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Fatty acid amide hydrolase (FAAH) inactivation confers enhanced sensitivity to nicotine-induced dopamine release in the mouse nucleus accumbens

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Abstract

Nicotine exerts its rewarding effects by promoting an increase in dopamine (DA) release in the nucleus accumbens (NAc) and this process is influenced by the endocannabinoid system. Fatty acid amide hydrolase (FAAH) is the main enzyme responsible for the degradation of the endocannabinoid anandamide and other non-cannabinoid *N*-acylethanolamines. Previous research has reported that both genetic deletion and pharmacological inhibition of FAAH enhance nicotine-induced conditioned place-preference (CPP) at low doses. We conducted a microdialysis study to characterize nicotine-induced changes in DA and serotonin (5-HT) levels in the NAc of FAAH knockout (KO) mice using a CPP-like paradigm with 3 nicotine doses (0.1, 1 and 10 mg/kg, s.c.). Additionally, the effects of the selective FAAH inhibitor PF-3845 (10 mg/kg, i.p.) were also examined. Our data indicated that compared to wild-type mice, genetic deletion of FAAH selectively enhanced the effect of low-dose nicotine on DA release ($p < 0.001$) and resulted in a strong post-nicotine elevation in DA levels ($p < 0.01$). However, there were no differences between the genotypes at higher doses. Furthermore, FAAH KO mice displayed a moderate enhancement of the effect of low-dose nicotine on NAc 5-HT release ($p < 0.05$), with no differences between the genotypes at higher doses. Compared with vehicle-pretreated mice, mice pretreated with PF-3845 displayed an enhancement of the effect of low-dose nicotine on NAc DA release ($p < 0.001$), which

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AUTHOR CONTRIBUTIONS

LHP was responsible for the study concept and design. BFC provided the FAAH KO mice and their WT counterparts. IP performed the nicotine studies in mice using a CPP test. FJP and AS conducted the microdialysis experiments in the NAc and collected dialysate samples. FJP and DS performed the quantification of DA and 5-HT from dialysate samples. FJP, NS and RM-F assisted with data analysis and interpretation of the findings. FJP and FRF drafted the manuscript. RM-F and FRF provided critical revision of the manuscript for important intellectual content. All authors critically reviewed the content and approved the final version for publication.

resulted in a sustained increase in DA levels ($p<0.05$). Similar to FAAH KO mice, PF-3845-pretreated mice displayed a moderate enhancement of the effect of low-dose nicotine on NAc 5-HT release ($p<0.01$). These observations in mice suggest that enhanced nicotine-induced NAc DA release might contribute to increased sensitivity to the conditioned rewarding effects of low-dose nicotine following FAAH inhibition, which has been previously reported. Future studies combining behavioral and neurochemical approaches are needed to elucidate the precise mechanism of these effects.

Keywords

Nicotine; FAAH; mice; PF-3845; microdialysis; monoamines

INTRODUCTION

Nicotine is the primary psychoactive constituent in tobacco smoke that initiates and maintains tobacco addiction. Nicotine exerts its rewarding and reinforcing effects in the central nervous system (CNS) by activating nicotinic acetylcholine receptors (nAChR) in the ventral tegmental area (VTA) to promote a sustained increase in dopamine (DA) release in the nucleus accumbens (NAc) that binds to DA receptors (e.g., D1R and D2R), which is considered pivotal to the development of nicotine addiction (Wonnacott et al., 2005). In this context, the VTA is primarily modulated by excitatory glutamatergic inputs that arise from the PFC, amygdala and pontomesencephalic tegmental nuclei and by inhibitory GABAergic interneurons and neurons arise from the NAc and other areas such as the rostromedial tegmental nucleus (RTMg) (Barrot et al., 2012; Feduccia et al., 2012) (Fig. 1a).

There are several different nAChR receptors expressed in the VTA, although the heteromeric receptors $\alpha 4\beta 2$ and the homomeric receptors $\alpha 7$ play a major role for nicotine-mediated DA effects in the VTA. The $\alpha 4\beta 2$ receptors are very expressed in dopaminergic and GABAergic neurons, while $\alpha 7$ nAChRs are densely localized on glutamatergic terminals in the VTA (Feduccia et al., 2012). Nicotine activation of nAChRs induces a cascade of interactions with several neurobiological systems involved in the regulation of nicotine addiction (Castane et al., 2005; Muldoon et al., 2013). Among these neurobiological systems, lipid signaling pathways such as the endocannabinoid system have acquired great importance due to their role in the etiology of drug addiction (Panlilio et al., 2013; Parsons and Hurd, 2015; Serrano and Parsons, 2011).

The endocannabinoid system consists of two well-characterized G protein-coupled receptors (CB_1 and CB_2), endogenous ligands (endocannabinoids) that bind to these receptors, enzymes responsible for endocannabinoid synthesis and metabolism, and processes involved in the cellular uptake of some endocannabinoids (Pertwee, 2015). CB_1 receptors are widely distributed throughout the peripheral nervous system and CNS, and CB_2 receptors are mainly associated with immune cells in the periphery. Regarding endocannabinoids, the most extensively studied endocannabinoids are anandamide (*N*-arachidonylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG), which are synthesized “on demand” through cleavage from membrane lipid precursors and immediately extruded from neurons through

calcium-independent molecular mechanisms to exert agonist activity at cannabinoid receptors and other receptor targets. Fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) are the main enzymes responsible for the degradation of AEA and 2-AG, but there are a growing number of additional enzymes that are reportedly involved in endocannabinoid catabolism, such as *N*-acylethanolamine-hydrolyzing acid amidases (NAAA), lipoxygenases, cyclooxygenases (COX-2), serine hydrolase α - β -hydrolases (ABHD6/12) and cytochrome P450 monooxygenases [for review (Fezza et al., 2014)]. The enzymatic degradation of AEA is primarily due to the activity of FAAH, but this hydrolase can also metabolize other fatty acid ethanolamides [e.g., *N*-oleoylethanolamine (OEA) and *N*-palmitoylethanolamine (PEA)] (Schmid et al., 1985) and 2-AG (Ahn et al., 2008).

