Endocannabinoid modulation of scratching response in an acute allergenic model: a new prospective neural therapeutic target for pruritus

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Abstract

Pruritus (itch) is a common cause of discomfort by dermatological disorders. Several peripherally and centrally mediated pathologies that induce pruritus do not generally respond to typical allergenic and anti-inflammatory treatments. Accordingly, we employed an acute allergenic murine model to determine whether the endogenous cannabinoid system could be targeted to treat pruritus. Subcutaneous administration of the mast cell degranulator compound 48/80 evoked an intense, concentration-dependent scratching response. Systemic THC reduced the scratching response, though this effect was accompanied with hypomotility. Complementary genetic and pharmacological approaches to target fatty acid amide hydrolase (FAAH), the primary enzyme responsible for the degradation of the endocannabinoid anandamide, were evaluated in the compound 48/80 model. FAAH (-/-) mice, as well as mice treated with the respective irreversible and reversible FAAH inhibitors, URB597 and OL-135, displayed comparable reductions in scratching to mice treated with common non-sedative allergenic treatments (loratadine & dexamethasone), but without affecting locomotor behavior. The anti-scratching phenotype of FAAH-compromised mice was completely blocked by either genetic deletion or pharmacological antagonism of the CB1 receptor. FAAH-NS mice, which have FAAH exclusively restricted to neural tissues, showed a similar magnitude of scratching as wild type mice. Importantly, URB597 reduced compound 48/80-induced scratching in FAAH-NS mice, but it did not produce any further reduction in FAAH (-/-) mice. These findings indicate that neuronal FAAH suppression reduces the scratching response through activation of CB1 receptors. More generally, these are the first preclinical data suggesting that FAAH represents a novel target to treat pruritus without eliciting overt side effects.
Introduction

Pruritus is the general clinical term for itch, characterized by a generally unpleasant sensation attributed to an area at, or just below, the skin. The sensation is closely related to the reflex desire and action to scratch the afflicted area. Pruritus can greatly reduce the quality of life of those inflicted and is a common symptom attributed to discomfort from dermatological conditions (Stante et al., 2005). While there are no collective data on the prevalence of patients seeking treatment for pruritus, the numbers on skin diseases alone estimates an incidence of one in three Americans afflicted at any given moment, costing a yearly $28.3 billion in medical treatment (Bickers et al., 2006). Due to the great variety of pathophysiological conditions (e.g., anemia, parasites, liver/kidney disease, thyroid disorders, HIV) underlying pruritus symptomology, common treatments are not always effective. Recent studies show pruritus shares much of its neural substrates and signaling characteristics with that of another nocifensive modality, pain. Many of the same receptor systems shown to modulate analgesic actions are also being examined for their effects on itch (e.g. serotonin and opioid), often with antinociception enhancing perception of itch (for review see Paus et al., 2006; Schmelz and Handwerker, 2006).

Δ9-tetrahydrocannabinol (THC), the primary psychoactive component in marijuana, and other cannabinoid receptor agonists produce analgesic effects through the activation of cannabinoid receptors in the periphery, spine, and brain (for review see Pacher et al., 2006). However, given the occurrence of motor suppression, abuse potential, physical dependence, and psychomimetic effects of THC and other direct-acting cannabinoid agonists, recent efforts in cannabinoid research are focused on examining drugs that prevent the catabolism of endogenous cannabinoids. The endocannabinoid system consists of CB1 and CB2 receptors as well as several phospholipid-derived endogenous ligands that bind to these receptors. The most characterized endogenous cannabinoid, anandamide, is rapidly degraded predominantly by the enzyme fatty acid amide hydrolase (FAAH). Inactivation or inhibition of FAAH greatly increases anandamide levels in brain, spinal cord, liver, and other tissues (Boger et al., 2005; Cravatt et al., 2001; Fegley et al., 2005). FAAH also degrades several other fatty acid amides with
known physiological functions, including oleamide (sleep), N-palmitoyl ethanolamine (PEA; anti-inflammatory), and oleylethanolamide (satiety) (Cravatt et al., 2001). FAAH (-/-) mice, as well as wild-type mice treated with FAAH inhibitors, possess cannabinoid receptor-mediated hypoalgesic and anti-inflammatory phenotypes, without any signs of significant sedation or muscle relaxation (Lichtman et al., 2004a; Lichtman et al., 2004b).

Though minimal study has been performed in the area of cannabinoids and pruritus, cannabinoid receptor agonists tend to decrease scratching behavior, while cannabinoid receptor antagonists, such as rimonabant, elicit a dose-dependent increase in scratching behavior in mice (Darmani and Pandya, 2000). Interestingly, several mixed CB1/CB2 receptor agonists (i.e., THC, WIN55212-2, CP55940, and HU-210) potently reversed rimonabant-induced scratching in an order similar to their potency to elicit known cannabinoid mediated behavioral effects (Janoyan et al., 2002). Additionally, each of these cannabinoids also reduced scratching elicited by the 5-HT2A/C receptor agonist, DOI (Darmani, 2001).

In limited case reports, dronabinol (oral THC) relieved intractable pruritus resulting from liver cholestasis (Neff et al., 2002), a common clinical cause of severe and prolonged pruritus. Finally, transdermal application of the potent cannabinoid receptor agonist, HU-210, significantly reduced the perception of itch elicited by histamine iontophoresis in a small group of healthy volunteers (Dvorak et al., 2003). Taken together, the above evidence suggests that cannabinoids may provide a novel therapeutic target for both peripheral and central inhibition of pruritus. Presently, there are no published studies of which we are aware that have examined the role of FAAH in an animal model of pruritus.

