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


†The Skaggs Institute for Chemical Biology and Departments of Cell Biology and Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037; and ‡Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298

Edited by Tomas Hökfelt, Karolinska Institutet, Stockholm, Sweden, and approved June 2, 2004 (received for review February 24, 2004)

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-6X4 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 heterozygotic for the FAAH-NSE transgene (NS+). All studies were conducted with littersmates 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 littersmates from inbred FAAH+/+NS+ and FAAH−/−NS− mice crosses generated by backcrossing both the FAAH-NSE and FAAH−/− transgenic lines onto the C57BL/6J 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

PNAS | July 20, 2004 | vol. 101 | no. 29 | 10821–10826

© 2004 by The National Academy of Sciences of the USA

This paper was submitted directly (Track II) to the PNAS office.

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-palmityl ethanolamine; OEA, N-oleoyl ethanolamine.

*To whom correspondence should be addressed. E-mail: cravatt@scripps.edu.
studies, tissue specimens were prepared and analyzed on a Zeiss Axiovert STV100 microscope with a Bio-Rad MRC100 confocal system as described (19). FAAH signals were detected by using an anti-rabbit IgG conjugated to Alexa 488 (Molecular Probes) and specimens were counterstained with propidium iodide. Imaging of FAAH+/−, FAAH−/−, and FAAH-NS specimens was performed with equivalent laser power and signal amplification settings. FAAH activity assays were conducted by incubating total tissue homogenates (100–400 μg of protein in 50 mM Tris buffer, pH 8.0) with 100 μM substrate [14C]oleoyl ethanolamine ([14C]OEA) at 37°C for 1 h. The conversion of [14C]OEA to oleic acid was quantified by thin layer chromatography as described (14) and expressed as pmol OEA per min per mg of protein.

**Determination of Tissue Levels of FAAs.** The levels of three representative FAAs [anandamide, OEA, and N-palmitoyl ethanolamine (PEA)] in mouse tissues were measured by isotope-dilution liquid chromatography mass spectrometry as described (20, 27) by using an Agilent (Palo Alto, CA) 1100 series HPLC coupled to an Agilent MSD mass spectrometer in the selected-ion monitoring mode.

**Behavioral Analysis of FAAH+/−, FAAH−/−, and FAAH-NS Mice.** Locomotor activity was assessed by placing each mouse in a clear Plexiglass cage (40 l × 22 w × 19 h cm) that was marked in 7 × 7-cm-square grids on the floor of the cage. The number of grids that was traversed by the hind paws was counted over a 5-min duration (for anandamide pharmacology studies, locomotor activity was measured 15–20 min postinjection). Nociception was assessed in two separate assays: tail immersion and hot plate. In the tail immersion assay, each mouse was hand-held with ~1 cm of the tip of the tail immersed into a water bath maintained at 56°C, and the latency for a mouse to withdraw its tail was scored (cutoff for a response was 10 s). For the anandamide pharmacology studies, the tail immersion assay was conducted at ~20 min postinjection, and the data were expressed as percent maximum possible effect (%MPE), where %MPE = 100 × (postinjection latency − preinjection latency)/(10 − preinjection latency). The hot plate assay was conducted at 56°C, and the latency to jump or lick/shake a hind paw was scored. For the anandamide pharmacology studies, the hot plate assay was conducted ~25 min postinjection, and the data were expressed as %MPE by using the same formula as described above with a 60-s cutoff. Catalepsy was evaluated by using the bar test, in which the front paws of each subject were placed on a horizontal metal rod (0.75-cm diameter) that was elevated 4.5 cm above the surface. Mice that remained motionless with their paws on the bar for 10 s were scored as cataleptic. Rectal temperature was determined by inserting a thermocouple probe 1.2 cm into the rectum. Both catalepsy and rectal temperature were recorded ~60 min postinjection. Inflammation was induced by giving an intraplantar injection of 0.3% λ-carrageenan (Sigma) in a 20-μl

![Fig. 1.](https://www.pnas.org/cgi/doi/10.1073/pnas.0401292101Cravatt et al.)
Materials and Methods

In vivo

... 

Results

Generation of Mice that Selectively Express FAAH in the Nervous System (FAAH-NS Mice).

To separately examine the function of FAAH transmitters in central and peripheral tissues, we created an animal model in which FAAH was selectively expressed in the nervous system. 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-

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 (23) with FAAH+/+ mice. Otherwise significant differences in FAAH activity 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).

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)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>FAAH+/−</th>
<th>FAAH−/−</th>
<th>FAAH-NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>133 ± 20</td>
<td>2 ± 1**</td>
<td>326 ± 25</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>72 ± 13</td>
<td>2 ± 1**</td>
<td>188 ± 32</td>
</tr>
<tr>
<td>Testis</td>
<td>30 ± 8</td>
<td>0.4 ± 0.1††</td>
<td>4.0 ± 4.1††</td>
</tr>
<tr>
<td>Liver</td>
<td>384 ± 69</td>
<td>4 ± 0.4††</td>
<td>4.0 ± 4.1††</td>
</tr>
<tr>
<td>Kidney</td>
<td>36 ± 5</td>
<td>0.6 ± 0.1††</td>
<td>0.5 ± 0.1††</td>
</tr>
<tr>
<td>Spleen</td>
<td>12 ± 3</td>
<td>0.2 ± 0.1††</td>
<td>0.1 ± 0.1††</td>
</tr>
</tbody>
</table>

** P < 0.01 for FAAH−/− mice vs. FAAH+/− mouse 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.

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.

Gravatt et al.
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>
<thead>
<tr>
<th>Table 2. Endogenous FAA levels in tissues from FAAH+/−, FAAH−/−, and FAAH-NS mice (pmol/g wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
</tr>
<tr>
<td>FAAH+/−</td>
</tr>
<tr>
<td>Anandamide</td>
</tr>
<tr>
<td>PEA</td>
</tr>
<tr>
<td>OEA</td>
</tr>
<tr>
<td>FAAH−/−</td>
</tr>
<tr>
<td>Anandamide</td>
</tr>
<tr>
<td>PEA</td>
</tr>
<tr>
<td>OEA</td>
</tr>
<tr>
<td>FAAH-NS</td>
</tr>
<tr>
<td>Anandamide</td>
</tr>
<tr>
<td>PEA</td>
</tr>
<tr>
<td>OEA</td>
</tr>
</tbody>
</table>

*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 < 0.01 for FAAH−/− 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 ± SE. n = 3–6 mice per group.
assays compared with FAAH
responses of FAAH
inflammation (Fig. 4
FAAH-reduced pain sensitivity in both the tail-immersion (Fig. 4
behavioral responses of FAAH
the blood
illuminate, for example, whether FAAH inhibitors must cross
lipid transmitters
importance of defining the anatomical site(s) of action for these
These findings, coupled with the myriad central and peripheral
phism in human FAAH associated with illicit drug abuse (34).
recently suggested by the identification of a functional polymor-
in rodents (33). Nonetheless, alterations in FAAH activity may
observed in FAAH
mice to pain and inflammatory stimuli. Strikingly, in both the
mice was completely abolished in
mice in the carrageenan model of
In contrast, FAAH-NS mice were found to exhibit reduced inflamma-
similar in magnitude to that observed in FAAH
mice (Fig. 4B). Interestingly, the reduced inflammatory responses of
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 (~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.

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). 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.