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Genetic Deletion of Fatty Acid Amide Hydrolase Alters Emotional Behavior and Serotonergic Transmission in the Dorsal Raphe, Prefrontal Cortex, and Hippocampus

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Pharmacological blockade of the anandamide-degrading enzyme, fatty acid amide hydrolase (FAAH), produces CB_1 receptor (CB_1R)-mediated analgesic, anxiolytic-like and antidepressant-like effects in murids. Using behavioral and electrophysiological approaches, we have characterized the emotional phenotype and serotonergic (5-HT) activity of mice lacking the FAAH gene in comparison to their wild type counterparts, and their response to a challenge of the CB_1R antagonist, rimonabant. FAAH null-mutant ($FAAH^{-/-}$) mice exhibited reduced immobility in the forced swim and tail suspension tests, predictive of antidepressant activity, which was attenuated by rimonabant. $FAAH^{-/-}$ mice showed an increase in the duration of open arm visits in the elevated plus maze, and a decrease in thigmotaxis and an increase in exploratory rearing displayed in the open field, indicating anxiolytic-like effects that were reversed by rimonabant. Rimonabant also prolonged the initiation of feeding in the novelty-suppressed feeding test. Electrophysiological recordings revealed a marked 34.68% increase in dorsal raphe 5-HT neural firing that was reversed by rimonabant in a subset of neurons exhibiting high firing rates (33.15% mean decrease). The response of the prefrontocortical pyramidal cells to the $5-HT_{2A/2C}$ agonist ($\pm$)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (($\pm$)-DOI) revealed desensitized $5-HT_{2A/2C}$ receptors, likely linked to the observed anxiolytic-like behaviors. The hippocampal pyramidal response to the $5-HT_{1A}$ antagonist, WAY-100635, indicates enhanced tonus on the hippocampal $5-HT_{1A}$ heteroreceptors, a hallmark of antidepressant-like action. Together, these results suggest that FAAH genetic deletion enhances anxiolytic-like and antidepressant-like effects, paralleled by altered 5-HT transmission and postsynaptic $5-HT_{1A}$ and $5-HT_{2A/2C}$ receptor function.

INTRODUCTION

Major depression and anxiety disorders are prevalent, debilitating neuropsychiatric conditions with high comorbidity and treatment options that fall short of optimal efficacy. About a third of depressive patients are treatment-resistant, and many others experience relapsing symptoms (Bambico et al, 2009a; Zisook et al, 2008). Accordingly, some types of anxiety disorder, such as posttraumatic stress disorder, are both chronic and have low remission rates

(Ohayon, 2006). Although the pathophysiology of mood disorders is not completely understood, it is widely accepted to be linked to impairments in the neurotransmission of 5-hydroxytryptamine (5-HT or serotonin) (Bambico et al, 2009a) produced by serotonergic neurons most densely found in the mesencephalic dorsal raphe (DR) nucleus (Dahlstrom and Fuxe, 1964; Descarries et al, 1982). Despite limited efficacies and a slow onset of action, drugs that augment 5-HT transmission, such as selective serotonin reuptake inhibitors (SSRIs), are the most preferred first-line treatments (Vaswani et al, 2003). Chronic antidepressant treatment facilitates exocytosis of 5-HT and/or increases its synaptic availability in corticolimbic regions extensively innervated by the DR, such as the prefrontal cortex, hippocampus, amygdala, and other forebrain structures implicated in mood regulation and stress adaptation (Steinbusch, 1981; Holmes, 2008). In contrast,

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5-HT-depleting manipulations, such as through the 5-HT synthesis inhibitor, parachlorophenylalanine, or a diet deprived of the 5-HT precursor, tryptophan, precipitate anxiety and depression-like symptoms in animals (Blokland *et al*, 2002; Zhou *et al*, 2008; Roiser *et al*, 2008), exacerbate anxiogenic responses in human participants (Miller *et al*, 2000), and inflict a relapse in depressive patients (Smith *et al*, 1997). The delay in therapeutic onset of antidepressants has been attributed to gradual neuroplastic adaptations at the presynaptic and postsynaptic levels that result from the progressive augmentation in 5-HT activity. These modifications include desensitization of auto-inhibitory $5-HT_{1A}$ receptors, sensitization or increased tonic activation of postsynaptic $5-HT_{1A}$ receptors (Haddjeri *et al*, 1998; Besson *et al*, 2000; Szabo and Blier, 2001b), and down-regulation of cortical $5-HT_{2A/2C}$ receptors (Hollander *et al*, 1991a; Kennett *et al*, 1994a, c; Quested *et al*, 1997). The receptor subtypes involved have been extensively explored for their roles in emotion and mood regulation, and for their modulatory impact on neurotrophic factor synthesis and neurogenesis, which are considered downstream biological signatures of antidepressant activity (Holmes, 2008; Bambico and Gobbi, 2008; Bambico *et al*, 2009a).

N-arachidonoylethanolamide (anandamide) and 2-arachidonoylglycerol, lipid endocannabinoid molecules that suppress excitatory and inhibitory transmission through retrograde signaling, are also present in the DR, along with CB_1 receptors (CB_1Rs)—their attendant $G_{i/o}$-protein-coupled receptor—and their catabolic enzyme, fatty acid amide hydrolase (FAAH) (Egertova *et al*, 1998; Haring *et al*, 2007; Moldrich and Wenger, 2000). Recent findings support a role for CB_1R signaling in the modulation of the DR 5-HT system. Direct CB_1R agonists modulate DR 5-HT neural firing in a biphasic manner *in vivo* (Bambico *et al*, 2007), inhibit 5-HT reuptake *ex vivo* (Johnson *et al*, 1976), and decrease 5-HT synthesis *in vivo* (Moranta *et al*, 2004). Inhibitors of FAAH also stimulate DR 5-HT neural firing by increasing anandamide-CB_1R signaling (Gobbi *et al*, 2005). The spatio-temporal pattern of this 5-HT neural stimulation in the DR likely depends on the complementary expression of FAAH and CB_1R and the on-demand characteristic of anandamide synthesis and release (Bambico and Gobbi, 2008; Bambico *et al*, 2009a). Consistent with the 5-HT-augmenting mechanism of antidepressants and anxiolytics, these drugs elicit identical effects detected in a wide range of behavioral tests and animal models for depression and anxiety (Bambico *et al*, 2007, 2009a; Bortolato *et al*, 2007; Gobbi *et al*, 2005; Hill and Gorzalka, 2005; Kathuria *et al*, 2003). The pharmacological properties of FAAH inhibitors relinquish much of the unwanted effects of direct CB_1R agonists, including their reward properties, therefore offering favorability for these inhibitor drugs as potential alternative therapeutic agents (Hill *et al*, 2009a; Bambico *et al*, 2009a; Bambico and Gobbi, 2008).

Transgenic mice lacking FAAH ($FAAH^{-/-}$) represent an invaluable tool in which to further evaluate the impact of increased endocannabinoid-CB_1R signaling on emotional reactivity. As they are severely impaired in the capacity to degrade anandamide, they exhibit more than 10-fold higher levels of this endocannabinoid than wild types (WT) (Cravatt *et al*, 2001), while retaining normal brain CB_1R density (Basavarajappa *et al*, 2006). Moreover, some of the

inconsistent outcomes reported on the anxiolytic-like and antidepressant-like activity of FAAH inhibitors and FAAH genetic inactivation have been attributed to variable experimental conditions (Naidu *et al*, 2007; Moreira *et al*, 2008). We therefore subjected $FAAH^{-/-}$ mice to a battery of tests for antidepressant-like and anxiolytic-like activity. We then compared $FAAH^{-/-}$ and WT mice on the basis of their spontaneous DR 5-HT firing activity and function of prefrontocortical and hippocampal $5-HT_{1A}$ and $5-HT_{2A/2C}$ receptors. We also examined whether changes in behavioral and electrophysiological phenotypes were due to increased endocannabinoid–CB_1R signaling by a challenge injection of the CB_1R antagonist, rimonabant.

MATERIALS AND METHODS

Maintenance and Preparation of Animals

All experiments were carried out on male, adult FAAH knockout ($FAAH^{-/-}$) mice and their WT counterparts ($FAAH^{+/+}$). All mice used were from the F9 generation and weighed 25–40 g during the time of the experiments. $FAAH^{-/-}$ mice were generated as described earlier (Cravatt *et al*, 2001), and were backcrossed into the C57BL6/6J background. Mutant and WT mice were bred and maintained in the animal facilities of the University of California, Irvine, under standard conditions (12:12 light–dark cycle, lights on at 07:30; temperature at $20 \pm 2°C$; 50–60% relative humidity, *ad libitum* access to food and water) until maturation. Thereafter, two batches from the colony were transported to the animal facilities of McGill University, where they were maintained under similar conditions, and caged in pairs or threes. The experimental procedures commenced about 2 months after arrival in the laboratory facility when the mice were approximately 5 months old. Additional experiments were performed on 8 to 9-month-old mice. Inspection of weight differences was carried out on 9- to 10-month old mice. All experiments on $FAAH^{-/-}$ mice were performed with the appropriate age-matched WT controls. All procedures were undertaken in compliance with the standards and ethical guidelines mandated by the local facility animal care committee of McGill University, the Canadian Institutes of Health Research, and the Canadian Council on Animal Care.

Drugs

The CB_1R antagonist, rimonabant (SR141716A, provided by Dr Daniele Piomelli) and the carbamate inhibitor of FAAH, URB597 (3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate, a kind gift from Dr Andrea Duranti and Dr Giorgio Tarzia, University of Urbino, Italy), were dissolved in a vehicle consisting of 5% Tween 80, 5% polyethylene glycol, and 90% saline (0.9% NaCl solution) to a final concentration of 300 and 90 µg/ml, respectively. All other drugs were obtained from Sigma-Aldrich Canada Ltd. The $5-HT_{1A}$ receptor antagonist, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridyl)cyclohexanecarboxamide (WAY-100635), was dissolved in saline (0.9% NaCl solution) to a final concentration of 150 µg/ml. The aforementioned drugs were administered intraperitoneally at a volume of 3.33 ml/kg body weight. For iontophoretic applications,

the following drugs were used: the 5-HT$_{1A}$ receptor agonist 8-hydroxy-*N,N*-dipropyl-2-aminotetralin HBr (8-OH-DPAT, 50 mM in 200 mM NaCl, pH 4.0–4.5), the 5-HT$_{2A/C}$ receptor agonist (±)-DOI (10 mM in 200 mM NaCl, pH 4.0–4.5), serotonin creatinine sulfate (5-HT, 50 mM in 200 mM NaCl, pH 4.0–4.5) and quisqualic acid (1.5 mM in 400 mM NaCl, pH 8.0). Chloral hydrate was used as an anesthetic in all electrophysiological experiments.

