



Mice lacking fatty acid amide hydrolase exhibit a cannabinoid receptor-mediated phenotypic hypoalgesia

Aron H. Lichtman^{a,*}, Christopher C. Shelton^a, Tushar Advani^a, Benjamin F. Cravatt^b

^aDepartment of Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298, USA

^bThe Skaggs Institute for Chemical Biology and Departments of Cell Biology and Chemistry, The Scripps Research Institute, 10550 N. Torrey Pines Rd, La Jolla, CA 92037, USA

Received 9 June 2003; received in revised form 1 December 2003; accepted 26 January 2004

Abstract

Although the *N*-arachidonoyl ethanolamine (anandamide) binds to cannabinoid receptors and has been implicated in the suppression of pain, its rapid catabolism *in vivo* by fatty acid amide hydrolase (FAAH) has presented a challenge in investigating the physiological functions of this endogenous cannabinoid. In order to test whether anandamide and other non-cannabinoid fatty amides modulate nociception, we compared FAAH (+/+) and (-/-) mice in the tail immersion, hot plate, and formalin tests, as well as for thermal hyperalgesia in the carrageenan and the chronic constriction injury (CCI) models. FAAH (-/-) mice exhibited a CB₁ receptor-mediated phenotypic hypoalgesia in thermal nociceptive tests. These mice also exhibited CB₁ receptor-mediated hypoalgesia in both phases of the formalin test accompanied with a phenotypic anti-edema effect, which was not blocked by either CB₁ or CB₂ antagonists. Additionally, FAAH (-/-) mice displayed thermal anti-hyperalgesic and anti-inflammatory effects in the carrageenan model that were mediated, in part, by CB₂, but not CB₁ receptors. In contrast, no genotype differences in pain behavior were evident following CCI, which was instead found to obliterate the phenotypic hypoalgesia displayed by FAAH (-/-) mice in the tail immersion and hot plate tests, suggesting that nerve injury may promote adaptive changes in these animals. Collectively, these findings demonstrate a cannabinoid receptor-mediated analgesic phenotype in FAAH (-/-) mice. In more general terms, these findings suggest that selective inhibitors of FAAH might represent a viable pharmacological approach for the clinical treatment of pain disorders.

© 2004 International Association for the Study of Pain. Published by Elsevier B.V. All rights reserved.

Keywords: Fatty acid amide hydrolase; Chronic constriction injury models; Endogenous cannabinoid

1. Introduction

The endogenous cannabinoid ('endocannabinoid') system consists of two cannabinoid receptor subtypes, CB₁ receptors present largely in the central nervous system (CNS) as well as the peripheral CB₂ receptor, and several postulated endogenous ligands, including *N*-arachidonoyl ethanolamine (anandamide; Devane et al., 1992), 2-arachidonoyl glycerol (2-AG; Mechoulam et al., 1995; Sugiura et al., 1995), noladin ether (Hanus et al., 2001), and *O*-arachidonoyl ethanolamine (virodhamine; Porter et al., 2002). The endocannabinoid system has been proposed to serve several important physiological functions, including the modulation of nociception (Calignano et al., 1998; Richardson et al., 1998b; Walker et al.,

1999), feeding (Di Marzo et al., 2001), cognition (Lichtman, 2000; Terranova et al., 1996), and drug dependence (Ledent et al., 1999). In particular, the analgesic effects of exogenously administered cannabinoids, coupled with the expression of CB₁ receptors and endogenous cannabinoid ligands in nociceptive circuits, suggest that pharmacological modulators of the endocannabinoid system may be useful for the treatment of pain disorders (Hohmann, 2002; Rice et al., 2002; Walker and Huang, 2002).

Anandamide, the most characterized endocannabinoid to date, is a member of a large class of endogenous fatty acid amides (FAAs) that are metabolized by a common enzyme, fatty acid amide hydrolase (FAAH; Cravatt et al., 1996, 2001). With the exception of anandamide, most FAAs do not bind CB receptors, but rather, appear to serve as neural signaling molecules that act through distinct receptor

* Corresponding author. Tel.: +1-804-828-8480; fax: 1-804-828-2117.
E-mail address: alichtma@hsc.vcu.edu (A.H. Lichtman).

systems in vivo (Calignano et al., 1998; Cravatt et al., 1995; Lichtman et al., 2002; Rodriguez de Fonseca et al., 2001). Due to rapid catabolism in the brain, anandamide and related FAAs produce only weak and transient pharmacological effects in vivo (Smith et al., 1994). Accordingly, efforts to understand the role that anandamide and the endocannabinoid system play in the regulation of pain behavior have produced mixed results (Calignano et al., 1998; Farquhar-Smith and Rice, 2001; Jaggar et al., 1998; Lichtman et al., 1996; Richardson et al., 1998a,c; Smith et al., 1998). Still, the observation that a subcutaneous injection of formalin elevates the levels of endogenous anandamide in the periaqueductal gray is consistent with the notion that this endocannabinoid tonically modulates nociception (Walker et al., 1999). The availability of FAAH ($-/-$) mice, which exhibit a profound reduction in hydrolysis activity for anandamide and other FAAs, accompanied by at least a 10-fold increase in endogenous brain levels of these lipids (Cravatt et al., 2001), provides a powerful model to investigate the function of FAA signaling pathways. When treated with exogenous anandamide, FAAH ($-/-$) mice exhibit a full spectrum of CB₁-dependent behavioral responses, including hypomotility, analgesia, catalepsy, and hypothermia. Moreover, FAAH ($-/-$) mice displayed a phenotypic anti-nociception compared to wild type controls in tail immersion, hot plate, and formalin tests. Notably, the CB₁ receptor antagonist, SR 141716 prevented the analgesic responses of FAAH ($-/-$) mice in the hot plate test, indicating that CB₁ receptor pathways are required for the expression of this phenotype. More recently, mice treated with irreversible FAAH inhibitors also exhibited significant CB₁ receptor-dependent hypoalgesia in the mouse hot plate test (Kathuria et al., 2003).

