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Role of the endocannabinoid system in a mouse model of Fragile X undergoing neuropathic pain

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Abstract:	Background: Neuropathic pain is a complex condition characterized by sensory, cognitive and affective symptoms that magnify the perception of pain. The underlying pathogenic mechanisms are largely unknown and there is an urgent need for the development of novel medications. The endocannabinoid system modulates pain perception and drugs targeting the cannabinoid receptor type 2 (CB2) devoid of psychoactive side effects could emerge as novel analgesics. An interesting model to evaluate the mechanisms underlying resistance to pain is the fragile X mental retardation protein knockout mouse (Fmr1KO), a model of fragile X syndrome that exhibits nociceptive deficits and fails to develop neuropathic pain. Methods: A partial sciatic nerve ligation was performed to wild-type (WT) and Fmr1KO mice having (HzCB2 and Fmr1KO-HzCB2, respectively) or not (WT and Fmr1KO mice) a partial deletion of CB2 to investigate the participation of the endocannabinoid system on the pain-resistant phenotype of Fmr1KO mice. Results: Nerve injury induced a strong hypersensitivity in WT and HzCB2, whereas this increased pain sensitivity was absent in Fmr1KO mice. Interestingly, Fmr1KO mice partially lacking CB2 lost this protection against neuropathic pain. Similarly, pain-induced depressive-like behavior was observed in WT, HzCB2 and Fmr1KO-HzCB2 mice, but not in Fmr1KO littermates. Nerve injury evoked different alterations in WT and Fmr1KO mice at spinal and supra-spinal levels that correlated with these nociceptive and emotional alterations. Conclusions: This work shows that CB2 is necessary for the protection against neuropathic pain observed in Fmr1KO mice, raising the interest of targeting this receptor for the treatment of neuropathic pain.
Additional Information:	
Question	**Response**
Significance Below please give a paragraph entitled **"Significance"**, indicating the main aspects where this work adds significantly to existing knowledge in the field, and if appropriate to clinical practice. The signifiance statement should be short, attention-grabbing,non-redundant with the	The current study reveals that the cannabinoid receptor type 2 participates in the protective phenotype displayed by Fmr1KO mice against the nociceptive and emotional manifestations triggered by persistent nerve damage, raising the interest of targeting this receptor for neuropathic pain treatment. Our findings add knowledge about pain-related molecules also involved at spinal and supra-spinal levels in this protective phenotype of Fmr1KO mice.

conclusions and rigorously in line with the contents of the full article. It should not exceed 80 words and will be added to the end of the abstract at the time of typesetting. This paragraph will NOT count to the abstract's total word limit of 250 words. The statement "Significance" also applies to Review papers.	

Dear Luis,

Thanks for your positive comments and for considering this study to be published in European Journal of Pain. We have addressed the comments of the Section Editor and Reviewer #1 by adding the requested modifications in the manuscript and the point-by-point list of responses is included below. We hope this new version of the manuscript will satisfy the requests.

SECTION EDITOR

We agree with de section editor on highlighting the limitations and openings of this study and for that purpose, we have included at the end of the discussion section the following paragraph:

'Nevertheless, it would be important in future investigations to include multidisciplinary techniques, such as high-resolution brain imaging or electrophysiological tools, which represent experimental approaches more closely related to human pain experience to potentially optimize preclinical drug discovery aimed at alleviating neuropathic pain.' *(page 17)*

We have also rebuilt the significance statement in order to make it more attention-grabbing:

'Significance: Neuropathic pain is a complex chronic pain condition and current treatments are limited by the lack of efficacy and the incidence of important side effects. Our findings show that the pain-resistant phenotype of Fmr1KO mice against nociceptive and emotional manifestations triggered by persistent nerve damage requires the participation of the cannabinoid receptor CB2, raising the interest of targeting this receptor for neuropathic pain treatment. Additional multidisciplinary studies more closely related to human pain experience should be conducted to explore the potential use of cannabinoids as adequate analgesic tools.' *(page 1)*

REVIEWER #1

Minor comments:

1. The section 'Experimental procedure' does not add any relevant information to fig 1 and can be deleted.

The 'Experimental procedure' description has been deleted from the Methods section. Pertinent information about the experimental procedure can now be found in Fig. 1 and its legend.

2. The following sentence does not read well (pg 13): NF-κB promotes the expression of genes encoding proinflammatory mediators, such as cytokines and brain-derived neurotrophic factor, which arise sensitization processes.

Corrected to: 'NF-κB promotes the expression of genes that participate in the sensitization processes by encoding proinflammatory mediators, such as cytokines and brain-derived neurotrophic factor'. *(page 12)*

3. About the last paragraph of results on 'Correlations between gene expression and affective disorders'. It is not sufficiently clear what is the need for this section, it doesn't seem to add substantially to the discussion and it complicates the results section.

The aim of the last paragraph of results was to emphasize the role of the left amygdala (contralateral to the site of injury) on the effect of Fmr1 gene promoting depressive-like behavior after nerve injury. The positive correlation between cannabinoid-related gene expression in the left amygdala and immobility time in the forced swim test maze point to the left amygdala as a supra-spinal site involved in the development of this chronic pain comorbidity. We have rebuilt the last paragraph of results to ease understand the meaning of this section, as follows:

'In order to provide additional findings in support of the role of the different brain areas on the effect of Fmr1 gene promoting depressive-like behavior after nerve injury, we evaluated the correlations between gene expression and immobility time of WT and Fmr1KO mice regardless of the type of surgery. Elevated time of immobility in the forced swim test was positively correlated with the expression in the left amygdala (contralateral to the site of injury) of Rela ($r = 0.567$; $p = 0.007$), Grm5 ($r = 0.507$; $p = 0.016$) and Homer1a ($r = 0.596$; $p = 0.003$), but not with Ppara expression ($r = 0.007$; $p = 0.976$) (Fig. 6A-D). These correlations and the observed decreased gene expression of Homer1a and Rela in the left amygdala of nerve-injured Fmr1KO mice (Fig. 5D) suggest a possible role of the left amygdala in the protective phenotype of Fmr1KO

mice against the nociceptive and emotional manifestations of neuropathic pain. No association was revealed between time of immobility and mPFC expression of Ppara (r = -0.067; p = 0.761), Rela (r = 0.256; p = 0.227), Grm5 (r = 0.163; p = 0.406) and Homera1 (r = -0.041; p = 0.834). Similarly, no significant associations were revealed in the right amygdala for Ppara (r = 0.088; p = 0.697), Rela (r = 0.162; p = 0.471), Grm5 (r = 0.357; p = 0.103) and Homera1 (r = -0.280; p = 0.207) expression (data not shown).' *(page 13)*

4. In addition, please consider substituting the expression 'affective disorders' for a more neutral formulation like 'immobility time in the water maze' (in this and other instances).

Replaced throughout the manuscript, as requested.

5. Furthermore, wouldn't it be necessary to exclude motor or activity/sedative impairments as part of the phenotype of Fmr1KO mice that may interfere with this test?

The forced swimming test (FST), is considered the most common behavioral paradigm to assess depressive-like behavior in rodents based on observations of immobility in the water maze. This form of behavioral despair reflects psychomotor activity of the animal and constitutes a reliable approach in antidepressant research (Unal and Canbeyli, 2019). Our data reveal that sham Frm1KO mice did not show an increased time of immobility in the FST in comparison to sham WT mice, excluding an impairment of basal activity associated with this genotype that would affect this behavioral response.

- Unal, G., Canbeyli, R. (2019). Psychomotor retardation in depression: A critical measure of the forced swim test. *Behav Brain Res* 372, 112047.

6. The following sentence does not read well (pg 17): suggesting that the expression of Grm5 could in the right amygdala participates in the antinociceptive phenotype observed in Fmr1KO mice.

We have corrected the sentence as follows: 'suggesting that the unaltered expression of Grm5 in the right amygdala of Fmr1KO mice could participate in the antinociceptive phenotype observed in these animals.' *(page 16)*

ABSTRACT

Background: Neuropathic pain is a complex condition characterized by sensory, cognitive and affective symptoms that magnify the perception of pain. The underlying pathogenic mechanisms are largely unknown and there is an urgent need for the development of novel medications. The endocannabinoid system modulates pain perception and drugs targeting the cannabinoid receptor type 2 (CB2) devoid of psychoactive side effects could emerge as novel analgesics. An interesting model to evaluate the mechanisms underlying resistance to pain is the fragile X mental retardation protein knockout mouse (Fmr1KO), a model of fragile X syndrome that exhibits nociceptive deficits and fails to develop neuropathic pain.

Methods: A partial sciatic nerve ligation was performed to wild-type (WT) and Fmr1KO mice having (HzCB2 and Fmr1KO-HzCB2, respectively) or not (WT and Fmr1KO mice) a partial deletion of CB2 to investigate the participation of the endocannabinoid system on the pain-resistant phenotype of Fmr1KO mice.

Results: Nerve injury induced a strong hypersensitivity in WT and HzCB2, whereas this increased pain sensitivity was absent in Fmr1KO mice. Interestingly, Fmr1KO mice partially lacking CB2 lost this protection against neuropathic pain. Similarly, pain-induced depressive-like behavior was observed in WT, HzCB2 and Fmr1KO-HzCB2 mice, but not in Fmr1KO littermates. Nerve injury evoked different alterations in WT and Fmr1KO mice at spinal and supra-spinal levels that correlated with these nociceptive and emotional alterations.

Conclusions: This work shows that CB2 is necessary for the protection against neuropathic pain observed in Fmr1KO mice, raising the interest of targeting this receptor for the treatment of neuropathic pain.

Keywords: Neuropathic pain, nociception, depressive-like behavior, Fmr1KO mice, protective phenotype, cannabinoid receptor type-2, N-acylethanolamines.

Role of the endocannabinoid system in a mouse model of Fragile X undergoing neuropathic pain

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Running head: Endocannabinoid system and pain-resistant phenotype.

Category: Original article.

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Disclosure statement: The authors have no conflicts of interest to declare.

Significance: Neuropathic pain is a complex chronic pain condition and current treatments are limited by the lack of efficacy and the incidence of important side effects. Our findings show that the pain-resistant phenotype of Fmr1KO mice against nociceptive and emotional manifestations triggered by persistent nerve damage requires the participation of the cannabinoid receptor CB2, raising the interest of targeting this receptor for neuropathic pain treatment. Additional multidisciplinary studies more closely related to human pain experience should be conducted to explore the potential use of cannabinoids as adequate analgesic tools.

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Background: Neuropathic pain is a complex condition characterized by sensory, cognitive and affective symptoms that magnify the perception of pain. The underlying pathogenic mechanisms are largely unknown and there is an urgent need for the development of novel medications. The endocannabinoid system modulates pain perception and drugs targeting the cannabinoid receptor type 2 (CB2) devoid of psychoactive side effects could emerge as novel analgesics. An interesting model to evaluate the mechanisms underlying resistance to pain is the fragile X mental retardation protein knockout mouse (Fmr1KO), a model of fragile X syndrome that exhibits nociceptive deficits and fails to develop neuropathic pain.

