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# Endocannabinoids decrease neuropathic pain-related behavior in mice through the activation of one or both peripheral $CB_1$ and $CB_2$ receptors

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ABSTRACT

The two most studied endocannabinoids are anandamide (AEA), principally catalyzed by fatty-acid amide hydrolase (FAAH), and 2-arachidonoyl glycerol (2-AG), mainly hydrolyzed by monoacylglycerol lipase (MGL). Inhibitors targeting these two enzymes have been described, including URB597 and URB602, respectively. Several recent studies examining the contribution of $CB_1$ and/or $CB_2$ receptors on the peripheral antinociceptive effects of AEA, 2-AG, URB597 and URB602 in neuropathic pain conditions using either pharmacological tools or transgenic mice separately have been reported, but the exact mechanism is still uncertain. Mechanical allodynia and thermal hyperalgesia were evaluated in 436 male C57BL/6, *cnr1*KO and *cnr2*KO mice in the presence or absence of cannabinoid $CB_1$ (AM251) or $CB_2$ (AM630) receptor antagonists in a mouse model of neuropathic pain. Peripheral subcutaneous injections of AEA, 2-AG, WIN55,212-2 (WIN; a $CB_1/CB_2$ synthetic agonist), URB597 and URB602 significantly decreased mechanical allodynia and thermal hyperalgesia. These effects were inhibited by both cannabinoid antagonists AM251 and AM630 for treatments with 2-AG, WIN and URB602 but only by AM251 for treatments with AEA and URB597 in C57BL/6 mice. Furthermore, the antinociceptive effects for AEA and URB597 were observed in *cnr2*KO mice but absent in *cnr1*KO mice, whereas the effects of 2-AG, WIN and URB602 were altered in both of these transgenic mice. Complementary genetic and pharmacological approaches revealed that the anti-hyperalgesic effects of 2-AG and URB602 required both $CB_1$ and $CB_2$ receptors, but only $CB_2$ receptors mediated its anti-allodynic actions. The antinociceptive properties of AEA and URB597 were mediated only by $CB_1$ receptors.



## 1. Introduction

Endocannabinoids, such as arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG), are endogenous lipid-signaling ligands produced on demand from phospholipid precursors (see (Luchicchi and Pistis, 2012) for a review). These promising pain modulators act through the activation of two distinct G-protein-coupled receptors, $CB_1$ (Devane et al., 1988) and $CB_2$ receptors (Munro et al., 1993). $CB_1$ cannabinoid receptors are present on primary afferent neurons and various peripheral tissues (Agarwal et al., 2007; Hohmann and Herkenham, 1999; Walczak et al., 2005, 2006). The expression of $CB_2$ cannabinoid receptors is generally limited to cells of the immune and hematopoietic systems, for recent reviews see (Pacher and Mechoulam, 2011; Rom and Persidsky, 2013), although the $CB_2$ receptor protein has also been identified on brainstem neurons (Van Sickle et al., 2005) and in microglial cultures (Beltramo et al., 2006). The endocannabinoid system also includes enzymes responsible for their synthesis and degradation. AEA is mainly hydrolyzed by fatty-acid amide hydrolase (FAAH) (Cravatt et al., 1996), whereas 2-AG is mainly metabolized by monoacylglycerol lipase (MGL) (Dinh et al., 2002; Goparaju et al., 1999) and to a lesser extent by FAAH (Bisogno et al., 2005), serine hydrolase α-β-hydrolase domain 6 (ABHD6) and 12 (ABHD12) (Blankman et al., 2007; Marrs et al., 2010), among others. The expression of these two main endocannabinoid catabolizing enzymes is found along pain pathways, specifically in the sensory peripheral nervous system, such as dorsal root ganglia

*Abbreviations:* AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; CB, cannabinoid; DMSO, dimethylsulfoxide; FAAH, fatty-acid amide hydrolase; MGL, monoacylglycerol lipase.

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(DRG), the sciatic nerve and spinal cord for FAAH (Lever et al., 2009), whereas the precise anatomical localization of MGL in the periphery has not been fully elucidated, although its activity in peripheral paw tissues has been reported (Guindon et al., 2011) and its expression has been co-localized in superficial dorsal horn microglia (Wilkerson et al., 2012).

Central and spinal cannabinoidergic transmission is well established (for a review see (Walker and Huang, 2002)), and peripheral cannabinoidergic transmission is crucial in pain control (Agarwal et al., 2007; Clapper et al., 2010; Marrs et al., 2010; Yu et al., 2010), involving different mechanisms of action. We have previously reported that the local antinociceptive effects of AEA were selectively blocked by a $CB_1$ receptor antagonist (Guindon and Beaulieu, 2006), whereas the peripheral effects of 2-AG in the partial sciatic nerve ligation (PNL) rat neuropathic pain model were alleviated by both $CB_1$ and $CB_2$ cannabinoid antagonists (Desroches et al., 2008). For pain treatment, an alternative approach to direct exogenous injections of AEA and 2-AG is to indirectly increase their levels in tissues through the injection of pharmacological inhibitors of the enzymatic degradation of endocannabinoids. Accordingly, we have already reported that mechanical allodynia and thermal hyperalgesia in the PNL neuropathic pain model were alleviated following peripheral administration of FAAH and MGL inhibitors (Desroches et al., 2008) *via* $CB_1$ (URB597; (Kathuria et al., 2003) and $CB_1/CB_2$ (URB602; (Hohmann et al., 2005)) mechanisms.

According to the recent failures of FAAH inhibitors in patients with painful knee osteoarthritis (Huggins et al., 2012), it appears important to increase the concentrations of endocannabinoids in a targeted manner only in specific tissues where such an increase would be desirable (Di Marzo, 2008; Pacher and Kunos, 2013). Thus, several studies have demonstrated that the administration of inhibitors of the enzymes involved in the degradation of endocannabinoids attenuates different pain states, including neuropathic pain (see (Roques et al., 2012) for a review). However, some of these studies present intriguing discrepancies between the relative contributions of each cannabinoid receptor following the administration of FAAH and MGL inhibitors, suggesting that distinct cannabinoid receptor mechanisms of action underlie the antinociceptive effects of FAAH and MGL inhibitors (Kinsey et al., 2009).

Therefore, the aim of this study was to understand the exact contribution of each cannabinoid receptor in mediating the peripheral antinociceptive effects of FAAH and MGL inhibitors in a mouse model of neuropathic pain. In contrast to most of the previous studies who used transgenic mice or pharmacological approaches separately to examine the underlying cannabinoid mechanisms of action, the present study used complementary pharmacological and genetic approaches to assess if (1) genetic inactivation of $CB_1$ or $CB_2$ receptors impacts on PNL-induced nociception, (2) anti-allodynic and anti-hyperalgesic effects of FAAH inhibition by URB597 are prevented when cannabinoid receptors are inactivated, and, (3) $CB_1$ and $CB_2$ receptors are involved in the antinociceptive effects following MGL inhibition using URB602.

## 2. Methods

### 2.1. Animals

The research protocol was approved by the Animal Ethics Committee of the Université de Montréal and all of the procedures conformed to the directives of the Canadian Council on Animal Care and guidelines of the International Association for the Study of Pain. Four hundred thirty-six male C57BL/6, *cnr1*KO and *cnr2*KO mice (20–25 g at the time of testing) were used in the current study. They were housed in groups of two to four in standard plastic cages with sawdust bedding in a climate-controlled room. The mice were allowed free access to food pellets and water. They were maintained in a 12-h light/dark cycle. All behavioral testing was performed during the light cycle. C57BL/6 mice were purchased from Charles River (St-Constant, Quebec, Canada). *Cnr1* transgenic mice were obtained from Pr. Beat Lutz (Institute of Physiological Chemistry and Pathobiochemistry, University of Mainz, Germany), which were generated and genotyped as previously described (Marsicano et al., 2002). *Cnr2* transgenic mice (005786; MGI 3604531) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). These colonies were maintained in-house. All of the animal experiments were designed to minimize the number of animals used and their suffering.