The overall objective of the present study was to elucidate the therapeutic potential of targeting the endocannabinoid system to treat pruritus. A model using the mast cell degranulator, compound 48/80, that directly activates signaling events characteristic of an acute allergenic response was adapted from Sugimoto et al. (1998). Local application of compound 48/80 elicits short-term and predictable levels of scratching behavior in the affected area. Clinical treatments of allergenic pruritus (e.g. antihistamines and glucocorticoids) effectively suppress scratching compound 48/80-induced scratching. The
prototypical cannabinoid receptor agonist, THC, was evaluated in the compound 48/80 pruritus model for both scratching attenuation and levels of behavioral locomotor inactivity. FAAH (-/-) mice and wild type mice treated with FAAH inhibitors were also assessed in the compound 48/80-induced scratching model. In addition, both pharmacological and transgenic tools were employed to elucidate cannabinoid receptor mechanisms as well as whether blockade of neuronal FAAH was critical in reducing scratching responses.
Methods

Subjects

Tests utilizing transgenic animals (i.e. FAAH, CB₁, and CB₂ knockout animals) and their controls were performed on mice from the Center Transgenic Colony at Virginia Commonwealth University (Richmond, VA) backcrossed onto either a C57BL/6J (13 generations) or DBA/1 (6 generations) background. All other experiments utilized adult male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), except one intravenous study employing male ICR mice (Harlan Laboratories, Indianapolis, IN). Mice were housed 4-6 per cage in a temperature (20–22°C) controlled environment, with food and water available ad libitum while in their home cages. Mice were kept on a 12-hour light/dark cycle, with all experiments performed during the animals’ light period. All experiments were performed with the approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the Guide for the Care and Use of Laboratory Animals.

Drugs

THC and rimonabant were obtained from the National Institute on Drug Abuse (Bethesda, MD) and OL-135 was synthesized as previously described (Boger et al., 2005). URB597 was purchased from Cayman Chemical (Ann Arbor, MI), while compound 48/80, diphenhydramine, dexamethasone, and loratadine were purchased from Sigma (St. Louis, MO). Compound 48/80, diphenhydramine, dexamethasone, and loratadine were dissolved in 0.9% saline, while all other drugs were dissolved in vehicle mixture of ethanol/alkamuls-620 (Rhone-Poulenc, Princeton, NJ)/saline in a ratio of 1:1:18. Drugs were diluted to an injection volume of 10 µl/g body mass and injected via intraperitoneal injection unless otherwise specified. Compound 48/80 was administered as a fixed quantity diluted per 200 µl subcutaneous injection.

All pretreatments and genotypic comparisons were made against a compound 48/80 challenge dose of 30 µg. The CB₁/CB₂ mixed receptor agonist THC, the first-generation antihistamine
diphenhydramine, the non-sedating antihistamine loratadine, and the glucocorticoid dexamethasone, were each administered 30 min prior to compound 48/80 injection. The reversible FAAH inhibitor OL-135 and the irreversible FAAH inhibitor URB597 were both administered 1 h before compound 48/80, based on previous reports showing anandamide levels peaked at that time (Fegley et al., 2005; Lichtman et al., 2004a). In one study, URB597 was administered intravenously 10 min after application of compound 48/80 to examine reversal of scratching. In all studies utilizing rimonabant, the selective CB<sub>1</sub> receptor antagonist was administered 15 min prior to compound 48/80.

**Behavioral Evaluation of Scratching Response**

Animals were pretreated with test drugs at times described for individual experiments. All animals were placed into white (for contrast) acrylic chambers (20 cm x 20 cm x 20 cm), with a clear acrylic front panel and a mirrored back panel, for 30 min to acclimate to the observational environment. The chambers were enclosed in sound-attenuating cabinets, designed and custom built at Virginia Commonwealth University, that contained an indirect filtered LED light source and fans for air circulation and white noise. At the 30 min time point, animals were briefly removed from the chambers, which were wiped clean with water. Subjects were then given an injection of compound 48/80 under the scruff at the most dorsal point of the back just beneath the head, and were returned to the chambers for a 30 min. Behavior was recorded through the clear front panel using a series of Fire-i™ digital cameras (Unibrain, San Ramon, CA) and the videos were processed and saved using ANY-maze™ video tracking software (Stoelting Co., Wood Dale, IL). Chambers were fully sanitized at the end of each testing day using ammonia-based cleansers and soap, then left to air dry at least two days to dissipate any odors.

The videos were subsequently placed in randomized order in a separate ANY-maze™ protocol for a trained observer to score using a keyboard-based behavioral tracking system, blinded to treatment group. Either ANY-maze™ or ODLog™2 (Macropod Software, Armidale, NSW, Australia) software was used
to track key presses assigned to specific behavioral endpoints for both time pressed and number of occurrences. The scratching response was tracked as hind leg scratching of the injection site and surrounding areas, excluding behaviors focused at areas within the ears or flicking and pulling at ear tags. Behavioral locomotor inactivity was also monitored, scored as complete lack of voluntary movement for any continuous period longer than 5 s.

Data Analysis

All data are reported as mean ± SEM, and represent the total number of seconds a specific behavior was scored from a total observational period of 1800 sec. Experiments with only two treatment groups were analyzed for statistical significance using Student’s t test. Experiments with more than two groups were analyzed using one- or two-way analysis of variance (ANOVA), with specified appropriate post-hoc test. Resulting p values of less than 0.05 were considered significant. ED$_{50}$ values (with 95% confidence intervals), as well as the potency ratios derived from the ED50 values, were calculated using the least-squares linear regression method. Significant differences in potency were determined by the 95% confidence intervals of the potency ratio not including a ratio of 1.
Results

Pruritic Response to Compound 48/80

In an initial experiment, we evaluated the compound 48/80 model as a reliable model of pruritus by examining a similar dose range as used by Sugimoto et al. (1998) in eliciting scratching. Injections of compound 48/80 in caudal regions of the back induced increased grooming behavior (data not shown), as well as intense scratching with the hind paws directed at the rostral area of the back. As shown in Figure 1, compound 48/80 dose-dependently increased time mice spent scratching the area of injection more than 100-fold above that of saline control injections \( F(5,30) = 55.3, p < 0.001 \). Based on these results, 30 µg compound 48/80 was used in all subsequent experiments because this was the lowest tested dose that significantly differed from saline at \( p < 0.01 \) (Dunnett’s post hoc test).