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Conclusions: This work shows that CB2 is necessary for the protection against neuropathic pain observed in Fmr1KO mice, raising the interest of targeting this receptor for the treatment of neuropathic pain.

Keywords: Neuropathic pain, nociception, depressive-like behavior, Fmr1KO mice, protective phenotype, cannabinoid receptor type-2, N-acylethanolamines.

ABBREVIATIONS

2-AG: 2-Arachidonoylglycerol; **AEA:** Anandamide; **CB1:** Cannabinoid receptor type 1; **CB2:** Cannabinoid receptor type 2; **FRMP:** Fragile X mental retardation protein; **HOMER1a:** Homer scaffolding protein 1a; **mGluR5:** Glutamate metabotropic receptor 5; **mPFC:** Medial prefrontal cortex; **NF-κB:** Nuclear factor-kappa B; **OEA:** Oleoylethanolamine; **PEA:** Palmitoylethanolamide; **PPARα:** Peroxisome proliferator-activated receptor alpha; **PSNL:** Partial sciatic nerve ligation; **WT:** Wild-type.

INTRODUCTION

Neuropathic pain is defined as an unpleasant sensory and emotional experience initiated by a lesion or disease of the somatosensory nervous system, mainly associated with spontaneous pain, hyperalgesia and allodynia (Scholz et al., 2019). Neuropathic pain patients often experience anxiety, depression and impaired cognitive functions that diminish their life quality (Descalzi et al., 2017). This clinical entity that affects millions of people worldwide (Colloca et al., 2017) has pathogenic mechanisms that remain largely unknown and current treatments are limited by the lack of efficacy and important side effects (Bouhassira and Attal, 2018). Therefore, there is an urgent need to develop novel therapeutic strategies to improve the quality of life of neuropathic pain patients.

The fragile X mental retardation protein (FMRP), required for the adequate development of neuronal connections, has raised new interest to clarify pain processing (Aloisi et al., 2017; Busquets-Garcia et al., 2014). The absence of FMRP in humans causes the most common monogenic condition of autistic spectrum disorders, the fragile X syndrome, and a prominent feature of this disorder is self-injurious behavior associated with an alteration of the nociceptive system (Peebles and Price, 2012). Likewise, the preclinical mouse model of fragile X syndrome, a knockout mouse lacking FMRP (Fmr1KO), shows profound deficits in nociceptive sensitization during neuropathic pain (Price et al., 2007). Therefore, the pain-resistant phenotype of the Fmr1KO mouse represents an appropriate model to investigate the mechanisms underlying these nociceptive deficits and its comorbid manifestations, including cognitive impairment and depressive-like behavior.

The endocannabinoid system is involved in several physiological processes including affective, cognitive and nociceptive functions (Donvito et al., 2018; Rácz et al., 2015). Cannabinoid receptors type 1 (CB1) and type 2 (CB2) are distributed in main central and peripheral nervous system areas involved in pain processing, such as medial prefrontal cortex (mPFC), amygdala and spinal cord (La Porta et al., 2015). The activation of both receptors reduces nociception and affective alterations produced by neuropathic pain (Klauke et al., 2014; Rácz et al., 2015). We have previously demonstrated that the pain-resistant phenotype of Fmr1KO mice in a model of inflammatory pain depends on CB1 presence (Busquets-Garcia et al., 2013). However, due to its widespread brain distribution, CB1 activation leads to important psychoactive, motor and cognitive effects, which represent major limitations for chronic pain treatment (Davis, 2014). Alternative approaches have been developed to overcome this problem by targeting CB2 that is predominantly expressed in peripheral immune cells, although it is also present at low levels in

neurons (Shang and Tang, 2017). Therefore, CB2 could be a potential therapeutic target for neuropathic pain treatment avoiding the risk of centrally-mediated side effects.

The aim of this study was to elucidate the neurobiological mechanisms involved in the pain-resistant phenotype of Fmr1KO mice in order to identify potential pharmacological targets for neuropathic pain. For this purpose, we evaluated the nociceptive, cognitive and affective manifestations associated to a peripheral nerve injury in Fmr1KO mice partially lacking CB2 to explore the therapeutic interest of this receptor for neuropathic pain.

METHODS

Animals

Taken into account that fragile X syndrome predominantly occurs in male individuals (Razak et al., 2020), all experiments were performed in male mice between 8 and 20 weeks of age. WT ($Fmr1^{+/y}$, $Cnr2^{+/+}$), Fmr1KO ($Fmr1^{-/y}$, $Cnr2^{+/+}$), WT heterozygous for CB2 (HzCB2) ($Fmr1^{+/y}$, $Cnr2^{+/-}$) and Fmr1KO heterozygous for CB2 (Fmr1KO-HzCB2) ($Fmr1^{-/y}$, $Cnr2^{+/-}$) littermates in C57BL/6J genetic background were used. The behavioral experiments were conducted in the animal facility at Universitat Pompeu Fabra-Barcelona Biomedical Research Park (UPF-PRBB; Barcelona, Spain). Mice were group-housed (2-4 animals) and maintained in a controlled temperature (21 ± 1 °C) and humidity (55 ± 10%) environment. Food and water were available ad libitum and mice were handled during the light phase of a 12 h light/dark cycle (light on at 8:00 a.m., light off at 8:00 p.m.). All behavioral experiments were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona) and were performed in accordance with the European Communities Council Directive (2010/63/EU). All the experiments were performed under blind and randomized conditions.

Neuropathic pain induction

Mice underwent a partial sciatic nerve ligation (PSNL) at mid-thigh level to induce neuropathic pain, as previously described (Malmberg and Basbaum, 1998) with minor modifications. Briefly, mice were anesthetized with isoflurane (induction, 5% V/V; surgery, 2% V/V) in oxygen and the sciatic nerve was exposed at the level of the mid-thigh of the right hind leg. At ~1 cm proximally to the nerve trifurcation, a tight ligature was created around 33–50% of the cranial side of the sciatic nerve using a 9–0 non-absorbable virgin silk suture (Alcon Cusí SA, Barcelona, Spain) and leaving the rest of the nerve untouched. The muscle was then stitched with 6-0 silk (Alcon Cusí), and the skin incision was closed with wound clips. Sham-operated mice underwent the same surgical procedure except that the sciatic nerve was not ligated.

Nociception

Sensitivity to mechanical and heat stimuli was used as nociceptive measures of neuropathic pain. Ipsilateral and contralateral hind paw withdrawal thresholds were evaluated the day before, 3, 7 and 14 days after the nerve injury. Mechanical allodynia was quantified by measuring the withdrawal response to von Frey filament stimulation through the up–down paradigm, as previously reported (Chaplan et al., 1994). Filaments equivalent to 0.04, 0.07, 0.16, 0.4, 0.6, 1 and 2 g were used, applying first the 0.4 g filament and increasing or decreasing the

strength according to the response. The filaments were bent and held for 5 s against the surface of the hind paws. Heat sensitivity was assessed by recording the hind paw withdrawal latency in response to radiant heat applied with the plantar test apparatus (Ugo Basile, Varese, Italy) as previously reported (Hargreaves et al., 1988). Clear paw withdrawal, shaking or licking was considered a nociceptive response.

Cognitive performance

The novel object-recognition test was performed on day 12 after the surgery as previously described (Puighermanal et al., 2009). Briefly, mice were habituated to a V-shaped maze for 9 min on day 1. The following day, mice were introduced in the maze where 2 identical objects (familiar objects) were presented in the extremes of the maze. For the memory test performed on the third day, 1 of the familiar objects was replaced with a new object (novel object), and the total time spent exploring each of the 2 objects (novel and familiar) was measured. Object exploration was defined as the orientation of the nose towards the object at a distance of <1 cm. A discrimination index (DI) was calculated as the difference between the time spent exploring either the novel (Tn) or familiar (Tf) object divided by the total time exploring both objects: (DI = (Tn − Tf)/(Tn + Tf)). A higher discrimination index is considered to reflect greater memory retention for the familiar object. Mice that explored <10 s both objects were excluded from the analysis.

Depressive-like behavior

Depressive-like behavior was evaluated 19 days after the surgery using the forced-swimming test (Porsolt and Bertin, 1977). Briefly, mice were individually placed into a glass cylinder (17.5 x 12.5 cm) filled 15 cm high with water (22 ± 1°C). Mice were subjected to forced swimming for 6 min and the total duration of immobility, disregarding small hind limb movements to keep the head above water, was measured during the last 4 min when mice show a sufficiently stable level of immobility.

Endocannabinoid quantification

Animals were sacrificed at the end of the experimental protocol and L3-L5 ipsilateral spinal cord dorsal horns were freshly dissected. Samples were rapidly frozen and stored at -80°C. The quantification of endocannabinoids and related compounds was based on the methodology previously described in plasma (Pastor et al., 2014), adapted for the extraction of endocannabinoids from spinal tissue. The following endocannabinoids and related compounds were quantified: 2-arachidonoyl glycerol (2-AG), N-arachidonoylethanolamine (AEA),

palmitoylethanolamide (PEA) and oleoylethanolamine (OEA). Frozen spinal cords (4 ± 1 mg) of mice were placed in a 1 ml Wheaton glass homogenizer and spiked with 25 µl of a mix of deuterated internal standards dissolved in acetonitrile. The mix contained 5 ng/ml of each compound. All internal standards were purchased from Cayman Chemical (Ann Harbor). Tissues were homogenized on ice with 700 µl a mixture of 50 mM Tris-HCl buffer (pH 7.4): methanol (1:1) and the homogenates were transferred to 12 ml glass tubes. The homogenizer was washed twice with 0.9 ml of the same mixture and the contents were combined into the tube giving an approximate volume of 2.5 ml of homogenate. The homogenates were kept on ice until organic extraction to minimize the ex-vivo generation of endocannabinoids. Next, homogenates were extracted with 5 ml chloroform over 20 min by placing the tubes in a rocking mixer. Tubes were centrifuged at 1700 g over 5 min at room temperature. The lower organic phase was transferred to clean glass tubes, evaporated under a stream of nitrogen in a 39 °C water bath and extracts were reconstituted in 100 µl of mixture water: acetonitrile (10:90, v/v) with 0.1% formic acid (v/v) and transferred to high performance liquid chromatography vials with glass micro-vials. Endocannabinoids were separated using an Agilent 6410 triple quadrupole Liquid-Chromatograph equipped with a 1200 series binary pump, a column oven and a cooled autosampler (4 °C). Chromatographic separation was carried out with a Waters C18-CSH column (3.1×100 mm, 1.8 µm particle size) maintained at 40°C with a mobile phase flow rate of 0.4 ml/min. The composition of the mobile phase was: A: 0.1% (v/v) formic acid in water; B: 0.1% (v/v) formic acid in acetonitrile. Endocannabinoids and related compounds were separated by gradient chromatography. The ion source was operated in the positive electrospray mode. The selective reaction monitoring mode was used for the analysis. Quantification was done by isotope dilution with the response of the deuterated internal standards and data were expressed as a percentage of the control group (WT sham).