### 2.2. Drug administration

Anandamide is an agonist with a 4-fold selectivity for $CB_1$ ($K_i = 89$ nM) over $CB_2$ ($K_i = 371$ nM) receptors (Pertwee et al., 1995). It was purchased from Tocris (Ellisville, MO, USA) in a liquid form of a water-dispersible emulsion and dissolved in 0.9% NaCl in water. 2-arachidonoyl glycerol is a full agonist of both the $CB_1$ and $CB_2$ receptors, with a 3-fold selectivity for $CB_1$ (Ki = 472 nM) over $CB_2$ (Ki = 1400 nM) receptors (Mechoulam et al., 1995). It was purchased from Sigma (St. Louis, MO, USA) as an acetonitrile solution and dissolved in normal saline. WIN 55,212-2 is a synthetic agonist for $CB_1$ (62 nM) and $CB_2$ (3 nM) receptors (Felder et al., 1995). It was purchased from Tocris (Ellisville, MO, USA) in a powder form and dissolved in a 1:1:18 mixture of ethanol:cremophor:saline (Costa et al., 2004). AM251 and AM630 are selective $CB_1$ and $CB_2$ receptor antagonists, respectively. AM251 exhibits a 306-fold selectivity for $CB_1$ over $CB_2$ receptors (Gatley et al., 1996), whereas AM630 exhibits 70–165-fold selectivity for $CB_2$ over $CB_1$ receptors (Pertwee et al., 1995; Ross et al., 1999). The doses for each of the substances used in this study were chosen according to previous reports from our lab and others and adjusted for mice. They were purchased from Tocris (Ellisville, MO, USA) and dissolved in normal saline containing 0.1% of dimethyl sulfoxide (DMSO). URB597 and URB602 were purchased from Cayman Chemical (Ann Arbor, MI, USA) and dissolved in normal saline containing 2.5% of DMSO for the experimental doses. Preliminary experiments performed in our laboratory with neuropathic rats ($n = 4$) have previously shown that there was no difference in neuropathic testing between 0.9% NaCl and 0.9% NaCl solution with 8% DMSO (Guindon and Beaulieu, 2006). All of the drugs were stored at −20 °C and protected from light, and fresh solutions were prepared on the day of the experiment with the appropriate vehicle.

### 2.3. Mouse model of neuropathic pain

The PNL model was used in this study to achieve unilateral hind-limb neuropathy (Seltzer et al., 1990). Briefly, mice were anaesthetized using isoflurane (induction, 3%; maintenance, 1–2%). The left sciatic nerve was isolated under aseptic conditions and exposed at high-thigh level, and a third to a half of the dorsal thickness of the nerve was then tightly ligated using an 8-0 monofilament nylon suture. After surgery, the wound was closed using 6–0 absorbable sutures for muscles and skin. The mice were allowed nine days to recover, and their posture and behavior were closely monitored for 48 h. Beginning on day 10, the mice were tested to assess the development of mechanical allodynia and thermal hyperalgesia. On the 11th day, drugs were administered 15 min prior to neuropathic behavioral testing.

### 2.4. Sensory testing

During sensory testing, the mice were placed in elevated Plexiglas boxes (10.5 × 8.5 × 14 cm) with a 0.7-cm-diameter mesh grid floor for mechanical allodynia testing and a dry glass floor for thermal hyperalgesia testing. The animals were allowed to acclimatize for 15 min or until their exploratory behavior ceased before beginning the behavioral testing. The effect of drugs with short half-lives, such as anandamide and 2-AG (Willoughby et al., 1997), may be influenced by the testing order. To minimize this effect, the sequence of testing was alternated between mechanical allodynia and thermal hyperalgesia. The maximum time to complete one battery of tests was approximately 40 min.

#### 2.4.1. Mechanical allodynia

Mechanical sensitivity of the ipsilateral and contralateral hindpaws was assessed using von Frey hairs (Senselab aesthesiometer, Somedic, Sweden). Innocuous mechanical punctuate stimuli were delivered to the plantar surface of the hind paw with a series of individual von Frey filaments of ascending forces (increasing range between 1.90 and 43.54 mN). For each filament, the stimulus was repeated five times with an interval of 1–2 s between each trial. The threshold was determined as the lowest force that evoked a withdrawal response to one of the five stimuli (Tal and Bennett, 1994).

#### 2.4.2. Thermal hyperalgesia

The thermal sensitivity of the ipsilateral and contralateral hindpaws was assessed using an infrared noxious heat stimuli of increasing temperature range, from the non-noxious to noxious range (Plantar test, Ugo Basile, Italy). Thermal punctuate stimuli were delivered to the plantar surface of the hind paw with a focused beam of radiant heat, and the withdrawal latency time was recorded. The results of each test are expressed as the mean of three withdrawal latencies in seconds (s). Three minutes was allowed between each test (Hargreaves et al., 1988). A cutoff of 10 s was established to prevent tissue damage.

### 2.5. Experimental design

The experiments were conducted in a randomized manner by the same experimenter. The experimenter was blind to the drug treatment during all of the testing. Mechanical allodynia and thermal hyperalgesia were evaluated the day before PNL and on the 10th day after PNL for the ipsilateral and contralateral paws to confirm that the mice were neuropathic.

On the 11th day, 16 different combinations were tested in C57BL/6, *cnr1*KO and *cnr2*KO mice: (1) 0.9% NaCl (control group), (2) AEA (10 μg), (3) 2-AG (10 μg), (4) WIN (10 μg), (5) AM251 (1 μg), (6) AEA + AM251, (7) 2-AG + AM251, (8) AM630 (1 μg), (9) AEA + AM630, (10) 2-AG + AM630, (11) URB597 (100 μg), (12) AEA + URB597, (13) 2-AG + URB597, (14) URB602 (100 μg), (15) AEA + URB602 and (16) 2-AG + URB602. Anandamide (AEA, 10 μg) (Khasabova et al., 2012), 2-arachidonoyl glycerol (2-AG, 10 μg) (Khasabova et al., 2011), WIN 55,212-2 (WIN, 10 μg), URB597 (25 μg) and URB602 (25 μg) (Jhaveri et al., 2006), AM251 (1 μg) and AM630 (1 μg) (Malan et al., 2001) were all dissolved in the same total volume of 10 μL (the injection volume) and administered subcutaneously (s.c.) in the dorsal surface of the operated left hind paw 15 min before the pain tests ($n = 6$ per group). To exclude any possible systemic effect of the drugs, they were also all administered s.c. on the dorsal surface of the contralateral side (right hind paw, $n = 4$ per group).

### 2.6. Statistical analysis

The mechanical threshold and withdrawal latency for each treatment group are expressed as the mean ± the Standard Error of the Mean (S.E.M.). Calculations were performed with Excel 2007. Graphs were generated and statistical analysis was performed using Prism 5.01 (Graph Pad Software, San Diego, CA, USA). Comparisons of the means between the ipsilateral and contralateral paws were performed using either a two-tailed paired *t*-test, a one-way or a two-way ANOVA followed by Bonferroni's multiple-comparison test. The contralateral side was analyzed separately, using a one-way ANOVA followed by Bonferroni's multiple comparison test. The critical level of significance was set at 5% ($P < 0.05$).

## 3. Results

All of the mice were neuropathic 10 days after PNL, as a statistically significant difference was present in the ipsilateral hind paw in comparison to the pre-lesion baseline values for mechanical allodynia ($P < 0.0001$, $t = 12.86$ and df = 5) (Fig. 1A) and thermal hyperalgesia ($P < 0.0001$, $t = 14.13$ and df = 5, two-tailed paired *t*-test) (Fig. 1B). However, there were no significant differences in the values for the contralateral hind paw [(Fig. 1A, inset; $P = 0.204$, $t = 1.460$ and df = 5) and (Fig. 1B, inset; $P = 0.096$, $t = 2.048$ and df = 5), two-tailed paired *t*-test].

### 3.1. Anti-allodynic effects of AEA, 2-AG and WIN 55,212-2 in the absence or presence of cannabinoid $CB_1$ and $CB_2$ receptor antagonists in C57BL/6, cnr1KO and cnr2KO mice

Overall, there were no significant differences between the three genotypes in the von Frey values (Table 1). Genetic deletion of either the $CB_1$ or $CB_2$ receptor did not impact the pre-lesion baseline values nor did the development of PNL-induced allodynic effects. Furthermore, nociceptive responses after PNL were not affected by treatment with saline, AM251 or AM630 in all genotypes. Importantly, emerging evidence suggests that AM251 presents direct antagonistic actions at the mu opioid peptide receptors (MOP) (Seely et al., 2012). Therefore, we cannot exclude any opioid off-target effects in our experiments using this compound. However, we did confirm that AM251 does not evoke hypernociceptive effects in naïve C57BL/6 mice, which indicates that the previous findings were not based on the MOP antagonist effects of AM251 (this is also true for results reported in Sections 3.3 and 3.4).