Previous studies in rodents have shown that whereas CB₁ and CB₂ agonists increase the rewarding effects of nicotine (Gamaledin et al., 2012), CB₁ antagonists/inverse agonists decrease nicotine intake and seeking in rodents, which also occurs in CB₁ deficient mice (Castane et al., 2002; Cohen et al., 2002). Consequently, elevation of endocannabinoid tone by inhibiting degradative enzymes, such as FAAH, may lead to an increase in the reinforcing effects of nicotine, but preclinical studies have provided contradictory data (Muldoon et al., 2013; Panlilio et al., 2013).

Previous research has reported that FAAH knockout (FAAH KO) mice display enhanced sensitivity to nicotine reward relative to wild-type (WT) mice in the conditioned place preference (CPP) procedure, although this genotypic effect is specific to low nicotine doses (Merritt et al., 2008). This enhanced sensitivity to the rewarding effects of low-dose nicotine is blocked by the CB₁ receptor antagonist rimonabant. Similarly, FAAH inhibition in WT mice by different FAAH antagonists, such as URB597 and PF-3845, enhances the rewarding effects of low-dose nicotine in CPP (Ahn et al., 2009; Merritt et al., 2008). Collectively, these results suggest that FAAH inhibition enhances nicotine reward in mice through a CB₁ receptor-dependent mechanism, which is most likely due to elevated levels of AEA.

Notably, no neurochemical evaluations of the effects of nicotine following FAAH inhibition have been performed in mice. Thus, the present study sought to characterize nicotine-induced increases in extracellular DA in the NAc of FAAH KO and WT mice. For comparison, nicotine-induced alterations in extracellular serotonin (5-HT) within the NAc were also evaluated. Additionally, the effects of the selective FAAH inhibitor, PF-3845, on nicotine-induced changes in extracellular DA and 5-HT in the NAc of WT mice were examined.

METHODS AND MATERIALS

Animals

Null FAAH allele mice (i.e., FAAH KO) were created using homologous recombination as previously described and were maintained on the original 129/SvJ $\times$ C57BL/6J genetic background (Cravatt et al., 2001). All *in vivo* microdialysis studies were performed on wild-type (WT) and homozygous FAAH KO littermates weighing 20–30 g that were generated from crosses between heterozygous animals. The mice were group housed (2–4 mice/cage,

based on sex) in a humidity- and temperature-controlled vivarium (22 °C) on a reverse 12 h/12 h light/dark cycle (lights off at 09:00 am) and were provided free access to food (PicoLab® Mouse Diet 20) and water. All experiments and procedures involving the care and use of animals were conducted in strict adherence to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. All efforts were made to minimize unnecessary suffering and distress.

Drugs

(–)-Nicotine hydrogen tartrate salt was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Nicotine was diluted in saline and administered subcutaneously (s.c.). *N*-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl)piperidine-1-carboxamide [PF-3845] was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). PF-3845 was dissolved at 10 mg/mL by vortex into a mixture of 1:1:18 v/v/v (ethanol: emulphor: saline) and administered intraperitoneally (i.p.). All injections were administered in a volume of 10 mL/kg body weight.

Nicotine studies

Based on data from Merritt et al. (Merritt et al., 2008), a nicotine conditioned place preference (CPP)-like procedure was designed to evaluate the effects of nicotine on monoamine levels in the NAc. During three consecutive days, FAAH KO and WT mice were injected s.c. once daily with nicotine (at doses of 0.1, 1 or 10 mg/kg) or saline and immediately transferred to special nicotine cages individually with different characteristics (e.g., pine bedding and painted walls) for a 20-min period every day before returning to their original cages. The last day (3rd day), the mice that were grouped according to their CPP doses were injected with nicotine or saline during a microdialysis session in nicotine cages, but they were not returned to their original cages.

Considering that these experiments were performed with null mice, an additional experiment was performed to evaluate the effect of pharmacological FAAH blockade on NAc monoamine levels using a subthreshold dose of nicotine for WT mice. Therefore, low-dose nicotine (0.1 mg/kg, s.c.) was used for this purpose in WT mice following the same CPP-like procedure described above. In this case, the last nicotine injection was administered 60 min after PF-3845 (10 mg/kg) or vehicle i.p. administration.

Nicotine treatments were performed during the dark cycle at 09:30 am. Each group consisted of a sample size of 6–9 mice.