To validate further the allergenic pruritus model, a pair of drugs representing the most common classes of clinically effective treatments for allergenic itch was tested against compound 48/80 (30 µg). The glucocorticoid compound, dexamethasone (2 mg/kg i.p.) significantly reduced scratching compared to saline pretreatment \( t(14) = 6.1, p < 0.001 \). In an initial study, we attempted to examine the dose-response relationship of diphenhydramine, an over-the-counter and commonly used H1 receptor antagonist. However, a significant reduction in scratching only occurred at doses that also elicited profound hypothermia and almost complete immobility (data not shown). The non-sedating second generation H1 receptor antagonist, loratadine (10 mg/kg i.p.), significantly reduced scratching behavior compared to vehicle controls \( t(10) = 4.8, p < 0.001 \). No significant effects of behavioral locomotor inactivity were found for either dexamethasone or loratadine. The results of these tests are summarized in Table 1.

Evaluation of THC Treatment

Intraperitoneal administration of THC dose-dependently attenuated compound 48/80-induced scratching \( F(4,35) = 15.3, p < 0.001 \); Figure 2A]. The resulting curve shows that THC possessed great
efficacy and potency in suppression of scratching behavior (ED50 = 0.66 mg/kg, 95% CI 0.52 to 0.82 mg/kg). However, this attenuation of scratching was accompanied with a concurrent significant suppression in behavioral locomotor activity [\(F(4,35) = 7.9, p < .001\)]. To determine if the therapeutic profile improves with local administration, THC was given subcutaneously at the site of compound 48/80 administration (injection concentration was increased to 5 µl/g body mass). As seen in Figure 2B, local administration of THC (0.3, 0.56, 0.75 mg/kg) produced a significant reduction in compound 48/80-induced scratching [\(F(3,20) = 11.4, p < 0.001\)] (\(p < 0.01\), Dunnett’s post-hoc), indicating an increase in potency compared to the i.p. route of administration. Additionally, significant hypomotility [\(F(3,20) = 3.7, p < 0.05\)] occurred only at the highest doses tested, demonstrating a dissociation between the suppressive effects of THC on scratching and its locomotor depressive effects.

As THC binds to both CB1 and CB2 receptors, we evaluated its receptor mechanism of action using mice lacking functional CB1 or CB2 receptors. As shown in Figure 3A, THC (1 mg/kg) significantly reduced scratching behavior in the CB1 (+/+ ) mice, but did not cause any significant changes in the CB1 (-/-) mice [\(F(1,28) = 8.9, p < 0.01\) for interaction between genotype and THC pretreatment]. Compound 48/80 produced significantly less scratching behavior in CB1 (-/-) mice than in CB1 (+/+ ) mice (\(p < 0.01\), Tukey’s post-hoc). In addition, there was a significant interaction between genotype and THC on the locomotor activity data [\(F(1,28) = 15.5, p < 0.001\)]. THC more than doubled the time that the CB1 (+/+ ) mice spent inactive (vehicle: 208 ± 62 sec, THC: 532 ± 46 sec), but did not significantly affect the CB1 (-/-) mice. However, CB1 (-/-) mice were significantly less active than the CB1 (+/+ ) mice under vehicle conditions (\(p < 0.01\), Tukey’s post-hoc). In contrast, CB2 (-/-) mice exhibited similar scratching responses and levels of inactivity compared to CB2 (+/+ ) mice under vehicle treatment (see Figure 3B). In addition, both genotypes showed equivalent responses to treatment with THC with respect to both scratching suppression and behavioral inactivity, with only a resulting main effect of THC treatment for scratching suppression [\(F(1,20) = 52.3, p < .001\)].
Evaluation of FAAH (-/-) Mice Scratching Response

FAAH (-/-) mice display impaired catabolic activity to anandamide and other fatty acid amides, resulting in at least 10-fold increases in endogenous levels of these lipid signaling molecules (Cravatt et al., 2001). The effects of compound 48/80 challenge in FAAH (-/-) mice backcrossed onto either C57BL/6 (C57) or DBA/1 (DBA) genetic backgrounds are shown in Figure 4. FAAH (-/-) mice demonstrated a significantly reduced scratching response to compound 48/80 compared to FAAH (+/+) mice in both C57 \( t(14) = 2.5, p < 0.05 \) and DBA \( t(14) = 2.3, p < 0.05 \) strains. Neither the C57 nor DBA strains of FAAH (-/-) mice showed any differences in time spent inactive during testing (Table 2). These two background strains were examined because C57 mice were used in most other behavioral tests presented here, and the neural specific FAAH knock-in (i.e., FAAH-NS) mice available for this research was developed on the DBA background.

While numerous biologically activated fatty acid amides are elevated in mice lacking functional FAAH activity, anandamide is the only mediator that has shown high affinity for activating cannabinoid receptors. To test whether anandamide and CB₁ receptors contribute to the FAAH (-/-) phenotypic reduction in scratching, a moderate dose of rimonabant (1 mg/kg, i.p.) was given 15 min prior to compound 48/80. As seen in Figure 5, the vehicle-treated FAAH (-/-) mice displayed a significant reduction in scratching behavior compared to the vehicle-treated FAAH (+/+) mice. The dose of rimonabant did not affect scratching in FAAH (+/+) mice, but significantly reversed the FAAH (-/-) phenotype \( F(1, 28) = 20.0, p < 0.001 \) for interaction between rimonabant and genotype], indicating a CB₁ receptor mechanism. There were no significant effects of either FAAH genotype or rimonabant pretreatment on locomotor activity (Table 2).