In C57BL/6 mice (Fig. 2A), in the absence of cannabinoid antagonists, the local administration of AEA (10 μg) and 2-AG (10 μg) produced significant anti-allodynic effects, equivalent to those of WIN (10 μg) vs. saline [$P < 0.05$, $F_{treatment}(9150) = 2.412$, two-way ANOVA]. In the presence of the $CB_2$ receptor antagonist AM630 (1 μg), AEA produced significant antinociceptive effects vs. saline ($P < 0.05$, two-way ANOVA), whereas it lost its anti-allodynic effect in the presence of AM251. Indeed, the administration of AEA alone produced significant anti-allodynic effects compared with the combination of AEA + AM251 [($P = 0.001$, $F(2,17) = 11.10$), one-way ANOVA]. In addition, the anti-allodynic effects of 2-AG were reversed by both AM251 and AM630 [($P = 0.004$, $F(2,17) = 8.182$), one-way ANOVA]. These results demonstrate that the effects of AEA are likely to be mediated through direct activation of $CB_1$ receptors, while 2-AG requires both cannabinoid receptors to mediate its anti-allodynic effects. Moreover, the antinociceptive responses following ipsilateral injections were not significantly different from any of the treatments vs. saline in the contralateral paw (Fig. 2A, inset) [$P = 0.062$, $F = 1.976$, one-way ANOVA].

In the *cnr1*KO mice (Fig. 2B), only peripheral 2-AG produced significant anti-allodynic effects vs. saline [$P = 0.049$, $F_{treatments}(7120) = 2.099$, two-way ANOVA]. Hence, AEA alone compared with saline did not produce any anti-allodynic effects, confirming that AEA requires the presence of $CB_1$ receptors, whereas 2-AG is still able to produce antinociceptive effects when $CB_1$ receptors are absent. Moreover, there was a statistically significant difference between 2-AG + AM630 vs. 2-AG alone [($P < 0.0001$, $F(2,17) = 24.64$), one-way ANOVA], suggesting that $CB_2$ receptors are involved in the effects of 2-AG. In contrast, when both receptors were genetically and pharmacologically (*cnr1*KO + AM630) invalidated, 2-AG produced no effect. Indeed, when combining complementary genetic and pharmacological approaches, only $CB_2$ receptors are essential for the anti-allodynic effects of 2-AG. Again, there were no significant differences in the von Frey values of any of the treatments vs. saline in the contralateral paw (Fig. 2B, inset) [$P = 0.508$, $F = 0.912$, one-way ANOVA].

In the *cnr2*KO mice (Fig. 2C), only peripheral AEA produced significant anti-allodynic effects vs. saline ($P < 0.05$, two-way ANOVA), whereas no other substances reached statistical significance [$P = 0.765$, $F_{treatments}(7120) = 0.588$, two-way ANOVA], again demonstrating that $CB_2$ receptors are necessary for 2-AG to produce antinociception but not mandatory for AEA. However, the involvement of $CB_1$ receptors in the anti-allodynic effects of both endocannabinoids was confirmed by a statistically significant difference between the combination of AEA + AM251 vs. AEA alone [($P < 0.0001$, $F(2,17) = 24.17$), one-way ANOVA] and 2-AG + AM251 vs. 2-AG alone [($P = 0.036$, $F(2,17) = 4.175$), one-way ANOVA]. Notably, the synthetic $CB_1/CB_2$ receptor agonist WIN55,212-2 lost its antinociceptive efficacy in both transgenic mice, as WIN55,212-2 is a synthetic agonist at both the $CB_1$ and $CB_2$ receptors. Once more, the scores between the contralateral paws were not significantly different using any of the treatments vs. saline (Fig. 2C, inset) [$P = 0.960$, $F = 0.276$, one-way ANOVA].

### 3.2. Anti-hyperalgesic effects of AEA, 2-AG and WIN 55,212-2 in the absence or presence of cannabinoid $CB_1$ and $CB_2$ receptor antagonists in C57BL/6, cnr1KO and cnr2KO mice

There were no significant differences between the three genotypes in their noxious heat stimulus values (Table 1). Genetic deletion of either $CB_1$ or $CB_2$ receptors did not impact the pre-lesion baseline values, indicating a lack of endocannabinoid tone following short-term injury to the sciatic nerve or the development of PNL-induced hyperalgesic effects. Furthermore, the nociceptive responses after PNL were not affected by treatment with saline, AM251 or AM630 in all genotypes.

In the C57BL/6 mice (Fig. 3A), in the absence of AM251 and AM630, the local administration of AEA, 2-AG and WIN produced significant anti-hyperalgesic effects vs. saline [$P < 0.001$, $F_{treatments}(9150) = 14.46$, two-way ANOVA] as well as the combination of 2-AG + AM630, 2-AG + AM251 and AEA + AM630 when

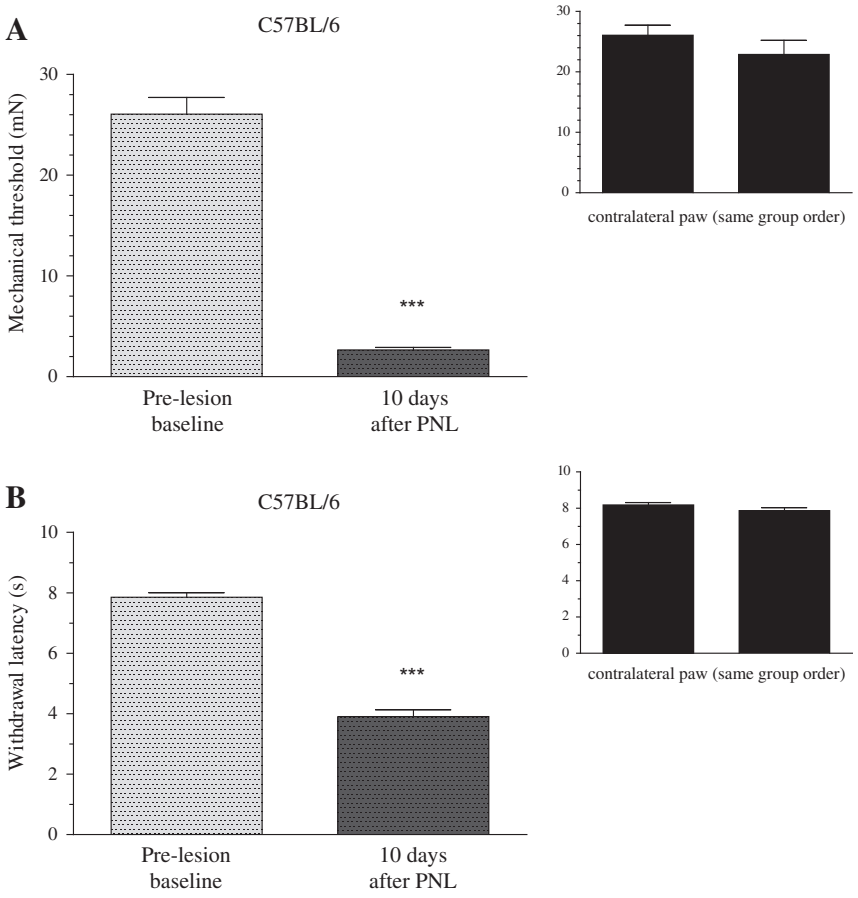


**Fig. 1.** Mechanical allodynia using von Frey hair (A) and thermal hyperalgesia using infrared noxious heat stimulus (B) before (pre-lesion baseline) and 10 days after surgery, for the ipsilateral and contralateral sides (inset) in C57BL/6 mice. The data are expressed as the means ± SEM ($n = 6$ per group). (***$P < 0.0001$ vs. pre-lesion baseline, two-tailed unpaired $t$ -test).

all of the treatments were compared with saline ($P < 0.05$, $P < 0.01$, $P < 0.001$, two-way ANOVA, respectively). Moreover, the administration of AEA alone also produced significant anti-hyperalgesic effects vs. AEA + AM251 [($P = 0.007$, $F(2,17) = 7.126$), one-way ANOVA]. These results demonstrate that the antinociceptive effects of AEA are likely to be mediated through direct activation of $CB_1$ receptors. In addition, the administration of 2-AG alone also produced significant anti-hyperalgesic effects when compared with the combination of 2-AG with both antagonists [($P = 0.006$, $F(2,17) = 7.499$), one-way ANOVA]. These results demonstrate that $CB_1$ and $CB_2$ receptors are involved in the anti-hyperalgesic effects of 2-AG. In addition, the antinociceptive responses following the ipsilateral injections were not significantly different for any of the treatments vs. saline in the contralateral paw (Fig. 3A, inset) [$P = 0.327$, $F = 1.183$, one-way ANOVA].