Behavioral Assay

FAAH$^{-/-}$ and FAAH$^{+/+}$ mice received a single injection of either the vehicle or rimonabant (1 mg/kg, intraperitoneal). Behavioral testing took place 30 min after drug/vehicle administration. We used a serial behavioral and electrophysiological testing procedure that has been validated and compared with single testing procedures in our laboratory, and by other investigators (Bambico *et al*, 2010; Walf and Frye, 2007). The novelty-suppressed feeding test (NSFT) and the open field test (OFT) were used to evaluate anxiety-like behaviors, whereas the forced swim test (FST) for antidepressant/depression-like coping behaviors. These were conducted on 5-month-old mice. To further validate behavioral data from these tests, we conducted additional ones, the elevated plus maze test (EPMT) for anxiety-like behavior and the tail suspension test (TST) for antidepressant-like behavior performed on 8- to 9-month-old mice. A 1- to 2-day interim period was interlaced between tests. On the day of testing, the mice were acclimated for about 60 min in the behavioral room before the procedures were initiated. The apparatus was cleaned with 70% alcohol and water after each run. The behaviors were recorded, stored, and analyzed as MPEG files using an automated behavioral tracking system (Videotrack, View Point Life Science, Montreal, Canada) equipped with infrared lighting-sensitive CCD cameras. The analog signals supplied by the camera were measurements of the luminosity of each point of the image scanned point-by-point and line-by-line at the rate of 25 images per second. These signals were transmitted to the Videotrack system and digitized on 8 bits by digital analog conversion. Before the experiments, animal/image background contrast detection thresholds were calibrated by visual inspection to distinguish diffferent behavioral patterns. Additional offline analyses were conducted by a rater who was blind to the experimental manipulations to further verify results.

Open field activity testing. Mice were each placed at the center of a white-painted open field arena (40 × 40 × 15 cm) and left to explore the whole field for 20 min of recording. The experiment took place under standard room lighting (350 lx); a white lamp (100 W) was suspended 2 m above the arena. Anxiety-like thigmotactic ('wall-following') behavior was measured by the frequency and total duration of central zone (30 × 30 cm) visits. Anxiogenics decrease these behavioral endpoints whereas anxiolytics conversely increase them and attenuate freezing episodes (Archer, 1973; Choleris *et al*, 2001). Other ethological measures analyzed included grooming, rearing, turning, and locomotor activity; modified after Choleris *et al* (2001). This experiment was performed at 1400 hours.

Elevated plus maze test. The EPMT was used to assess anxiety-like reactivity as induced suppression of exploratory behavior. The maze was made of white Plexiglass and consisted of two open arms (16 × 5 cm) opposite each other and two walled arms (16 × 5 × 12 cm) opposite each other. The plus maze was raised 50 cm above ground and had a 5 × 5 cm central platform forming the intersection of the four arms. The mice were each placed on the central platform facing one of the open arms. EPMT behavior was recorded for 5 min under bright white light (100 W). Behavioral endpoints that were analyzed included time spent in the open *vs* closed arms, frequency of open arm *vs* closed arm entries, time spent (s) on the central platform, frequency of stretch-attend postures (SAP), and head dips into the open arms and beyond the borders of the open arms. Arm entries were defined as crossing of both forepaws into the predefined borders of each of the open and closed arms (modified after Rago *et al* (1988); Pellow and File (1986)). Anxiolytic agents are known to significantly increase open arm visits, head dips, and rearing behaviors while decreasing SAP and freezing behaviors. Anxiogenics increase closed arm visits, SAP, and freezing behaviors while decreasing head dips and rearing behaviors (Walf and Frye, 2007; Cole and Rodgers, 1995; Rodgers *et al*, 1995). To further enhance the procedure's sensitivity to anxiolytic agents and manipulations, the mice were singly caged for at least 3 days before the test, with the test itself conducted at 1400 hours (Hogg, 1996).

Forced swim test. The FST examines the dynamics of transition from an active (swimming) to a passive (immobility) mode of coping in an inescapable water-filled pool. Enhancement of immobility normally ensues after exposure; a phenomenon argued to reflect learned behavioral despair (Porsolt *et al*, 1977) and prevented by antidepressant treatment. The mice were placed individually into Plexiglas cylindrical bins (20 cm diameter, 50 cm high) filled with water (25–27°C water temperature) to a depth of 20 cm. This depth did not allow the tail and hindpaws to touch the floor of the bin. The test was conducted at 1900 hours when mice were allowed to swim for 6 min in a dimly lit environment and under minimal anxiogenic conditions (Kelliher *et al*, 2000). Custom-made plates arrayed with infrared light-emitting diodes were suspended above the bins. Infrared light-sensitive CCD cameras allowed for the capture and storage of images in the Videotrack system. After recording, the mice were rescued using a plastic grid and caged near a heat source (lamp). The behavioral tracking system was calibrated so that a mouse was considered immobile when making only those movements necessary to keep its head above water. The total duration of activity was determined during the last 4 min.

Tail suspension test. This procedure, which is also used to assess antidepressant-like activity, involved suspending the mouse by the tail from a lever in a 30 × 30 × 30 cm white-painted enclosure. The test was conducted at 1900 hours; movements were recorded and quantified for 6 min (the last 5 min were used for analysis) in a dimly lit environment and under minimal anxiogenic conditions. These experimental conditions were similar to those described by Gobbi *et al* (2005). Antidepressant treatment

reduces the total time the mouse remains immobile (Steru et al, 1985).

Novelty-suppressed feeding test. This procedure was used to measure novelty-induced anxiety-like behaviours (neo-hypophagia, the inhibition of feeding upon exposure to an anxiety-provoking novel environment) and has been used to validate the acute effects of putative anxiolytics. It has also been used to predict the therapeutic effects of putative antidepressants upon long-term treatment (Bodnoff et al, 1988; Gross et al, 2000). The mice were food-deprived for 48 h, then each mouse was placed in a brightly illuminated (100 W, 350 lx) open arena (40 × 40 white-painted floor with walls 30 cm in height covered with blue textile) containing lab chow (12 pellets) evenly spread across the floor. The latency to initiate feeding (in seconds) was noted, and used as an index of anxiety/depression-like behavior. The cut-off time was 600 s (Bodnoff et al, 1988). Feeding latency was also observed in the home cage containing 12 pellets spread on the floor; the session was terminated immediately after the mice initiated feeding. The test was conducted at 1400 hours.

In Vivo Electrophysiology

Preparation for recording procedures. Three days after the last behavioral test, in vivo single-unit extracellular recordings of DR 5-HT neurons were performed as described earlier (Bambico et al, 2007; Gobbi et al, 2001, 2005). Another cohort of mice, naive to behavioral testing, was also used for these experiments. First, the mice were anesthetized with chloral hydrate (chloral hydrate, 400 mg/kg, 2% solution, intraperitoneal) then mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, California) with the skull positioned horizontally (incisor bar at −3.3 mm). Anesthesia was confirmed by the absence of nociceptive reflex reaction to a tail or paw pinch and lack of an eye blink response to pressure. During the course of the experiments, anesthesia was maintained by a periodic intraperitoneal injection of supplemental doses of chloral hydrate (100 mg/kg) upon recovery of tail/paw nociceptive reflexes and eye blink response. Body temperature was maintained at $37 \pm 0.5°C$ throughout the experiment using a thermistor-controlled heating pad (Seabrook Medical Instrument, Inc.). Recordings were carried out using single-barreled (R&D Scientific Glass, Spencerville, MD) or microiontophoresis multi-barreled (Harvard/Applied Scientific Instrumentation, OR, USA) glass micropipettes pulled on a Narashige (Tokyo, Japan) PE-2 pipette puller. The micropipettes were preloaded with fiberglass strands to promote capillary filling with 2% Pontamine Sky Blue solution in 2 M NaCl and their tips were broken down to diameters of 1–3 μm for single-barreled and 10–15 μm for multi-barreled recordings. The impedances ranged from 2 to 6 MΩ. The stereotaxic brain coordinate system by Paxinos and Franklin (2001) was used in all electrophysiological experiments. Using a hydraulic micropositioner (model 650; David Kopf Instruments, Tujunga, California), the electrode was advanced slowly into the brain structure at approximately 0.15 mm/min to minimize the probability of missing slow-spiking neurons. To maximize sampling without introducing considerable tissue damage, three to five electrode descents

were achieved. Single-unit activity was recorded as discriminated action potentials amplified by a Tennelec (Oakridge, TN) TB3 MDA3 amplifier, post-amplified and filtered by a Realistic 10 band frequency equalizer, digitized by a CED1401 interface system (Cambridge Electronic Design, Cambridge, UK), processed online, and analyzed off-line by Spike2 software version 5.20 for Windows PC (Microsoft, Seattle, WA). An Npi electronic Gmbh Micro-iontophoretic System (Tamm, Germany) was used for local (iontophoretic) drug applications. The spontaneous single-spike activity of neurons was recorded for at least 2 min; the first 30 s immediately after detecting the neuron was not considered to eliminate mechanical artifacts due to electrode displacement. For experiments requiring acute drug injection, a catheter was inserted intraperitoneally prior to electrophysiological recording to facilitate intra-peritoneal administration. Drug response was considered inhibitory or excitatory if changes in neural activity exceeded 10% of the basal firing rate. At the end of each recording session, the recording site was marked by iontophoretic ejection (1–10 μA, negative current for 10 min) of Pontamine Sky Blue for later histological verification of recording sites. All recordings were carried out between 1400 and 2200 hours.

Extracellular recordings of spontaneous DR 5-HT single-spike and burst firing activity. The DR is the main source of 5-HT innervation in the brain. To record from single-unit 5-HT neurons in the DR, a burr hole was drilled on the cranial midline subtending regions above the entire rostrocaudal medial extent of the DR presumed to be richest in 5-HT neurons (Descarries et al, 1982). The electrode was lowered into the region of the DR (0.5–1.0 mm posterior to the interaural line on the midline, 2.5–3.5 mm from the dura mater, just beneath the Sylvian aqueduct). Under physiological conditions, spontaneously active 5-HT neurons exhibit characteristic electrophysiological properties distinguishable from non-5-HT neurons. These 5-HT neurons exhibit a slow (0.1–4 Hz) and a prominently regular firing rate (coefficient of variation (C.O.V.), ranges from 0.12 to 0.87), a broad biphasic (positive–negative) or triphasic waveforms (0.8–3.5 ms; 1.4 ms first positive and negative deflections) (Bambico et al, 2007; Allers and Sharp, 2003; Baraban and Aghajanian, 1980). Although these criteria may vary in response to pharmacological or ecological conditions (Bambico et al, 2009b), some spike features (waveform shape and spike duration) have been shown to be stable across conditions, and are therefore reliable indicators for 5-HT neurons (Aghajanian et al, 1978; Urbain et al, 2006; VanderMaelen and Aghajanian, 1983). When the regularity of firing was apparently altered by drug exposure/genetic manipulation (exceeding C.O.V. 0.87), the neurons were rigidly discriminated based on firing rates, spike shape, and duration. The regularity of neural firing activity was represented using inter-stimulus interval histograms or autocorrelograms. Burst firing activity was analyzed offline using a script designed for Spike 2 (Cambridge Electronic Design, Cambridge, UK). These analyses generated four parameters: mean ratio of the number of spikes within bursts to the sum total of recorded spikes (%), mean number of spikes contained in a burst, mean of inter-spike intervals (ISIs) in a burst, and mean

burst length. The parameters used for analyzing the bursts were based on the criteria of Gobbi et al (2005), such that a train of at least two spikes with an onset defined by a maximum initial ISI of 20 ms within a regular low-frequency firing pattern was categorized as a burst. The longest ISI allowed within a burst was 40 ms.

