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Short communication

Anandamide effects on 5-HT_3 receptors in vivo

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ABSTRACT

Studies in knockout mouse strains have shown that some cannabimimetic effects persist in animals lacking cannabinoid CB_1 and CB_2 receptors. These residual effects are thought to result, in part, from a cannabinoid-modulation of ion channels. This study investigates the role of 5-HT_3 receptors as a potential *in vivo* target for cannabinoids. Mice deficient in CB_1 and CB_2 receptors were treated with Δ^9-tetrahydrocannabinol and anandamide, in the presence of the 5-HT_3 antagonist ondansetron. We show that the cannabinoid receptor-independent anandamide analgesia, but not catalepsy, is completely blocked by ondansetron. Thus, 5-HT_3 receptors seem to be involved in cannabinoid analgesia.

1. Introduction

Endogenous cannabinoids exert most of their physiological effects through the activation of G-protein coupled cannabinoid receptors CB_1 and CB_2. The CB_2 receptors are mostly located on immune cells (Munro et al., 1993), while cannabinoid CB_1 receptors are prominently expressed in the brain on presynaptic terminals in excitatory as well as inhibitory neurons (Freund et al., 2003). Activation of cannabinoid CB_1 receptors through postsynaptically generated endocannabinoids inhibits neurotransmitter release. Interestingly, anandamide still produces behavioural and physiological effects in mice lacking CB_1 and CB_2 receptors. These findings led to the suggestion that there is an other, yet undiscovered cannabinoid receptor. Recently, it has been shown that GPR55 may constitute such a receptor (Brown, 2007). In addition, there is evidence that anandamide is not only an agonist at G-protein coupled cannabinoid receptors, but also binds to and modulates the activity of ion channels, such as $TRPV_1$, serotonin$_3$ (5-HT_3), NMDA/glutamate and acetylcholine receptors (Hampson et al., 1998; Kimura et al., 1998; Lagalwar et al., 1999; Starowicz et al., 2007).

Of particular interest to this study is the observation by Fan that cannabinoid CB_1 receptor agonists stereoselectively inhibited serotonin induced, 5-HT_3 receptor mediated currents in rat nodose ganglion neurons (Fan, 1995). The same *in vitro* effect was found in HEK 293 cells expressing recombinant human 5-HT_3 receptors (Barann et al., 2002) and in Xenopus oocytes expressing cloned mouse 5-HT_3 receptors (Oz et al., 2002). There are *in vivo* evidences of 5-HT_3-mediated effects of cannabinoid agonists. Przegalinski and coworkers demonstrated that cannabinoid receptor agonists inhibit the cocaine-evoked hyperlocomotion, which is blocked by 5-HT_3 receptor antagonists (Reith, 1990), in a cannabinoid receptor-independent way (Przegalinski et al., 2005). Godlewski et al. substantiated that cannabinoid receptor agonists modulate the von Bezold-Jarisch reflex by inhibiting 5-HT_3 receptors in rats (Godlewski et al., 2003).

The aim of the present study was to examine the possible role of 5-HT_3 receptors as *in vivo* targets for the pharmacological effects of anandamide. We therefore tested the effects of Δ^9-tetrahydrocannabinol (THC) and anandamide in double knockout mice deficient in cannabinoid CB_1 and CB_2 receptors, in the presence of the 5-HT_3 receptor antagonist ondansetron.

2. Material and Methods

2.1. Materials

Δ^9-tetrahydrocannabinol (THC) was obtained from the National Institute on Drug Abuse (Bethesda, MD, USA). Anandamide and ondanstron were purchased from Sigma Aldrich Company.

THC and anandamide were dissolved in a cocktail of ethanol, Cremophor (Sigma) and saline (1:1:18), and were administered to the animals intravenously. Ondanstron (0.1 mg/kg) was diluted in saline and injected intraperitoneally in a volume of 0.1 ml/10 g 30 min before the THC (3 mg/kg) or anandamide (20 mg/kg) administration.