**Table 1**
On the 11th day after partial sciatic nerve ligation (PNL) surgery, the C57BL/6, *cnr1*KO and *cnr2*KO mice received saline, AM251 (cannabinoid $CB_1$ antagonist; 1 μg) and AM630 (cannabinoid $CB_2$ antagonist; 1 μg) on the ipsilateral side. The data are expressed as the means ± SEM (n = 6 per group).

| | | Mechanical threshold (mN) | | Withdrawal latency (s) | |
|---|---|---|---|---|---|
| | | Mean | ±SEM | Mean | ±SEM |
| C57BL/6 | Pre-lesion baseline | 26.062 | 1.656 | 7.857 | 0.151 |
| | 10 Days after PNL | 2.661 | 0.244 | 3.907 | 0.224 |
| | 11 Days after PNL – NaCl | 2.543 | 0.240 | 3.417 | 0.298 |
| | 11 Days after PNL – AM251 | 2.955 | 0.240 | 3.278 | 0.084 |
| | 11 Days after PNL – AM630 | 3.160 | 0.161 | 3.493 | 0.369 |
| *cnr1*KO | Pre-lesion baseline | 19.950 | 1.569 | 7.678 | 0.296 |
| | 10 Days after PNL | 2.856 | 0.220 | 3.833 | 0.304 |
| | 11 Days after PNL – NaCl | 2.656 | 0.210 | 3.170 | 0.244 |
| | 11 Days after PNL – AM251 | 2.747 | 0.288 | 3.395 | 0.107 |
| | 11 Days after PNL – AM630 | 2.744 | 0.248 | 3.217 | 0.085 |
| *cnr2*KO | Pre-lesion baseline | 23.970 | 2.090 | 7.695 | 0.210 |
| | 10 Days after PNL | 2.919 | 0.291 | 3.877 | 0.306 |
| | 11 Days after PNL – NaCl | 2.477 | 0.224 | 3.433 | 0.179 |
| | 11 Days after PNL – AM251 | 2.754 | 0.250 | 3.067 | 0.097 |
| | 11 Days after PNL – AM630 | 2.903 | 0.300 | 3.495 | 0.210 |

In the *cnr1*KO mice (Fig. 3B), the local injection of 2-AG and WIN in the absence of cannabinoid antagonists produced significant anti-hyperalgesic effects vs. saline [$P < 0.05$, $P < 0.01$, $F_{treatments}(7120) = 1.529$, two-way ANOVA, respectively]. A noteworthy observation is that AEA alone compared with saline did not produce any effect, confirming that AEA requires the presence of $CB_1$ receptors to produce antinociception. Interestingly, 2-AG combined with AM630 lost its anti-hyperalgesic effect vs. 2-AG alone [($P = 0.031$, $F(2,17) = 4.403$), one-way ANOVA], confirming that 2-AG requires $CB_2$ receptors to produce anti-hyperalgesic effects. Moreover, when both receptors were genetically and pharmacologically (*cnr1*KO + AM630) inhibited, 2-AG did not produce any effect. As expected, there were again no significant differences in the noxious heat stimulus values of any of the treatments compared with saline in the contralateral paw (Fig. 3B, inset) [$P = 0.207$, $F = 1.467$, one-way ANOVA].

In the *cnr2*KO mice (Fig. 3C), peripheral AEA, 2-AG and WIN produced significant anti-hyperalgesic effects vs. saline [$P < 0.05$, $P < 0.01$, $P < 0.001$, $F_{treatments}(7120) = 5.786$, two-way ANOVA,

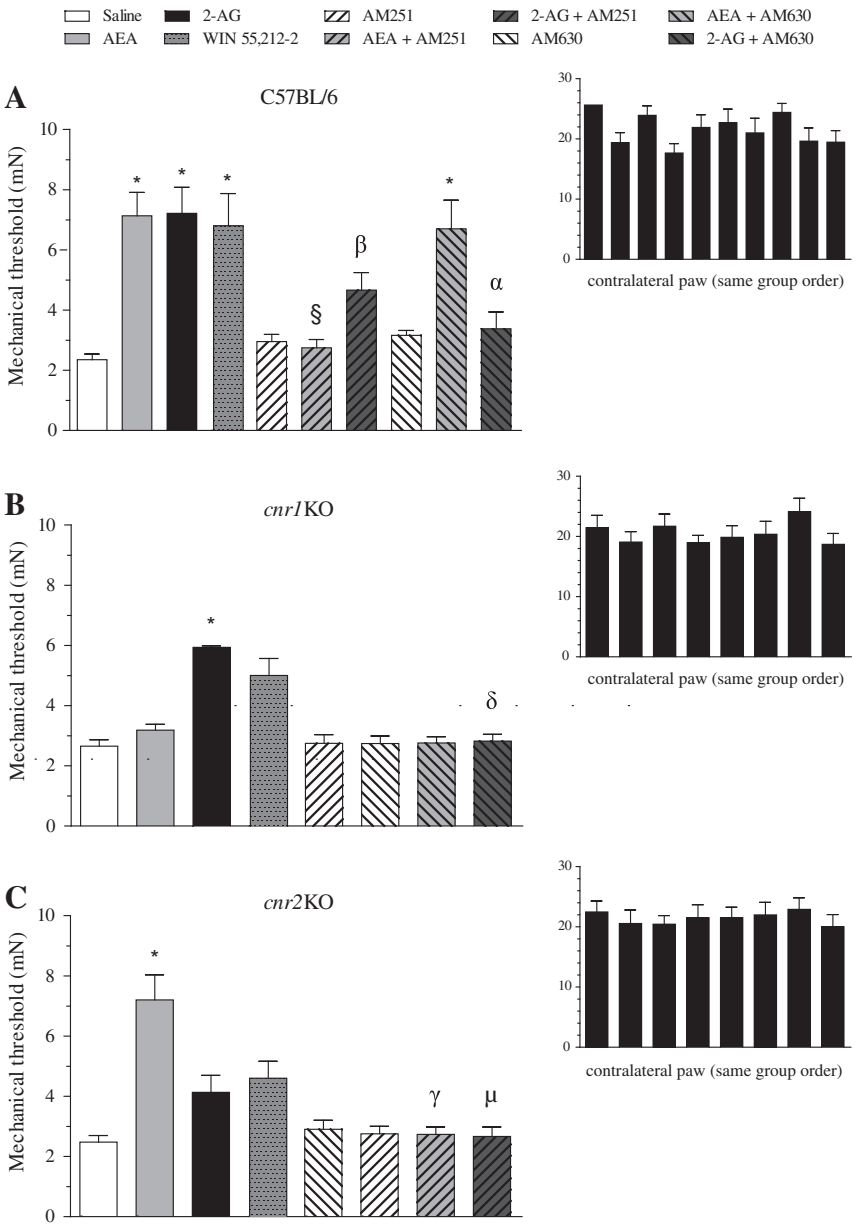


**Fig. 2.** Mechanical allodynia using von Frey hair 11 days after surgery for the ipsilateral and contralateral sides (inset) in (A) C57BL/6, (B) *cnr1*KO and (C) *cnr2*KO mice. On that day, the mice received either 0.9% NaCl, AEA (10 μg), 2-AG (10 μg) or WIN (10 μg), in the absence or presence of cannabinoid $CB_1$ and $CB_2$ receptor antagonists, AM251 (1 μg) and AM630 (1 μg). The data are expressed as the means ± SEM ($n = 6$ per group). (*$P < 0.05$ vs. saline, two-way ANOVA with Bonferroni's *post hoc* test); (A) (§$P < 0.01$ AEA + AM251 vs. AEA alone; β $P < 0.05$ 2-AG + AM251 vs. 2-AG alone; α $P < 0.01$ 2-AG + AM630 vs. 2-AG alone); (B) (δ $P < 0.001$ 2-AG + AM630 vs. 2-AG alone); (C) (γ $P < 0.001$ AEA + AM251 vs. AEA alone; μ $P < 0.05$ 2-AG + AM251 vs. 2-AG alone, one-way ANOVA with Bonferroni's *post hoc* test). Identification of the substances has been voluntarily omitted in the inset of all of the figures to simplify the presentation. The data in the contralateral graph are presented following the same order as the data in the ipsilateral graph.

respectively]. Once again, the involvement of $CB_1$ receptors in the anti-hyperalgesic effects of AEA and 2-AG was confirmed by the absence of antinociceptive effects following the administration of AEA + AM251 vs. AEA alone [($P < 0.0001$, $F(2,17) = 33.89$), one-way ANOVA] and 2-AG + AM251 vs. 2-AG alone [($P = 0.001$, $F(2,17) = 10.80$), one-way ANOVA]. Notably, these results confirm that 2-AG also requires $CB_1$ receptors to produce anti-hyperalgesic effects. However, when both receptors were genetically and pharmacologically (*cnr2*KO + AM251) inhibited, 2-AG produced no effect. On the contralateral side, the scores were not significantly different for any of the treatments compared with saline (Fig. 3C, inset) [$P = 0.580$, $F = 0.816$, one-way ANOVA].

