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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 μ 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 ± 0.17 g
233 to 0.07 ± 0.02 g for the vehicle group and from 0.58 ± 0.15 g to 0.09 ± 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 ± 0.10 g vs 0.07 ± 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 ± 0.25 g to 0.10 ± 0.05 g for vehicle group and
240 from 0.47 ± 0.17 g to 0.13 ± 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 ± 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 ± 25.8 s to 4.3 ± 9.6 s in SNI ($p < 0.01$; Mann-Whitney; **Fig. 2A**
261 **left**) and from 9.0 ± 9.3 s to 1.4 ± 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 ± 26.7 s, $p < 0.001$; OIPN: 137.2 ± 111.6 s, $p <$
265 0.01 ; Mann-Whitney; **Fig. 2B and Fig. 2D**) compared to their respective baseline (25.8 ± 12.5
266 s and 24.6 ± 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 ± 11.9 s, $p < 0.05$; Mann-Whitney; **Fig. 2A left**) and in OIPN
269 (14.2 ± 17.3 s, $p < 0.05$; Mann-Whitney; **Fig. 2C left**) compared to vehicle (SNI: 4.3 ± 9.5 s and
270 OIPN: 1.4 ± 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 ± 49.0 s) and the OIPN (16.3 ± 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 ± 34.5 s vs 143.7 ± 41.7 s, $p < 0.01$, baseline vs post-
285 SNI; OIPN: 186.3 ± 67.4 s vs 300.0 ± 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 ± 21.4 s vs 164.0 ± 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 ± 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 ± 0.07 g vs 0.40 ± 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 ± 12.4 s vs 0.8 ± 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 ± 3.8 vs 0.8 ± 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 ± 12.4 s vs 126.4 ± 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 ± 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 ± 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 ± 58.3 s; **Fig. 5D**) and
335 the immobility time (TST: 198.6 ± 25.6 s; FST: 230.4 ± 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 ± 36.0 s, $p < 0.001$; Mann-Whitney; **Fig 5D**)
339 and total immobilization time (TST: 156.9 ± 48.0 s, $p < 0.05$; *t*-test; and FST: 174.5 ± 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 Ca²⁺-activated K⁺ 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
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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.**

768 The effect of ethosuximide (ETX, 200 mg/kg, i.p.) on chronic
769 inflammatory pain (monoarthritic model, 14 days after periarticular injection of CFA) was
770 evaluated by the von Frey test to evidence the symptoms of mechanical hypersensitivity (A).
771 This test was assessed before CFA injection (baseline) and after vehicle (Veh) or ETX
772 treatments realized 14 days after CFA injection. 50% paw withdrawal threshold (PWT) was
773 determined using an adaptation of the Dixon up-down method. Area under the time-course
774 (0-120 min) of PWT variations are represented in the right side. Data are shown as mean \pm
775 SD (n = 10 per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two way ANOVA followed by
776 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.**

779 To assess the impact of ethosuximide (ETX, 200
780 mg/kg, i.p.) on anxiety-like behavior developed during chronic inflammatory pain
(monoarthritic model, 14 days after periarticular injection of CFA), two tests were

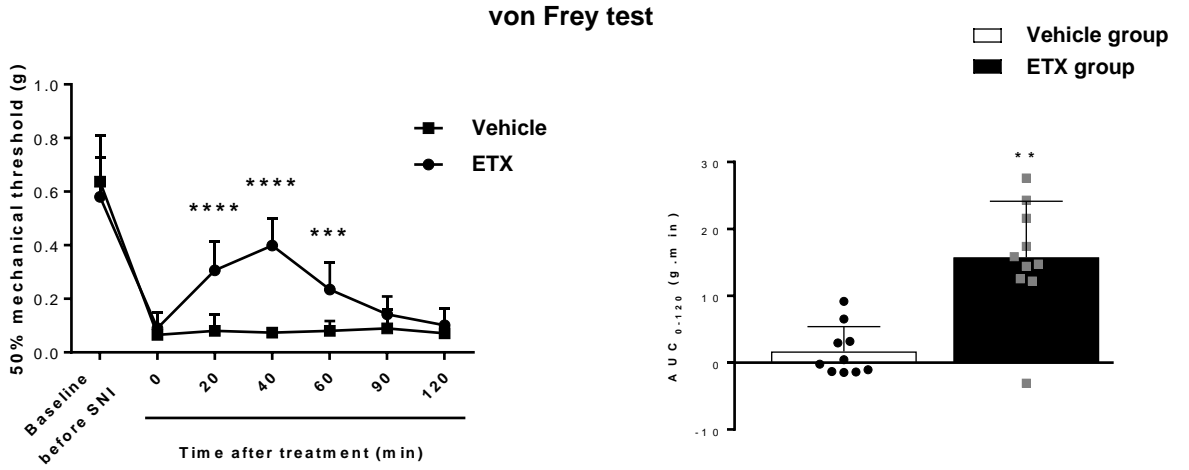
781 performed: the elevated plus maze test in which the total time spent in the anxious area
782 (open arms) **(A)** and the and the number of entries in the open arms **(B)** were recorded over
783 5 min; and **(C)** the novelty suppress feeding test (anxious modality) in which the time to go
784 to 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