Extracellular recordings and microiontophoresis from the CA_3 dorsal hippocampus and the ventromedial prefrontal cortex. This procedure was modified after Gobbi et al (2001) and Labonte et al (2009). The multi-barreled micropipette was lowered into the CA_3 regions of the dorsal hippocampus (2–3 mm lateral and 2.5–2.7 mm posterior to bregma) or the infralimbic (IL) region of the ventromedial prefrontal cortex (mPFCv) (1.5–2.0 mm anterior to bregma; 2.5–3.5 mm ventral to the dura mater; 0.25 mm from the midline, within layer 5 of the cortex). The side barrels had impedances ranging from 50 to 150 MΩ and contained 5-HT, 8-OH-DPAT, DOI, or quisqualic acid, and a NaCl solution (2 M) for automatic current balancing. As most of the pyramidal neurons are not spontaneously active under chloral hydrate anesthesia, prolonged low-current quisqua-late ejections (from -2 to -5 nA) were introduced to activate them within their physiological firing rates (8–15 Hz in the hippocampus and 0.5–10 Hz in the mPFCv) (Ashby et al, 1990; ElMansari and Blier, 1997; Gobbi et al, 2001) and were retained with a current of $+10$ nA. It has been shown that there is no response difference between pharmacologically induced and spontaneously firing pyramidal neurons (Ashby et al, 1990). Neurons were also selected based on their steady response to standard short pulses of quisqualate and by large amplitudes, long durations, and single-action potential patterns alternating with complex spike discharges (Bartho et al, 2004; Gobbi and Janiri, 2006; Labonte et al, 2009). 5-HT was used to assess the function of the 5-HT transporter that is mainly responsible in the clearance of synaptic 5-HT through the tranmitter's reuptake. This was achieved by calculating the time required by neurons to recover 50% of the basal (initial) firing rate from the termination of the micro-iontophoretic application (designated as recovery time-50 (RT_{50})) (Gobbi et al, 2001; Pineyro et al, 1994). DOI was used to assess the sensitivity of $5\text{-HT}_{2A/2c}$ receptors and was ejected as a cation ($+5$ to $+50$ nA 1–2 min-currents) and retained with a current of -11 nA. 8-OH-DPAT was used to assess the sensitivity of 5-HT_{1A} receptors and was ejected as a cation ($+2$, $+4$, $+6$ and $+8$ nA in the hippocampus, $+1$, $+2$ and $+3$ nA in the mPFCv, 1–2 min currents) and retained with a current of -11 nA. Tonic hippocampal 5-HT_{1A} receptor activity was assessed by a single injection of 0.5 mg/kg WAY-100635. This dose was fivefold greater than the maximal intravenous dose validated earlier and used in our laboratory (Besson et al, 2000) as the administration route used here was intraperitoneal. On this parameter, we also compared FAAH$^{-/-}$ mice response to that of WT that have received chronic (12 days; once-a-day) treatment with the FAAH inhibitor URB597 (0.3 mg/kg, intraperitoneal) with or without cotreatment with rimonabant (1 mg/kg, intraperitoneal, once daily during the last 7 days of treatment). Pyramidal activity was identified by large amplitudes (0.5–1.2 mV), long durations (0.8–1.2 ms), and as single action potentials alternating with complex spike

discharges (Kandel and Spencer, 1961). Pyramidal neural response to systemic or microiontophoretic drug application was expressed as percentage increase/decrease from pre-drug (baseline) activity.

Euthanasia and histological verification. At the end of electrophysiological experiments, the animals were euthanized and the brains were harnessed and stored in paraformaldehyde. Coronal brain slices (20 µm) through regions of interests were prepared to inspect the location and extent of electrode lesions under a light microscope.

Data Analyses and Statistics

Data are presented as mean ± standard error of the mean. All data were analyzed using SPSS version 17 (SPSS Inc., Chicago, Illinois) and Datasim version 1.2 (D.R. Bradley, Bates College, Lewiston, Maine). After testing for assumptions of normality of data distribution and of homogeneity of variance, the behavioral data were accordingly submitted to three-way or two-way analyses of variance (ANOVA) with genotype (FAAH$^{-/-}$ vs WT) and CB_1R antagonist challenge (rimonabant vs vehicle) as between-group factors and time as a within-group factor, when appropriate. Electrophysiological data were submitted to Mann–Whitney U-tests for between-group comparisons or to two-way mixed design ANOVA (genotype × current) when accounting for repeated microiontophoretic current ejections. Tukey's honestly significant difference (HSD) test was used for multiple *post hoc* comparisons. Probability value of $p \leqslant 0.05$ was considered to be statistically significant.

RESULTS

Behavioral Assay

Effect of genetic FAAH deletion on locomotor activity and spontaneous behaviors in the open field. Figure 1 displays data obtained from the OFT. Only a main effect of genotype on distance covered was obtained after a three-way ANOVA with repeated measures across 5-min time bins ($F(1, 28) = 4.46$, $p = 0.044$) or a two-way ANOVA test on the area under the curve (AUC) ($F(1, 28) = 4.27$, $p = 0.048$); there was neither a significant main effect of drug (rimonabant) challenge nor genotype–rimonabant challenge interaction (Figure 1b). However, the administration of rimonabant resulted in an apparent decrease in locomotor activity regardless of genotype, attenuating distance covered by FAAH$^{-/-}$ mice to the level of vehicle-treated WT controls during the first 15 min. A significant main effect of time on distance covered was calculated ($F(3, 84) = 18.37$, $p < 0.001$). The interaction among factors (genotype, rimonabant challenge, and time) yielded no significance whereas rimonabant challenge-time interaction was marginally significant ($F(3, 84) = 2.34$, $p = 0.079$), indicating that the genetic and pharmacological interventions did not block habituation of locomotor activity. FAAH$^{-/-}$ mice, compared with the WT controls, displayed decreased thigmotactic behavior as determined by ANOVA on central zone duration (three-way ANOVA, main effect of genotype on central zone duration, $F(1, 28) = 4.91$, $p = 0.035$) or on AUC for central zone duration (two-way ANOVA, main
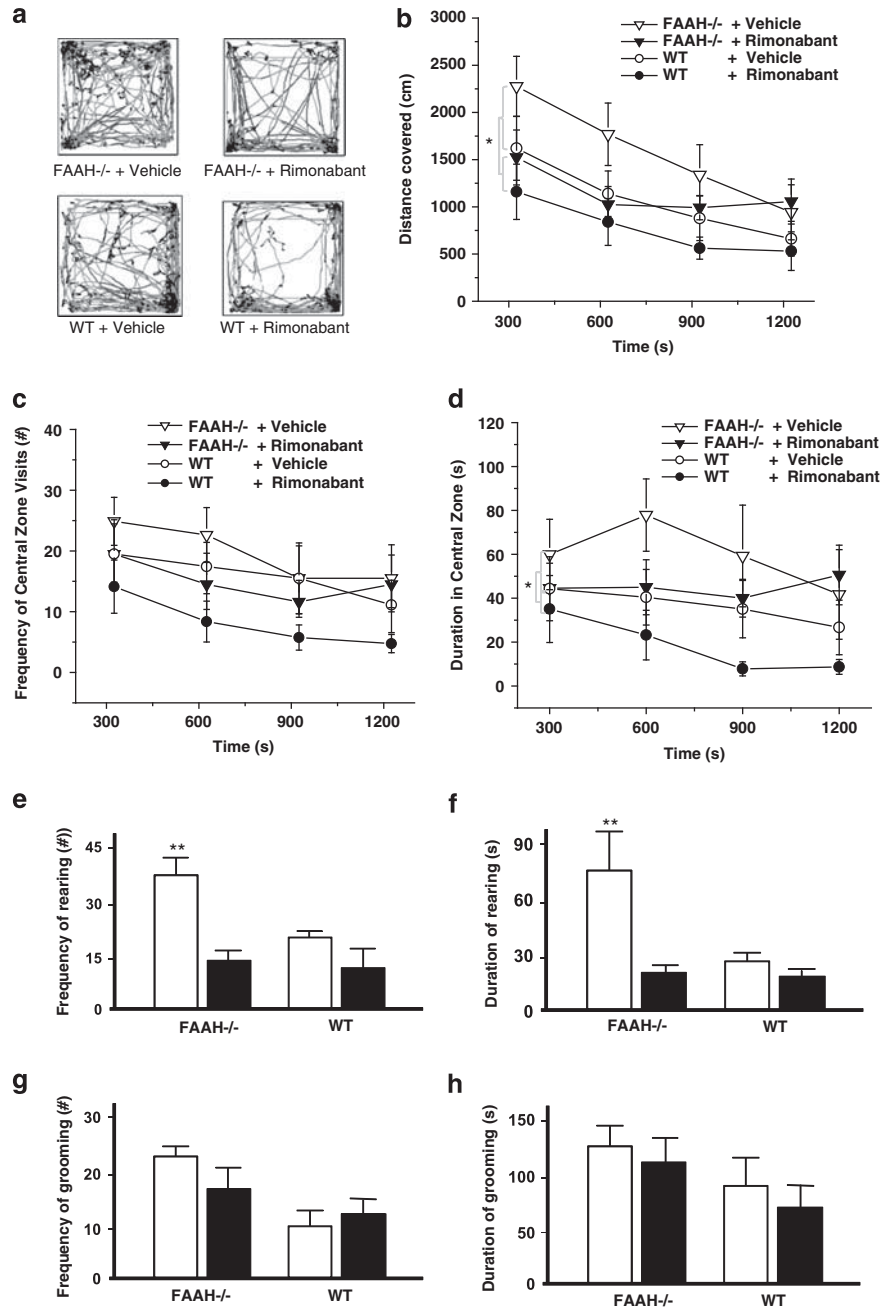
Figure 1 Effect of genetic deletion of FAAH on locomotor and exploratory activity in the open field test (OFT). (a) Horizontal movement traces of FAAH knockout mice (FAAH$^{-/-}$) and wild type (WT) controls after intraperitoneal injection of vehicle or the CB$_1$ receptor antagonist rimonabant (1 mg/kg) 30 min before the test. (b) Distance covered (cm) in horizontal locomotion. Rimonabant induced an apparent (nonsignificant) attenuation to the level of WT controls during the first 15 min of recording. Habituation of locomotor activity was not abolished by genetic FAAH deletion or by rimonabant. (c, d) FAAH$^{-/-}$ mice showed less thigmotaxis and longer times spent in the central zone; rimonabant induced an apparent nonsignificant attenuation to the level of WT controls. Reduced thigmotaxis in the open field indicated antianxiety effects. (e, f) Vertical exploratory activity (rearing) was less inhibited in FAAH$^{-/-}$ mice than in WT controls. This effect was reversed by rimonabant. (g, h) Genotype did not significantly influence grooming behaviour. Each trace in (a) is scaled from a 40 × 40 cm field dimension. Data points in (b–d) represent mean values ± standard error of mean (SEM) drawn from FAAH$^{-/-}$ mice treated with vehicle (unshaded triangle) or rimonabant (shaded triangle) and from WT controls treated with vehicle (unshaded circle) or rimonabant (shaded circle). *$p < 0.05$, main effect of genotype. Bar graphs in (e–h) represent mean values ± SEM after treatment with vehicle (unshaded) or rimonabant (shaded); **$p < 0.01$ *vs* rest of the groups, $N = 8$ animals per group.

effect of genotype, $F(1, 28) = 5.40$, $p = 0.028$) (Figure 1d). There was neither any significant main effect of time nor of rimonabant challenge, nor significant interactions among factors. No significant differences were calculated from the frequency of the central zone visits (Figure 1c). Ethological

analyses of anxiety-related and exploratory behaviors revealed a significant main effect of genotype and rimonabant challenge, as well as significant interaction effect, on rearing frequency (genotype, $F(1, 28) = 9.14$, $p = 0.005$; rimonabant challenge, $F(1, 28) = 15.23$, $p < 0.001$; interaction, $F(1, 28) = 5.94$,

$p = 0.021$; Figure 1e), and duration (genotype, $F(1, 28) = 15.01$, $p < 0.001$; rimonabant challenge, $F(1, 28) = 24.94$, $p < 0.001$; interaction, $F(1, 28) = 13.26$, $p = 0.01$; Figure 1f), but not on grooming (Figure 1g and h), turning, and immobility episodes (see Supplementary Table 1). *Post hoc* analyses on both frequency and duration of rearing indicated significantly lower values among drug-naive WT controls in comparison with drug-naive FAAH$^{-/-}$ mice ($p < 0.01$); this difference was completely abolished by rimonabant challenge (Figure 1e and f). Rimonabant only attenuated the rearing behavior when administered in FAAH$^{-/-}$ mice ($p < 0.01$).

