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# A pro-nociceptive phenotype unmasked in mice lacking fatty-acid amide hydrolase

**Lawrence M Carey, BA, MS[1,2], Richard A Slivicki, BS[1,2], Emma Leishman, BS[1,2], Ben Cornett, BS[1], Ken Mackie, MD[1,2,3], Heather Bradshaw, PhD[1,2] and Andrea G Hohmann, PhD[1,2,3]**

**Abstract**

Fatty-acid amide hydrolase (FAAH) is the major enzyme responsible for degradation of anandamide, an endocannabinoid. Pharmacological inhibition or genetic deletion of FAAH (FAAH KO) produces antinociception in preclinical pain models that is largely attributed to anandamide-induced activation of cannabinoid receptors. However, FAAH metabolizes a wide range of structurally related, biologically active lipid signaling molecules whose functions remain largely unknown. Some of these endogenous lipids, including anandamide itself, may exert pro-nociceptive effects under certain conditions. In our study, FAAH KO mice exhibited a characteristic analgesic phenotype in the tail flick test and in both formalin and carrageenan models of inflammatory nociception. Nonetheless, intradermal injection of the transient receptor potential channel V1 (TRPV1) agonist capsaicin increased nocifensive behavior as well as mechanical and heat hypersensitivity in FAAH KO relative to wild-type mice. This pro-nociceptive phenotype was accompanied by increases in capsaicin-evoked Fos-like immunoreactive (FLI) cells in spinal dorsal horn regions implicated in nociceptive processing and was attenuated by $CB_1$ (AM251) and TRPV1 (AMG9810) antagonists. When central sensitization was established, FAAH KO mice displayed elevated levels of anandamide, other fatty-acid amides, and endogenous TRPV1 agonists in both paw skin and lumbar spinal cord relative to wild-type mice. Capsaicin decreased spinal cord 2-AG levels and increased arachidonic acid and prostaglandin E2 levels in both spinal cord and paw skin irrespective of genotype. Our studies identify a previously unrecognized pro-nociceptive phenotype in FAAH KO mice that was unmasked by capsaicin challenge. The heightened nociceptive response was mediated by $CB_1$ and TRPV1 receptors and accompanied by enhanced spinal neuronal activation. Moreover, genetic deletion of FAAH has a profound impact on the peripheral and central lipidome. Thus, genetic deletion of FAAH may predispose animals to increased sensitivity to certain types of pain. More work is necessary to determine whether such changes could explain the lack of efficacy of FAAH inhibitors in clinical trials.

**Keywords**

Fatty-acid amide hydrolase, FAAH knockout, endocannabinoid, endovanilloid, anandamide, capsaicin, cannabinoid $CB_1$ receptor, TRPV1

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## Introduction

Fatty-acid amide hydrolase (FAAH) is the primary catabolic enzyme responsible for the metabolism of anandamide (*N*-arachidonoyl ethanolamine, AEA),[1] an endogenous ligand for cannabinoid receptors (i.e., endocannabinoid).[2] Since its discovery in 1993,[3] FAAH has gained considerable attention as a therapeutic target.[4,5] Inhibitors of FAAH produce therapeutic effects in a variety of pathophysiological conditions including anxiety,[5] depression,[6] and neuropathic and inflammatory pain.[7, 8]

[1]Department of Psychological and Brain Sciences, Indiana University, Bloomington, IN, USA
[2]Program in Neuroscience, Indiana University, Bloomington, IN, USA
[3]Gill Center for Biomolecular Science, Indiana University, Bloomington, IN, USA

**Corresponding author:**
Andrea G Hohmann, Department of Psychological and Brain Sciences, Gill Center for Biomolecular Science, Indiana University, Bloomington, IN 47405-2204, USA.
Email: hohmanna@indiana.ed

Additionally, mice with a genetic deletion of FAAH (FAAH KO) display decreased pain responsiveness to acute thermal stimulation and formalin-induced tonic pain,[9] as well as decreased hypersensitivity in response to both carrageenan inflammation and chronic constriction injury.[10] However, despite producing antinociceptive efficacy in preclinical pain models,[11] the FAAH inhibitor PF-04457845 failed to produce symptom relief in human patients with pain due to osteoarthritis.[12] The failure of FAAH inhibition to produce antinociceptive effects in these subjects occurred even though FAAH activity was decreased by 96% and plasma AEA levels increased nearly 12-fold versus placebo. Although a mechanistically unexplained, unexpected fatality and severe toxicity associated with high dose administration of an FAAH inhibitor in a recent Phase 1 clinical trial is unlikely to be attributable to FAAH inhibition, more work is clearly necessary to understand physiological impact of FAAH inhibition in vivo.[13,14]

Pharmacological inhibition or genetic deletion of FAAH has been postulated to exert antinociceptive effects via elevations of AEA and subsequent activation of cannabinoid $CB_1$ receptors; the antinociceptive effects of both pharmacological inhibition,[7,8] and genetic deletion of FAAH[10] are generally prevented by $CB_1$ antagonists. However, other fatty-acid amides (also known as *N*-acyl amides or lipoamines) that are also degraded by FAAH, such as *N*-palmitoyl ethanolamine (PEA), produce antinociception through $CB_1$-independent mechanisms.[15] The relationship between endocannabinoids and $CB_1$-mediated inhibition of pain processing is likely to be even more complex than previously thought. While $CB_1$ receptor activation is typically thought of as a mechanism to promote analgesia, in some instances, it has been suggested to elevate pain responsiveness.[16]

AEA is presumed to act as both an endocannabinoid (that produces antinociception via metabotropic $CB_1$ receptors) and as an endovanilloid (that produces hyperalgesia via ionotropic transient receptor potential channel V1 (TRPV1) receptors).[17] C-fiber stimulation via application of capsaicin, a TRPV1 agonist, produces both an immediate primary hyperalgesia at the site of injection, and a secondary hyperalgesia in the surrounding healthy tissue.[18] The secondary hyperalgesia produced by capsaicin may occur in part as a result of homosynaptic or heterosynaptic depression of GABAergic inhibitory neurotransmission in the spinal cord that is mediated by endocannabinoid stimulation of $CB_1$ receptors.[16] Intraplantar (i.pl.) injection of exogenous AEA excites C-fibers and increases nocifensive behavior in a dose dependent fashion, and these effects are prevented by pretreatment with TRPV1 antagonists.[19] In addition to binding to and activating TRPV1 receptors, AEA influences the function of TRPV1 receptors in many other ways (see Ross[20] for review). However, whether such changes induced by high concentrations of AEA are actually relevant under physiological conditions remains unresolved. Moreover, FAAH serves as the major enzyme inactivating a large number of biologically active lipid signaling molecules other than AEA and PEA. These include *N*-oleoyl ethanolamine (OEA), which also acts as a TRPV1 agonist,[21] and other fatty-acid amides that do not bind to $CB_1$.[22] Yet changes in the lipid profiles produced by pharmacological inhibition or genetic deletion of FAAH remain incompletely characterized and mechanisms underlying alterations in pain phenotypes remain poorly understood. To our knowledge, effects of the TRPV1 receptor agonist capsaicin in FAAH KO mice have never been reported in the published literature. The present study, therefore, sought to confirm the analgesic phenotype previously reported in FAAH KO mice and to determine whether FAAH KO mice display heightened behavioral and neuronal sensitization in response to capsaicin in comparison to wild-type (WT) littermates. We also sought to define the impact of i.pl. injection of capsaicin or its vehicle on the lipid profiles of these mice at the site of peripheral tissue injury and at the level of the central nervous system. Additionally, the contribution of cannabinoid and vanilloid receptors to capsaicin-evoked behavioral hypersensitivities was examined using selective $CB_1$ and TRPV1 antagonists.

## Materials and methods

### Subjects

A total of 246 mice weighing 17–48 g were used in these experiments. FAAH KO and WT littermates were bred and genotyped (by BC) in the Mackie laboratory and transferred to the Hohmann lab for in vivo procedures and tissue extractions. Additional C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). All procedures were approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC) of Indiana University. Animals were housed in groups of 3–4 separated by sex in a temperature controlled facility with free access to food and water in their home cages on a regular 12 h light/dark cycle. Evaluation of responsiveness to mechanical and heat stimulation was always performed in different sets of animals to prevent the development of stimulus sensitization.

### Drugs and chemicals

Capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide), AMG9810 (2E-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-Propenamide), λ-carrageenan, and formaldehyde (37% in $H_2O$) were purchased

from Sigma–Aldrich (St. Louis, MO). The $CB_1$ selective antagonist/inverse agonist AM251 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1H-pyrazole-3-carboxamide) was purchased from Cayman Chemical Company (Ann Arbor, MI). Capsaicin was dissolved (1 μg/10 μl) in a vehicle of 7% Tween 80 in 0.9% saline, sonicated, and filtered with a 0.22-μm Millipore syringe filter. Formalin was diluted from formaldehyde stock (100% formalin) in sterile saline to a final concentration of 2.5%. All other drugs were dissolved in a vehicle of 20% dimethyl sulfoxide (Sigma–Aldrich), with the remaining 80% consisting of 95% ethanol (Sigma–Aldrich), emulphor (Alkamuls EL 620L; Solvay) and 0.9% saline (Aquilite System; Hospira, Inc., Lake Forest, IL) at a ratio of 1:1:8, respectively, for intraperitoneal (i.p.) administration and administered in a volume of 5 ml/kg.

### Tail immersion

FAAH KO and WT mice ($n = 6$, 6) were gently restrained in a towel and handheld. Approximately 1 cm of the tip of the tail was submerged in a hot water bath maintained at 56°C and the latency to withdraw the tail was measured. Measurements were taken in triplicate with 20 min separating each trial.

### Carrageenan-evoked thermal hyperalgesia

FAAH KO ($n = 6$) and WT ($n = 6$) mice received a unilateral intradermal injection of 20 μl of 0.3% λ-carrageenan (% weight/volume in 0.9% saline) into the superficial plantar surface of the hind paw. Sensitivity to thermal stimulation was evaluated prior to, and 5 h post-carrageenan. Responsiveness to heat stimulation was assessed in triplicate in paws ipsilateral and contralateral to carrageenan administration.

### Formalin test

FAAH KO ($n = 6$) and WT ($n = 6$) mice were placed in an elevated clear observation chamber and allowed to habituate for 30 min. Following habituation, all mice received a unilateral intradermal injection (20 μl) of 2.5% formalin into the superficial plantar surface of the hind paw. Nociceptive behaviors were scored for 40 min immediately following formalin injection. Composite pain scores (CPS) were calculated for every 5-min bin for the total duration of 40 min using the following scoring criteria: no behavior was scored 0, lifting was scored 1, and shaking/biting/flinching was scored as 2. The area under the curve (AUC) of pain behavior was calculated for both the early phase (0–10 min) and late phase (10–40 min) for each subject.

### Behavioral assessment of hyperalgesia following capsaicin administration

*Assessment of nocifensive behavior and heat hyperalgesia following intradermal capsaicin administration.* Thermal hyperalgesia was assessed using radiant heat with the Hargreaves method.[23] For the initial characterization of capsaicin-induced thermal hyperalgesia, male and female FAAH KO ($n = 6$, 6) and WT ($n = 6$, 6) mice were placed in Plexiglas cages on an elevated glass platform and acclimated to the testing environment for 1 h. A focused beam of infrared radiant heat (thermal intensity: infrared 29.0) was applied to the midplantar region of the hind paw utilizing a commercial apparatus (Ugo Basile, Varese, Italy). Thermal stimulation was terminated upon withdrawal of the hind paw, or after 20 s if the animal failed to produce a withdrawal response to prevent tissue damage. Baseline withdrawal latencies were measured in duplicate, allowing 7 min between stimulations. Following acquisition of stable baseline measurements, the mice received a unilateral intradermal injection of vehicle (10 μl) or capsaicin (1 μg/10 μl) into the superficial plantar surface of the hind paw. Nocifensive behavior (lifting, shaking, biting, licking, or otherwise tending to the paw) was scored over the course of 5 min in the same mice used to evaluate heat responsiveness. Thus, thermal paw withdrawal latencies were measured in duplicate at 10, 30, 60, 90, and 120 min post-capsaicin administration.

As no significant differences were observed between male and female mice of the same genotype, male and female mice were pooled for each antagonist treatment group. Following determination of baseline responding, mice received a single i.p injection (5 ml/kg) of AMG9810 (3 mg/kg, $n = 5$ per group), AM251 (3 mg/kg, $n = 5$ per group), or vehicle ($n = 6$ per group). I.p. injections were performed 30 min prior to i.pl. capsaicin or vehicle administration. Paw withdrawal latencies were assessed before and 10, 30, 60, 90 and 120 min after intradermal injection of capsaicin or vehicle. Paw withdrawal latencies were measured in duplicate in each paw at each time point, and are reported as the mean of the two duplicate determinations from each animal, averaged across subjects.

*Assessment of paw withdrawal thresholds to mechanical stimulation.* Paw withdrawal thresholds to mechanical stimulation were measured using an electronic von Frey anesthesiometer (IITC model Alemo 2390–5, Woodland Hills, CA) as described previously.[24] Mice were placed on an elevated metal mesh table where they were habituated under individual, inverted plastic cages for at least 20 min until exploratory behavior had ceased. After the habituation period, a force was applied to the midplantar region of the hind paw with a semiflexible tip connected to the anesthesiometer. Mechanical stimulation was terminated when the animal withdrew its paw and the value of the

applied force was recorded in grams. Mechanical paw withdrawal thresholds were obtained twice for each paw, and are reported as the mean of two duplicate determinations obtained from each animal, averaged across subjects. Mechanical thresholds were taken at 5, 30, 60, and 120 min post-capsaicin injection. AM251 (3 mg/kg), AMG9810 (3 mg/kg), or vehicle were administered 30 min prior to i.pl. capsaicin or vehicle injection.

## Tissue preparation for immunohistochemistry

Male FAAH KO ($n = 4$) and WT ($n = 4$) mice received a unilateral i.pl. injection of capsaicin (1 μg/10 μl) into the superficial plantar surface of the hind paw. One hour after capsaicin administration, mice were deeply anesthetized with isoflourane, then transcardially perfused with 0.1% heparinized 0.1 M phosphate-buffered saline (PBS) followed by ice cold 4% paraformaldehyde. Spinal cord tissue was extracted and kept in the same fixative for 24 h, then cryoprotected in 30% sucrose for three days prior to sectioning.