### 3.3. Anti-allodynic effects of AEA and 2-AG in the absence or presence of FAAH and MGL inhibitors in C57BL/6, cnr1KO and cnr2KO mice

In the C57BL/6 mice, the local administration of AEA, 2-AG, URB597 and URB602 produced significant anti-allodynic effects vs. saline [$P < 0.05$, $F_{treatments}(8129) = 1.415$, two-way ANOVA] as well as the combination of AEA + URB597, AEA + URB602, 2-AG + URB597 and 2-AG + URB602 when all of the treatments were compared with saline ($P < 0.05$, $P < 0.01$, two-way ANOVA) (Fig. 4A). However, the combination of either AEA or 2-AG with either an FAAH or MGL inhibitor did not show a greater anti-allodynic effect [($P = 0.471$, $F(2,16) = 0.795$) and ($P = 0.598$,

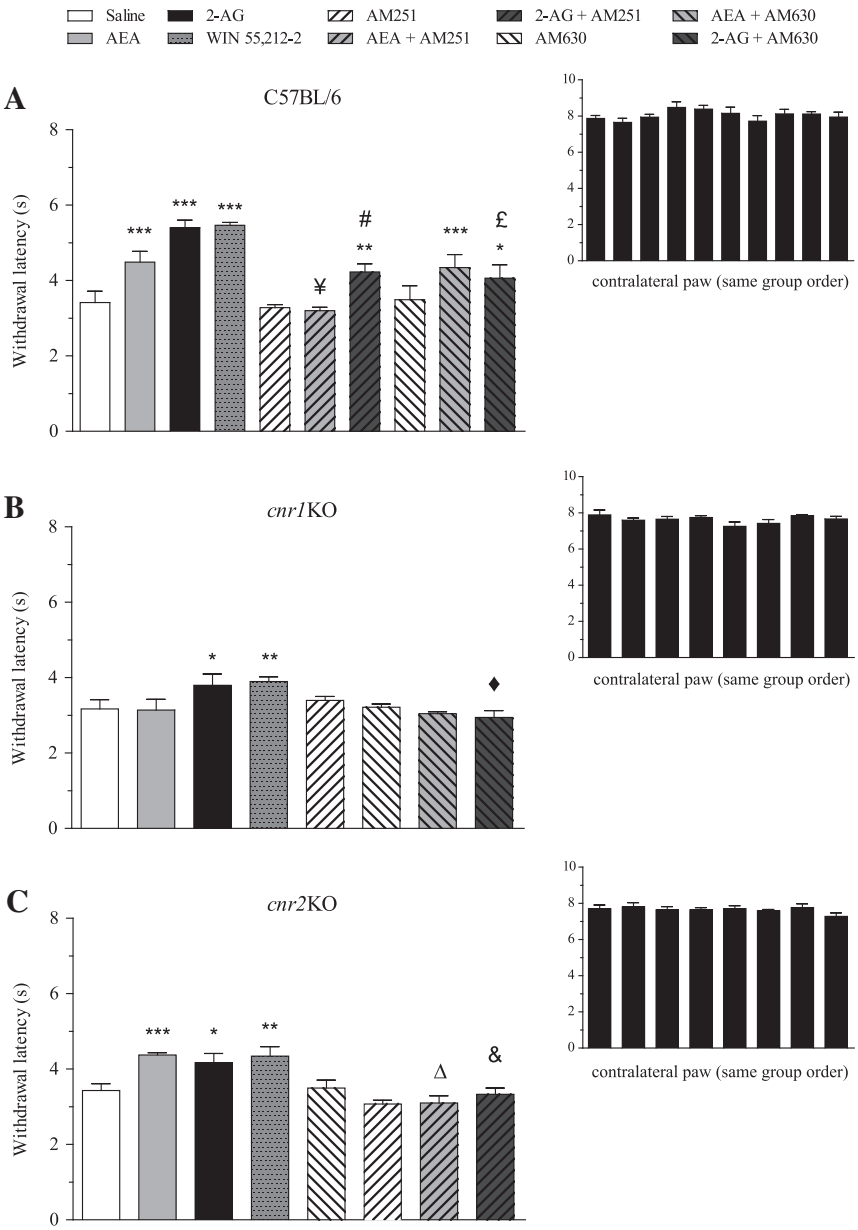


**Fig. 3.** Thermal hyperalgesia using infrared noxious heat stimuli 11 days after surgery for the ipsilateral and contralateral sides (inset) in (A) C57BL/6, (B) *cnr1*KO and (C) *cnr2*KO mice. The animals received either 0.9% NaCl, AEA (10 μg), 2-AG (10 μg) or WIN (10 μg), in absence or presence of cannabinoid $CB_1$ and $CB_2$ receptor antagonists, AM251 (1 μg) and AM630 (1 μg). The data are expressed as the means ± SEM ($n = 6$ per group). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. saline, two-way ANOVA with Bonferroni's *post hoc* test); (A) (¥ $P < 0.05$ AEA + AM251 vs. AEA alone; #$P < 0.05$ 2-AG + AM251 vs. 2-AG alone; £ $P < 0.01$ 2-AG + AM630 vs. 2-AG alone); (B) ( ◆ $P < 0.05$ 2-AG + AM630 vs. 2-AG alone); (C) (Δ $P < 0.001$ AEA + AM251 vs. AEA; & $P < 0.01$ 2-AG + AM251 vs. 2-AG alone, one-way ANOVA with Bonferroni's *post hoc* test).

$F(2,16) = 0.534$), respectively, one-way ANOVA]. The antinociceptive responses following ipsilateral injections were not significantly different for any of the treatments vs. saline in the contralateral paw (Fig. 4A, inset) [$P = 0.754$, $F = 0.623$, one-way ANOVA].

In the *cnr1*KO mice, peripheral 2-AG and URB602 produced significant anti-allodynic effects vs. saline [$P < 0.05$, $F_{treatments}(8135) = 1.902$, two-way ANOVA] as well as the combination AEA + URB602, 2-AG + URB597 and 2-AG + URB602 when all of the treatments were compared with saline ($P < 0.05$, $P < 0.01$, two-way ANOVA) (Fig. 4B). Hence, AEA and URB597 did not produce any effects, confirming that AEA and URB597 both require the presence of $CB_1$ receptors. In contrast, the combination of AEA + URB602 produced significant anti-allodynic effects vs. AEA alone [($P = 0.0009$, $F(2,17) = 11.70$), one-way ANOVA] indicating that URB602 may produce its effects through indirect activation of $CB_2$ receptors. Interestingly, the combination of either an FAAH or MGL inhibitor with 2-AG did not show a greater anti-allodynic effect than 2-AG alone [($P = 0.616$, $F(2,17) = 0.501$), one-way ANOVA]. Moreover, there were no significant differences between the von Frey values of any of the treatments compared with saline in the contralateral paw (Fig. 4B, inset) [$P = 0.586$, $F = 0.825$, one-way ANOVA].