Gene expression analysis

Animals were sacrificed at the end of the experimental protocol and mPFC, amygdala (left and right, separately) and spinal ipsilateral dorsal horns were freshly dissected. The samples were rapidly placed in individual tubes and stored at -80°C. Total RNA was isolated from frozen samples with RNeasy Micro kit (74004, Qiagen, Stokach, Germany) and subsequently reverse-transcribed to cDNA with a High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real Time-Polymerase Chain Reaction (RT-PCR) was carried out in triplicate with a QuantStudio 12K Flex RT-PCR System (4471134, Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix (04707516001, Roche, Basel, Switzerland). The expression of the following genes

was analysed: nuclear factor NF-Kappa-B P65 subunit (*Rela*), glutamate metabotropic receptor 5 (*Grm5*), homer scaffolding protein 1a (*Homer1a*) and peroxisome proliferator-activated receptor alpha (*Ppara*). Levels of the target genes were normalized against the housekeeping gene beta-2-microglobulin (*B2m*) and compared using the ΔΔCt method (Livak and Schmittgen, 2001). The following specific primers were used: 5'-CTTCCTCAGCCATGGTACCTCT-3' (*Rela* forward); 5'-CAAGTCTTCATCAGCATCAAACTG-3' (*Rela* reverse), 5'-GTCCTGGCCCACTGACGA-3' (*Grm5* forward); 5'-GGTCACCCCATCGAAGATAC-3' (*Grm5* reverse), 5'-GGGAGGATGGAGACACAGC-3' (*Homer1a* forward); 5'-CGGTCCGTCCCTTTTTCCTT-3' (*Homer1a* reverse), 5'-AGAGGGCTGAGCGTAGGTAA-3' (*Ppara* forward); 5'-ATTGGGCCGGTTAAGACCAG-3' (*Ppara* reverse), 5'-TTCTGGTGCTTGTCTCACTGA-3' (*B2m* forward); 5'-CAGTATGTTCGGCTTCCCATTC-3' (*B2m* reverse).

Statistics

All the data were first subjected to a Shapiro-Wilk test of normality. The time course of nociceptive thresholds was analysed using a linear mixed model with three factors (surgery, genotype, time and their interactions) considering the presence of non-gaussian distribution in some of the experimental days. Bonferroni *post hoc* analysis was performed when pertinent. Long-term memory and endocannabinoid quantification and RT-PCR data were analysed with a two-way ANOVA (genotype, surgery) followed by Bonferroni *post hoc*. Depressive-like behavior and endocannabinoid quantification and RT-PCR data were analysed with a Kruskal-Wallis followed by U Mann Whitney with Bonferroni adjustment for multiple comparisons taken into account that these data did not follow a normal distribution. A probability of 0.05 or less was considered statistically significant. IBM SPSS 19 (SPSS Inc., Chicago, USA) was used to analyse the data. Detailed statistical analysis is presented in Supplementary Tables S1-S4.

RESULTS

1. Role of CB2 in the nociceptive manifestations of neuropathic pain in Fmr1KO mice.

Nociceptive sensitivity to mechanical and heat stimulation was evaluated under basal conditions and 3, 7 and 14 days after PSNL or sham surgery (**Fig. 1**).

1.1 Mechanical allodynia.

The von Frey test was used to assess sensitivity to mechanical stimuli in sham and neuropathic pain conditions. All mice showed similar nociceptive thresholds in naïve conditions in both ipsilateral and contralateral paws (**Fig. 2A**). After surgery, nerve-injured animals developed mechanical allodynia, as revealed by significant differences between ipsilateral and contralateral paw sensitivity. As expected, Fmr1KO mice revealed decreased mechanical allodynia following nerve injury when compared to WT mice. Interestingly, the WT mice phenotype on the mechanical nociceptive responses was rescued when CB2 was partially removed in Fmr1KO mice (**Fig. 1A**). No significant differences in mechanical thresholds following sham surgery were observed between WT and the other genotypes (**Fig. 2A**). Therefore, the deletion of the Fmr1 gene decreases the mechanical allodynia associated to the nerve injury and CB2 is involved in this phenotype.

1.2 Heat hyperalgesia.

Sensitivity to thermal stimuli in the plantar test was also used as a nociceptive measure of neuropathic pain. All mice showed similar withdrawal latencies of both ipsilateral and contralateral paws in naïve conditions (**Fig. 2B**). The nerve injury induced heat hyperalgesia in all genotypes in comparison to the contralateral paw, but this nociceptive behavior was significantly attenuated on Fmr1KO mice. These mutants showed decreased heat sensitivity on the ipsilateral paw following PSNL-surgery compared to WT littermates. When CB2 was partially removed from Fmr1KO mice (Fmr1KO-HzCB2 mutants), thermal hypersensitivity was similar to WT mice (**Fig. 2B**). Sham surgery did not alter the plantar withdrawal latencies of any group (**Fig. 2B**). Thus, the deletion of the Fmr1 gene decreases the heat hypersensitivity associated to the nerve injury and CB2 is also involved in this phenotype.

2. Role of CB2R in memory and emotional alterations associated with neuropathic pain in Fmr1KO mice.

Cognitive performance and depressive-like behavior were evaluated under sham and neuropathic pain conditions 12 and 19 days after the surgery, respectively (**Fig. 1**).

2. 1. Cognitive performance.

The novel object recognition test was used to assess long-term memory after the induction of neuropathic pain or the sham surgery. Long-term memory impairment was revealed in WT and HzCB2 mice exposed to PSNL by a decrease in the discrimination index in comparison to sham littermates, whereas no significant effect was observed in Fmr1KO and Fmr1KO-HzCB2 mice. These mutants already showed a low discrimination index compared to WT mice regardless the surgery (**Fig. 3A**). Hence, both nerve injury and lack of Fmr1 gene produce cognitive impairment, that is not modified by the partial deletion of CB2.

2. 2. Depressive-like behavior.

The forced swimming test was used to assess depressive-like behavior associated to neuropathic pain. As expected, nerve injury induced depressive-like behavior revealed by a significant increase in the time of immobility of WT and HzCB2 animals in comparison to sham-operated mice. Interestingly, HzCB2 mutants subjected to the sham surgery showed increased depressive-like behavior when compared to WT sham mice, suggesting a possible anti-depressive effect of CB2. On the other hand, Fmr1KO mice did not develop depressive-like behavior associated to the PSNL surgery and showed different responses to nerve-injured WT and HzCB2 mice. However, the depressive-like behavior was rescued in Fmr1KO mutants partially lacking CB2 (**Fig. 3B**). Therefore, the inhibition of depressive-like behavior in Fmr1KO mice depends on CB2 presence, which also modifies this behavior under basal conditions.

3. Effects of nerve injury on modulating the endocannabinoid tone at spinal cord level.

To explore the possible mechanisms underlying the effects of Fmr1 deletion on the nociceptive manifestations of neuropathic pain, we evaluated the levels of endocannabinoids and related lipids in the spinal cord dorsal horn of WT and Fmr1KO mice. 2‑arachidonoylglycerol (2-AG) and N‑arachidonoyl‑ethanolamine (AEA) are the main endogenous ligands of CB1 and CB2, whereas palmitoylethanolamide (PEA) and oleoylethanolamid (OEA) belong to the N-acylethanolamines family and display anti-inflammatory properties that could participate in the protective phenotype of Fmr1KO mice mainly through peroxisome proliferator-activated receptors (PPARs) (Di Marzo, 2018). Nerve injury did not change the expression of 2-AG, AEA, PEA or OEA in WT mice compared to sham littermates at spinal level (**Fig. 4A-B**). In contrast, nerve-injured Fmr1KO mice showed increased levels of PEA and OEA, but not 2-AG or AEA when compared to sham mutants (**Fig. 4C-D**). Sham-operated Fmr1KO mice did not show significant alterations in any of these endocannabinoids in comparison to control WT mice (**Fig. 4A-D**).

These findings reveal an association between N-acylethanolamines levels and the nociceptive changes observed in Fmr1KO mice.

4. Molecular changes in somatosensory pain-related areas of nerve-injured WT and Fmr1KO mice.

RT-PCR analysis was used to evaluate gene expression levels of pain-related proteins in the spinal cord dorsal horn, mPFC, right and left amygdalae of WT and Fmr1KO mice 21 days after nerve injury.

4.1. N-acylethanolamines signaling pathway.

PPARs are activated by ligands of different chemical structure, including the endocannabinoid-like compounds PEA and OEA (O'Sullivan, 2016). Considering the increased levels of PEA and OEA in the spinal cord of nerve-injured Fmr1KO mice, we evaluated *Ppara* (PPARα encoding gene) expression in spinal cord and brain areas involved in pain and emotional processing.

WT and Fmr1KO animals did not reveal significant alterations of *Ppara* expression in the spinal cord dorsal horn, although a trend to enhance *Ppara* levels was observed after nerve injury in Fmr1KO mice (**Fig. 5A**). However, nerve-injured Fmr1KO mice showed enhanced *Ppara* expression in the mPFC when compared to sham littermates or nerve-injured WT mice (**Fig. 5B**). In the right amygdala, a global decrease of *Ppara* expression was revealed in Fmr1KO animals compared to WT mice (**Fig. 5C**).

Additionally, PPARα exerts anti-inflammatory effects by modulating the activity of several pro-inflammatory transcription factors, including the nuclear factor-Kappa B (NF-κB) (Rakhshandehroo et al., 2010). NF-κB promotes the expression of genes that participate in the sensitization processes by encoding proinflammatory mediators, such as cytokines and brain-derived neurotrophic factor (Grace et al., 2014). The expression of *Rela* (NF-κB encoding gene) was not modified in the spinal cord dorsal horn and right amygdala of WT and Fmr1KO mice regardless of the surgery (**Fig. 5A, C**). In contrast, nerve-injured Fmr1KO animals revealed increased levels of *Rela* expression in the mPFC in comparison to sham littermates and nerve-injury WT mice (**Fig. 5B**), whereas this gene expression was diminished in the left amygdala of Fmr1KO after nerve injury (**Fig. 5D**).

