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THE NONPSYCHOACTIVE CANNABINOID CANNABIDIOL INHIBITS 5-HT_{3A} RECEPTOR-MEDIATED CURRENTS IN *XENOPUS* OOCYTES

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Running title: Cannabidiol on 5-HT_{3A} receptor

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ABBREVIATIONS: CBD, cannabidiol; CB, cannabinoid; 5-HT, 5-hydroxytryptamine; ActD, actinomycin D; ANOVA, analysis of variance; BAPTA, 1,2-bis (*o*-aminophenoxy) ethane-*N*, *N*, *N'*, *N'*-tetraacetic acid; DMSO, dimethyl sulfoxide; GR65630, 3-(5-methyl-1H-imidazol-4-yl)-1-(1-methylindol-3-yl)propan-1-one; HEPES, 4-(2-hydroxyethyl) piperazineethane sulfonic acid; MBS, modified Barth's solution; MDL72222, Tropanyl 3,5-dichlorobenzoate; PMSF, phenylmethylsulfonyl fluoride; PTX, pertussis toxin;

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Abstract

The effect of the plant-derived nonpsychotropic cannabinoid, cannabidiol (CBD), on the function of hydroxytryptamine (5-HT) type 3A (5-HT_{3A}) receptors expressed in *Xenopus* oocytes was investigated using two-electrode voltage clamp techniques. CBD reversibly inhibited 5-HT (1 µM)-evoked currents in a concentration-dependent manner (IC_{50}=0.6 µM). CBD (1 µM) did not alter specific binding of the 5-HT_{3A} antagonist [^{3}H]GR65630, in oocytes expressing 5-HT_{3A} receptors. In the presence of 1 µM CBD, the maximum 5-HT-induced currents were also inhibited. The EC_{50} values were 1.2 µM and 1.4 µM, in the absence and presence of CBD, indicating that CBD acts as a noncompetitive antagonist of 5-HT_3 receptors. Neither intracellular BAPTA injection nor pertussis toxin pretreatment (5 µg/ml) altered the CBD-evoked inhibition of 5-HT-induced currents. CBD inhibition was inversely correlated with 5-HT_{3A} expression levels and mean 5-HT_3 receptor current density. Pretreatment with actinomycin D, which inhibits protein transcription, decreased the mean 5-HT_3 receptor current density and increased the magnitude of CBD inhibition. These data demonstrate that CBD is an allosteric inhibitor of 5-HT_3 receptors expressed in *Xenopus* oocytes. They further suggest that allosteric inhibition of 5-HT_3 receptors by CBD may contribute to its physiological roles in the modulation of nociception and emesis.

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Introduction

The serotonin type three ($5\text{-}HT_3$) receptor, a member of the ligand-gated ion channel family, mediates rapid and transient membrane depolarizing effect of 5-HT in the central and peripheral nervous system (Yakel and Jackson, 1988). An involvement of $5\text{-}HT_3$ receptors in pain transmission, mood disorders, and drug abuse has been reported (for reviews, Riering et al., 2004; Faerber et al., 2007; Engleman et al., 2008). Furthermore, $5\text{-}HT_3$ receptor antagonists are effective therapeutic agents for the treatment of chemotherapy-induced nausea and vomiting (Slatkin 2007; Thompson and Lummis, 2007).

Earlier studies showed that $5\text{-}HT_3$-receptor antagonists and cannabinoids produce similar pharmacological effects such as non-opioid receptor-mediated analgesia and antiemesis (for reviews; Tramer et al., 2001; Martin and Wiley, 2004; Riering et al., 2004). In fact, synthetic Δ^9- tetrahydrocannabinol (THC), dronabinol, (Marinol), and THC analogs such as nabilone (Cesamet) are approved by the US Food and Drug Administration for use in chemotherapy-induced nausea and vomiting refractory to conventional antiemetic therapy (for reviews, Tramer et al., 2001; Martin and Wiley, 2004; Slatkin, 2007).

The limitation of the therapeutic utility of THC and its above-mentioned chemical analogs is the potential development of psychoactive effects through central nervous system cannabinoid 1 receptor (CB1) Cannabidiol (CBD) is one of the most abundant cannabinoids of *Cannabis sativa* with reported antioxidant, anti-inflammatory, and antiemetic effects. It is well tolerated and is without side effects when chronically administered to humans (for reviews Mechoulam et al., 2007; Scuderi et al., 2009; Izzo et

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al., 2009). Furthermore, CBD is devoid of psychoactive properties due to a low affinity for the CB1 and CB2 receptors (Mechoulam et al., 2007; Izzo et al., 2009; Pertwee, 2009). Thus, pharmaceutical interest in this compound has risen significantly in recent years (for reviews, Scuderi et al., 2009; Izzo et al., 2009; Pertwee, 2009).