## Immunohistochemistry

Transverse sections (30 μm) of the L4–L5 lumbar spinal cord were cut on a cryostat and kept in an antifreeze solution (50% sucrose in ethylene glycol and 0.1 M PBS) prior to immunostaining. Tissue was taken so that every fourth section would be processed for immunohistochemistry. Free-floating sections were washed three times in 0.1 M PBS, then immersed in 0.3% $H_20_2$ for 30 min. To prevent nonspecific binding, sections were pretreated for 1 h with blocking buffer consisting of 5% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS, followed by incubation with rabbit polyclonal Fos protein antibody (1:1500, Santa Cruz Biotechnology, Dallas, TX) for 24 h at 4°C. Fos-like immunoreactivity was visualized using the avidin-biotin peroxidase method using diaminobenzidine as the chromogen. Three sections per animal displaying the highest levels of Fos-like immunoreactivity were selected and cells were counted under light microscopy by an investigator blinded to genotype. Fos protein expression was localized to the spinal dorsal horn ipsilateral to the capsaicin-treated paw at the time point used for immunohistochemical analysis. Fos protein expression was largely absent following i.pl. injection of vehicle in both the current study (data not shown) and in our previously published work.[25]

## Liquid chromatography-tandem mass spectrometry (LC/MS/MS)

*Tissue preparation.* During our initial characterization of the hyperalgesic phenotype displayed by FAAH KO mice, sensitization to mechanical and heat stimulation was present at 1 h post-capsaicin for both modalities. For this reason, lumbar spinal cords and paw skin were dissected 1 h post-capsaicin for use in LC/MS/MS experiments. Male and female mice received bilateral injections of capsaicin (1 μg/10 μl), or bilateral injections of vehicle (10 μl) to ensure that both hemispheres of the spinal cord would receive identical nociceptive inputs. Mice were decapitated and spinal cord and paw skin tissue was dissected 1 h following intradermal injection and snap frozen in 2-methylbutane. Samples were kept frozen at −80°C until being prepared for extraction.

*Lipid extraction.* Tissue extracts were performed as previously described.[26–28] In brief, samples were placed in 50 volumes of HPLC-grade methanol (Avantor Performance Materials, Center Valley, PA, USA) then spiked with 500 pmols deuterium-labeled *N*-arachidonoyl glycine ($d_8$NAGly; Cayman Chemical, Ann Arbor, MI, USA) as an internal standard. Samples were placed on ice in darkness for 2 h then individually homogenized. Homogenates were then centrifuged at 19,000 *g* for 20 min at 20°C. Supernatants were decanted and diluted with HPLC water (purified in house) to make a 75:25 water to supernatant solution. Partial purification was achieved using C-18 solid phase extraction columns (Agilent, Palo Alto, CA, USA). A series of four elutions with 1.5 ml of 60%, 75%, 85%, and 100% methanol were collected for analysis.

*HPLC/MS/MS.* Samples were analyzed in the Bradshaw laboratory using an Applied Biosystems API 3000 triple quadrupole mass spectrometer with electrospray ionization (Foster City, CA, USA). Twenty microliters from each elution were chromatographed using XDB-C18 reversed phase HPLC analytical column (Agilent) and optimized mobile phase gradients. Mobile phase A: 20% / 80% (v/v) methanol/water and 1 mM ammonium acetate (Sigma–Aldrich). Mobile phase B: 100% methanol, 1 mM ammonium acetate. Two Shimadzu 10ADvp pumps (Columbia, MD, USA) provided the pressure for gradient elution. Levels of each compound were determined by running each sample using a multiple reactions monitoring method tailored for each amide family of compounds as previously described.[27]

## Data analysis and statistical procedures

Analysis of the HPLC/MS/MS data was performed using Analyst software (Applied Biosystems, Framingham, MA, USA) as previously described.[26–28] One way or two-way repeated measures ANOVA were used, as appropriate, to assess lipid levels, levels of nocifensive behaviors and the time course of mechanical allodynia or heat hyperalgesia. One-way ANOVA was

subsequently used to identify the source of significant interactions, followed by Newman–Keuls multiple comparisons tests for comparisons between groups. Planned comparisons were made using one- and two-tailed $t$ tests as appropriate. All statistical analyses and figures were generated using GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was defined as $p < 0.05$ and results were labeled trending if $0.05 \leqslant p \leqslant 0.10$.

## Results

### *FAAH KO mice display an analgesic phenotype in the hot water tail immersion test and in carrageenan and formalin models of inflammatory pain*

FAAH KO mice displayed reduced sensitivity to thermal stimulation in the hot water tail immersion test ($t_{10} = 2.286$, $p < 0.05$; Figure 1(a)). I.pl. injection of carrageenan reduced paw withdrawal latencies to heat stimulation in both FAAH KO and WT mice, consistent with development of thermal hyperalgesia ($F_{1,20} = 160.2$, $p < 0.0001$; Figure 1(b)). However, FAAH KO mice displayed reductions in carrageen-induced thermal hyperalgesia relative to their WT counterparts, as manifested by elevated thermal paw withdrawal latencies relative to WT mice in the carrageenan-injected paw ($F_{1,20} = 10.83$, $p < 0.01$; Figure 1(b)), and the interaction between genotype and carrageenan was significant ($F_{1,20} = 26.19$, $p < 0.0001$; Figure 1(b)). Thermal paw withdrawal latencies did not differ in the paw contralateral to carrageenan injection in either FAAH KO or WT mice. I.pl. formalin increased composite pain scores in a biphasic manner in both FAAH KO and WT mice ($F_{8,10} = 52.09$, $p < 0.0001$; Figure 1(c)). However, FAAH KO mice displayed decreased formalin-evoked pain behavior (i.e., hypoalgesia) relative to WT mice ($F_{1,10} = 29.08$, $p < 0.001$; Figure 1(c)), and the interaction between genotype and formalin-evoked pain was significant ($F_{8,10} = 3.571$, $p < 0.01$; Figure 1(c)). I.pl. formalin increased the area under the curve in a phase ($F_{1,20} = 110.8$, $p < 0.0001$; Figure 1(d)) and genotype ($F_{1,20} = 32.21$, $p < 0.0001$; Figure 1(d)) dependent manner and the interaction between phase and genotype was also significant ($F_{1,20} = 20.50$, $p < 0.001$; Figure 1(d)); the AUC of phase 2 pain behavior was lower in FAAH KO relative to WT mice, whereas the AUC of phase 1 pain behavior did not differ between genotypes.

### *Intraplantar administration of capsaicin increases nocifensive behaviors in FAAH KO relative to WT mice*

I.pl.) injection of capsaicin (1 μg) increased nocifensive behavior in FAAH KO mice of both sexes relative to WT mice ($F_{3,20} = 11.46$, $p < 0.0001$; Figure 2(a)). While genotype impacted nocifensive behavior ($F_{1,20} = 34.27$, $p < 0.0001$; Figure 2(a)), sex did not ($p > 0.08$; Figure 2(a)) and the interaction between sex and genotype was not significant. Moreover, levels of nocifensive behavior in response to i.pl. vehicle did not differ between FAAH KO and WT mice ($p > 0.4$; Figure 2(b)).

### *FAAH KO mice develop increased heat hyperalgesia in response to capsaicin relative to WT mice*

Intradermal capsaicin reduced paw withdrawal latencies to heat stimulation ($F_{5,20} = 41.91$, $p < 0.0001$; Figure 3(a)), consistent with development of robust thermal (heat) hyperalgesia. FAAH KO mice displayed increased capsaicin-evoked heat hypersensitivity, as manifested by lower thermal paw withdrawal latencies relative to WT mice ($F_{3,20} = 12.55$, $p < 0.0001$; Figure 3a); the interaction between time and genotype was not significant ($p > 0.1$; Figure 3(a)). Paw withdrawal latencies did not differ as a function of sex in the paw ipsilateral ($p > 0.5$; Figure 3(a)) or contralateral to capsaicin injection ($p > 0.1$, Figure 3(b)). Capsaicin injection also altered paw withdrawal latencies in the noninjected paw ($F_{5,20} = 10.27$, $p < 0.0001$; Figure 3(b)). Paw withdrawal latencies in the contralateral paw were not affected by genotype ($p > 0.4$; Figure 3(b)), although the interaction between genotype and time was significant ($F_{15,20} = 1.827$, $p < 0.05$; Figure 3(b)). Intraplantar vehicle injection produced modest alterations in paw withdrawal latencies ipsilateral ($F_{5,10} = 5.89$, $p < 0.0001$; Figure 3(c)), but not contralateral, to the injection ($p > 0.8$; Figure 3(d)). However, genotype did not impact paw withdrawal latencies either ipsilateral ($p > 0.5$; Figure 3(c)) or contralateral ($p > 0.5$; Figure 3(d)) to the intraplantar vehicle injection and the interaction between genotype and time was not significant (ipsilateral: $p > 0.3$; Figure 3(c); or contralateral: $p > 0.8$; Figure 3(d)).

### *FAAH KO mice develop increased mechanical hypersensitivity in response to capsaicin*

Intradermal capsaicin reduced paw withdrawal thresholds to mechanical stimulation in all mice ($F_{4,20} = 100.3$, $p < 0.0001$; Figure 4(a)). FAAH KO mice exhibited increases in capsaicin-evoked mechanical hypersensitivity relative to WT mice throughout the observation interval ($F_{1,8} = 35.63$, $p < 0.001$; Figure 4(a)), and these changes in paw withdrawal thresholds were time dependent ($F_{4,8} = 3.496$, $p < 0.05$; Figure 4(a)). Enhanced capsaicin-evoked hypersensitivities were observed relative to WT mice at 30 ($p < 0.01$; Figure 4(a)), 60 ($p < 0.05$; Figure 4(a)) and 120 ($p < 0.01$; Figure 4(a)) minutes following injection. No changes in mechanical paw withdrawal thresholds were observed in the paw contralateral to

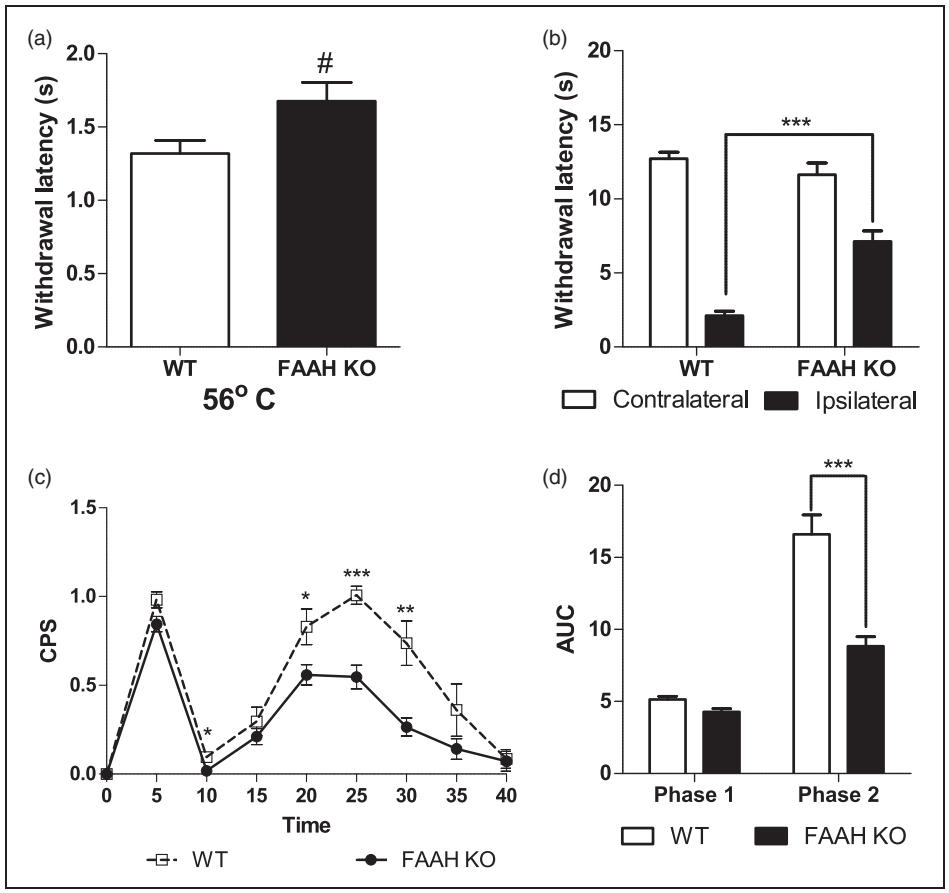


**Figure 1.** Genetic deletion of FAAH produces an analgesic phenotype in response to hot water tail immersion, intraplantar carrageenan and intraplantar formalin. FAAH KO mice display longer withdrawal latencies in response to hot water tail immersion relative to WT mice (a). $^{\#}p < 0.05$ versus WT, 2-tailed *t* test. Five hours post i.pl. carrageenan, FAAH KO mice show reduced thermal hyperalgesia in the paw ipsilateral, but not contralateral, to carrageenan injection relative to WT mice (b). Data are expressed as ± SEM ($n = 6$ per group). $^{***}p < 0.001$ versus WT ipsilateral, 2-way ANOVA, Bonferroni post-hoc. I.pl. formalin produces increased composite pain scores in WT animals relative to FAAH KO animals (c). FAAH KO mice displayed decreased levels of pain behavior from 10 to 30 min post-injection relative to WT mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus FAAH KO, 2-tailed *t* test. FAAH KO mice displayed decreases in the area under the curve in phase 2 of formalin-evoked pain behavior but no change during phase 1 (d). $^{***}p < 0.001$, 2-way ANOVA, Bonferroni post-hoc. FAAH KO: FAAH knockout; WT: wildtype; CPS: composite pain score; AUC: area under curve.