In vivo microdialysis

Microdialysis probes with a cellulose membrane (1 mm active length) were constructed as previously described (Frantz et al., 2002) with minor modifications. During the probe implantation procedures, the probes were continuously perfused with artificial cerebrospinal fluid (aCSF; 149 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 0.25 mM ascorbic acid and 5.4 mM d-glucose [pH 7.2–7.4]). After the second session of nicotine-CPP (approximately 12 h prior to the microdialysis session), each mouse was anesthetized with an isoflurane/oxygen vapor mixture (1.5 %) and mounted on a stereotaxic apparatus (David

Kopf Instruments, Tujunga, CA, USA) for implantation of a probe aimed at the NAc. The stereotaxic coordinates relative to Bregma for the NAc were as follows: +1.5 mm AP, ± 0.8 mm ML and -5.0 mm DV from the skull surface (Paxinos and Franklin, 2001). The probe was secured to the skull using a single skull screw anchor and a thin layer of Geristore[®] resin ionomer cement (Den-Mat Corp., Santa Maria, CA, USA). Following probe implantation, each mouse was immediately transferred to a standard mouse cage with an adapted cover for microdialysis, and allowed to regain consciousness.

During a 12-h postsurgical recovery period, the dialysis probes were perfused with aCSF at $0.15 \mu\text{L}/\text{min}$. After this period, the perfusion flow rate was increased to $0.6 \mu\text{L}/\text{min}$ for 120 min to reach interstitial equilibrium before sample collection. Dialysate fractions were subsequently collected at 10-min intervals following two different experimental protocols (see *Nicotine studies*) during 180 min (60-min baseline period, nicotine s.c. injection and 120-min post-injection period) or 240 min (60-min baseline period, pretreatment with PF-3845 or vehicle i.p. injection, 60 min post-pretreatment, nicotine s.c. injection and 10-min post-injection period). In both cases, all samples were collected and subsequently stored at -80°C until monoamine content analysis. One FAAH KO mouse that received nicotine at a dose of $10 \text{ mg}/\text{kg}$ died during microdialysis.

High-performance liquid chromatography coupled with electrochemical detection

Dialysis samples were analyzed for DA and 5-HT using high-performance liquid chromatography coupled with electrochemical detection (HPLC-ECD) (model HTEC-500, from EiCOM Co., Kyoto, Japan). Aliquots of dialysate ($5 \mu\text{L}$) were injected onto an analytical column (particle size 2 mm , PP-ODS $4.6 \times 30 \text{ mm}$ maintained at 25°C) and eluted using an isocratic mobile phase consisting of a 100 mM NaH_2PO_4 buffer containing $134 \mu\text{M}$ 2Na-EDTA , 3.27 mM SDS and 0.75% (v/v) Methanol (pH 6.0) delivered at $400 \mu\text{L}/\text{min}$. Both monoamine molecules were detected electrochemically using a graphite working electrode set at $+450 \text{ mV}$ against a silver-silver chloride reference electrode. Quantification was performed using external calibration curves that were constructed daily using freshly prepared standards. All chemicals for the mobile phase and chromatographic standards were of the highest obtainable grade from Sigma-Aldrich Co. (St. Louis, MO, USA) except for SDS, which was purchased from TCI-ACE, Tokyo Chemical Industry Co. (Tokyo, Japan).

Histology

Proper placement of the active dialysis membrane within the NAc was verified histologically for each experimental subject. Brains were sliced ($20 \mu\text{m}$), and the placement of microdialysis probes was verified using a mouse brain atlas (Paxinos and Franklin, 2001).

Statistical analysis

Baseline DA and 5HT concentrations were determined for each mouse from the mean of dialysate samples collected before the first injection. Mean basal DA and 5-HT values were compared between groups by unpaired Student's *t*-tests. Data were converted to the percent change from the average baseline concentration obtained prior to nicotine/saline administration, and statistical analysis was performed on the percentage data. Two-way repeated measures analysis of variance (ANOVA) was conducted to determine the relative

effect of time (during 120 min post-nicotine at 10-min intervals) and genotype (FAAH KO and WT mice) as factors on nicotine-induced DA and 5-HT levels. *F*-tests for simple main effects and pairwise *post hoc* (Bonferroni) comparisons were performed to examine interactions between factors. Similarly, a two-way repeated ANOVA was performed to determine the relative effect of time (during 120 min post-nicotine at 1-min intervals) and pretreatment (PF-3845 and vehicle) as factors on low-dose nicotine-induced DA and 5-HT levels in WT mice.

Area under the curve (AUC) measures were used to indicate the overall effects of nicotine challenge on monoamine concentrations in both genotypes. Additionally, AUC measures were used to illustrate the effects of PF-3845 pretreatment on nicotine-induced monoamine alterations in WT mice. The AUC was calculated for each animal by subtracting 100 from the percent of the baseline value for each data point following nicotine administration during 60 min and 120 min and subsequently summing all these data points. Significant differences in AUC measures were determined using unpaired Student's *t*-tests.

Test statistic values and degrees of freedom are indicated in the results when appropriate, and *p* value less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA, USA)

RESULTS

Saline injection did not affect the monoamines DA and 5-HT in the NAc of FAAH KO and WT mice

As a reference for nicotine studies, we conducted *in vivo* microdialysis in FAAH KO and WT mice to assess monoamine responses in the NAc following an acute saline challenge (Fig. 1b and 1c). Saline injection (s.c.) induced no changes in dialysate DA and 5-HT levels compared with the baseline levels in both genotypes. However, there was a 5-HT elevation at 10 min post-injection in FAAH KO (110.3 ± 3.6 % of baseline) and WT (111.9 ± 4.2 % of baseline) mice, but these elevations were not statistically significant. Furthermore, no significant differences in baseline monoamine levels were observed between FAAH KO and WT mice [DA: FAAH KO, 2.91 ± 0.47 nM and WT, 2.81 ± 0.27 nM; 5-HT: FAAH KO, 0.45 ± 0.07 nM and WT, 0.41 ± 0.06 nM].