4.2. Glutamate signaling.

An altered glutamate transmission mainly due to modifications in the metabotropic glutamate receptor 5 (mGluR5) has been implicated in the pathophysiological processes leading to chronic

pain and associated affective states (Chung et al., 2017). mGluR5 form a complex with the Homer Scaffolding Protein 1a (HOMER1a) that plays an important role in glutamate-mediated cellular signaling and nociception (Obara et al., 2013b). We have evaluated the expression levels of the gene coding for mGluR5 protein (*Grm5*) and the HOMER1A encoding gene (*Homer1a*). *Grm5* gene expression was not altered in the spinal cord and left amygdala of WT and mutant mice with or without nerve ligation (**Fig. 5A, D**). However, sham and nerve-injured Fmr1KO mice showed downregulated mRNA levels of *Grm5* in the mPFC compared to the respective WT groups (**Fig. 5B**). On the other hand, WT mice showed increased levels of *Grm5* expression after nerve injury in the right amygdala when compared to sham littermates and nerve-injured Fmr1KO mice (**Fig. 5C-D**). The expression of *Homer1a* was not modified in the spinal cord dorsal horn nor right amygdala of WT and Fmr1KO mice regardless of the surgery (**Fig. 5A, C**). In the mPFC, a global decrease of *Homer1a* expression was revealed in Fmr1KO animals in comparison to WT mice (**Fig. 5B**). However, *Homer1a* expression was decreased in the left amygdala after PSNL surgery in Fmr1KO mice compared to nerve-injured WT animals (**Fig. 5D**).

5. Correlations between gene expression and the time of immobility in the water maze.

In order to provide additional findings in support of the role of the different brain areas on the effect of Fmr1 gene promoting depressive-like behavior after nerve injury, we evaluated the correlations between gene expression and immobility time of WT and Fmr1KO mice regardless of the type of surgery. Elevated time of immobility in the forced swim test maze was positively correlated with the expression in the left amygdala (contralateral to the site of injury) of Rela ($r = 0.567$; $p = 0.007$), Grm5 ($r = 0.507$; $p = 0.016$) and Homer1a ($r = 0.596$; $p = 0.003$), but not with Ppara expression ($r = 0.007$; $p = 0.976$) (Fig. 6A-D). These correlations and the observed decreased gene expression of Homer1a and Rela in the left amygdala of nerve-injured Fmr1KO mice (Fig. 5D) suggest a possible role of the left amygdala in the protective phenotype of Fmr1KO mice against the nociceptive and emotional manifestations of neuropathic pain. No association was revealed between time of immobility and mPFC expression of Ppara ($r = -0.067$; $p = 0.761$), Rela ($r = 0.256$; $p = 0.227$), Grm5 ($r = 0.163$; $p = 0.406$) and Homera1 ($r = -0.041$; $p = 0.834$). Similarly, no significant associations were revealed in the right amygdala for Ppara ($r = 0.088$; $p = 0.697$), Rela ($r = 0.162$; $p = 0.471$), Grm5 ($r = 0.357$; $p = 0.103$) and Homera1 ($r = -0.280$; $p = 0.207$) expression (data not shown).

DISCUSSION

This study provides novel findings to clarify the mechanisms involved in the resistant phenotype of Fmr1KO mice, a mouse model of Fragile X syndrome, against the nociceptive, cognitive and affective manifestations of neuropathic pain. We reveal the participation of CB2 on the protective effects of this phenotype in the development of neuropathic pain and identify specific changes in the endocannabinoid system and the expression of pain-related genes in spinal and supra-spinal areas in this mouse model protected against chronic pain manifestations.

Nerve injury induced the expected mechanical allodynia and heat hypersensitivity in WT mice (La Porta et al., 2016), whereas both nociceptive manifestations were attenuated in Fmr1KO mice during at least 2 weeks after nerve ligation. In agreement, previous studies have reported that Fmr1KO mice failed to show enhanced mechanical and thermal hypersensitivity in response to nerve injury (Price et al., 2007; Wang et al., 2016). We investigated for the first time the emotional and cognitive manifestations of neuropathic pain in Fmr1KO mice. Neuropathic pain impaired long-term memory and produced depressive-like behavior in WT mice, as previously reported (Chung et al., 2017; Martínez-Navarro et al., 2019). The lack of *Fmr1* gene has been widely related to memory deficits in mice (Gomis-González et al., 2016). Accordingly, Fmr1KO mice showed cognitive impairment in sham and neuropathic pain conditions. Furthermore, Fmr1KO mice did not develop depressive-like behavior associated to nerve injury, suggesting a participation of FRMP in this emotional manifestation of chronic pain.

Fmr1KO mice with a partial deletion of CB2 and their WT littermates were used to evaluate the involvement of this receptor in the neuropathic pain-resistant phenotype displayed by Fmr1KO mice. CB2 presence was required to obtain the protective phenotype on nociceptive and emotional responses, since the WT phenotype induced by PSNL was rescued when CB2 was partially removed in Fmr1KO mice. In addition, sham mice partially lacking CB2 showed an enhanced depressive-like behavior, suggesting an antidepressant function of CB2 under basal conditions. Accordingly, previous studies targeting CB2 with pharmacological tools or genetic deletion have also shown antidepressant effects of CB2 activation (Ishiguro et al., 2018; Liu et al., 2017). On the other hand, sham mice partially lacking CB2 did not show cognitive deficits under basal conditions, as other studies have reported when this receptor was pharmacologically blocked (Busquets-Garcia et al., 2013). Similarly, CB2 deletion did not modify the cognitive impairment associated to the nerve injury or to the lack of FMRP, ruling out a function of CB2 promoting cognitive impairment. Altogether, these results highlight that CB2 participates in the protective phenotype displayed by Fmr1KO mice against the nociceptive and

emotional manifestations of neuropathic pain and prevents depressive-like behavior in WT mice under basal conditions.

Sciatic nerve injury increased PEA and OEA levels in the spinal cord of Fmr1KO mice, but not in WT mice. This enhanced tone of spinal N-acylethanolamines could participate in the attenuation of nociceptive responses observed in Fmr1KO mice after the nerve injury. In agreement, previous studies have shown that increased levels of PEA and OEA alleviate different chronic pain states (Gugliandolo et al., 2018; Suardíaz et al., 2007). On the contrary, neuropathic pain was not associated in our study with altered levels of 2-AG or AEA at spinal level, neither in WT or Fmr1KO mice. Previous studies showed that changes of 2-AG and AEA at spinal level were dependent on time at early periods after nerve injury (from day 3 to 7) (Petrosino et al., 2007), whereas no major changes of endocannabinoids were revealed by other authors at early stages (Starowicz et al., 2013; Thomas et al., 2020). However, previous studies did not investigate the changes in endocannabinoid levels at late periods, such as in our study (21 days after nerve injury).

Taken into account these changes on N-acylethanolamines, we investigated the expression of specific genes related to ethanolamines pathways and pain processing including *Ppara*, *Rela*, *Grm5* and *Homer1a*. PPARα, the protein encoded by *Ppara*, is mainly activated by PEA and OEA and is involved in nociception modulating the activity of pro-inflammatory factors such as NF-κB, encoded by *Rela* (Iannotti et al., 2016; Di Marzo, 2018). Nerve ligation did not modify *Ppara* and *Rela* gene expression in the spinal dorsal horn of WT or Fmr1KO mice. In accordance, previous studies did not reveal alterations in *Ppara* expression in the spinal cord of mice after peripheral nerve injury (Okine et al., 2015). We presume that the absence of alterations in *Rela* expression in our experimental conditions could be explained by the absence of changes in *Ppara* gene expression (Rakhshandehroo et al., 2010).

The mGluR5-Homer1a complex, formed by the proteins encoded by these genes, is implicated in the development of chronic pain and associated negative affective states through glutamate-mediated cellular signaling (Chung et al., 2017; Obara et al., 2013a). The expression levels of *Grm5* and *Homer1a* in the spinal dorsal horn of WT or Fmr1KO mice also remained unaltered after nerve injury. Accordingly, no alterations of mGluR5 or Homer1a expression were previously reported following peripheral nerve injury (Michot et al., 2017; Obara et al., 2013b). This absence of expression changes highlights the complex nature of pain processing and prompted us to search for molecular mechanisms in other somatosensory areas that could explain the protective phenotype of Fmr1KO mice.

The expression of *Ppara* and *Rela* in the mPFC was enhanced after nerve injury in Fmr1KO, but not in WT mice. This cortical *Ppara* enhancement may potentially prevent the nociceptive manifestations of neuropathic pain in Fmr1KO mice considering the antinociceptive effects reported by PPARα activation (Di Marzo, 2018). Although PPARα induces antinociceptive effects by preventing upregulation of the protein product of *Rela* (NF-κB) (Iannotti et al., 2016), a previous study demonstrated that PPARα agonist administration did not modify mRNA levels of *Rela* (Nakano et al., 2018). Therefore, *Rela* modifications in Fmr1KO mice could be independent of *Ppara*. On the other hand, Fmr1KO mice exhibited diminished *Grm5* and *Homer1a* expression in the mPFC under sham and neuropathic pain conditions. The complex mGluR5-Homer1a increases glutamatergic input and nociceptive transmission under neuropathic pain conditions (Obara et al., 2013b) and the blockade of cortical mGluR5 decreases mechanical and thermal hypersensitivity after spinal nerve injury (Chung et al., 2017). Hence, the reduced cortical expression of the genes encoding for the pronociceptive mGluR5-Homer1a complex may also contribute to the attenuated nociceptive manifestations of Fmr1KO mice after nerve ligation.

Nerve-injured WT mice showed increased Grm5 expression restricted to the right amygdala, in agreement with previous studies describing enhanced activity of the right amygdala in chronic pain conditions (Allen et al., 2020). Indeed, previous reports described increased mGlur5 function in the right amygdala of mice subjected to different pain models (Crock et al., 2012; Kolber et al., 2010). Interestingly, Fmr1KO mice did not show this increase in Gmr5 expression and exhibited decreased Ppara expression in the same area. Previous studies showed that mGluR5 inhibition in this brain area decreased nociceptive responses in different chronic pain models (Crock et al., 2012; Kolber et al., 2010), suggesting that the unaltered expression of Grm5 in the right amygdala of Fmr1KO mice could participate in the antinociceptive phenotype observed in these animals. Unexpectedly, nerve ligation induced also significant changes in the left amygdala of Fmr1KO mice, involving significant downregulations of *Rela* and *Homer1a*. Positive correlations between the expression of the pronociceptive genes *Rela* and *Homer1a* (Obara et al., 2013b; Paterniti et al., 2017) and the time of immobility in the forced swimming test were obtained exclusively in the contralateral left amygdala of WT and Fmr1KO mice. In agreement, preclinical animal models have demonstrated divergent functions of the left and right amygdala (Cooper et al., 2018; Sadler et al., 2017). Thus, our data is compatible with a participation of the left amygdala in the depressive-like phenotype associated to chronic pain conditions, suggesting that decreased expression of *Rela* and *Homer1a* in the contralateral left amygdala could prevent this emotional manifestation of chronic pain.