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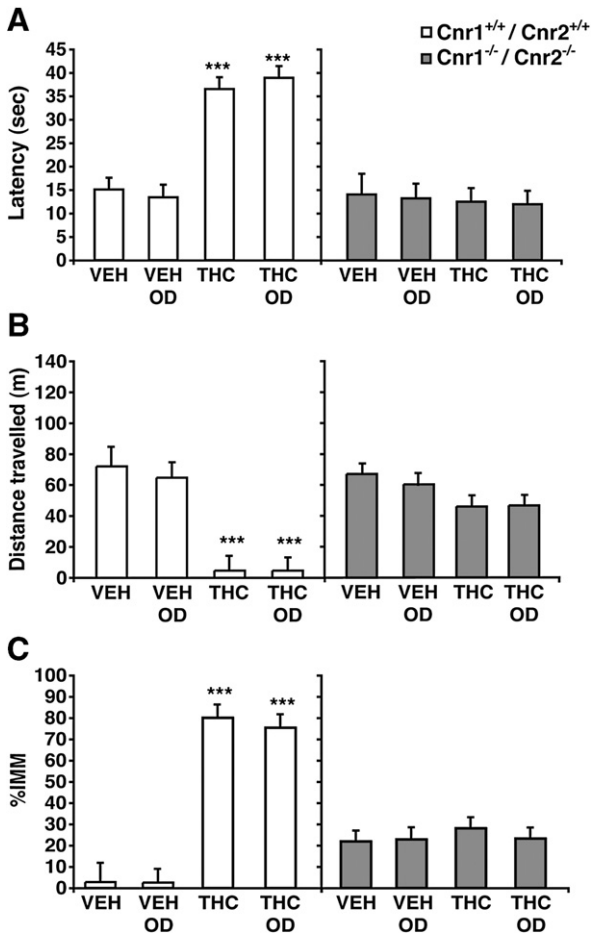

Fig. 1. Pharmacological effects of THC in the hotplate test (A), on spontaneous activity (B), and in the ring-catalepsy test (C). THC (3 mg/kg) significantly increased the reaction latency in the hot plate, decreased the locomotion and increased the immobility time in $Cnr1^{+/+}/Cnr2^{+/+}$ ($n=10$) but not in $Cnr1^{-/-}/Cnr2^{-/-}$ ($n=12$) animals. Ondanstroen (OD) treatment did not influence the THC effect. %IMM = percent of the time in a 5 min test session in which the mice were motionless. *** = $P<0.0001$.

2.2. Animals

Three to five months old, male CB_1/CB_2 receptor deficient mice ($Cnr1^{-/-}/Cnr2^{-/-}$) (Karsak et al., 2007) – which were generated by crossing congenic heterozygous $Cnr1^{-/-}$ and $Cnr2^{-/-}$ knockout animals – and their wild type controls C57BL6/J animals ($Cnr1^{+/+}/Cnr2^{+/+}$) were bred in our animal facility. Animals were housed in groups of 3–5 under reversed light–dark conditions (lights on — 7 pm, lights off — 9 am) at least two weeks before testing. During the experiments the animals had free access to food and water. Each animal was used only once and was naïve to the test. Experiments were carried out in the active (dark) phase between 10 and 17 h. Animal procedures followed the guidelines of the German Animal Protection Law.

2.3. Pharmacological studies

The experiments were carried out as previous described (Di Marzo et al., 2000). Briefly, the animals received THC and anandamide by tail-vein injection and locomotion, nociceptive reaction and immobility were tested. We studied THC effects successively in the same animal. For anandamide we used new animals for the catalepsy, because the short half-life of this compound did not permit the serial testing of all responses.

Locomotor activity was evaluated 5 min after THC injection in a dimly illuminated open-field system (20 lx at the ground level of the arena) in a sound-attenuated room. The animals were placed in the center of the open-field arena (45×45×22 cm). Spontaneous activity of animals was monitored and recorded for 10 min with an automatic system (TSE Systems GmbH, Germany). Antinociceptive drug effects were determined 20 min after the injection on a hotplate (55 °C) apparatus (TSE Systems GmbH, Germany). The first sign of pain was evaluated. The cut-off time was at 30 s. Catalepsy was determined 30 min after the THC treatment. The mice were placed on a ring (5.5 cm diameter, 16 cm height), the immobility time was measured during a 5 min testing period by an observer, who was blind for treatment strategy. Mice that jumped or fell from the ring were allowed five more trial.

Because of the shorter duration of action of anandamide, we modified the protocol (Adams et al., 1998; Smith et al., 1994) as follows: Antinociception was determined 5 min after the anandamide injection and locomotor activity immediately after the hotplate test for 10 min. Another group of mice were injected with anandamide for investigating catalepsy. The immobility was measured 5 min after the treatment during a five-min testing period.