The purpose of the present study was to characterize the behavioral responses of FAAH ($-/-$) mice in a broader series of nociceptive models of acute and chronic nociception. In addition to the tail immersion (Ledent et al., 1999), hot plate (Fride and Mechoulam, 1993), and formalin tests (Calignano et al., 1998; Guhring et al., 2001; Moss and Johnson, 1980), exogenous administration of cannabinoids has been shown to produce analgesia in a wide range of other nociceptive tests. For example, cannabinoids have reliable anti-hyperalgesic and anti-inflammatory effects in the extensively used carrageenan model of inflammatory nociception (Clayton et al., 2002; Richardson et al., 1998c; Sofia et al., 1973) and block hyperalgesic effects to thermal or mechanical stimulation following chronic constriction injury (CCI) of the sciatic nerve (Fox et al., 2001; Herzberg et al., 1997; Mao et al., 2000), a neuropathic model of nociception. The second objective of the present study was to elucidate molecular mechanisms of action underlying the anti-nociceptive phenotype of FAAH ($-/-$) mice through the use of selective CB₁ and CB₂ receptor antagonists.

2. Methods

2.1. Subjects

Male and female FAAH ($+/+$), ($+/-$), and ($-/-$) mice used in this study were sixth generation offspring backcrossed onto a C57BL/6 background (Cravatt et al., 2001). All mice were derived from FAAH ($+/-$) breeding pairs and were born in the Virginia Commonwealth University vivarium from breeding pairs. Subjects weighed between 20 and 30 g, and were housed six animals per cage in a temperature-controlled (20–22 °C) facility. The Institutional Animal Care and Use Committee at Virginia Commonwealth University approved all experiments. Mice were given unlimited access to food and water and were maintained on a 12/12-h light/dark cycle.

2.2. Drugs

SR 141716 and SR 144528, selective receptor antagonists for the CB₁ and CB₂ receptor, respectively (Rinaldi-Carmona et al., 1994, 1998), were provided by the National Institute on Drug Abuse (Bethesda, MD). The vehicle consisted of a mixture of 1:1:18 ethanol:alkamuls-620 (Rhone-Poulenc, Princeton, NJ):saline. The dose of each antagonist was 3 mg/kg and was given through the i.p. route administration in a volume of 10 μ l/g body weight. We have previously found that this dose of SR 141716 effectively blocked the pharmacological effects of CB₁ receptor agonists (Lichtman and Martin, 1996, 1997). This dose of SR 144528 displaced specific cannabinoid binding to mouse spleen homogenates, but not to mouse whole brain (Rinaldi-Carmona et al., 1998), as well as prevented the anti-edema and anti-hyperalgesic effects of cannabinoids in the rat carrageenan model (Conti et al., 2002). Both drugs were administered 30 min prior to the tail immersion and hot plate tests as well as 30 min prior to intraplantar injections of formalin or carrageenan.

2.3. Manipulations

2.3.1. Induction of inflammation

Inflammation was induced by giving an intraplantar injection of 0.3% λ -carrageenan (Sigma, St Louis) in a 20 μ l volume into the right hind paw using a 30-gauge needle. In a preliminary time course study, we found that the hyperalgesic and inflammatory responses following 0.3% carrageenan administration peak at approximately 5 h and return to normal within 24 h (data not shown). Therefore, the 5 h time point was used for these studies. The diameter of each paw was measured both prior to and 5 h following carrageenan using electronic digital calipers (Traceable Calipers, Friendswood, TX) and expressed to the nearest \pm 0.01 mm. Similarly, sensitivity to a thermal stimulus was evaluated using a plantar stimulator

(see Section 2.4.3) both prior to and 5 h following carrageenan (Hargreaves et al., 1988).

2.3.2. Chronic constriction injury

Subjects were administered atropine (0.1 mg/kg, i.p.) 10 min prior to pentobarbital (40 mg/kg, i.p.). Bupivacaine (0.2 ml, 0.25%) was given immediately prior to incision. As previously described, an incision was made in the skin above the joint of leg bone to body, and the sciatic nerve was isolated following separation of the muscle (Malmberg and Basbaum, 1998; Malmberg et al., 1997). A suture was then placed around the nerve and a knot was tied three times. The surrounding muscle and skin were then sutured. Subjects were evaluated for thermal hypersensitivity using a plantar stimulator beginning 1–2 weeks later.

2.4. Behavioral assessments

2.4.1. Thermal nociception assays

Subjects were assessed for basal responses in the tail immersion and hot plate assays. In the tail immersion test, each mouse was hand-held, with approximately 1 cm of the tip of the tail immersed into a water bath maintained at 50.0, 52.0, 54.0, 56.0, or 58.0 °C and the latency for the animal to withdraw its tail was scored (Cravatt et al., 2001). A cutoff time of 10 s was employed for tests in which the water temperature was at least 52 °C and a 20 s cutoff was used for the 50 °C test, as response latencies were substantially elevated using this mild stimulus. In the hot plate test, each mouse was placed on a hot plate that was maintained at 50.0, 52.0, 54.0, 56.0, or 58.0 °C, and the latency to jump or lick/shake a hind paw within a 60 s observation period was scored (Cravatt et al., 2001). The same subjects were evaluated in each test using the same temperature stimulus, with the tail withdrawal latency always evaluated first. A period of at least 48 h was used between baseline test days.

2.4.2. Formalin nociception assay

Naïve subjects were given an intraplantar injection into the right hind paw containing 20 µl of a 2.5% formalin solution (Cravatt et al., 2001). The total amount of time spent licking or lifting the afflicted paw was recorded for both the early phase (i.e. 0–5 min) and the late phase (i.e. 10–25 min). Peak pain behavior was observed during each respective time period in wild type mice.

2.4.3. Thermal hypersensitivity

Mice in the CCI and carrageenan models were evaluated for thermal hypersensitivity as described by Hargreaves et al. (1988). Subjects were placed in transparent lucite cubicles (24.6 × 7.5 × 7.5 cm³), which allowed minimal movement, for an approximately 30 min acclimation period. Sensitivity to noxious heat was assessed using a plantar stimulator, which was positioned under a glass substrate, directly beneath the hind paws. The time from the initiation of the radiant heat until paw withdrawal was measured

automatically [paw withdrawal latency (PWL)] to the nearest 0.1 s. A maximal cutoff of 20 s was used to prevent tissue damage. Each paw was tested three times, and the mean withdrawal latency was calculated.

2.5. Data analysis

Analysis of variance (ANOVA) was used to analyze the effect of genotype in each nociceptive assay. Scheffe's test was used for post-hoc analysis and *t*-tests were used for planned comparisons. Differences were considered significant at the $P < 0.05$ level.