Overall, we show that CB2 is required to obtain a protective phenotype against the nociceptive and emotional manifestations of neuropathic pain in Fmr1KO mice. Increased spinal levels of PEA and OEA and decreased expression of glutamate-transmission genes at supra-spinal levels are associated to the attenuated nociceptive responses of Fmr1KO mice. In addition, our findings support the lateralized activity of the left and right amygdala to modulate the affective dimension of chronic pain and highlight the role of glutamate transmission-related genes in this area in the prevention of depressive-like behavior associated with chronic pain conditions. The present data underlines the interest of CB2 as a target to treat neuropathic pain since its low presence in neuronal cells (Shang and Tang, 2017) could avoid the psychoactive side effects of cannabinoid drugs (Davis, 2014). Nevertheless, it would be important in future investigations to include multidisciplinary techniques, such as high-resolution brain imaging or electrophysiological tools, which represent experimental approaches more closely related to human pain experience to potentially optimize preclinical drug discovery aimed at alleviating neuropathic pain.

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AUTHORS CONTRIBUTIONS

R.M. and A.O. designed the study and obtained grants to fund it. C.LP. performed preliminary behavioral experiments. A.R-L. performed the behavioral and molecular experiments and statistical analysis. D.C. contributed to data analysis. A.P. and R.T. carried out and provided resources for the HPLC analysis. A.R-L. wrote the manuscript and R.M. and D.C. reviewed and revised the manuscript and contributed to the interpretation of findings. All authors have read and approved the final manuscript.

FIGURE LEGENDS

Fig. 1. Experimental procedure. Baseline mechanical and heat nociceptive thresholds were measured the day before PSNL or sham surgery. Mechanical and heat nociception were assessed again at days 3, 7 and 14 after the surgery. Cognitive performance and affective behavior were also evaluated under sham and neuropathic pain conditions. Long-term memory was evaluated at day 12 and depressive-like behavior was assessed at day 19 after the surgery. Mice were sacrificed 21 days after the surgery and tissue collection was performed for molecular analysis.

Fig. 2. Nociceptive sensitivity to mechanical and heat stimulation in sham and neuropathic pain conditions. Mice were tested on the ipsilateral and contralateral paws to evaluate mechanical allodynia (mechanical thresholds in grams) in the von Frey test and heat hyperalgesia (plantar latency in seconds) in the plantar test under basal conditions and on day 3, 7 and 14 after PSNL or sham surgery. **(A)** The development of mechanical allodynia after nerve injury mice was demonstrated by significant differences between contralateral and ipsilateral paw sensitivity, which was significantly attenuated on the ipsilateral paw of Fmr1KO mice. No differences in mechanical thresholds following sham surgery were observed between the different genotypes. **(B)** Heat hyperalgesia after nerve injury was also confirmed by significant differences between contralateral and ipsilateral paw withdrawal latencies. Fmr1KO mice also showed decreased heat sensitivity on the ipsilateral paw following PSNL-surgery in comparison to WT mice. No differences in heat hypersensitivity following sham surgery were observed between the different genotypes. Data are expressed as mean ± SEM (n = 13-19 per group). ***p<0.001 between contralateral and ipsilateral paws; +p<0.05, +++p<0.001 vs. WT ipsilateral paw (Linear mixed model, Bonferroni test). Detailed statistical analysis is presented in Supplementary Table S1.

Fig. 3. Cognitive and emotional behaviors in mice exposed to sham or PSNL surgery. Long-term memory impairment (discrimination index in the novel recognition test) and depressive-like behavior (time of immobility in seconds in the forced swimming test) were evaluated 12 and 19 days after the induction of the neuropathy, respectively. **(A)** Nerve injury impaired memory significantly in WT and HzCB2 mice compared to sham mice, whereas Fmr1KO and Fmr1KO-HzCB2 mice show a low discrimination index regardless the surgery. **(B)** PSNL significantly increased the depressive-like behavior in WT, HzCB2 and Fmr1KO-HzCB2 mice, but not in Fmr1KO mice, as indicated by the time of immobility. HzCB2 mice showed a pro-depressive phenotype under sham conditions in comparison to WT sham animals. Data are expressed as mean ± SEM (n = 11-22 per group). Novel object recognition test: ***p<0.001 vs. Sham mice of

each genotype; ###p<0.001 vs. WT sham (ANOVA, Bonferroni test). Forced swimming test: *p<0.05, **p<0.01, ***p<0.001 vs. Sham mice of each genotype; #p<0.05 vs. WT sham; +p<0.05, +++p<0.001 vs. Fmr1KO PSNL mice. (Kruskal-Wallis, U Mann Whitney). Detailed statistical analysis is presented in Supplementary Table S2.

Fig. 4. Quantification of spinal endocannabinoid levels of WT and Fmr1KO mice 21 days after sham or PSNL surgery. High performance liquid chromatography was used to assess the concentration of 2-AG (nmol/g) **(A)**, AEA (pmol/g) **(B)**, PEA (pmol/g) **(C)** and OEA (pmol/g) **(D)** in the spinal dorsal horn ipsilateral to the site of injury of WT and Fmr1KO mice after a sham of PSNL surgery. Nerve-injured Fmr1KO mice showed upregulated spinal levels of PEA and OEA compared to sham mutants, while 2-AG and AEA spinal levels were no altered. Data are expressed as mean ± SEM (n = 8-11 per group). *p<0.05 vs. Fmr1KO sham mice (ANOVA, Bonferroni test). Detailed statistical analysis is presented in Supplementary Table S3.

Fig. 5. Molecular changes in somatosensory pain-related areas of WT and Fmr1KO mice 21 days after sham or PSNL surgery. RT-PCR analysis of *Ppara, Rela, Grm5 and Homer1a* gene expression in the spinal ipsilateral dorsal horn, mPFC, right and left amygdala were measured by using the ΔΔCt method. Expression of all these targets was first standardized by expression of B2M and expressed relative to the data taken from the WT sham control group. **(A)** In the spinal cord, RT-PCR analysis of mRNA *Ppara, Rela, Grm5 and Homer1a* expression revealed no significant differences among genotypes. **(B)** In the mPFC, nerve-injured Fmr1KO mice showed upregulated levels of *Ppara* and *Rela* expression compared to WT PSNL and Fmr1KO sham mice, while *Grm5* expression was reduced in mutants. **(C)** In the right amygdala, WT mice with the neuropathy exhibited increased levels of *Grm5* expression in comparison to sham littermates and nerve-injured Fmr1KO mice. **(D)** In the left amygdala, Fmr1KO mice after PSNL surgery showed decreased expression of *Rela* and *Homer1a* compared to nerve-injured WT animals. Data are expressed as mean ± SEM (n = 6-10 per group). *p<0.05, **p<0.01, ***p<0.001 (ANOVA, Bonferroni test; Kruskal-Wallis, U Mann Whitney). Detailed statistical analysis is presented in Supplementary Table S4.

Figure 6. Correlations between gene expression in the left amygdala and depressive-like behavior of WT and Fmr1KO mice 21 days after sham or PSNL surgery. Correlation between the time of immobility in the forced swimming test and the gene expression of *Ppara* **(A)***, Rela* **(B)***, Grm5* **(C)** *and Homer1a* **(D)** in the left amygdala of WT and Fmr1KO mice. *Rela, Grm5 and Homer1a* gene expression in the left amygdala, but no *Ppara*, showed significant positive

correlations with depressive-like behaviors. Orange dots represent PSNL Fmr1KO mice. Data are expressed as mean (n = 5-7 per group). *p<0.05, **p<0.01 (Pearson Correlation).

Supplementary material

Role of the endocannabinoid system in a mouse model of Fragile X undergoing neuropathic pain

Á. Ramírez-López[1], A. Pastor[2], R. de la Torre[2], C. La Porta[1,a], A. Ozaita[1], D. Cabañero[1,b] and R. Maldonado[1,2,*]

Supplementary table S1. Detailed statistical evaluation for Fig. 1.

Tests of normality

Von-Frey PSNL	Shapiro-Wilk significance
Baseline	0.005
Day 3	0.000
Day 7	0.000
Day 14	0.000

Von-Frey sham	Shapiro-Wilk significance
Baseline	0.000
Day 3	0.000
Day 7	0.000
Day 14	0.000

Plantar PSNL	Shapiro-Wilk significance
Baseline	0.478
Day 3	0.001
Day 7	0.000
Day 14	0.003

Plantar sham	Shapiro-Wilk significance
Baseline	0.824
Day 3	0.003
Day 7	0.064
Day 14	0.038

Linear mixed model with three factors for nociceptive thresholds. Only when F was significant, Linear mixed model was followed by Bonferroni *post hoc.*

Between genotypes **(Von-Frey PSNL)**	**Pressure**
Genotype	F (3, 520.922) = 3.315; p<0.05
Paw	F (1, 520.922) = 457.620; p<0.001
Genotype x Paw	F (3, 520.922) = 9.419; p < 0.001
Time	F (3, 260.160) = 58.798; p<0.001
Time x Genotype	F (9, 260.160) = 1.241; p = 0.270
Time x Paw	F (3, 260.160) = 77.498; p<0.001
Time x Genotype x Paw	F (9, 260.160) = 1.569; p = 0.125

Between genotypes **(Von-Frey sham)**	**Pressure**
Genotype	F (3, 498.569) = 4.911; p<0.01
Paw	F (1, 498.569) = 1.972; p = 0.161
Genotype x Paw	F (3, 498.569) = 5.108; p = 0.741
Time	F (3, 245.633) = 2.394; p = 0.069
Time x Genotype	F (9, 245.633) = 1.258; p = 0.261
Time x Paw	F (3, 245.633) = 1.774; p = 0.153
Time x Genotype x Paw	F (9, 245.633) = 0.527; p = 0.854

Between genotypes **(Plantar PSNL)**	**Heat**
Genotype	F (3, 526.262) = 23.258; p<0.001
Paw	F (1, 526.262) = 617.584; p<0.001
Genotype x Paw	F (3, 526.262) = 23.049; p<0.001
Time	F (3, 255.117) = 94.555; p<0.001
Time x Genotype	F (9, 255.117) = 5.113; p<0.001
Time x Paw	F (3, 255.117) = 84.631; p<0.001
Time x Genotype x Paw	F (9, 255.117) = 2.575; p<0.01

Between genotypes **(Plantar sham)**	**Heat**
Genotype	F (3, 495.040) = 3.534; p < 0.05
Paw	F (1, 495.040) = 0.240; p = 0.625
Genotype x Paw	F (3, 495.040) = 0.164; p = 0.920
Time	F (3, 237.012) = 1.374; p = 0.251
Time x Genotype	F (9, 237.012) = 1.218; p = 0.284
Time x Paw	F (3, 237.012) = 0.564; p = 0.652
Time x Genotype x Paw	F (9, 237.012) = 0.693; p = 0.715

Supplementary table S2. Detailed statistical evaluation for Fig. 2.