Because CB₁ receptors are expressed in both the CNS and periphery, we next evaluated the contribution of FAAH activity in neural tissues. FAAH-NS mice are conditional knock-in mice with FAAH expression linked to the promoter for neural-specific enolase, resulting in mice that express FAAH exclusively in neuronal tissue (i.e., brain, spinal cord, and dorsal root ganglia). Thus, these mice
possess wild type levels of anandamide and noncannabinoid fatty acid amide levels in brain and spinal cord, but have greatly elevated levels of these lipid signaling molecules in peripheral organs (Cravatt et al., 2004). A comparison of FAAH-NS mice to FAAH (-/-) and FAAH (+/-) controls (on a DBA/1 background strain) is shown in Figure 6. FAAH (+/-) mice exhibited a similar response to compound 48/80 as wild-type animals, while FAAH (-/-) mice continued to display a significant reduction in scratching \[F(2, 26) = 9.7, p < 0.001; \text{Figure 6A}\]. The observation that the FAAH-NS mice showed a similar magnitude of scratching to compound 48/80 as wild-type mice indicates that neuronal fatty acid amides regulate this response. Despite scratching significantly more than FAAH (-/-) mice, and the same as FAAH (+/-) mice, FAAH-NS showed a significant increase in time spent inactive (Table 2). This pattern of findings shows that scratching and hypomotility can be functionally uncoupled.

**Treatment of Scratching by FAAH Inhibitors**

In the final series of experiments, we evaluated the effects of the selective FAAH inhibitors, URB597 and OL-135, in the compound 48/80 pruritus model. As seen in Figure 7, and summarized in Table 1, both compounds significantly reduced scratching responses, with no significant effects on behavioral activity. URB597, shown in the black line, significantly reduced the scratching response compared to vehicle at doses above 1 mg/kg \([F(4,35) = 8.9, p < 0.001]\), with an ED$_{50}$ dose of 0.8 mg/kg (95% C.I. 0.4 - 1.6 mg/kg). Increasing doses of URB597 had no significant effect on behavioral locomotor inactivity in the mice (shown in figure 7B), including doses that elicited significant attenuation of scratching [ANOVA, \(p = 0.50\)]. Similarly, OL-135, shown in the gray line, significantly reduced scratching compared to vehicle \([F(3,28) = 8.1, p < 0.001]\), with an ED$_{50}$ dose of 3.9 mg/kg (95% C.I. 2.1 – 7.2 mg/kg). OL-135 also did not affect locomotor activity (figure 7B; ANOVA, \(p = 0.62\)). Both FAAH inhibitors displayed equivalent efficacy in reducing scratching behavior. The potency ratio (95% confidence limits) for the anti-scratching effects between URB597 and OL-135 was
4.1 (95% C.I. 1.8 – 9.1), indicating a significant difference because the confidence limits did not include the ratio of 1.

Because pruritic conditions are treated after persistent itching and are not treated prophylactically, we next investigated whether URB597 would reverse scratching after the allergenic response was already initiated. Accordingly, ICR mice were given compound 48/80 and monitored before and after either vehicle or URB597 (1 mg/kg) intravenous treatment. As shown in Table 3, both groups showed equivalent levels of scratching response over the initial 10 min after compound 48/80 (i.e., before drug treatment). At the conclusion of the first 10 min time block, the mice were removed from the observation cage, immediately given an injection of either vehicle or URB597 (10 mg/kg) into the tail vein, and returned to the test chamber by 11 min. Mice were then scored again for the final 10 min of the compound 48/80 scratching (i.e., from 20 to 30 min after compound 48/80 administration), allowing 10 min without scoring for onset of drug action. After the i.v. injection, both groups showed less scratching behavior than during the first 10 min time block, in line with a typical compound 48/80 time-course. Importantly, URB597 injected after the induction of compound 48/80-induced scratching significantly attenuated scratching behavior compared to vehicle-treated mice. Additionally, the vehicle- and URB597-treated groups displayed a similar degree of hypomotility at each respective time block.

To examine the receptor mechanism of action, we evaluated the effects of URB597 on compound 48/80-induced scratching in CB1 (-/-) and CB2 (-/-) mice (see Figure 8). URB597 significantly decreased scratching in CB1 (+/+) mice, but was without effect in CB1 (-/-) mice [F(1,30) = 5.9, p < 0.05 for interaction between URB597 and genotype; Figure 8A]. There was also a significant increase in behavioral locomotor inactivity, regardless of treatment, in the CB1 (-/-) mice [F(1,30) = 15.0, p < 0.001 for genotype; Table 2]. Because vehicle-treated CB1 (-/-) mice displayed a reduced scratching response compared to vehicle-treated CB1 (+/+) mice (p = 0.05, Tukey’s post hoc), we next evaluated whether rimonabant would block the reduction of scratching behavior caused by URB597. As depicted in Figure
8B, rimonabant completely reversed the reduction of scratching caused by URB597 \( F(1, 20) = 16.2, p < 0.001 \) for interaction between rimonabant and URB597]. Rimonabant and URB597 did not alter behavioral activity levels either alone or in combination (see Table 2). In contrast, URB597 elicited a similar attenuation of scratching in both CB2 (+/+ and (-/-) mice \( F(1,20) = 23.9, p < 0.001 \) for main effect of URB597; Figure 8C]. In addition, both CB2 (+/+ and (-/-) mice displayed a similar degree of scratching behavior as well as activity levels (Table 2). These data indicate that CB1 receptors are critical for URB597’s attenuation of compound 48/80-induced scratching.

Finally, to validate the specificity of both the transgenic and pharmacological tools, the effects of vehicle or URB597 (10 mg/kg) were evaluated in FAAH (+/-), FAAH (-/-), and FAAH-NS mice to ascertain the locus of action. In a replication of the previous experiment shown in Figure 6, FAAH (-/-) mice showed a phenotypic attenuation in scratching compared to FAAH (+/-) controls, while FAAH-NS mice displayed wild type levels of compound 48/80-induced scratching. URB597-treated FAAH (-/-) mice exhibited no further suppression of scratching beyond the phenotypic reduction, but this FAAH inhibitor significantly reduced scratching behavior in both FAAH (+/-) and FAAH-NS mice \( F(2,43) = 3.92, p < 0.05 \) for interaction between genotype and URB597]. Thus, systemic pharmacological inhibition of FAAH by URB597, in mice with either ubiquitous FAAH activity or only neuronal FAAH activity, produces a similar magnitude in the suppression of scratching in response to compound 48/80. This scratching suppression is equivalent to that seen as a phenotypic characteristic of FAAH (-/-) animals, mice with globally inactive FAAH.
Discussion

Pruritus represents a significant clinical problem that can produce great suffering in affected patients that includes 70% of patients suffering from liver cholestasis, as well as approximately 20% in kidney failure and most patients with atopic dermatitis. In fact, upwards of 50% of all pruritus cases requiring medical treatment may result from a systemic, chronic underlying pathology (Bernhard, 1994). None of the aforementioned conditions have pharmacotherapies available that consistently alleviate the pruritus. The research presented in the present manuscript is the first to examine the therapeutic and mechanistic roles of the endogenous cannabinoid system in the modulation of pruritus. Here, we demonstrate that THC, as well as pharmacological blockade or genetic deletion of FAAH, reduces scratching in a murine model of pruritus.

