

Functional disassociation of the central and peripheral fatty acid amide signaling systems

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Fatty acid amides (FAAs) constitute a large class of endogenous signaling lipids that modulate several physiological processes, including pain, feeding, blood pressure, sleep, and inflammation. Although FAAs have been proposed to evoke their behavioral effects through both central and peripheral mechanisms, these distinct signaling pathways have remained experimentally challenging to separate. Here, we report a transgenic mouse model in which the central and peripheral FAA systems have been functionally uncoupled. Mice were generated that express the principle FAA-degrading enzyme FAA hydrolase (FAAH) specifically in the nervous system (FAAH-NS mice) by crossing FAAH^{-/-} mice with transgenic mice that express FAAH under the neural specific enolase promoter. FAAH-NS mice were found to possess wild-type levels of FAAs in the brain and spinal cord, but significantly elevated concentrations of these lipid transmitters in peripheral tissues. This anatomically restricted biochemical phenotype correlated with a reversion of the reduced pain sensitivity of FAAH^{-/-} mice, consistent with the FAA anandamide producing this effect by acting on cannabinoid receptors in the nervous system. Interestingly, however, FAAH-NS mice still exhibited an antiinflammatory phenotype similar in magnitude to FAAH^{-/-} mice, indicating that this activity, which was not blocked by cannabinoid receptor antagonists, was mediated by peripherally elevated FAAs. These data suggest that the central and peripheral FAA signaling systems regulate discrete behavioral processes and may be targeted for distinct therapeutic gain.

Historically considered to serve as structural components of cell membranes, lipids have more recently been recognized to act as fundamental signaling molecules that regulate a diversity of physiological processes (1). In this regard, the fatty acid amide (FAA) class of signaling lipids, which includes the endogenous cannabinoid (“endocannabinoid”) *N*-arachidonoyl ethanolamine (anandamide) (2), is particularly intriguing in that it has been implicated in the modulation of a large number of behavioral processes, including pain (3–6), feeding (7, 8), blood pressure (9), sleep (10), and inflammation (6, 11). Interestingly, FAAs have been proposed to produce their behavioral effects by acting in both the central nervous system (CNS) (3, 8, 10) and peripheral tissues (4–6, 7, 9). Nonetheless, to date, uncoupling the central and peripheral functions of endogenous FAAs has proven experimentally difficult to accomplish, leaving fundamental questions unanswered regarding the mechanism of action of these lipid transmitters.

The levels of FAAs are controlled *in vivo* by a balance between enzymatic biosynthesis and degradation (12, 13). One key protein involved in FAA metabolism is the integral membrane enzyme FAA hydrolase (FAAH) (14), which degrades a wide range of endogenous FAAs to their corresponding acids (15–18). Mice in which FAAH has been genetically deleted (FAAH^{-/-} mice) (19) possess dramatically elevated endogenous levels of FAAs in several brain regions (20), confirming the primary role that this enzyme plays in FAA catabolism in the CNS. FAAH is also expressed in several peripheral tissues (14), suggesting that this enzyme may regulate FAA signaling

throughout the organism. Consistent with this notion, the peripheral administration of anandamide to FAAH^{+/+} and FAAH^{-/-} mice leads to significantly higher levels of this FAA in all FAAH^{-/-} tissues examined (21).

To separately examine the function of endogenous FAAs in the nervous system and peripheral tissues, we report here the generation and characterization of a transgenic mouse model in which the expression of FAAH has been restricted to the nervous system. These animals display a discrete subset of the biochemical and behavioral phenotypes observed in FAAH^{-/-} mice, thus providing key insights into the distinct functions played by the central and peripheral FAA signaling systems *in vivo*.