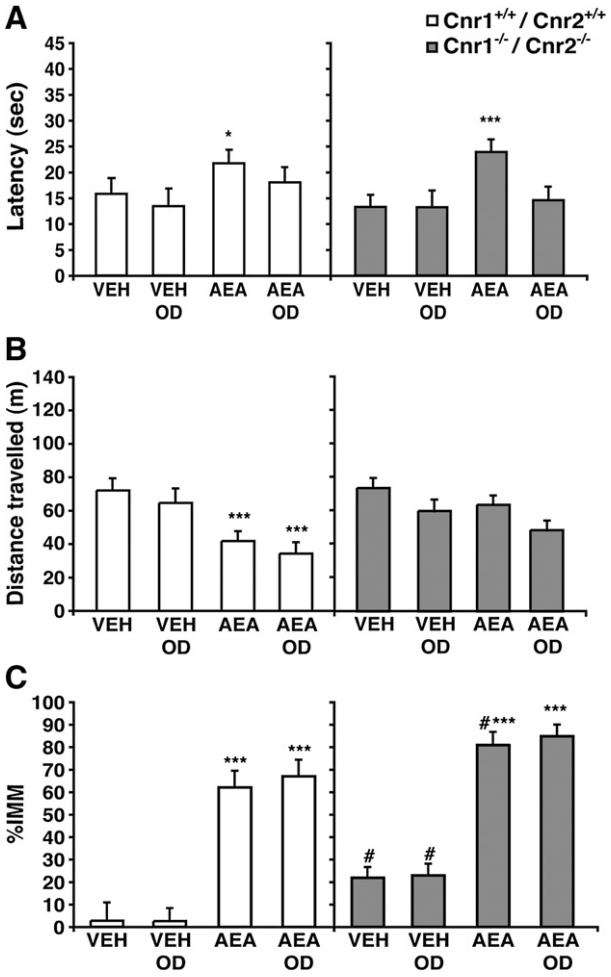

Fig. 2. Pharmacological effects of anandamide in the hotplate test (A), on spontaneous activity (B), and in ring-catalepsy test (C). Anandamide (20 mg/kg) significantly increased the reaction latency in the hot plate in both strains, and ondansetron (OD) treatment significantly reduced this analgetic effect. Anandamide (AEA) significantly decreased the locomotor activity in $Cnr1^{+/+}/Cnr2^{+/+}$ but not in $Cnr1^{-/-}/Cnr2^{-/-}$ mice. Ondansetron treatment had no effect on the locomotion. Anandamide injection increased the immobility time in both strains. Ondansetron treatment had not influenced this effect. The significant differences were indicated with asterisk between the treated groups and with double crosses between the strains. $Cnr1^{+/+}/Cnr2^{+/+}$: $n=12$; $Cnr1^{-/-}/Cnr2^{-/-}$: $n=15$; #, * = $P<0.05$; *** = $P<0.0001$.

2.4. Statistical analysis

Statistical analyses were performed with a STATISTICA software package (version 7.1 Statsoft, Inc. (2005)). Two-way ANOVA was employed to assess the treatment effect in the knockout and in the wild type strains (strain and treatment were the between group factors), followed by corresponding one-way ANOVA. For post hoc comparison Fischer's LSD (Least Significant Difference) test was used. All significance-tests were two tailed and considered as significant at $P<0.05$.

3. Results

3.1. Antinociception

A dose of 3 mg/kg of THC produced significant antinociception in $Cnr1^{+/+}/Cnr2^{+/+}$ animals ($P<0.001$), but not in $Cnr1^{-/-}/Cnr2^{-/-}$ mice (Fig. 1A; $P=0.38$). Treatment with the 5-HT_3 receptor antagonist ondansteron had no influence on THC-induced analgesia. In contrast, 20 mg/kg anandamide produced significant analgesia in both $Cnr1^{+/+}/Cnr2^{+/+}$ and $Cnr1^{-/-}/Cnr2^{-/-}$ animals (Fig. 2A; $P<0.001$), thus demonstrating that anandamide analgesia is not exclusively mediated by CB_1 and or CB_2 receptor activation. In order to determine, whether the analgesic effects of anandamide are mediated through 5-HT_3 receptor modulation, mice were pre-treated with ondansteron. Strikingly, ondansteron completely blocked anandamide analgesia (Fig. 2A; $P<0.001$).

3.2. Locomotor effects

THC administration profoundly reduced locomotor activity in wild type mice, which were immobile almost during the entire test-period ($P<0.001$). In $Cnr1^{-/-}/Cnr2^{-/-}$ mice, THC treatment had no significant effect on locomotor activity ($P=0.59$). Ondansteron pre-treatment did not change the THC effects (Fig. 1B). Anandamide significantly reduced open-field activity in $Cnr1^{+/+}/Cnr2^{+/+}$ mice ($P<0.001$), but not in $Cnr1^{-/-}/Cnr2^{-/-}$ animals. Ondansteron pre-treatment had no influence on anandamide-hypolocomotion (Fig. 2B).