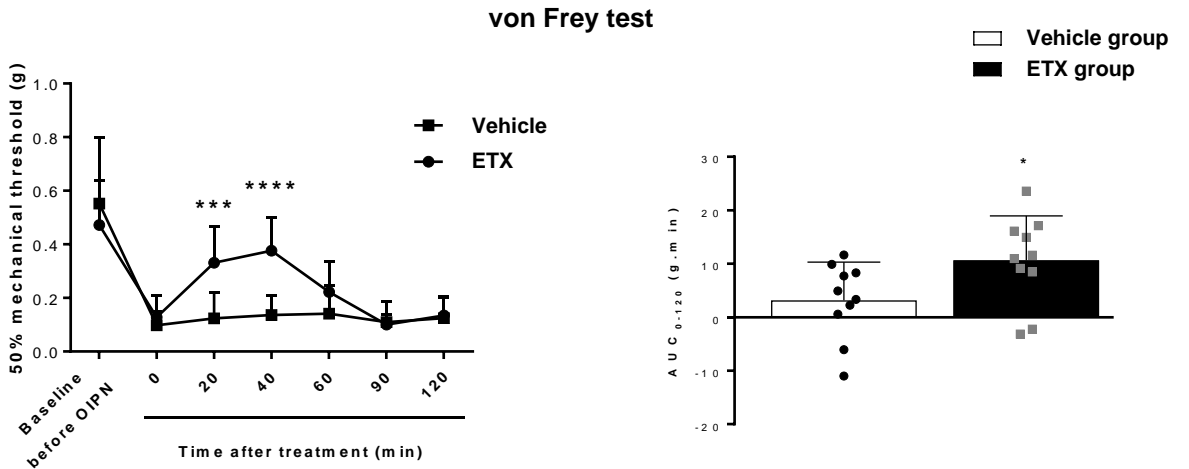
794 **Table 1: Ethosuximide reduced hypersensitivity, anxiety and depressive-like behaviors**
795 **resulting from neuropathic pain and chronic inflammatory pain.** This table summarizes the
796 effect of ethosuximide on nociception, anxiety and depressive-like behaviors in two models
797 of neuropathic 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

