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Genetic Loss of *Faah* Compromises Male Fertility in Mice[1]

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ABSTRACT

Marijuana is the most commonly used illicit drug. Although there is some indication that reproductive functions in males are impaired in chronic marijuana users, the genetic evidence and underlying causes remain largely unknown. Herein we show that genetic loss of *Faah*, which encodes fatty acid amide hydrolase (FAAH), results in elevated levels of anandamide, an endocannabinoid, in the male reproductive system, leading to compromised fertilizing capacity of sperm. This defect is rescued by superimposing deletion of cannabinoid receptor 1 (*Cnr1*). Retention of *Faah*$^{-/-}$ sperm on the egg zona pellucida provides evidence that the capacity of sperm to penetrate the zona barrier is hampered by elevated anandamide levels. Collectively, the results show that aberrant endocannabinoid signaling via CNR1 impairs normal sperm function. Besides unveiling a new regulatory mechanism of sperm function, this study has clinical significance in male fertility.

anandamide, CNR1, FAAH, male fertility, mouse, sperm, sperm capacitation, sperm motility and transport

INTRODUCTION

There is some evidence that male fertility in humans is negatively regulated by long-term exposure to marijuana extracts (reviewed by Wang et al. [1]). The major psychoactive component of marijuana is Δ^9-tetrahydrocannabinol (THC). Although in vitro experiments have shown that THC exerts adverse effects on sperm function (reviewed by Rossato et al. [2]), there is no in vivo or genetic evidence that cannabinoids impair male fertility. After THC was identified in 1964 [3], research on cannabinoids exploded with the discovery and cloning of two G protein-coupled cannabinoid receptors, brain-type *Cnr1* encoding CNR1 [4, 5] and spleen-type *Cnr2* encoding CNR2 [6]. Around the same time, several endogenous lipid molecules targeting CNR1 and CNR2 were identified, collectively called endocannabinoids. Two of the most studied endocannabinoids are *N*-arachidonoylethanolamide (known as anandamide) and 2-arachidonoylglycerol (2-AG) [7–9]. Anandamide levels are regulated by a balance between the rates of its synthesis and degradation. Anandamide was thought to be produced primarily from *N*-arachidonoylphosphatidylethanolamine (NAPE) by NAPE-hydrolyzing phospholipase D (NAPEPLD) [10]. However, genetic investigations in NAPEPLD-deficient mice [11] and recent identification of other anandamide synthetic pathways [12, 13] demonstrate that regulation of anandamide synthesis is more complex than previously thought. Anandamide is degraded to ethanolamine and arachidonic acid by a membrane-bound fatty acid amide hydrolase (FAAH) [14, 15]. Although FAAH can hydrolyze other endocannabinoids, including 2-AG [16], investigations in *Faah*$^{-/-}$ mice show that FAAH has a major role in regulating the magnitude and duration of anandamide signaling [12, 17].

Sperm undergo a long journey to acquire fertilization capacity [18–20]. Through the process of spermatogenesis, spermatogonia differentiate into highly polarized sperm, which then undergo maturation in the epididymis before capacitation, acquiring motility in the female reproductive tract. After traveling through the uterine lumen and reaching ovulated eggs in the oviduct ampulla, capacitated sperm navigate through cumulus cells surrounding the egg to contact the zona pellucida, the outermost membrane of the egg. On binding to the zona, sperm undergo a Ca^{++}-dependent exocytotic event known as the acrosome reaction, which is essential for their zona penetration and homing into the perivitelline space. After a sperm binds to an egg plasma membrane, the two gametes unite, resulting in egg activation, pronuclear formation, and syngamy. Each step in the process is essential for successful fertilization.

There are reports that endocannabinoids and their receptors are present in the testis and sperm of invertebrates and vertebrates, including sea urchins, frogs, rats, mice, boars, and humans [21]. This conserved expression across species suggests that endocannabinoid signaling has important roles in male reproduction. In vitro studies also showed that endocannabinoid signaling inhibits capacitation of boar sperm in a cAMP-dependent pathway and prevents the acrosome reaction [22] and that anandamide reduces human sperm motility by quenching mitochondrial activity [21]. However, there is no in vivo genetic

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evidence of endocannabinoid signaling affecting male reproductive functions, to our knowledge.

In this study, we used gene-targeted mice for *Faah* to mimic the conditions of long-term exposure to marijuana. We explored roles of cannabinoid and endocannabinoid signaling in male fertility.

MATERIALS AND METHODS

Mice

Targeted deletion of *Faah*, *Cnr1*, or *Cnr2* in mice (129/SvJ-C57BL/6J) has been previously described [17, 23, 24]. Double mutants for *Faah*/*Cnr1* or *Faah*/*Cnr2* were generated using appropriate breeding strategies. Adult wild-type (WT), $Faah^{-/-}$, $Faah^{-/-}/Cnr1^{-/-}$, and $Faah^{-/-}/Cnr2^{-/-}$ mice were housed at an institutional animal care facility according to National Institutes of Health and institutional guidelines. Experiments were conducted on mice between 3 and 4 mo of age. Testes and epididymis from $Faah^{-/-}$ and WT males were processed for anandamide measurement and in situ hybridization.