Tests of normality

Novel object recognition test	Shapiro-Wilk significance
WT	0.623
Fmr1KO	0.945
HzCB2	0.119
Fmr1KO-HzCB2	0.227

Forced swimming test	Shapiro-Wilk significance
WT	0.002
Fmr1KO	0.053
HzCB2	0.171
Fmr1KO-HzCB2	0.017

ANOVA model with two factors for cognitive manifestations of neuropathic pain. Only when F was significant, ANOVA was followed by Bonferroni *post hoc.*

Among 8 groups	**Discrimination Index**
Genotype	F (3, 124) = 10.037; p<0.001
Surgery	F (1, 124) = 15.915; p<0.001
Genotype x Surgery	F (3, 124) = 15.891; p<0.001

Kruskal-Wallis for emotional manifestations of neuropathic pain. Only when H was significant, Kruskal-Wallis was followed by U Mann Whitney with Bonferroni adjustment.

Among 8 groups	**Time of immobility**
Time of immobility	H (7) = 40.141, p<0.001

Supplementary table S3. Detailed statistical evaluation for Fig. 3.

Tests of normality

2-AG	Shapiro-Wilk significance
WT	0.729
Fmr1KO	0.073
AEA	
WT	0.388
Fmr1KO	0.598
PEA	
WT	0.078
Fmr1KO	0.663
OEA	
WT	0.328
Fmr1KO	0.885

ANOVA model with two factors for High performance liquid chromatography data. Only when F was significant, ANOVA was followed by Bonferroni *post hoc.*

2-AG	
Genotype	F (1, 34) = 2.701; p = 0.109
Surgery	F (1, 34) = 2.456; p = 0.126
Genotype x Surgery	F (1, 34) = 0.191; p = 0.665
AEA	
Genotype	F (1, 34) = 0.351; p = 0.558
Surgery	F (1, 34) = 0.641; p = 0.429
Genotype x Surgery	F (1, 34) = 1.063; p = 0.310
PEA	

Genotype	F (1, 34) = 0.0.29; p = 0.865
Surgery	F (1, 34) = 4.539; p = 0.040
Genotype x Surgery	F (1, 34) = 4.602; p<0.05
OEA	
Genotype	F (1, 34) = 0.812; p = 0.374
Surgery	F (1, 34) = 2.253; p = 0.143
Genotype x Surgery	F (1, 34) = 15.891; p<0.05

Supplementary table S4. Detailed statistical evaluation for Fig. 4.

Tests of normality

SPINAL CORD

Ppara	Shapiro-Wilk significance
WT	0.023
Fmr1KO	0.005
Rela	
WT	0.138
Fmr1KO	0.034
Grm5	
WT	0.020
Fmr1KO	0.013
Homer1a	
WT	0.432
Fmr1KO	0.007

mPFC

Ppara	Shapiro-Wilk significance
WT	0.813
Fmr1KO	0.196
Rela	
WT	0.654
Fmr1KO	0.346
Grm5	
WT	0.041
Fmr1KO	0.768
Homer1a	
WT	0.553
Fmr1KO	0.081

RIGHT AMYGDALA

Ppara	Shapiro-Wilk significance
WT	0.876
Fmr1KO	0.700
Rela	
WT	0.001
Fmr1KO	0.017
Grm5	
WT	0.803
Fmr1KO	0.700
Homer1a	
WT	0.165
Fmr1KO	0.001

LEFT AMYGDALA

Ppara	Shapiro-Wilk significance
WT	0.865
Fmr1KO	0.339
Rela	
WT	0.281
Fmr1KO	0.398
Grm5	
WT	0.906
Fmr1KO	0.484
Homer1a	
WT	0.799
Fmr1KO	0.093

ANOVA model with two factors for RT-PCR data. Only when F was significant, ANOVA was followed by Bonferroni *post hoc.*

mPFC

Ppara	
Genotype	F (1, 26) = 7.117; p<0.05
Surgery	F (1, 26) = 3.500; p = 0.073
Genotype x Surgery	F (1, 26) = 9.287; p<0.01
Rela	
Genotype	F (1, 34) = 22.377; p<0.001
Surgery	F (1, 34) = 13.576; p<0.01
Genotype x Surgery	F (1, 34) = 23.729; p<0.001
Homer1a	
Genotype	F (1, 31) = 10.292; p<0.01
Surgery	F (1, 31) = 4.410; p<0.05
Genotype x Surgery	F (1, 31) = 2.486; p = 0.125

LEFT AMYGDALA

Ppara	
Genotype	F (1, 20) = 0.470; p = 0.501
Surgery	F (1, 20) = 6.564; p<0.05
Genotype x Surgery	F (1, 20) = 0.560; p = 0.463
Rela	
Genotype	F (1, 19) = 12.907; p<0.01
Surgery	F (1, 19) = 0.870; p = 0.363
Genotype x Surgery	F (1, 19) = 4.474; p<0.05
Grm5	
Genotype	F (1, 20) = 1.995; p = 0.173
Surgery	F (1, 20) = 0.533; p = 0.474
Genotype x Surgery	F (1, 20) = 0.501; p = 0.487
Homer1a	
Genotype	F (1, 19) = 7.742; p<0.05
Surgery	F (1, 19) = 0.041; p = 0.840
Genotype x Surgery	F (1, 19) = 4.930; p<0.05

RIGHT AMYGDALA

Ppara	
Genotype	F (1, 20) = 5.194; p<0.05
Surgery	F (1, 20) = 3.927; p = 0.061
Genotype x Surgery	F (1, 20) = 3.254; p = 0.086
Grm5	
Genotype	F (1, 20) = 5.900; p<0.05
Surgery	F (1, 20) = 10.349; p<0.01
Genotype x Surgery	F (1, 20) = 6.390; p<0.05

Kruskal-Wallis for RT-PCR data. Only when H was significant, Kruskal-Wallis was followed by U Mann Whitney with Bonferroni adjustment.

SPINAL CORD	
Ppara	H (3) = 5.507, p = 0.138
Rela	H (3) = 5.539, p = 0.136
Grm5	H (3) = 1.294, p = 0.731
Homer1a	H (3) = 3.497, p = 0.321
MPFC	
Grm5	H (3) = 14.125, p<0.01
RIGHT AMYGDALA	
Rela	H (3) = 0.087, p = 0.993
Homer1a	H (3) = 7.167, p = 0.067

Figure 1

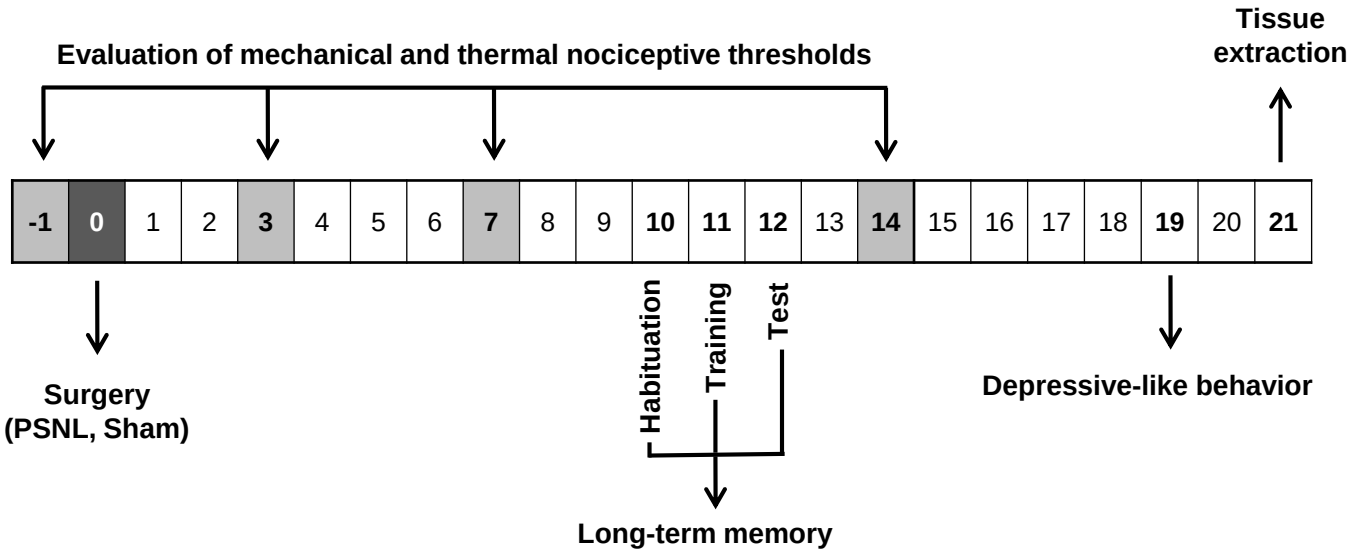
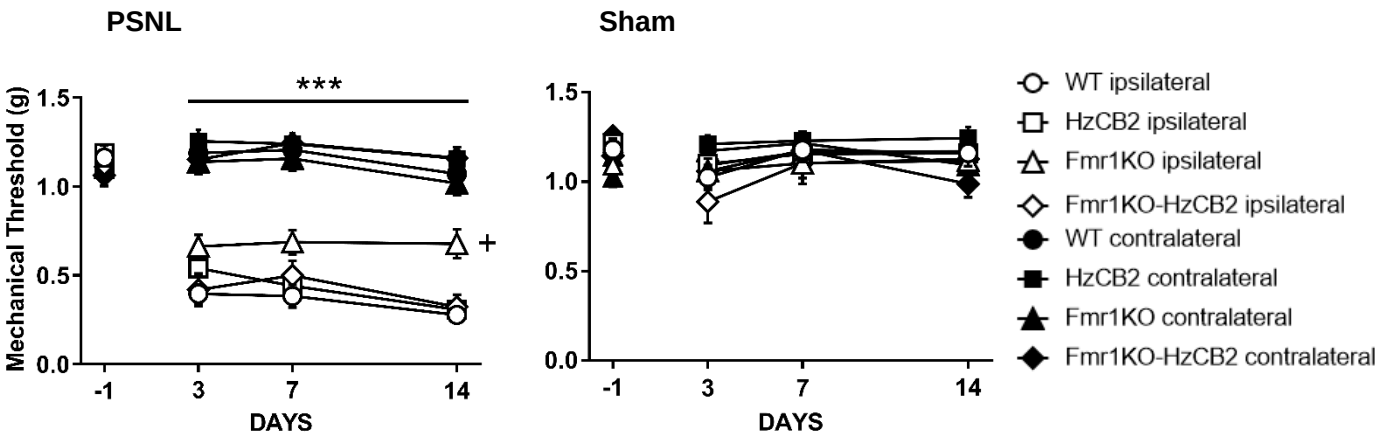