Materials and Methods

Generation of FAAH-Nervous System (FAAH-NS) Mice. The full-length mouse FAAH cDNA was excised from the pcDNA3 vector (22) and subcloned into the pNSE-Ex4 vector by blunt end cloning for expression under the neural-specific enolase (NSE) promoter (23). This FAAH-pNSE construct was linearized and microinjected into CByB6F2 embryos (F₂ hybrids of BALB/cByJ and C57BL/6J mice). Transgenic FAAH-neural-specific enolase (FAAH-NSE) mice were identified by Southern blotting by using a probe corresponding to base pairs 184–955 of the mouse FAAH cDNA. FAAH^{+/-} and FAAH^{-/-} mice were generated as described (19) and intercrossed with mice heterozygous for the FAAH-NSE transgene (NS⁺). All studies were conducted with littermates from outbred crosses of FAAH^{+/-}/NS⁺ and FAAH^{-/-}/NS⁻ mice, with the exception of the behavioral studies shown in Fig. 4, which were carried out with littermates from inbred FAAH^{+/-}/NS⁺ and FAAH^{-/-}/NS⁻ mice crosses generated by backcrossing both the FAAH-NSE and FAAH^{+/-} transgenic lines onto the C57BL/6 strain for five generations. FAAH^{+/-}/NS⁺ and FAAH^{-/-}/NS⁻ crosses provided sufficient mice of each relevant group for analysis: FAAH^{+/-}/NS⁻ (FAAH^{+/-}), FAAH^{-/-}/NS⁻ (FAAH^{-/-}), and FAAH^{-/-}/NS⁺ (FAAH-NS) mice. FAAH^{+/-}/NS⁺ mice were not analyzed in these studies. Previous studies have shown that FAAH^{+/-} mice exhibit biochemical and behavioral properties similar to those of FAAH^{+/+} mice (19, 24) and are therefore a suitable control group for analysis.

Determination of FAAH Expression and Activity. FAAH Western blots were conducted with total tissue homogenates by using polyclonal anti-FAAH antibodies (25) as described (19). Neutrophils were isolated from mouse femur bone marrow following previously described methods (26). For immunofluorescence

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Abbreviations: CB1 receptor, central cannabinoid receptor; CB2 receptor, peripheral cannabinoid receptor; FAA, fatty acid amide; FAAH, FAA hydrolase; FAAH-NS, FAAH-nervous system; NSE, neural-specific enolase; PEA, *N*-palmitoyl ethanolamine; OEA, *N*-oleoyl ethanolamine.

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Table 1. FAAH activities in tissues from FAAH^{+/-}, FAAH^{-/-}, and FAAH-NS mice as measured by the hydrolysis of the FAAH substrate OEA (pmol/min per mg)

	Brain	Spinal cord	Testis	Liver	Kidney	Spleen
FAAH ^{+/-}	133 ± 20	72 ± 13	30 ± 8	384 ± 69	36 ± 5	12 ± 3
FAAH ^{-/-}	2 ± 1**	2 ± 1**	0.4 ± 0.1 ^{††}	4 ± 0.4 ^{††}	0.6 ± 0.1 ^{††}	0.2 ± 0.1 ^{††}
FAAH-NS	326 ± 25	188 ± 32	0.4 ± 0.1 ^{††}	4 ± 0.4 ^{††}	0.5 ± 0.1 ^{††}	0.1 ± 0.1 ^{††}

***P* < 0.01 for FAAH^{-/-} mice vs. FAAH^{+/-} mice or FAAH-NS mice; ^{††}*P* < 0.01 for FAAH^{-/-} mice or FAAH-NS mice vs. FAAH^{+/-} mice. Data are reported as means ± SE. *n* = 3 mice per group.

volume (saline) into the right paw by using a 30-gauge needle. Previous studies have shown that, under these conditions, inflammation peaks at ≈5 h postinjection (24), and therefore inflammation was recorded at this time point. The diameter of each paw was measured both before and 5 h after carrageenan by using electronic digital calipers (Mitutoyo, Aurora, IL). No genotype differences were noted in baseline paw diameters (average baseline paw diameter = 2.45 ± 0.04 mm; *P* = 0.77). Inflammation data were expressed as the ipsilateral paw diameter at 5 h – contralateral paw diameter at 5 h (expressed to the nearest ±0.01 mm). For pharmacological studies, anandamide (50 mg/kg), SR141716 (3 mg/kg), and SR144528 (3 mg/kg) were administered i.p. in a mixture of 1:1:18 ethanol:Emulphor:saline (vehicle, 10 μl/g of body weight). For all studies, the data reported were from a combination of male and female mice from 2–5 months of age (no significant sex differences were observed for any genotype).