The effects of THC, synthetic cannabinoid receptor agonists such as WIN55,212-2, CP55,940, and JWH-015 (Fan, 1995; Barann et al., 2002), and the endocannabinoid anandamide (Barann et al., 2002; Oz et al., 1995; 2002a; Xion, 2008) on the functional properties of 5-HT_3-receptors have been shown in earlier *in vitro* studies. However, whether nonpsychotropic cannabinoids such as cannabidiol affect 5-HT_{3A}-receptor function is unknown. In the present study, we have tested the hypothesis that CBD may produce its pharmacological effects, at least in part, via 5-HT_3 receptors. For this purpose, the cRNA encoding the mouse 5-HT_3 subunit A of the receptor was expressed in *Xenopus* oocytes and the effect of CBD on receptor function was investigated.

Methods

Mature female *Xenopus laevis* frogs were purchased from *Xenopus* leavis I, Ann Arbor, MI and were housed in dechlorinated tap water at 18 ^{0}C with 12/12 hours light-dark lighting and fed with beef liver at least twice a week. Clusters of oocytes were removed surgically under tricaine (Sigma, St.Louis, MO) anesthesia (0.15 %) and individual oocytes were manually dissected away in a solution containing (in mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $MgSO_4$, 0.8; HEPES, 10 (pH 7.5). Dissected oocytes were stored 2-7 days in modified Barth's solution containing (in mM): NaCl 88; KCl 1; $NaHCO_3$ 2.4; $Ca(NO_3)_2$ 0.3; $CaCl_2$; 0.9; $MgSO_4$ 0.8; HEPES 10 (pH 7.5), supplemented with sodium pyruvate 2 mM, penicillin 10,000 IU/L, streptomycin 10 mg/L, gentamicin 50 mg/L, and theophylline 0.5 mM. Oocytes were placed in a 0.2 ml recording chamber and superfused at a constant rate of 3-5 ml/min. The bathing solution consisted of : 95 mM NaCl; 2 mM KCl; 2 mM $CaCl_2$; and 5 mM HEPES 5 (pH 7.5). The amount of 5-HT_{3A} receptor cRNA injected into oocytes varied from 1 to 30 ng, as indicated. However, the injection volume of diethylpyrocarbonate-treated distilled water was kept at 30 nl throughout the experiments. The cells were impaled with two standard glass microelectrodes filled with a 3 M KCl (1-3 MΩ). The oocytes were routinely voltage clamped at a holding potential of -70 mV using an GeneClamp-500B amplifier (Axon Instruments Inc., Burligame, CA). Current responses were digitized by A/D converter and analyzed using pClamp 8 (Axon Instruments Inc.) run on an IBM/PC or directly recorded on a Gould 2400 rectilinear pen recorder (Gould Inc., Cleveland, OH). Current-voltage curves were generated by holding each membrane potential in a series for 50-60 s, followed by a return to -70 mV for 5 min. Oocyte capacitance was measured by a paired-ramp method described earlier (Oz et

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al., 2004a). Briefly, voltage-ramps were employed to elicit constant capacitive current, I_{cap}, and the charge associated with this current was calculated by the integration of I_{cap}. Ramps had slopes of 2 V/s and durations of 20 ms and started at a holding potential of –90 mV. A series of 10 paired-ramps was delivered at 1 s intervals and averaged traces were used for charge calculations. In each oocyte, the averages of 5-6 measurements were used to obtain values for membrane capacitance; C_m. Currents for I_{cap} recordings were filtered at 20 kHz and sampled at 50 kHz. Current density was calculated by normalizing the average of 3 consecutive control currents to the oocyte capacitance.

Compounds were applied by addition to the superfusate. All chemicals used in preparing the solutions were from Sigma-Aldrich (St. Louis, MO). Pertussis toxin, BAPTA, actinomycin D, 5-HT and MDL72222 (Tropanyl 3,5-dichlorobenzoate) were purchased from Tocris Cookson (St.Louis, MO). Cannabidiol was generously provided by NIDA Drug Supply System/NIH, Rockville, MD. Procedures for the injections of pertussis toxin (PTX, 50 nl, 50 µg/ml) or BAPTA (50 nl, 200 mM) were performed as described previously (Oz et al., 1998). Injections were performed 1 h prior to recordings using oil-driven ultra microsyringe pumps (Micro4, WPI, Inc. Sarasota, FL). Stock solutions of CBD were prepared in dimethylsulfoxide (DMSO) at a concentration of 30 mM. DMSO alone did not affect 5-HT_{3A} receptor function when added at concentrations as high as 0.2 % (v/v), a concentration twice greater than the most concentrated application of the agents used. Electrophysiological recordings from oocytes were conducted 3 to 4 days after cRNA injections and both control and treatment (PTX, BAPTA) groups were recorded on the same days.

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Synthesis of cRNA: The cDNA clone of the mouse and human 5-HT_{3A} subunits were provided by Dr. David Julius (University of California, San Francisco, CA) and OriGen Inc. (Rockville, MD), respectively. Complementary RNAs (cRNAs) were synthesized *in vitro* using a mMessage mMachine RNA transcription kit (Ambion Inc., Austin, TX). The quality and sizes of synthesized cRNAs were confirmed by denatured RNA agarose gels..