Thermal place preference test

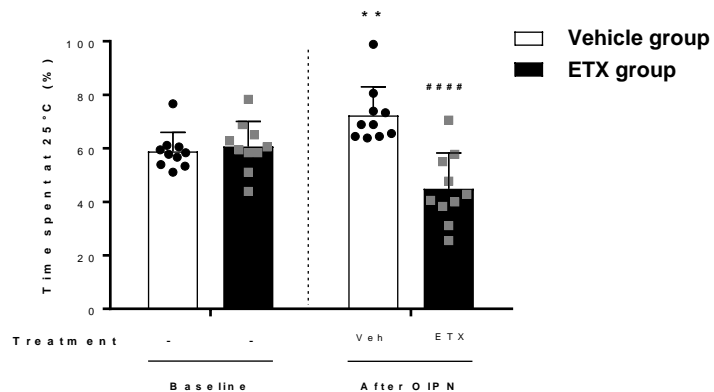


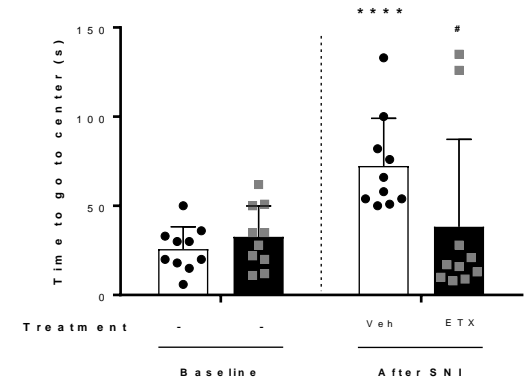
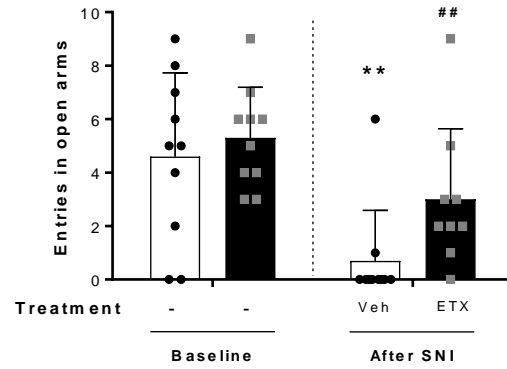
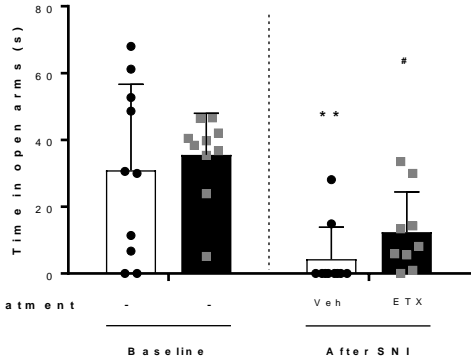
Figure 2

Elevated plus maze test

Novelty suppressed feeding test

A

B



Elevated plus maze test

Novelty suppressed feeding test

C

D

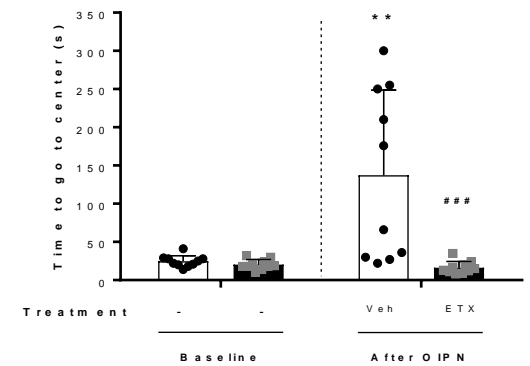
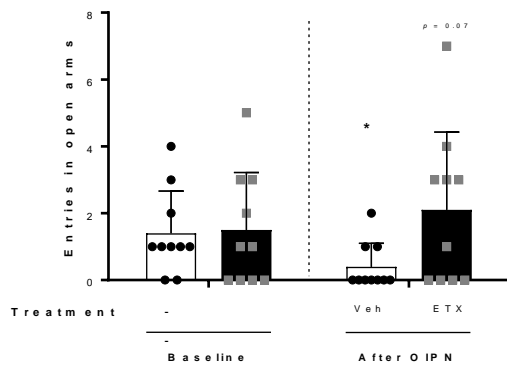
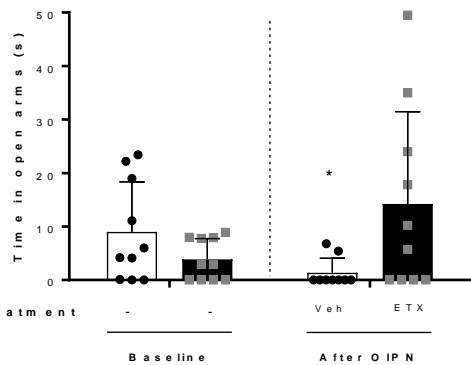