3. Results

3.1. FAAH (–/–) mice exhibit a CB₁ receptor-mediated phenotypic hypoalgesia in acute nociceptive models

As shown in Fig. 1a, FAAH (–/–) mice exhibited a moderate phenotypic hypoalgesia in the tail immersion test, $F(2, 4) = 6.4$, $P < 0.01$. Specifically, the FAAH (–/–) mice had significantly elevated response latencies compared to FAAH (+/–) and (+/+) mice at 54, 56, and 58 °C, but not at 50 and 52 °C. SR 141716 completely prevented the elevated response latencies in the tail immersion test using a water stimulus of 56 °C, $F(2, 27) = 17.4$, $P < 0.001$ (Fig. 1b), indicating a CB₁ receptor mechanism of action. FAAH (–/–) mice also exhibited a moderate phenotypic hypoalgesic response in the hot plate test, $F(2, 4) = 4.2$, $P < 0.05$ (Fig. 1c). Similar to the tail immersion data, the FAAH (–/–) mice had significantly elevated response latencies compared to FAAH (+/–) and (+/+) mice at 54, 56, and 58 °C. Again, SR 141716 completely prevented the elevated response latencies in the hot plate test when set at 56 °C, $F(2, 27) = 11.7$, $P < 0.001$ (Fig. 1d).

In contrast to the tail immersion and hot plate tests, which evaluate phasic nociceptive responses to a thermal stimulus, the formalin test reflects a chemical noxious stimulus that elicits two phases of nociceptive responses, an immediate phasic component and a delayed tonic nociceptive response related to inflammation. FAAH (–/–) mice exhibited significantly less pain-related behavior than FAAH (+/–) and (+/+) mice in both the early and late phases of the formalin test, $F(2, 15) = 45$, $P < 0.001$ (Fig. 2a), and late phase, $F(2, 15) = 19$, $P < 0.001$ (Fig. 2b). The effects of pre-treatment of SR 141716 and SR 144528 on pain-related behavior during each respective phase are shown in Fig. 2c and d. Two-way ANOVA revealed a significant genotype by drug interaction for both the early phase, $F(2, 30) = 15$, $P < 0.001$, and late phase, $F(2, 30) = 8$, $P < 0.001$. As shown in Fig. 2c and d, the anti-nociception exhibited in each respective phase was completely blocked by SR 141716, but was unaffected by SR 144528, again indicating a CB₁ receptor mechanism of action. In response to formalin treatment, FAAH (–/–)

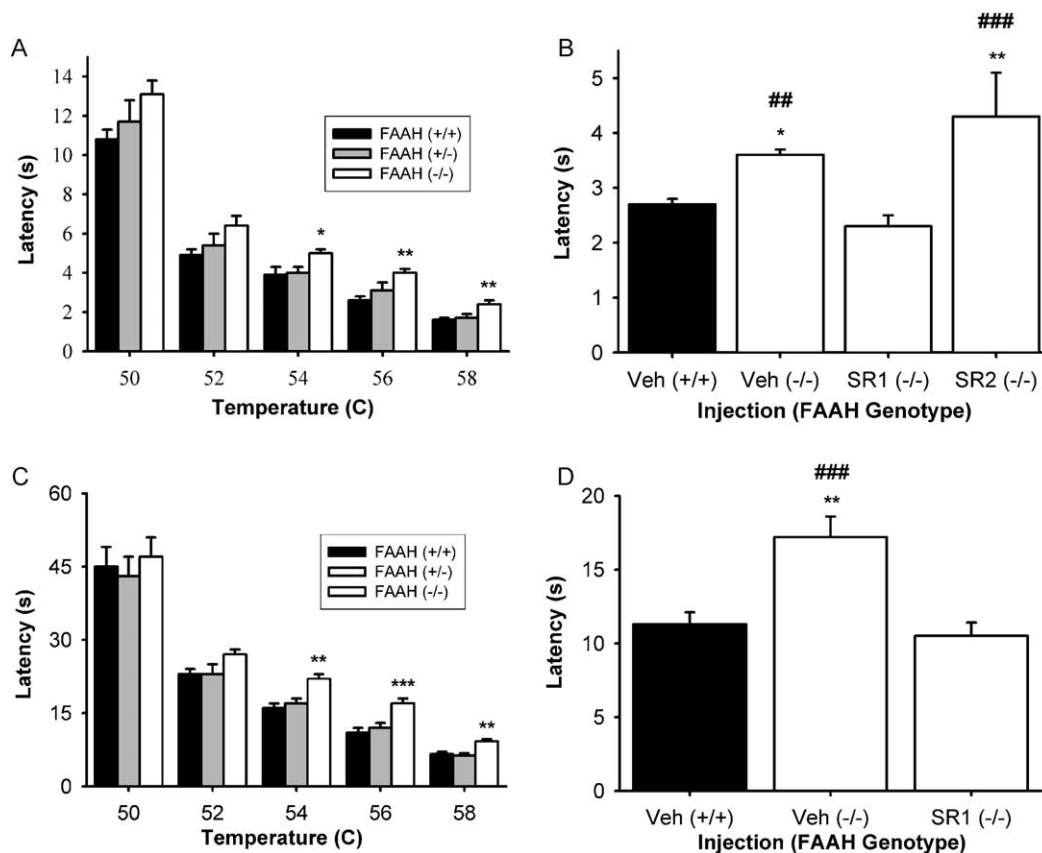


Fig. 1. Reduced thermal pain responses in FAAH (-/-) mice. FAAH (-/-) mice exhibited prolonged response latencies in the tail immersion (A) and hot plate (C) tests relative to FAAH (+/+) and (+/-) mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, for FAAH (-/-) mice versus FAAH (+/+) or (+/-) mice at each respective temperature (planned comparisons). This phenotypic thermal hypoalgesia of FAAH (-/-) mice was mediated by a CB₁ receptor-dependent mechanism of action in the tail immersion (B) and hot plate (D) tests, set at 56°C. ** $P < 0.01$, for FAAH (-/-) mice treated with vehicle or SR 144528 versus FAAH (+/+) vehicle-treated mice; *** $P < 0.001$ for FAAH (-/-) mice treated with vehicle or SR 144528 versus FAAH (-/-) SR 141716-treated mice (Scheffé post hoc test); $n = 6-10$ mice/group. Results shown as mean \pm SE.

mice were also found to show significantly smaller increases in paw diameter (i.e. 0.32 ± 0.04 mm, mean \pm SE) compared with the FAAH (+/+) mice (i.e. 0.73 ± 0.07 mm, mean \pm SE), $t(15) = 4.8$, $P < 0.001$. However, this anti-edema phenotype did not appear to be mediated via cannabinoid receptor mechanism of action, as the mean \pm SE differences in paw diameter of FAAH (-/-) mice treated with vehicle (i.e. 0.35 ± 0.06 mm), 3 mg/kg SR 141716 (i.e. 0.41 ± 0.07 mm), and 3 mg/kg SR 144528 (i.e. 0.33 ± 0.03 mm) failed to differ significantly from each other ($P = 0.68$).