Radioligand Binding Studies: Oocytes were injected with 10 ng mouse 5-HT_{3A} cRNA and functional expression of the receptors was tested by electrophysiology on day three. Isolation of oocyte membranes were carried out by modification of a method described earlier (Oz et al., 2004b). Briefly, oocytes were suspended (20 μl/oocyte) in a homogenization buffer (HB) containing HEPES 10 mM, EDTA 1 mM, 0.02% NaN_3 , 50 μg/mL bacitracin, and 0.1 mM PMSF (pH 7.4) at 4 °C on ice and homogenized using a motorized Teflon homogenizer (six strokes, 15 s each at high speed). The homogenate was centrifuged for 10 min at 800 g. The supernatant was collected and the pellet was resuspended in HB and recentrifuged at 800 g for 10 min. Supernatants were then combined and centrifuged for 1 h at 36,000 g. The membrane pellet was resuspended in HB at the final protein concentration of 0.5 - 0.7 mg/ml and used for the binding studies.

Binding assays were performed in 500 μL of 10 mM HEPES (pH 7.4) containing 50 μL of oocyte preparation and 1 nM [^{3}H]GR65630 (58.7 Ci/mmol; Perkin-Elmer, Inc. Waltham, MA). Nonspecific binding was determined using 100 μM MDL72222. Oocyte membranes were incubated with [^{3}H]GR65630 in the absence and presence of CBD at 4 °C for 1 h before bound radioligand was separated by rapid filtration onto GF/B filters presoaked in 0.3% polyethylenemine. Filters were then washed with two 5 mL washes of

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ice-cold HEPES buffer and left in 3 mL of scintillation fluid for at least 4 h before scintillation counting was conducted to determine amounts of membrane-bound radioligand.

Data analysis: For the nonlinear curve fitting and regression fits of the radioligand binding data, the computer software Origin™ (Originlab Corp. Northampton, MA) was used. In functional assays, average values were calculated as mean ± standard error means (S.E.M.). Statistical significance was analyzed using ANOVA or Student's *t* test. Concentration-response curves were obtained by fitting the data to the logistic equation,

1) $$y = \{ (E_{max}-E_{min})/(1+[x/EC_{50}]^{n}) \} + E_{min},$$

where x and y are concentration and response, respectively, E_{max} is the maximal response, E_{min} is the minimal response, EC_{50} is the half-maximal concentration, and n is the slope factor.

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Results

Bath application of neither 5-HT (50 µM) nor CBD (10 µM) produced detectable currents in oocytes injected with diethylpyrocarbonate-treated distilled water (30 nl per oocyte, n=6). Application of CBD (10 µM) for 20 min did not affect membrane resistance (R_m), membrane capacitance (C_m), or resting membrane potential (V_m) in oocytes injected with 3 ng cDNA encoding the 5-HT_{3A} receptor (Table I). Currents evoked by 5-HT (1 µM) were maximally inhibited by CBD within 10-15 minutes after the initiation of CBD perfusion. Following CBD washout, recovery was slow (e.g., 20 - 30 min) (Figure 1A). Time course studies assessing the effects of 25 min CBD application on the mean amplitude of 5-HT-induced currents from 6 oocytes are presented in Figure 1B.

In the next series of experiments we examined the concentration-response relationship of the CBD effects on the function of 5-HT_3 receptors (Fig 1C). The threshold concentration for inhibition by CBD was 0.1 µM and maximal inhibition was achieved in concentrations ranging between 10 to 30 µM. The inhibition of 5-HT (1 µM)-induced current by 25 min CBD application was concentration-dependent with an IC_{50} of 0.6 ± 0.1 µM and a slope value of 0.9 (Fig. 1C).

As the participation of $G_{i/o}$-proteins in the signalling of the receptors activated by the cannabinoids and certain CBD analogs have been reported (Jarai et al., 1999), we tested the effect of CBD in control (distilled-water injected) and pertussis toxin (PTX)-injected oocytes expressing 5-HT_3 receptors. There was no significant difference in CBD inhibition of 5-HT responses between controls and PTX-injected cells (Figure 2A; $P<0.001$; $F_{3, 18}=130.9$; ANOVA, n=5-6 for the effect of CBD compared to controls in

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distilled water injected and PTX groups; Bonferroni test, $P > 0.05$ for the significance of CBD inhibition between controls and PTX group).

Since CBD has been shown to increase intracellular Ca^{2+} levels in neurons and glia (Drysdale et al., 2006; Ryan et al., 2006), we investigated the effect of the Ca^{2+} chelator BAPTA on CBD inhibition of 5-HT responses. In oocytes injected with BAPTA, the inhibition of 5-HT responses by 20 min CBD application was not significantly different from controls (Figure 2B; $P<0.001$, $F_{3, 20}= 110.7$; ANOVA, n=5-7, for the effect of CBD compared to controls in distilled water injected and PTX groups; Bonferroni test, $P > 0.05$ for the significance of CBD inhibition between distilled water injected and PTX group).