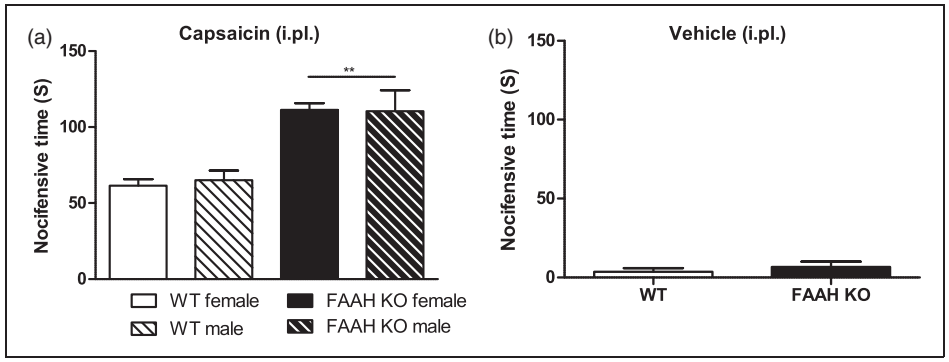


**Figure 2.** FAAH KO mice show increases in capsaicin-evoked nocifensive behavior compared to WT littermates. FAAH KO mice display increased levels of nocifensive behavior relative to WT mice (a) in response to intraplantar administration of capsaicin (1 μg i.pl.). Responding did not differ between FAAH KO and WT animals that received local injections of vehicle (10 μl i.pl.) (b). Data are expressed as mean ± SEM ($n = 6$ per group). $^{**}p < 0.01$ KO male and KO female versus WT male and WT female, One-way ANOVA followed by Newman–Keuls post-hoc test. FAAH KO: FAAH knockout; i.pl: intraplanar; WT: wildtype.

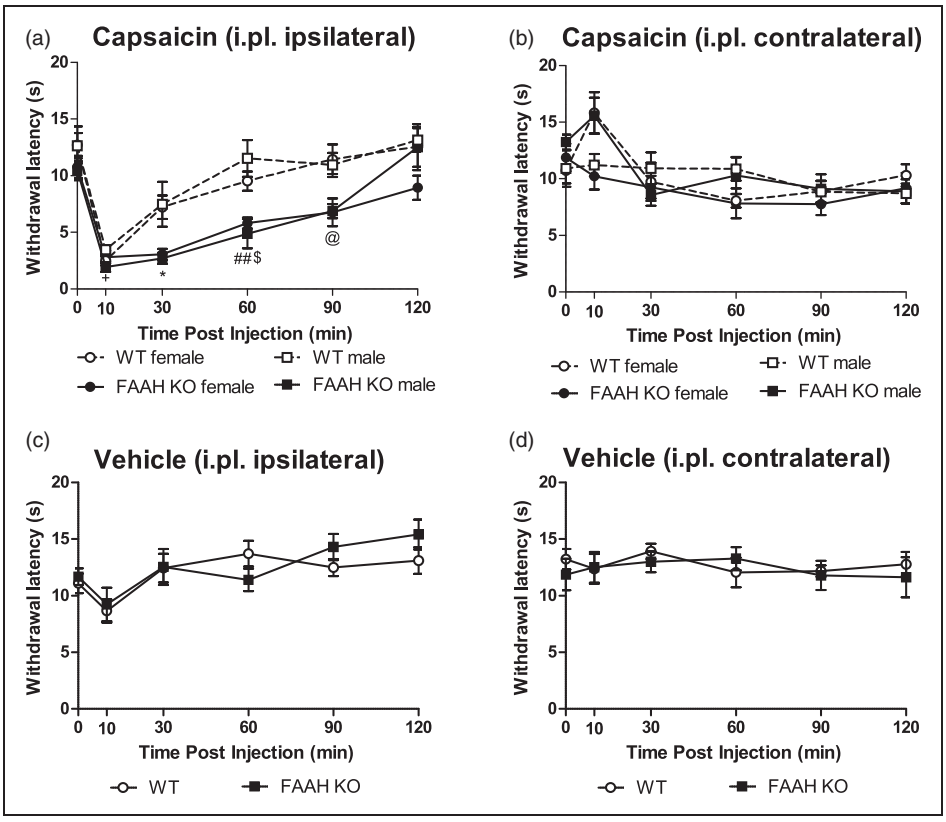


**Figure 3.** FAAH KO mice show increased capsaicin-evoked heat hyperalgesia compared to WT littermates. FAAH KO mice exhibit a delayed resolution of capsaicin-induced heat hyperalgesia relative to WT mice (a). Heat hyperalgesia does not develop in the noninjected (contralateral) paw in either genotype (b). Paw withdrawal latencies produced by i.pl. vehicle administration do not differ in FAAH KO mice in the paw ipsilateral to (c) or contralateral to vehicle injection (d). Data are expressed as mean ± SEM ($n = 6$ per group). + $p < 0.05$ KO male versus WT female, one-way ANOVA followed by Newman–Keuls post hoc test. * $p < 0.05$ KO male and KO female versus WT male and WT female, $^{\$}p < 0.05$ WT female versus KO male and KO female, $^{\#\#}p < 0.01$ WT male versus KO male and KO female one-way ANOVA followed by Newman–Keuls post hoc test. @ $p < 0.05$ KO female versus WT male and WT female, one-way ANOVA followed by Newman–Keuls post-hoc test. FAAH KO: FAAH knockout; i.pl: intrplanar; WT: wildtype.

capsaicin injection ($p > 0.4$; Figure 4(b)). Moreover, no differences in paw withdrawal thresholds were observed between FAAH KO and WT mice following vehicle (i.pl.) injection in either ipsilateral ($p > 0.8$; Figure 4(c)) or contralateral ($p > 0.06$; Figure 4(c)) paws.

## *FAAH KO mice display increases in capsaicin-evoked Fos-like immunoreactivity in lumbar spinal dorsal horn*

FAAH KO mice showed increased numbers of FLI cells in the lumbar spinal dorsal horn ipsilateral to i.pl. capsaicin administration ($F_{1,18} = 29.25$, $p < 0.01$; Figure 5(a to c)). Capsaicin-evoked FLI cells were distributed in lumbar spinal cord in a lamina-dependent manner ($F_{3,18} = 61.01$, $p < 0.0001$; Figure 5(a)). FAAH KO mice also exhibited a lamina-dependent increase in the number of FLI cells ($F_{3,18} = 8.272$, $p < 0.01$). FAAH KO mice exhibited elevations in FLI cells in the superficial dorsal horn (lamina I and II); $t(6) = 6.644$, $p < 0.001$), the nucleus proprius (lamina III and IV; $t(6) = 2.783$, $p < 0.05$), and the neck region (lamina V and VI) ($t(6) = 2.926$, $p < 0.05$) of the dorsal horn relative to WT mice. By contrast, capsaicin-evoked Fos protein expression did not differ between genotypes in the ventral horn ($p > 0.05$). Moreover, intradermal injection of vehicle did not reliably alter Fos protein expression in the lumbar spinal cord (data not shown).

## *Effects of the $CB_1$ antagonist AM251 and the TRPV1 antagonist AMG9810 on capsaicin-evoked nocifensive behavior*

The $CB_1$ antagonist AM251 (3 mg/kg, i.p.) decreased capsaicin-evoked nocifensive behavior ($F_{1,18} = 28.45$, $p < 0.0001$; Figure 6(a)). This suppressive effect was genotype dependent ($F_{1,18} = 14.83$, $p < 0.01$; Figure 6(a)), and the interaction between the effects of genotype and AM251 approached significance ($F_{1,18} = 4.704$, $p = 0.0587$, Figure 6(a)). Planned comparisons revealed that AM251 reduced nocifensive behaviors in FAAH KO mice ($p < 0.01$) but

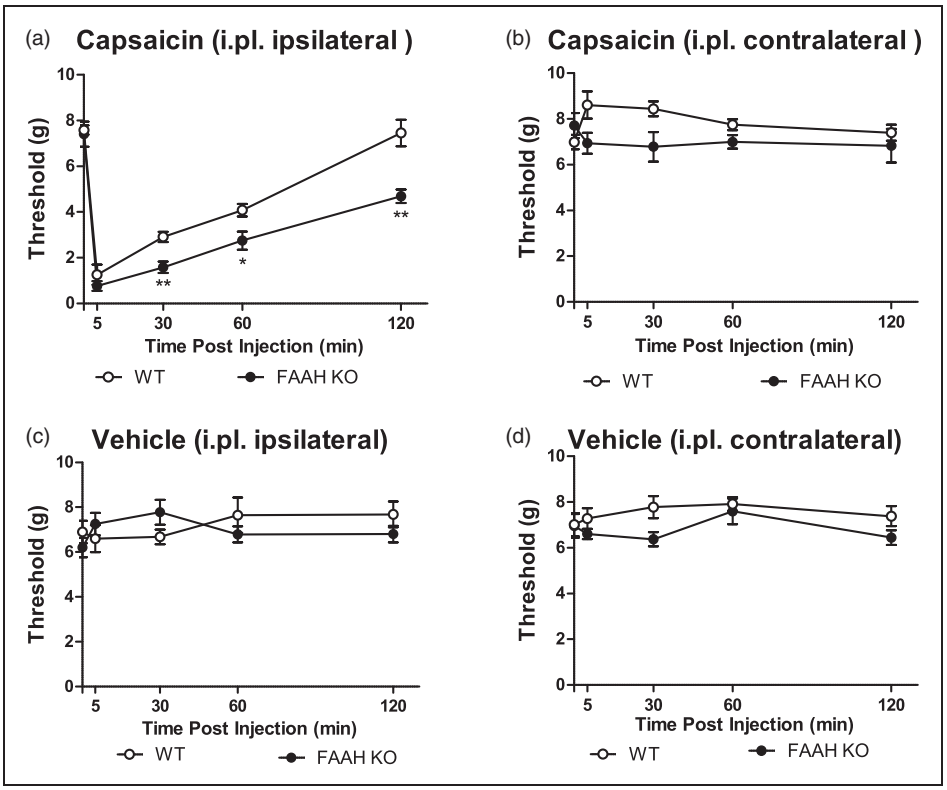


**Figure 4.** FAAH KO mice show enhanced capsaicin-evoked mechanical hypersensitivity relative to WT littermates. FAAH KO mice display, relative to WT mice, a prolongation of mechanical hypersensitivity in the paw ipsilateral (capsaicin-injected) (a), but not contralateral (b), to local injection of capsaicin (1 μg i.pl.). FAAH KO mice displayed an enhanced mechanical hypersensitivity relative to WT littermates at 30, 60, and 120 minutes post-capsaicin administration. I.pl. injection of vehicle did not alter mechanical paw withdrawal thresholds in the paw ipsilateral (c) or contralateral (d) to injection in either genotype. Data are expressed as mean ± SEM ($n = 5$–6 per group). *$p < 0.05$, **$p < 0.01$ FAAH KO versus WT, Two-tailed *t* test. FAAH KO: FAAH knockout; i.pl: intraplanar; WT: wildtype.

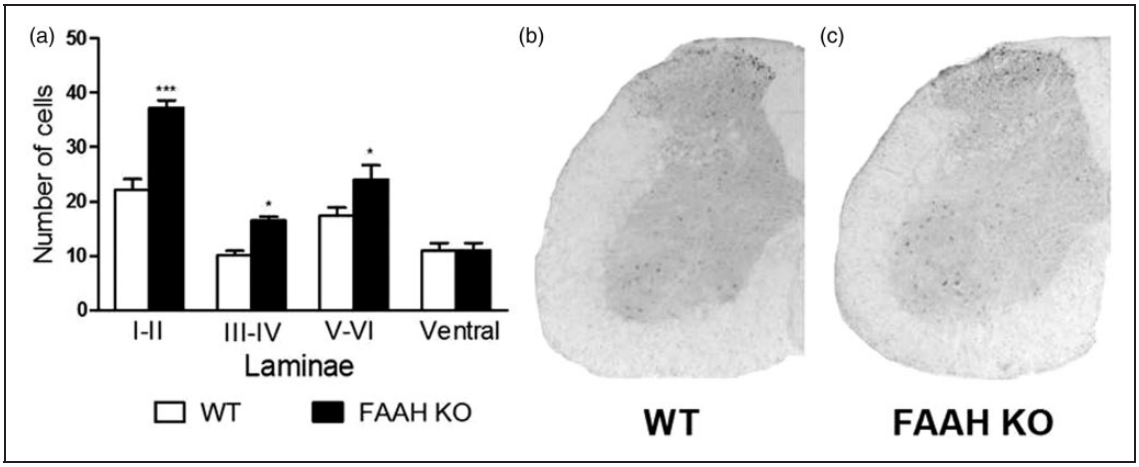


**Figure 5.** FAAH KO mice display increases in capsaicin-evoked Fos-like immunoreactive cells in the spinal dorsal horn. Compared to WT mice, FAAH KO mice exhibit increased numbers of Fos-like immunoreactive cells in the superficial dorsal horn (lamina I and II), the nucleus proprius (lamina III and IV), and the neck region of the dorsal horn (lamina V-VI) but not in the ventral horn (a). Representative photomicrographs of capsaicin treated (i.pl.) WT (b) and FAAH KO mice (c). Data are expressed as mean ± SEM ($n = 4$ per group). ***$p < 0.001$, *$p < 0.05$ versus WT, 2-way ANOVA, Bonferroni post-hoc. FAAH KO: FAAH knockout; WT: wildtype.

failed to alter nocifensive behavior in WT mice ($p > 0.2$) relative to their respective vehicle controls.

As expected, intradermal capsaicin increased nocifensive behavior in FAAH KO relative to WT mice ($F_{1,18} = 19.04$, $p < 0.001$; Figure 6(b)) similarly receiving vehicle (i.p.). The TRPV1 antagonist AMG9810 (3 mg/kg, i.p.) alone did not alter nocifensive behavior as the main effect of AMG9810 treatment was not significant ($p > 0.4$; Figure 6(b)). Nonetheless, AMG9810 produced a genotype-dependent change in capsaicin-evoked nocifensive behavior ($F_{1,18} = 10.15$, $p < 0.01$; Figure 6(b)). Post-hoc analysis revealed that WT mice receiving vehicle exhibited less nocifensive behavior than FAAH KO mice receiving either vehicle ($p < 0.001$; Figure 6(b)) or AMG9810 (3 mg/kg, $p < 0.01$; Figure 6(b)). Additionally, AMG9810 increased nocifensive behavior in WT mice relative to WT mice receiving vehicle ($p < 0.05$; Figure 6(b)). This AMG9810-induced increase in capsaicin-evoked nocifensive behavior exhibited by WT mice was absent in FAAH KO mice, which already exhibit a hypersensitive nocifensive response to capsaicin. AMG9810 did not reliably decrease capsaicin-evoked nocifensive behavior in FAAH KO mice. Thus, AMG9810 selectively increased capsaicin-induced nocifensive behavior in WT mice to the sensitized levels exhibited by FAAH KOs receiving the same capsaicin challenge.