Figure 3

□ Vehicle group
■ ETX group

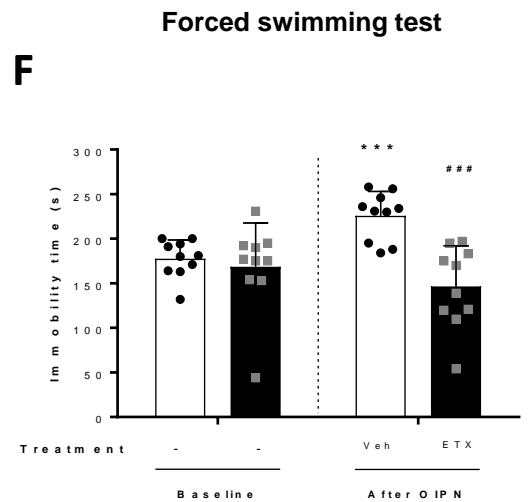
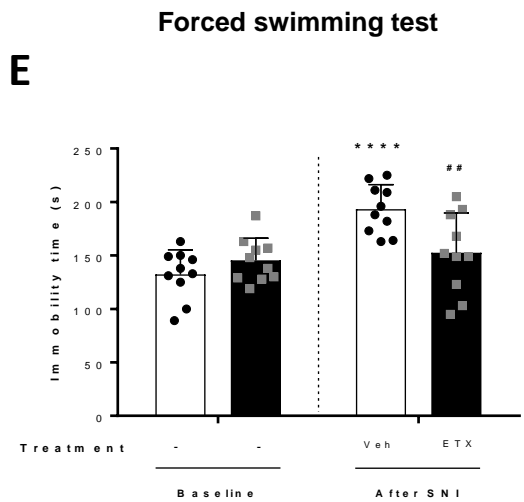
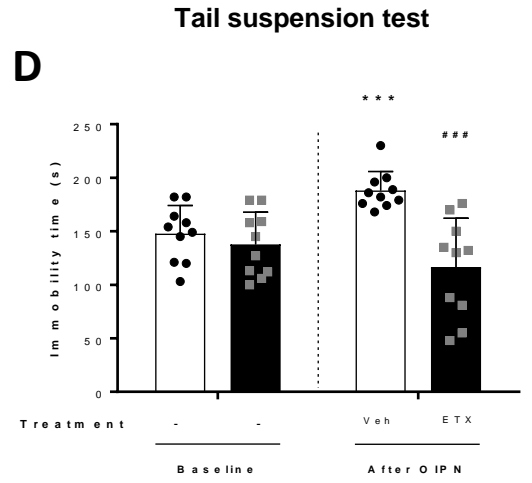
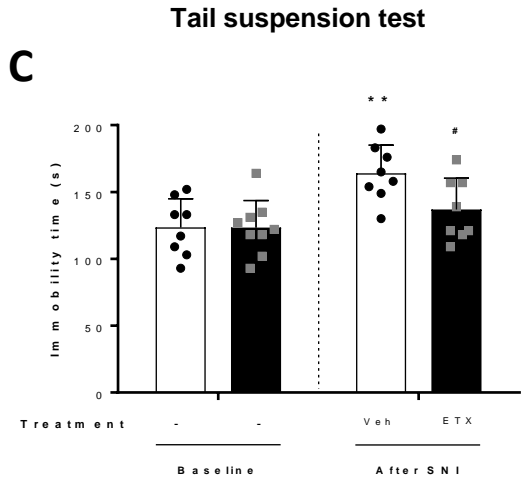
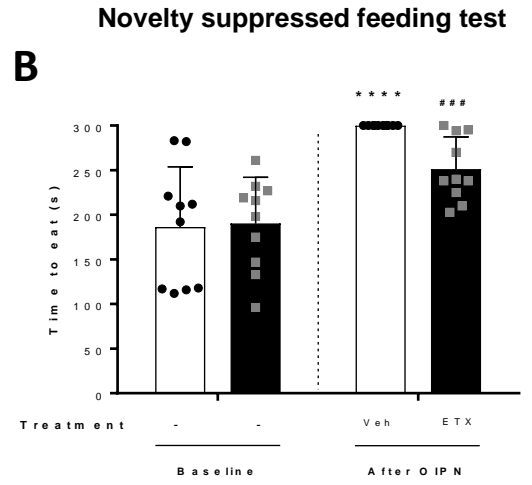
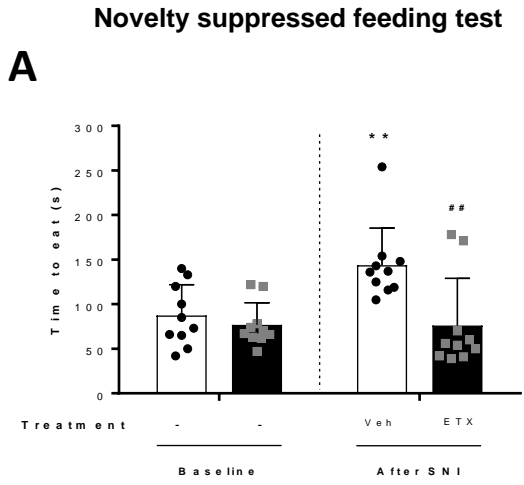