FAAH (-/-) mice, as well as mice treated with FAAH inhibitors, displayed a significant decrease in compound 48/80-induced scratching with the absence of motor suppression. The converging evidence from FAAH (-/-) mice and two separate classes of FAAH inhibitors suggests that inactivation of this enzyme, resulting in elevated levels of several fatty acid amides, leads to inhibition of pruritic responses. The ED_{50} values of URB597 and OL-135 in attenuation of scratching response closely matched those previously reported for inhibition of FAAH and/or enhancement of activity from exogenously administered anandamide (Lichtman et al., 2004a; Piomelli et al., 2006). The FAAH inhibitors URB597 and OL-135 show similar efficacy in scratching suppression as current treatments of choice for allergenic pruritus (Table 1). Importantly, neither of these agents elicited hypomotility. Intravenous administration of URB597 demonstrated rapid reversal of pruritic activity after initiation of mast cell degranulation and allergenic response, suggesting further a non-immune blockade of itch signaling with potential therapeutic application. Reversal of scratching is of clinical relevance because pruritus is rarely treated prophylactically.

The mechanism underlying FAAH influence on pruritus is not directly clear, due to the wide variety of receptor systems and lipid mediators affected by FAAH inactivation. While inhibition of FAAH
leads to increased endogenous anandamide levels, it also increases content of other fatty acid amides important to cellular signaling (Cravatt et al., 2001). Of note, topical creams containing a noncannabinoid substrate of FAAH, PEA, suppressed ratings of pruritus in a small clinical study of volunteers with various chronic dermatological conditions (Stander et al., 2006). Although no mechanistic studies were performed, most evidence points to PEA providing anti-inflammatory action through PPARα nuclear receptors (Lo Verme et al., 2005). The FAAH (−/−) phenotypic reduction in scratching was completely reversed by 1 mg/kg of the CB1 antagonist, rimonabant. Similarly, the FAAH inhibitor, URB597, failed to attenuate scratching behavior in CB1 (−/−) mice or rimonabant-treated wild type mice. Conversely, URB597 continued to elicit full antipruritic effects in CB2 (−/−) mice. Moreover, these findings are consistent with the notion that elevated levels of anandamide mediate this phenotype because it is the only known substrate of FAAH that binds to CB1 receptors.

Through the use of FAAH-NS mice, we evaluated whether FAAH activity in the nervous system plays an important role in modulating scratching behavior in the compound 48/80 model. Interestingly, FAAH-NS mice that express wild type levels of fatty acid amides in the nervous system, but elevated levels in peripheral organs (Cravatt et al., 2004) exhibited a similar scratching response as wild-type mice. Thus, despite possessing elevated levels of fatty acid amides in the peripheral organs, these mice have no phenotypic attenuation of pruritus. Furthermore, administration of URB597 to FAAH-NS mice, which inhibited the remaining active FAAH in the nervous tissues, suppressed the scratching response in a fashion similar to wild-type controls. These suppressed scratching responses mimicked that of FAAH knockout mice in magnitude, and no further reductions of URB597 were seen in global FAAH knockout mice. The above results show the apparent specificity of the scratching phenotype of both the drug and transgenic models to their FAAH activity, and indicate that FAAH inhibition within the nervous system is essential for antipruritic action. These findings suggest that this enzyme may be targeted for therapeutic gain to treat a generalized pruritus from a wide variety of etiologies, including immunological, neuropathic, and psychogenic.
This study also demonstrated that even though systemic THC is capable of near full suppression of pruritic scratching response to compound 48/80, its effects are accompanied with overt CNS behavioral effects. Subcutaneous THC administered locally to the site of compound 48/80 potently suppressed scratching response, supporting previous evidence that locally applied cannabinoid agonists have antipruritic action (Dvorak et al., 2003), though suppression was observed in a narrow dose range before also eliciting central inhibitory effects. The profile of THC in the compound 48/80 model is similar to animals pretreated with systemic diphenhydramine, a well-established antihistamine that has sedative and adverse CNS effects, limiting its use when patient sedation and cognition are considerations (for review see Banerji et al., 2007). Pretreatment with dexamethasone and loratadine, representing commonly used non-sedative treatments of allergenic pruritus, produced effective suppression of scratching response to compound 48/80 with minimal effects on locomotor activity and sedation. The suppression of scratching observed following THC administration was absent in CB1 knockout mice, but equally effective in CB2 knockout mice, suggesting that at least most of THC’s antipruritic actions are attributable to activation of CB1 receptors. However, the observation that THC also increased hypomotility suggests that at least a portion of the suppression seen may be attributed to central side-effects at higher doses.

Therapeutic use of THC is hindered by many issues associated with chronic use. THC has been shown to have potential for abuse, as THC has been shown to be self-administered in primates (Justinova et al., 2008), and shows a withdrawal syndrome indicative of physical dependence in humans (Haney et al., 1999). In contrast, the FAAH inhibitor URB597 appear to lack abuse potential, with no conditioned place preference in rodents (Gobbi et al., 2005), and no self-administration in primates (Justinova et al., 2008). In addition, URB597 does not appear to elicit cannabimimetic subjective effects, as it fails to substitute for THC in drug discrimination tests in rodents (Solinas et al., 2007). However, FAAH inhibition has therapeutic efficacy in numerous preclinical models of anxiety, depression, pain, and inflammation (for review see Cravatt and Lichtman, 2004; Piomelli et al., 2006).
CB₁ (-/-) mice generally displayed phenotypic reductions in compound 48/80-induced scratching response as well as increases in time spent inactive under vehicle conditions, complicating interpretation of the results. This CB₁ (-/-) phenotypic reduction in scratching may be the result of compensatory adaptations across ontogeny, epistasis in which the effect of gene disruption is modified by the genetic background on which it is placed, or pleiotropic effects in which other consequences of gene disruption indirectly affects the behavior of interest (Mogil and Grisel, 1998). Importantly, the CB₁ receptor antagonist, rimonabant, also blocked phenotypic decreases in compound 48/80-induced scratching caused by genetic deletion or pharmacological inhibition of FAAH. These complementary approaches indicate that CB₁ receptor activation is a crucial downstream signaling event for the antipruritic phenotype of FAAH-compromised mice.

