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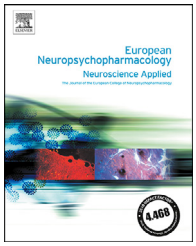

Ethosuximide improves chronic pain-induced anxiety- and depression-like behaviors

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Abstract
Chronic pain is a heavy burden disease. Current treatments are generally weakly effective or associated with adverse effects. New therapeutic approaches are therefore needed. Recent studies have suggested T-type calcium channels as an attractive target for the treatment of chronic pain. In this perspective, it was decided to perform a preclinical evaluation of the efficacy of ethosuximide, a T-type channel blocker used clinically as an antiepileptic, as a novel pharmacological treatment for chronic pain. Assessment of the effect of ethosuximide was thus made in both nociception and pain-related comorbidities as anxiety and depression are frequently encountered in chronic pain patients. Our results show that such symptoms occurred in three animal models of chronic pain designed to reflect traumatic neuropathic, chemotherapy-induced neuropathic and inflammatory pain conditions. Administration of ethosuximide reduced both chronic pain and comorbidities with a marked intensity ranging from partial reduction to a complete suppression of symptoms. These results make ethosuximide, and more broadly the inhibition of T-type calcium channels, a new strategy for the

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

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management of uncontrolled chronic pain, likely to improve not only pain but also the accompanying anxiety and depression.

1. Introduction

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

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

Several studies have shown the interest of T-type calcium channels in different pain condition (Choi et al., 2007; Francois et al., 2013; Snutch and Zamponi, 2018; Picard et al., 2019), especially neuropathic (Bourinet et al., 2016) and inflammatory pain (Kerckhove et al., 2014). Ethosuximide, a non-specific blocker of T-type channels, has an analgesic effect on neuropathic (Dogrul et al., 2003; Flatters and Bennett, 2004; Hamidi et al., 2012; Kawashiri et al., 2012; Okubo et al., 2011) and inflammatory pain (Barton et al., 2005; Cheng et al., 2007; Munro et al., 2007; Shannon et al., 2005), but no study has yet assessed its effect, and more broadly that of T-type calcium channel inhibition, on anxiety and depression resulting from chronic pain.

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

2. Materials and methods

2.1. Animals

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

2.2. Products

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

2.3. Animal models

2.3.1. Spared nerve injury (SNI) model

Mice were anesthetized with i.p. injection of xylazine hydrochloride (10 mg/kg) and ketamine (100 mg/kg). The tibial and peroneal nerves were ligatured with non-resorbable suture. One centimeter

of nerve was cut distal of the ligature (Shields et al., 2003). The sural nerve was kept intact. The behaviors tests were performed before and 21 days after surgery.

2.3.2. Oxaliplatin-Induced peripheral neuropathic (OIPN) pain model

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

2.3.3. Monoarthritic model

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

2.3.4. Restraint stress-induced depression model

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

2.4. Behavioral tests

2.4.1. von Frey test

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

2.4.2. Thermal place preference

Mice were placed in an arena containing two identical platforms placed side by side such that the adjacent thermal surfaces at different temperatures were enclosed in a single chamber (32 × 16 cm, Bioseb, France) (Pereira et al., 2014). The temperature of one platform was maintained at 25 °C and the other at 23 °C. The movement of the mice between the two plates was recorded with a video tracking system over 3 min and the parameter evaluated was the time spent on each platform. The percentage of time spent at 25 °C was recorded.

2.4.3. Elevated plus maze (EPM) test

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

2.4.4. Tail suspension test (TST)

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

2.4.5. Forced swimming test (FST)

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

2.4.6. Novelty suppressed feeding (NSF) test

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

2.5. Experimental protocol

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

2.6. Statistical analysis

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

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

Component		Chronic neuropathic pain		Chronic inflammatory pain
	Tests	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

3. Results

3.1. Ethosuximide reduced hypersensitivity in two different neuropathic pain models

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

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

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

We first investigated the effect of ethosuximide on the anxiety-like behavior by measuring the time spent and the number of entries in open arms in the elevated plus maze (EPM) test and the time needed to reach the center of the arena in the novelty suppressed feeding (NSF) test were measured.