FAAH deletion enhanced the effects of low-dose nicotine on the monoamines DA and 5-HT in the NAc

The effects of an acute nicotine challenge (0.1 mg/kg, s.c.) on NAc dialysate DA and 5-HT levels in FAAH KO and WT mice are shown in Fig. 2. Baseline DA and 5-HT levels for both genotypes were as follows: [DA: FAAH KO, 2.67 ± 0.43 nM and WT, 2.89 ± 0.29 nM; 5-HT: FAAH KO, 0.48 ± 0.06 nM and WT, 0.47 ± 0.05 nM]. No differences in baseline monoamine levels were observed between FAAH KO and WT mice.

Acute nicotine challenge increased NAc DA levels in mice of both genotypes, but this increase was significantly potentiated and prolonged in FAAH KO mice to a maximum of 127.7 ± 7.1 % of baseline DA at 10 min post-injection and returned to basal DA levels at 80

min post-injection (Fig. 2a). Therefore, a two-way repeated measures ANOVA during 120 min after nicotine exposure detected a main effect of genotype [$F_{(1,192)}=38.60$, $p<0.001$] and time [$F_{(11,192)}=3.732$, $p<0.001$] on DA levels but not an interaction. This genotype difference was clearly evident in the AUC analysis for DA after 60 min and 120 min of nicotine injection (Fig. 2b). Indeed, compared with WT mice, FAAH KO mice displayed significantly greater AUC of dialysate DA levels following the nicotine injection [60 min: $t_{(16)}=3.498$, $p=0.003$; 120 min: $t_{(16)}=2.946$, $p=0.009$]. Interestingly, compared with the baseline DA, this DA elevation was statistically significant as [60 min: $t_{(8)}=4.628$, $p=0.002$; 120 min: $t_{(8)}=2.820$, $p=0.022$] in FAAH KO mice but not in WT mice, which revealed that 0.1 mg/kg nicotine was a subthreshold dose in WT mice.

With respect to 5-HT, the acute nicotine challenge produced a modestly enhanced increase in NAc 5-HT levels of FAAH KO mice compared with those of WT mice (138.9 ± 13.9 % and 123.3 ± 3.4 % respectively at 10 min post-injection), and both genotypes returned to basal levels at 50 min post-injection (Fig. 2c). Thus, a two-way repeated measures ANOVA revealed that 5-HT responses after nicotine challenge were significantly affected by genotype [$F_{(1,180)}=5.334$, $p=0.022$] and time [$F_{(11,180)}=7.890$, $p<0.001$], but there was no interaction. As shown in Fig. 2d, there was a trend toward increased 5-HT levels (AUC) after the acute nicotine challenge in FAAH KO mice compared with WT mice, but this increase was not statistically significant after 60 min or 120 min. Furthermore, nicotine-induced 5-HT elevations were statistically significant compared with baseline 5-HT levels during the first 60 min in FAAH KO [60 min: $t_{(7)}=2.419$, $p=0.046$] and WT [60 min: $t_{(8)}=2.370$, $p=0.045$] mice.

The dialysis probe placements for FAAH KO and WT mice in this experiment with nicotine at a dose of 0.1 mg/kg are shown in Fig. 6a.

Effects of moderate-dose nicotine on the monoamines DA and 5-HT are unaltered by FAAH deletion in the NAc

The effects of acute nicotine exposure at 1.0 mg/kg s.c. on dialysate DA and 5-HT levels in the NAc are shown in Fig. 3. Baseline monoamine concentrations in NAc dialysates were as follows: [DA: FAAH KO, 2.69 ± 0.36 nM and WT, 2.30 ± 0.32 nM; 5-HT: FAAH KO, 0.46 ± 0.04 nM and WT, 0.46 ± 0.06 nM]. No significant differences in baseline monoamine levels were observed between FAAH KO and WT mice.

Acute nicotine challenge increased NAc DA levels in a similar manner in FAAH KO and WT mice, reaching a maximum of 133.46 ± 6.04 % and 142.81 ± 6.92 % at 10 min post-injection, respectively. After 60 min post-injection, both genotypes returned to basal DA levels (Fig. 3a). A two-way ANOVA revealed a main effect of time [$F_{(11,169)}=15.52$, $p<0.001$] with a sustained decrease in nicotine-induced DA elevations but no effect of genotype or an interaction between the factors. Thus, both genotypes displayed comparable total dialysate DA levels following the acute nicotine challenge, and no AUC differences were detected between the mice. Relative to baseline levels, post-nicotine AUC values of DA were significantly increased in FAAH KO mice [60 min: $t_{(7)}=3.325$, $p=0.013$] and WT mice [60 min: $t_{(7)}=3.663$, $p=0.008$; 120 min: $t_{(7)}=2.469$, $p=0.043$] as illustrated in Fig. 3b.