Previous studies show pruritus may be gated by inhibitory input from nociceptive pathways. Noxious cooling and heating, along with scratching itself, produce increases in pain ratings with associated decreases in itch (Ward et al., 1996; Yosipovitch et al., 2005; Yosipovitch et al., 2007). Compared to either histamine or noxious cold alone, human subjects had selective neural activation of the periaqueductal gray (PAG) during induction of both pruritus and pain together (Mochizuki et al., 2003). The PAG having such a pronounced role in the inhibition of nociception suggests that this brain region may also inhibit pruritus (Waters and Lumb, 2007). Considering that opioid (Rosenfeld, 1994) and cannabinoid (Lichtman et al., 1996) receptor activation in the PAG plays a prominent role in antinociception, their respective roles in modulation of pruritus are similarly anticipated. Indeed, opioid drugs such as the μ receptor antagonist naltrexone and the κ receptor agonist nalfurafine are being clinically tested as modulators of pruritus (Bigliardi et al., 2007; Mansour-Ghanaei et al., 2006; Wikstrom et al., 2005). Unlike the μ-opioid receptor, CB₁ receptor activation appears to have the benefit of inhibiting both nociceptive and pruriceptive signals. Further study of the important neural substrates in endocannabinoid inhibition of prurition are necessary to understand the endocannabinoid system’s differential function.
In conclusion, the present study demonstrates that activation of the CB₁ cannabinoid receptor, via either direct-acting receptor agonist (i.e., THC) or inhibiting degradation of anandamide, can suppress scratching response to a pruritic agent. While direct agonists show cannabimimetic central effects that included hypomotility, elevating endogenous cannabinoids through FAAH inhibition produces efficacious suppression of scratching response. Suppression of compound 48/80-induced scratching through FAAH inactivation is comparable to currently available treatments, with no apparent effect on sedation and coordination. FAAH produces scratching suppression through activation of CB₁ receptors within neural tissues, representing a potential therapeutic target for blockade of pruritic signaling. The potential of a signaling-based neural target to treat pruritus, regardless of the causal pathology, makes FAAH inhibitors a promising prospective tool for the treatment of itch.
Acknowledgements

We would like to acknowledge the technical assistance of Ashley Crockett and Darby Fleming in performing video observations for some of the experiments. We also thank all the members of Lichtman laboratory group for their creative input and feedback on this project. Finally, we are indebted to our dear friend and mentor, the late Dr. Billy R. Martin, for his great insight and guidance that helped shape this project.
References


Darmani NA and Pandya DK. (2000) Involvement of other neurotransmitters in behaviors induced by the cannabinoid CB1 receptor antagonist SR 141716A in naive mice. *J Neural Transm* **107**:931-945.


Footnotes

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Legends for Figures

Figure 1. Dose-response of time spent hind leg scratching induced by subcutaneous compound 48/80 (1, 3, 10, 30, 100 µg). Totals represent time scratching within 30 min post-injection. ★p<0.05, ★★p<0.01 versus saline (Dunnett’s post-hoc). n = 8 mice per dose.

Figure 2. Evaluation of THC in attenuating scratching response induced by compound 48/80. Filled black squares (■) and line represent hind leg scratching time and is quantified on the left axis, while open gray circles (○) and gray dashed line represent time animals spent inactive quantified on the right axis. (A) THC dose-response (0.3, 0.56, 1, 3 mg/kg) following 30 min i.p. pretreatment. (B) THC dose-response (0.3, 0.56, 0.75 mg/kg) following 30 min s.c. pretreatment. ★p<0.05, ★★p<0.01 versus vehicle (Dunnett’s post-hoc). n = 6-8 mice per dose.

Figure 3. Effect of THC (1 mg/kg) on attenuating scratching response induced by compound 48/80 in CB1 and CB2 knockout animals and wild type controls. (A) THC (striped bars) significantly attenuated scratching response compared to 1:1:18 vehicle control in CB1 (+/+), but not in CB1 (-/-) mice. CB1 (-/-) mice also show attenuated (p≤0.05) scratching response with vehicle pretreatment. (B) THC elicits a similar attenuation of compound 48/80-induced scratching in CB2 (+/+) and CB2 (-/-). ★★p<0.01, ★★★p<0.001 versus corresponding vehicle-treated mice. n = 6-8 mice per group.

Figure 4. FAAH (-/-) mice display a phenotypic reduction in compound 48/80-induced scratching. Graph shows a reduced response in mice lacking FAAH enzyme activity (checkered bars) bred onto either C57BL/6 or DBA/1 background strains. Significance was determined between genotypes for each strain using Student’s t test; ★p<0.05 versus respective FAAH (+/+ ) group (Tukey’s post-hoc). n = 8-12 mice per group.
Figure 5. The FAAH (-/-) antipruritic phenotype is mediated through a CB₁ receptor mechanism of action. FAAH (+/+) and FAAH (-/-) mice on a C57Bl/6 background were given an i.p. injection of rimonabant (1 mg/kg) 15 min before compound 48/80. The rimonabant (hatched bars) did not affect scratching behavior in FAAH (+/+) mice compared to 1:1:18 vehicle controls, but reversed the phenotypic reduction in FAAH (-/-) mice. ★★p<0.01 versus FAAH (+/+) vehicle group, ###p<0.01 versus FAAH (-/-) vehicle group (Tukey’s post-hoc). n = 8 mice per group.