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

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

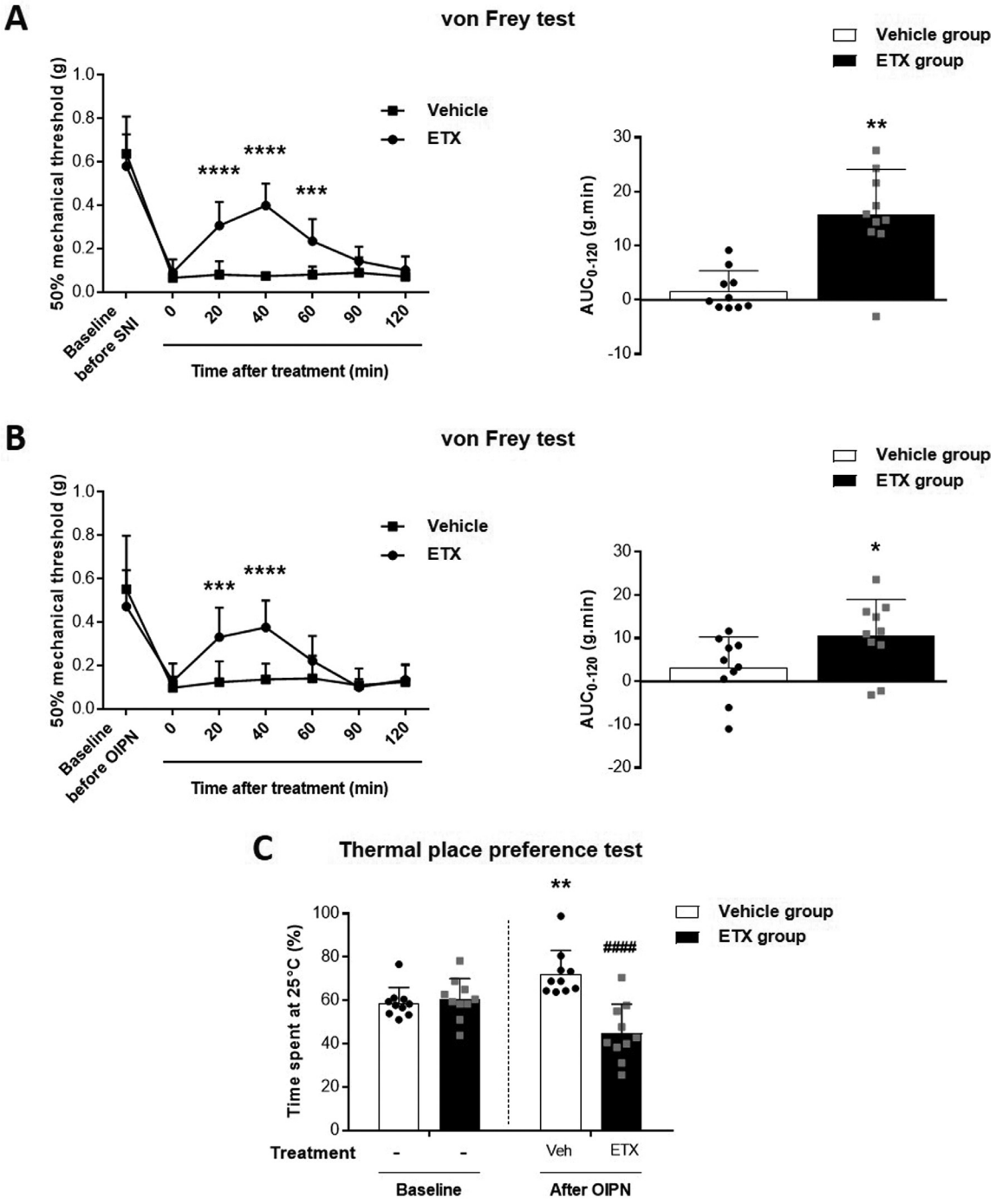

Fig. 1 Ethosuximide reduced neuropathic pain induced in SNI and OIPN models. To assess the impact of ethosuximide (ETX, 200 mg/kg, i.p.) on mechanical hypersensitivity in neuropathic pain induced by the spared nerve injury (SNI) model (**A**) and on oxaliplatin-induced peripheral neuropathic pain (OIPN) model (**B**), von Frey test were performed. This test was assessed before the surgery (baseline) and after vehicle (Veh) or ETX treatments realized 21 days after the surgery. 50% paw withdrawal threshold (PWT) was determined using an adaptation of the Dixon up–down method. Area under the time-course (0–120 min) of PWT variations are represented in the right side. To assess the impact of ethosuximide on cold allodynia in OIPN model, the thermal place preference test was performed (**C**). This test was assessed before surgery (baseline) and after vehicle (Veh) or ETX treatments realized five days after the last injection of oxaliplatin. Mice were placed for 3 min in an arena containing two identical platforms, one at 25 °C and one at 23 °C, and the percentage of time spent at 25 °C was measured. Data are shown as mean ± 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–Whithney for AUC mean comparing. **C**: $^{***}p < 0.001$, compared with the baseline/vehicle group, $^{\#\#\#}p < 0.001$, compared with the OIPN/vehicle group; *t*-test.

