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 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 lux). White noise and ventilation were supplied by a PC fan. Prior to each test, twenty 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 $[F_{(3,28)} = 37.3; p < 0.0001;$ Figure 1A]. Post hoc analyses revealed that marble burying was decreased at 1 and 3 mg/kg doses. However, the 3 mg/kg dose also caused a significant increase in immobility, indicating sedation $[F_{(3,532)} = 18.6; p < 0.0001;$ Figure 1B]. Overall immobility increased over time during testing $[F_{(19,532)} = 3.8; p < 0.0001]$.

Similarly, the plant-derived cannabinoid THC reduced marble burying $[F_{(7,64)} = 31.1; p < 0.0001;$ Figure 1C], but caused profound hypomotility at doses greater than 3 mg/kg $[F_{(7,1216)} = 70.6; p < 0.0001;$ Figure 1D]. Post hoc analyses revealed that marble burying was decreased at 0.3, 10, and 30 mg/kg, but not at 1 or 3 mg/kg, THC.

3.2 Inhibition of eCB catabolic enzymes attenuates anxiety-like behavior in the marble burying test

The highly selective FAAH inhibitor PF-3845 (10 mg/kg) significantly reduced marble burying $[F_{(3,44)} = 3.199; P < 0.05;$ Figure 2A], but had no effect on immobility at any dose tested ($p = 0.31;$ Figure 2B).

The selective MAGL inhibitor JZL184 significantly reduced marble burying $[F_{(3,28)} = 46.3; p < 0.0001;$ Figure 2C] at doses of 16 and 40 mg/kg. However, 40 mg/kg JZL184 also caused a statistically significant increase in immobility $[F_{(57,532)} = 2.26; p < 0.0001;$

Figure 2D]. Unlike THC or diazepam treatment, however, this decrease in locomotor activity did not manifest until after 10 min in the test apparatus.

3.3 A CB_1 receptor mechanism of action mediates the reduction of marble burying caused by FAAH and MAGL inhibitors

The anxiolytic-like effects of eCBs and exogenous cannabinoids have been previously shown to occur via the CB_1 receptor, although the possible role of CB_1 in marble burying is unknown. As reported above, PF-3845 (10 mg/kg) reduced marble burying [$F(1,43) = 14.2$; $p < 0.001$; Figure 3A], and rimonabant pretreatment blocked this reduction in marble burying. None of the treatments affected locomotor activity ($p = 0.38$; Figure 3B).

Similarly, JZL184 (16 mg/kg) reduced marble burying [$F(1,44) = 11.4$; $p < 0.01$; Figure 3C] and rimonabant prevented this reduction in marble burying. Neither JZL184 nor rimonabant affected immobility ($p = 0.60$; Figure 3D).

Finally, the possible role of the CB_1 receptor in benzodiazepine suppression of marble burying was tested. Diazepam (1 mg/kg) reduced marble burying [$F(1,41) = 44.03$; $p < 0.0001$; Figure 3E], however, rimonabant pretreatment had no effect on diazepam suppression of marble burying ($p = 0.58$). Neither diazepam nor rimonabant affected locomotor activity ($p = 0.65$; Figure 3F).

4. Discussion

In the present study, we investigated the consequences of inhibiting eCB catabolic enzymes on anxiety-like behavior in the marble burying test. Specifically, we tested the effects of pharmacological inhibition of FAAH or MAGL, as well as Δ^9-tetrahydrocannabinol (THC) and the benzodiazepine diazepam, on marble burying and locomotor activity. As reported previously (Broekkamp et al., 1986; Njung'e and Handley, 1991b), diazepam dose-dependently decreased marble burying. The FAAH inhibitor PF-3845 and the MAGL inhibitor JZL184 decreased marble burying, at doses that did not affect locomotor activity, suggesting that eCBs may reduce compulsive or anxiety-like behavior in the marble burying test. The observation that rimonabant completely blocked reductions in marble burying, following FAAH or MAGL inhibition, indicates a CB_1 receptor mechanism. The overall pattern of marble burying in response to THC indicated sedative effects in the range of doses tested. It is noteworthy that, as in previous studies, FAAH inhibition had no effect on locomotor activity.