In the *cnr2*KO mice, peripheral AEA and URB597 produced significant anti-allodynic effects vs. saline [$P < 0.05$, $F_{treatments}(8135) = 1.501$, two-way ANOVA] as well as the combination of AEA + URB597, 2-AG + URB597 and AEA + URB602 when

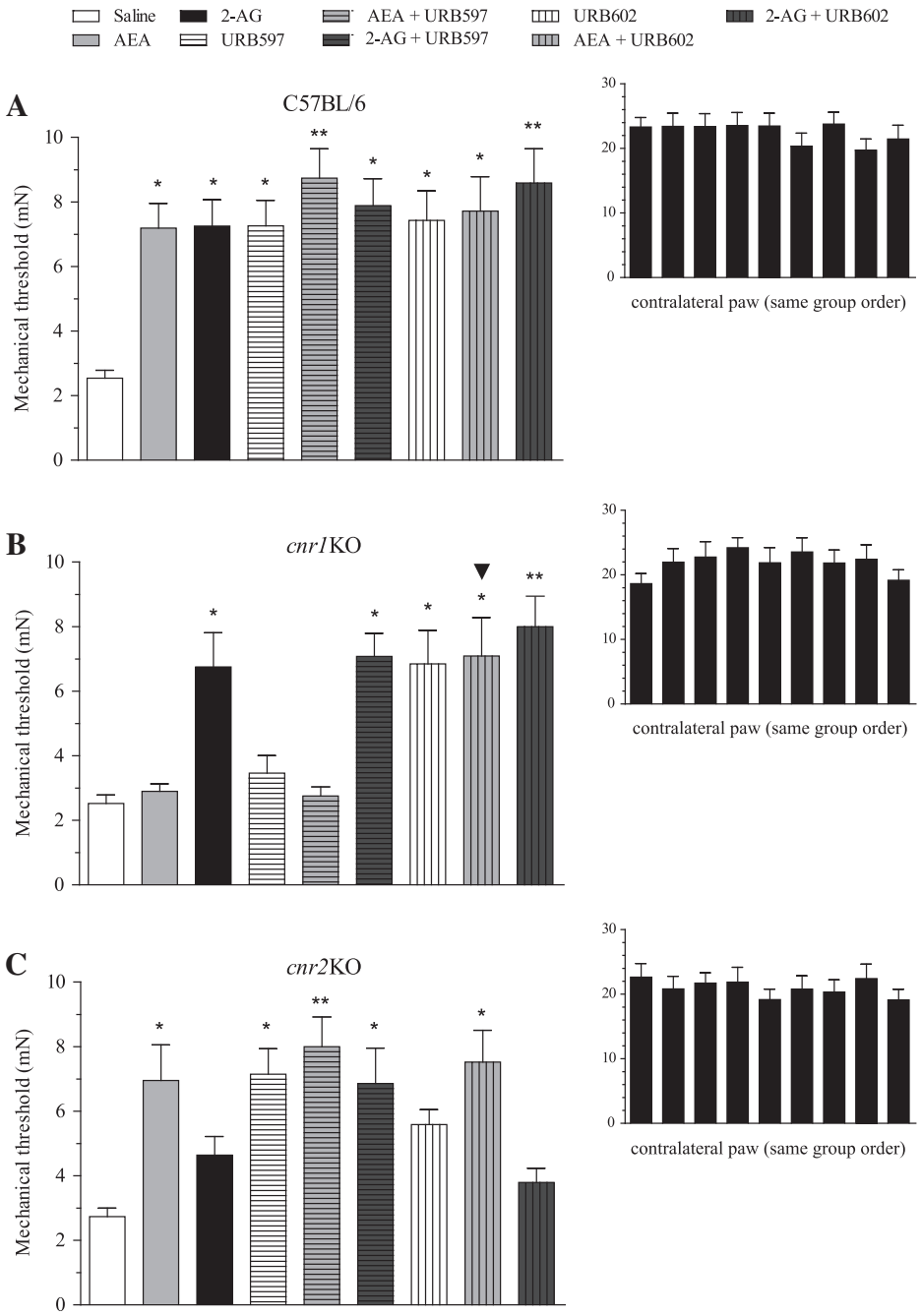


**Fig. 4.** Mechanical allodynia using von Frey hair 11 days after surgery for the ipsilateral and contralateral sides (inset) in (A) C57BL/6, (B) *cnr1*KO and (C) *cnr2*KO mice. On that day, the mice received either 0.9% NaCl, AEA (10 μg) or 2-AG (10 μg), in absence or presence of FAAH and MGL inhibitors, URB597 (100 μg) and URB602 (100 μg). The data are expressed as the means ± SEM ($n$ = 5-6 per group). (*$P < 0.05$, **$P < 0.01$ vs. saline, two-way ANOVA with Bonferroni's *post hoc* test); (B) (§ $P < 0.01$ AEA + URB602 vs. AEA alone, one-way ANOVA with Bonferroni's *post hoc* test).

all of the treatments were compared with saline ($P < 0.05$, $P < 0.01$, two-way ANOVA) (Fig. 4C). Because no effect was observed in the *cnr2*KO animals, 2-AG and URB602 seem to require the presence of $CB_2$ receptors to produce any effect. However, the combination of either AEA or 2-AG with either an FAAH or MGL inhibitor did not show any greater anti-allodynic effect, respectively; [($P = 0.768$, $F(2,17) = 0.269$) and ($P = 0.598$, $F(2,17) = 0.534$), one-way ANOVA]. Moreover, the anti-allodynic scores between contralateral paws were not significantly different in any of the treatments vs. saline (Fig. 4C, inset) [$P = 0.889$, $F = 0.443$, one-way ANOVA].

### *3.4. Anti-hyperalgesic effects of AEA and 2-AG in the absence or presence of FAAH and MGL inhibitors in C57BL/6, cnr1KO and cnr2KO mice*

In the C57BL/6 mice, the local administration of AEA, 2-AG, URB597 and URB602 produced significant anti-hyperalgesic effects vs. saline [$P < 0.001$, $F_{treatments}(8129) = 5.192$, two-way ANOVA] as well as the combination of AEA + URB597, 2-AG + URB597, AEA + URB602 and 2-AG + URB602 when all of the treatments were compared with saline ($P < 0.001$, two-way ANOVA) (Fig. 5A).

Moreover, the administration of AEA + URB597 and AEA + URB602 revealed superior anti-hyperalgesic effects of these combinations vs. AEA alone [($P = 0.006$, $F(2,16) = 7.538$), one-way ANOVA]. Similarly, the administration of 2-AG + URB597 and 2-AG + URB602 produce significant anti-hyperalgesic effects vs. 2-AG alone [($P = 0.0002$, $F(2,16) = 16.43$), one-way ANOVA]. Hence, the combination of AEA or 2-AG with both inhibitors produced significantly greater anti-hyperalgesic effects. On the contralateral side, the antinociceptive responses following ipsilateral injections were not significantly different using any of the treatments vs. saline (Fig. 5A, inset) [$P = 0.144$, $F = 1.632$, one-way ANOVA].

In the *cnr1*KO mice, the local injection of 2-AG and URB602 produced significant anti-hyperalgesic effects vs. saline [$P < 0.01$, $P < 0.001$, $F_{treatments}(8135) = 6.930$, two-way ANOVA] as well as the combination of 2-AG + URB597, AEA + URB602 and 2-AG + URB602 ($P < 0.001$, two-way ANOVA) (Fig. 5B). However, AEA alone vs. saline did not produce any effect, confirming that AEA requires the presence of $CB_1$ receptors to produce its anti-hyperalgesic effects. Moreover, the administration of AEA + URB602 and 2-AG + URB602 produced significant anti-nociceptive effects when compared with AEA and 2-AG alone, respectively; [($P = 0.0004$, $F(2,17) = 13.54$) and ($P = 0.001$, $F(2,17) = 11.13$), one-way ANOVA], whereas the combination of either AEA or 2-AG with URB597 did not produced a greater effect in the *cnr1*KO mice. These results further confirm that the analgesic effect of URB597 was likely to be mediated through the indirect activation of $CB_1$ receptors. Again, there were no significant differences between the noxious heat stimulus values of any of the treatments vs. saline in the contralateral paw (Fig. 5B, inset) [$P = 0.270$, $F = 1.295$, one-way ANOVA].

In the *cnr2*KO mice, peripheral AEA, 2-AG, URB597 and URB602 produced significant anti-hyperalgesic effects vs. saline [$P < 0.05$, $P < 0.01$, $P < 0.001$, $F_{treatments}(8135) = 5.667$, two-way ANOVA] as well as the combination of AEA + URB597, 2-AG + URB597, AEA + URB602 and 2-AG + URB602 ($P < 0.05$, $P < 0.001$, two-way ANOVA) (Fig. 5C). Moreover, the administration of AEA + URB597 produced significant anti-hyperalgesic effects when compared with AEA alone [($P = 0.005$, $F(2,17) = 7.851$), one-way ANOVA], whereas the combination of 2-AG with both of the inhibitors did not produce a greater effect [($P = 0.057$, $F(2,17) = 3.483$), one-way ANOVA]. Overall, these results confirm that the antinociceptive effects of URB602 were likely to be mainly mediated through an indirect activation of $CB_2$ receptors, although $CB_1$ receptors are certainly also involved because URB602 can still lead to antinociception in *cnr2*KO mice. Again, the anti-hyperalgesic scores between the contralateral paws were not significantly different using any of the treatments vs. saline (Fig. 5C, inset) [$P = 0.762$, $F = 0.613$, one-way ANOVA].