Examination of the voltage-dependence of the CBD inhibition indicated that the degree of inhibition of the 5-HT (1 µM)-induced currents did not vary with membrane potential (Figure 2C). In addition, there was no change of the reversal potential of the 5-HT-activated ion currents (control: 2 ± 2 mV in controls; CBD (1 µM): 4 ± 3 mV), indicating that neither the ion selectivity of the channel nor the driving force on Na^{+} and Ca^{2+} were affected by CBD. Moreover, quantitative evaluation of data for the inhibitory effect of CBD at different membrane potentials (Figure 2D) showed no statistically significant differences on the effect of CBD at different holding potentials (among -20, -40, -60, and -80 mV groups; P=0.953, $F_{3,16}$=0,11, n=5 for each group, ANOVA).

By definition, an open-channel blockade requires the opening of the channel by the binding of the agonist to the receptor. Thus, in the absence of an agonist, the degree of blockade should be related to the frequency of channel activation. Therefore, the extent of CBD inhibition of the $5\text{-}HT_{3A}$ receptors was compared in cells exposed to 5-HT at 5-

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min intervals with those exposed at 10- and 20-min intervals (Figure 3A). During application of 1 μM CBD for 20 min, CBD was equally effective in inhibiting currents activated at 5, 10, and 20 min intervals (Figure 3B; between 5, 10, and 20 min interval groups *P*=0.746, $F_{2, 14}$=0,29, n=5-7, ANOVA), indicating that the frequency of channel opening does not alter the extent of CBD inhibition and that the channel does not need to be opened by the agonist for CBD to be effective. Recovery from an open channel blocker would be facilitated by the increases in opening frequencies. Therefore, we analyzed the extent of recovery from CBD inhibition at different 5-HT stimulation intervals (Figure 3C). The recovery from CBD inhibition was not altered by 5-HT stimulation intervals, suggesting that CBD is not trapped in the channel when the channel closes, as can occur with open channel blocking drugs (between 5, 10, and 20 min interval groups *P*=0.379, $F_{2, 14}$=1,06, n=5-7, ANOVA).

CBD may alter 5-HT_3 receptor function via competitive inhibition of 5-HT binding to the receptor. To examine this issue, we performed radioligand binding assays (Figure 4A-B). In competition experiments, 5-HT concentration-dependently inhibited the specific binding of 1 nM [^{3}H]GR65630 (Figure 4A). The concentration-dependent inhibition of [^{3}H]GR65630 binding by 5-HT was not altered by 1 μM CBD (Figure 4A). The IC_{50} values in the absence and presence of CBD were 0.7 ± 0.3 and 0.6 ± 0.2 μM, respectively (Student's *t* test, *P*=0.24; *t*=1.2; *df*=17; n=8-11). Similarly, increasing CBD concentrations did not reduce specific [^{3}H]GR65630 binding to membranes of oocytes expressing 5-HT_{3A} receptor cDNA (Figure 4B).

In oocytes expressing 5-HT_{3A} receptor, the concentration-response curve of 5-HT was examined in the absence and presence of 1 μM CBD. The EC_{50} values in the absence

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and presence of CBD were 1.2 ± 0.2 and 1.4. ± 0.1 μM (means ± S.E.M.), respectively (Student's *t* test, P=0.36; t=-0.96; df=7, n=4-5). As shown in figure 4C, CBD did not significantly alter EC_{50} values and inhibited the maximal 5-HT-responses to the same percentage of control values (n=5), suggesting that CBD inhibits 5-HT-activated ion currents in a noncompetitive manner.

Since our experiments were conducted using mouse 5-HT_{3A} receptor cRNA, we compared the effect of 1 μM CBD on the function of mouse and human 5-HT_{3A} receptors expressed in *Xenopus* oocytes (3 ng cRNA per oocyte). Application of CBD for 20 min caused a significant inhibition of currents induced by 1 μM 5-HT. The magnitude of CBD inhibition did not differ between mouse (61 ± 4% inhibition, n=5) and human 5-HT_{3A} receptors (65 ± 5% inhibition, n=7; Student's *t* test, P=0.54; t=-0.63; df=10, n=5-7; Figure 4D)

In a recent study, it was demonstrated that the magnitude of inhibition induced by the endocannabinoid anandamide is inversely correlated with the amount of 5-HT_3 receptor cRNA injected into *Xenopus* oocytes (Xiong et al., 2008). For this reason, we compared the effects of CBD on 5-HT_3 receptors in *Xenopus* oocytes injected with increasing concentrations of cRNA encoding for this receptor. Increasing the concentration of 5-HT_3 receptor cRNA reversed the inhibitory effect of CBD at this receptor (Figure 5A). For instance, the maximal inhibition induced by 1 μM CBD was 81 ± 5% (n=6) in oocytes injected with 1 ng of 5-HT_{3A} receptor cRNA, whereas the maximal inhibition was only 11 ±3% (n=5) in oocytes injected with 30 ng of 5-HT_{3A} receptor cRNA. These values were significantly different (Figure 5B; Student's *t* test, P<0.001; t=-12.8, df=9, n=5-6). The IC_{50} values of CBD inhibition differed by nearly