Effect of genetic FAAH deletion on anxiety-related behaviors in the elevated plus maze. Data from the EPMT are presented in Figure 2a–d. Two-way ANOVA performed on the percentage of time spent in the open arm showed a

significant main effect of genotype ($F(1, 25) = 4.36$, $p = 0.047$) and rimonabant challenge ($F(1, 25) = 11.81$, $p = 0.002$), and a marginally significant interaction between these two factors ($F(1, 25) = 3.62$, $p = 0.069$) (Figure 2a). *Post hoc* comparisons indicated significantly lesser time spent in the open arms among drug-naive WT controls in comparison with drug-naive FAAH$^{-/-}$ mice ($p < 0.01$); this difference was completely abolished by rimonabant challenge. Rimonabant also exhibited attenuating effects on this measure when administered in FAAH$^{-/-}$ mice ($p < 0.01$). Rimonabant failed to elicit significant effects in WT controls. A similar pattern of effects was observed from the frequency of open arm entries with significant main effects of genotype ($F(1, 25) = 7.12$, $p = 0.013$) and rimonabant challenge ($F(1, 25) = 8.04$, $p = 0.008$), and a significant interaction effect ($F(1, 25) = 5.45$, $p = 0.027$) (Figure 2b). Two-way ANOVA on head dips failed to reveal
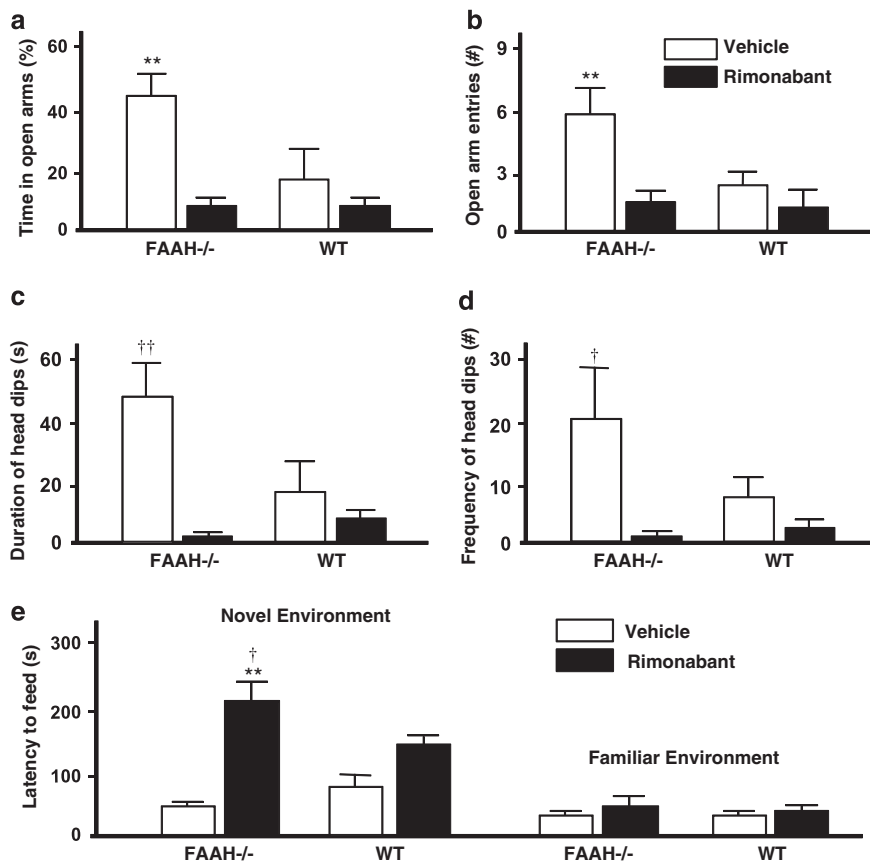

Figure 2 Effect of genetic deletion of FAAH on anxiety-like behaviours in the elevated plus maze test (EAMT) (a–d) and novelty-suppressed feeding test (NFST) (e). (a, b) Vehicle-treated FAAH knockout (FAAH$^{-/-}$) mice stayed significantly longer (a) and entered more frequently (b) in the open arms than did the vehicle-treated wild type (WT) controls. This resistance-like behaviour against the anxiogenic properties of the open arms were reversed by the intraperitoneal injection of the CB$_1$ receptor antagonist rimonabant (1 mg/kg) 30 min before the test. (c, d) No significant effect of genotype was noted on head dipping behaviour. Rimonabant significantly attenuated duration (c) and frequency (d) of head dips only in vehicle-treated FAAH$^{-/-}$ mice. Bar graphs represent mean values ± standard error of mean (SEM) after treatment with vehicle (unshaded) or rimonabant (shaded); **$p < 0.01$ vehicle-treated FAAH$^{-/-}$ mice *vs* rest of the groups; $^\dagger p < 0.05$, $^{\dagger\dagger}p < 0.01$ vehicle-treated FAAH$^{-/-}$ mice *vs* rimonabant-treated FAAH$^{-/-}$ mice. N = 7–8 animals per group. (e) Impact of genetic deletion of FAAH on the suppressant effect of novelty on feeding behaviour. Left portion: latency to feed in a novel environment was slightly prolonged in vehicle-treated FAAH$^{-/-}$ mice as compared with vehicle-treated WT controls, indicating reduced anxiety in FAAH$^{-/-}$ mice. Intraperitoneal injection rimonabant (1 mg/kg) 30 min before the test slightly prolonged latency to feed in WT, suggesting that a constitutive activity of the CB$_1$ receptor participates in the tonic inhibitory control of novelty-induced anxiety. The same dose of rimonabant led to an enormous and significant increase in the latency to feed in FAAH$^{-/-}$ mice, pointing to an increase in this CB$_1$ receptor-mediated tonic inhibitory control on novelty-induced anxiety. Right portion: There were no noticeable differences in feeding behaviour among experimental groups when tested in a familiar environment. Bar graphs represent mean latency to feed in seconds ± standard error of mean (SEM) after treatment with vehicle (unshaded) or rimonabant (shaded). **$p < 0.01$ *vs* vehicle-treated FAAH$^{-/-}$ mice, $^\dagger p < 0.05$ *vs* vehicle-treated and rimonabant-treated WT. N = 8 animals per group.

a significant effect of genotype (Figure 2c and d). A significant main effect of rimonabant challenge was calculated from head dip duration ($F_{(1, 25)} = 8.46$, $p = 0.008$), accompanied by a significant genotype–rimonabant challenge interaction ($F_{(1, 25)} = 5.76$, $p = 0.024$) (Figure 2c). Analysis of head dip frequency also yielded significant main effect of the rimonabant challenge ($F_{(1, 25)} = 8.91$, $p = 0.006$), accompanied by a marginally significant genotype–rimonabant challenge interaction ($F_{(1, 25)} = 3.5$, $p = 0.073$) (Figure 2d). *Post hoc* analyses indicated significantly lower head dip duration ($p < 0.01$) and frequency ($p < 0.05$) among drug-naive WT controls in comparison with drug-naive FAAH$^{-/-}$ mice. No difference was observed after rimonabant injection. Furthermore, when administered to FAAH$^{-/-}$ mice, rimonabant produced a robust attenuation in head dip duration and frequency falling below WT levels.

Effect of genetic FAAH deletion on anxiety-related behaviors in the NSFT. Figure 2e shows the suppressant effect of novelty on feeding behavior. Two-way ANOVA on the latency to feed in the novel environment showed a significant main effect of rimonabant challenge ($F_{(1, 60)} = 29.83$, $p < 0.01$), but not of genotype, and a significant genotype-rimonabant challenge interaction ($F_{(1, 60)} = 8.29$, $p < 0.006$) (Figure 2e, left portion). The onset to feed was not any more augmented in vehicle-injected FAAH$^{-/-}$ mice than in vehicle-injected WT mice. However, cohorts injected with rimonabant showed longer

delays in feeding onset regardless of the genotype, which were dramatically prolonged in FAAH$^{-/-}$ mice (74.27% longer delay relative to vehicle-injected FAAH$^{-/-}$ mice, $p < 0.001$, 58.74% relative to vehicle-injected WT controls, $p < 0.001$; and 35.74% relative to the rimonabant-injected WT controls, $p = 0.031$; Tukey HSD test). Rimonabant administration in WT mice was already sufficient to prolong the onset to feed (35.79% longer delays relative to vehicle-injected WT controls) albeit failing to reach significance. When feeding behavior was examined in the familiar home cage environment, no observable differences were noted among the experimental groups (Figure 2e, right portion).