NAc 5-HT concentrations were also increased to similar levels in both genotypes after nicotine administration, resulting in maximum values at 10 min post-injection in FAAH KO (122.83 ± 4.80 %) and WT (128.28 ± 5.75 % of baseline 5-HT) mice, and both genotypes returned to basal levels after 60 min post-nicotine. Similar to DA, there was a significant effect of time [$F_{(11,180)}=10.06$, $p<0.001$] with a decrease in nicotine-induced 5-HT elevations but no effect of genotype or an interaction (Fig. 3c). Thus, mice of both genotypes displayed equivalent dialysate 5-HT levels expressed as AUC after the acute nicotine challenge. However, AUC analysis also revealed that these NAc 5-HT elevations were significantly different from their baseline levels in FAAH KO [60 min: $t_{(8)}=5.378$, $p<0.001$; 120 min: $t_{(8)}=3.112$, $p=0.014$] and WT [60 min: $t_{(7)}=3.521$, $p=0.010$] mice as shown in Fig. 3d.

The dialysis probe placements for FAAH KO and WT mice in this experiment with nicotine at a dose of 1 mg/kg are shown in Fig. 6b.

Effects of high-dose nicotine on the monoamines DA and 5-HT are modestly altered by FAAH deletion in the NAc

Fig. 4 represents the effect of an acute nicotine treatment (10.0 mg/kg, s.c.) on the NAc dialysate DA and 5-HT levels of FAAH KO and WT mice. Baseline monoamine concentrations were as follows: [DA: FAAH KO, 3.02 ± 0.59 nM and WT, 2.51 ± 0.23 nM; 5-HT: FAAH KO, 0.42 ± 0.02 nM and WT, 0.39 ± 0.04 nM]. As in other cases, there were no significant differences in baseline monoamine levels between FAAH KO and WT mice.

As represented in Fig. 4a, a high dose nicotine challenge produced a biphasic increase in NAc DA levels that peaked at approximately 10–20 min and 60–80 min post-injection in mice of both genotypes, with a maximum level of 161.02 ± 21.71 % of baseline DA at 10 min post-injection in FAAH KO mice and 150.15 ± 9.35 % at 70 min post-injection in WT mice. These nicotine-induced effects on DA concentrations were not significantly different between both genotypes, but there was a main effect of time [$F_{(11,108)}=1.894$, $p=0.048$]. In fact, FAAH KO and WT mice displayed comparable total dialysate DA levels following the acute nicotine challenge, and the AUC analysis of both genotypes revealed no significant differences in DA increases. Similar to the 1 mg/kg nicotine data, the post-nicotine AUC values of DA were also significantly increased in FAAH KO [60 min: $t_{(4)}=3.933$, $p=0.017$; 120 min: $t_{(4)}=4.180$, $p=0.013$] and WT [60 min: $t_{(5)}=3.760$, $p=0.013$; 120 min: $t_{(5)}=3.062$, $p=0.028$] mice (Fig. 4b).

With respect to 5-HT levels, the acute nicotine challenge produced a very high elevation in FAAH KO and WT mice, with a maximum increase at 10 min post-injection (241.23 ± 64.32 % and 174.88 ± 52.37 % of baseline 5-HT, respectively) (Fig. 4c). Overall, compared with WT mice, FAAH KO mice exhibited a modestly enhanced increase in NAc 5-HT levels. Thus, statistical analysis revealed a main effect of time [$F_{(11,108)}=4.224$, $p<0.001$] on the 5-HT response, but there was no effect of genotype or an interaction. When AUC was evaluated, compared with WT mice, FAAH KO mice displayed modestly greater total dialysate 5-HT levels following the acute nicotine injection at a high dose, but these differences in AUC were not significant at 60 min or 120 min (Fig. 4d). Although FAAH KO

had elevated 5-HT levels as indicated by AUC analysis, this post-nicotine increase was not significantly different from baseline levels.

The dialysis probe placements for FAAH KO and WT mice in this experiment with nicotine at a dose of 10 mg/kg are shown in Fig. 6c.

Acute FAAH inhibition potentiates the effect of low-dose nicotine on the monoamines DA and 5-HT in the NAc

The effect of acute pharmacological blockade of FAAH by PF-3845 (10 mg/kg, i.p.) on nicotine-induced (0.1 mg/kg, s.c.) changes in the NAc dialysate DA and 5-HT levels of WT mice is represented in Fig. 5. Baseline monoamine concentrations in mice pretreated with PF-3845 or vehicle were as follows: [DA: PF-3845, 3.19 ± 0.40 nM and vehicle, 2.46 ± 0.25 nM; 5-HT: PF-3845, 0.56 ± 0.06 nM and vehicle 0.68 ± 0.04 , nM]. No significant differences in baseline monoamine levels were observed between PF-3845-pretreated and vehicle-pretreated mice.

The acute nicotine challenge increased NAc DA levels in both pretreatment groups, but this increase was significantly potentiated and prolonged for 120 min following PF-3845 pretreatment, with a maximum increase of 138.38 ± 5.53 % compared with vehicle-pretreatment with 118.16 ± 5.87 % of baseline DA at 10 min nicotine post-injection (Fig. 5a). Therefore, a two-way repeated measures ANOVA showed a main effect of pretreatment [$F_{(1,192)}=22.02$, $p<0.001$] on nicotine-induced DA levels but no effect of time or an interaction. Regarding the AUC analysis (Fig. 5b), PF-3845-pretreated mice displayed greater dialysate DA levels than vehicle-treated mice following nicotine exposure, resulting in a significant increase [60 min: $t_{(16)}=2.158$, $p=0.046$; 120 min: $t_{(16)}=2.296$, $p=0.036$]. When the AUC of 5-HT levels was compared with baseline levels, we observed significant elevations in PF-3845-pretreated [60 min: $t_{(8)}=3.837$, $p=0.005$; 120 min: $t_{(8)}=4.445$, $p=0.002$] and vehicle-pretreated [60 min: $t_{(8)}=3.048$, $p=0.016$] mice.