Although irreversible carbamate FAAH inhibitors (e.g., URB597; (Kathuria et al., 2003)) and inhibit FAAH and increase brain levels of anandamide *in vivo*, they also inhibit other serine hydrolases, calling into question their selectivity for FAAH (Lichtman et al., 2004). Reversible α-ketoheterocycle FAAH inhibitors (e.g. OL-92, OL-135; (Boger et al., 2005; Lichtman et al., 2004)) are more selective for FAAH than URB597, but cause milder elevations of anandamide *in vivo*. Recently, a new class of highly selective and potent piperidine urea FAAH inhibitors (e.g. PF-3845; (Ahn et al., 2009)) has been synthesized. PF-3845 has a 10-fold higher binding affinity for FAAH than URB597 (Ahn et al., 2009) and inhibits FAAH for at least 24 h *in vivo*. Similarly, although several compounds inhibit MAGL, these compounds have been criticized because they lack selectivity for MAGL. For example, URB602 lacks potency to systemically elevate 2-AG *in vivo* (King et al., 2007) and has similar selectivity for both MAGL and FAAH (Vandevoorde et al., 2007). However, JZL184 (Long et al., 2009) potently inhibits MAGL in vivo and is highly selective (200-fold higher for MAGL than for FAAH) and long lasting (> 24 h). Systemic administration of JZL184 significantly increases anandamide in mouse brain and spinal cord and has antihyperalgesic and anti-allodynic effects (Kinsey et al., 2009; Long et al., 2009).

In the present studies, JZL184 caused a significant decrease in marble burying but also increased immobility at the highest dose tested (i.e., 40 mg/kg). Unlike diazepam or THC, however, this increase in immobility was not apparent until 10 min into the test session. These data somewhat contrast with our previous report that JZL184 causes a decrease in spontaneous locomotor activity (Long et al., 2009), however, this discrepancy is likely due to differences in the vehicle used. The original study used a vehicle consisting of 4:1 parts polyethylene glycol (PEG200):TWEEN80) and the present study used a vehicle consisting of 1:1:18 parts ethanol, Alkamuls-620, and saline. Supporting this notion, when dissolved in the same vehicle used in the present study, JZL184 had no effect on muscle coordination in the rotarod test (Schlosburg et al., 2009). Moreover, in the same experiment, high doses of THC disrupted motor coordination. Thus, high doses of JZL184 may produce hypomotility, but not sedation or loss of muscle coordination.

AEA elevation, via FAAH inhibition or genetic deletion, has been reported to have anxiolytic-like effects in exploratory models of anxiety, such as the zero maze (Kathuria et al., 2003), elevated plus maze (Moreira et al., 2008; Naidu et al., 2007; Patel and Hillard, 2006), and light/dark box (Moreira et al., 2008). However, the effects of 2-AG on anxiety have been difficult to ascertain, due to its rapid *in vivo* metabolism, until the recent synthesis of JZL184. Although MAGL is the primary enzyme responsible for 85% of 2-AG catabolism, other enzymes such as ABHD6 and ABHD12 contribute to 2-AG degradation, and these enzymes have distinct intracellular distributions (Blankman et al., 2007). Thus, it is possible that inhibition of these minor 2-AG catabolic enzymes may also have consequences on anxiety-related behavior that differ from MAGL inhibition.

In humans, the dose response to THC is biphasic with regard to anxiety and anxiety-like behavior. That is, at low to moderate doses, THC elicits anxiolytic effects, whereas at high doses it has anxiogenic effects (D'Souza et al., 2004; Gonzalez, 2007). In the present studies, although THC caused a decrease in marble burying at 0.3 mg/kg, this effect was not dose-related. Higher doses (i.e., 10 and 30 mg/kg), which reduced marble burying behavior, also lead to profound hypomotility, indicating sedation, as reported previously (Martin et al., 1987). Previously, Onaivi et al. (1990) reported that THC produced dose-related, anxiogenic-like effects in both mice and rats, as assessed in the elevated plus maze test. The marble burying test, however, is not designed to assess increased anxiogenic-like behavior.

Endocannabinoids have been shown to have both anxiolytic and anti-depressant properties in clinical and preclinical modes (Hill and Gorzalka, 2009; Moreira et al., 2009). CB_1 receptors are expressed throughout the central nervous system, with high densities in the amygdala, prefrontal cortex, hippocampus, and hypothalamus (Herkenham et al., 1991). Activation of CB_1 inhibits the presynaptic release of GABA and glutamate (Mackie, 2008). Thus, it is posited that differences in endocannabinoid effects on emotionality occur through regional differences in receptor activation (Hill and Gorzalka, 2009) and/or through differential activation of CB_1 and off-target receptors, such as TRPV1 (Rubino et al., 2008).

Although marble burying has long been used as a model to assess anxiolytics, the data derived from this assay do not necessarily correlate directly with exploratory models of anxiety-like behavior, such as the open field test or the light/dark box test (Thomas et al., 2009). Marble burying is also reduced by selective serotonin reuptake inhibitors and tricyclic antidepressants and anti-psychotics (Broekkamp et al., 1986; Njung'e and Handley, 1991a), calling into question its specificity as a test of anxiety. Despite these caveats, given its sensitivity to anxiolytics, marble burying is generally accepted as a model of anxiety disorders marked by compulsive and repetitive behaviors, such as obsessive-compulsive disorder (Thomas et al., 2009).