Western Blotting

Tissue samples were homogenized in lysis buffer (150 mmol/L of NaCl, 1% nonionic detergent, 0.5% deoxycholate, 0.1% SDS, and 50 mmol/L Tris [pH 8]) containing protease and phosphatase inhibitors. The lysates were centrifuged at $9880 \times g$ for 10 min at 4°C. Supernatants (25 μg) were boiled for 5 min in SDS sample buffer. Samples were run on 10% SDS-PAGE gels under reducing conditions and transferred onto nitrocellulose membranes. Membranes were blocked with 10% carnation milk in Tris-buffered saline with 0.1 Tween-20) and probed with antibodies against mouse FAAH (1:1000; custom made by the laboratory of Cravatt et al. [17], CNR1 (1:2000) [25], CNR2 (1:250; Cayman), and β-actin (1:100; Santa Cruz Biotechnology) overnight at 4°C. After thorough washings, blots were incubated in peroxidase-conjugated donkey/anti-goat IgG (1:2000) or donkey/anti-rabbit IgG (1:2000; Jackson/ImmunoResearch), followed by washings. Protein signals were detected using chemiluminescent reagents (Amersham).

Immunohistochemistry

Immunostaining in Bouin solution-fixed paraffin-embedded sections (6 μm) was performed using antibodies specific to FAAH (1:200) [17], CNR1 (1:200) [25], or CNR2 (1:250; Cayman) following antigen retrieval in citrate buffer (pH 6.0) for 10 min in an autoclave. A Histostain-Plus (DAB) kit (Zymed) was used to visualize the antigen. Reddish brown deposits indicate sites of positive immunostaining.

Immunofluorescence

Sperm were isolated from the epididymis of mature WT males and thoroughly washed in PBS. Sperm were fixed with 1% formaldehyde at room temperature for 15 min. After blocking in 1% BSA/PBS containing 0.05% Tween-20, sperm were incubated with CNR1 antibody (1:200; ~500 ng/ml of IgG) [25] with or without blocking peptide overnight at 4°C. After thorough washings, secondary antibodies conjugated with Cy3 (Jackson/ImmunoResearch) were used to detect immunofluorescence signaling. SYTO13 green fluorescence dye (Invitrogen) was used for nuclear staining.

Anandamide Assay

Testis and sperm (100 mg) were pooled separately from five WT or $Faah^{-/-}$ mice in each group ($n = 3–6$) and were assayed for anandamide as previously described [26]. Briefly, the preweighed samples were homogenized in ethyl acetate with 0.5% acetic acid. Immediately before homogenization, $^2H_8$-labeled anandamide was added as an internal standard to a mortar. The homogenate was centrifuged, and the supernatant was dried, reconstituted in chloroform, and purified on a silica-based solid-phase extraction cartridge. The eluent was dried, reconstituted in 1:8 of aqueous silver acetate-methanolic silver acetate, and analyzed by reverse-phase positive-ion electrospray ionization-HPLC-tandem mass spectrometry. Quantification was performed by stable isotope dilution against the octadeuterated internal standard.

In Situ Hybridization

Frozen sections (12 μm) were hybridized with ^{35}S-labeled cRNA probes for mouse *Cnr1* or *Cnr2* as described previously [27]. Sections hybridized with sense probes served as negative controls and showed no positive signals.

In Vitro Fertilization

In vitro fertilization (IVF) was performed as previously described [28]. Briefly, WT females were superovulated by intraperitoneal injections of 5 IU of eCG (Sigma), followed by injections of 5 IU of hCG (Sigma) 48 h later. Cumulus-oocyte complexes were collected from the oviduct ampulla 12–14 h after hCG injection and placed in 100-μl droplets of human tubal fluid (HTF) medium (Chemicon). In most IVF experiments, zona-intact eggs were used. In some IVF experiments, zona-free eggs were used. Cumulus-oocyte complexes were treated with hyaluronidase (Sigma), and cumulus-free eggs were then exposed to acidic Tyrode solution and passed through a pipette several times until zona pellucidae were dissolved. Eggs were washed three times in HTF medium and incubated longer than 1 h to allow surface proteins to recover [29]. Sperm were collected from the cauda of the epididymis and placed into 400 ml of HTF medium to allow capacitation for 2.5 h in a humidified 5% CO_2 incubator at 37°C. Sperm (~$1.2–1.5 \times 10^6$ sperm/ml) were then coincubated with eggs to allow fertilization. After 6 h, sperm were removed, and putative zygotes were placed in 200-μl droplets of potassium simplex optimized medium (Chemicon) and incubated in a humidified 5% CO_2 incubator at 37°C. The cleavage rate (two-cell stage) after 24 h was used as an index of fertilization. Formation of blastocysts at 120 h indicated developmental potential of fertilized embryos.

Evaluation of Sperm-Zona Binding in IVF

After sperm were incubated with eggs for 2 h in IVF experiments, eggs were removed. Attached sperm were stained with propidium iodide and fluorescein isothiocyanate (FITC)-conjugated antibody specific to Izumo protein, generated in the laboratory of Inoue et al. [30].

Analysis of the Acrosome Reaction by Flow Cytometry

Wild-type and $Faah^{-/-}$ caudal sperm were incubated in HTF medium with anti-Izumo antibody conjugated with FITC to monitor spontaneous acrosome reaction by flow cytometry at 30-min intervals for up to 3 h. Sperm were stained with propidium iodide (10 μg/ml) 2 min before flow cytometry analysis. Viable sperm were selected by propidium iodide staining, while acrosome-reacted sperm were identified by anti-Izumo antibody staining [30].