After analyzing 5-HT concentrations in these mice, PF-3845 pretreatment resulted in an enhanced nicotine-induced increase in 5-HT levels at maximum levels of 132.66 ± 5.22 % compared with 116.96 ± 7.21 % of baseline 5-HT in vehicle-pretreated mice at 10 min nicotine post-injection (Fig. 5c). A two-way ANOVA revealed a significant effect of pretreatment [$F_{(1,192)}=20.01$, $p=0.001$] and time [$F_{(11,192)}=2.600$, $p=0.004$] on nicotine-induced 5-HT levels. In agreement with the genetic deletion of FAAH, PF-3845 induced greater total dialysate 5-HT levels (AUC figure) following the acute nicotine challenge (Fig. 5d), but this increase in NAc 5-HT resulted in no significant differences with respect to the vehicle pretreatment. Finally, only PF-3845-pretreated mice displayed significant elevations in the post-nicotine 5-HT response, expressed as AUC, relative to baseline levels [60 min: $t_{(8)}=3.007$, $p=0.017$; 120 min: $t_{(8)}=2.350$, $p=0.047$].

The dialysis probe placements for WT mice in this experiment with PF-3845 or vehicle pretreatment and nicotine at a dose of 0.1 mg/kg are shown in Fig. 6d.

DISCUSSION

The inhibition of FAAH increases the levels of *N*-acylethanolamines and consequently affects the activity of different receptors, mainly cannabinoid receptors and PPAR, which modulate the neurochemical and behavioral effects of nicotine and other addictive substances (Panlilio et al., 2013; Serrano and Parsons, 2011). Since substantial evidence indicates a critical influence of the *N*-acylethanolamines on the rewarding effects of nicotine, the present study in mice was designed to characterize nicotine-induced alterations in NAc monoamine levels in the absence of FAAH activity using genetic and pharmacological approaches. The main findings are summarized as follows: 1) Genetic deletion of FAAH selectively enhances the effect of low-dose nicotine (0.1 mg/kg, s.c.) on NAc DA release with no differences between genotypes at higher doses (1 or 10 mg/kg, s.c.); 2) WT mice pretreated with a FAAH inhibitor (10 mg/kg, i.p.) display an enhancement of the effect of low-dose (0.1 mg/kg, s.c.) nicotine on NAc DA release; 3) FAAH deletion produces a moderate enhancement of the effect of low-dose nicotine (0.1 mg/kg, s.c.) on NAc 5-HT release with no differences between genotypes at higher doses (1 or 10 mg/kg, s.c.); 4) Similarly, WT mice pretreated with a FAAH inhibitor (10 mg/kg, i.p.) display a moderate enhancement of the effect of low-dose (0.1 mg/kg, s.c.) nicotine on NAc 5-HT release. These observations in mice suggest that enhanced nicotine-induced NAc monoamine release, especially DA, might contribute to increased sensitivity to the conditioned rewarding effects of low-dose nicotine following FAAH inhibition, which was previously described using a nicotine-CPP procedure in mice (Merritt et al., 2008).

We used FAAH KO mice and PF-3845 to induce an increase in *N*-acylethanolamines, and the nicotine exposure was conducted using a CPP-like procedure with 3 nicotine injections, although the last injection was performed during the microdialysis session. Our results are consistent with previous studies in mice that showed that FAAH inhibition enhances the rewarding properties of nicotine (Merritt et al., 2008). FAAH KO mice have been reported to display nicotine preference in nicotine-CPP with 0.1 mg/kg, a subthreshold dose in WT mice. Consistently, we found a significant increase in NAc DA following this low dose of nicotine in FAAH KO mice but no changes in WT mice. Regarding the use of FAAH inhibitors, recent studies in pretreated mice have reported similar results to those from FAAH KO mice in nicotine-CPP tests. Thus, mice pretreated with FAAH inhibitors (URB597 or PF-3845) display an enhanced nicotine-CPP with low-dose nicotine (Merritt et al., 2008; Muldoon et al., 2013). Since PF-3845 is a highly selective inhibitor of FAAH and induces higher brain levels of AEA, OEA and PEA than URB597 (Ahn et al., 2009), we used PF-3845 to evaluate monoamine release in the NAc following a nicotine challenge. Our microdialysis experiments revealed a strong enhancement of nicotine-induced DA release in mice pretreated with PF-3845, and this DA response pattern was similar to that of FAAH KO mice exposed to 0.1 mg/kg of nicotine. In parallel, we also observed a moderate increase in the 5-HT response when FAAH KO mice and PF-3845-pretreated WT mice were exposed to low-dose nicotine, although the enhancement of 5-HT was lower than that of DA. Therefore, these findings extend previous results from nicotine-CPP tests, indicating that FAAH inactivation (using FAAH KO mice or FAAH inhibitors) confers enhanced sensitivity to nicotine-induced monoamine release in the NAc.