### *Capsaicin-evoked heat and mechanical hypersensitivity is preserved in FAAH KO mice receiving intraperitoneal injections of vehicle*

Intradermal capsaicin decreased thermal paw withdrawal latencies ($F_{5,10} = 34.31$, $p < 0.0001$; Figure 7(a)) in response to heat stimulation in vehicle (i.p.)-treated FAAH KO and WT mice, analogous to the pronociceptive phenotype unmasked by capsaicin in otherwise naïve animals (i.e., mice that did not receive i.p. injections) (Figures 3 and 4). FAAH KO mice displayed a prolonged duration of capsaicin-evoked heat hyperalgesia relative to WT mice ($F_{1,10} = 17.57$, $p < 0.01$, Figure 7(a)), and the interaction between genotype and capsaicin/vehicle treatment was significant ($F_{5,10} = 6.635$, $p < 0.0001$; Figure 7(a)). Post-hoc analyses revealed that FAAH KO mice receiving vehicle (i.p.) display decreased withdrawal latencies at 30 ($p < 0.01$; Figure 7(a)), 60 ($p < 0.001$; Figure 7(a)), and 90 ($p < 0.001$, Figure 7(a)) minutes post-capsaicin relative to WT mice similarly receiving vehicle (i.p.). Capsaicin did not affect paw withdrawal latencies in the paw contralateral to capsaicin administration ($p > 0.5$, data not shown).

Capsaicin decreased mechanical paw withdrawal thresholds in FAAH KO and WT mice receiving vehicle ($F_{4,8} = 132.9$, $p < 0.0001$; Figure 7(b)). FAAH KO mice displayed a prolonged duration of capsaicin-evoked mechanical hypersensitivity relative to WT mice ($F_{1,8} = 17.59$, $p < 0.01$, Figure 7(b)), and the interaction between genotype and the effects of capsaicin was significant ($F_{4,8} = 4.438$, $p < 0.01$; Figure 7(b)). Post-hoc analyses revealed that capsaicin-treated FAAH KO mice receiving vehicle (i.p.) displayed increased mechanical hypersensitivity 30 ($p < 0.01$; Figure 7(b)), 60 ($p < 0.05$; Figure 7(b)), and 120 ($p < 0.01$, Figure 7(b)) but not at 5 minutes post-capsaicin relative to identically-treated WT mice.

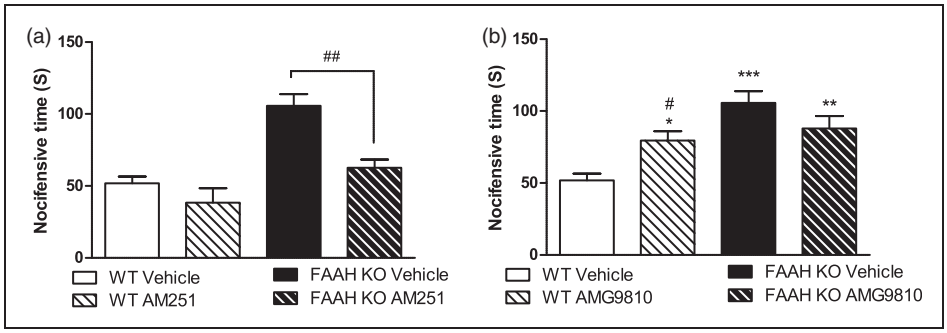


**Figure 6.** AM251 attenuates capsaicin-evoked nocifensive behavior in FAAH KO but not WT mice whereas AMG9810 increases it in WT but not FAAH KO mice. FAAH KO mice receiving vehicle (i.p.) show increased levels of capsaicin-evoked nocifensive behavior relative to FAAH KO mice receiving the CB1 antagonist AM251 (3 mg/kg i.p.) (a). AM251 (3 mg/kg, i.p.) pretreatment eliminated the increase in capsaicin-evoked nocifensive behavior in FAAH KO mice at a dose that did not reliably alter responding in WT mice (a). Data are expressed as mean ± SEM ($n = 5$–6 per group). Capsaicin-treated WT mice receiving vehicle (i.p.) display lower levels of nocifensive behavior compared to all other groups tested (b). Capsaicin-treated WT mice receiving TRPV1 agonist AMG9810 (3 mg/kg, i.p.) display lower levels of nocifensive behavior than capsaicin-treated FAAH KO mice receiving vehicle (i.p.) (b). Data are expressed as mean ± SEM ($n = 5$–6 per group). $^{\#\#}p < 0.01$ versus FAAH KO vehicle. Planned comparison, 2-tailed $t$ test. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ versus wt vehicle, $^{\#}p < 0.05$ versus FAAH KO vehicle. ANOVA, Newman–Keuls post-hoc. FAAH KO: FAAH knockout; WT: wildtype.

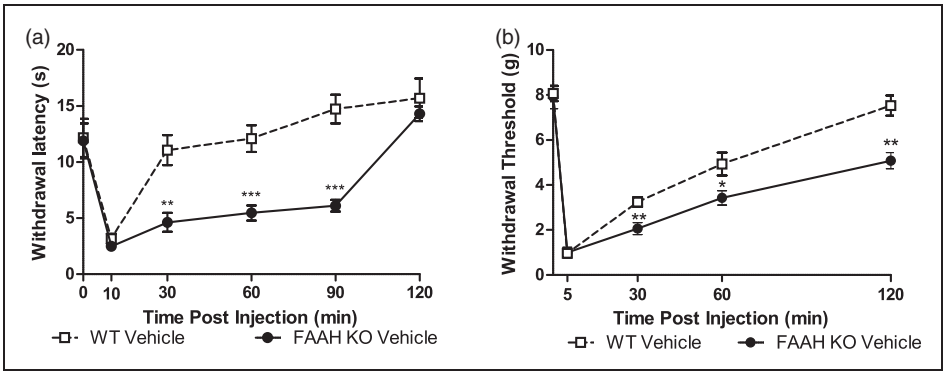


**Figure 7.** FAAH KO mice receiving i.p. vehicle display a delayed resolution of capsaicin-evoked heat and mechanical hypersensitivity. FAAH KO mice receiving vehicle (i.p.) display a prolongation of capsaicinevoked heat hypersensitivity, as measured by thermal paw withdrawal latencies, relative to WT mice receiving vehicle (i.p.) (a). FAAH KO mice receiving vehicle (i.p.) display a prolongation of capsaicin-evoked mechanical hypersensitivity relative to WT animals similarly receiving vehicle (i.p.) (b). Data are expressed as mean ± SEM ($n = 5$–6 per group).***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus FAAH KO vehicle, Two-tailed $t$ test. FAAH KO: FAAH knockout; WT: wildtype.

## *The $CB_1$ antagonist AM251 suppresses capsaicin-evoked thermal hyperalgesia and mechanical allodynia in FAAH KO mice*

Intradermal capsaicin lowered thermal paw withdrawal latencies in FAAH KO ($F_{5,9} = 32.21$, $p < 0.0001$; Figure 8(a)) and WT ($F_{5,9} = 25.39$, $p < 0.0001$; Figure 8(b)) mice. AM251 (3 mg/kg, i.p.) reduced the duration of heat hypersensitivity in FAAH KO ($F_{1,9} = 21.43$, $p < 0.01$; Figure 8(a)) but not WT mice ($p > 0.3$; Figure 8(b)). AM251 suppressed capsaicin-evoked heat hypersensitivity in a time-dependent manner in FAAH KO ($F_{5,9} = 4.349$, $p < 0.01$; Figure 8(a)) but not in WT mice ($p > 0.3$; Figure 8(b)). Post-hoc analysis revealed that FAAH KO mice receiving vehicle (i.p.) displayed heightened thermal hypersensitivity at 30 ($p < 0.05$), 60 ($p < 0.05$), and 90 ($p < 0.001$) minutes post-capsaicin in comparison to FAAH KO animals receiving AM251 (Figure 8(a)). While capsaicin reduced thermal paw withdrawal latencies in both AM251-treated FAAH KO mice and vehicle-treated WT mice ($F_{5,9} = 25.33$, $p < 0.0001$; Figure 8(c)), withdrawal latencies did not differ between groups ($p > 0.1$; Figure 8(c)), and there was no significant interaction between drug and genotype ($p > 0.8$; Figure 8(c)). Thus, in FAAH KO mice, AM251 treatment normalized capsaicin-evoked heat hyperalgesia to levels observed in WT mice treated with vehicle (i.p.).

Thermal paw withdrawal latencies in the paw contralateral to capsaicin administration did not differ in FAAH KO mice receiving either vehicle or AM251 ($p > 0.1$; data not shown), or WT mice receiving either vehicle or AM251 ($p > 0.4$; data not shown). However, thermal paw withdrawal latencies differed in AM251-treated FAAH KO mice and vehicle-treated WT mice in the paw contralateral to capsaicin administration ($F_{5,9} = 2.742$, $p < 0.05$; Supplemental Figure 1). AM251-treated FAAH KO mice displayed lower thermal paw withdrawal latencies relative to vehicle-treated WT mice in the paw contralateral to capsaicin administration ($F_{1,9} = 6.995$, $p < 0.05$; Supplemental Figure 1), and the interaction was significant ($F_{5,9} = 3.258$, $p < 0.05$; Supplemental Figure 1).

Capsaicin altered mechanical paw withdrawal thresholds in FAAH KO mice receiving AM251 (3 mg/kg, i.p.) or vehicle ($F_{4,8} = 48.25$, $p < 0.0001$; Figure 8(d)) and WT mice receiving AM251 or vehicle ($F_{4,8} = 128.9$, $p < 0.0001$; Figure 8(e)). AM251 attenuated capsaicin-induced decreases in mechanical paw withdrawal thresholds in FAAH KO ($F_{1,8} = 12.59$, $p < 0$ .01; Figure 8(d)), but not in WT mice ($p > 0.5$; Figure 8(e)). The interaction between the effects of AM251 and capsaicin was significant in FAAH KO ($F_{4,8} = 3.321$, $p < 0.05$; Figure 8(d)) but not WT mice ($p > 0.5$; Figure 8(e)). While capsaicin administration significantly lowered mechanical paw withdrawal thresholds in AM251-treated FAAH KO mice and vehicle (i.p.)-treated WT mice ($F_{4,8} = 56.76$, $p < 0.0001$; Figure 8(f)), withdrawal thresholds did not differ between groups ($p > 0.2$; Figure 8(f)), and the interaction was not significant ($p > 0.4$; Figure 8(f)). Post-hoc analysis revealed that AM251 reduced mechanical hypersensitivities in FAAH KO compared to their vehicle-treated counterparts at 30 ($p < 0.05$), 60 ($p < 0.05$), and 120 ($p < 0.01$) minutes post-capsaicin administration (Figure 8(d)). No differences were observed in the contralateral paw at any time point for any condition ($p > 0.4$; data not shown).

## *The TRPV1 antagonist AMG9810 suppresses capsaicin-evoked thermal hyperalgesia and mechanical allodynia in FAAH KO but not WT mice*

Intradermal capsaicin decreased thermal paw withdrawal latencies in FAAH KO ($F_{5,9} = 20.57$, $p < 0.0001$; Figure 9(a)) and WT ($F_{5,9} = 25.78$, $p < 0.0001$; Figure 9(b)) mice treated with vehicle or AMG9810