3.2. Evaluation of FAAH (-/-) mice for thermal hyperalgesia in carrageenan and CCI models

In contrast to the nociceptive tests employed in the above experiments that evaluated decreases in responsivity to noxious stimuli, FAAH (-/-) mice were also evaluated in two models that induce a state of increased sensitivity to noxious stimuli (i.e. hyperalgesia), the carrageenan and CCI models. In the former assay, an intraplantar injection of carrageenan induces a time-dependent edema and hyperalgesia. Prior to carrageenan, FAAH (-/-) and (+/+) mice

had virtually identical paw withdrawal latencies in the plantar stimulator test (mean \pm SE; 10.5 ± 0.6 and 10.3 ± 0.3 s, respectively) and paw diameters (mean \pm SE; 2.1 ± 0.02 and 2.1 ± 0.02 mm, respectively). When evaluated 5 h following carrageenan, separate ANOVAs revealed significant effects in the ipsilateral paw for both hyperalgesia in the plantar stimulator test, $F(3, 39) = 6.4$, $P = 0.001$ (Fig. 3a), and edema, $F(3, 39) = 6.4$, $P = 0.001$ (Fig. 3b). FAAH (-/-) mice exhibited significantly less hyperalgesia and inflammation than the FAAH (+/+) mice. In fact, the vehicle-treated FAAH (-/-) mice failed to exhibit hyperalgesia, as there was no significant difference between their pre-injection and 5 h post-injection paw withdrawal latencies, $t(11) = 1.98$, $P = 0.07$ (paired t -test). SR 141716 failed to attenuate the anti-hyperalgesic and anti-inflammatory phenotypes. In contrast, SR 144528 partially attenuated both of these effects (~50%), as pain behavior and paw diameter did not differ significantly between SR 144528-treated FAAH (-/-) mice and vehicle-treated FAAH (+/+) mice. Conversely, no significant differences were found between the genotypes for either withdrawal latency ($P = 0.96$) or paw diameter ($P = 0.86$) in the contralateral paw.

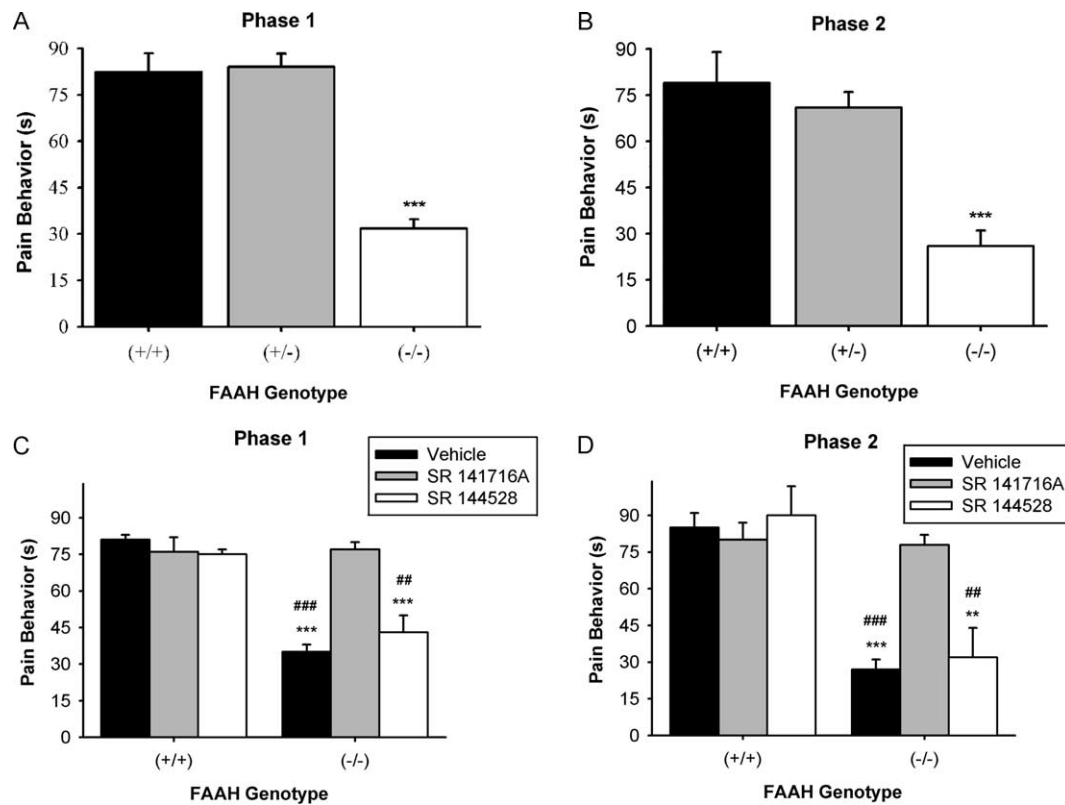


Fig. 2. FAAH (-/-) mice exhibited a phenotypic anti-nociception during both phase 1 (0–5 min) (A) and phase 2 (10–25 min) (B) of the formalin test. *** $P < 0.001$ for FAAH (-/-) versus FAAH (+/-) and (+/+) mice (Scheffe post-hoc comparison). The CB₁ receptor antagonist SR 141716, but not the CB₂ antagonist SR 144528 normalized pain responses in FAAH (-/-) mice in both phases of the formalin test (C and D). ** $P < 0.01$ and *** $P < 0.001$ for vehicle- or SR 144528-treated FAAH (-/-) mice versus SR 141716-treated FAAH (-/-) mice; ### $P < 0.01$ and #### $P < 0.001$ for FAAH (-/-) mice versus respective FAAH (+/+) mice (Scheffe post-hoc test); $n = 6$ mice/group. Results shown as mean \pm SE.