Evaluation of Sperm Motility

After capacitation for 30 and 90 min, 20 μl of media containing sperm (2×10^6 sperm/ml) was placed on a prewarmed slide under a coverslip. Sperm motility was recorded in 12 frames/sec for 20 sec at a resolution of 640×512 pixels. The total travel distance and linear travel distance (linear distance from the starting point to the end point) and the travel time were measured using the Nikon Nis-elements object tracking function. The curvilinear velocity was calculated from the total distance traveled divided by the travel time. The linear velocity was calculated from the linear travel distance divided by the travel time, whereas linearity was calculated from the linear velocity divided by the curvilinear velocity.

RESULTS

$Faah^{-/-}$ Males Have Compromised Fertility

We have previously shown that FAAH is a key metabolic regulator of anandamide levels in mice [17] and that FAAH deficiency results in higher anandamide levels in the female reproductive tract, impairing normal oviductal embryo transport and embryo development [12]. In the course of these studies, analysis of breeding results showed that litter sizes generated by mating WT females with $Faah^{-/-}$ males are 13% smaller than those generated by mating WT females with WT males (Table 1). These results suggested that FAAH deficiency compromises male fertility. This is further evident from our findings of significantly reduced litter sizes generated by mating $Faah^{-/-}$ females with $Faah^{-/-}$ males compared with those generated by mating $Faah^{-/-}$ females with WT males. These breeding results prompted us to further examine fertility of $Faah^{-/-}$ males. We used WT females mated with $Faah^{-/-}$ or WT males. Females were killed on the morning of Day 2 of pregnancy, and oviducts were flushed to record fertilized (two-cell embryos) and

TABLE 1. Reproductive performance of *Faah*$^{-/-}$ males.

Genotype		No. of litters examined*	Average litter size (mean ± SEM)
Female	Male		
WT	WT	30	8.1 ± 0.4
	Faah$^{-/-}$	23	7.0 ± 0.4†
Faah$^{-/-}$	WT	21	6.3 ± 0.2
	Faah$^{-/-}$	39	4.1 ± 0.3‡

* Litters were sired by different males.
† $P = 0.06$; unpaired *t*-test between litters from WT female × *Faah*$^{-/-}$ male crossings and those from WT female × WT male crossings.
‡ $P < 0.001$; unpaired *t*-test between litters from Faah$^{-/-}$ female × *Faah*$^{-/-}$ male crossings and those from *Faah*$^{-/-}$ female × WT male crossings.

unfertilized eggs. We observed that WT females mated with *Faah*$^{-/-}$ males have significantly fewer fertilized eggs compared with those recovered from WT females mated with WT males. In addition, fewer WT females yielded fertilized egg (Fig. 1). These data corroborate the breeding data that FAAH deficiency impairs male fertility. Collectively, our findings show that *Faah*$^{-/-}$ sperm underperform even in the WT female reproductive tract and that function of null sperm is further compromised in the *Faah*$^{-/-}$ female reproductive tract. These observations provide evidence that paternal FAAH deficiency is a cause for compromised fertility.

Endocannabinoid Signaling Is Present in the Male Reproductive System

The extent and duration of anandamide signaling via CNR1 or CNR2 are mainly regulated by FAAH [17]. Therefore, we examined the expression of CNR1, CNR2, and FAAH in the testis and epididymis to study potential roles of anandamide in regulating male fertility. Western blotting analysis showed that FAAH, CNR1, and CNR2 are present in the testis and epididymis of WT mice (Fig. 2a). We next examined cell-specific localization of FAAH and cannabinoid receptors in the testis and epididymis of WT mice by immunohistochemistry (Fig. 2b). While CNR1 was present in Leydig cells and epididymal epithelial cell surfaces, testicular spermatocytes and spermatids showed modest positive staining. In contrast, CNR2 was localized in spermatocytes and Sertoli cells encircling spermatocytes and spermatids in the testis. In the epididymis, epithelial cell surfaces demonstrated CNR2 immunostaining, whereas signals were undetectable in interstitial cells. FAAH was present in spermatocytes and spermatids, while spermatogonia had little or no positive signal. Sertoli cells and Leydig cells also showed positive staining of FAAH. The localization of FAAH was evident on cell surfaces of the epididymal epithelium. The antibody specificity was confirmed using *Faah*$^{-/-}$ tissues (Supplemental Figure 1 available online at www.biolreprod.org). The presence of FAAH on the testis and epididymis suggests that endocannabinoid levels are tightly regulated by FAAH in these tissues.

The presence of CNR1 and CNR2 on sperm was also examined by immunofluorescence. As shown in Figure 2c, CNR1 immunofluorescence is primarily noted in anterior regions of sperm heads, the site of the acrosomal sac, but also in the midpiece. CNR1 is undetectable in the principal piece and endpiece of sperm tails. Sperm incubated with CNR1 antibody preabsorbed with an antigenic peptide showed that, while the signal in the anterior region of sperm heads is specific, the signal is nonspecific in the midpiece. CNR2 was undetectable in sperm (Supplemental Figure 2 available online at www.biolreprod.org). Our findings of the presence of FAAH, CNR1, and CNR2 in the testis and epididymis and the presence