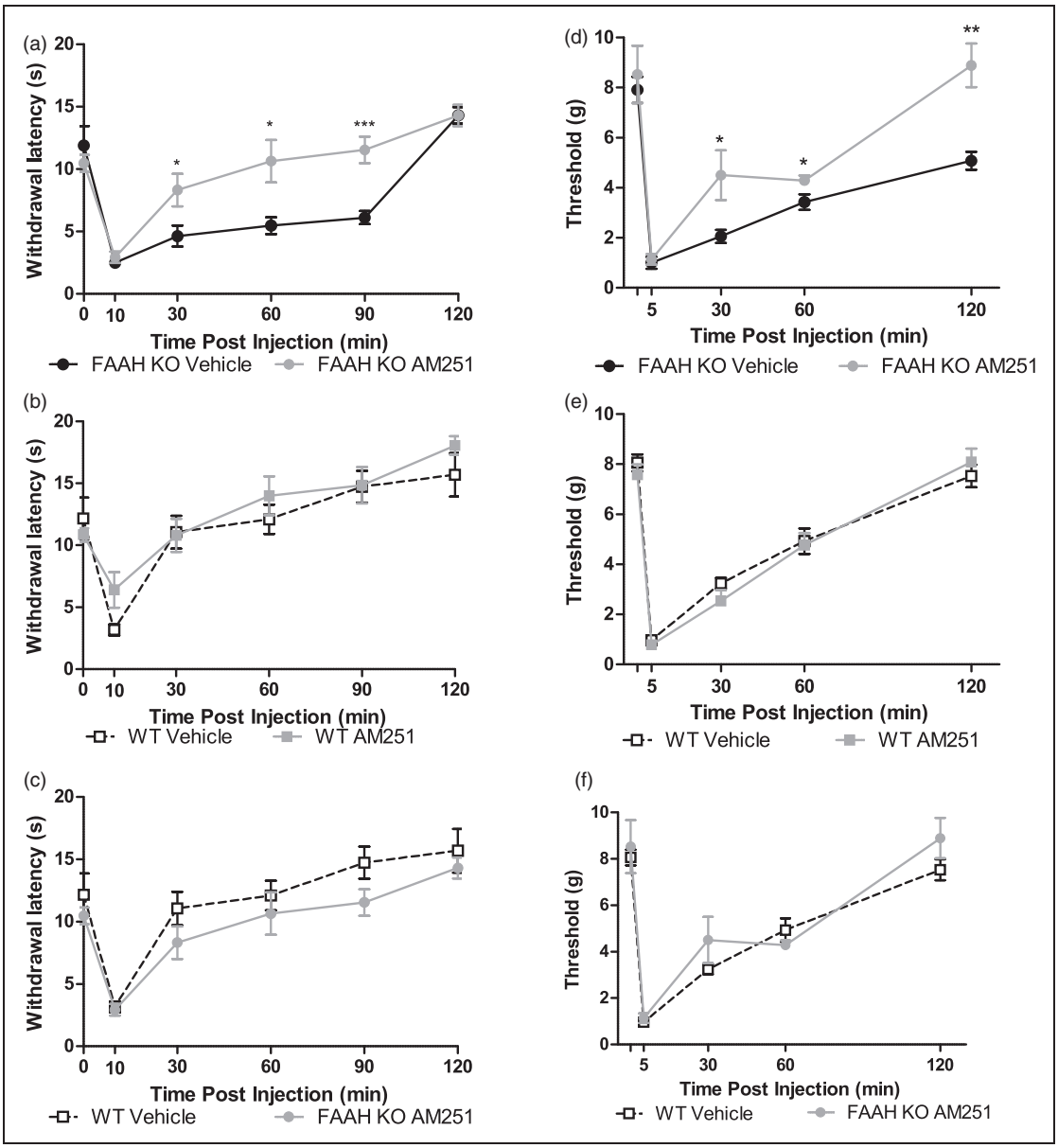


**Figure 8.** AM251 eliminates the pro-nociceptive phenotype of FAAH KO mice as measured by capsaicinevoked heat and mechanical hypersensitivity. The CB1 antagonist AM251 (3 mg/kg, i.p.) attenuates the heightened capsaicin-evoked heat hypersensitivity observed in FAAH KO mice compared to FAAH KO mice pretreated with vehicle (i.p.) (a). AM251 (3 mg/kg i.p.) does not alter capsaicin-evoked heat hyperalgesia in WT mice relative to WT mice receiving vehicle (i.p) (b). AM251 (3 mg/kg, i.p.) restores paw withdrawal latencies of FAAH KO mice to levels observed in WT mice (c). In FAAH KO mice, AM251 (3 mg/kg i.p.) reduced capsaicin-evoked mechanical hypersensitivity relative to vehicle treatment at 30, 60, and 120 minutes post-capsaicin administration (d). In WT mice, AM251 (3 mg/kg, i.p.) does not alter mechanical hypersensitivity relative to vehicle (i.p.) treatment (e). In FAAH KO mice, AM251 (3 mg/kg i.p.) restores paw withdrawal thresholds to levels observed in WT mice (f). Data are expressed as mean ± SEM ($n = 5$–6 per group). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus FAAH KO vehicle, two-tailed $t$ test. FAAH KO: FAAH knockout; WT: wildtype.

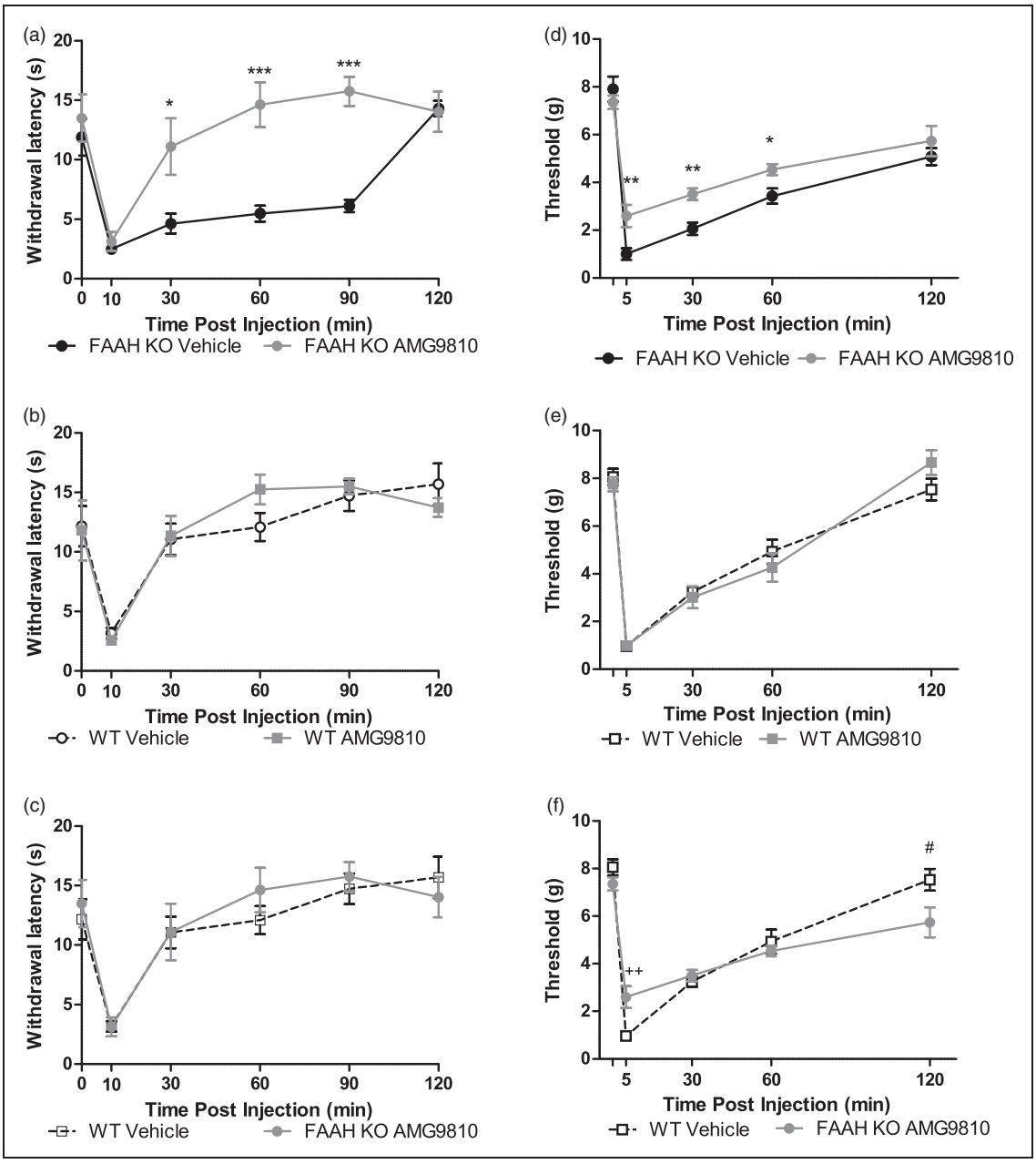


**Figure 9.** AMG9810 eliminates the pro-nociceptive phenotype of FAAH KO mice as measured by capsaicin-evoked heat and mechanical hypersensitivity. In FAAH KO mice, the TRPV1 antagonist AMG9810 (3 mg/kg, i.p.) pretreatment promotes the resolution of capsaicin-evoked heat hypersensitivity relative to vehicle (i.p) treatment (A). In WT mice, AMG9810 (3 mg/kg, i.p.) does not alter capsaicin-evoked heat hypersensitivity relative to vehicle (i.p.) treatment (B). AMG9810 pretreatment in FAAH KO mice restores thermal paw withdrawal latencies to levels observed in vehicle-treated WT mice (C). In FAAH KO mice, AMG9810 (3 mg/kg i.p.) reduced capsaicin-evoked mechanical hypersensitivity relative to vehicle (i.p.) treatment (D). In WT mice, AMG9810 (3 mg/kg, i.p.) does not alter capsaicin-evoked mechanical hypersensitivity relative to vehicle (i.p.) treatment (E). FAAH KO mice receiving AMG9810 (3 mg/kg, i.p.) exhibit a lower magnitude of mechanical hypersensitivity at 5 min but enhanced mechanical hypersensitivity at 120 minutes post capsaicin relative to WT mice receiving vehicle (i.p) (F). Data are expressed as mean ± SEM (n = 5-6 per group). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. FAAH KO vehicle, ++$p < 0.01$ vs. WT vehicle, #$p < 0.05$ vs. FAAH KO AMG9810, Two-tailed t-test. FAAH KO: FAAH knockout; WT: wildtype.

(i.p.). AMG9810 (3 mg/kg, i.p.) shortened the duration of the capsaicin-induced heat hyperalgesia in FAAH KO ($F_{1,9} = 29.09$, $p < 0.001$; Figure 9(a)) but not WT ($p > 0.8$; Figure 9(b)) mice. A significant interaction between the effects of AMG9810 and those of capsaicin was similarly observed in FAAH KO ($F_{5,9} = 6.207$, $p < 0.001$; Figure 9(a)) but not WT mice ($p > 0.4$; Figure 9(b)). While capsaicin reduced thermal paw withdrawal latencies in both AMG9810-treated FAAH KO mice and vehicle-treated WT mice ($F_{5,9} = 20.62$, $p < 0.0001$; Figure 9(c)), withdrawal latencies did not differ between the two groups ($p > 0.6$) and there was no significant interaction ($p > 0.7$). Post-hoc analysis revealed that vehicle-treated FAAH KO mice displayed lower paw withdrawal latencies relative to AMG9810-treated FAAH KO mice at 30 ($p < 0.05$), 60 ($p < 0.001$), and 90 ($p < 0.001$) minutes post-capsaicin but not at 5 or 120 min post-capsaicin (Figure 9(a)). Thus, the TRPV1 and CB1 antagonists normalized capsaicin-evoked responding in FAAH KO mice to levels observed in capsaicin-treated WT mice (Figures 8 and 9).

Intradermal capsaicin administration did not affect thermal withdrawal latencies in the paw contralateral to capsaicin administration in AMG9810-treated relative to vehicle-treated FAAH KO mice ($p > 0.4$; data not shown). Similarly, contralateral paw responding in AMG9810-treated and vehicle-treated WT mice ($p > 0.4$; data not shown) did not differ between groups. Contralateral paw responding was also similar in AMG9810-treated FAAH KO mice and vehicle-treated WT mice ($p > 0.5$; data not shown).

Capsaicin administration significantly altered mechanical thresholds in FAAH KO mice receiving AMG9810 (3 mg/kg, i.p.) or vehicle ($F_{4,8} = 83.94$, $p < 0.0001$; Figure 9(d)) and WT mice receiving AMG9180 or vehicle ($F_{4,8} = 126.1$, $p < 0.0001$; Figure 9(e)). AMG9810 (3 mg/kg, i.p.) attenuated capsaicin-induced decreases in mechanical paw withdrawal thresholds in FAAH KO mice ($F_{1,8} = 8.576$, $p < 0.05$; Figure 9(d)) but not in WT ($p > 0.97$; Figure 9(e)) mice. AMG9810 elevated capsaicin-evoked paw withdrawal thresholds in FAAH KO ($F_{4,8} = 2.992$, $p < 0.05$; Figure 9(d)) but not in WT mice ($p > 0.2$; Figure 9(e)). Post-hoc analysis revealed that AMG9810 reduced capsaicin-evoked mechanical hypersensitivities in FAAH KO compared to their vehicle-treated counterparts at 5 ($p < 0.05$), 30 ($p < 0.01$), 60 ($p < 0.05$), but not 120 min post-capsaicin. While capsaicin administration lowered paw withdrawal thresholds in both AMG9810-treated FAAH KO mice and vehicle-treated WT mice ($F_{4,8} = 102.3$, $p < 0.0001$; Figure 9(f)), capsaicin differentially altered responding in these groups ($F_{4,8} = 7.57$, $p < 0.01$; Figure 9(f)). Post-hoc analysis revealed that AMG9810-treated FAAH KO mice exhibited less hypersensitivity relative to vehicle-treated WT mice at 5 ($p < 0.01$) minutes but more hypersensitivity at 120 ($p < 0.05$) minutes post-capsaicin. No differences were observed in the contralateral paw at any time point for any condition ($p > 0.4$; data not shown).

Thus, the TRPV1 and $CB_1$ antagonists largely normalized capsaicin-evoked responding in FAAH KO mice to levels observed in capsaicin-treated WT mice for both modalities (heat, mechanical) of cutaneous stimulation (Figures 8 and 9).

### *AMG9810 and AM251 do not alter thermal paw withdrawal latencies, mechanical paw withdrawal thresholds or produce nocifensive behavior in FAAH KO mice in the absence of capsaicin*

Pretreatment (i.p.) with vehicle, AM251, or AMG9810 did not alter nocifensive behavior ($p > 0.9$; data not shown), thermal paw withdrawal latencies ($p > 0.1$; data not shown) or mechanical paw withdrawal thresholds ($p > 0.9$; data not shown) in FAAH KO mice receiving vehicle (i.pl.) in lieu of capsaicin.

### *N-acyl ethanolamine levels are increased in paw skin and lumbar spinal cords of FAAH KO vs. WT mice*

Paw skin levels of AEA, LEA, PEA, and SEA were higher in FAAH KO relative to WT mice at 1 h following intradermal injection of capsaicin (Figure 10(a, c, e, and g), see Table 1 for summary). Capsaicin differentially affected paw skin levels of SEA in FAAH KO mice in the same samples; FAAH KO mice treated with vehicle displayed higher levels of SEA compared to all other groups (Figure 10(g)).

Similarly, lumbar spinal cord levels of AEA, LEA, PEA, and SEA were higher in FAAH KO relative to WT mice irrespective of vehicle or capsaicin treatment (Figure 1(b d, f, and h), see Table 1 for summary).

### *Arachidonic acid derivatives are elevated in both paw skin and lumbar spinal cords 1 hour post intradermal capsaicin*

Intradermal capsaicin increased, relative to (i.pl.) vehicle injection, AA and $PGE_2$ (Figure 11(c and e)) levels in paw skin derived from both FAAH KO and WT mice that was dissected 1 h post i.pl. injection (see Table 2 for summary). Only $PGF_{2\alpha}$ levels were differentially altered by capsaicin treatment as a function of genotype; intradermal capsaicin, but not vehicle, treatment increased paw skin $PGF_{2\alpha}$ levels in FAAH KO but not WT mice (Figure11(g)).

Intradermal capsaicin decreased levels of 2-AG (Figure 11(b)) and increased levels of AA (Figure 11(d)) and $PGE_2$ (Figure 11(f)) in lumbar spinal cords of FAAH KO and WT mice (see Table 2 for summary). PGF2α was not reliably increased in the same lumbar spinal cord samples (Figure 11(h)).