The withdrawal latencies of FAAH (-/-) and (+/+) mice in the plantar stimulator test following CCI of the sciatic nerve are presented in Fig. 4. A significant main effect of surgery collapsed across genotype was found, $F(1, 13) = 59$, $P < 0.001$, indicating that the withdrawal latencies for the ipsilateral paw were shorter than those for the contralateral paw (Fig. 4a). However, there was no

significant main effect of genotype and no significant genotype by paw interaction, indicating that both FAAH (+/+) and (-/-) mice exhibited an equivalent degree of hyperalgesia in the ipsilateral paw. Given that the phenotypic hypoalgesia of the FAAH (-/-) mice in the tail immersion and hot plate tests is dependent on the intensity of the radiant heat stimulus (see Fig. 1a and c), it is plausible

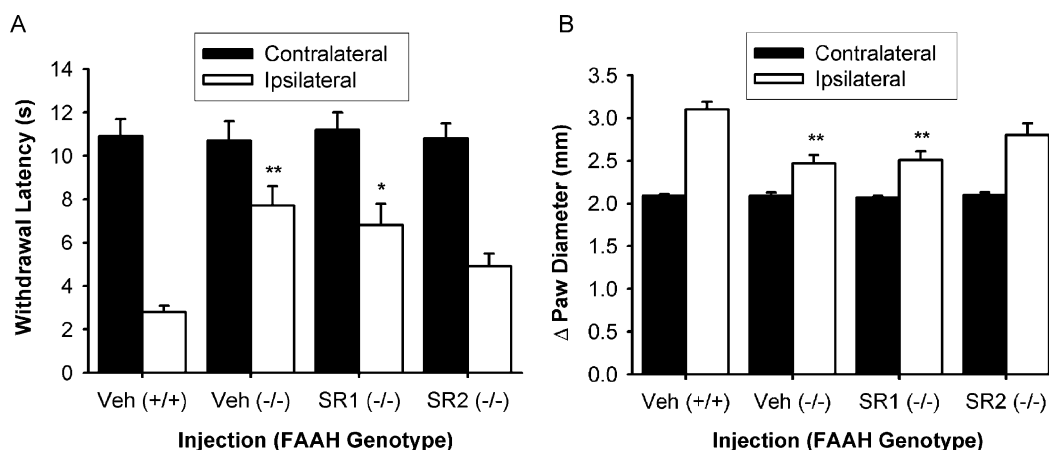


Fig. 3. FAAH (-/-) mice exhibited anti-hyperalgesic (A) and anti-inflammatory effects (B) in the carrageenan model. Subjects were given an intraplantar injection of λ -carrageenan into a hind paw and tested 5 h later. * $P < 0.05$ and ** $P < 0.01$ for each group vs. vehicle-treated FAAH (+/+) mice (Scheffe post-hoc test); $n = 9$ –12 mice/group. Results shown as mean \pm SE.

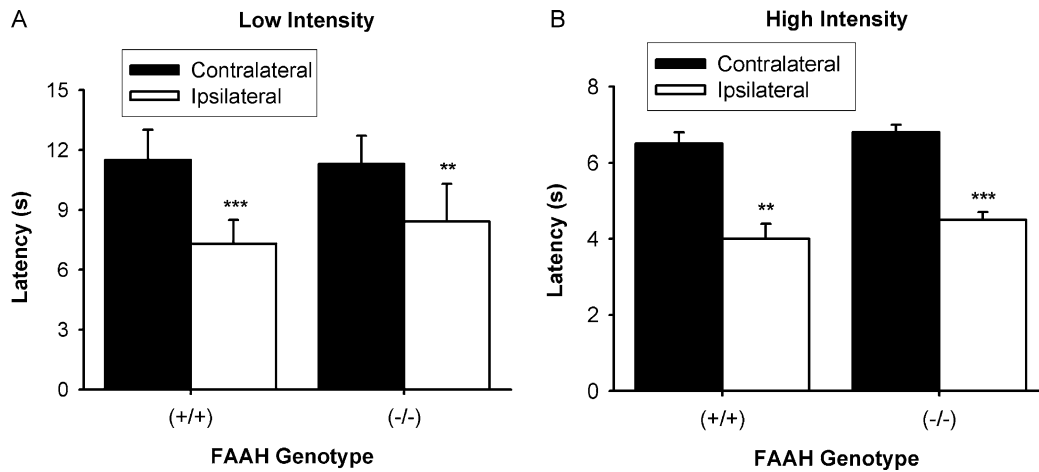


Fig. 4. FAAH (+/+) and (-/-) mice exhibit similar hyperalgesic responses to either a low (A) or high (B) intensity of noxious heat applied to the plantar surface of the rear paws following ligation of the sciatic nerve (CCI model). ** $P < 0.01$, *** $P < 0.001$, for ipsilateral paw versus contralateral paw within each genotype (planned comparisons); $n = 8$ mice/group. Results shown as mean \pm SE.

that a more intense thermal stimulus in the plantar test might better reveal genotype differences than the mild thermal stimulus used. In order to evaluate this possibility, we tested the CCI mice in the plantar stimulator test after increasing the intensity of the radiant heat stimulus (Fig. 4b). Again, only a significant main effect of paw was found, $F(1,9) = 72$, $P < 0.001$, indicating that FAAH (-/-) and (+/+) mice exhibit a similar hypersensitive response to a mild and more intense thermal stimulus in the CCI model.

One possibility for the lack of genotype differences is that adaptive changes following CCI of the sciatic nerve in the FAAH (-/-) mice could have normalized their responses to noxious stimuli. In order to evaluate this possibility, FAAH (-/-) and (+/+) CCI mice used in the plantar stimulator experiments were evaluated in the tail immersion and hot plate tests. The data in Table 1 depict the response latencies of both groups using a 56 °C thermal stimulus in each test. Strikingly, FAAH (-/-) mice that had received a unilateral sciatic nerve ligation, no longer exhibited a phenotypic hypoalgesic response in either the tail immersion test or hot plate test. These data suggest that adaptive changes may have occurred in FAAH (-/-) mice following CCI that ameliorate their phenotypic hypoalgesia compared to FAAH (+/+) mice.