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230-fold between oocytes previously injected with 1 ng and 30 ng of cRNAs (Figure 5C); the IC_{50} values for CBD inhibition were 121 ± 11 nM, 587 ± 62 nM, and 29 ± 4 µM (means ± S.E.M.) in cells injected with 1 ng, 3 ng, and 30 ng of cRNA, respectively (Figure 5C; Student's *t* test, $P<0.001$; $t=-7.1$, $df=10$, n=5-7). Similarly, the magnitude of inhibition produced by 1 µM CBD was highly correlated with the amount of the cRNA injected into the oocytes (Figure 5D; $r = -0.99$). As would be expected, the amplitude of current activated by 1 µM 5-HT also increased with the amount of cRNA injected (Figures 5 and 6A), indicating that the levels of functional receptor expression correlate with the amount of cRNA expressed in the oocytes. The magnitude of inhibition produced by 1 µM CBD was also highly correlated with the mean current density of the receptor (Figure 6A; $r = -0.96$). To further confirm the relationship between receptor expression and CBD inhibition, we pretreated oocytes for 24 h before recordings with 15 µg/ml actinomycin D (ActD), which inhibits RNA transcription. Pretreatment with ActD significantly reduced mean current density (activated by 30 µM 5-HT) from 4.9 ± 0.6 nA/pF to 1.6 ± 0.4 nA/pF (Figure 6B on the right; n=6-8, ANOVA, $F_{1, 12}=25.9$, $P<0.001$) suggesting that ActD reduces the functional expression of 5-HT_{3A} receptors. On the other hand, ActD significantly increased the magnitude of CBD inhibition from 48 ± 4% to 81 ± 6% (Figure 6B on the left; n=6-8, ANOVA, $F_{1,12}=18.2$; $P<0.002$).

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Discussion

The results presented indicate that the plant-derived nonpsychoactive cannabinoid CBD inhibits the function of both mouse and human 5-HT_3 receptors expressed in *Xenopus* oocytes. The inhibitory effect of CBD on 5-HT-induced currents was concentration-dependent and related to 5-HT_3 receptor expression. The IC_{50} values varied from 121 nM to 29 μM in oocytes injected with 1 to 30 ng of cRNA, respectively. Increasing the concentration of 5-HT did not overcome CBD inhibition of 5-HT-induced ion currents, ie., the maximal amplitudes of 5-HT-induced currents were also inhibited, suggesting that CBD inhibition is noncompetitive.

Cannabidiol (CBD) is a major nonpsychotropic constituent of Cannabis sativa. Unlike THC, it is virtually inactive at both CB1 and CB2 receptors (for reviews Izzo et al., 2009; Pertwee, 2009). CB1 and CB2 are not expressed in *Xenopus* oocytes (Hejazi et al., 2005; Oz et al., 2007). Therefore, it is unlikely that the effect of CBD on 5-HT_3 receptors is mediated by the activation of CB1 or CB2 receptors. CBD analogues such as abnormal-cannabidiol are reported to activate nonCB1 and nonCB2 receptor by a PTX-sensitive G-protein (Jarai et al., 1999). However, CBD inhibited 5-HT_3 receptor function in PTX-treated oocytes, indicating that the PTX-sensitive receptors do not mediate the functional interaction of CBD with the 5-HT_3 receptor.

Cannabidiol increases intracellular Ca^{2+} levels in neurons and glia (Drysdale et al., 2006; Ryan et al., 2006). However, the magnitude of CBD inhibition of 5-HT_{3A} currents was not significantly altered by intracellular injection of BAPTA, a high affinity Ca^{2+} chelator. Furthermore, during our experiments, application of CBD in the highest concentration of used (30 μM) in this study, did not modify base-line currents, indicating

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that intracellular Ca^{2+} concentration was not affected by CBD. Since Ca^{2+}-activated Cl^- channels are highly sensitive to intracellular levels of Ca^{2+} (for a review Dascal, 1988), the release of Ca^{2+} from internal stores of this ion would be reflected by changes in holding current in voltage-clamp conditions. This was not seen. In addition, other passive membrane properties such as membrane capacitance and oocyte input resistance were not significantly altered (Table 1), suggesting that CBD, at the concentrations used in this study, also did not disrupt the integrity of the lipid membrane.

CBD suppresses nausea and vomiting in animal models. In shrews, pretreatments with CBD suppress lithium chloride-induced vomiting (Parker et al.2004) and reduces conditioned retching elicited by a lithium-paired context (Parker et al. 2006). In rats, CBD interferes with nausea elicited by lithium chloride and with conditioned nausea elicited by a flavor paired with lithium chloride (Parker et al. 2002). Because CBD does not activate known CB receptors (Izzo et al., 2009; Pertwee, 2009), and the effect of CBD was not reversed by the CB1 receptor antagonist SR-141617A, this suppression of nausea and vomiting does not appear to be linked to activity of the CB1 or CB2 receptors (Kwiatkowska et al. 2004).