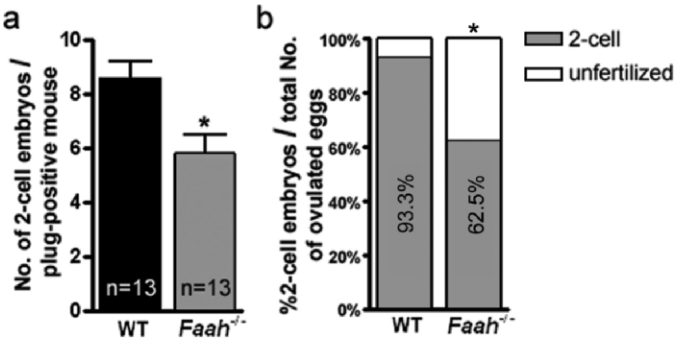

FIG. 1. FAAH deficiency impairs sperm fertility. **a**) Number of two-cell embryos per plug-positive WT females mated with WT or *Faah*$^{-/-}$ males. Numbers of plug-positive mice used are shown within the bars (* $P < 0.05$, unpaired Student *t*-test). **b**) Percentage of two-cell embryos and unfertilized eggs retrieved from the same groups. Thirteen mice are used in each group (* $P < 0.01$, Chi-square test).

of FAAH and CNR1 in sperm suggest that endocannabinoid signaling has a role in spermatogenesis and sperm maturation.

FAAH Deficiency Elevates Anandamide Levels in the Testis and Epididymis

To provide genetic evidence for function of FAAH in the male reproductive system, we measured anandamide and 2-AG levels in the testis and epididymis of WT and *Faah*$^{-/-}$ mice using reverse-phase HPLC-tandem mass spectrometry. As shown in Figure 3a, testis and epididymis from *Faah*$^{-/-}$ mice had significantly increased anandamide levels, suggesting that FAAH is a primary enzyme that regulates anandamide turnover in these tissues. Higher testicular anandamide levels in *Faah*$^{-/-}$ males corroborate our previous observation [31]. However, levels of 2-AG in the testis and epididymis were comparable between *Faah*$^{-/-}$ and WT males (Fig. 3a). These results are consistent with our previous data in the uterus showing unaltered 2-AG levels in the absence of FAAH [12].

Higher anandamide levels in the *Faah*$^{-/-}$ testis and epididymis prompted us to speculate that reduced fertility in these males is due to persistent or elevated endocannabinoid signaling. However, it is possible that there is a negative feedback loop to downregulate the expression of cannabinoid receptors to counter the consequence of high anandamide levels. To address this possibility, we examined the status of cannabinoid receptors in the testis and epididymis of WT and *Faah*$^{-/-}$ mice by Western blotting. As shown in Figure 3b, levels of CNR1 and CNR2 protein in these tissues were comparable between *Faah*$^{-/-}$ and WT males. These results suggest that higher anandamide levels do not appreciably downregulate CNR1 or CNR2 expression. To further confirm that expression of CNR1 and CNR2 is not altered in *Faah*$^{-/-}$ males, in situ hybridization and immunohistochemistry were performed. Expression patterns of CNR1 and CNR2 were similar in WT and *Faah*$^{-/-}$ epididymis (data not shown). Collectively, the data suggest that the status of cannabinoid receptors is not altered by higher anandamide levels and that heightened signaling via CNR1 or CNR2 occurs in the presence of increased anandamide levels.

FAAH Deficiency Impairs Sperm Fertilizing Capacity

Our in vivo breeding data led us to speculate that higher anandamide levels in males lacking FAAH results in their reduced fertility. To examine this, we first compared histology of