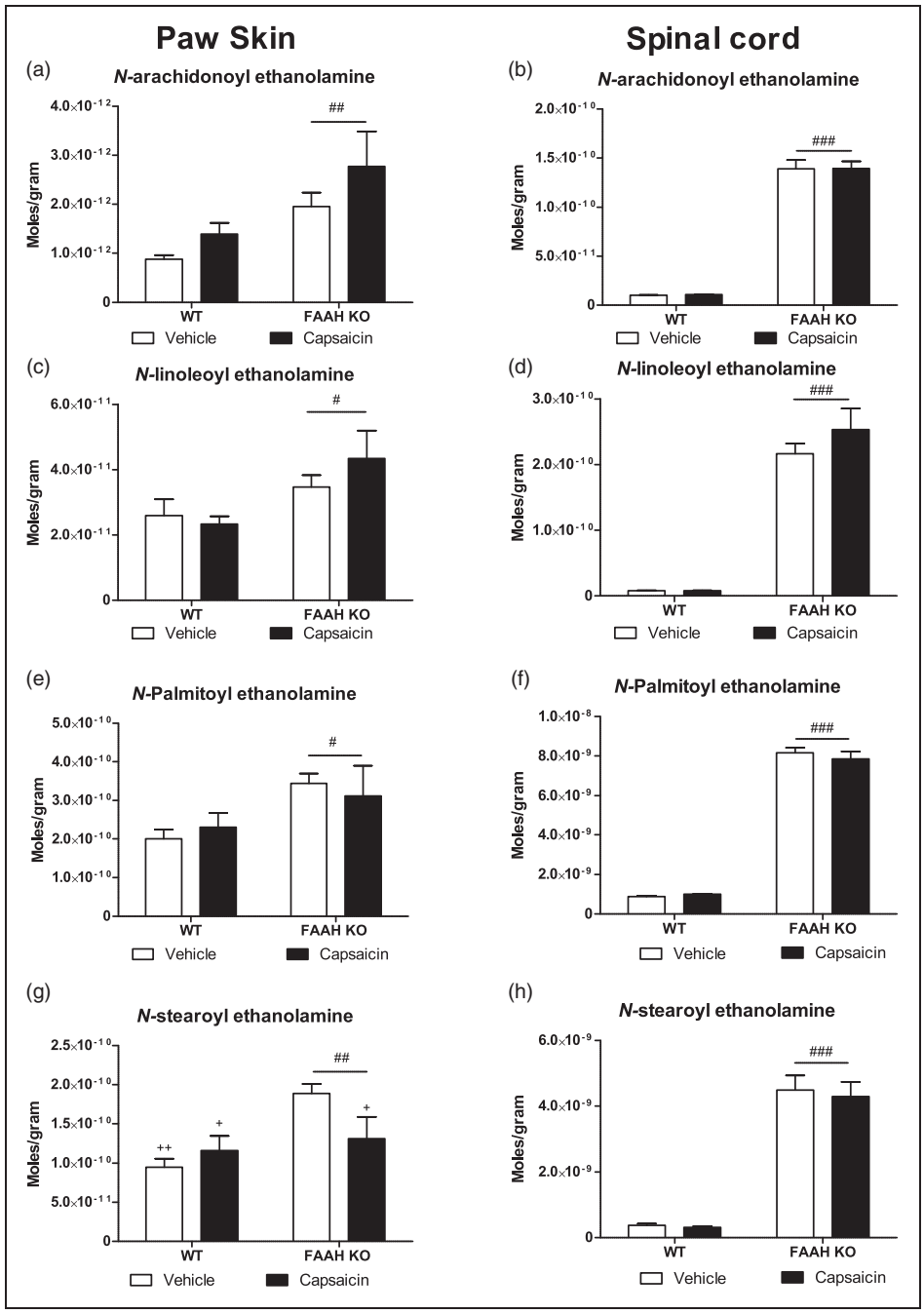


**Figure 10.** Capsaicin decreases, whereas genetic deletion of FAAH increases, N-acyl ethanolamines in paw skin and lumbar spinal cord. Paw skin levels of AEA, LEA, PEA, and SEA were elevated in FAAH KO relative to WT mice irrespective of capsaicin or vehicle (i.pl.) treatment (a, c, e, g). Paw skin SEA levels are preferentially lowered by capsaicin in FAAH KO but not WT mice. Whereas FAAH KO mice in general display higher levels of SEA than WT mice, capsaicin lowered paw skin SEA levels in FAAH KO but not WT mice (g). FAAH KO mice displayed elevated spinal cord levels of AEA, LEA, PEA and SEA relative to WT mice irrespective of capsaicin or vehicle (i.pl.) treatment (b, d, f, h). Data are expressed as mean + SEM ($n = 6$). Tissue was dissected 1 h following intradermal injection of vehicle or capsaicin. $^{###}p < 0.0001$, $^{##}p < 0.01$, $^{#}p < 0.05$ versus WT. Two-way ANOVA.$^{++}p < 0.01$, $^{+}p < 0.05$ versus FAAH KO vehicle (K), one-way ANOVA followed by Newman–Keuls post hoc. FAAH KO: FAAH knockout; WT: wildtype.

**Table 1.** Statistical summary: Impact of capsaicin and genotype (FAAH KO vs. WT) on N-acyl ethanolamine levels in paw skin and lumbar spinal cord.

| Compound | Tissue | Capsaicin (vs. vehicle) | Genotype (vs. WT) | Interaction | Figure |
|---|---|---|---|---|---|
| AEA | Paw skin | NS | $F_{1,20} = 9.146$, $p < 0.01$ ↑ | NS | 10(a) |
| | Spinal cord | NS | $F_{1,20} = 503.4$, $p < 0.0001$ ↑ | NS | 10(b) |
| LEA | Paw skin | NS | $F_{1,20} = 7.53$, $p < 0.05$ ↑ | NS | 10(c) |
| | Spinal cord | NS | $F_{1,20} = 162.9$, $p < 0.0001$ ↑ | NS | 10(d) |
| PEA | Paw skin | NS | $F_{1,20} = 5.823$, $p < 0.05$ ↑ | NS | 10(e) |
| | Spinal cord | NS | $F_{1,20} = 904.9$, $p < 0.0001$ ↑ | NS | 10(f) |
| SEA | Paw skin | NS | $F_{1,20} = 8.682$, $p < 0.01$ ↑ | $F_{1,20} = 4.489$, $p < 0.05$ | 10(g) |
| | Spinal cord | NS | $F_{1,20} = 163.6$, $p < 0.0001$ ↑ | NS | 10(h) |

AEA: *N*-arachidonoyl ethanolamine; LEA: *N*-linoleoyl ethanolamine; NS: not significant; PEA: *N*-palmitoyl ethanolamine; SEA: *N*-stearoyl ethanolamine; ↑: increase.

### *FAAH KO mice display elevated endovanilloid levels in lumbar spinal cords 1 h post-injection*

Capsaicin increased paw skin levels of DEA in both FAAH KO and WT mice relative to vehicle treatment 1 h following intradermal injection (Figure 12(a)). FAAH KO mice displayed higher levels of both DEA and OEA in lumbar spinal cord relative to WT mice, irrespective of treatment with vehicle or capsaicin (Figures 12(b and d), see Table 3 for summary).

### *Levels of endovanilloids and orphan endocannabinoid receptor ligands are altered in the spinal cords of FAAH KO mice 1 h post-capsaicin injection*

Several endovanilloids and GPR18 ligands that were not present at detectable levels in paw skin were altered in the lumbar spinal cord. The GPR18 ligand *N*-arachidonoyl serine was elevated (Figure 13(a)) whereas the GPR18 ligand, and AEA metabolite, *N*-arachidonoyl glycine,[29] was decreased (Figure 13(b)) in FAAH KO relative to WT mice irrespective of treatment with capsaicin or vehicle. Most notably, capsaicin preferentially decreased the levels of *N*-arachidonoyl taurine (NAT) in the lumbar spinal cords of capsaicin- versus vehicle (i.pl.)-treated FAAH KO mice (Figure 13(c)). Post-hoc analysis revealed that NAT levels were higher in the spinal cords of vehicle-treated FAAH KO mice than all other groups (Figure 13(c)). NAT levels were also higher in lumbar spinal cords of FAAH KO mice treated with i.pl. capsaicin than WT mice treated with either i.pl. vehicle or i.pl. capsaicin (Figure 13(c)). FAAH KO mice exhibited elevated levels of the endovanilloids *N*-docosahexaenoyl GABA (Figure 13(d)), *N*-linoleoyl GABA (Figure 13(e)), and decreased levels of the endovanilloid *N*-arachidonoyl GABA[30] (Figure 13(f), see Table 4 for summary). By contrast, neither genotype nor capsaicin treatment reliably altered *N*-docosahexaenoyl glycine or *N*-docosahexaenoyl serine levels in the lumbar spinal cord (Figure 13(g and h)).

### *Capsaicin and genotype alter levels of many lipid signaling molecules with unknown protein targets in paw skin and lumbar spinal cord*

Capsaicin increased paw skin levels of *N*-stearoyl glycine in both FAAH KO and WT mice at 1 h post-intradermal injection (Supplemental Figure 2(c)). FAAH KO mice displayed decreased levels of *N*-oleoyl glycine relative to WT mice in paw skin (Supplemental Figure 2(e)) and elevated levels of *N*-palmitoyl glycine, *N*-stearoyl glycine, *N*-oleoyl glycine, *N*-stearoyl serine, and *N*-oleoyl serine in lumbar spinal cord dissected 1 h post-injection (Supplemental Figure 2(b, d, f, h, and j), see Supplemental Table 1 for summary).

Capsaicin increased paw skin levels of *N*-stearoyl alanine in both FAAH KO and WT mice 1 h following intradermal injections (Supplemental Figure. 3(c)). Capsaicin differentially altered the levels of *N*-palmitoyl serine in FAAH KO paw skin (Supplemental Figure 3(g)); capsaicin lowered paw skin levels of *N*-palmitoyl serine in FAAH KO but not WT mice; *N*-palmitoyl serine levels were also elevated in paw skin derived from vehicle (i.pl.)-treated FAAH KO mice relative to vehicle-treated WT mice (Supplemental Figure 3(g), see Supplemental Table 1 for summary).

FAAH KO mice displayed increased lumbar spinal cord levels of *N*-palmitoyl alanine, *N*-stearoyl alanine, *N*-oleoyl alanine, *N*-palmitoyl serine relative to WT mice in tissue dissected 1 h following intradermal injections (Supplemental Figure 3(b, d, f, and h)). Capsaicin administration (i.pl.) decreased *N*-palmitoyl serine and *N*-linoleoyl serine levels in lumbar spinal cord derived from FAAH KO and WT mice (Supplemental Figure 3(h and j), see Supplemental Table 1 for summary).

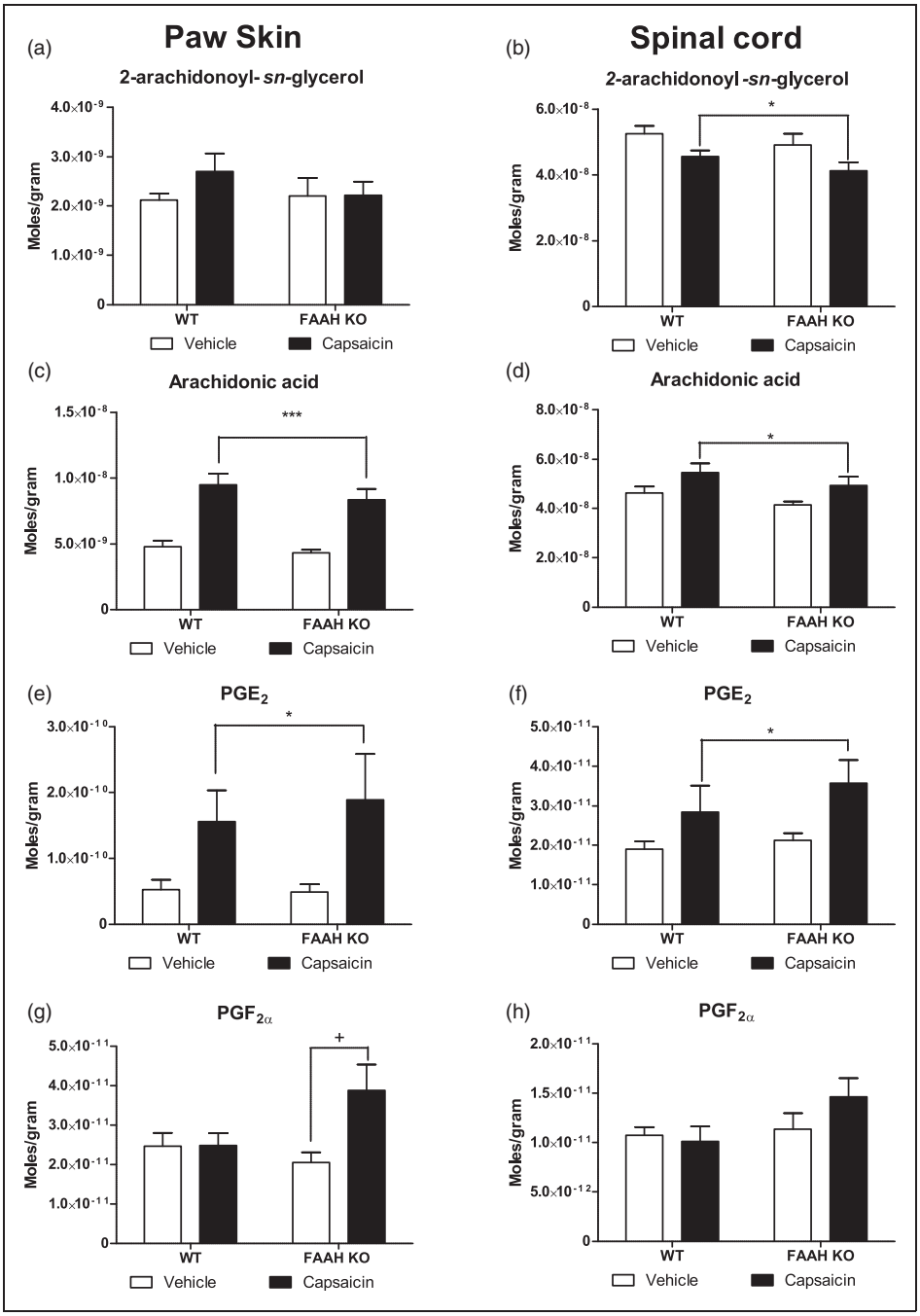


**Figure 11.** Capsaicin alters levels of arachidonic acid derivatives in both paw skin and lumbar spinal cord. Capsaicin increases paw skin levels of arachidonic acid (AA) and prostaglandin E2 (PGE2) in FAAH KO and WT mice (c, e). Capsaicin selectively increases paw skin PGF2$\alpha$ levels in FAAH KO but not WT mice (g). Capsaicin decreased 2-AG levels in lumbar spinal cord (b) but not paw skin (a). Capsaicin increased levels of AA and PGE2 in both paw skin (c, e) and lumbar spinal cord (d, f). Data are expressed as mean + SEM ($n = 6$). Tissue was dissected 1 h following intradermal injection of vehicle or capsaicin. $^{***}p < 0.0001$, $^{*}p < 0.05$ versus vehicle. Two-way ANOVA. $^{+}p < 0.05$ versus FAAH KO vehicle, one-way ANOVA followed by Newman–Keuls post hoc. FAAH KO: FAAH knockout; WT: wildtype.