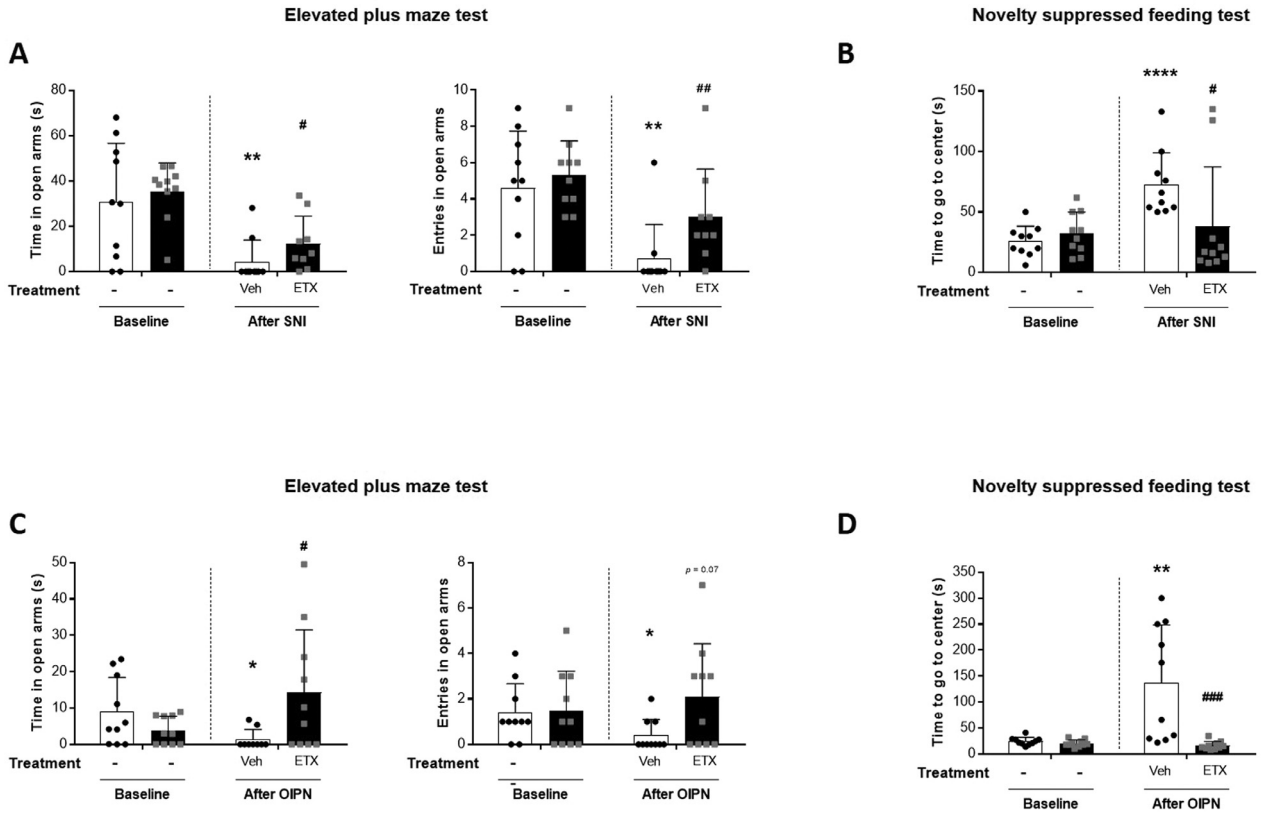

Fig. 2 Ethosuximide reduced anxiety-like behavior in SNI and OIPN models. Two tests were performed, the elevated plus maze test (**A and C**) in which the total time spent in the anxious area (open arms (**left**)) and the and the number of entries in the open arms (**right**) were recorded over 5 min; and (**B and D**) the novelty suppress feeding test (anxious modality) in which the time to reach the center of the arena (anxious zone) was measured, to assess the impact of ethosuximide (ETX, 200 mg/kg, i.p.) on anxiety-like behavior developed in (**A and B**) spared nerve injury (SNI) and (**C and D**) oxaliplatin-induced peripheral neuropathy (OIPN) models. In the SNI model, tests were performed before the surgery (baseline) and after vehicle (Veh) or ETX treatments realized 21 days after the surgery. In the OIPN model, tests were performed before the first oxaliplatin injection (baseline) and after vehicle (Veh) or ETX treatments realized five days after the last injection of oxaliplatin. Data are shown as mean ± SD ($n = 10$ *per* group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, compared with the baseline/vehicle group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the SNI or OIPN/vehicle group; Mann–Whitney test.

ability of ethosuximide in this test to reverse anxiety-like symptoms.