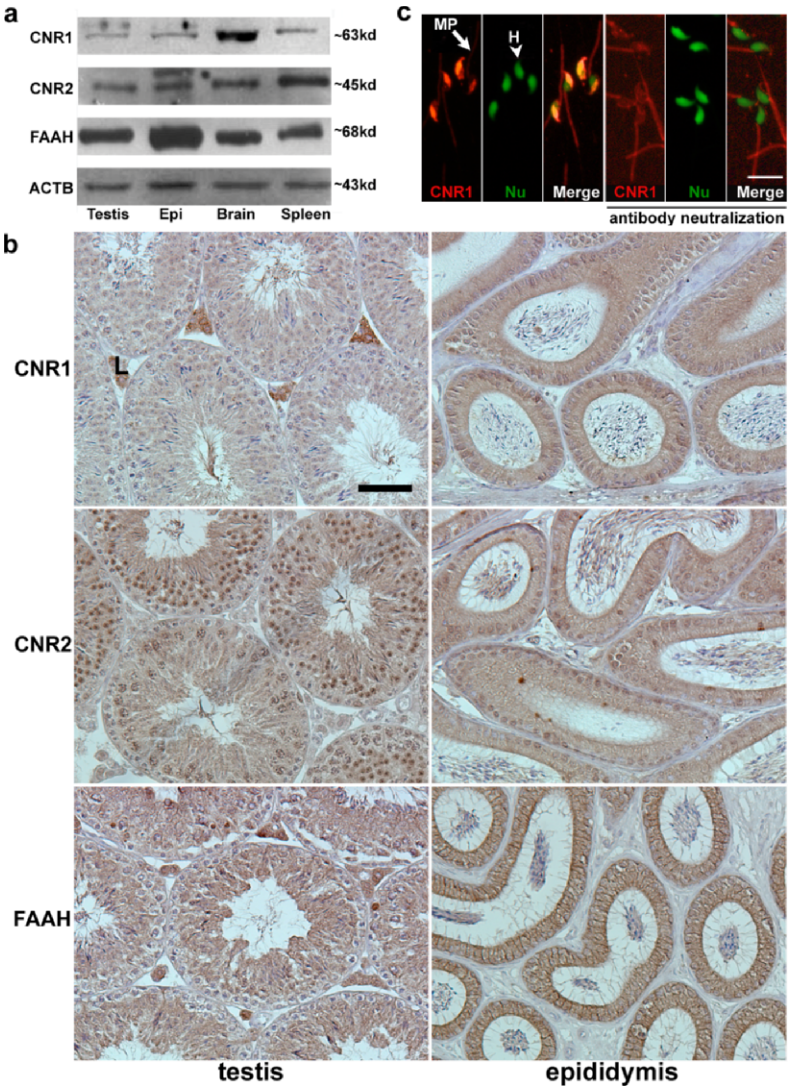

FIG. 2. FAAH and cannabinoid receptors are expressed in the male reproductive tract. **a**) Western blotting of CNR1, CNR2, and FAAH in the WT testis and epididymis. Brain tissue extracts served as positive controls for CNR1 and FAAH, while spleen tissue samples served as positive controls for CNR2. β-Actin (ACTB) is a loading control. Epi, epididymis. **b**) Immunolocalization of CNR1, CNR2, and FAAH in the testis and epididymis. L, Leydig cells. Bar = 50 μm. **c**) CNR1 immunostaining (red) in sperm (left three panels) and in sperm exposed to CNR1 antibody preabsorbed with an antigenic peptide (right three panels). In each group, CNR1 staining, nuclear staining, and merged pictures are shown from left to right. Nuclei were counterstained with SYTO13 (green). MP (arrow), sperm midpiece; H (arrowhead), sperm head; Nu, nuclear. Bar = 10 μm.

the testis and epididymis, as well as sperm morphology, between *Faah*$^{-/-}$ and WT males at the age of 3–4 mo. To our surprise, no apparent histological abnormalities were observed in these tissues missing *Faah* (Supplemental Figure 3 available online at www.biolreprod.org). We next explored whether FAAH deficiency in males impairs the fertilizing capacity of sperm by performing IVF experiments using *Faah*$^{-/-}$ or WT sperm with WT eggs. Sperm retrieved from the caudal epididymis were subjected to capacitation in vitro for 2 h before placing them with eggs in culture. The fertilization rate was calculated by counting the number of two-cell embryos developed on the second day after IVF. As summarized in Table 2, sperm retrieved from WT males showed a 75% fertilization rate, with 97% of two-cell embryos developing to blastocysts (evaluated on the fifth day of culture). In contrast, *Faah*$^{-/-}$ sperm showed a remarkably reduced fertilization rate (42%), although development of fertilized eggs into blastocysts was comparable (89%) to that in WT animals (97%). These results suggest that the fertilizing capacity of *Faah*$^{-/-}$ sperm is compromised because of impairment in the male reproductive tract before ejaculation.

Deletion of Cnr1 *Reverses Impaired Fertilizing Capacity of* Faah$^{-/-}$ *Sperm*

Sustained higher anandamide levels in the male reproductive tract lacking FAAH are capable of exerting endocanna-

TABLE 2. Higher anandamide levels impair sperm fertilizing capacity in vitro via CNR1.

Genotypes	No. of eggs used for IVF	IVF rate		Development	
		Percentage	No. of 2-cell embryos/ total no. of eggs used	Percentage	No. of blastocysts/ total no. of 2-cell embryos used
WT	624	75	466/624	97	450/466
Faah$^{-/-}$	528	42*	221/528*	89	197/221
Faah$^{-/-}$/*Cnr1*$^{-/-}$	177	70	124/177	93	115/124
Faah$^{-/-}$/*Cnr2*$^{-/-}$	118	11*	13/118*	84.6	11/13

* $P < 0.01$; chi-square analysis.

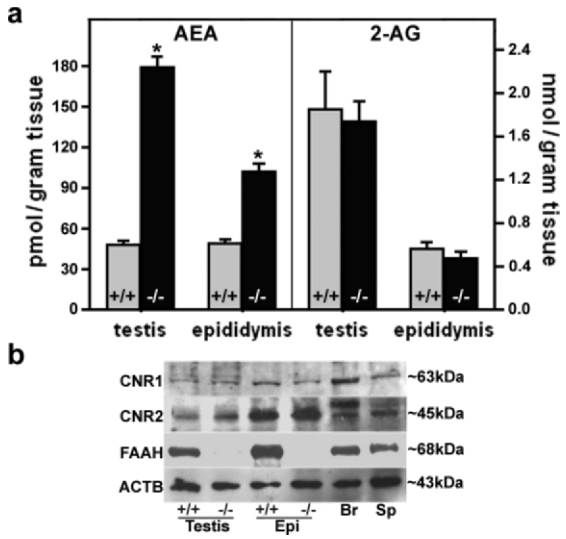

FIG. 3. FAAH deficiency elevates anandamide levels. **a**) Anandamide (AEA) levels, but not 2-AG levels, in *Faah*$^{-/-}$ testis and epididymis were higher than those in WT males (n = 10; * $P < 0.05$, unpaired Student *t*-test). **b**) Western blotting of CNR1, CNR2, and FAAH in the testis and epididymis of WT and *Faah*$^{-/-}$ males. Brain and spleen samples served as positive controls, while β-actin (ACTB) served as a loading control. Epi, epididymis; Br, brain; Sp, spleen.

