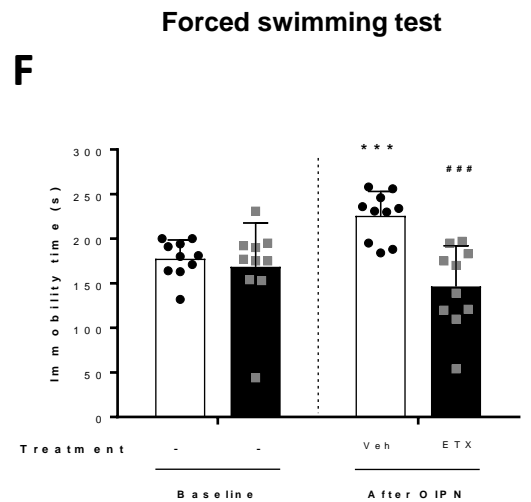
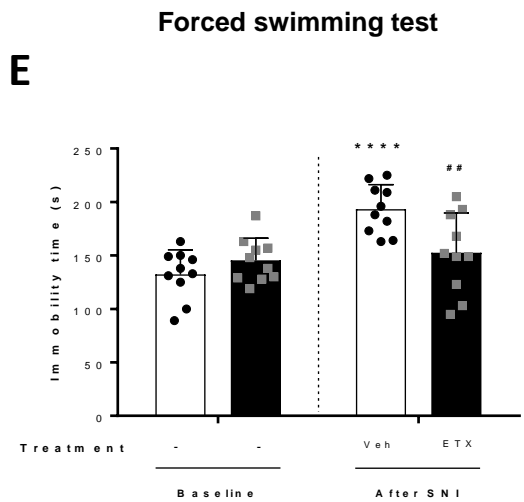
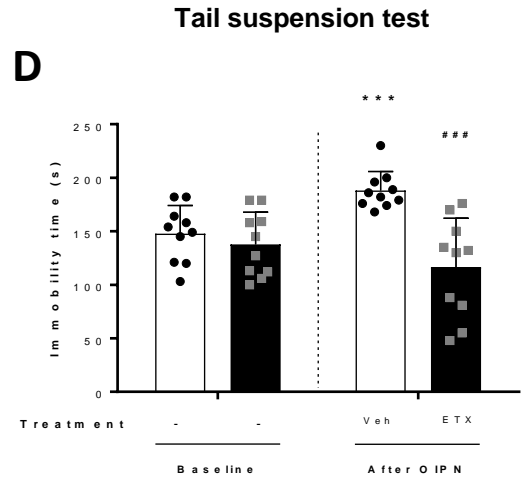
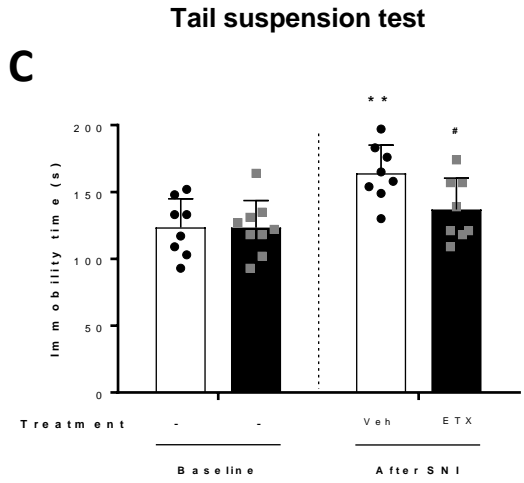
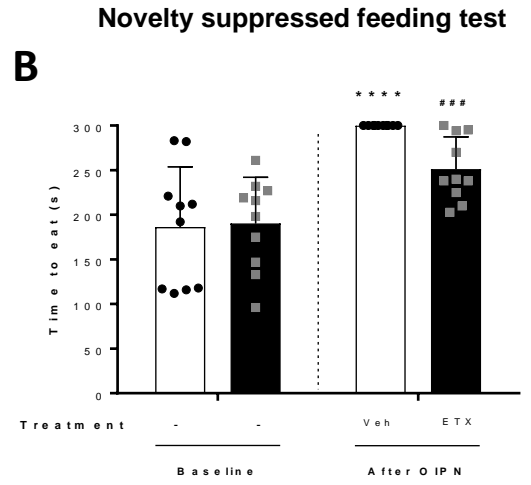
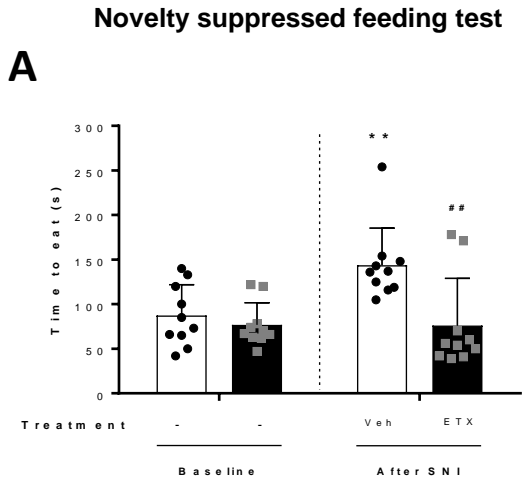
**C**