Effect of genetic FAAH deletion on stress-coping behavior in the FST. Figure 3a presents the behavioral profile in the FST, comparing differences in immobility, swimming, and struggling episodes predictive of antidepressant-like related activity. Two-way ANOVA on the duration of behaviors revealed that compared with their WT counterparts, FAAH$^{-/-}$ mice spent significantly less time immobile (main effect of genotype, $F_{(1, 31)} = 5.02$, $p = 0.032$); the main effect of drug (rimonabant) challenge was not significant whereas the genotype–rimonabant challenge interaction was marginally significant ($F_{(1, 31)} = 3.48$, $p = 0.071$). *Post hoc* comparisons showed that immobility duration was significantly lower ($p = 0.037$) in vehicle-treated FAAH$^{-/-}$ mice than in vehicle-treated WT controls. The administration of rimonabant attenuated the decrease in immobility
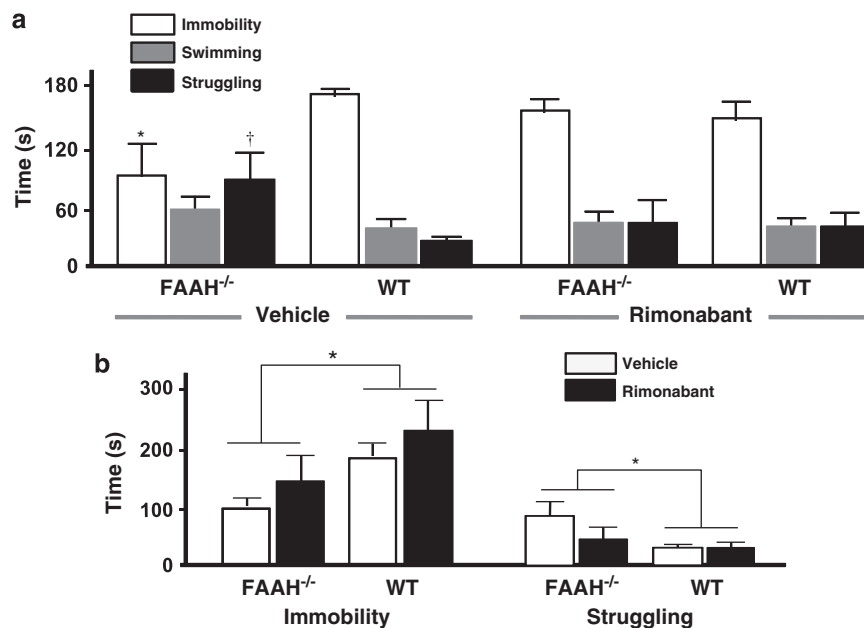

Figure 3 Antidepressant-like effects of genetic deletion of FAAH in the forced swim test (FST) and the tail suspension test (TST). (a) In the FST, vehicle-treated FAAH knockouts (FAAH$^{-/-}$ mice, left portion) displayed shorter and longer times in immobility and struggling, respectively, as compared with vehicle-treated wild type (WT) controls (right portion). As with treatment with standard antidepressants, this enhanced activity is predictive of antidepressant effects. Intraperitoneal injection of the CB$_1$ antagonist rimonabant (1 mg/kg) 30 min before the test attenuated this antidepressant-like effect. Bar graphs represent mean total time spent immobile (white), swimming (gray) and struggling (black), expressed in seconds ± standard error of mean (SEM) after treatment with vehicle or rimonabant as indicated. *$p < 0.05$, immobility duration of vehicle-treated FAAH$^{-/-}$ mice vs vehicle-treated WT controls; †$p < 0.05$, struggling duration of vehicle-treated FAAH$^{-/-}$ mice vs vehicle-treated WT controls. $N = 7$–10 animals per group. (b) In the TST, FAAH$^{-/-}$ mice spent shorter times immobile as compared with WT controls, regardless of rimonabant administration. Rimonabant (1 mg/kg), intraperitoneally injected 30 min before the test slightly increased immobility. Bar graphs represent mean total time spent immobile expressed in seconds ± SEM after treatment with vehicle (unshaded) or rimonabant (shaded). *$p < 0.05$, immobility duration of FAAH$^{-/-}$ mice vs WT controls. $N = 7$–12 animals per group.

duration observed in FAAH$^{-/-}$ mice. Analysis of the duration of struggling revealed a significant main effect of genotype (F(1,31) = 4.67, $p = 0.039$), a nonsignificant main effect of rimonabant challenge and a marginally significant genotype–rimonabant challenge interaction (F(1,31) = 3,69, $p = 0.064$). *Post hoc* comparisons showed that the struggling duration was significantly higher ($p = 0.038$) in vehicle-treated FAAH$^{-/-}$ mice than in vehicle-treated WT controls. The administration of rimonabant attenuated this increase in struggling duration observed in FAAH$^{-/-}$ mice. No significant effects were calculated from swimming duration. Two-way ANOVA on the frequency of behaviors revealed only a marginally significant main effect rimonabant challenge on the frequency of immobility episodes (F(1,31) = 3.37, $p = 0.076$, data not shown); there were no other significant differences obtained from swimming or struggling frequencies.

Effect of genetic FAAH deletion on stress-coping behavior in the TST. Figure 3b presents the behavioral profile in the TST, comparing differences in immobility and struggling episodes predictive of antidepressant-related activity. Two-way ANOVA on the duration of behaviors showed that FAAH$^{-/-}$ mice, compared with their WT counterparts, exhibited significantly less time being immobile and reciprocally more time in struggling, regardless of rimonabant challenge (main effect of genotype, F(1,33) = 6.54, $p = 0.015$) in the TST. The main effect of rimonabant challenge was marginally significant (F(1,33) = 3.17, $p = 0.084$), although rimonabant, but not the vehicle, appeared to slightly increase the duration of immobility, regardless of genotype. No significant genotype–rimonabant challenge interaction was found. Two-way ANOVA on the frequency of behaviors showed no significant differences in immobility or struggling (data not shown).

Effect of genetic FAAH deletion on weight gain. There was no significant difference between the mean weight of FAAH$^{-/-}$ mice and WT controls (see Supplementary Table 1).

In Vivo Electrophysiology

Effect of genetic FAAH deletion on spontaneous single-spike and burst firing activity of DR 5-HT neurons. No significant differences between FAAH$^{-/-}$ and WT mice were observed in terms of the burst firing activity of 5-HT neurons in the DR (percentage of spikes in bursts, number of spikes within bursts, and ISI) except for an increase in mean burst length from 3.82 ± 1.29 ms in WT controls to 7.20 ± 0.68 ms in FAAH$^{-/-}$ mice ($p = 0.048$, Mann–Whitney U-test). An inspection of ISI histograms generated from WT controls showed a Gausssian profile (Figure 4b top), and the autocorrelograms of WT single-spike activity indicated clear multiple peaks (Figure 4b bottom); these indicate a prominently regular neural firing among WT controls. By contrast, FAAH$^{-/-}$ mice exhibited positively skewed ISI histograms (Figure 4b, top) and flat autocorrelograms (Figure 4b, bottom), suggesting that the genetic FAAH deletion disrupts the regularity or rhythmicity of firing. In contrast, the mean spontaneous single-spike firing activity

recorded from FAAH$^{-/-}$ mice was significantly greater than that of the WT controls (+ 35%, $p = 0.008$, Mann–Whitney U-test; Figure 4a). *k*-means clustering aimed at grouping all 5-HT neurons recorded from FAAH$^{-/-}$ mice into two clusters segregated the neural population into fast-spiking neurons with a mean firing rate of 3.66 Hz ($n = 37$) and normal-spiking neurons with a mean firing rate of 1.78 Hz ($n = 36$) (F(1,71) = 138.12, $p < 0.001$). The mean firing rate of WT controls (1.74 ± 0.15 Hz) was significantly different from that of the high-spiking neurons ($p < 0.01$) but not from that of the normal-spiking neurons of FAAH$^{-/-}$ mice. In an attempt to characterize the effect of acute rimonabant administration, the response of 5-HT neurons sampled across the rostrocaudal extent of the DR of FAAH$^{-/-}$ mice and WT controls was monitored. In WT mice, all eight neurons recorded were normal spiking, two of which were moderately inhibited by a challenge of 1 mg/kg rimonabant (22.15–33.33%; representative, Figure 4c, lower left) whereas the rest were not responding. From among the responding neurons, higher rimonabant doses tested (> 1.5 mg/kg, intraperitoneal) attenuated firing activity until complete inhibition. In FAAH$^{-/-}$ mice, six out of nine neurons recorded were fast spiking and exhibited more profound inhibitory responses (23.99–52.69%; representative, Figure 4c, upper right) except for one non-responder, two of the nine were normal spiking and were non-responsive (representative, Figure 4c, lower right), and one of the nine was normal spiking and showed a slight excitatory response (inhibitory, excitatory, non-responders: FAAH$^{-/-}$ mice *vs* WT control, $\chi^2(2) = 6.0$, $p = 0.049$). Two-way ANOVA on rimonabant-induced decrease in firing activity from inhibited and non-responsive neurons showed a significant main effect of the rimonabant challenge (F(1,14) = 13.23, $p = 0.003$) and a slight genotype effect (F(1,13) = 3.58, $p = 0.079$), and interaction (F(1,13) = 3.31, $p = 0.090$) (Figure 4d). Administration of vehicle did not elicit a response from both genotypes. In contrast, rimonabant, but not the vehicle, elicited a dramatic decrease in 5-HT neural firing in FAAH$^{-/-}$ mice ($p < 0.01$), which was more profound than rimonabant's effect in WT controls ($p < 0.05$) (Figure 4d). Interestingly, 5-HT neurons that were profoundly inhibited by rimonabant were localized toward the rostral segment of the DR (Figure 4e). The responsive neurons from the WT controls were also located in the rostral DR.