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

Compared to baseline, mice submitted to SNI surgery or treated with oxaliplatin took 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-SNI; OIPN: 186.3 ± 67.4 s *vs* 300.0 ± 0.0 s, $p < 0.0001$; Mann–Whitney; baseline *vs* post-OIPN; Fig. 3(A) and (B)). In addition, we observed an increased immobility time in the TST (SNI: 123.5 ± 21.4 s *vs* 164.0 ± 21.1 s, $p < 0.01$; *t*-test; baseline *vs* post-SNI, Fig. 3(C); OIPN: 147.8 ± 26.4 s *vs* 188.0 ± 17.7 s, $p < 0.001$; *t*-test; baseline *vs* post-OIPN; Fig. 3(D)) and in the FST (SNI: 132.4 ± 22.9 s *vs* 193.3 ± 22.9 s, $p < 0.0001$; *t*-test; baseline *vs* post-SNI, Fig. 3(E); OIPN: 177.6 ± 21.1 s *vs* 225.8 ± 27.3 s, $p < 0.001$; *t*-test; baseline *vs* post-OIPN, Fig. 3(F)). These results indicated depression-like behavior in both SNI and OIPN models.

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

Interestingly, in naïve mice, compared to the vehicle, ethosuximide reduced anxiety- and depression-like behaviors, in the EPM test (time in open arms: 6.7 ± 8.0 s *vs* 30.7 ± 20.9 s, $p < 0.01$; Mann-Whitney; entries number: 3.3 ± 2.8 *vs* 9.3 ± 3.6, $p < 0.001$; *t*-test), in the 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* 165 ± 18.4 s to eat, $p = 0.078$;