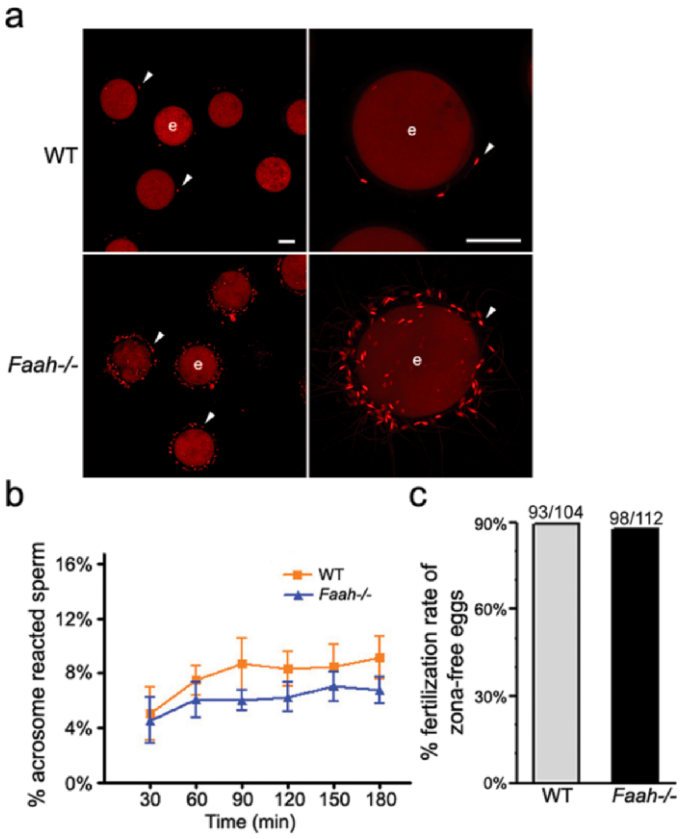

FIG. 4. Zona-penetrating capacity of *Faah*$^{-/-}$ sperm is inferior. **a**) Sperm-egg interactions using zona-intact WT eggs. After 2 h of incubation with eggs, *Faah*$^{-/-}$ sperm were still attached to zona pellucidae. Arrowhead, sperm on the zona-intact egg surface; e, egg. Bar = 40 μm. **b**) Spontaneous acrosome reaction as assessed by flow cytometry. The rate (%) of acrosome-reacted WT and *Faah*$^{-/-}$ sperm at each time point was analyzed by flow cytometry as described in *Materials and Methods*, and no statistically significant difference was noted between the two groups as analyzed by Student *t*-test. **c**) The IVF rates of zona-free WT eggs fertilized by WT or *Faah*$^{-/-}$ sperm. Numbers above the bars indicate the number of fertilized eggs/total zona-free eggs used for IVF.

binoid signaling through CNR1, CNR2, or both. To address this, we generated *Faah*$^{-/-}$/*Cnr1*$^{-/-}$ and *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ double-mutant mice. We again performed IVF using sperm retrieved from *Faah*$^{-/-}$/*Cnr1*$^{-/-}$ or *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ males with eggs isolated from WT females. As summarized in Table 2, sperm isolated from *Faah*$^{-/-}$/*Cnr1*$^{-/-}$ males exhibited a 70% fertilization rate, with 93% of fertilized eggs developing to the blastocyst stage, but sperm isolated from *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ males showed a remarkably low fertilization rate (11%). These data show that, in the absence of CNR1, *Faah*$^{-/-}$ sperm escape the deleterious effects of higher anandamide levels. The inferior fertilizing capacity of *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ sperm exceeded that of *Faah*$^{-/-}$ sperm. The results provide genetic evidence that higher anandamide levels work through CNR1 in the *Faah*$^{-/-}$ male reproductive tract to impair sperm fertilizing capacity.

Faah$^{-/-}$ *Sperm Have Poor Zona-Penetrating Ability*

Our next objective was to see which step in the fertilization process is impaired in *Faah*$^{-/-}$ sperm. We first examined whether *Faah*$^{-/-}$ sperm can adhere to zona pellucidae and, if so, whether they can undergo the acrosome reaction. Izumo, a recently discovered protein, is absent from plasma membranes of acrosome-intact sperm [30]. Following the acrosome reaction, Izumo is exposed and participates in sperm-egg fusion. Therefore, only acrosome-reacted sperm are stained by Izumo antibody.