In contrast to these findings in mice, other studies have reported that FAAH inhibitors reduce the rewarding effects of nicotine in rats and non-human primates. Thus, while URB597 attenuated the reinstatement of nicotine-CPP and nicotine intravenous (i.v.) self-administration in rats (Forget et al., 2009; Scherma et al., 2008), Justinova and colleagues reported that pharmacological FAAH inhibition reduced nicotine seeking and relapse using a self-administration paradigm in squirrel monkeys (Justinova et al., 2015). Furthermore, URB597 prevented nicotine-induced DA release in the NAc, suppressing nicotine-induced excitation of dopaminergic neurons in the VTA of rats (Melis et al., 2008). Unlike these studies, we observed an enhancement of nicotine-induced DA release in the NAc with PF-3845, although we are aware that we used only one effective dose of this FAAH inhibitor based on previous studies (Ahn et al., 2009). Indeed, this limitation might prevent the observation of the opposite effects on nicotine-induced monoamine release in the NAc, considering the biphasic effects of endocannabinoids on emotional behaviors (i.e., anxiety) and addiction (Parsons and Hurd, 2015; Serrano and Parsons, 2011).

The disparity between these findings mainly suggests species differences (mice, rats and nonhuman primates) in the influence of FAAH inhibition on nicotine-related behaviors and neurochemistry due to the differential and even opposite effects of *N*-acylethanolamines acting on multiple targets. Indeed, these contradictory data found in animal models are in line with human studies that reported the existence of genetic variants, suggesting an association of polymorphisms in the FAAH gene with addictive disorders and indicating endophenotypic variability (Lopez-Moreno et al., 2012).

As previously noted, FAAH inactivation promotes an increase of *N*-acylethanolamines, including endocannabinoids (e.g., AEA) and non-cannabinoid lipids (e.g., OEA and PEA); therefore, the rewarding properties of nicotine might be influenced by effects mainly resulting from the combination and/or interaction of CB₁- and PPAR α -mediated mechanisms (Panlilio et al., 2013). Thus, these CB₁- and PPAR α receptors may regulate the dopaminergic VTA neurons through direct and indirect mechanisms involving synaptic inputs from GABAergic, glutamatergic and cholinergic neurons (Fig.7). In fact, the effects of URB597 on dopaminergic cells have been reported to require both CB₁ and PPAR α receptors (Luchicchi et al., 2010).

A growing body of evidence in rodents has demonstrated that nicotine-induced rewarding properties are mediated through a cannabinoid CB₁-related mechanism because animals treated with CB₁ antagonists (e.g., rimonabant or AM251) and CB₁ KO mice display a decrease in nicotine-CPP and nicotine self-administration (Biala et al., 2009; Budzynska et al., 2009; Castane et al., 2002; Le Foll and Goldberg, 2004). Furthermore, rats injected with a full CB₁ agonist (i.e., WIN55,212-2) show greater nicotine self-administration (Gamaledin et al., 2012). Consistent with these findings based on CB₁, endocannabinoids are increased following nicotine exposure, and studies conducted in rats have reported that rimonabant attenuates nicotine-evoked DA release in the NAc (Cohen et al., 2002). In fact, CB₁ receptors modulate excitatory glutamatergic and inhibitory GABAergic inputs that control dopaminergic neurons because endocannabinoids serve as retrograde signaling molecules. In addition to direct activation of CB₁, increased levels of AEA might affect the

function of nAChR subtypes in the VTA, where dopaminergic neurons project to the NAc (Castane et al., 2005; Viveros et al., 2006).

In contrast to a CB₁-related mechanism, recent studies in rodents and monkeys have shown that PPAR α is involved in the reinforcing properties of nicotine (Panlilio et al., 2013). PPAR α agonists (WY14643 and synthetic OEA) reduce nicotine self-administration and nicotine-induced reinstatement in rats (Mascia et al., 2011). Furthermore, these agonists also attenuate nicotine-induced excitation of dopaminergic neurons in the VTA and nicotine-induced DA release in the NAc of rats (Mascia et al., 2011). Thus, a possible PPAR α -mediated mechanism that regulates the nicotine-induced DA response is the phosphorylation of nAChRs (Melis et al., 2008). Finally, recent results have revealed that WY14643 prevents the rewarding effects of nicotine in the CPP test in mice (Muldoon et al., 2013).

Consequently, as illustrated in Figure 7, whereas increased levels of endocannabinoid *N*-acylethanolamines might promote the reinforcing effects of nicotine through CB₁ activation in glutamatergic and GABAergic inputs with a net excitatory effect on dopaminergic VTA neurons, non-cannabinoid *N*-acylethanolamines might attenuate these rewarding effects through PPAR α with an inhibitory effect on dopaminergic neurons via nAChRs (Muldoon et al., 2013). Here, we observed increased sensitivity to low-dose nicotine-induced DA release following FAAH inhibition with genetic deletion or pharmacological blockade in mice. We speculate that FAAH inhibition might facilitate and amplify nicotine-induced monoamine release (DA and 5-HT) in the NAc with subthreshold-dose nicotine. The precise mechanism will have to be elucidated in future studies combining behavioral and neurochemical approaches, although the present data are more likely to be linked to a CB₁-mediated mechanism rather than PPAR α -mediated mechanisms, but we cannot exclude other molecular targets (e.g., TRPV1 vanilloid receptors and other PPAR subtypes).

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We dedicate this manuscript to Dr. Loren H Parsons, who passed away suddenly on June 21, 2016. He was a recognized leader and researcher in the fields of neurochemistry and addiction, a fantastic mentor and a well-loved friend.

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Figure 1.

