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Inhibition of endocannabinoid catabolic enzymes elicits anxiolytic-like effects in the marble burying assay

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ABSTRACT

Cannabinoids have long been shown to have a range of potential therapeutic effects, including antiemetic actions, analgesia, and anxiolysis. However, psychomimetic and memory disruptive side effects, as well as the potential for abuse and dependence, have restricted their clinical development. Endogenous cannabinoids (i.e., endocannabinoids; eCBs), such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are produced throughout the limbic system and other brain regions associated with emotionality and are believed to modulate behavioral responses to stress-related conditions. AEA and 2-AG are rapidly metabolized by the respective enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL). Accordingly, inhibition of each enzyme increases brain levels of the appropriate eCB. Although FAAH inhibition has been established to decrease anxiety-like behavior, the role of 2-AG has been difficult to ascertain until the recent synthesis of JZL184, a potent and selective MAGL inhibitor. In the present study, we investigated the effects of inhibiting FAAH or MAGL on anxiety-like behavior in marble burying, a model of repetitive, compulsive behaviors germane to anxiety disorders such as obsessive–compulsive disorder. The FAAH inhibitor PF-3845, the MAGL inhibitor JZL184, and the benzodiazepine diazepam decreased marble burying at doses that did not affect locomotor activity. In contrast, Δ^9-tetrahydrocannabinol (THC), the primary psychoactive constituent of marijuana, did not consistently reduce marble burying without also eliciting profound decreases in locomotor behavior. The CB_1 cannabinoid receptor antagonist rimonabant blocked the reduction in marble burying caused by FAAH and MAGL inhibitors, but not by diazepam, indicating a CB_1 receptor mechanism of action. These data indicate that elevation of AEA or 2-AG reduces marble burying behavior and suggest that their catabolic enzymes represent potential targets for the development of new classes of pharmacotherapeutics to treat anxiety-related disorders.

1. Introduction

Marijuana is commonly smoked to reduce feelings of stress and anxiety (Kogan and Mechoulam, 2007), though paradoxically its primary psychoactive constituent, Δ^9-tetrahydrocannabinol (THC), can also increase anxiogenic-like behaviors (Onaivi et al., 1990). Other adverse side effects of cannabis use, including cognitive deficits, abuse potential, and dependence liability, have dampened enthusiasm for the therapeutic development of THC and other cannabinoid receptor agonists. Instead, much interest has been generated by the discovery of the endogenous cannabinoid (i.e. endocannabinoid; eCB) system as a source of targets for the development of new therapeutic treatments of a range of ailments including anxiety and depression (Pacher et al., 2006).

The eCB system is comprised of lipid signaling molecules, anandamide (*N*- arachidonoylethanolamide; AEA (Devane et al., 1992)) and 2-arachidonoylglycerol (2-AG (Mechoulam et al., 1995)), the receptors for these lipids, CB_1 and CB_2 (Matsuda et al., 1990; Munro et al., 1993), and biosynthetic and catabolic enzymes that regulate eCB levels. AEA is hydrolyzed by the enzyme fatty acid amide hydrolase (FAAH (Cravatt et al., 1996; Deutsch and Chin, 1993)), and 2-AG is predominantly metabolized by monoacylglycerol lipase (MAGL (Blankman et al., 2007; Dinh et al., 2002)). While eCBs are rapidly metabolized *in vivo*, limiting the efficacy of exogenous administration, inhibition of their catabolic enzymes results in

Abbreviations: 2-AG, 2-Arachidonoylglycerol; AEA, Anandamide, *N*-arachidonoyl ethanolamine; CB_1, Cannabinoid receptor type 1; CB_2, Cannabinoid receptor type 2; DZ, Diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-1,4-benzodiazepin-2(3*H*)-one; eCB, Endocannabinoid; FAAH, Fatty acid amide hydrolase; JZL184, 4-Nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate; MAGL, Monoacylglycerol lipase; PF-3845, N-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl)-piperdine-1-carboxamide; Rim, Rimonabant, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl; THC, Δ^9-Tetrahydrocannabinol.

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elevated eCB brain levels (Kathuria et al., 2003; Long et al., 2009). Thus, the availability of selective FAAH and MAGL inhibitors has made it possible to study the impact of elevating eCB levels in the whole animal.

A growing body of evidence indicates that CB_1 receptor agonists reduce anxiety-like behavior (Moreira et al., 2009). The high levels of CB_1 receptor expression in the amygdala, hippocampus, hypothalamus, and prefrontal cortex (Mackie, 2006), coupled with evidence that CB_1 receptor activation results in decreased release of glutamate and GABA (Steiner and Wotjak, 2008), support the idea that cannabinoids affect anxiety and anxiety-like behavior. FAAH inhibition as well as genetic deletion of FAAH have been reported to reduce anxiety-like behavior in the elevated plus maze (Moreira et al., 2008; Naidu et al., 2007; Patel and Hillard, 2006), zero maze (Kathuria et al., 2003), and light/dark box (Moreira et al., 2008), particularly under stressful conditions (Haller et al., 2009). However, investigation of the consequences of elevating endogenous 2-AG levels has not been possible until the recent development of the highly selective MAGL inhibitor, JZL184 (Long et al., 2009). Thus, little is known about the possible anxiolytic and/or anxiogenic effects of 2-AG modulation *in vivo*.