Effect of genetic FAAH deletion on hippocampal 5-HT$_{1A}$ and 5-HT$_{2A/2C}$ function. To assess between-genotype differences in the overall transmission of 5-HT in the hippocampus, we examined the disinhibitory response of hippocampal CA$_3$ pyramidal neurons to a single bolus of WAY-100635. If 5-HT tonus on 5-HT$_{1A}$ receptors is increased, pyramidal firing activity will be greatly attenuated through the agonism of 5-HT at these inhibitory 5-HT$_{1A}$ receptors. Blockading these receptors by the 5-HT$_{1A}$ antagonist, WAY-100635, will accordingly neutralize inhibition, increasing pyramidal firing from basal level (Besson *et al*, 2000; Haddjeri *et al*, 1998). As shown in Figure 5a and b, the systemic administration of WAY-100635 potently disinhibited pyramidal activity in FAAH$^{-/-}$ mice fivefold more efficiently than in WT controls (one-way ANOVA, F(2,9) = 72.28, $p < 0.001$; Tukey's test, $p < 0.001$).
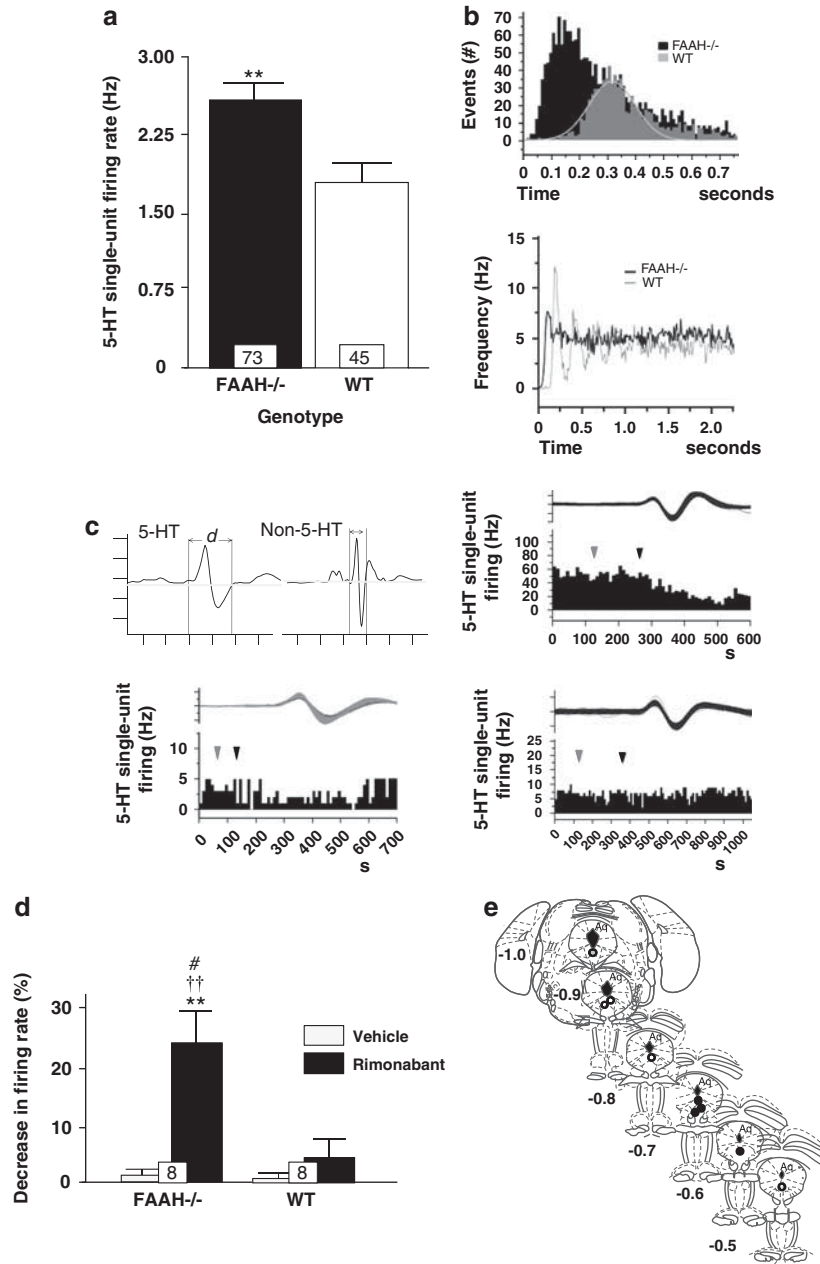
Figure 4 Effect of genetic deletion of FAAH on 5-HT neural activity in the DR nucleus. (a) FAAH knockout (FAAH$^{-/-}$) mice (shaded bar) exhibited significantly elevated mean spontaneous DR 5-HT neural firing activity relative to wild type (WT) control levels (unshaded bar). (b) Representative ISI histograms (top) and autocorrelograms (bottom) generated from a 5-HT neural activity of a FAAH$^{-/-}$ mouse (black bars/black trace) and a WT control (gray bars/gray trace) show profiles indicating irregular firing activity in FAAH$^{-/-}$ mice (skewed ISI profile, flat autocorrelogram) as opposed to the regular or rhythmic firing activity in WT controls (normally distributed ISI, multiple autocorrelogram peaks). (c) Representative integrated 5-HT neural firing rate histograms from a fast-spiking FAAH$^{-/-}$ mouse neuron (upper right), normal-spiking FAAH$^{-/-}$ mouse neuron (lower right) and from WT control neuron (lower left). Systemic injection of rimonabant (1 mg/kg, intraperitoneal, black arrows) was inhibitory to the fast-spiking FAAH$^{-/-}$ mouse neuron, was without effect on the normal-spiking FAAH$^{-/-}$ mouse neuron and slightly attenuative on the WT control neuron; vehicle injection (gray arrows) was without effect in all neurons. Upper left: typical spike characteristics of presumed 5-HT neurons (left) and non-5-HT neurons (right) encountered during electrophysiological recordings from the DR. Ordinate scale unit = 1 mV, abscissa scale unit = 1 ms, d = spike duration. (d) Intraperitoneal injection of rimonabant (shaded bars) more profoundly decreased mean DR 5-HT neural firing activity in FAAH$^{-/-}$ mice than in WT controls. (e) Postmortem histological verification of electrode descents against the recorded coordinates (based on the brain atlas of Paxinos and Franklin, 2001; numbers indicate millimeter distance of tissue section posterior to the interaural line, Aq = cerebral aqueduct) revealed that in FAAH$^{-/-}$ mice, 5-HT neurons inhbited by rimonabant (black circles) were localized towards the rostral segment of the DR while the nonresponders (white circles) and slightly stimulated ones (gray circle) were found towards the caudal segment of the DR. Bar graphs in (a) and (d) represent mean values ± SEM; the value at the bottom of each bar denotes the number of neurons recorded for that respective group. **$p<0.01$ *vs* WT control; $^{††}p<0.01$ *vs* vehicle-treated FAAH$^{-/-}$ mice or vehicle-treated WT control, $^{#}p<0.05$ *vs* rimonabant-treated WT control.
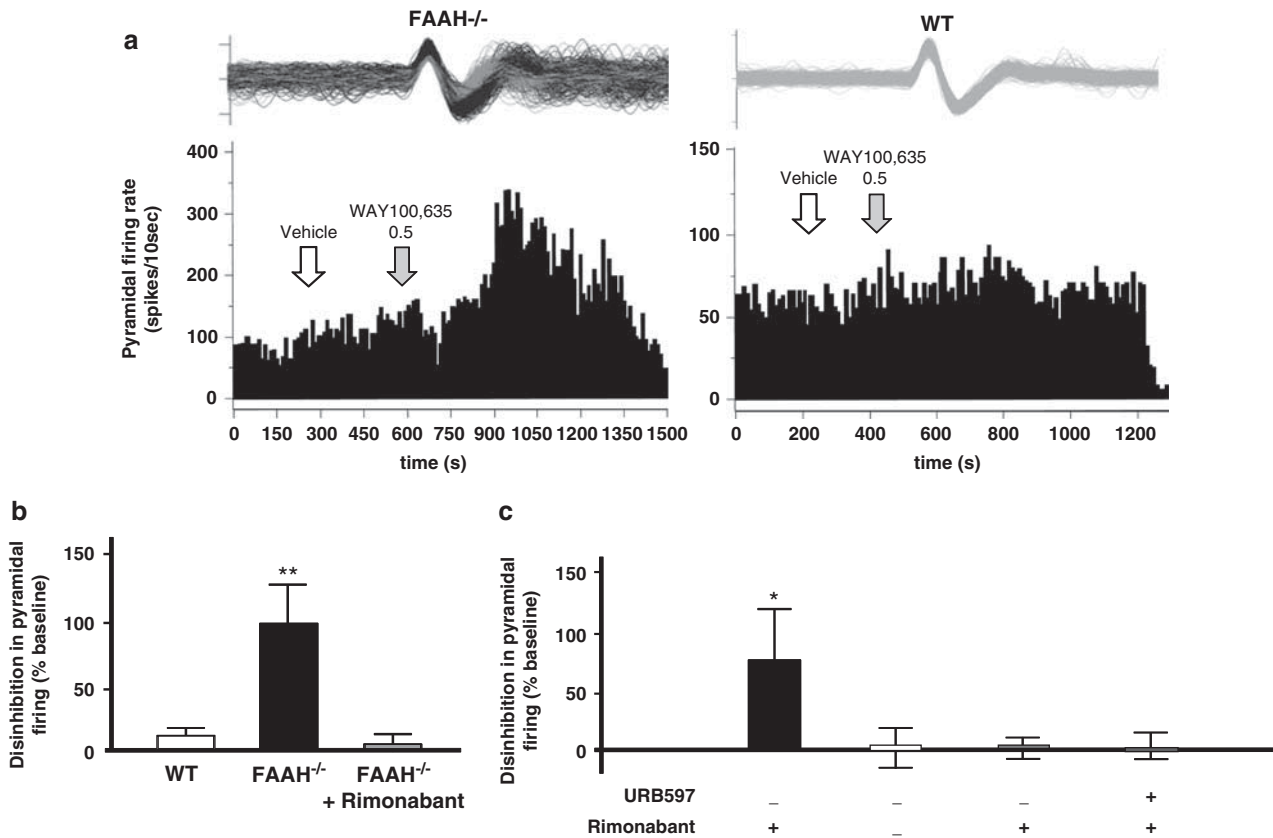
Figure 5 Effects of genetic and pharmacological deactivation of FAAH on postsynaptic 5-HT$_{1A}$ and 5-HT$_{2A/2C}$ receptors. Representative integrated hippocampal CA$_3$ pyramidal firing rate histograms (a) showing that intraperitoneal administration of the 5-HT$_{1A}$ antagonist WAY-100635 (0.5 mg/kg) (shaded arrows in a) but not of vehicle (unshaded arrows in a) potently disinhibited hippocampal pyramidal neural activity in FAAH knockout (FAAH$^{-/-}$) mice but not wild type (WT) controls. Bar graphs in (b) representing mean percentage of disinhibition (increase) ± SEM in the firing of hippocampal CA$_3$ pyramidal neurons in response to WAY-100635; this response in FAAH$^{-/-}$ mice (black bars in b) was significantly greater than in WT controls (white bars in b), indicating enhanced 5-HT tonus on hippocampal 5-HT$_{1A}$ receptors in FAAH$^{-/-}$ mice. This effect was abrogated by rimonabant treatment (1.0 mg/kg, 7 days, once-daily intraperitoneal administrations) (gray bars in b). $N = 4$ neurons per group. **$p < 0.001$ vs response of rimonabant-treated FAAH$^{-/-}$ mice or of WT control. (c) Bar graphs representing mean percentage of disinhibition (increase) ± SEM in the firing of hippocampal CA$_3$ pyramidal neurons in response to WAY-100635 (0.5–1.0 mg/kg, intraperitoneal) after chronic URB597 treatment (0.3 mg/kg, intraperitoneal, 12 dyas, once-daily, black bars in c) with or without cotreatment with rimonabant (1.0 mg/kg, intraperitoneal, during the last 7 days of URB597 treatment) in comparison with control (vehicle, white bars) or rimonabant alone (1.0 mg/kg, intraperitoneal, 7 days, once daily, gray bar). Chronic URB597 treatment produced a significant increase in tonic 5-HT$_{1A}$ activity that was prevented by rimonabant. Plus and minus signs below the bars indicate administration or nonadministration, respectively, of the drug indicated. $N = 4$–5 neurons per group. *$p < 0.05$ vs response of URB597 plus rimonabant, of rimonabant alone or of control (vehicle).