**D**

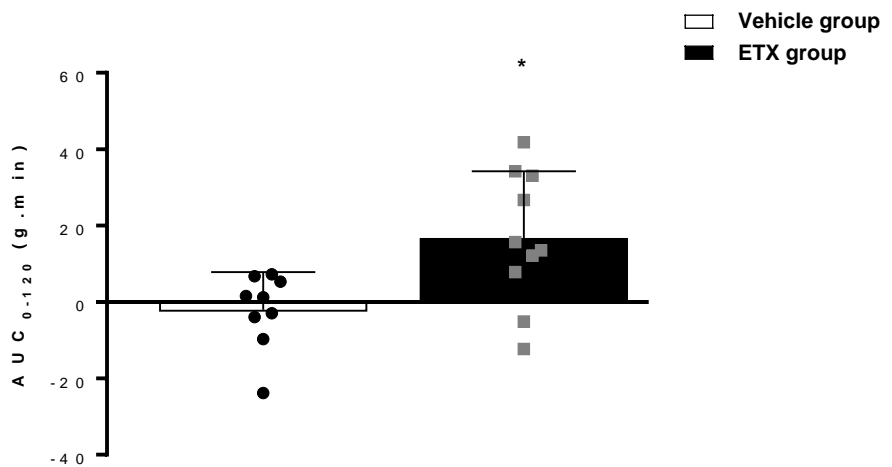
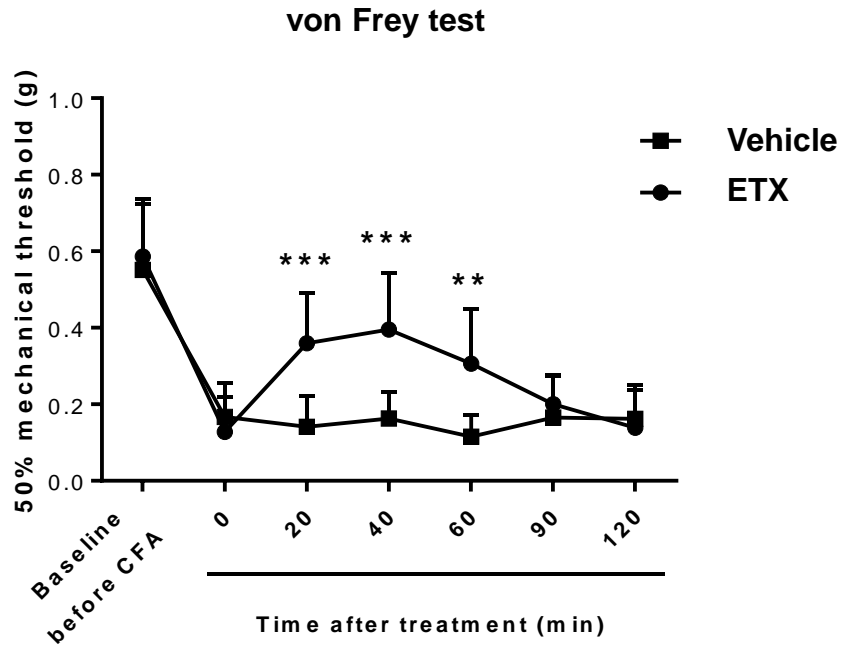