Wild-type or *Faah*$^{-/-}$ sperm were incubated with WT eggs for 2 h and then stained with propidium iodide to label cell nuclei. After 2 h of incubation, most WT sperm detached from the zona surface (Fig. 4a), whereas numerous *Faah*$^{-/-}$ sperm were still attached to the zona. Even after several washings, *Faah*$^{-/-}$ sperm remained adherent to the zona, indicating good binding of *Faah*$^{-/-}$ sperm to the zona. These results suggested that most eggs were fertilized by WT sperm but that eggs incubated with *Faah*$^{-/-}$ sperm were still unfertilized. We then stained the sperm attached to eggs with Izumo antibody. Many *Faah*$^{-/-}$ sperm remaining on the zona surface showed positive signal by Izumo antibody (Supplemental Figure 4 available online at www.biolreprod.org), indicating that they underwent the acrosome reaction. To further confirm that *Faah*$^{-/-}$ sperm undergo normal acrosome reaction, we examined the spontaneous acrosome reaction rate of *Faah*$^{-/-}$ sperm. The acrosome reaction, which occurs during sperm penetration through the zona, can also occur spontaneously without binding to the zona. Analysis of spontaneous acrosome reaction is used to assess the fertilizing ability of human [32] and mouse [33] sperm. We compared the status and time course of spontaneous acrosome reaction of WT and *Faah*$^{-/-}$ sperm in the fertilization medium by flow cytometry. While viable sperm were selected by propidium iodide staining, acrosome-reacted sperm were identified by Izumo staining. As shown in Figure 4b, the percentage of acrosome-reacted *Faah*$^{-/-}$ sperm is somewhat lower than that of acrosome-reacted WT sperm, but the difference is not statistically significant. Collectively, these data suggest that *Faah*$^{-/-}$ sperm can bind to the zona and undergo the acrosome reaction but still have difficulty in fertilizing eggs.

The acrosome reaction is not the only prerequisite for zona penetration. Sperm motility and acrosomal release of proteases are also involved in this process [20]. To examine whether *Faah*$^{-/-}$ sperm can penetrate the zona successfully, we performed IVF using sperm from *Faah*$^{-/-}$ or WT mice

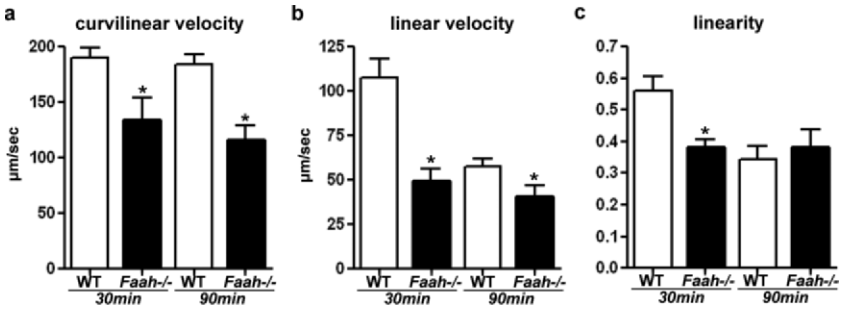

FIG. 5. Motility of *Faah*$^{-/-}$ sperm is inferior. **a**) Curvilinear velocity of WT and *Faah*$^{-/-}$ sperm. Curvilinear velocities of *Faah*$^{-/-}$ sperm were significantly lower than those of WT sperm at 30 and 90 min of capacitation (* $P < 0.01$, unpaired Student *t*-test). **b**) Linear velocity of WT and *Faah*$^{-/-}$ sperm. Linear velocities of *Faah*$^{-/-}$ sperm were significantly lower than those of WT sperm (* $P < 0.05$, unpaired Student *t*-test). **c**) Linearity of WT and *Faah*$^{-/-}$ sperm. Linearity of *Faah*$^{-/-}$ sperm was significantly lower than that of WT sperm at 30 min of capacitation (* $P < 0.05$, unpaired Student *t*-test).

incubated with zona-free WT eggs. To our surprise, *Faah*$^{-/-}$ sperm exhibited fertilizing capacity comparable to that of WT sperm (Fig. 4c), indicating that the zona is a major barrier for normal fertilization by *Faah*$^{-/-}$ sperm.

Sperm Motility Is Attenuated in Faah$^{-/-}$ *Males*

It is generally accepted that robust sperm motility is an important component of normal male fertility [34] and that hyperactivated motility of sperm is correlated with sperm's fertilizing ability of zona-intact eggs [35]. In a low-viscosity medium, motility of hyperactivated sperm is characterized by asymmetrical flagellar bends with large amplitude and curvature, and moving trajectories are irregular and highly curved [36]. We often observed sluggish motility of *Faah*$^{-/-}$ sperm when they were incubated in the capacitation medium. We speculated that the reduced zona-penetrating ability of *Faah*$^{-/-}$ sperm could be due to their reduced motility or hyperactivation. Therefore, we assayed motility of WT and *Faah*$^{-/-}$ sperm after capacitation for 30 and 90 min in vitro. In this measurement, the curvilinear velocity was calculated from the total distance traveled divided by the travel time; this parameter indicates the swimming ability of sperm. The linear velocity was calculated from the distance between the start and end points divided by the travel time. The linearity is the linear velocity-curvilinear velocity ratio; this is an indicator of straightness of sperm movement. The movement of *Faah*$^{-/-}$ sperm was significantly slower than that of WT sperm at 30 and 90 min of incubation in the capacitation medium (Fig. 5a). The movement of WT sperm was primarily straight at 30 min of capacitation, with symmetrical flagellar beats. After 90 min of capacitation, WT sperm showed hyperactivated movement pattern, resulting in reduced linear velocity (Fig. 5b) and linearity (Fig. 5c); the curvilinear velocity was not significantly changed (Fig. 5a). However, *Faah*$^{-/-}$ sperm demonstrated irregular movement from 30 min of capacitation, distinguished by decreased linear velocity and linearity (Fig. 5, b and c). Although their moving trajectories were erratic, the seemingly hyperactivated movement of *Faah*$^{-/-}$ sperm was not the consequence of harder beat of flagellum after capacitation, as the moving speed of *Faah*$^{-/-}$ sperm stayed at low levels. These results show that heightened anandamide signaling in the male reproductive tract compromises motility of *Faah*$^{-/-}$ sperm, leading to reduced zona penetration and fertilization.