In the present study, whereas the CB_1 receptor antagonist rimonabant blocked the anxiolytic effects of PF-3845 and JZL184, it had no effect on benzodiazepine suppression of marble burying. This is in contrast to a recent report that that the anxiolytic effects of the benzodiazepine alprazolam were blocked by pretreatment with the CB_1 antagonist AM251 in the light/dark box (Garcia-Gutierrez and Manzanares, 2010). This apparent discrepancy may be due to intrinsic differences between exploratory tests, such as the light/dark box, and marble burying, as described above.

In conclusion, inhibition of the eCB catabolic enzymes FAAH and MAGL produced anxiolytic-like effects in the marble burying assay. This decrease in marble burying behavior was blocked by rimonabant pretreatment, indicating that the CB_1 receptor plays a necessary role in mediating this observed decrease in marble burying. Together with previous reports of the anxiolytic-like effects of FAAH inhibition, these data indicate that elevation of AEA or 2-AG can reduce anxiety-like behavior in mice. Collectively, these results suggest that both FAAH and MAGL are potential therapeutic targets for the development of new classes of anxiolytic drugs.

Acknowledgments

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Abbreviations

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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
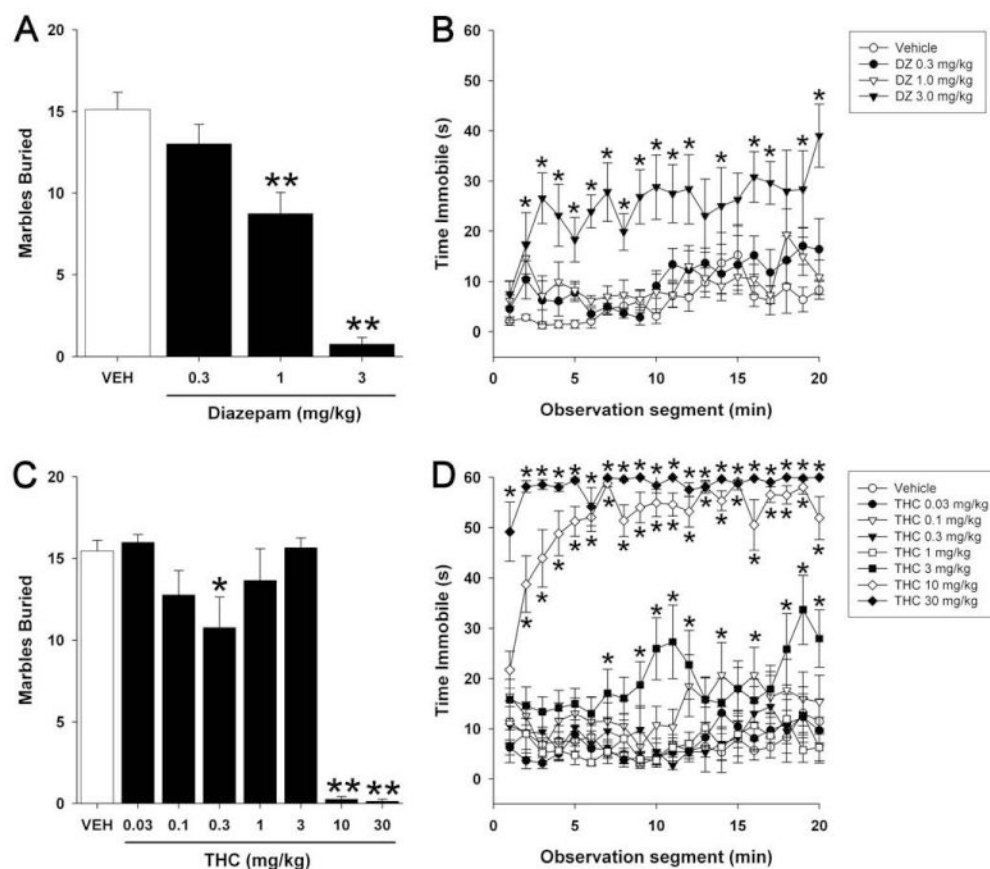
Figure 1.
Diazepam and Δ^9-THC reduce marble burying behavior. Mice were injected (i.p.) with diazepam (DZ), THC, or vehicle and tested for anxiety-like behavior in the marble burying assay. Locomotor activity was simultaneously recorded and was quantified as seconds per minute spent immobile. (**A,C**) Diazepam and THC dose-dependently reduced marble burying. (**B,D**) Both diazepam and THC also caused sedation at higher doses than required to reduce marble burying. Data are presented as the mean ± SEM (n = 8-11). * $p < 0.05$, ** $p < 0.01$ vs. vehicle.
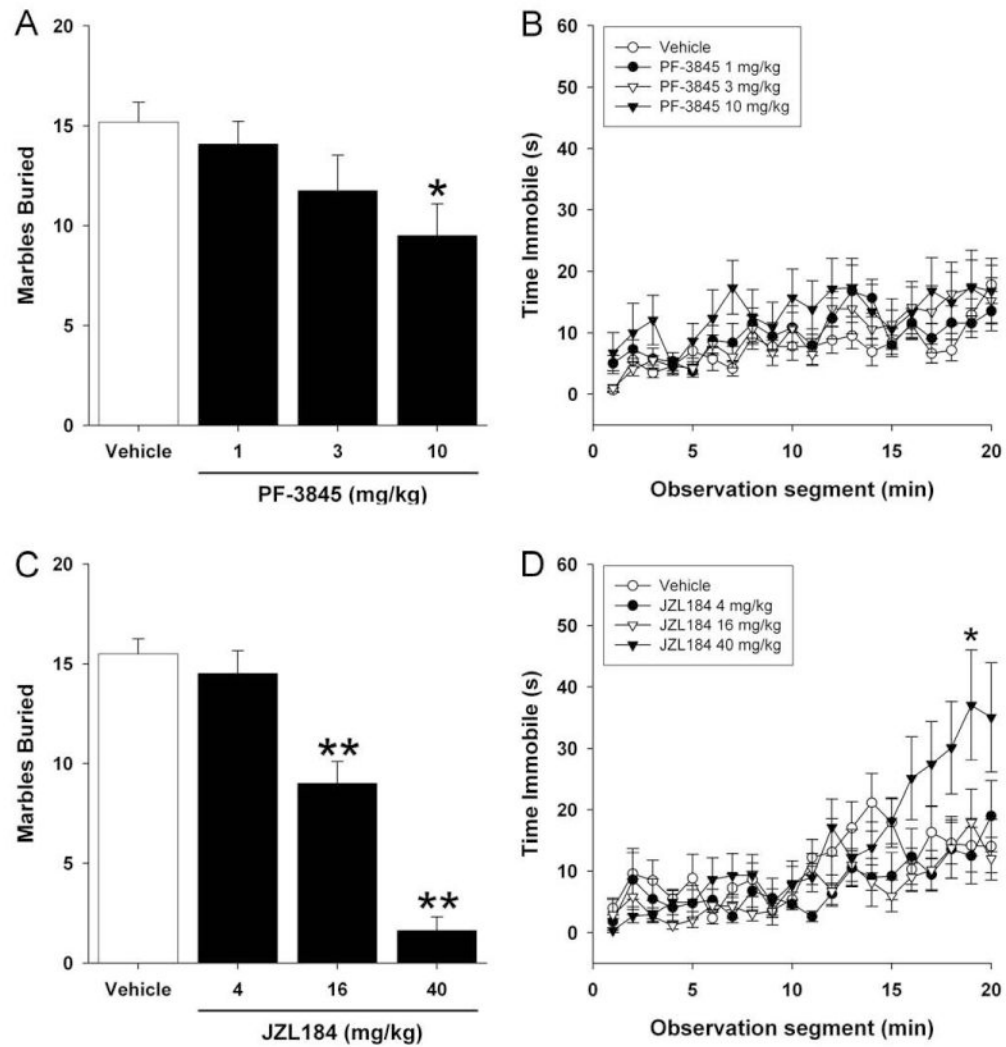
Figure 2.
Endocannabinoids reduce marble burying behavior. Mice were injected (i.p.) with the
FAAH inhibitor PF-3845, the MAGL inhibitor JZL184, or vehicle and tested in the marble
burying assay. Locomotor activity was simultaneously recorded and was quantified as