Systemic administration of the vehicle in either FAAH$^{-/-}$ mice or WT controls did not at all affect pyramidal firing activity. To examine locally possible changes in the sensitivity of hippocampal 5-HT$_{1A}$ and 5-HT$_{2A/2C}$ receptors, iontophoretic ejections of the 5-HT$_{1A}$ agonist 8-OH-DPAT and 5-HT$_{2A/2C}$ agonist DOI were performed while recording from pyramidal neurons. Both these agonists inhibited pyramidal firing, the degree of which was proportional to the amount of current ejected. However, between-genotype pyramidal response did not significantly differ (Figure 6b and c) indicating that the sensitivity of hippocampal 5-HT$_{1A}$ and 5-HT$_{2A/2C}$ receptors was no different in FAAH$^{-/-}$ mice than in WT controls. Figure 6a shows the stereotactic location of recording sites on one coronal section. Similarly, there was no significant difference between the RT$_{50}$ of 5-HT-induced pyramidal inhibition in FAAH$^{-/-}$ mice and WT controls suggesting that the function of the 5-HT transporter system was not affected by the genetic deletion of FAAH (data not shown). We further ascertained whether

the enhanced hippocampal 5-HT$_{1A}$ receptor tonic activity was truly because of increased anandamide-CB$_1$R signaling and not merely to non-specific compensatory mechanisms resulting from a life-long condition of increased anandamide levels. First, we found that rimonabant (1.0 mg/kg, 7 days, once-daily, intraperitoneal) in FAAH$^{-/-}$ mice abrogated the disinhibitory response of pyramidal neurons to WAY-100635 (one-way ANOVA, $F(2, 9) = 72.28$, $p < 0.001$; FAAH$^{-/-}$ vs FAAH$^{-/-}$ plus rimonabant or vs WT, Tukey's test, $p < 0.001$, Figure 5b). Interestingly, chronic (12 days, once daily) treatment with the FAAH inhibitor, URB597, at a dose (0.3 mg/kg, intraperitoneal) shown earlier by Bortolato et al (2007) to exhibit a potent antidepressant-like effect in the mouse chronic unpredictable mild stress model displayed enhanced disinhibitory response of pyramidal neurons to WAY-100635, achieving a significant difference from control (vehicle) levels in response to 1.0 mg/kg WAY-100635 (one-way ANOVA, $F(4, 16) = 4.44$, $p = 0.013$; URB597 vs control (vehicle), Tukey's test,
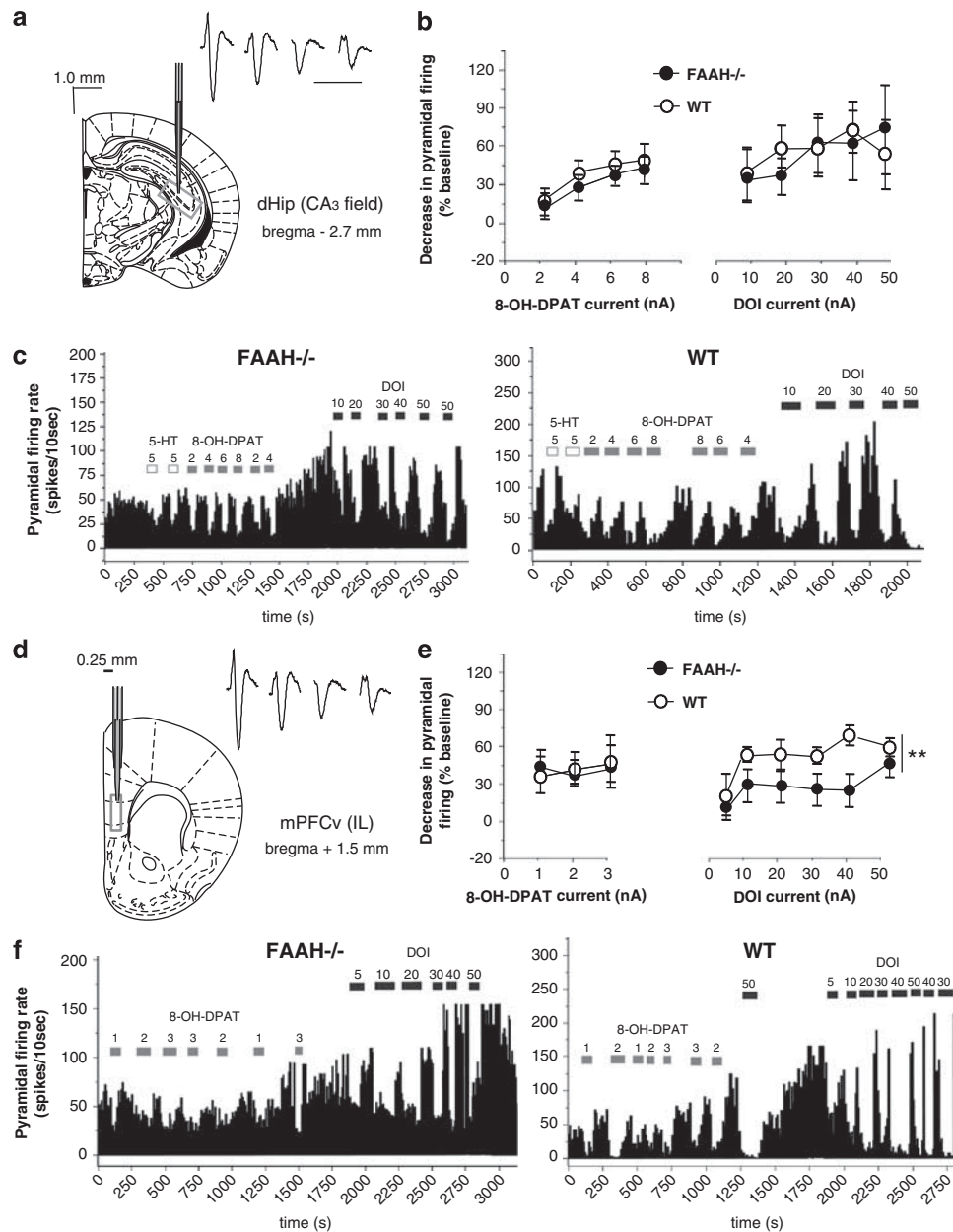
Figure 6 Effect of genetic deletion of FAAH on postsynaptic 5-HT$_{1A}$ and 5-HT$_{2A/2C}$ receptors. Stereotactic location of the recording sites in the CA$_3$ field (boxed area, scale bar on the upper left of figure) of the dorsal hippocampus (dHip, a) or the ventromedial prefrontal cortex (mPFCv) (d), infralimbic (IL) region, layer 5 (0.25 mm lateral to midline, boxed area) and a representation of the complex spike waveform of pyramidal neurons (black bar = 10 ms duration, scaled). (b) Line graphs showing the lack of difference in the mean decrease ($\pm$ SEM) in hippocampal CA$_3$ pyramidal neural activity (ordinate) between fatty acid amid hydrolase knockout (FAAH$^{-/-}$) mouse (black circles) and a wild type (WT) control (white circles) in response to increasing doses of 8-OH-DPAT (abscissa, left panel) and DOI (abscissa, right panel). (c) Representative integrated firing rate histograms showing the activity of hippocampal CA$_3$ pyramidal neurons (ordinate, spikes/10 s) plotted against time (abscissa). There is no difference between a FAAH$^{-/-}$ mouse (left panel) and a WT control (right panel) in the degree of response to microiontophoretic currents (values on top of horizontal bars) of 5-HT (white horizontal bars), the 5-HT$_{1A}$ agonist 8-OH-DPAT (gray horizontal bars) and the 5-HT$_{2A/2C}$ agonist DOI (black horizontal bars), meaning that both 5-HT$_{1A}$ and 5-HT$_{2A/2C}$ receptors remained normosensitive. (e) Line graphs showing between-genotype difference (FAAH$^{-/-}$ mouse: black circles; WT controls: white circles) in the response of mPFCv pyramidal neurons (ordinate, spike/10 s) to increasing microiontophoretic currents of DOI (abscissa, right panel) but not to those of 8-OH-DPAT (abscissa, left panel). **$P<0.01$. (f) Representative integrated firing rate histograms showing the activity of mPFCv pyramidal neurons (ordinate, spikes/10 s) plotted against time (abscissa). The response to microiontophoretic currents (values on top of horizontal bars) of DOI (back horizontal bars) but not of 8-OH-DPAT (gray hrizontal bars) is significantly blunted in FAAH$^{-/-}$ mouse (left panel) in comparison with a WT control (right panel).

$p<0.031$, Figure 5c); this effect was similar to that observed in FAAH$^{-/-}$ mice. We then found that similar to that observed in FAAH$^{-/-}$ mice, rimonabant (1.0 mg/kg, 7 days, once daily, intraperitoneal) co-applied with URB597 during the last 7 of the 12-day chronic URB597 regimen blocked the disinihbitory response of pyramidal neurons to WAY-100635 (URB597 *vs* URB597 plus rimonabant, Tukey's test, $p<0.016$, Figure 5c).

Effect of genetic FAAH deletion on prefrontocortical 5-HT$_{1A}$ and 5-HT$_{2A/2C}$ function. Microiontophoretic ejection of increasing 8-OH-DPAT currents failed to show significant differences in the response of pyramidal neurons in the IL prefrontal cortex (layer 5) of FAAH$^{-/-}$ mice and WT controls (Figure 6e and f). This indicates a lack of difference in the sensitivity of prefrontocortical 5-HT$_{1A}$ receptors of FAAH$^{-/-}$ and WT mice. In contrast, iontophoretic ejection of DOI showed a significant main effect of current (two-way ANOVA, $F(5, 32) = 4.12$, $p = 0.005$) and genotype $(F(1, 32) = 13.68$, $p < 0.001)$, but without significant interaction between these two factors (Figure 6e and f). These observations indicate a current-dependent inhibition of prefrontocortical pyramidal neurons through 5-HT$_{2A/2C}$ receptor activation and that genetic FAAH deletion downregulates this 5-HT$_{2A/2C}$ receptor-mediated inhibition. Figure 6d shows the stereotactic location of recording sites on one coronal section.

DISCUSSION

This study provides evidence that genetic deletion of FAAH confers resistance to anxiety-like and depression-like behavioral responses, along with an enhancement in the spontaneous activity of DR 5-HT neurons. This phenotype is accompanied by the desensitization of prefrontocortcal 5-HT$_{2A/2C}$ receptors and increased tonic hippocamapal 5-HT$_{1A}$ activity, likely representing postsynaptic adaptations to increased impulse-dependent 5-HT release.

Compared with wild types (WT), FAAH knockout (FAAH$^{-/-}$) mice were found to be less avoidant of the aversive open arms of the elevated plus maze and the central zone of the open field, and displayed significantly greater exploratory rearings—all indications of antianxiety. They were also more resistant to depression-like behavior in the forced swim and tail suspension tests, indicated by greater time spent in active coping. These observations are consistent with those already made by others in these FAAH$^{-/-}$ mice (Moreira *et al*, 2008; Naidu *et al*, 2007), and with results obtained from the pharmacological enhancement of anandamide levels through FAAH inhibitors (eg, URB597) and reuptake blockers (eg, AM404) (Gobbi *et al*, 2005; Moreira *et al*, 2008; Naidu *et al*, 2007; Patel and Hillard, 2006). Interestingly, similar to the effects of URB597 (Haller *et al*, 2009), the anxiolytic-like and antidepressant-like reactivity of FAAH$^{-/-}$ mice can be effectively evoked under certain laboratory and handling conditions (Moreira *et al*, 2008; Naidu *et al*, 2007). This is not surprising as other studies propose that optimization of experimental conditions, such as adjusting anxiogenic testing conditions in tests for anxiety-like reactivity (eg, the elevated plus maze) (Hogg, 1996) and behavioral despair tests (eg, the forced swim test) (Kelliher *et al*, 2000) more potently unmasks or enhances the sensitivity to even the conventionally used anxiolytics and antidepressants. Under highly anxiogenic conditions (eg, high-lighting) in tests for anxiety-related behavior and under less anxiogenic conditions (eg, dim-lighting) in behavioral despair tests, we were able to elicit potent genotype-dependent responses. All the anxiolytic-like responses elicited here were completely reversed by rimonabant, and are therefore mediated by an increased