# Figure 3

□ Vehicle group  
■ ETX group



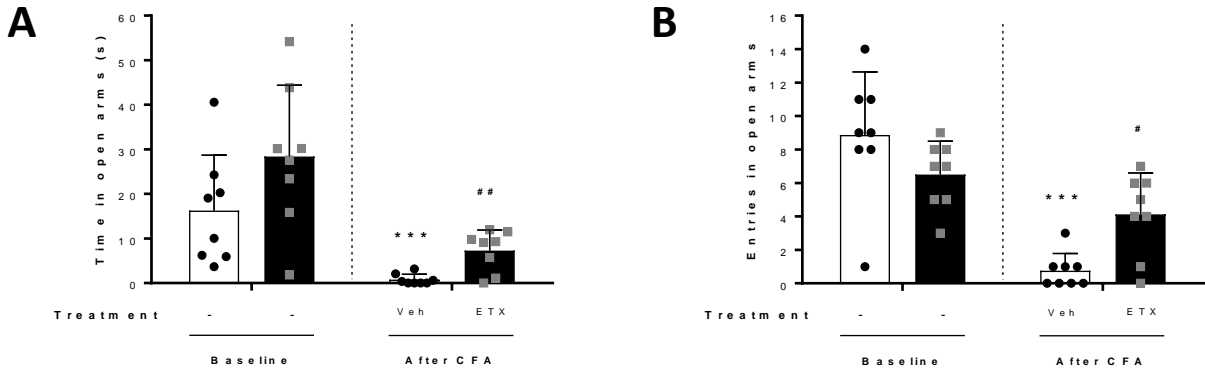
# Figure 4



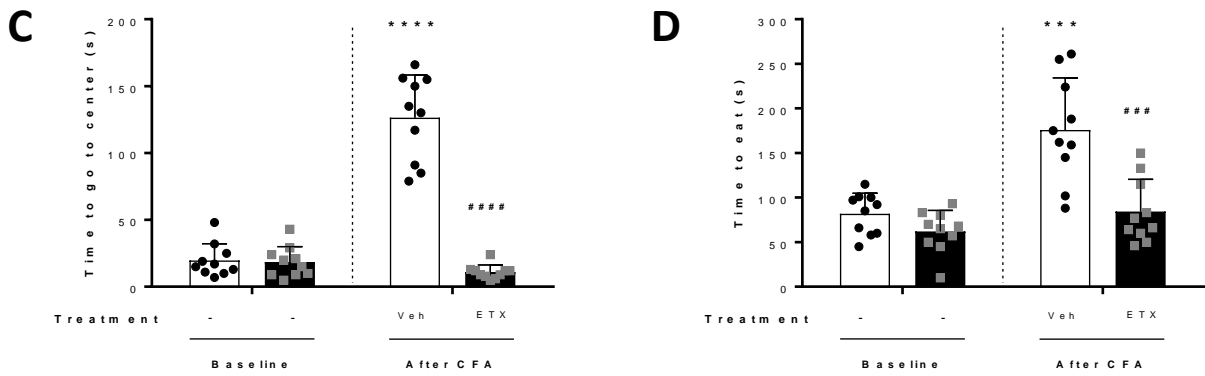
# Figure 5

□ Vehicle group  
■ ETX group

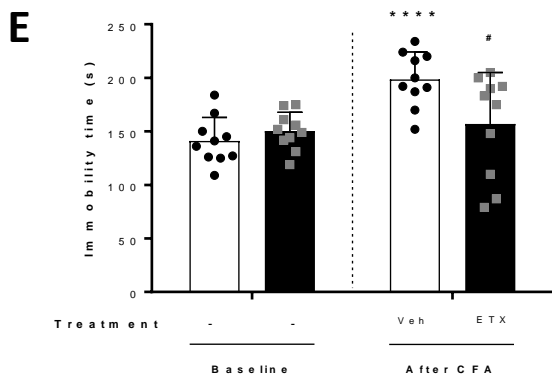
## Elevated plus maze test



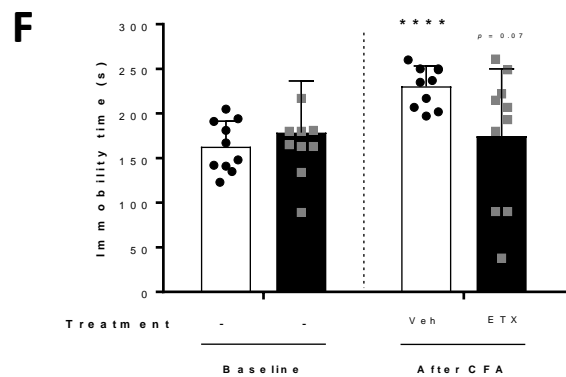
## Novelty suppressed feeding test



## Tail suspension test



## Forced swimming test



**Table 1**

Component	Models Tests	Chronic neuropathic pain		Chronic inflammatory pain
		SNI	OIPN	CFA
Nociception	Von Frey test (at 40 min)	- 68.4%	- 83.6%	- 50.0%
	Thermal place preference	/	Complete	/
Anxiety-like behavior	Elevated plus maze test	- 13.2%	Complete	- 36.1%
	Novelty suppressed feeding test (anxiety modality)	- 87.8%	Complete	Complete
Depressive-like behavior	Novelty suppressed feeding (depressive modality)	Complete	- 46.4%	- 76.3%
	Tail suspension test	- 66.2%	Complete	- 88.5%
	Forced swimming test	- 88.3%	Complete	Complete