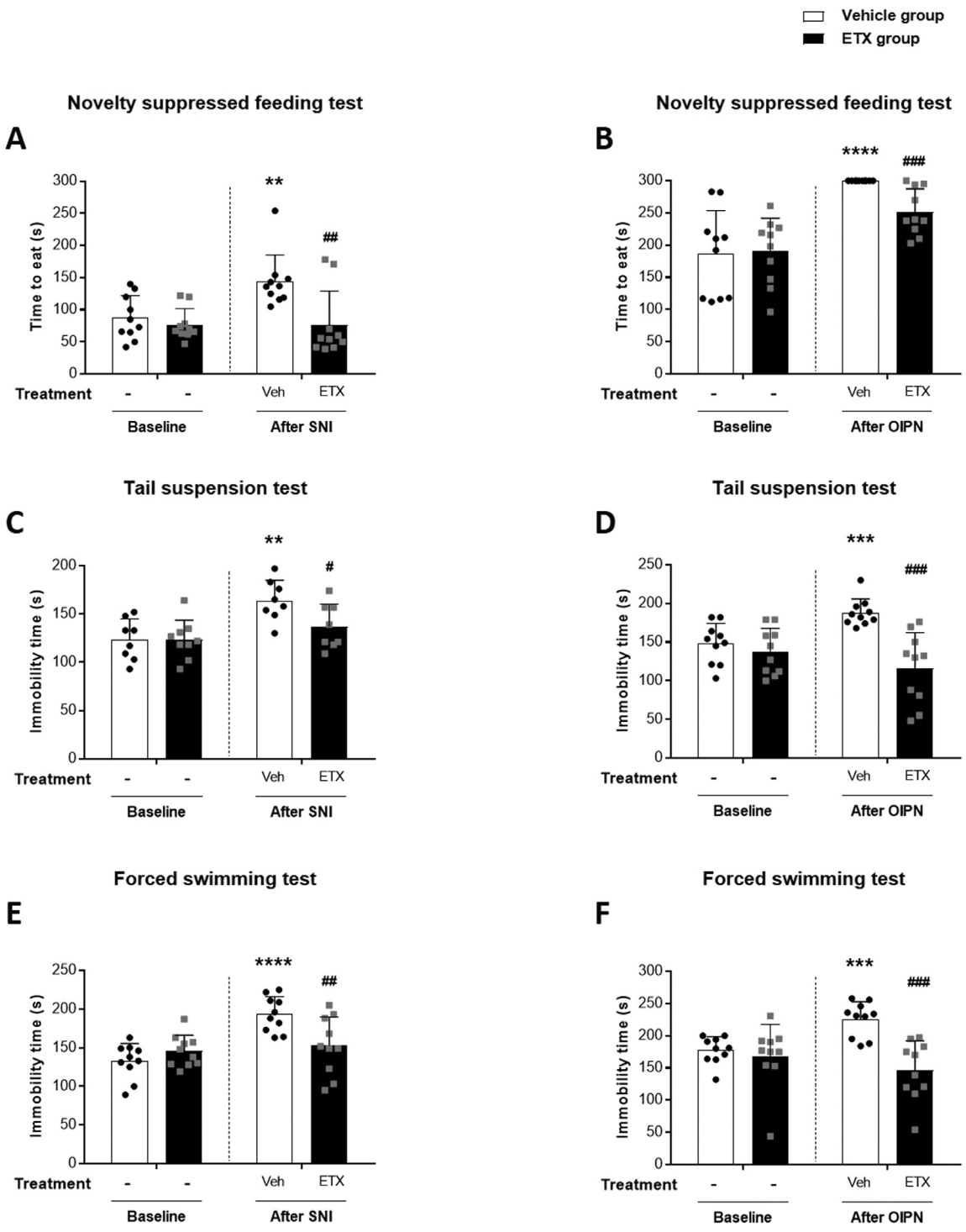

Fig. 3 Ethosuximide reduced the depressive-like behavior in SNI and OIPN mice models. To assess the impact of ethosuximide (ETX, 200 mg/kg, i.p.) on depressive-like behaviors in (**A, C and E**) spared nerve injury (SNI) and in (**B, D, and F**) oxaliplatin-induced peripheral neuropathy (OIPN) models, various tests were performed: (**A and B**) the novelty suppressed feeding test (latency to feed, depressive modality), (**C and D**) the tail suspension test (time of total immobility) and (**E and F**) the forced swimming test (time of total immobility). In the SNI model, tests were performed before the surgery (baseline) and after vehicle (Veh) or ETX treatments realized 21 days after the surgery. In the OIPN model, tests were performed before the first oxaliplatin injection (baseline) and after vehicle (Veh) or ETX treatments realized five days after the last injection of oxaliplatin. Data are shown as mean ± SD ($n = 10$ *per* group). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared with the baseline/vehicle group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with the SNI or OIPN/vehicle group; Mann–Whitney or *t*-test.

t-test),in the TST (151.8 ± 16.0 s *vs* 60.9 ± 43.5 s, $p < 0.0001$; Mann–Whitney) and in the FST (243.0 ± 34.3 s *vs* 50.7 ± 47.2 s, $p < 0.0001$; *t*-test) (Suppl. Fig. 1).

Finally, the anti-depressive effect of ethosuximide was investigated in a murine model of depression (Suppl. Fig. 2). Compared to baseline, chronic restrain stress induced a statistical significant increase of immobility time (135.6 ± 22.7 s *vs* 176.1 ± 18.2, $p < 0.001$; *t*-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 (Suppl. Fig. 2B), respectively. After stress, mice treated with ethosuximide showed a significant reduction of immobility time in both tests compared to vehicle (Suppl. Fig. 2).