Results

Generation of Mice that Selectively Express FAAH in the Nervous System (FAAH-NS Mice). To separately examine the function of FAA transmitters in central and peripheral tissues, we created an animal model in which FAAH was selectively expressed in the nervous system. These animals, referred to here as FAAH-NS mice, were created by crossing transgenic mice that express FAAH under the NSE promoter (23) with FAAH^{-/-} mice (19) (Fig. 1A). In all of the studies described below, FAAH-NS mice were compared with FAAH^{+/-} and FAAH^{-/-} littermates lacking the FAAH-NSE transgene (see *Materials and Methods* for more details).

Western blotting (Fig. 1B) and immunofluorescence analysis (Fig. 1C) revealed that FAAH-NS mice strongly expressed FAAH throughout the nervous system but exhibited no detectable FAAH protein in any of the peripheral tissues examined. Likewise, the brains and spinal cords of FAAH-NS mice expressed high levels of FAAH activity [≈3- to 4-fold greater activity than FAAH^{+/-} mice (Table 1) and 1.8-fold greater activity than FAAH^{+/+} mice (brain^{+/+} = 194 ± 8 pmol of OEA/min per mg; spinal cord^{+/+} = 101 ± 34 pmol of OEA/min per mg)], whereas peripheral tissues displayed negligible FAAH activity (equivalent to FAAH^{-/-} tissues) (Table 1).

The Behavioral Effects of Anandamide in FAAH^{+/-}, FAAH^{-/-}, and FAAH-NS mice. To test whether the FAAH protein expressed in the nervous system of FAAH-NS mice was functional *in vivo*, we compared the behavioral effects of anandamide in these animals with those observed in FAAH^{+/-} and FAAH^{-/-} mice. Previous studies have shown that FAAH^{-/-} mice display highly exaggerated central cannabinoid (CB1) receptor-dependent behavioral responses to anandamide in the tetrad test for cannabinoid activity (19). Consistent with these findings, anandamide was found to produce strong hypomotility, catalepsy, hypothermia, and analgesia in FAAH^{-/-} mice (Fig. 2). In contrast, in FAAH^{+/-} and FAAH-NS mice, anandamide failed to produce significant effects in any of these assays, with the exception of locomotor activity, where a moderate reduction in motility was observed in both genotypes (Fig. 2). All of the behavioral effects of anandamide in FAAH^{-/-} mice were blocked by pretreatment with CB1 antagonist SR141716, but not the peripheral canna-