Evaluation of mechanical and thermal nociceptive thresholds
Tissue extraction
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
Surgery (PSNL, Sham)
Habituation
Training
Test
Long-term memory
Depressive-like behavior

Figure 2

(A) Mechanical nociception



(B) Heat nociception

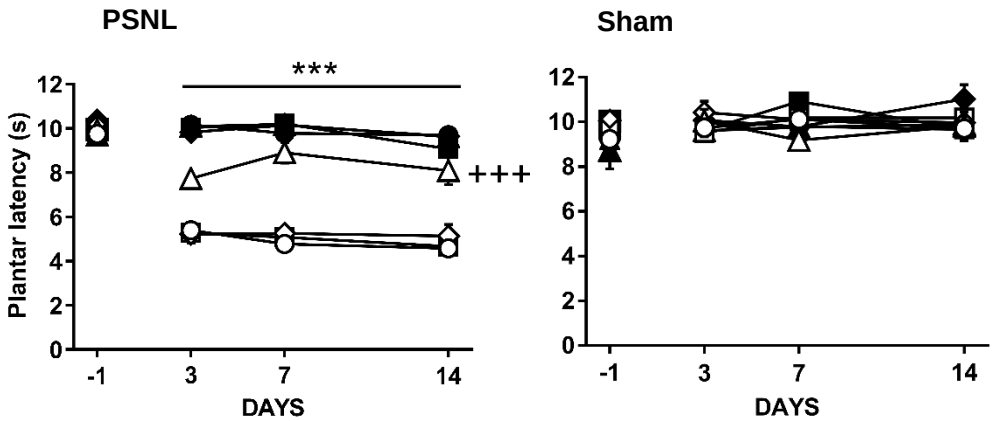


Figure 3

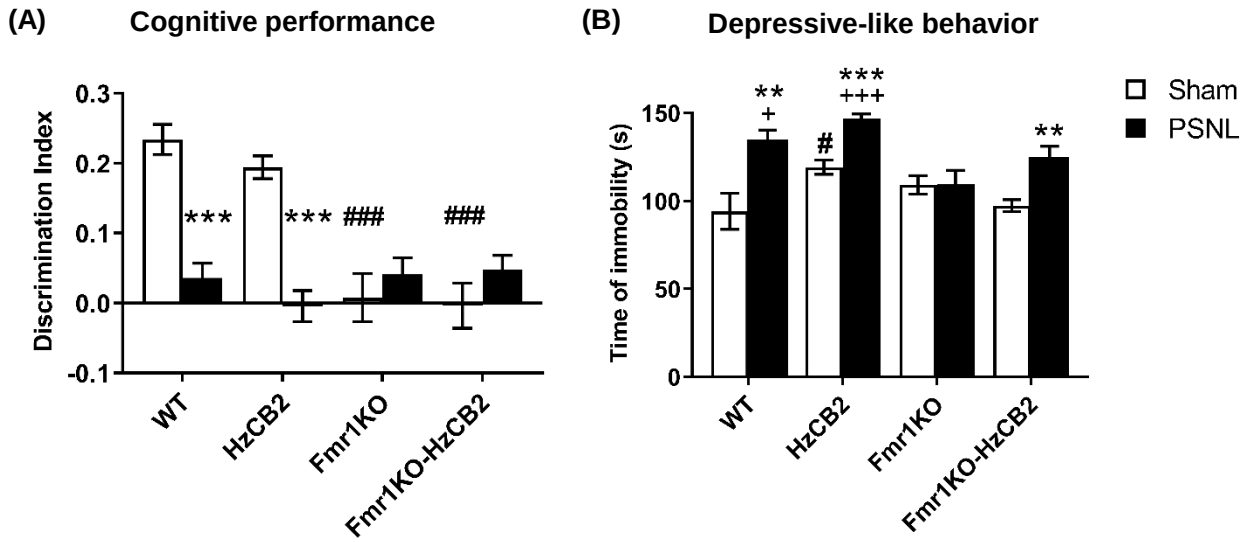

(A)
Cognitive performance
Discrimination Index
0.3
0.2
0.1
0.0
-0.1
***
***
###
###
WT
HzCB2
Fmr1KO
Fmr1KO-HzCB2
(B)
Depressive-like behavior
Time of immobility (s)
150
100
50
0
**
+
#
***
+++
**
Sham
PSNL
WT
HzCB2
Fmr1KO
Fmr1KO-HzCB2

Figure 4

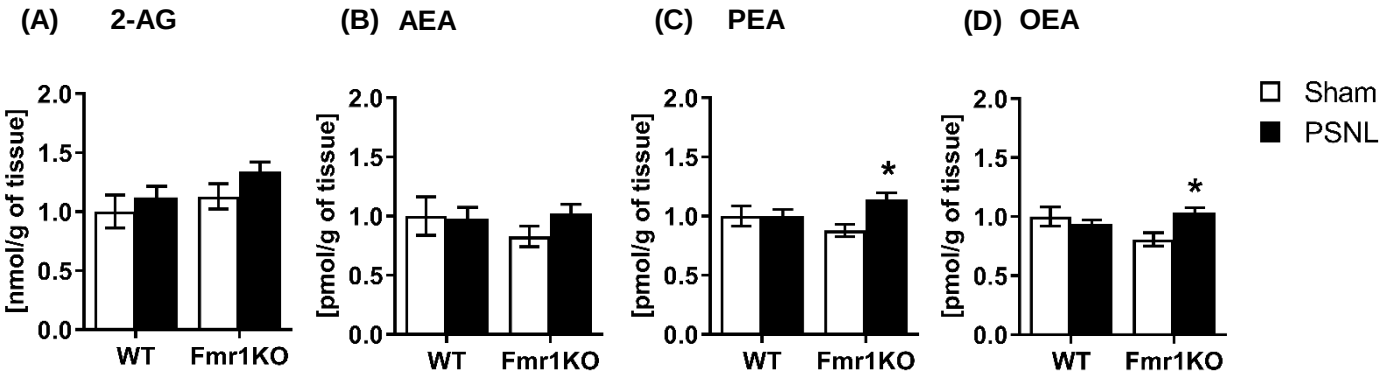

SPINAL CORD
(A) 2-AG
(B) AEA
(C) PEA
(D) OEA
[nmol/g of tissue]
[pmol/g of tissue]
2.0
1.5
1.0
0.5
0.0
WT
Fmr1KO
*
Sham
PSNL

Figure 5

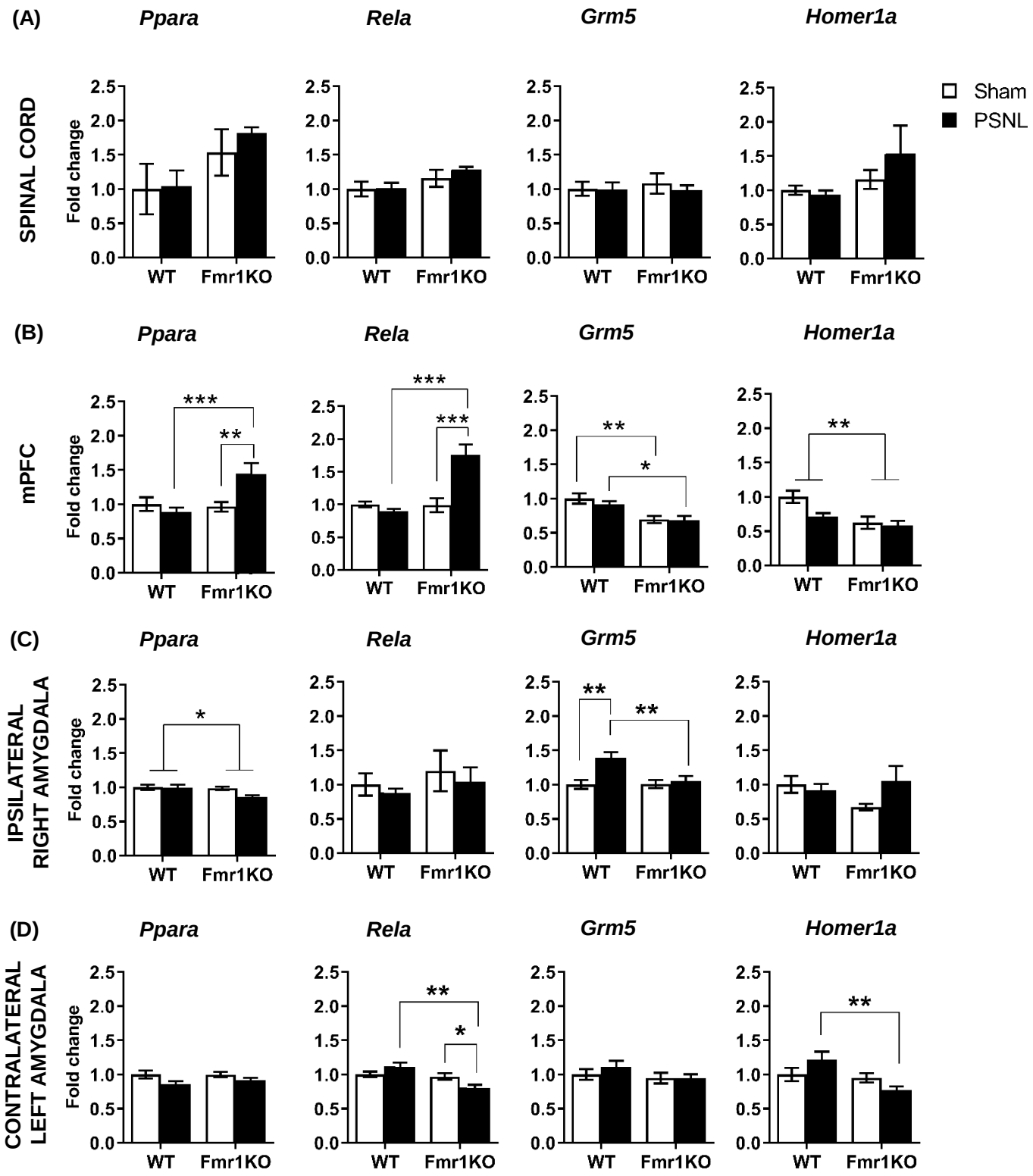
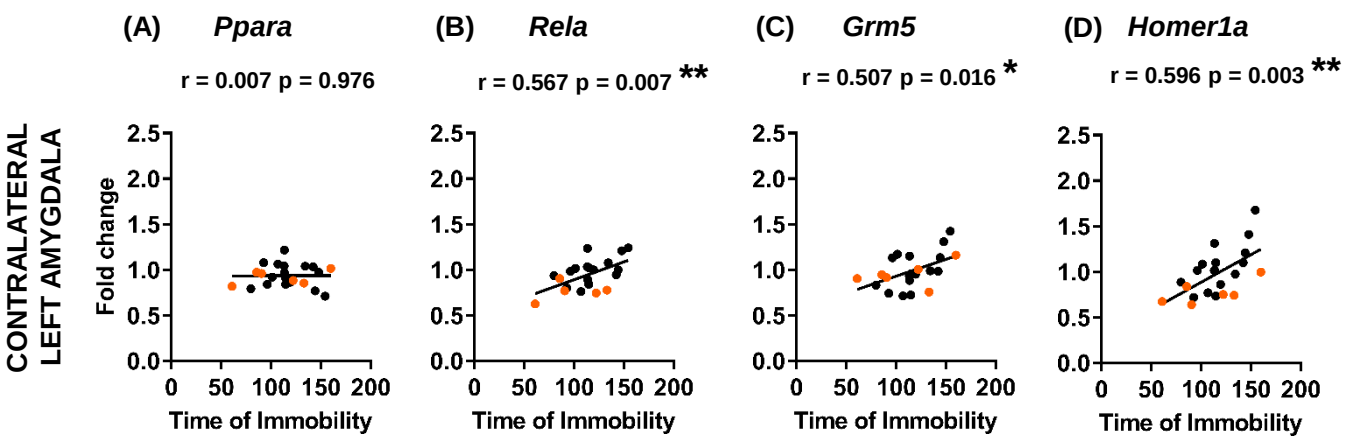