### 3.5. Anti-allodynic and anti-hyperalgesic effects of drugs are mediated locally in C57BL/6 mice

Mechanical allodynia and thermal hyperalgesia were assessed following injections of the drugs into the contralateral hind paw to confirm that the antinociceptive effects observed after the drug injections were mediated by a local site of action. When the drugs were administered on the contralateral side to mice 11 days after PNL, there was a significant difference in the anti-allodynic effects observed during the days after surgery (days 10 and 11) compared to the day before surgery (pre-lesion baseline) [$P < 0.0001$, $F(7,31) = 46.91$, one-way ANOVA]. However, there was no significant difference in the anti-allodynic effects between days 10 and 11 [$P = 0.986$, $F(6,27) = 0.157$, one-way ANOVA] on the ipsilateral side (Fig. 6A), confirming that the previously observed anti-allodynic effects of the drugs were locally rather than systemically mediated.

When the drugs were given on the contralateral side to mice 11 days after PNL, there was a significant difference in the anti-hyperalgesic effects between days 10 and 11 compared to the pre-lesion baseline [$P < 0.0001$, $F(7,31) = 93.62$, one-way ANOVA]. Although, there was no significant difference in the anti-hyperalgesic effects between days 10 and 11 [$P = 0.943$, $F(6,27) = 0.275$, one-way ANOVA] on the ipsilateral side (Fig. 6B), confirming that the previously observed anti-hyperalgesic effects of the drugs were locally mediated. No anti-allodynic or anti-hyperalgesic effects were observed on the contralateral side when the drugs were injected contralaterally to the surgery (data not shown).

## 4. Discussion

The present study was designed to address the contrasting cannabinoid receptor mechanisms through which either direct (exogenous endocannabinoids) or indirect (FAAH and MGL inhibitors) approaches mediate their peripheral antinociceptive effects. Using a local route of administration and a combination of complementary pharmacological and genetic tools, we sought to determine the exact involvement of $CB_1$ and $CB_2$ receptors in the local antinociceptive effects of endocannabinoids in Seltzer's mouse model of neuropathic pain. Our results indicate that exogenous AEA and URB597 inhibited mechanical allodynia and thermal hyperalgesia through a local $CB_1$-receptor-mediated mechanism. On the other hand, our results demonstrated that both peripheral $CB_1$ and $CB_2$ receptors contribute to the anti-hyperalgesic properties of exogenous 2-AG and URB602, whereas only $CB_2$ receptors were involved in the anti-allodynic effects of 2-AG and URB602.

The current literature indicates that FAAH and MGL inhibitors reduce nerve-injury-related nociceptive states through distinct cannabinoid receptor mechanisms of action. Intriguingly, most of the experiments are actually not conclusive on the exact contribution of both of the cannabinoid receptors on the antinociceptive effects of endogenous cannabinoids. Both of the cannabinoid receptors seem to be necessary for FAAH inhibitors to have an effect, while the actions of MGL inhibitors are only mediated *via* a $CB_1$ mechanism of action, suggesting that in these studies, $CB_2$ receptors are not necessary for 2-AG antinociceptive effects (Caprioli et al., 2012; Chang et al., 2006; Gattinoni et al., 2010; Kinsey et al., 2009; Russo et al., 2007). For example, the inhibition of FAAH using the new, peripherally acting inhibitor URB937 was lost in the FAAH KO and *cnr1*KO mice in the chronic constriction injury (CCI) model of neuropathic pain (Clapper et al., 2010). In addition, a recent study using transgenic mice has demonstrated that i.p. PF-3845, a FAAH selective inhibitor, lacked anti-allodynic efficacy in both cannabinoid knockout lines, whereas i.p. JZL184, an MGL inhibitor, did not produce anti-allodynic effects in *cnr1*KO mice, but retained its anti-allodynic effects in *cnr2*KO mice using the CCI model (Kinsey et al., 2010). Interestingly, our results do not agree with the results described in some previous studies. From this perspective, we examined the results based on the apparent complexity of the two endocannabinoid-hydrolyzing enzyme inhibitors presented by previous studies by combining transgenic mice and pharmacological approaches. Therefore, we aimed at mimicking $CB_1/CB_2$ double KO mice (Jarai et al., 1999); *cnr1*KO mice were administered AM630, a $CB_2$ receptor antagonist, whereas *cnr2*KO mice were given AM251, a $CB_1$ receptor antagonist, assuming that both cannabinoid receptors were thus invalidated under these conditions.

As some authors have reported, the variability in the observations is most likely dependent on several factors such as the sensory modality, the level of endocannabinoid tone produced by the injury, the compound used, the strain and gender of mice and rats,

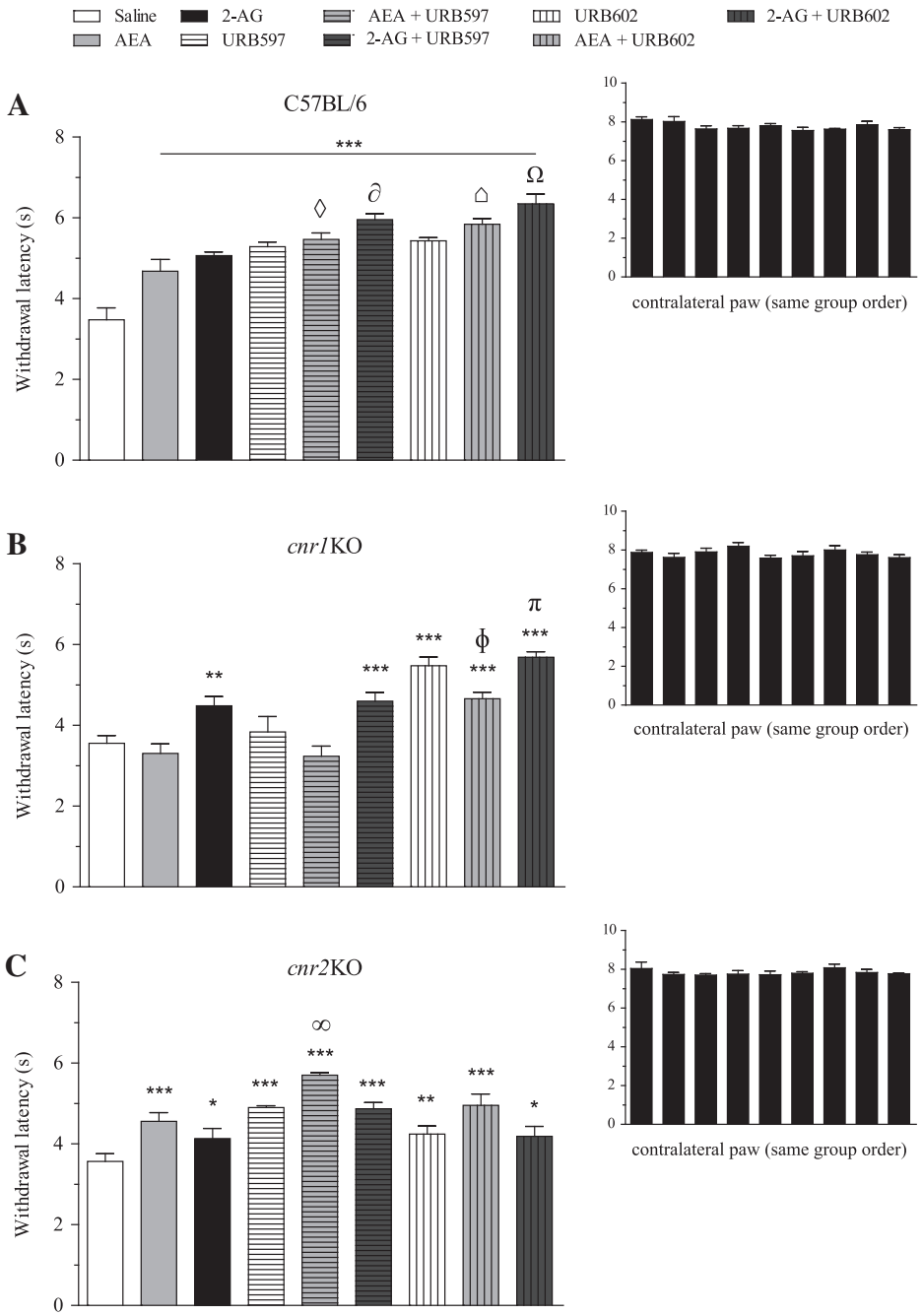


**Fig. 5.** Thermal hyperalgesia using infrared noxious heat stimuli 11 days after surgery for the ipsilateral and contralateral sides (inset) in (A) C57BL/6, (B) *cnr1*KO and (C) *cnr2*KO mice. The animals received either 0.9% NaCl, AEA (10 μg) or 2-AG (10 μg), in absence or presence of FAAH and MGL inhibitors, URB597 (100 μg) and URB602 (100 μg). The data are expressed as the means ± SEM ($n = 5$-6 per group). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. saline, two-way ANOVA with Bonferroni's *post hoc* test); (A) ($\diamond$ $P < 0.05$ AEA + URB597 vs. AEA alone; $\frown$ $P < 0.01$ AEA + URB602 vs. AEA alone; $\partial$ $P < 0.01$ 2-AG + URB597 vs. 2-AG alone; $\Omega$ $P < 0.001$ 2-AG + URB602 vs. 2-AG alone); (B) ($\phi$ $P < 0.01$ AEA + URB602 vs. AEA alone; $\pi$ $P < 0.01$ 2-AG + URB602 vs. 2-AG alone); (C) ($\infty$ $P < 0.01$ AEA + URB597 vs. AEA alone, one-way ANOVA with Bonferroni's *post hoc* test).