FAAH KO mice also displayed lower levels of 2-oleoyl-*sn*-glycerol in lumbar spinal cord relative to WT mice, irrespective of vehicle or capsaicin treatment, whereas paw skin levels were not reliably altered. By contrast, 2-lineolyl-*sn*-glycerol levels were not reliably altered in either paw skin or lumbar spinal cord by genotype or capsaicin treatment (Supplemental Figure 4(a to d), see Supplemental Table 1 for summary).

**Table 2.** Statistical summary: Impact of capsaicin and genotype (FAAH KO vs. WT) on levels of arachidonic acid derivatives in paw skin and lumbar spinal cord.

| Compound | Tissue | Capsaicin (vs. vehicle) | Genotype (vs. WT) | Interaction | Figure |
|---|---|---|---|---|---|
| 2-AG | Paw skin | NS | NS | NS | 11(a) |
| | Spinal cord | $F_{1,20} = 7.897$, $p < 0.05$ ↓ | NS | NS | 11(b) |
| AA | Paw skin | $F_{1,20} = 45.56$, $p < 0.0001$ ↑ | NS | NS | 11(c) |
| | Spinal cord | $F_{1,20} = 7.897$, $p < 0.05$ ↑ | NS | NS | 11(d) |
| $PGE_2$ | Paw skin | $F_{1,20} = 7.837$, $p < 0.05$ ↑ | NS | NS | 11(e) |
| | Spinal cord | $F_{1,20} = 6.488$, $p < 0.05$ ↑ | NS | NS | 11(f) |
| $PGF_{2\alpha}$ | Paw skin | $F_{1,20} = 4.943$, $p < 0.05$ ↑ | NS | $F_{1,20} = 4.762$, $p < 0.05$ | 11(g) |
| | Spinal cord | NS | NS | NS | 11(h) |

2-AG: 2-arachidonoyl-*sn*-glycerol; AA: arachidonic acid; NS: not significant; ↑: increase, ↓: decrease.

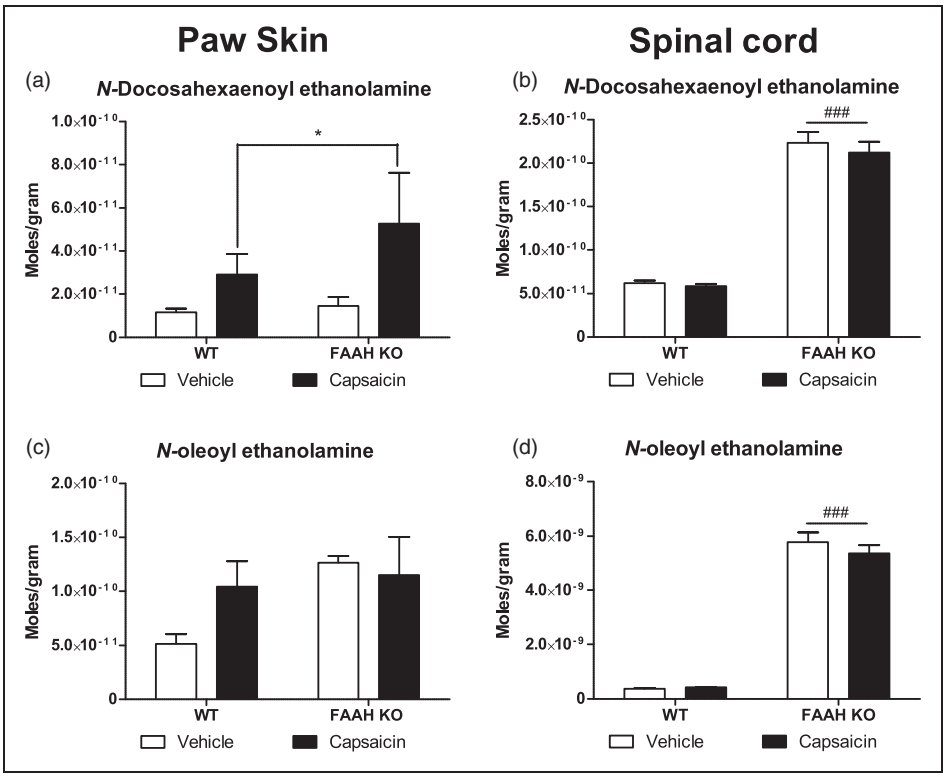


**Figure 12.** Genetic deletion of FAAH increases endovanilloids in lumbar spinal cord. Capsaicin increases paw skin DEA levels in both FAAH KO and WT mice (a). FAAH KO mice display higher spinal cord levels of DEA and OEA relative to WT mice irrespective of capsaicin or vehicle (i.pl.) treatment (b, d). Data are expressed as mean + SEM ($n = 6$). Tissue was dissected 1 h following intradermal injection of vehicle or capsaicin. $^{*}p < 0.05$ versus vehicle, $^{\#\#\#}p < 0.0001$ versus WT. Two-way ANOVA; FAAH KO: FAAH knockout; WT: wildtype.

### *Genotype and intradermal capsaicin alter levels of lipid signaling molecules with unknown protein targets that were detectable in lumbar spinal cord but not paw skin*

FAAH KO mice displayed elevated spinal cord levels of *N*-arachidonoyl alanine, *N*-stearoyl GABA, and *N*-oleoyl GABA relative to WT mice in tissue dissected 1 h post-i.pl. injection (Supplemental Figure 5(a to c), see Supplemental Table 2 for summary). Capsaicin administration (i.pl.) decreased lumbar spinal cord levels of *N*-palmitoyl methionine, *N*-stearoyl methionine, and *N*-oleoyl proline in both FAAH KO and WT relative to i.pl. vehicle treatment (Supplemental Figure 6(a to c), see Supplemental Table 3 for summary). Capsaicin differentially altered levels of *N*-oleoyl proline in the lumbar

**Table 3.** Statistical summary: Impact of capsaicin and genotype (FAAH KO vs. WT) on endovanilloid levels in paw skin and lumbar spinal cord.

| Compound | Tissue | Capsaicin (vs. vehicle) | Genotype (vs. WT) | Interaction | Figure |
|---|---|---|---|---|---|
| DEA | Paw skin | $F_{1,20} = 4.633$, $p < 0.05$ ↑ | NS | NS | 12(a) |
| | Spinal cord | NS | $F_{1,20} = 309.8$, $p < 0.0001$ ↑ | NS | 12(b) |
| OEA | Paw skin | NS | $F_{1,20} = 3.825$, $p = 0.0646$ ↑ | NS | 12(c) |
| | Spinal cord | NS | $F_{1,20} = 497.1$, $p < 0.0001$ ↑ | NS | 12(d) |

DEA: *N*-docosahexaenoyl ethanolamine; NS: not significant; OEA: *N*-oleoyl ethanolamine; WT: wildtype; ↑: increase. Gray shading denotes a trend toward significance.

spinal cords of FAAH KO mice; FAAH KO mice treated with vehicle (i.pl.) displayed higher levels of *N*-oleoyl proline than all other groups (Supplemental Figure 6(c)).

## Discussion

To our knowledge, the present study provides the first evidence that genetic deletion of FAAH can increase pain responsiveness (i.e., produce hyperalgesia), as unmasked by challenge with the TRPV1 agonist capsaicin. These results are noteworthy as previous reports have indicated that pharmacological inhibition and genetic deletion of FAAH decreases nociceptive responding (i.e., produces hypoalgesia or antinociception). Indeed, our studies confirm that FAAH KO mice exhibit an analgesic phenotype in the hot water tail immersion test, and in models of inflammatory nociception induced by either carrageenan or formalin.[9,10] Nonetheless, a FAAH inhibitor was ineffective in decreasing pain in a human osteoarthritis trial.[12] Strikingly, in our study, FAAH KO mice exhibited *increased* sensitivity to pain induced by the TRPV1 agonist capsaicin; FAAH KO mice displayed profound increases in nocifensive behavior, thermal (i.e., heat) hyperalgesia and mechanical allodynia evoked by intradermal capsaicin administration. The magnitude of the capsaicin-evoked nocifensive behavior was enhanced in FAAH KO mice compared to WT mice. Moreover, a delayed resolution of capsaicin-evoked sensitization to mechanical and heat stimulation was apparent in FAAH KO relative to WT mice. These observations are consistent with heightened central sensitization, evoked by capsaicin challenge, in FAAH KO relative to WT mice. Consistent with this hypothesis, we observed increases in capsaicin-evoked Fos protein expression, a marker of neuronal activation, at the level of the lumbar spinal dorsal horn in FAAH KO relative to WT mice. FAAH KO mice exhibited the greatest increase in number of capsaicin-evoked FLI cells in the superficial dorsal horn (i.e., lamina I and II) of the spinal cord. Increases in Fos protein–like immunoreactive cells in FAAH KO mice were also restricted to the dorsal horn, a spinal cord region implicated in nociceptive processing, and were not observed in FAAH KO mice receiving intradermal injection of vehicle. These latter observations also highlight the importance of the nociceptive challenge (i.e., capsaicin) in evoking central sensitization in mice with a genetic deletion of FAAH. However, the pro-nociceptive phenotype unmasked in the present study, and lack of analgesic efficacy in human osteoarthritis pain[12] could indicate the therapeutic effects of FAAH inhibition may only extend to specific pain states.

Systemic administration of either the $CB_1$ antagonist AM251 or the TRPV1 antagonist AMG9810 effectively removed the capsaicin-induced hyperalgesic phenotype displayed by FAAH KO mice. Thus, both $CB_1$- and TRPV1-mediated components underlie the observed pro-nociceptive phenotype. Interestingly, the antagonist treatments preferentially blunted the enhanced nociceptive responding exhibited by FAAH KO mice, effectively restoring capsaicin-evoked responding to levels observed in capsaicin-treated WT mice. Moreover, the same pharmacological treatments, in general, failed to alter capsaicin-evoked responsiveness in WT mice. AMG9810 did not reliably decrease capsaicin-evoked nocifensive behavior, heat hypersensitivity or mechanical thresholds in any of the WT groups tested. In fact, AMG9810-treated WT animals displayed increases in capsaicin-evoked nocifensive behavior relative to their vehicle-treated WT counterparts. Furthermore, AMG9810 did not further increase capsaicin-evoked nocifensive behavior in FAAH KO mice, which already exhibit a heightened nocifensive response to capsaicin. A ceiling effect in responding in FAAH KO mice could possibly account for the failure to observe further AMG9810-induced changes in nocifensive behavior in FAAH KO mice. More work is necessary to determine whether FAAH KO animals display alterations in TRPV1 protein expression and whether such changes could contribute to the observed behavioral phenotype.

Our studies analyzing the lipidome demonstrate that the biological actions of FAAH extend far beyond termination of AEA signaling and suggest that these broad changes in lipid levels may enhance susceptibility to