3.3. Catalepsy

THC produced a strong ring-catalepsy in $Cnr1^{+/+}/Cnr2^{+/+}$, but not in $Cnr1^{-/-}/Cnr2^{-/-}$ mice ($P<0.0001$). Ondansteron pre-treatment did not change the cataleptic effect of THC (Fig. 1C). Anandamide treatment produced a profound immobility in both strains, which was not affected by ondansteron (Fig. 2C).

4. Discussion

The aim of this study was to determine the role of 5-HT_3 receptors in the *in vivo* pharmacological effects of anandamide. For this purpose, we utilized $Cnr1^{-/-}/Cnr2^{-/-}$ mice that lack cannabinoid CB_1 and CB_2 receptors. The mice were pre-treated with ondansetron at a very high dose in order to "pharmacologically knock out" the 5-HT_3 receptors by blockade of their orthosteric binding site. Thus, the 5-HT_3 receptors were prevented from being activated by endogenous serotonin released tonically from the adjacent serotoninergic nerve terminals. Stimulation of the 5-HT_3 receptors at their orthosteric site is a prerequisite for inhibitory effect of anandamide exerted via its allosteric binding site, which is probably located in one of the transmembrane domains of the receptor. Evidence for such a mechanism can be derived from the *in vitro* experiments (Barann et al., 2002) on which the present study was based; in electrophysiological experiments on outside-out patches of the cell membrane of the 5-HT_3 receptor expressing HEK293 cells, the anandamide-induced inhibition was observed only when the 5-HT_3 receptors were stimulated by serotonin. We showed that THC has little or no effect in three paradigms in $Cnr1^{-/-}/Cnr2^{-/-}$ mice, while anandamide still produced analgesia and ring-catalepsy in the absence of cannabinoid receptors. Our results are consistent with previous findings, which have demonstrated that anandamide, but not THC, exerts a cannabimimetic effect in $Cnr1^{-/-}$ animals (Di Marzo et al., 2000). In contrast, Wise et al. (Wise et al., 2007) found anandamide analgesia, catalepsy and hypothermia in FAAH deficient mice, but not in CB_1 deficient animals. However, they used an even higher dose of anandamide (50 mg/kg), and the behavioural assessments were performed 60 min after the treatment, which is longer than the supposed duration of anandamide action (Adams et al., 1998; Smith et al., 1994).

In basal locomotion activity (without any treatment) we found no difference between wild type and $Cnr1^{-/-}/Cnr2^{-/-}$ mice. Previous studies found an elevated or reduced open-field activity, depending on the experimental conditions (Di Marzo et al., 2000; Jardinaud et al., 2005; Ledent et al., 1999; Martin et al., 2000; Steiner et al., 1999; Valverde et al., 2005). THC and anandamide effect on spontaneous activity was completely absent in $Cnr1^{-/-}/Cnr2^{-/-}$ animals, consistent with previous results (Di Marzo et al., 2000; Zimmer et al., 1999). However, anandamide, but not THC, still produced catalepsy in the absence of cannabinoid receptors. Together these findings show that the analgesic and cataleptic effects of anandamide are not exclusively mediated by CB_1 and CB_2 receptors.

Although the in-vivo targets of anandamide have not been unambiguously identified yet, there is substantial evidence suggesting that anandamide modulates the activity of ion channels. As described anandamide is an allosteric modulator of 5-HT_{3A} receptors (Barann et al., 2002). It inhibits 5-HT_{3A} currents in HEK293 cells and Xenopus oocytes, in a cannabinoid receptor-independent manner (Barann et al., 2002; Oz et al., 2002). It is not a competitive inhibitor, because it did not shift the dose response curve to the right but depressed the maximum response to a representative cannabinoid CB_1 receptor agonist and it did not affect the binding of the 5-HT_3 receptor antagonist [^{3}H]-GR65630 (Barann et al., 2002). Anandamide may selectively change the 5-HT_{3A} receptor desensitization properties without altering the activation and deactivation kinetics (Adams et al., 1998).

Taken together we show here that the cannabinoid receptor-independent analgesic effect of anandamide was prevented by blockade of the orthosteric site of the 5-HT_3 receptor by ondanstron. Due to the lack of the physiological stimulation of the 5-HT_3 receptor by endogenous serotonin — a prerequisite for the modulatory effect of anandamide via the allosteric site — anandamide can no longer inhibit the function of the 5-HT_3 receptor. In contrast, ondansteron did not influence the cannabinoid receptor-independent cataleptic effect of anandamide. These findings strongly suggest that 5-HT_3 receptors contribute to anandamide analgesia, but not catalepsy.

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