etc. (Ahmed et al., 2010; Guindon et al., 2011; Khasabova et al., 2011). The discrepancies cannot only be based on differences related to the pharmacological tools because we were using transgenic mice; therefore, the differences could also be caused by the route of administration or the animal model used. Indeed, in contrast to most of the studies presented above, our experiments focused on the peripheral level. Accordingly, studies using a local route of administration present similar conclusions (Guindon et al., 2011; Spradley et al., 2010), indicating possible region-specific elevation of endocannabinoids. However, the specificity of either URB597 or URB602 for inhibiting FAAH or MGL, respectively, at the concentrations used in the present study cannot be determined without measuring endocannabinoid levels in peripheral paw tissues. Therefore, we cannot confirm that URB597 and URB602 did indeed elevate endocannabinoid levels in our study; however, it is reasonable to assume that this was the mechanism of action because a number of studies have shown specific elevation of the endocannabinoid levels after the administration of these inhibitors (Guindon et al., 2011; Kathuria et al., 2003; Lichtman et al., 2002).

Additionally, the differences may reside in the pathophysiology of the models of peripheral injury. For example, a recent study using paired genetic and pharmacological approaches has revealed that

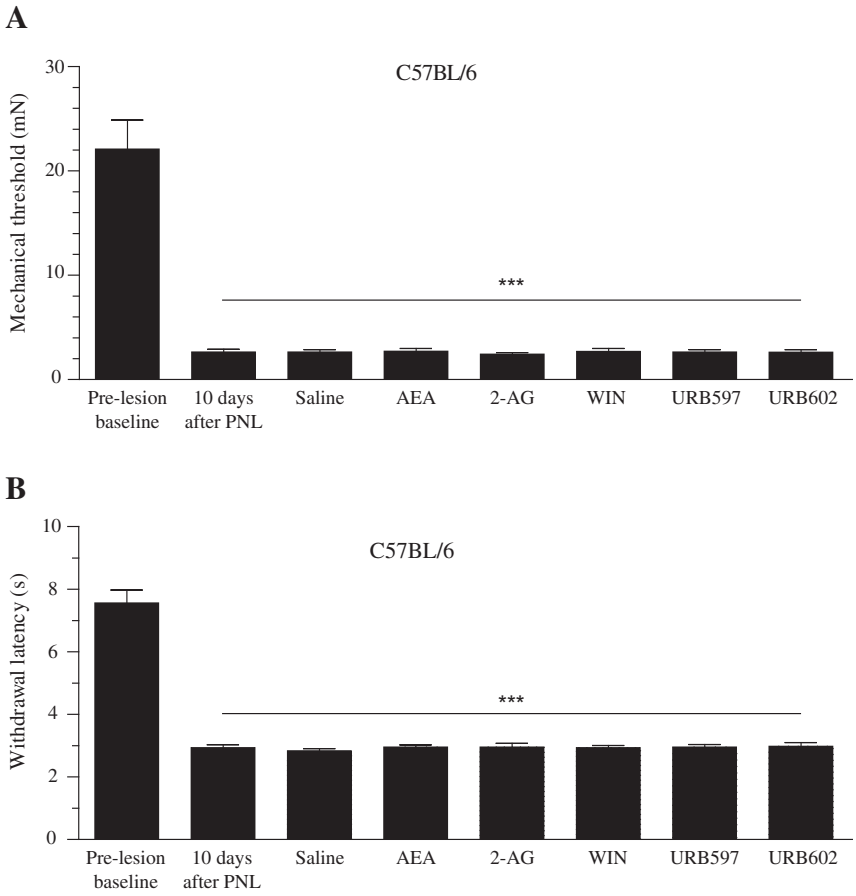


**Fig. 6.** Mechanical allodynia using von Frey hair (A) and thermal hyperalgesia using infrared noxious heat stimuli (B) before (pre-lesion baseline), 10 and 11 days after partial sciatic nerve ligation (PNL) surgery for the ipsilateral side in C57BL/6 mice. On the 11th day, the mice received either 0.9% NaCl, AEA (10 μg), 2-AG (10 μg), WIN (10 μg), URB597 (100 μg) or URB602 (100 μg) on the contralateral side. The data are expressed as the means ± SEM ($n = 4$ per group). (***$P < 0.0001$ vs. pre-lesion baseline, one-way ANOVA with Bonferroni's *post hoc* test).

the anti-allodynic effects of i.p. JZL184 in the carrageenan model required both $CB_1$ and $CB_2$ receptors (Ghosh et al., 2013). Indeed, in some models involving inflammation, such as carrageenan (Ghosh et al., 2013), capsaicin-induced thermal hyperalgesia (Spradley et al., 2010) and formalin (Guindon et al., 2011), $CB_1$ and $CB_2$ receptors are both involved in MGL inhibition, while FAAH inhibition requires only $CB_1$ receptors. One possible hypothesis related to this issue is the degree to which inflammatory responses contribute to nociception, which explains the existing discrepancies between the studies depending on the nociceptive model. It is important to note that there is also a significant inflammatory component in the development of painful neuropathy. Under inflammatory conditions, the endocannabinoid system has been shown to function differently compared to its physiological state (Rani Sagar et al., 2012). Therefore, models or surgical techniques producing more inflammation may have a role in these inconsistencies. However, some authors have reported that the PNL neuropathic pain model involves minimal inflammatory processes compared with the most commonly used CCI model (Bridges et al., 2001); thus, the level of inflammation may not explain all of the discrepancies.

Several studies have questioned the selectivity of the first reversible MGL inhibitor URB602 and concluded that this compound cannot be used systemically as a selective MGL inhibitor because it can suppress FAAH *in vitro* (Saario et al., 2005; Vandevoorde et al., 2007). However, following site-specific injection in the brain, this MGL-preferring inhibitor increased 2-AG levels with no impact on AEA levels *in vivo* (Hohmann et al., 2005) and *in vitro* (King et al., 2007). Accordingly, a recent study confirmed this previous finding and demonstrated that peripheral injection of URB602 increased hind paw 2-AG levels without altering AEA levels (Guindon et al., 2011), validating URB602 as an effective *in vivo* research tool for the inhibition of 2-AG hydrolysis. More recently, novel, selective MGL inhibitors have been generated, such as OMDM169 (Bisogno et al., 2009) and JZL184 (Long et al., 2009). Nevertheless, in regards to these discrepancies, future studies should concentrate on improved FAAH and MGL inhibitors, such as KML29 (Chang et al., 2012), or the use of either FAAH KO (Cravatt et al., 2001) or MGL KO mice (Schlosburg et al., 2010).

## 5. Conclusions

We have demonstrated that the local administration of AEA, 2-AG, WIN, URB597 and URB602 induced anti-allodynic and anti-hyperalgesic effects in a murine model of neuropathic pain. These antinociceptive effects were locally mediated. Although potential discrepancies exist, the use of complementary pharmacological and genetic tools revealed that both $CB_1$ and $CB_2$ receptors are necessary for the peripheral anti-hyperalgesic effects of 2-AG and URB602, whereas only $CB_2$ receptors are required for the anti-allodynic effects. The peripheral antinociceptive effects of AEA and URB597 are mediated through $CB_1$ receptors in the mouse PNL model. Targeted modulation of AEA and 2-AG levels in peripheral tissues through interference with their degrading enzymes represents a promising approach to the treatment of neuropathic pain.

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