Cannabinoid receptor-independent actions of various cannabinoids on the function of 5-HT_3 receptors have been demonstrated in several earlier investigations (for a review Oz, 2006). In *in vitro* electrophysiological studies, direct effects of the cannabinoid receptor ligands THC, anandamide, WIN55,212-2, and CP55,940 on the function of 5-HT_3 receptors have been reported (Fan, 1995; Barann et al., 2002; Oz et al; 1995;2002; Xiong et al., 2008). In *in vivo* studies, 5-HT-induced cardiovascular responses such as bradycardia mediated by 5-HT_3 receptors located on the terminals of

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cardiopulmonary afferent C-fibers, were inhibited by the synthetic cannabinoid receptor agonists WIN55,212-2 and CP55,940 in anesthetized and SR141716A pretreated rats (Godlewski et al., 2003). Our results provide the first demonstration that nonpsychotropic phytocannabinoids such CBD also modulate the function of 5-HT_3 receptors.

Commonly used doses of CBD (3-10 mg/kg) produce brain levels of 200 nM - 3 μM, respectively (Varvel et al., 2005). Therefore, functional modulation of 5-HT_{3A} receptors demonstrated in this study (IC_{50}= 121 nM and 587 nM for 1 ng and 3 ng cRNA injected oocytes, respectively) can mediate some of the cannabinoid-receptor independent actions of CBD. In earlier studies, direct actions of CBD on several integral membrane proteins including various subtypes of glycine receptors (Ahrens et al., 2009), 5-HT receptors (Russo et al., 2005), opioid receptors (Kathmann et al., 2006), transient receptor potential channels (Bisogno et al., 2001; De Petrocellis et al., 2008; Qin et al., 2008), and T-type Ca^{2+} channels (Ross, 2008) have also been demonstrated (for a recent review Izzo et al., 2009). In addition anti-inflammatory, analgesic, and antiepileptic actions of CBD are mediated by mechanisms independent of known cannabinoid receptors (for a review; Izzo et al., 2009).

Open-channel blockade is a widely used model to describe the block of ligand-gated ion channels. However, this model cannot account for the results of the present study. Firstly, for open channel blockers, the presence of the agonist is required to let the blocker enter the channel after the receptor has undergone an agonist-induced conformational change to open the channel. In contrast to open channel blockers, preincubation of CBD caused a further inhibition (Figs 1A and 3A), indicating that CBD can interact with the closed state of the 5-HT_{3A} receptor. Secondly, inhibition by CBD is

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not voltage sensitive, suggesting that the CBD-binding site is not charged and that the site is not within the transmembrane electric field. Similarly, there was an absence of use-dependent blockade (Fig. 3A) and CBD had little effect when co-administered with 5-HT without CBD preincubation (data not shown). Thirdly, recovery from CBD inhibition occurred independent of agonist application intervals (Fig. 3C), indicating that CBD is not trapped in the channel when the channel closes, as can occur with open channel blocking drugs. Finally, CBD did not significantly affect the reversal potential of 5-HT-induced currents, indicating that current inhibition is not due to an alteration in the ion selectivity of the channels.

Allosteric modulators alter the functional properties of ligand-gated-ion channels by interacting with site(s) that are topographically distinct from the ligand binding sites (for a review; Onaran and Costa, 2009). In electrophysiological studies, although the potency of the 5-HT, a natural ligand (agonist) for this receptor, was not altered, its efficacy was significantly inhibited by CBD, indicating that CBD did not compete with the 5-HT binding site on the receptor. In agreement with these findings, radioligand binding studies indicated that displacement of [^{3}H]GR65630 by 5-HT was not significantly affected by CBD, further suggesting that CBD does not interact with 5-HT binding site on the receptor. These findings indicate that CBD acts as an allosteric modulator of 5-HT$_3$ receptor. In earlier studies, CBD has been reported to be an allosteric modulator of several structurally different ion channels (Izzo et al., 2009); i.e., CBD binds to site(s) topographically distinct from the 5-HT binding sites on the receptor-ion channel complex. The non-competitive property of the allosteric CBD inhibition puts it in an advantageous position, since the increases in concentration of endogenous agonist (5-

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HT) in synaptic cleft cannot alter the efficacy of CBD.

It is likely that CBD, a highly lipophilic agent, first dissolves into the lipid membrane and then diffuses into a non-annular lipid space to inhibit the ion channel-receptor complex. Consistent with this idea, the effect of CBD on 5-HT_3 receptor reached to a maximal level within 10-15 min of application time. Similarly, actions of several hydrophobic allosteric modulators such as endocannabinoids (Oz et al., 2002a; Spivak et al., 2007; Xiong et al., 2008), fatty acids (Oz et al., 2004b), steroids (Oz et al., 2002b), and general anesthetics (Zhang et al., 1995) on ligand-gated ion channels require 5-20 min to reach their maxima (for a review Oz, 2006), suggesting that the binding site(s) for these allosteric modifiers is located inside the lipid membrane and require a relatively slow (in minutes) time course to modulate the function of the receptor. It is likely that these hydrophobic agents act as gating modifiers (for a review Oz, 2006) affecting the energy requirements for the gating-related conformational changes in ligand-gated ion channels (Spivak et al., 2007).