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

Paw withdrawal threshold was decreased 14 days after CFA injection from 0.55 ± 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 ethosuximide group (Fig. 4). Ethosuximide administration after CFA significantly increased the PWT from 20 min to 60 min after the injection ($p < 0.01$; 2-way ANOVA; Fig. 4) with a maximum effect at 40 min (PWT of 0.16 ± 0.07 g *vs* 0.40 ± 0.15 g, vehicle *vs* ethosuximide, $p < 0.001$; Sidak *post-hoc*; Fig. 4).

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

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

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

4. Discussion

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

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

Thus, this broad spectrum of action of ethosuximide makes it a potential interesting in the treatment of patients with chronic pain, whose management requires a holistic approach that in particular integrates comorbidities. The relationship between depression and chronic pain is frequent and bi-directional in nature (Zhuo, 2016). Symptoms of depression or anxiety can exacerbate the severity of pain (Klauenberg et al., 2008; Ploghaus et al., 2001; Wilson et al., 2002), contribute to the persistence of chronic pain (Boogaard et al., 2011) and have an impact on response to treatments (Hider et al., 2009; Jamison et al., 2013; Wasan et al., 2015). Chronic pain therapy is thus not limited in clinical practice to the prescription of analgesics and treatments targeted at emotional manifestations are often necessary even if pain reduction by analgesics can, by itself, help to reduce emotional manifestations. In this respect, the decrease in nociceptive hypersensitivity achieved by ethosuximide in this study could have resulted from both the intrinsic effect of ethosuximide on nociception and the inhibition of comorbidities: the two mechanisms are not exclusive. We demonstrated that ethosuximide presented an anxiolytic- and an antidepressant-like effects in naïve mice and an antidepressant-like effect in a murine model of depression devoided of pain which plaid for an intrinsic action. However, this question remains to be more investigated.