seconds per minute immobile. (**A**) FAAH dose-dependently reduced marble burying, but (**B**)
had no effect on immobility. (**C**) JZL184 dose-dependently reduced marble burying,
although (**D**) caused a statistically significant increase in immobility at 40 mg/kg. Data are
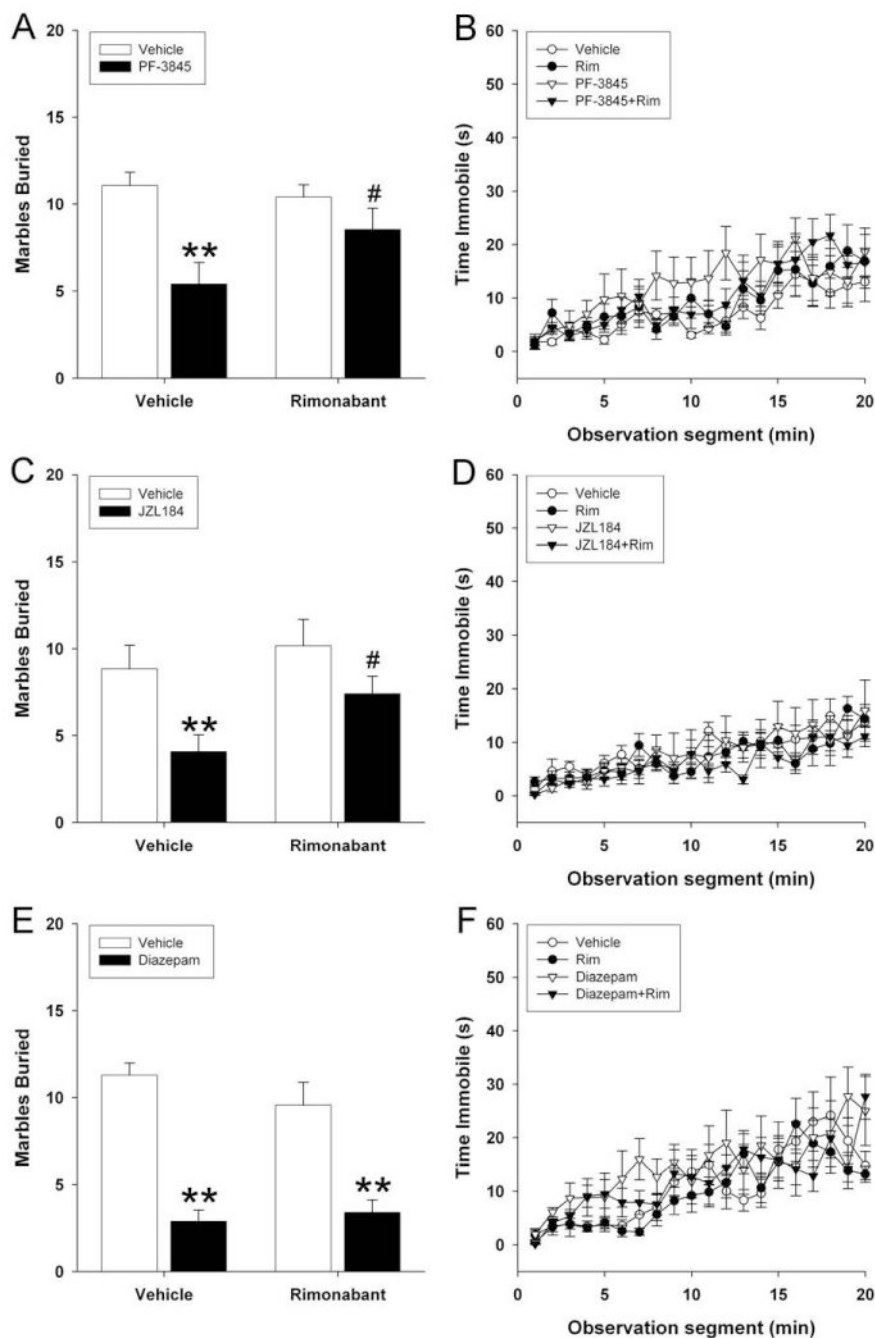presented as the mean ± SEM (n = 8). * $p < 0.05$, ** $p < 0.01$ vs. vehicle.

Figure 3.
The anxiolytic-like effects of FAAH and MAGL inhibition in the marble burying test are mediated by the CB_1 receptor. Mice were injected (i.p.) with PF-3845 (10 mg/kg), JZL184 (16 mg/kg), diazepam (DZ; 1 mg/kg), or vehicle and tested in the marble burying assay. Locomotor activity was simultaneously recorded and was quantified as seconds per minute spent immobile. (**A, C, D**) Pretreatment with the CB_1 receptor selective antagonist rimonabant (Rim) reversed the anxiolytic effects of PF-3845 and JZL184, but had no effect on diazepam. (**B, D, E**) Rimonabant had no effect on locomotor activity by itself or in conjunction with PF-3845, JZL184, or diazepam. Data are presented as the mean ± SEM (n = 9-16). ** $p < 0.01$ vs. vehicle; # $p < 0.05$ vs. PF-3845 or JZL184.