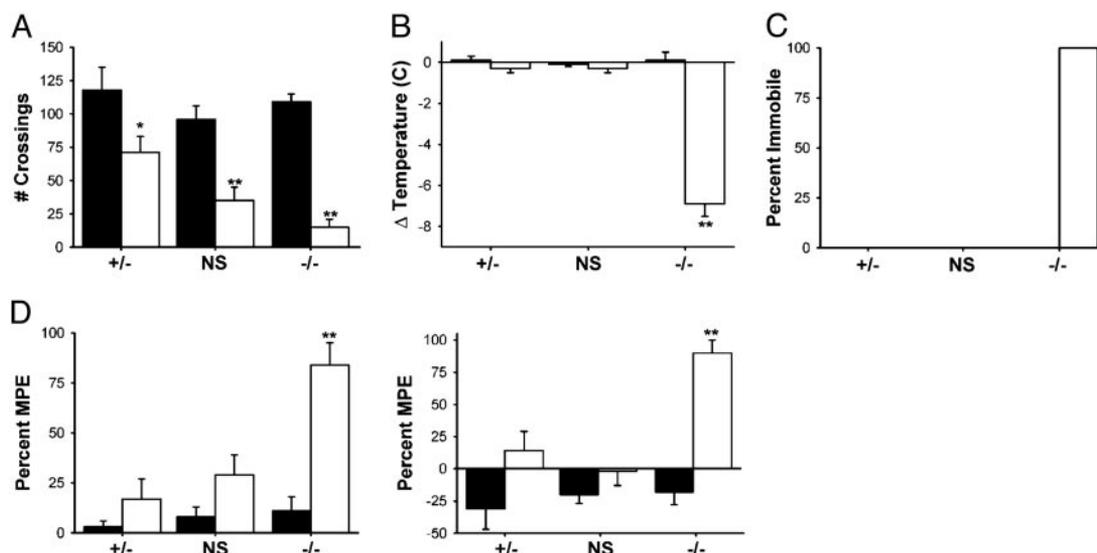


Fig. 2. Pharmacological effects of anandamide in FAAH^{+/-}, FAAH^{-/-}, and FAAH-NS mice. In FAAH^{-/-} mice, anandamide (50 mg/kg, i.p., open bars) elicited significant hypomotility (A), hypothermia (B), catalepsy (C), and analgesia (D Left, tail immersion test; Right, hot plate test) compared with treatment of vehicle (filled bars). In contrast, in FAAH^{+/-} and FAAH-NS mice, anandamide only produced hypomotility (A), but not hypothermia (B), catalepsy (C), or analgesia (D). *, *P* < 0.05, **, *P* < 0.01 for anandamide-treated vs. vehicle-treated mice of the same genotype (planned comparison). Results are presented as means ± SE. *n* = 6–11 mice per group. MPE, maximum possible effect.

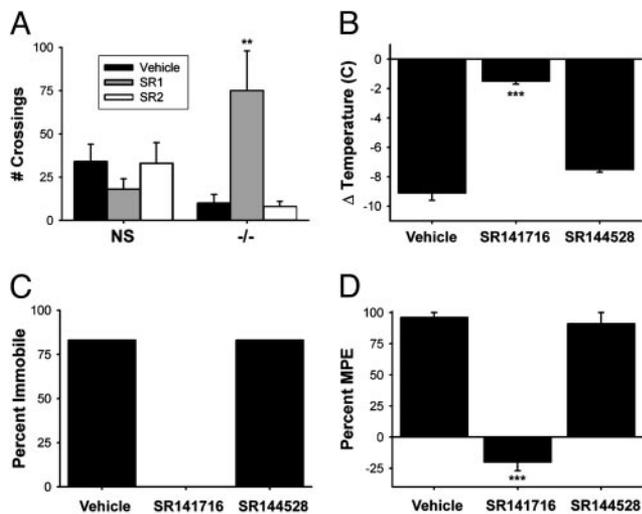


Fig. 3. The effects of cannabinoid receptor antagonists on the pharmacological activity of anandamide in *FAAH*^{-/-} and *FAAH-NS* mice. The hypomotility (A), hypothermia (B), catalepsy (C), and antinociception (D, hot plate test) induced by anandamide (50 mg/kg, i.p.) in *FAAH*^{-/-} mice were blocked by pretreatment (10 min) with the CB1 antagonist SR141716 (SR1, 3 mg/kg, i.p.), but not the CB2 antagonist SR144528 (SR2, 3 mg/kg, i.p.) or vehicle. Neither SR141716 nor SR144528 blocked the hypomotility induced by anandamide in *FAAH-NS* mice (A). **, $P < 0.01$, ***, $P < 0.001$ for SR141716-treated vs. vehicle- or SR144528-treated *FAAH*^{-/-} mice (planned comparisons). Results are presented as means \pm SE. $n = 6$ mice per group.

binoid (CB2) receptor antagonist SR144528 (Fig. 3), providing further evidence that these activities occurred through a central mechanism. Curiously, neither the CB1 nor CB2 antagonist blocked the hypomotility induced by anandamide in *FAAH-NS* (Fig. 3A) or *FAAH*^{+/-} (data not shown) mice, indicating that these effects were due to a noncannabinoid mechanism. One potential explanation for these findings is that the hypomotility effects of anandamide in *FAAH-NS* and *FAAH*^{+/-} mice were due to the rapid FAAH-mediated conversion of this FAA to arachidonic acid in the nervous system. Consistent with this notion, previous studies have shown that arachidonic acid, which does not bind cannabinoid receptors, reduces locomotor activity in mice (28). Collectively, these results demonstrate that the amplified CB1-dependent behavioral effects of anandamide

observed in *FAAH*^{-/-} mice are eradicated in *FAAH-NS* mice, indicating that specific expression of FAAH in the nervous system is sufficient to block the neuropharmacological activities of this endocannabinoid.