4. Discussion

In the present investigation, FAAH (-/-) mice were found to display decreased pain behavior compared with FAAH (+/+) and (+/-) mice in a variety of nociceptive assays, including the tail immersion, hot plate, and formalin tests, as well as decreases in thermal hypersensitivity in the carrageenan model. Additionally, FAAH (-/-) mice exhibited significantly reduced inflammatory responses compared with wild type mice in both the formalin and carrageenan models. These phenotypes are associated with greatly elevated levels of FAAs in the spinal cords and brains of FAAH (-/-) mice (Clement et al., 2003; Cravatt et al., 2001). SR 141716 completely blocked the phenotypic decrease in pain behavior in the hot plate, tail immersion, and formalin tests, indicating the involvement of CB₁ receptors. In contrast, SR 144528 failed to attenuate the hypoalgesia of FAAH (-/-) mice in thermal and formalin nociceptive tests, arguing against CB₂ receptor involvement in these phenotypes. Collectively, these findings, in conjunction with the greatly elevated endogenous brain levels of anandamide in FAAH (-/-) mice, indicate that much of the reduced pain behavior of these animals is likely mediated by an enhanced, CNS-based endocannabinoid tone.

Table 1

The phenotypic analgesic responses of FAAH (-/-) mice in the tail immersion and hotplate tests are obliterated following chronic constriction injury (CCI) of the sciatic nerve

Group	Tail withdrawal latency (s)		Hot plate latency (s)	
	FAAH (+/+)	FAAH (-/-)	FAAH (+/+)	FAAH (-/-)
Non operated mice ^a	2.6 \pm 0.2	4.0 \pm 0.2***	10.6 \pm 0.8	16.7 \pm 0.8***
CCI	2.3 \pm 0.2	2.2 \pm 0.2	10.5 \pm 1.3	10.9 \pm 0.7

The radiant heat stimulus was set to 56 °C in both tests. Values represent mean \pm SE. $n = 6-8$ per group. *** $P < 0.001$ for non-operated FAAH (-/-) mice versus non-operated FAAH (+/+) mice or CCI FAAH (-/-) mice (planned comparisons).

^a Data taken from Fig. 1a and c for each respective test.

Conversely, the anti-hyperalgesic and anti-inflammatory responses of FAAH (–/–) mice in the carrageenan model were unaffected by SR 141716, but were significantly attenuated by SR 144528 (see Fig. 3a and b), suggesting the partial involvement of CB₂ receptors. It is noteworthy that FAAH (–/–) mice possess increased brain levels of non-cannabinoid-binding FAAs, including *N*-palmitoyl ethanolamine (PEA) (Clement et al., 2003), any of which could contribute to this phenotype. Of consequence, PEA has been well described to have anti-inflammatory actions (Conti et al., 2002; Mazzari et al., 1996). Thus it is plausible that elevated levels of other FAAs in addition to anandamide may contribute to the anti-inflammatory and anti-hyperalgesic phenotype observed in FAAH (–/–) mice.

The phenotypic hypoalgesic responses to radiant heat appear to be dependent upon the intensity of the thermal stimulus. As we previously reported (Cravatt et al., 2001), significant differences were found between the genotypes in both the tail immersion and hot plate tests when the thermal stimulus was at least 54 °C, but did not occur when lower temperatures were used. Consistent with this interpretation is that both genotypes exhibited nearly identical baseline withdrawal responses in the plantar stimulator assay, which employed a comparatively mild heat stimulus as reflected by relatively long withdrawal latencies.

In contrast to their behavior in the other nociceptive assays, both FAAH (+/+) and (–/–) mice exhibited an equivalent degree of thermal hyperalgesia in the CCI model, regardless of whether a low or high intensity of noxious heat was used. There are several explanations that may account for the lack of genotype differences in this model. First, deletion of FAAH might not effectively alleviate pathological states in which the organism is hypersensitive to noxious stimuli. This explanation can be ruled out because FAAH (–/–) mice exhibited a decrease in thermal hyperalgesia in the carrageenan model. Second, it is possible that deletion of FAAH leads to a quicker recovery from the hyperalgesic state than in wild type mice. However, the thermal hyperalgesia of the CCI mice used in the present study persisted for at least 3 months (data not shown). Third, the elevated levels of anandamide and/or other FAAs in FAAH (–/–) mice may have been insufficient to block thermal hyperalgesia in this neuropathic pain model. Fourth, adaptive changes may accompany deletion of FAAH during development in the transgenic animals and these changes specifically may alter the response to nerve injury. This possibility is consistent with the observations that an FAAH inhibitor reduced mechanical hyperalgesia in the Chung model of neuropathic nociception (Sit and Xie, 2003). On the other hand, the apparent disparity between the results of Sit and Xie and the present study may also reflect species differences or the different stimuli evaluated (i.e. mechanical versus thermal). Thus, it will be important to evaluate whether FAAH inhibitors also reduce thermal hyperalgesia in the Chung model, as well as evaluate FAAH (–/–) mice for mechanical hyperalgesia following CCI. A fifth

possibility is that nerve ligation may lead to adaptive changes in the nociceptive circuits of FAAH (–/–) mice that reduce the influence of endogenous FAAs over pain behavior. The observation that FAAH (–/–) mice, which had received a unilateral sciatic nerve ligation, no longer exhibited a phenotypic hypoalgesia in either the tail immersion or hot plate test (see Table 1) is consistent with the last possibility. In addition, it has also been demonstrated that exogenous cannabinoids have anti-hyperalgesic effects in a chronic malignant pain model (Kehl et al., 2003). Accordingly, it will be of interest to characterize the responses of FAAH (–/–) mice, as well as mice treated with FAAH inhibitors in models of both malignant and non-malignant chronic nociception. The possible mechanism(s) that may account for these functional changes in nerve injury-afflicted FAAH (–/–) mice, including molecular adaptations in the endocannabinoid system, will be a subject of future studies.

The fact that FAAH (–/–) mice exhibit decreases in pain behavior in a variety of acute nociceptive assays is quite remarkable considering that endogenous levels of anandamide and other FAAs are constitutively elevated in these animals. These results indicate that CB₁ receptor-mediated responses in FAAH (–/–) mice do not undergo tolerance throughout ontogeny, a finding that is also supported by other data. For example, both the binding affinity of [³H]CP55,940 to the CB₁ receptor and CB₁ receptor density were nearly identical in FAAH (–/–) and (+/+) mouse brains (Lichtman et al., 2002). Moreover, both genotypes exhibited similar hypomotility, anti-nociception, and hypothermia Δ^9 -THC dose–response profiles, indicating that CB₁ receptor pathways in FAAH (–/–) and (+/+) mice were functionally equivalent (Cravatt et al., 2001).