Figure 4

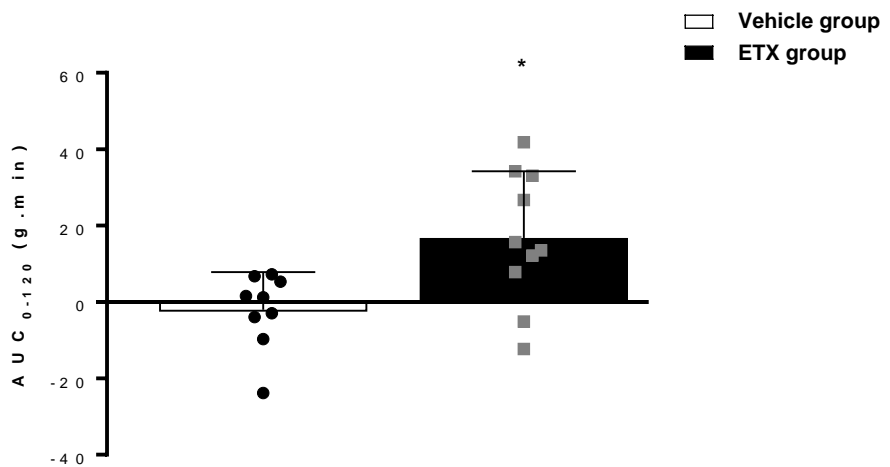
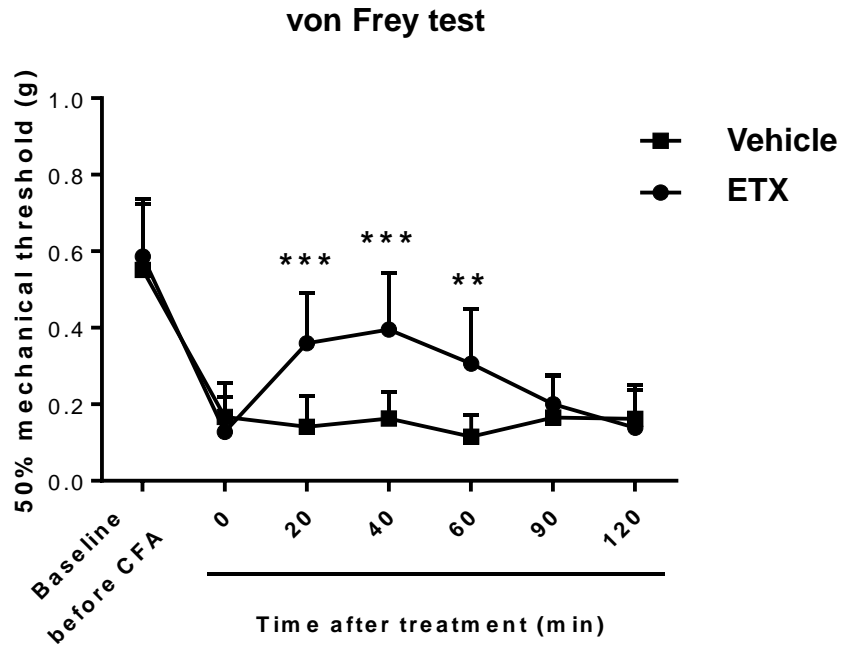
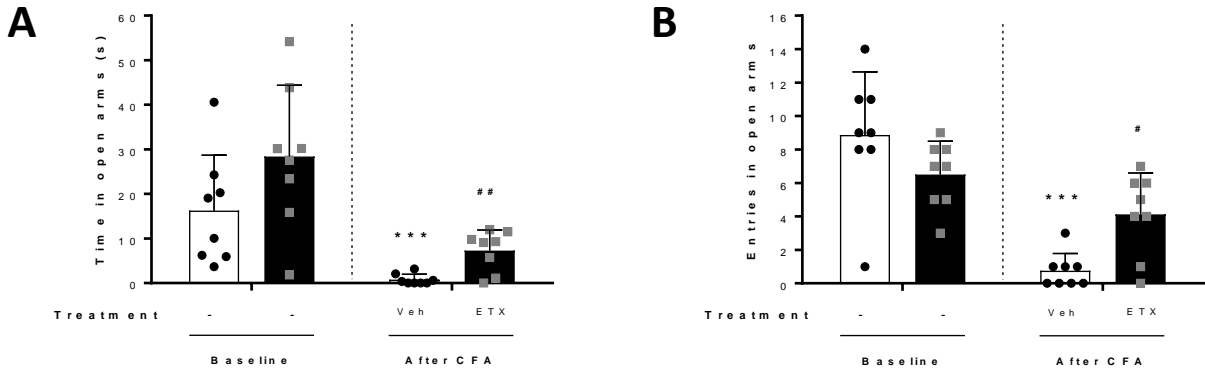