(A)
Ppara
Rela
Grm5
Homer1a
SPINAL CORD
Fold change
Sham
PSNL
WT
Fmr1KO
(B)
mPFC
(C)
IPSILATERAL RIGHT AMYGDALA
(D)
CONTRALATERAL LEFT AMYGDALA

Figure 6


(A) Ppara
r = 0.007 p = 0.976
(B) Rela
r = 0.567 p = 0.007 **
(C) Grm5
r = 0.507 p = 0.016 *
(D) Homer1a
r = 0.596 p = 0.003 **
CONTRALATERAL LEFT AMYGDALA
Fold change
0.0
0.5
1.0
1.5
2.0
2.5
0
50
100
150
200
Time of Immobility

Supplementary material

Role of the endocannabinoid system in a mouse model of Fragile X undergoing neuropathic pain

Á. Ramírez-López[1], A. Pastor[2], R. de la Torre[2], D. Cabañero[1,a] and R. Maldonado[1,2,*]

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Tests of normality

Von-Frey PSNL	Shapiro-Wilk significance
Baseline	0.005
Day 3	0.000
Day 7	0.000
Day 14	0.000

Von-Frey sham	Shapiro-Wilk significance
Baseline	0.000
Day 3	0.000
Day 7	0.000
Day 14	0.000

Plantar PSNL	Shapiro-Wilk significance
Baseline	0.478
Day 3	0.001
Day 7	0.000
Day 14	0.003

Plantar sham	Shapiro-Wilk significance
Baseline	0.824
Day 3	0.003
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Linear mixed model with three factors for nociceptive thresholds. Only when F was significant, Linear mixed model was followed by Bonferroni *post hoc.*

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Genotype	F (3, 520.922) = 3.315; p<0.05
Paw	F (1, 520.922) = 457.620; p<0.001
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Paw	F (1, 498.569) = 1.972; p = 0.161
Genotype x Paw	F (3, 498.569) = 5.108; p = 0.741
Time	F (3, 245.633) = 2.394; p = 0.069
Time x Genotype	F (9, 245.633) = 1.258; p = 0.261
Time x Paw	F (3, 245.633) = 1.774; p = 0.153
Time x Genotype x Paw	F (9, 245.633) = 0.527; p = 0.854

Between genotypes **(Plantar PSNL)**	**Heat**
Genotype	F (3, 526.262) = 23.258; p<0.001
Paw	F (1, 526.262) = 617.584; p<0.001
Genotype x Paw	F (3, 526.262) = 23.049; p<0.001
Time	F (3, 255.117) = 94.555; p<0.001
Time x Genotype	F (9, 255.117) = 5.113; p<0.001
Time x Paw	F (3, 255.117) = 84.631; p<0.001
Time x Genotype x Paw	F (9, 255.117) = 2.575; p<0.01

Between genotypes **(Plantar sham)**	**Heat**
Genotype	F (3, 495.040) = 3.534; p < 0.05
Paw	F (1, 495.040) = 0.240; p = 0.625
Genotype x Paw	F (3, 495.040) = 0.164; p = 0.920
Time	F (3, 237.012) = 1.374; p = 0.251
Time x Genotype	F (9, 237.012) = 1.218; p = 0.284
Time x Paw	F (3, 237.012) = 0.564; p = 0.652
Time x Genotype x Paw	F (9, 237.012) = 0.693; p = 0.715

Supplementary table S2. Detailed statistical evaluation for Fig. 2.

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Novel object recognition test	Shapiro-Wilk significance
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Fmr1KO	0.945
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Forced swimming test	Shapiro-Wilk significance
WT	0.002
Fmr1KO	0.053
HzCB2	0.171
Fmr1KO-HzCB2	0.017

ANOVA model with two factors for cognitive manifestations of neuropathic pain. Only when F was significant, ANOVA was followed by Bonferroni *post hoc.*

Between 8 groups	**Discrimination Index**
Genotype	F (3, 124) = 10.037; p<0.001
Surgery	F (1, 124) = 15.915; p<0.001
Genotype x Surgery	F (3, 124) = 15.891; p<0.001

Kruskal-Wallis for emotional manifestations of neuropathic pain. Only when H was significant, Kruskal-Wallis was followed by U Mann Whitney with Bonferroni adjustment.

Between 8 groups	**Time of immobility**
Time of immobility	H (7) = 40.141, p<0.001

Supplementary table S3. Detailed statistical evaluation for Fig. 3.

Tests of normality

2-AG	Shapiro-Wilk significance
WT	0.729
Fmr1KO	0.073
AEA	
WT	0.388
Fmr1KO	0.598
PEA	
WT	0.078
Fmr1KO	0.663
OEA	
WT	0.328
Fmr1KO	0.885

ANOVA model with two factors for High performance liquid chromatography data. Only when F was significant, ANOVA was followed by Bonferroni *post hoc.*

2-AG	
Genotype	F (1, 34) = 2.701; p = 0.109
Surgery	F (1, 34) = 2.456; p = 0.126
Genotype x Surgery	F (1, 34) = 0.191; p = 0.665
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Genotype	F (1, 34) = 0.351; p = 0.558
Surgery	F (1, 34) = 0.641; p = 0.429
Genotype x Surgery	F (1, 34) = 1.063; p = 0.310
PEA	

Genotype	F (1, 34) = 0.0.29; p = 0.865
Surgery	F (1, 34) = 4.539; p = 0.040
Genotype x Surgery	F (1, 34) = 4.602; p<0.05
OEA	
Genotype	F (1, 34) = 0.812; p = 0.374
Surgery	F (1, 34) = 2.253; p = 0.143
Genotype x Surgery	F (1, 34) = 15.891; p<0.05

Supplementary table S4. Detailed statistical evaluation for Fig. 4.

Tests of normality

SPINAL CORD

Ppara	Shapiro-Wilk significance
WT	0.023
Fmr1KO	0.005
Rela	
WT	0.138
Fmr1KO	0.034
Grm5	
WT	0.020
Fmr1KO	0.013
Homer1a	
WT	0.432
Fmr1KO	0.007

mPFC

Ppara	Shapiro-Wilk significance
WT	0.813
Fmr1KO	0.196
Rela	
WT	0.654
Fmr1KO	0.346
Grm5	
WT	0.041
Fmr1KO	0.768
Homer1a	
WT	0.553
Fmr1KO	0.081

RIGHT AMYGDALA

Ppara	Shapiro-Wilk significance
WT	0.876
Fmr1KO	0.700
Rela	
WT	0.001
Fmr1KO	0.017
Grm5	
WT	0.803
Fmr1KO	0.700
Homer1a	
WT	0.165
Fmr1KO	0.001

LEFT AMYGDALA

Ppara	Shapiro-Wilk significance
WT	0.865
Fmr1KO	0.339
Rela	
WT	0.281
Fmr1KO	0.398
Grm5	
WT	0.906
Fmr1KO	0.484
Homer1a	
WT	0.799
Fmr1KO	0.093

ANOVA model with two factors for RT-PCR data. Only when F was significant, ANOVA was followed by Bonferroni *post hoc.*

mPFC

Ppara	
Genotype	F (1, 26) = 7.117; p<0.05
Surgery	F (1, 26) = 3.500; p = 0.073
Genotype x Surgery	F (1, 26) = 9.287; p<0.01
Rela	
Genotype	F (1, 34) = 22.377; p<0.001
Surgery	F (1, 34) = 13.576; p<0.01
Genotype x Surgery	F (1, 34) = 23.729; p<0.001
Homer1a	
Genotype	F (1, 31) = 10.292; p<0.01
Surgery	F (1, 31) = 4.410; p<0.05
Genotype x Surgery	F (1, 31) = 2.486; p = 0.125

LEFT AMYGDALA

Ppara	
Genotype	F (1, 20) = 0.470; p = 0.501
Surgery	F (1, 20) = 6.564; p<0.05
Genotype x Surgery	F (1, 20) = 0.560; p = 0.463
Rela	
Genotype	F (1, 19) = 12.907; p<0.01
Surgery	F (1, 19) = 0.870; p = 0.363
Genotype x Surgery	F (1, 19) = 4.474; p<0.05
Grm5	
Genotype	F (1, 20) = 1.995; p = 0.173
Surgery	F (1, 20) = 0.533; p = 0.474
Genotype x Surgery	F (1, 20) = 0.501; p = 0.487
Homer1a	
Genotype	F (1, 19) = 7.742; p<0.05
Surgery	F (1, 19) = 0.041; p = 0.840
Genotype x Surgery	F (1, 19) = 4.930; p<0.05

RIGHT AMYGDALA

Ppara	
Genotype	F (1, 20) = 5.194; p<0.05
Surgery	F (1, 20) = 3.927; p = 0.061
Genotype x Surgery	F (1, 20) = 3.254; p = 0.086
Grm5	
Genotype	F (1, 20) = 5.900; p<0.05
Surgery	F (1, 20) = 10.349; p<0.01
Genotype x Surgery	F (1, 20) = 6.390; p<0.05

Kruskal-Wallis for RT-PCR data. Only when H was significant, Kruskal-Wallis was followed by U Mann Whitney with Bonferroni adjustment.

SPINAL CORD	
Ppara	H (3) = 5.507, p = 0.138
Rela	H (3) = 5.539, p = 0.136
Grm5	H (3) = 1.294, p = 0.731
Homer1a	H (3) = 3.497, p = 0.321
MPFC	
Grm5	H (3) = 14.125, p<0.01
RIGHT AMYGDALA	
Rela	H (3) = 0.087, p = 0.993
Homer1a	H (3) = 7.167, p = 0.067