The Endogenous Levels of FAAs in Central and Peripheral Tissues from *FAAH*^{+/-}, *FAAH*^{-/-}, and *FAAH-NS* Mice. We next measured the endogenous levels of representative FAAs in central and peripheral tissues of *FAAH*^{+/-}, *FAAH*^{-/-}, and *FAAH-NS* mice by isotope dilution liquid chromatography-mass spectrometry (20, 27). In all tissues examined, *FAAH*^{-/-} mice possessed significantly elevated levels of FAAs compared with *FAAH*^{+/-} mice (Table 2). In the brain and spinal cord, FAAs were, in general, elevated at least 10-fold and, in the most dramatic cases (e.g., anandamide in the spinal cord), elevated over 30-fold in *FAAH*^{-/-} mice compared with *FAAH*^{+/-} mice. Strikingly, *FAAH-NS* mice were found to express CNS levels of FAAs that were equivalent to those in *FAAH*^{+/-} mice, indicating that the reintroduction of FAAH into the brain and spinal cord completely reverted the elevated expression of FAAs observed in these tissues from *FAAH*^{-/-} mice. In contrast, in each peripheral tissue examined, *FAAH-NS* mice possessed FAA levels that were significantly elevated compared with *FAAH*^{+/-} mice and were similar to the FAA levels observed in *FAAH*^{-/-} mice. The relative magnitude of expression of FAAs in peripheral tissues depended on both the individual FAA and tissue under examination, ranging from modest (≈ 1.5 -fold) to dramatic (>10 -fold) elevations in *FAAH*^{-/-} mice and *FAAH-NS* mice compared with *FAAH*^{+/-} mice. The basis for these differences in the relative levels of FAAs in peripheral tissues from *FAAH*^{+/-}, *FAAH*^{-/-}, and *FAAH-NS* mice is unknown, but may reflect the tissue-specific expression of enzymes involved in FAA biosynthesis [e.g., FAA biosynthetic enzymes are preferentially expressed in the testis and nervous system (29)] or additional enzymes that contribute to FAA catabolism (30, 31). Regardless, these data provide strong evidence that the central and peripheral FAA systems have been functionally uncoupled in *FAAH-NS* mice because these animals possess wild-type levels of FAAs in the nervous system, but constitutively elevated levels of these signaling lipids in peripheral tissues.

Comparative Analysis of the Pain and Inflammatory Responses of *FAAH*^{+/-}, *FAAH*^{-/-}, and *FAAH-NS* Mice. *FAAH*^{-/-} mice display a number of intriguing phenotypes, including reductions in pain

Table 2. Endogenous FAA levels in tissues from *FAAH*^{+/-}, *FAAH*^{-/-}, and *FAAH-NS* mice (pmol/g wet tissue)

	Brain	Spinal cord	Testis	Liver	Kidney
<i>FAAH</i> ^{+/-}					
Anandamide	10 \pm 2	5.6 \pm 1.3	11 \pm 4	2.2 \pm 0.1	ND
PEA	241 \pm 63	1036 \pm 45	149 \pm 33	104 \pm 23	221 \pm 28
OEA	102 \pm 17	411 \pm 36	76 \pm 10	43 \pm 5	116 \pm 10
<i>FAAH</i> ^{-/-}					
Anandamide	62 \pm 6**	173 \pm 9**	197 \pm 28 ^{††}	12 \pm 1 ^{††}	ND
PEA	2650 \pm 151**	9455 \pm 806**	564 \pm 38 ^{††}	441 \pm 35 ^{††}	312 \pm 11 [†]
OEA	1543 \pm 136**	6402 \pm 236**	239 \pm 12 ^{††}	137 \pm 7 ^{††}	204 \pm 19 [†]
<i>FAAH-NS</i>					
Anandamide	11 \pm 3	4.8 \pm 0.5	109 \pm 12 ^{††}	9.4 \pm 1.2 ^{††}	ND
PEA	230 \pm 44	1412 \pm 80	525 \pm 23 ^{††}	285 \pm 52 [†]	376 \pm 41 [†]
OEA	88 \pm 11	543 \pm 56	224 \pm 6 ^{††}	105 \pm 13 ^{††}	231 \pm 31 [†]