(a) Neurocircuitry and receptors involved in the reinforcing effects of nicotine in the mouse brain. Amyg, amygdala; HPC, hippocampus; LDT/PPTg, laterodorsal tegmental/pedunculopontine tegmental nucleus; NAc, nucleus accumbens; PFC, prefrontal cortex; RMTg, rostromedial tegmental nucleus; VP, ventral pallidum; VTA, ventral tegmental area. (b) Effect of an acute saline challenge (s.c.) on NAc dialysate DA levels in FAAH KO and WT mice. (c) Effect of an acute saline challenge (s.c.) on NAc dialysate 5-HT levels in FAAH KO and WT mice. DA and 5-HT levels are expressed as the percentage change from baseline levels (mean $\pm$ SEM) and saline injection is indicated by the red arrow at time point zero.

Figure 2.

Effect of an acute nicotine challenge (0.1 mg/kg, s.c.) on NAc dialysate DA and 5-HT levels in FAAH KO and WT mice. **(a)** DA levels are expressed as the percentage change from baseline levels (mean $\pm$ SEM). **(b)** The area under the curve (AUC) for DA summarizes the effect of nicotine on dialysate DA. **(c)** 5-HT levels are expressed as the percentage change from baseline levels (mean $\pm$ SEM). **(d)** The AUC for 5-HT summarizes the effect of nicotine on dialysate 5-HT. Nicotine injection is indicated by the red arrow at time point zero. $^{++}p < 0.01$ denotes significant differences in FAAH KO *versus* WT mice. $^{*}p < 0.05$ and $^{**}p < 0.01$ denote significant differences *versus* baseline (AUC=0).

Figure 3.

Effect of an acute nicotine challenge (1 mg/kg, s.c.) on NAc dialysate DA and 5-HT levels in FAAH KO and WT mice. **(a)** DA levels are expressed as the percentage change from baseline levels (mean $\pm$ SEM). **(b)** The area under the curve (AUC) for DA summarizes the effect of nicotine on dialysate DA. **(c)** 5-HT levels are expressed as the percentage change from baseline levels (mean $\pm$ SEM). **(d)** The AUC for 5-HT summarizes the effect of nicotine on dialysate 5-HT. Nicotine injection is indicated by the red arrow at time point zero. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ denote significant differences *versus* baseline (AUC=0).

Figure 4.

Effect of an acute nicotine challenge (10 mg/kg, s.c.) on NAc dialysate DA and 5-HT levels in FAAH KO and WT mice. **(a)** DA levels are expressed as the percentage change from baseline levels (mean $\pm$ SEM). **(b)** The area under the curve (AUC) for DA summarizes the effect of nicotine on dialysate DA. **(c)** 5-HT levels are expressed as the percentage change from baseline levels (mean $\pm$ SEM). **(d)** The AUC for 5-HT summarizes the effect of nicotine on dialysate 5-HT. Nicotine injection is indicated by the red arrow at time point zero. * $p < 0.05$ denotes significant differences *versus* baseline (AUC=0).

Figure 5.

Effect of acute pharmacological blockade of FAAH by PF-3845 (10 mg/kg, i.p.) on nicotine-induced (0.1 mg/kg, s.c.) changes in NAc dialysate DA and 5-HT levels in WT mice. **(a)** DA levels are expressed as the percentage change from baseline levels (mean $\pm$ SEM). **(b)** The area under the curve (AUC) for DA summarizes the effect of nicotine on dialysate DA in pretreated mice. **(c)** 5-HT levels are expressed as the percentage change from baseline levels (mean $\pm$ SEM). **(d)** The AUC for 5-HT summarizes the effect of nicotine on dialysate 5-HT in pretreated mice. PF-3845 or vehicle injection is indicated by the black arrow at -60 min and nicotine injection is indicated by the red arrow at time point zero. $^+p < 0.05$ denotes significant differences in PF-3845-treated *versus* vehicle-treated mice. $^*p < 0.05$ and $^{**}p < 0.01$ denote significant differences *versus* baseline (AUC=0).

Figure 6.

Histological representation of coronal brain sections (reproduced from Paxinos and Franklin, 2001) illustrating the placement of the microdialysis probe in the NAc of mice. A set of FAAH KO and WT mice were treated with the following doses of nicotine (s.c.): **(a)** 0.1 mg/kg, **(b)** 1 mg/kg and **(c)** 10 mg/kg. Another set of WT mice were pretreated with PF-3845 (10 mg/kg, i.p.) or vehicle and nicotine (0.1 mg/kg, s.c.) **(d)**. Probes were implanted at random in the left or right hemisphere. Lines represent the 1-mm active area of the probe.

Figure 7. Mechanisms of action for *N*-acylethanolamines and 2-AG in the dopaminergic VTA neuron. *N*-acylethanolamines and 2-AG regulate synaptic transmission in DA neurons via CB₁ and PPAR α mainly, modulating the nicotinic cholinergic transmission directly or indirectly via GABA and glutamate. 2-AG, 2-arachidonoylglycerol; ABHD6/12, serine hydrolase α - β -hydrolase; AEA, *N*-arachidonylethanolamine; FAAH, fatty acid amide hydrolase; MAGL, Monoacylglycerol lipase; NAc, nucleus accumbens; OEA, *N*-oleoylethanolamine; PEA, *N*-palmitoylethanolamine; PPAR α , peroxisome proliferator-activated receptor subtype alpha; RMTg, rostromedial tegmental nucleus; VTA, ventral tegmental area.