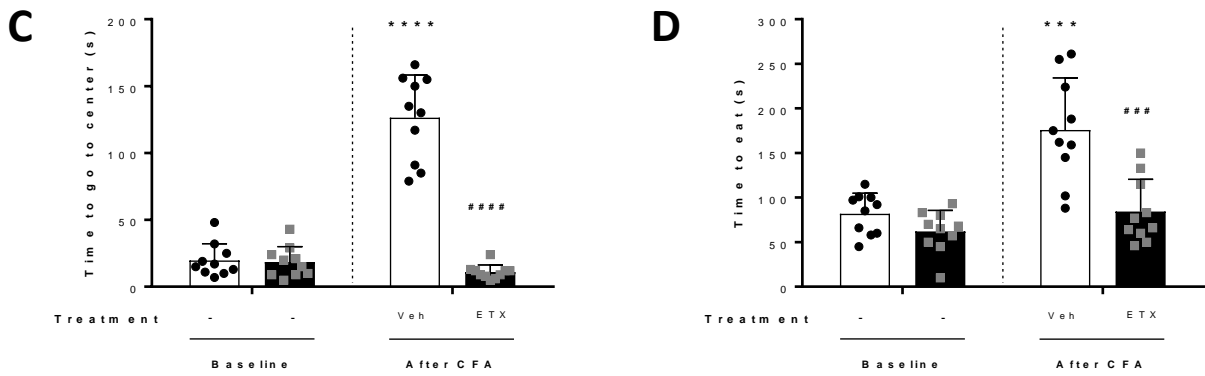
Figure 5

□ Vehicle group
■ ETX group

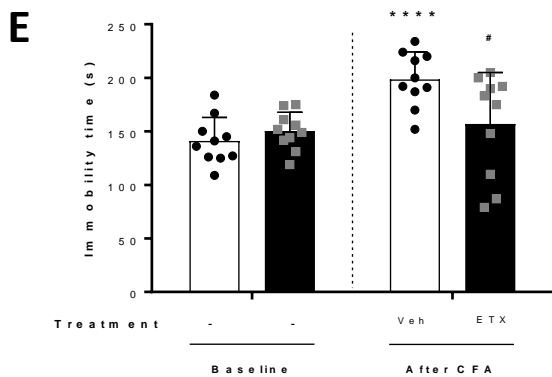
Elevated plus maze test



Novelty suppressed feeding test



Tail suspension test



Forced swimming test

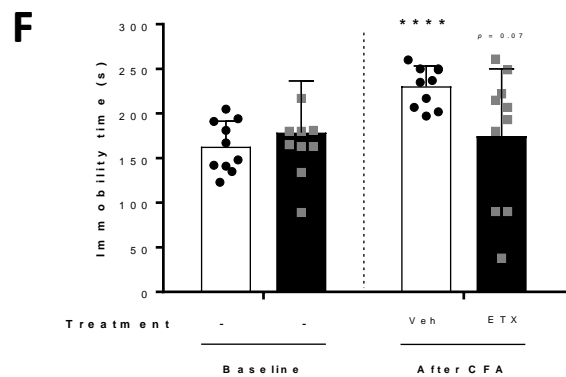


Table 1

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