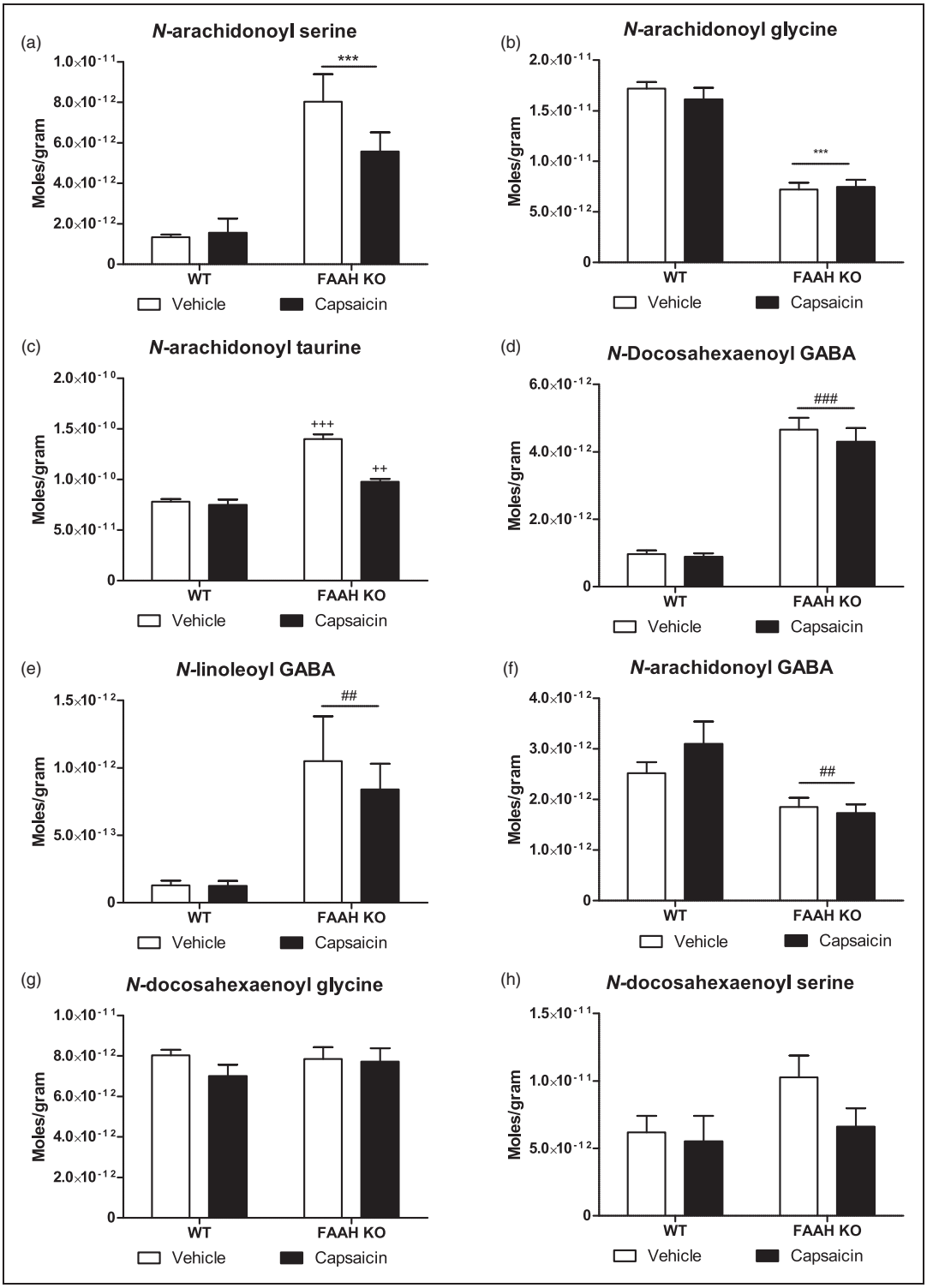


**Figure 13.** Genetic deletion of FAAH alters levels of several orphan cannabinoid receptor ligands and endovanilloids detectable in lumbar spinal cord but not paw skin. In FAAH KO mice, lumbar spinal cord levels of *N*-arachidonoyl serine are increased (a) whereas *N*-arachidonoyl glycine levels are decreased relative to WT mice (b). Capsaicin differentially affects levels of the endovanilloid *N*-arachidonoyl taurine in FAAH KO mice. FAAH KO mice receiving capsaicin (i.pl.) display lower levels of *N*-arachidonoyl taurine compared to FAAH KO mice receiving vehicle. Capsaicin decreased spinal *N*-arachidonoyl taurine levels in FAAH KO mice but not WT mice. Levels of *N*-arachidonoyl taurine were higher in FAAH KO mice receiving vehicle than all other groups tested (c). FAAH KO mice display increased spinal cord levels of the endovanilloids *N*-docosahexaenoyl GABA (d) and *N*-linoleoyl GABA (e), and decreased levels of the endovanilloid Narachidonoyl GABA (f) relative to WT mice. Data are expressed as mean + SEM ($n = 6$). $^{\#\#\#}p < 0.0001$, $^{\#\#}p < 0.01$ versus WT. Two-way ANOVA. $^{+++}p < 0.0001$ versus all other groups, $^{++}p < 0.01$ versus WT capsaicin and WT vehicle, one-way ANOVA followed by Newman–Keuls post hoc. FAAH KO: FAAH knockout; WT: wildtype.

**Table 4.** Statistical summary: Impact of capsaicin and genotype (FAAH KO vs. WT) on orphan cannabinoid receptor ligands and endovanilloid levels in lumbar spinal cord.

| Compound | Capsaicin (vs. vehicle) | Genotype (vs. WT) | Interaction | Figure |
|---|---|---|---|---|
| *N*-arachidonoyl serine | NS | $F_{1,20} = 35.59$, $p < 0.0001$ ↑ | NS | 13(a) |
| *N*-arachidonoyl glycine | NS | $F_{1,20} = 129.5$, $p < 0.0001$ ↓ | NS | 13(b) |
| NAT | $F_{1,20} = 32.91$, $p < 0.0001$ ↓ | $F_{1,20} = 115.9$, $p < 0.0001$ ↑ | $F_{1,20} = 24.91$, $p < 0.0001$ | 13(c) |
| *N*-docosahexaenoyl GABA | NS | $F_{1,20} = 165.6$, $p < 0.0001$ ↑ | NS | 13(d) |
| *N*-linoleoyl GABA | NS | $F_{1,20} = 18.05$, $p < 0.001$ ↑ | NS | 13(e) |
| *N*-arachidonoyl GABA | NS | $F_{1,20} = 13.81$, $p < 0.01$ ↓ | NS | 13(f) |
| *N*-docosahexaenoyl glycine | NS | NS | NS | 13(g) |
| *N*-docosahexaenoyl serine | NS | NS | NS | 13(h) |

NAT: *N*-arachidonoyl taurine; NS: not significant; ↑: increase, ↓: decrease.

capsaicin-evoked pain (see Piomelli et al.[31] for review). Moreover, effects of intradermal capsaicin are unlikely to be attributed to TRPV1 activation in isolation, given the broad spectrum of changes in the lipidome observed here at both central and peripheral levels. Therefore, it is unsurprising that a TRPV1 antagonist did not block capsaicin-induced nocifensive behavior in our study. Several mechanisms could account for the hyperalgesic phenotype produced by genetic deletion of FAAH that was unmasked by intradermal capsaicin treatment in our studies. In the current study, genetic deletion of FAAH had a profound impact on the lipidome, altering endocannabinoid and non-cannabinoid associated lipid-signaling molecules at central (i.e., lumbar spinal cord) as well as peripheral (i.e., paw skin) levels. In vitro studies suggest that AEA can activate TRPV1 receptors,[32] the primary target of capsaicin, albeit at higher concentrations than those that engage $CB_1$ receptors. Additionally, many of the other members of the *N*-acyl ethanolamine (NAE) family of lipids have been reported to act as agonists at the TRPV1 receptor including OEA,[33,34] LEA, and DEA,[30] all of which were found to be elevated in lumbar spinal cord of FAAH KO versus WT mice in both vehicle (i.pl.) and capsaicin (i.pl.) treatment conditions. Levels of AEA, LEA, PEA, and SEA were also elevated in FAAH KO paw skin tissue, suggesting that these lipids could potentially modulate nociceptor function in peripheral tissue as well as in the central nervous system. However, it is also important to note that FAAH is not the only enzyme that can degrade AEA and other NAEs. AEA can undergo oxidative metabolism by cyclooxygenase-2 (COX-2) to produce proalgesic metabolites such as prostamide $F_{2\alpha}$, application of which increases the firing rate of dorsal horn nociceptive neurons and produces thermal hyperalgesia.[35] COX-2 expression is inducible at sites where inflammation occurs,[36] so it is possible that following capsaicin administration, COX-2 mediated generation of proalgesic prostamides could also occur. Intravenous administration of exogenous AEA to FAAH KO mice leads to a dramatic increase in the formation of several prostamide signaling molecules relative to WT mice and non-treated controls,[37] but this observation does not imply that endogenous AEA behaves similarly under physiological conditions. In the absence of enhanced FAAH activity, excess levels of AEA could result in increased prostamide production in FAAH KO mice. By contrast, in WT mice, AEA is likely to be preferentially degraded by FAAH instead of being shunted into prostamide synthesis.

A striking observation of our studies was that capsaicin decreased levels of 2-AG in lumbar spinal cord. In this study, lumbar spinal cords were dissected 1 h post-capsaicin, to represent a time point associated with maximal capsaicin-evoked central sensitization to mechanical and heat stimulation. Such changes could potentially contribute to the development of secondary hyperalgesia. As inhibition of 2-AG metabolism in the spinal cord promotes analgesia,[38] a loss in 2-AG-mediated antinociceptive tone could potentially contribute to the increased pain behaviors observed in FAAH KO mice.

Apart from potentially increasing AEA availability for direct binding to TRPV1, FAAH inhibition can also modulate the function of TRPV1 in ways that may increase pain responsiveness. The agonist binding site for TRPV1 is presumed to be intracellular,[39] and, consequently, raising intracellular concentrations of AEA has been postulated to increase its potency at TRPV1 by increasing levels of endogenous ligand available to bind at this binding site.[40] As AEA is rapidly metabolized, inhibition of FAAH may increase the efficacy of AEA at TRPV1, again, by increasing ligand available to bind at this site.[41–43] Other NAE's that were increased in the lumbar spinal cords of FAAH KO versus WT mice may also produce an entourage effect, increasing the ability of AEA to activate TRPV1.[44] AEA may also alter the sensitivity of TRPV1 to agonists besides AEA through phosphorylation of TRPV1 via protein kinase C (PKC). AEA enhances the activation of PKC by phosphatidyl serine,[45] independent of $CB_1$ receptors. In vitro, in *Xenopus* oocytes expressing TRPV1 receptors, repeated

administration of AEA progressively enhances TRPV1-mediated currents, which is preventable by PKC inhibitors.[46] Although controversy exists as to the participation of the different PKC isozymes in facilitation of TRPV1 activity, and whether such effects occur under physiological conditions, supra-elevations of AEA levels produced by genetic deletion of FAAH could potentially lead to PKC activation and phosphorylation of TRPV1 in FAAH KO mice. Heightening the ability of TRPV1 to respond to agonists like capsaicin or AEA produced upon demand in response to capsaicin stimulation, could render FAAH KO mice more susceptible to TRPV1-mediated central sensitization. Consistent with this hypothesis, in the present study, the development of central sensitization and possibly secondary hyperalgesia, was indeed found to be TRPV1 dependent. AMG9810 eliminated heat and mechanical hypersensitivity in FAAH KO mice, but not in WT mice. This genotype-specific blockade of the capsaicin-evoked pro-nociceptive phenotype indicates that deletion of FAAH may have somehow altered TRPV1 receptor function, and, consequently, antagonizing TRPV1 receptors produces a return to WT levels of capsaicin-responsiveness in FAAH KO mice.

While $CB_1$ agonists have traditionally been thought to promote analgesia, emerging lines of experimental evidence also indicate that, under certain circumstances, activation of the $CB_1$ receptor may elevate pain responsiveness. $CB_1$-mediated inhibition of inhibitory interneuron circuitry within the dorsal horn has been postulated to account for the development of secondary hyperalgesia following capsaicin administration.[16] Upon endocannabinoid production, such as that generated by intense activation of metabotropic glutamate receptor 1/5 (mGluR1/5) during nociceptive processing, endocannabinoid mediated stimulation of $CB_1$ receptors may decrease release of GABA and glycine, preventing the generation of inhibitory post-synaptic currents (IPSCs). By removing this inhibitory input, glutamatergic nociceptive neurons within the dorsal horn may be rendered more excitable to otherwise non-painful stimuli. In vitro, applications of mixed $CB_1/CB_2$ agonists, and mGluR1/5 agonists reduce the generation of IPSCs, effects that are reversed by $CB_1$ and mGluR1/5 antagonists.[16] These results indicate mGluR1/5-mediated production of endocannabinoids and subsequent activation of $CB_1$ receptor activation can effectively silence inhibitory input within dorsal horn interneurons. In vivo, intrathecal delivery of mlGuR1/5 and $CB_1$ antagonists attenuates capsaicin-evoked mechanical hypersensitivity, whereas inhibition of endocannabinoid reuptake or metabolism via FAAH prolongs hyperalgesia.[16] Under other circumstances, activation of mGluR5 at the spinal level has been shown to enhance endogenous analgesia (evoked by exposure to a foot shock stressor) in a $CB_1$-dependent manner in a rat model of endocannabinoid-mediated stress-induced analgesia under conditions in which 2-AG is also elevated.[47] Both global deletion of $CB_1$ and selective deletion of $CB_1$ receptors on inhibitory interneuron circuitry prevents the development of mechanical hypersensitivity in response to capsaicin administration.[16] As the increased susceptibility to capsaicin-evoked pain observed in FAAH KO mice was $CB_1$ receptor dependent, endocannabinoid-mediated inhibition of IPSCs coupled with decreased clearance of endocannabinoids in FAAH KO mice could account for the FAAH KO-dependent increases in nocifensive behavior as well as thermal and mechanical hypersensitivity observed in the current study. The lack of effects of antagonist treatments in WT mice indicates that this pro-nociceptive phenotype is specific for FAAH KO mice, and further suggests that decreasing endocannabinoid clearance via FAAH has the potential to increase pain responsiveness in the capsaicin model. The analgesic effects of $CB_1$ receptor stimulation are likely more complex than previously thought. While $CB_1$ antagonists have been reported to attenuate formalin-evoked pain at low doses,[48] higher doses potentiate formalin-evoked pain (i.e., under conditions in which endocannabinoids are also likely to be mobilized).[49] $CB_1$ antagonists also reduce heat and mechanical hyperalgesia produced by burn injury,[50] indicating the pro-nociceptive effects of $CB_1$ receptor activation may extend to other TRPV1-dependent pain states beyond capsaicin-evoked pain. Furthermore, while the $CB_1$ antagonist SR141716A can also act as an antagonist at TRPV1,[42] AM251 does not,[51] indicating that in the current study, the antinociceptive effects of AM251 cannot be attributed to TRPV1 antagonism. It is also important to note that AM251 did not alter capsaicin-evoked hypersensitivity in WT mice in the present study, suggesting that genetic deletion of FAAH produced a cellular milieu in which a pro-nociceptive phenotype was eliminated by either $CB_1$ or TRPV1 antagonists.

## Conclusions

In conclusion, our studies suggest that the therapeutic efficacy of FAAH inhibition may be restricted to specific types of pain. While we confirm that genetic deletion of FAAH produces hypoalgesia in response to hot water tail immersion, and formalin- and carrageenan-induced pain, our studies also suggest that genetic deletion of FAAH drastically alters the lipidome and enhances both nocifensive behavior and behavioral sensitization to mechanical and heat stimulation that is induced by challenge with the TRPV1 agonist capsaicin. This pro-nociceptive phenotype was observed to both mechanical and heat stimulation. Whether similar changes in pain responsiveness also occur under conditions of chronic

dosing with pharmacological inhibitors of FAAH, though plausible, remains to be determined. Additional research is needed to determine the exact mechanisms by which genetic deletion of FAAH produces elevated pain responsiveness to capsaicin and whether or not this behavioral phenotype is specific for TRPV1-initiated states of behavioral sensitization. Regardless, the increase in pain responsiveness observed in FAAH KO mice in the present study merits further consideration if chronic pharmacological inhibition of FAAH is to be pursued as an avenue for the treatment of disease states in humans, especially for inflammatory pain conditions.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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