The magnitude of the genotype differences is much greater in the carrageenan and formalin models than in the tail immersion and hot plate tests. It is particularly striking that carrageenan failed to elicit thermal hyperalgesia in the FAAH (–/–) mice, as reflected by a lack of statistical significance between their pre- and post-injection paw withdrawal latencies in the plantar stimulator test. The hypoalgesic and anti-hyperalgesic phenotypes of FAAH (–/–) mice in the formalin and carrageenan assays were comparable in magnitude to the effects following exogenous cannabinoid administration (Kehl et al., 2003; Moss and Johnson, 1980), but without the disruptive effects on motor behavior. Moreover, FAAH (–/–) mice exhibited a similar magnitude of anti-edema effects following intraplantar injection of formalin or carrageenan as that following 20 mg/kg Δ^9 -THC in the rat carrageenan model (Sofia et al., 1973). On the other hand, the elevated latencies of FAAH (–/–) mice in the hot plate and tail immersion tests were markedly less in magnitude than the analgesic effects of exogenously administered Δ^9 -THC in these assays (Martin, 1985; Sofia et al., 1973). This pattern of results is consistent with the observation that the tail-flick test is considerably

less sensitive to the analgesic effects of exogenously administered cannabinoids than the formalin test (Guhring et al., 2001). Consequently, it is likely that the concentration of endogenous anandamide at the CB₁ receptor in the FAAH (–/–) mice was insufficient to achieve maximal analgesia in the tail immersion and hot plate tests. The formalin and carrageenan assays have been argued to be more reflective of clinical pain than the tail withdrawal and hot plate tests (Le Bars et al., 2001). The observations that FAAH (–/–) mice exhibit normal motility and thermoregulation (Cravatt et al., 2001), two behavioral systems that are dramatically affected by exogenously administered CB₁ agonists, suggests that selective inhibitors of FAAH may have potential promise to treat inflammatory pain disorders without side-effects that are generally associated with CB₁ agonists.

In conclusion, the present study demonstrates that FAAH (–/–) mice exhibit a CB₁ receptor-mediated hypoalgesic phenotype in a variety of acute nociceptive assays accompanied by elevated levels of anandamide in both the spinal cord and brain. Though the anti-inflammatory phenotype of these animals does not involve CB₁ receptors, there does appear to be a CB₂ receptor component, for carrageenan. Collectively, these findings suggest that inhibitors of FAAH may augment both central CB₁ and peripheral CB₂ endocannabinoid tone and thereby may offer a unique strategy for the treatment of inflammatory and non-inflammatory pain disorders.

Acknowledgements

This work was supported by National Institute of Drug Abuse of the National Institutes of Health Grants DA015197, DA03672, and DA09789, Allergan Inc., and the Helen L. Dorris Institute for the Study of Neurological and Psychiatric Disorders of Children and Adolescents.

References

- Calignano A, La Rana G, Giuffrida A, Piomelli D. Control of pain initiation by endogenous cannabinoids. *Nature* 1998;394:277–81.
- Clayton N, Marshall FH, Bountra C, O'Shaughnessy CT. CB₁ and CB₂ cannabinoid receptors are implicated in inflammatory pain. *Pain* 2002; 96:253–60.
- Clement AB, Hawkins EG, Lichtman AH, Cravatt BF. Increased seizure susceptibility and proconvulsant activity of anandamide in mice lacking fatty acid amide hydrolase. *J Neurosci* 2003;23:3916–23.
- Conti S, Costa B, Colleoni M, Parolaro D, Giagnoni G. Antiinflammatory action of endocannabinoid palmitoylethanolamide and the synthetic cannabinoid nabilone in a model of acute inflammation in the rat. *Br J Pharmacol* 2002;135:181–7.
- Cravatt B, Prospero-Garcia O, Siuzdak G, Gilula N, Henriksen S, Boger D, Lerner R. Chemical characterization of a family of brain lipids that induce sleep. *Science* 1995;268:1506–9.
- Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 1996;384:83–7.
- Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, Lichtman AH. Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci USA* 2001;98:9371–6.
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 1992;258:1946–9.
- Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, Fezza F, Miura GI, Palmiter RD, Sugiura T, Kunos G. Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* 2001;410:822–5.
- Farquhar-Smith WP, Rice AS. Administration of endocannabinoids prevents a referred hyperalgesia associated with inflammation of the urinary bladder. *Anesthesiology* 2001;94:507–13. discussion 6A.
- Fox A, Kessingland A, Gentry C, McNair K, Patel S, Urban L, James I. The role of central and peripheral cannabinoid1 receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. *Pain* 2001;92:91–100.
- Fride E, Mechoulam R. Pharmacological activity of the cannabinoid receptor agonist, anandamide, a brain constituent. *Eur J Pharmacol* 1993;231:313–4.
- Guhring H, Schuster J, Hamza M, Ates M, Kotalla CE, Brune K. HU-210 shows higher efficacy and potency than morphine after intrathecal administration in the mouse formalin test. *Eur J Pharmacol* 2001;429: 127–34.
- Hanus L, Abu-Lafi S, Frider E, Breuer A, Vogel Z, Shalev DE, Kustanovich I, Mechoulam R. 2-Arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB₁ receptor. *Proc Natl Acad Sci USA* 2001;98: 3662–5.
- Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988;32:77–88.
- Herzberg U, Eliav E, Bennett GJ, Kopin IJ. The analgesic effects of R(+)-WIN 55,212-2 mesylate, a high affinity cannabinoid agonist, in a rat model of neuropathic pain. *Neurosci Lett* 1997;221:157–60.
- Hohmann AG. Spinal and peripheral mechanisms of cannabinoid antinociception: behavioral, neurophysiological and neuroanatomical perspectives. *Chem Phys Lipids* 2002;121:173–90.
- Jaggari SI, Hasnie FS, Sellaturay S, Rice AS. The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB₂ receptor agonist palmitoylethanolamide in visceral and somatic inflammatory pain. *Pain* 1998;76:189–99.
- Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, Rana GL, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D. Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med* 2003;9:76–81.
- Kehl LJ, Hamamoto DT, Wacnik PW, Croft DL, Norsted BD, Wilcox GL, Simone DA. A cannabinoid agonist differentially attenuates deep tissue hyperalgesia in animal models of cancer and inflammatory muscle pain. *Pain* 2003;103:175–86.
- Le Bars D, Gozariu M, Cadden SW. Acute pain measurement in animals. Part 1. *Ann Fr Anesth Reanim* 2001;20:347–65.
- Ledent C, Valverde O, Cossu G, Petitot F, Aubert JF, Beslot F, Bohme GA, Imperato A, Pedrazzini T, Roques BP, Vassart G, Fratta W, Parmentier M. Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB₁ receptor knockout mice. *Science* 1999;283:401–4.
- Lichtman AH. SR 141716A enhances spatial memory as assessed in a radial-arm maze task in rats. *Eur J Pharmacol* 2000;404:175–9.
- Lichtman AH, Martin BR. Δ⁹-Tetrahydrocannabinol impairs spatial memory through a cannabinoid receptor mechanism. *Psychopharmacology* 1996;126:125–31.
- Lichtman AH, Martin BR. The selective cannabinoid antagonist, SR 141716A, blocks cannabinoid-induced antinociception in rats. *Pharmacol Biochem Behav* 1997;57:7–12.