anandamide-CB$_1$R signaling in FAAH$^{-/-}$ mice. Indeed, these mice have been reported to express 10-fold higher levels of intrinsic anandamide than those of WTs, without significant changes in the density of cerebral CB$_1$R (Basavarajappa *et al*, 2006; Cravatt *et al*, 2001). Furthermore, CB$_1$R appears to participate in a tonic inhibitory control of anxiety, evidenced by the rimonabant-induced reversal of responses of FAAH$^{-/-}$ mice below WT levels. In further support of this hypothesis, we also noted that rimonabant produced a slight anxiogenic-like response when administered to WTs, corroborating earlier reports of its anxiogenic-like effects in the defensive withdrawal test and elevated plus maze (Navarro *et al*, 1997). It is also possible that the anxiety-like response to rimonabant expressed by both genotypes observed here, which was particularly exaggerated in the novelty-suppressed feeding test is due to the interaction of the antagonism on tonic inhibition of anxiety and rimonabant's inverse agonistic property. This role on the tonic inhibitory control of anxiety is consistent with observations reported earlier that stress and anxiogenic stimuli lead to an increase in the hydrolytic activity of FAAH and a reduction in forebrain anandamide levels (Patel *et al*, 2005; Hill *et al*, 2009b). The attenuation of anandamide signaling would gate physiological responses associated with anxiety, including the activation of the hypothalamic-pituitary-adrenal axis and increased corticosterone production (for review, see Hill and McEwen (2009)). Under conditions of stable FAAH inactivity, as in FAAH$^{-/-}$ mice, the resultant elevation in anandamide tone would buffer stress-related responses; the behavioral impact of this buffering should be most evident under more aversive or anxiety-provoking conditions. The recruitment specifically of the CB$_1$R by anandamide in this tonic regulation of anxiety-related processes is further supported by the fact that transgenic mice deficient in CB$_1$R exhibit increased anxiety-like behavior in the light/dark test (Martin *et al*, 2002; Uriguen *et al*, 2004), elevated plus maze and social interaction test that persists after treatment with an otherwise effective dose of standard anxioytics (Uriguen *et al*, 2004). These CB$_1$R knockout mice also show exaggerated increases in plasma corticosterone in response to restraint stress (Uriguen *et al*, 2004). In the forced swim and tail suspension tests, rimonabant also attenuated the antidepressant-like phenotype of FAAH$^{-/-}$ mice, convergent with that observed with URB597 in rats (Gobbi *et al*, 2005), implicating the involvement of the CB$_1$R mediating the antidepressant-like response in FAAH$^{-/-}$ mice. However, caution must be taken in interpreting these data as under the testing conditions adopted here, FAAH$^{-/-}$ mice also exhibited moderate locomotor stimulation, possibly confounding results in these tests. Nevertheless, our electrophysiological experiments revealed a significantly elevated mean DR 5-HT neural activity in FAAH$^{-/-}$ mice that could provide the neurochemical substrate by which anxiolytic-like and antidepressant-like reactivity may be conveyed. Such an impact on 5-HT activity has been observed earlier with URB597 (Gobbi *et al*, 2005) and a low dose of the CB$_1$R agonist, WIN55,212-2 (Bambico *et al*, 2007), and is consistent with the reported augmentation of the postsynaptic 5-HT content by cannabinoid agonists (Miczek and Dixit, 1980; Holtzman *et al*, 1969; Sagredo *et al*, 2006) or URB597 (Gobbi *et al*, 2005). This increase in 5-HT

neural activity has been reliably replicated in other transgenic models of depression and antidepressant activity. For example, TREK-1 background potassium channel (Heurteaux *et al*, 2006) and NK$_1$ receptor (Santarelli *et al*, 2001) null-mutant mice have been shown to exhibit a similar augmentation in spontaneous DR 5-HT neural firing associated with antidepressant-like behavior. Standard 5-HT-acting antidepressants, such as SSRI and monoamin-oxidase inhibitors, initially suppress DR 5-HT neural firing but gradually enhance it thereafter, coinciding with the desensitization of 5-HT$_{1A}$ autoreceptors. This late 5-HT-increasing phase is linked to the onset of antidepressant effect (Bambico and Gobbi, 2008). Other new-generation antidepressants purportedly possessing faster onset of therapeutic action, such as reboxetine (Linner *et al*, 2004) and mirtazapine (Haddjeri *et al*, 1996), and other novel antidepressant treatments, such as NK-1 antagonists (Haddjeri and Blier, 2001) and vagus nerve stimulation (Dorr and Debonnel, 2006), also increase DR 5-HT neural firing. In contrast, 5-HT$_4$ receptor (Conductier *et al*, 2006) and 5-HT transporter (Gobbi *et al*, 2001) null-mutant mice, and neonatal clomipramine treatment (Kinney *et al*, 1997), drug withdrawal (Pistis *et al*, 1997), and high-frequency subthalamic stimulation (Temel *et al*, 2007) have all been shown to be associated with decrements in spontaneous DR 5-HT firing and enhanced depression-like behaviors. Recently, genetic CB$_1$R deletion and prolonged rimonabant treatment in mice have been shown to result in increased DR 5-HT neural firing *ex vivo*, instigated by impaired 5-HT$_{1A}$ autoreceptor negative feedback. As these mice simultaneously display enhanced depression-like behavior, this increase in 5-HT firing is interpreted to represent an adaptive response to depression-related neurobiological sequelae (Aso *et al*, 2009).

From our data, it is important to note the presence of fast-spiking and normal-spiking subsets of 5-HT neurons recorded along the midline axis of the DR in FAAH$^{-/-}$ mice. Interestingly, fast-spiking 5-HT neurons appear to be localized in the rostral DR subdivisions and are profoundly inhibited by a challenge of rimonabant, whereas normal-spiking neurons appear to be localized in the caudal regions and are unresponsive to rimonabant. This topographical pattern of reactivity to rimonabant is consistent with the presence of CB$_1$Rs mainly on 5-HT neural terminals in the caudal DR, and on many non-5-HT, likely GABAergic interneurons in the rostral DR (Haring *et al*, 2007) that synapse with and impose tonic inhibition on neighboring 5-HT neurons. In these FAAH$^{-/-}$ mice, an increase in intrinsic anandamide leads to CB$_1$R-mediated attenuations of GABAergic interneuronal activity and/or GABA efflux and a consequent disinhibition of 5-HT neurons. Rimonabant may conceivably reverse this disinhibition of 5-HT neurons by competing with anandamide on interneuronal CB$_1$Rs. Alternatively, 5-HT-modulating CB$_1$Rs may also be localized in the ventromedial prefrontal cortex (Bambico *et al*, 2007) or on glutamatergic terminals (Haj-Dahmane and Shen, 2009) that synapse with GABAergic interneurons or 5-HT neurons in the DR (Celada *et al*, 2001). The existence of this segregable 5-HT neural subpopulations may partly explain the region-selective alterations in postsynaptic 5-HT basal levels and evoked efflux we reported elsewhere (Bambico *et al*, 2009a).

Prefrontocortical pyramidal responses in FAAH$^{-/-}$ mice to microiontophoretically ejected agonists at 5-HT$_{2A/2C}$ and 5-HT$_{1A}$ receptors, DOI and 8-OH-DPAT respectively, showed normosensitive 5-HT$_{1A}$ receptors but subnormal 5-HT$_{2A/2C}$ receptor sensitivity. This 5-HT$_{2A/2C}$ receptor desensitization corroborates reported attenuations in 5-HT$_{2A}$ binding, 5-HT$_{2A}$-associated signal transduction and associated behavioral responses (for review, see Hill *et al* (2009a)). Chronic treatment with antidepressants, such as SSRIs, that potentiate 5-HT efflux in the prefrontal cortex can similarly lead to a downregulation of 5-HT$_{2A/2C}$ receptors in this region (Hill *et al*, 2009a; Hollander *et al*, 1991b; Kennett *et al*, 1994b; Quested *et al*, 1997). This downregulation has been associated with the anxiolytic efficacy of these drugs, as the antagonism of these receptors possesses identical therapeutic effects (Deakin, 1988; Griebel *et al*, 1997; Adamec *et al*, 2004) or augments the effects of antidepressants (Marek *et al*, 2003; Hill *et al*, 2009a), whereas agonism produces panic and anxiety in otherwise healthy humans (Germine *et al*, 1994). Furthermore, prolonged corticosterone exposure results in an enhancement of frontocortical 5-HT$_2$-related functions, an effect that was found to be reversed by chronic antidepressant treatment (Zahorodna and Hess, 2006). In contrast, hippocampal CA$_3$ pyramidal neurons of FAAH$^{-/-}$ mice exhibited supranormal responses to the disinhibitory effects of systemically injected 5-HT$_{1A}$ antagonist WAY-100635, indicating an increase in the tonus on hippocampal 5-HT$_{1A}$ receptors in FAAH$^{-/-}$ mice, which were similarly observed after chronic URB597 treatment. The similarity of effects of genetic and pharmacological FAAH deactivation and the fact that both were blocked by prolonged co-application with rimonabant strengthen the notion that postsynaptic 5-HT receptor adaptations are due to the common mechanism of increased anandamide-CB$_1$R signaling, and that the effects observed in FAAH$^{-/-}$ mice are not merely because of non-specific compensatory mechanisms resulting from a life-long condition of increased anandamide levels. These data are reminiscent of the hallmark enhancement in hippocampal 5-HT$_{1A}$ tonus that follows long-term administration of different classes of antidepressant treatments, including electroconvulsive shock (Haddjeri *et al*, 1998; Besson *et al*, 2000; Szabo and Blier, 2001b). This similar observation made in FAAH$^{-/-}$ mice may be explained by the increase in the depolarization-induced efflux of 5-HT in this structure, which has been shown in this transgenic mouse (Bambico *et al*, 2009a). We could also consider the possible involvement of an augmentation of noradrenergic neurotransmission, which has been reported to be associated with CB$_1$R activation and FAAH inhibition (for review, see Bambico *et al*, 2009a). Furthermore, as enhanced hippocampal 5-HT$_{1A}$ function has been linked to the induction of cell proliferation and neurogenesis in the dentate gyrus (Jacobs *et al*, 2000) such as that shown with fluoxetine (Santarelli *et al*, 2003), this increase in tonic hippocampal 5-HT$_{1A}$ activity in FAAH$^{-/-}$ mice may contribute, at least in part, to the enhanced hippocampal neurogenic or proliferative processes observed in these mice (Aguado *et al*, 2005, 2006).

Although these data altogether highlight the role of increased anandamide-CB$_1$R signaling in the anxiolytic-like and antidepressant-like phenotype of FAAH$^{-/-}$ mice, we

cannot completely rule out the potential involvement of other neurobiological factors that are found to be similarly altered in these mice. For instance, FAAH inactivation has been documented to elicit hormonal changes and increased production of neurosteroids or other lipidic molecules, such as palmithoylethanolamide and oleoylethanolamide (Gobbi et al, 2005), whose behavioral functions are less explored, and whose interactions with the monoaminergic systems remain largely unknown. Nevertheless, the findings at hand strengthen the notion that FAAH is a potential pharmacological and genetic target for the development of treatment strategies for depression and anxiety. Through this approach, the interaction of the endocannabinoid and monoaminergic systems may be harnessed to modulate 5-HT neurotransmission. The resultant prefrontocortical $5\text{-HT}_{2A/2C}$ desensitization and hippocampal 5-HT_{1A} functional upregulation starkly contrast with $5\text{-HT}_{2A/2C}$ upregulation and 5-HT_{1A} downregulation observed after long-term treatment with direct CB_1R agonists (Cheer et al, 1999; Hill et al, 2006), which are contrariwise asssociated with anxiogenic-like or depressogenic-like sequelae (Maes and Meltzer, 1995), further arguing for the favorability of indirect CB_1R agonism through FAAH inactivation over direct CB_1R agonism.

ACKNOWLEDGEMENTS

DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)