Figure 6. Mice that only express FAAH in neural tissues do not display a phenotypic reduction in compound 48/80-induced scratching. Heterozygous mice (+/-; white bar), global FAAH knockout mice (-/-; black bar), and conditional knockout mice expressing FAAH isolated to neural tissues (NS; grey bar). FAAH (-/-) mice display typical phenotypic reduction in scratching response (★★p<0.01; Tukey’s post-hoc) compared to FAAH (+/-) controls, however this phenotype is absent in mice with FAAH expression active in neural tissues (###p<0.01 versus FAAH (-/-) group). n = 10 mice per group.

Figure 7. FAAH inhibitors dose-dependently reduce compound 48/80 scratching without hypomotility. (A) Dose-response of FAAH inhibitors in attenuating scratching response to compound 48/80. The irreversible FAAH inhibitor URB597 (0.3, 1, 3, 10 mg/kg; i.p.) was given 1 h before compound 48/80, shown as closed black squares (■) with solid black line. The reversible FAAH inhibitor OL-135 (1, 3, 10, 30 mg/kg; i.p.) was also given 1 h before compound 48/80, shown as open gray circles (○) with dashed gray line. Inset. the structure and descriptions of both FAAH inhibitors. (B) The dose response of behavioral inactivity of the FAAH inhibitors in response to compound 48/80. ★p<0.05, ★★p<0.01 versus vehicle (Dunnett’s post-hoc). n = 8 mice per dose.

Figure 8. The FAAH inhibitor, URB597, attenuates compound 48/80-induced scratching through a CB₁ receptor mechanism of action. Mice were given either 1:1:18 vehicle or 10 mg/kg URB597 1 h before
compound 48/80. (A) URB597 reduced scratching in CB₁ (+/+), but not CB₁ (-/-), mice. (B) Rimonabant (1 mg/kg) blocked the anti-scratching effects of URB597. (C) URB597 reduced scratching in both CB₂ (+/+ and CB₂ (-/-) mice. ★★p<0.01, ★★★p<0.001 versus each respective vehicle control group, ###p<0.001 versus URB597 with vehicle pretreatment (Tukey’s post-hoc). n = 6-8 mice per group.

**Figure 9.** URB597 reduced scratching in FAAH-NS and FAAH (+/-) mice to the same magnitude as the FAAH (-/-) anti-scratching phenotypic response. However, URB597 did not further reduce scratching in FAAH (-/-) mice. ★★p<0.01 versus the corresponding vehicle group for each respective genotype, ###p<0.001 versus FAAH (+/-) vehicle controls (Tukey’s post-hoc). n = 8 mice per group.
Table 1. Maximal Effect of Scratching Attenuation by Non-sedating Pruritus Treatments

<table>
<thead>
<tr>
<th>Drug Pretreatment</th>
<th>Vehicle Control</th>
<th>Control Scratching (mean ± SEM s)</th>
<th>Treatment Scratching (mean ± SEM s)</th>
<th>% Scratching Reduction</th>
<th>Behavioral Locomotor Inactivity p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mg/kg Dexamethasone</td>
<td>Saline</td>
<td>279.7 ± 14.1</td>
<td>150.6 ± 16.0</td>
<td>46%</td>
<td>0.40</td>
</tr>
<tr>
<td>10 mg/kg Loratadine</td>
<td>Saline</td>
<td>299.0 ± 14.9</td>
<td>143.5 ± 15.4</td>
<td>52%</td>
<td>0.33</td>
</tr>
<tr>
<td>10 mg/kg URB597a</td>
<td>1:1:18</td>
<td>256.7 ± 14.7</td>
<td>133.2 ± 8.5</td>
<td>48%</td>
<td>0.96</td>
</tr>
<tr>
<td>30 mg/kg OL-135</td>
<td>1:1:18</td>
<td>264.5 ± 32.2</td>
<td>116.5 ± 17.1</td>
<td>56%</td>
<td>0.27</td>
</tr>
</tbody>
</table>

a) Data from four experiments with identical treatment conditions were combined.
Table 2. Behavioral Inactivity for Experiments Investigating FAAH Mechanism of Action. Mean time spent inactive (± SEM) for each group in the studies examining the mechanism of action of the FAAH reduction of pruritus. *p* values are given for the appropriate ANOVA or t-test of each experiment.