Marble burying is used as an assay to infer compulsive, anxiety-like behavior and is widely used as a model of obsessive–compulsive disorder (Broekkamp et al., 1986; Njung'e and Handley, 1991a, b). In this test, a mouse is placed into a clean cage filled with a level layer of bedding, covered with glass marbles. The marbles are disturbed and become covered as the mouse digs into the bedding. Thus, the number of marbles buried correlates with the frequency of digging bouts (Deacon, 2006; Thomas et al., 2009). As with other models of anxiety, marble burying is decreased by traditional anxiolytics, such as benzodiazepines (Broekkamp et al., 1986; Njung'e and Handley, 1991b). Marble burying offers advantages over exploratory models in that it measures a repetitive, possibly goal-directed form of anxiety, such as obsessive–compulsive disorder. Importantly, mice do not readily habituate to the assay (Thomas et al., 2009), making within-subjects designs possible and thereby reducing overall animal numbers.

The studies presented herein were designed to test the hypothesis that exogenous and endogenous cannabinoids reduce anxiety-like behavior in the marble burying test. First, we tested whether the plant-derived cannabinoid THC affected marble burying. Next, we tested the consequences of elevating eCB brain levels on marble burying by administering selective inhibitors of MAGL and FAAH. Finally, we used the CB_1 receptor antagonist rimonabant to test whether the observed anxiolytic-like effects of MAGL and FAAH inhibitors occurred via a cannabinoid receptor mechanism of action.

2. Materials and methods

2.1. Animals

Subjects consisted of male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) that were approximately 10 weeks of age and weighed approximately 25 g at the beginning of the study. Mice were group housed 4–6 per cage in a temperature (20–22 °C) and humidity controlled, AAALAC-approved facility maintained on a 12:12 light: dark cycle and were provided with *ad libitum* access to food and water. Mice were randomly assigned to treatment groups. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

2.2. Drugs

Diazepam (DZ) was purchased from Sigma-Aldrich (St. Louis, MO). Rimonabant (Rim) and Δ^9-tetrahydrocannabinol (THC) were obtained from the National Institute on Drug Abuse (Bethesda, MD). The MAGL inhibitor JZL184 (Long et al., 2009) and the FAAH inhibitor PF-3845 (Ahn et al., 2009) were synthesized as described previously. All drugs were dissolved in a vehicle consisting of equal parts ethanol and Alkamuls-620 (Rhone-Poulenc, Princeton, NJ), then diluted with normal saline to a final ratio of 1:1:18. All compounds were administered intraperitoneally (i.p.), at a volume of 10 µl/g body mass. All solutions were warmed to room temperature prior to injection.

2.3. Behavioral testing

Mice were allowed to acclimate to the test room for at least 1 h before experimental manipulation. Prior to testing, each mouse was weighed and injected intraperitoneally (i.p.) with drug or vehicle. For the diazepam and THC experiments, pretreatment time was 1 h. For the JZL184 and PF-3845 experiments, pretreatment time was 2 h, based on previous reports that endocannabinoid levels peak 2 h after treatment with either compound (Ahn et al., 2009; Long et al., 2009). The selective CB_1 receptor antagonist rimonabant (0.3 mg/kg, i.p.) was administered 10 min prior to JZL184 or PF-3845 treatment. This dose of rimonabant was chosen based on pilot data, which indicated deficits in locomotor activity at higher doses.

Marble burying behavior was assessed based on published methods (Deacon, 2006; Thomas et al., 2009). The testing apparatus consisted of a polycarbonate mouse cage (internal dimensions: 33 cm long × 21 cm wide × 19 cm high) filled to a depth of 5 cm with pine wood bedding (Harlan Sani-Chip, Indianapolis, IN), and placed in a sound-attenuating chamber lighted by a bank of white LEDs (75 lx). White noise and ventilation were supplied by a PC fan. Prior to each test, 20 clear, glass marbles (10 mm diameter) were evenly spaced and arranged in a grid-like fashion across the surface of the bedding. Then, individual mice were placed into the observation cage, which was then covered with a transparent, Plexiglas lid with air holes. At the conclusion of the 20 min test, the mice were carefully removed from the chamber and the number of buried marbles (50% or more of the marble was covered by bedding) was determined. Inter-observer reliability for assessing marble burying was >98%.

Locomotor activity was simultaneously captured during the test, using Unibrain Fire-I digital cameras and analyzed in real time using ANY-maze software (Stoelting, Kiel, WI). Immobility was defined as a lack of movement for 1250 ms or longer, and was analyzed in 1 min bins.

2.4. Data analyses

All data are reported as mean ± SEM and were analyzed using one-way between-subjects analysis of variance (ANOVA), with the exception of the antagonist studies, which were analyzed using two-way factorial ANOVA, with antagonist and enzyme inhibitor as the factors. Post hoc comparisons of dose–response data used Dunnett's test to compare each dose to vehicle. Planned comparisons between rimonabant and vehicle were made using *t* tests. Immobility data were analyzed using repeated-measures ANOVA, with time as the within-subjects variable. Differences were considered statistically significant at $p<0.05$.

3. Results

3.1. Evaluation of diazepam and THC on marble burying

Mice were injected (i.p.) with various doses of diazepam, THC, or vehicle and then tested in the marble burying assay. Locomotor activity was simultaneously recorded and was quantified as seconds per minute spent immobile. As reported previously, diazepam dose-dependently reduced marble burying [$F(3,28)=37.3$; $p<0.0001$; Fig. 1A]. Post hoc analyses revealed that marble burying was