PEA, *N*-palmitoyl ethanolamine; OEA, *N*-oleoyl ethanolamine. **, $P < 0.01$ for *FAAH*^{-/-} mice vs. *FAAH*^{+/-} mice or *FAAH-NS* mice; †, $P < 0.05$, ††, $P \leq 0.01$ for *FAAH*^{-/-} mice or *FAAH-NS* mice vs. *FAAH*^{+/-} mice. ND, not determined (kidney samples possessed an abundant contaminating metabolite of equivalent mass to the sodium adduct of anandamide that obscured detection of this FAA). Data are reported as means \pm SE. $n = 3$ –6 mice per group.

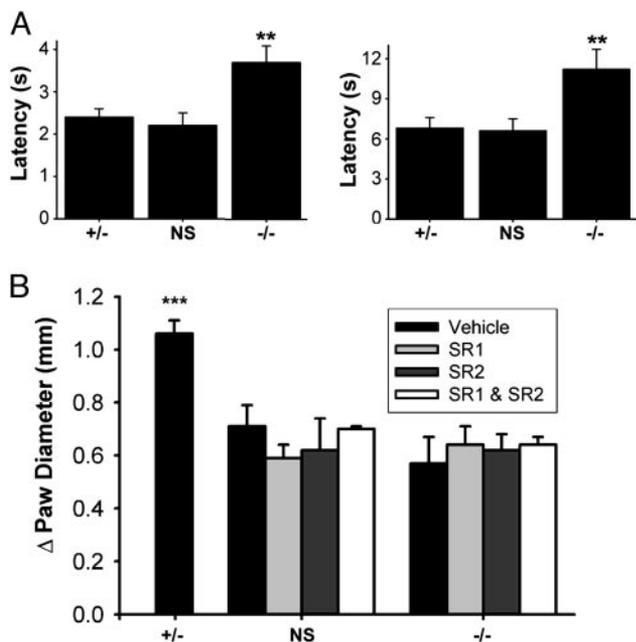


Fig. 4. Analysis of the nociceptive and inflammatory responses of FAAH^{+/-}, FAAH^{-/-}, and FAAH-NS mice. (A) FAAH^{-/-} mice, but not FAAH-NS mice, show reduced pain sensitivity in both the tail-immersion (Left) and hot plate (Right) assays compared with FAAH^{+/-} mice. **, $P < 0.01$ for FAAH^{-/-} mice vs. FAAH^{+/-} or FAAH-NS mice (planned comparison). (B) Both FAAH^{-/-} and FAAH-NS mice exhibit reduced paw edema in the carrageenan inflammation assay compared with FAAH^{+/-} mice. The reduced inflammatory responses of FAAH^{-/-} and FAAH-NS mice were not reversed by the CB1 antagonist SR141716 (SR1) or CB2 antagonist SR144528 (SR2) [or a combination of both antagonists (SR1 and SR2)]. Experiments were performed with equal numbers of male and female littermates from 2–5 months of age. ***, $P < 0.001$ for FAAH^{+/-} mice vs. each of the groups of FAAH^{-/-} and FAAH-NS mice (Bonferroni *t* test). Results shown as means \pm SE, $n = 6$ –10 mice per group.