- Lichtman AH, Cook SA, Martin BR. Investigation of brain sites mediating cannabinoid-induced antinociception in rats: evidence supporting periaqueductal gray involvement. *J Pharmacol Exp Ther* 1996;276:585–93.
- Lichtman AH, Hawkins EG, Griffin G, Cravatt BF. Pharmacological activity of fatty acid amides is regulated, but not mediated, by fatty acid amide hydrolase in vivo. *J Pharmacol Exp Ther* 2002;302:73–9.
- Malmberg AB, Basbaum AI. Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. *Pain* 1998;76:215–22.
- Malmberg AB, Chen C, Tonegawa S, Basbaum AI. Preserved acute pain and reduced neuropathic pain in mice lacking PKC γ . *Science* 1997;278:279–83.
- Mao J, Price DD, Lu J, Keniston L, Mayer DJ. Two distinctive antinociceptive systems in rats with pathological pain. *Neurosci Lett* 2000;280:13–16.
- Martin BR. Structural requirements for cannabinoid-induced antinociceptive activity in mice. *Life Sci* 1985;36:1523–30.
- Mazzari S, Canella R, Petrelli L, Marcolongo G, Leon A. *N*-(2-hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation. *Eur J Pharmacol* 1996;300:227–36.
- Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski N, Schatz A, Gopher A, Almog S, Martin B, Compton D, Pertwee R, Griffin G, Bayewitch M, Barg J, Vogel Z. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* 1995;50:83–90.
- Moss DE, Johnson RL. Tonic analgesic effects of Δ^9 -tetrahydrocannabinol as measured with the formalin test. *Eur J Pharmacol* 1980;61:313–5.
- Porter AC, Sauer JM, Knierman MD, Becker GW, Berna MJ, Bao J, Nomikos GG, Carter P, Bymaster FP, Leese AB, Felder CC. Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor. *J Pharmacol Exp Ther* 2002;301:1020–4.
- Rice AS, Farquhar-Smith WP, Nagy I. Endocannabinoids and pain: spinal and peripheral analgesia in inflammation and neuropathy. *Prostaglandins Leukot Essent Fatty Acids* 2002;66:243–56.
- Richardson JD, Aanonsen L, Hargreaves KM. Antihyperalgesic effects of spinal cannabinoids. *Eur J Pharm* 1998a;345:145–53.
- Richardson JD, Aanonsen L, Hargreaves KM. Hypoactivity of the spinal cannabinoid system results in NMDA-dependent hyperalgesia. *J Neurosci* 1998b;18:451–7.
- Richardson JD, Kilo S, Hargreaves KM. Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB1 receptors. *Pain* 1998c;75:111–9.
- Rinaldi-Carmona M, Barth F, Héaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Néliat G, Caput D, Ferrara P, Soubrié P, Brelière JC, Le Fur G. SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *Fed Eur Biochem Soc Lett* 1994;350:240–4.
- Rinaldi-Carmona M, Barth F, Millan J, Derocq JM, Casellas P, Congy C, Oustric D, Sarrau M, Bouaboula M, Calandra B, Portier M, Shire D, Brelière JC, Le Fur GL. SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J Pharmacol Exp Ther* 1998;284:644–50.
- Rodriguez de Fonseca F, Navarro M, Gomez R, Escuredo L, Nava F, Fu J, Murillo-Rodriguez E, Giuffrida A, LoVerme J, Gaetani S, Kathuria S, Gall C, Piomelli D. An anorexic lipid mediator regulated by feeding. *Nature* 2001;414:209–12.
- Sit S-Y, Xie K. Bisarylimidazolyl fatty acid amide hydrolase inhibitors. United States patent application, 128480; 2003.
- Smith PB, Compton DR, Welch SP, Razdan RK, Mechoulam R, Martin BR. The pharmacological activity of anandamide, a putative endogenous cannabinoid, in mice. *J Pharmacol Exp Ther* 1994;270:219–27.
- Smith FL, Fujimori K, Lowe J, Welch SP. Characterization of delta9-tetrahydrocannabinol and anandamide antinociception in nonarthritic and arthritic rats. *Pharmacol Biochem Behav* 1998;60:183–91.
- Sofia RD, Nalepa SD, Harakal JJ, Vassar HB. Anti-edema and analgesic properties of delta9-tetrahydrocannabinol (THC). *J Pharmacol Exp Ther* 1973;186:646–55.
- Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* 1995;215:89–97.
- Terranova JP, Storme JJ, Lafon N, Perio A, Rinaldi-Carmona M, Le Fur G, Soubrie P. Improvement of memory in rodents by the selective CB1 cannabinoid receptor antagonist, SR 141716. *Psychopharmacology* 1996;126:165–72.
- Walker JM, Huang SM. Cannabinoid analgesia. *Pharmacol Ther* 2002;95:127–35.
- Walker JM, Huang SM, Strangman NM, Tsou K, Sanudo-Pena MC. Pain modulation by release of the endogenous cannabinoid anandamide. *Proc Natl Acad Sci USA* 1999;96:12198–203.