DISCUSSION

Emerging evidence shows that endocannabinoid signaling has critical roles in male reproduction. Endocannabinoid signaling is operative in the oviduct, uterus, and embryo, and aberrant endocannabinoid signaling adversely affects oviductal transport of embryos and their development [1]. Consistent with our present findings, endocannabinoids and their receptors were reported to be present in the testis and sperm of invertebrates and vertebrates [21, 22, 37–40]. However, our findings of the endocannabinoid system in different regions along the male reproductive tract suggest that endocannabinoid signaling has diverse physiological functions. In this respect, Sertoli cells exposed to higher anandamide levels were shown to undergo apoptosis [41], and FAAH activity is regulated by FSH in mouse Sertoli cells [42]. In addition, sperm fertility and the acrosome reaction were reported to be adversely affected if exposed in vitro to high anandamide levels [21, 43].

Our experiments were designed to evaluate in vivo effects of sustained higher anandamide levels in the male reproductive tract on various aspects of sperm function. We used *Faah*$^{-/-}$ mice with high anandamide levels as a model system to mimic the conditions of long-term exposure to marijuana use to explore the role of cannabinoid and endocannabinoid signaling in male fertility. Results of our IVF experiments with *Faah*$^{-/-}$ sperm show a resemblance to reduced sperm fertilizing capacity and motility in marijuana users [44–46]. Our findings of compromised fertilizing capacity of *Faah*$^{-/-}$ sperm in vivo and in vitro, as well as their inability to recover in normal capacitating medium, provide strong evidence that functional impairment of sperm exposed to high anandamide levels in vivo persists for a prolonged period or becomes irreversible. Our results are clinically relevant because long-term in vivo exposure to marijuana is implicated in reduced male fertility [44–46].

The use of zona-free eggs in IVF experiments is an established method to study cellular mechanisms of gamete adhesion and fusion [47]. Using this strategy, we have shown that *Faah*$^{-/-}$ sperm are capable of adhering to and fusing with zona-free eggs in a manner similar to that of WT sperm. The fact that the fertilization rate of *Faah*$^{-/-}$ sperm increased from 42% with zona-intact eggs to 90% with zona-free eggs suggests that the zona is a major barrier to *Faah*$^{-/-}$ sperm, as these null sperm display spontaneous acrosome reaction comparable to that of WT sperm based on Izumo staining and flow cytometry analysis. We speculate that factors other than the acrosome reaction weaken the penetrating capacity of sperm through the zona. Sperm motility and acrosomal enzymes are involved in zona penetration [20]. It is possible that contents released from the acrosomal sac lack appropriate protease activity required for penetration or that *Faah*$^{-/-}$ sperm cannot acquire hypermotility following capacitation. Our results suggest that reduced motility is a contributing factor for reduced zona-penetrating ability of *Faah*$^{-/-}$ sperm. However, other factors

such as protease activity may contribute to reduced capacity of sperm for zona penetration. *Faah*$^{-/-}$ sperm show asymmetric flagellar beat at 30 min of capacitation. We do not know whether *Faah*$^{-/-}$ sperm show straightforward moving trajectory before 30 min of capacitation. Although it would be helpful to know the motility of *Faah*$^{-/-}$ sperm immediately after they are placed in the capacitation medium, we were unable to obtain this information because of the time necessary for sperm manipulation and counting.

Reversal of the defects of FAAH deficiency in the absence of CNR1 suggests that anandamide signaling exerts its effects on sperm through CNR1. Because CNR1 is expressed in the testis, epididymis, and sperm, it is unclear where and how CNR1-mediated signaling regulates sperm fertility. Because sperm display CNR1, it is possible that higher anandamide levels directly target sperm to alter their function. Alternatively, heightened signaling via CNR1 in the presence of higher anandamide levels in *Faah*$^{-/-}$ testis and epididymis changes the internal milieu to affect sperm maturation, influencing sperm fertility. Our findings that *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ sperm show much inferior fertilizing capacity than sperm deleted of *Faah*$^{-/-}$ only suggest that anandamide working via CNR2 is important for normal sperm fertility. Alternatively, in the absence of CNR2, higher levels of anandamide are exclusively available to CNR1, further enhancing its adverse effects on sperm function. The latter speculation seems more probable because homozygous crossings of *Cnr2*$^{-/-}$ mice have an average litter size of about seven, whereas homozygous crossings of *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ mice produce an average of four pups per litter. Although breeding data are more confounded by maternal factors than IVF results, this observation suggests that CNR2 has limited roles in sperm function under physiological anandamide levels.

The present investigation has physiological significance because sperm in *Faah*$^{-/-}$ mice and those in chronic marijuana users are subjected to enhanced cannabinoid and endocannabinoid signaling. Beneficial effects of anandamide in neurodegeneration, cancer, pain, and anxiety [48–51] have prompted heightened interest in and effort to develop FAAH inhibitors as novel therapeutic drugs. Therefore, adverse effects of anandamide should be carefully weighed against its beneficial effects. This study provides insights into male fertility regulation by endocannabinoid signaling and may shed light on improving male fertility.

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