(19, 24) and inflammation (24, 32), suggesting that the chemical inhibition of this enzyme may prove useful for treating several human pathologies (15–18). Consistent with this notion, FAAH inhibitors have been found to produce analgesia and anxiolysis in rodents (33). Nonetheless, alterations in FAAH activity may also lead, in certain instances, to unwanted effects, as has been recently suggested by the identification of a functional polymorphism in human FAAH associated with illicit drug abuse (34). These findings, coupled with the myriad central and peripheral functions ascribed to FAAH's FAA substrates, emphasize the importance of defining the anatomical site(s) of action for these lipid transmitters *in vivo* to guide drug discovery efforts and illuminate, for example, whether FAAH inhibitors must cross the blood–brain barrier to achieve a desired pharmacological effect. With this objective in mind, we compared the naive behavioral responses of FAAH^{+/-}, FAAH^{-/-}, and FAAH-NS mice to pain and inflammatory stimuli. Strikingly, in both the tail-immersion and hot plate assays, the reduced pain behavior observed in FAAH^{-/-} mice was completely abolished in FAAH-NS mice (Fig. 4A), indicating that these analgesic phenotypes, which have previously been shown to be dependent on CB1 (19, 24), but not CB2 receptors (24), are mediated by the central FAA system. In contrast, both FAAH^{-/-} mice and FAAH-NS mice showed equivalent reductions in paw edema compared with FAAH^{+/-} mice in the carrageenan model of inflammation (Fig. 4B). Interestingly, the reduced inflammatory responses of FAAH^{-/-} and FAAH-NS mice were unaffected by pretreatment with the CB1 antagonist SR141716 or the CB2 antagonist SR144528 (or a combination of both antagonists).

These results suggest that the antiinflammatory phenotype observed in FAAH^{-/-} and FAAH-NS mice is due to peripherally elevated FAAs acting through a cannabinoid receptor-independent mechanism.

Discussion

We describe herein the generation and characterization of a transgenic mouse model in which the central and peripheral FAA signaling systems have been functionally uncoupled. The restricted expression of the primary FAA-degrading enzyme FAAH in the nervous system produced animals (FAAH-NS mice) that possess wild-type levels of FAAs in the brain and spinal cord, but significantly elevated concentrations of these signaling lipids in peripheral tissues. This anatomically constrained biochemical phenotype correlated with a reversion of both the reduced pain responses and supersensitivity to anandamide observed in FAAH^{-/-} mice. These data, combined with previous studies showing that the altered pain sensitivity and pharmacological effects of anandamide in FAAH^{-/-} mice are blocked by CB1 antagonists (19, 24), argue that these behavioral responses are mediated by FAAs (likely, anandamide) acting on CB1 receptors expressed in the nervous system. In contrast, FAAH-NS mice were found to exhibit reduced inflammation similar in magnitude to that observed in FAAH^{-/-} mice (Fig. 4B). Interestingly, the reduced inflammatory responses of FAAH^{-/-} and FAAH-NS mice were not blocked by the CB1 antagonist SR141716 or the CB2 antagonist SR144528 (Fig. 4B). In previous studies (24), we also found no effect of SR141716 on carrageenan-induced inflammation in FAAH^{-/-} mice, but observed a partial ($\approx 50\%$) reversal of this phenotype with SR144528; however, this latter effect did not achieve statistical significance. Collectively, these results indicate that the antiinflammatory phenotype exhibited by FAAH^{-/-} and FAAH-NS mice is due to peripherally elevated FAAs acting through a cannabinoid receptor-independent mechanism. In this regard, the FAA PEA, which was elevated in each peripheral tissue examined in FAAH-NS and FAAH^{-/-} mice, has been known for several decades to possess antiinflammatory properties and has even entered clinical trials for the treatment of inflammatory disorders (35). Although PEA has been suggested to act on CB2-like receptors (4, 5), this FAA also produces pharmacological effects that are unaffected by the CB2 antagonist SR144528, including potentiation of microglial migration (11) and reduction of inflammation in the rat carrageenan model (36). Further studies will be required to determine whether endogenously produced PEA (or possibly another FAA) is responsible for the antiinflammatory phenotype observed in FAAH^{-/-} and FAAH-NS mice. Regardless of the precise molecular mechanism(s), the findings reported herein suggest an attractive therapeutic alternative in which peripherally active FAAH inhibitors may be used to reduce inflammation without causing potentially undesired nervous system side effects.

More generally, FAAH-NS mice should constitute a valuable animal model for mechanistic investigations of the FAA signaling system. For example, in addition to pain and inflammation, several other physiological processes, such as feeding (7, 8) and blood pressure (9, 37), seem to be regulated by both central and peripheral FAAs. Studies with FAAH-NS mice should help to dissect the relative contribution of these anatomically distinct signaling pathways, thereby increasing our understanding of the specific functions played by FAA transmitters in the nervous system and peripheral tissues.

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