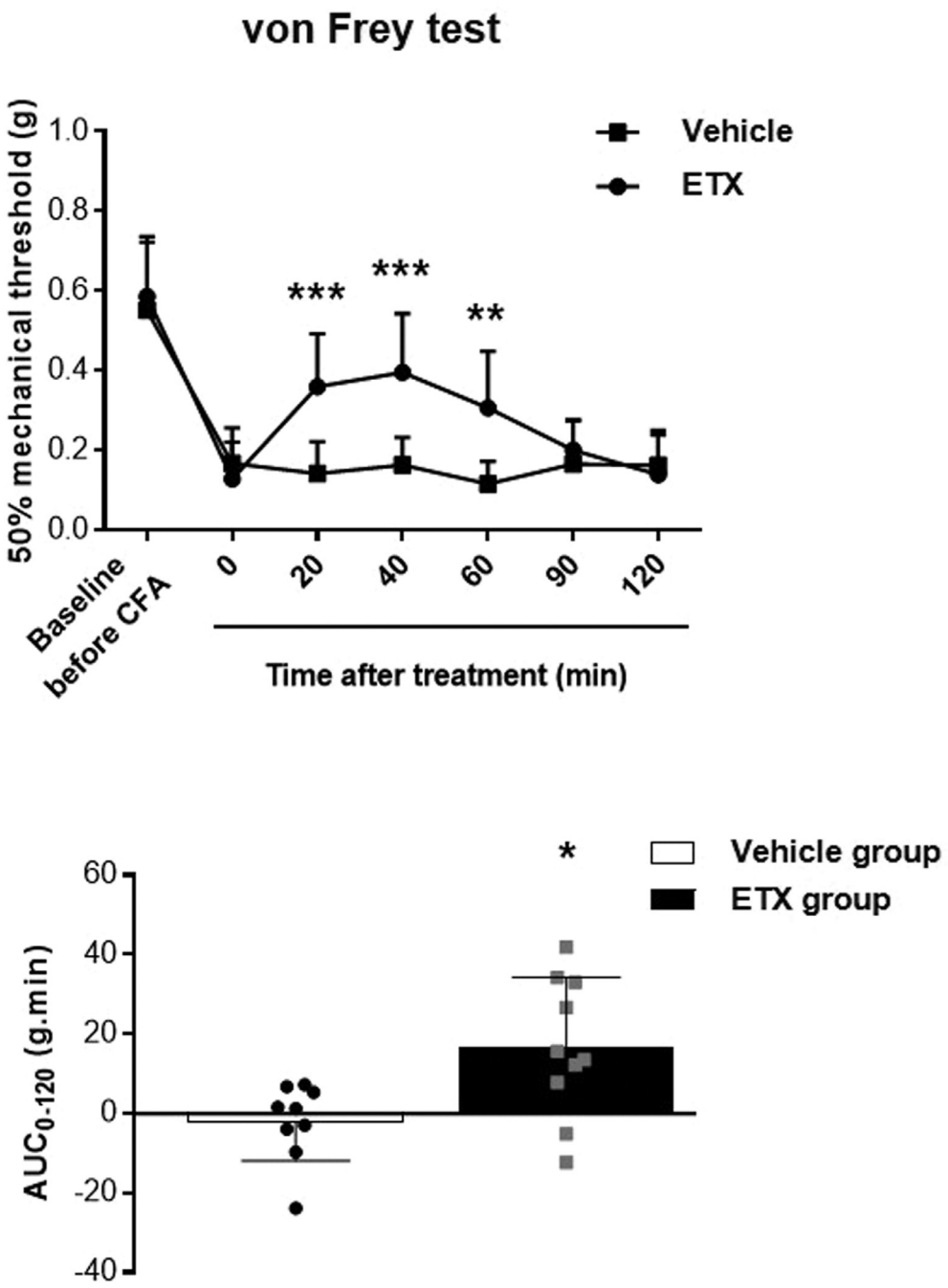

Fig. 4 Ethosuximide relieved nociceptive behavior of mice in a chronic inflammatory context induced by CFA. The effect of ethosuximide (ETX, 200 mg/kg, i.p.) on chronic inflammatory pain (monoarthritic model, 14 days after periarticular injection of CFA) was evaluated by the von Frey test to evidence the symptoms of mechanical hypersensitivity (**A**). This test was assessed before CFA injection (baseline) and after vehicle (Veh) or ETX treatments realized 14 days after CFA injection. 50% paw withdrawal threshold (PWT) was determined using an adaptation of the Dixon up-down method. Area under the time-course (0–120 min) of PWT variations are represented in the right side. Data are shown as mean ± SD ($n = 10$ *per* group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; two way ANOVA followed by Sidak's *post hoc* test and *t*-test for AUC mean comparing.

The effects of ethosuximide observed in our study can be explained by its mobilization of low voltage activated, also called T-type, calcium channels. The location of these channels on nociceptive message transmission pathways or in message transfer and modulation structures (for reviews, see Bourinet et al., 2016; Snutch and Zamponi, 2018) could explain the analgesia induced by their inhibition. These channels are also largely expressed in the brain structures involved in anxiety and depression (Talley et al., 1999). However, few studies have explored the involvement of T-type channels in anxiety and the results are conflicting (Choi et al., 2007; Gangarossa et al., 2014). Among ionic