<table>
<thead>
<tr>
<th></th>
<th>Time Inactive (s ± SEM)</th>
<th><em>p</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FAAH Genotype Comparison</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57 FAAH (+/+)</td>
<td>84.4 ± 42.3</td>
<td>0.66</td>
</tr>
<tr>
<td>C57 FAAH (-/-)</td>
<td>58.4 ± 39.1</td>
<td></td>
</tr>
<tr>
<td>DBA FAAH (+/+)</td>
<td>36.7 ± 24.9</td>
<td>0.82</td>
</tr>
<tr>
<td>DBA FAAH (-/-)</td>
<td>43.6 ± 17.6</td>
<td></td>
</tr>
<tr>
<td><strong>Rimonabant FAAH Reversal</strong></td>
<td></td>
<td>0.20 (interaction between genotype and rimonabant)</td>
</tr>
<tr>
<td>FAAH (+/+, vehicle)</td>
<td>131.4 ± 36.5</td>
<td></td>
</tr>
<tr>
<td>FAAH (+/+), rimonabant (1 mg/kg)</td>
<td>171.4 ± 35.5</td>
<td></td>
</tr>
<tr>
<td>FAAH (-/-), vehicle</td>
<td>273.0 ± 51.1</td>
<td></td>
</tr>
<tr>
<td>FAAH (-/-), rimonabant (1 mg/kg)</td>
<td>183.8 ± 66.3</td>
<td></td>
</tr>
<tr>
<td><strong>Neuronal FAAH Comparison</strong></td>
<td></td>
<td>0.034</td>
</tr>
<tr>
<td>FAAH (+/-)</td>
<td>126.2 ± 65.0</td>
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<tr>
<td>FAAH (-/-)</td>
<td>143.1 ± 44.3</td>
<td></td>
</tr>
<tr>
<td>FAAH-NS</td>
<td>364.2 ± 87.6</td>
<td></td>
</tr>
<tr>
<td><strong>URB597-treated FAAH transgenic mice</strong></td>
<td></td>
<td>0.51 (interaction between URB597 and genotype)</td>
</tr>
<tr>
<td>FAAH (+/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>217.4 ± 33.7</td>
<td></td>
</tr>
<tr>
<td>URB597 (10 mg/kg)</td>
<td>319.5 ± 47.2</td>
<td></td>
</tr>
<tr>
<td>FAAH (-/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>143.4 ± 35.8</td>
<td></td>
</tr>
<tr>
<td>URB597 (10 mg/kg)</td>
<td>262.5 ± 46.9</td>
<td></td>
</tr>
<tr>
<td>FAAH-NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>320.8 ± 56.8</td>
<td></td>
</tr>
<tr>
<td>URB597 (10 mg/kg)</td>
<td>344.1 ± 35.8</td>
<td></td>
</tr>
<tr>
<td><strong>URB597-treated CB1 and CB2 (-/-) mice</strong></td>
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<td>0.84 (interaction between URB597 and genotype)</td>
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<tr>
<td>CB1 (+/+)</td>
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<tr>
<td>Vehicle</td>
<td>140.5 ± 45.7</td>
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</tr>
<tr>
<td>URB597 (10 mg/kg)</td>
<td>197.4 ± 46.5</td>
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</tr>
<tr>
<td>CB1 (-/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>418.7 ± 89.6</td>
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</tr>
<tr>
<td>URB597 (10 mg/kg)</td>
<td>447.9 ± 87.2</td>
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</tr>
<tr>
<td>CB2 (+/+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>247.1 ± 49.7</td>
<td></td>
</tr>
<tr>
<td>URB597 (10 mg/kg)</td>
<td>247.4 ± 25.5</td>
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</tr>
<tr>
<td>CB2 (-/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>283.8 ± 39.5</td>
<td></td>
</tr>
<tr>
<td>URB597 (10 mg/kg)</td>
<td>311.3 ± 17.7</td>
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</tr>
<tr>
<td><strong>Rimonabant Reversal of URB597</strong></td>
<td></td>
<td>0.98 (interaction between URB597 and Rimonabant)</td>
</tr>
<tr>
<td>Vehicle, Vehicle</td>
<td>252.6 ± 47.2</td>
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</tr>
<tr>
<td>Vehicle, Rimonabant (1 mg/kg)</td>
<td>214.8 ± 21.2</td>
<td></td>
</tr>
<tr>
<td>URB597 (10 mg/kg), Vehicle</td>
<td>273.0 ± 51.1</td>
<td></td>
</tr>
<tr>
<td>URB597 (10 mg/kg), Rimonabant (1 mg/kg)</td>
<td>226.8 ± 47.3</td>
<td></td>
</tr>
</tbody>
</table>

a) *p*<0.05 vs FAAH (+/-)  
b) *p*<0.001 (CB1 genotype)
Table 3. URB597 Reversal of Compound 48/80 Induced Scratching

URB597 (10 mg/kg) reverses scratching when given intravenously 10 min after administration of compound 48/80. All values are represented as mean ± SEM. N = 7 mice/group

<table>
<thead>
<tr>
<th></th>
<th>Pre-injection</th>
<th>Post-injection</th>
<th>Pre-injection</th>
<th>Post-injection</th>
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<tbody>
<tr>
<td><strong>Scratching (s)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>38.6 ± 4.4</td>
<td>19.5 ± 4.1</td>
<td>4.5 ± 2.9</td>
<td>33.7 ± 10.2</td>
</tr>
<tr>
<td>URB597</td>
<td>45.1 ± 7.2</td>
<td>4.7 ± 0.2**</td>
<td>1.1 ± 0.8</td>
<td>47.0 ± 14.7</td>
</tr>
</tbody>
</table>

**, p < 0.01 versus vehicle-treated post-injection group**
**Figure 3**

(A) Hind Leg Scratching (s) for CB1 (+/+) and CB1 (-/-) genotypes in response to Vehicle and THC (1 mg/kg).

(B) Hind Leg Scratching (s) for CB2 (+/+) and CB2 (-/-) genotypes in response to Vehicle and THC (1 mg/kg).
Figure 5

The graph shows the hind leg scratching time in seconds for different genotypes and treatments. The x-axis represents the genotypes: FAAH(+/+), FAAH(-/-). The y-axis represents the hind leg scratching time in seconds, ranging from 0 to 360.

- **Vehicle**
  - FAAH(+/+): ~240 seconds
  - FAAH(-/-): ~120 seconds

- **Rimonabant**
  - FAAH(+/+): ~240 seconds
  - FAAH(-/-): ~360 seconds

Significance levels are indicated: **##** for FAAH(-/-) Rimonabant compared to FAAH(+/+) Rimonabant.
Figure 6

Hind Leg Scratching (s)

FAAH (+/-)  
FAAH (-/-)  
FAAH-NS

Genotype

* * *  
##

Error bars represent standard error of the mean.
Figure 7

(A) Hind Leg Scratching (s) vs. Dose (mg/kg).

(B) Inactivity (s) vs. Dose (mg/kg).

Molecular structures and annotations:
- OL-135, $K_i = 4.7\ \text{nM}$, reversible & competitive.
- URB597, $IC_{50} = 4.6\ \text{nM}$, irreversible: acylate active site serine.
Figure 8

A

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vehicle</th>
<th>URB597 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB1(+/+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB1(−/−)</td>
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Hind Leg Scratching (s)

B

<table>
<thead>
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<th>Pretreatment</th>
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<th>URB597 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>URB597 (10 mg/kg)</td>
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<td></td>
</tr>
</tbody>
</table>

Hind Leg Scratching (s)

C

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vehicle</th>
<th>URB597 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB2(+/+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB2(−/−)</td>
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</tbody>
</table>

Hind Leg Scratching (s)