Interestingly, we found an inverse correlation between the magnitude of CBD inhibition and the amount of cRNA injected into oocytes. In a recent study, biotinylation experiments indicated that the increase in the amount of cRNA injected into *Xenopus* oocytes enhances the surface expression of 5-HT_{3A} receptors and attenuates the magnitude of anandamide inhibition of 5-HT_{3A} receptor (Xiong et al., 2008). This phenomenon has been suggested to be due to the increased tendency of 5-HT_{3A} receptors to desensitize at low expression levels. Various conditions that decrease the desensitization of the receptor also attenuate anandamide inhibiton (Xiong et al., 2008). By definition, receptors are required to be open prior to their transition into a desensitized

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state. However, as mentioned earlier, in the majority of reports, the effects of highly lipophilic substances such as cannabinoid receptor ligands (Barann et al., 2002; Oz et al., 2002a) and steroids (Oz et al., 2002b), require a long (several seconds)- lasting exposure time before the opening of the channel by agonist application (for a review Oz, 2006). Thus, it appears that cannabinoids can interact with 5-HT$_{3A}$ receptors during the closed state and facilitate desensitization during agonist activation of the receptor.

It is plausible to predict that CBD, similar to the effect of anandamide on nicotinic acetylcholine receptors (Spivak et al., 2007), reduces current amplitude by lowering the energy barrier for receptors to enter a desensitized state. In addition, 5-HT$_{3A}$ receptor density can contribute to the free-energy barrier required for conformational changes during a receptor desensitization process and facilitate the effect of CBD on the desensitization of 5-HT$_{3A}$ receptor. Clearly, further investigations in which receptor kinetics can be studied in a more detailed and precise manner are required to delineate the mechanisms by which CBD affects 5-HT$_{3A}$ receptor function.

In conclusion, our results indicate that CBD inhibits the function of homomerically expressed 5-HT$_{3A}$ receptor by a noncompetitive (allosteric) mechanism and that the expression level of 5-HT$_{3A}$ receptors significantly influences the sensitivity of the receptor to the inhibitory effect of CBD. These data add to a growing body of evidence (Izzo et al., 2009) indicating that cannabinoid-receptor independent targets can contribute to pharmacological actions of CBD.

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Figure legends

Figure 1. The effect of cannabidiol on 5-HT_3 receptor-mediated ion currents. **(A)** Records of currents activated by 1 µM 5-HT in control (*left*), coapplication of 1 µM CBD and 5-HT after 20 min CBD application (*middle*), 30 min recovery (*right*). **(B)** Time-course of the effect of CBD application (25 min) on the maximal amplitudes of the currents induced by 1 µM 5-HT at 5 min intervals. Data points represent means ± S.E.M. of 6 cells. **(C)** Concentration-response curve for cannabidiol inhibition of 5-HT_3 receptor-mediated ion currents. For all concentrations used, CBD was applied for 25 min. Data points are the mean ± S.E.M. (n=6-7); error bars not visible are smaller than the size of the symbols. The curve is the best fit of the data to the logistic equation described in the Methods. The IC_{50} value for CBD was 0.6 ± 0.1 µM with a slope value of 0.9.

Figure 2. Effects of pertussis toxin treatment, calcium chelator, BAPTA injection, and membrane potential changes on cannabidiol inhibition of 5-HT_3 receptor-mediated ion currents. **(A)** Bar presentation of the effects of 1 µM CBD application (20 min) on the maximal amplitudes of 5-HT-induced currents in oocytes injected with 50 nl distilled-water, controls (n-6) or 50 nl of PTX (50 µg/ml, n=5). Bars represent the means ± S.E.M. **(B)** Bar presentation of the effects of 1 µM CBD application (20 min) on the maximal amplitudes of 5-HT-induced currents in oocytes injected with 50 nl distilled-water, controls (n-5) or BAPTA (50 nl, 200 mM, n=7). Bars represent the means ± S.E.M. **(C)** Current-voltage relationship of 5-HT-activated current in the absence (*open circles*) and presence (*filled circles*) of 1 µM CBD. Currents were activated by 1 µM 5-

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HT in the same oocyte. **(D).** Percentage inhibition of 5-HT-activated current by 1 µM CBD at different membrane potentials; there are no significant differences among these values at different membrane potentials ($P>0.05$, ANOVA; $n=5$).