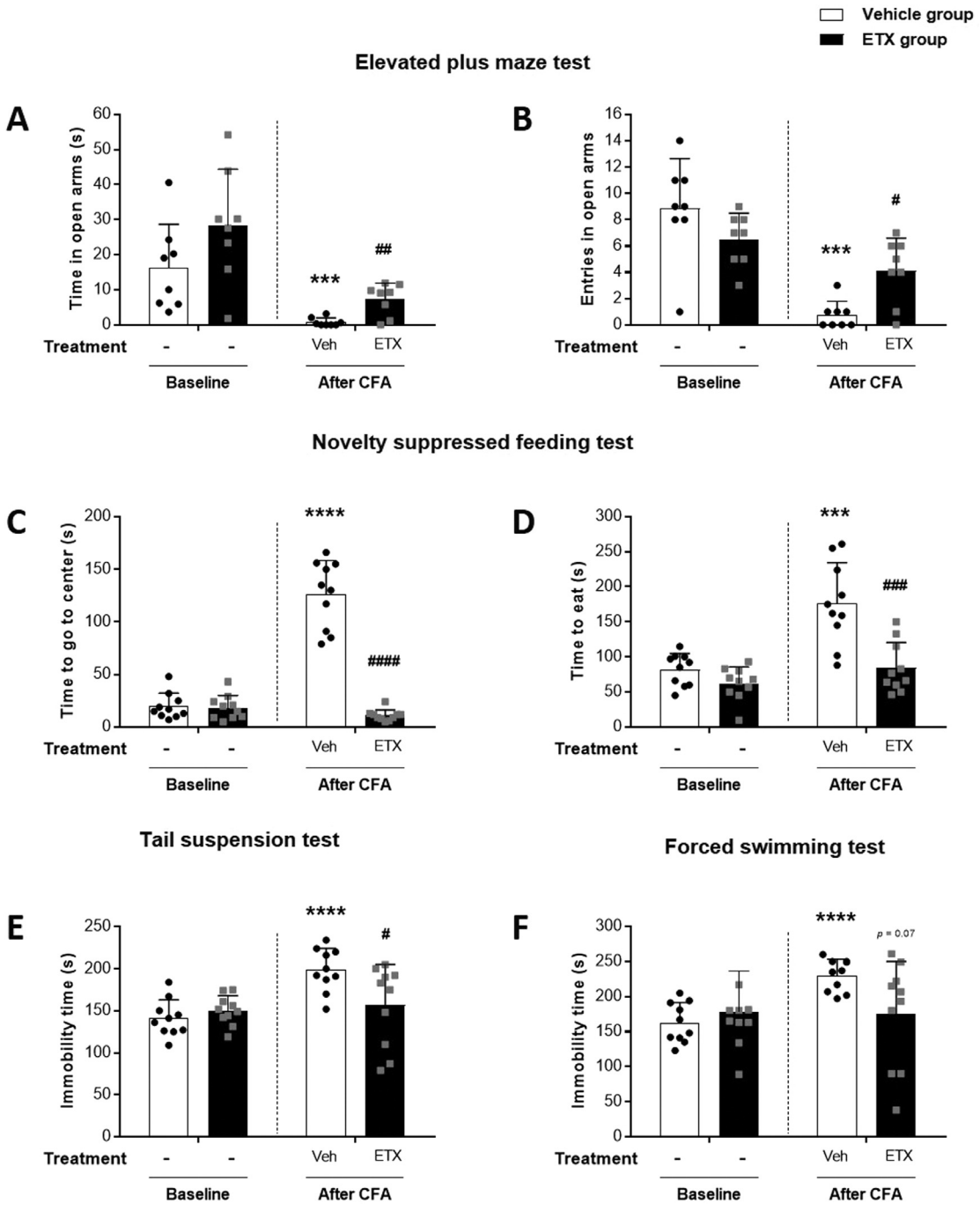

Fig. 5 Ethosuximide abolished the anxiety- and depressive-like behavior resulting from chronic inflammatory pain induced by CFA**.** To assess the impact of ethosuximide (ETX, 200 mg/kg, i.p.) on anxiety-like behavior developed during chronic inflammatory pain (monoarthritic model, 14 days after periarticular injection of CFA), two tests were performed: the elevated plus maze test in which the total time spent in the anxious area (open arms) (**A**) and the and the number of entries in the open arms (**B**) were recorded over 5 min; and (**C**) the novelty suppress feeding test (anxious modality) in which the time to go to the center of arena was measured. To assess the effect of ethosuximide (ETX, 200 mg/kg, i.p.) on depressive-like suppress feeding test (latency to feed, depressive modality), (**E**) the tail suspension test (time behaviors, (**D**) the novelty of total immobility) and (**F**) the forced swimming test (time of total immobility) were performed before (baseline) and 14 days after CFA injection. All tests were assessed before CFA injection (baseline) and after vehicle (Veh) or ETX treatments realized 14 days after CFA injection. Data are shown as mean ± SD ($n = 10$ *per* group). $^{***}p < 0.001$, $^{****}p < 0.0001$, compared with the baseline/vehicle group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ compared with the CFA/vehicle group; Mann–Whitney or *t*-test.

channels, ethosuximide inhibits Cav3.1/3.2/3.3 channels, sodium and Ca^{2+}-activated K^+ channels (Leresche et al., 1998; Todorovic and Lingle, 1998). The Cav3.2 isoform could be involved because Cav3.2 channels play a role in pain (Okubo et al., 2011; Sekiguchi and Kawabata, 2013), anxiety (Gangarossa et al., 2014) and depression (Llinás et al., 1999). However, certain questions remain concerning the mechanism of the anxiolytic effect of ethosuximide following the publication of Gangarossa et al. which showed that Cav3.2 knock-out mice exhibited an anxious phenotype (Gangarossa et al., 2014). Hypothesis explaining this duality were proposed in a recent review (Kaur et al., 2019).

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

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

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Contributors

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

Conflict of interest

The authors have no conflict of interest to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2019.10.012.

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