Figure 3. The effect of 5-HT stimulation-interval alterations on the inhibition of 5-HT_{3A} receptor-mediated responses by cannabidiol. **(A)** Time-course of the effect of CBD on the maximal amplitudes of the currents induced by 1 µM 5-HT at 5 (*open circles*), 10 (*filled circles*), and 20 (*filled triangles*) min intervals. Data points represent means ± S.E.M of 5-7 cells. **(B)** The percentage of cannabidiol (CBD) inhibition on the 5-HT_{3A} receptor-mediated currents recorded at the end of a 20-min application period was not different among oocytes stimulated with 5-HT application at 5,10, and 20 min intervals ($P>0.05$, ANOVA, n=5-7). **(C)** The percentage of controls (recovery) from cannabidiol (CBD) inhibition of the 5-HT_{3A} receptor-mediated currents recorded at the end of a 20-min recovery period was not different between oocytes stimulated and not stimulated with 5-HT application every 10 min ($P>0.05$, ANOVA, n=5-7). Duration of CBD application (20 min) is indicated by the horizontal bar in the figure.

Figure 4. The effects of cannabidiol and 5-HT on the specific binding of $[^3H]$GR65630 and the effect of cannabidiol on 5-HT concentration-response curves from *Xenopus* oocytes expressing 5-HT_{3A} receptors. **(A)** Inhibition of specific $[^3H]$GR65630 binding by nonlabeled 5-HT in membranes after 1 hour preincubation with 1 µM cannabidiol. The concentration of $[^3H]$GR65630 was 1 nM. The inhibition curve shows pooled data from 8-11 measurements from 3 experiments. **(B)** Effects of increasing concentration of cannabidiol on the specific binding of $[^3H]$GR65630. Experiments were conducted in the

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presence of 1 nM of [^{3}H]GR65630. The results present data from 9-11 measurements. Data points indicate mean ± S.E.M. **(C)** Concentration-response curves for 5-HT-activated current in the absence (*open circles*) and presence (*filled circles*) of 1 µM CBD. Currents were activated by 5-HT concentrations ranging from 0.1 to 100 µM. Cannabidiol was applied for 20 min, and 5-HT and CBD were then co-applied for 10 to 15 sec. Data points are the mean ± S.E.M. (n=4-5); error bars are not visible are smaller than the size of the symbols. The curve is the best fit of the data to the logistic equation described in the methods. The control concentration-response curve is normalized to the maximal response. The CBD concentration-response curve is the percentage of the maximal control. **(D)** Comparison of the 1 µM CBD effect on the mouse and human 5-HT_{3A} receptors expressed in oocytes (3 ng/oocyte, recorded on post-injection day 3). Bar graph shows average inhibition (mean ± SEM) of 5-HT (1 µM)-induced currents by 20 min CBD application in 5 oocytes expressing mouse 5-HT_{3A} receptors and 7 oocytes expressing 5-HT_{3A} receptors.

Figure 5. Cannabidiol inhibition is inversely correlated with the amount of 5-HT_{3A} receptor cRNA injected into *Xenopus* oocytes. **(A)** Current traces demonstrating CBD inhibition of 5-HT-activated currents in cells previously injected with 1 ng (top) and 30 ng (bottom) 5-HT_{3A} receptor cRNAs. **(B)** Time course of CBD inhibition of maximal currents induced by 1 µM 5-HT in oocytes previously injected with 1, 3, and 30 ng of the 5-HT_{3A} receptor cRNAs. The solid bar indicates the duration (20 min) of 1 µM CBD application. Each data point represents mean ± S.E.M. from the average of 5-6 cells. **(C)** Concentration-response curves of CBD inhibition of 5-HT-activated current in oocytes

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injected with 1, 3, and 30 ng of 5-HT_{3A} receptor cRNAs. The curves were best fit to the Logistic equation as described under *Materials and Methods*. Each data point represents mean ± S.E. from 5-7 oocytes. **(D)** Correlation between the magnitude of inhibitory effect induced by 1 μM CBD and increasing concentrations of 5-HT_{3A} cRNAs injected into oocytes (linear regression, r = -0.99, n=5-7).

Figure 6. Cannabidiol inhibition of 5-HT_{3A} receptors is inversely correlated with the mean current density and actinomycin D (ActD) treatment-induced decreases in 5-HT-induced currents. **(A)** Correlation between the percent CBD inhibition of mean current density (MCD; linear regression, r = -0.96). **(B)** Bar graphs of average amplitude of current induced by maximal concentration of 5-HT (30 μM) with and without ActD treatment of *Xenopus* oocytes expressing mouse 5-HT_{3A} receptors. Each bar represents mean ± S.E.M. from 6 to 8 cells **(C)** Bar graphs represents average percentage of CBD inhibition of 5-HT_{3A} receptors without and with ActD treatment. Each bar represents mean ± S.E.M. from 6 to 8 cells. * indicates a significant difference compared with control (P<0.05, ANOVA).

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Table I.

The effects of Cannabidiol (10 μM) on the passive membrane properties of the *Xenopus* oocytes expressing 5-HT_3 receptors.

	R_m (MΩ)	C_m (nF)	V_m (mV)
Control (n=11)	1.1 ± 0.3	193 ± 17	-35.2 ± 3.4
20 min CBD (n=9)	1.4 ± 0.3	197 ± 14	-36.9 ± 3.8

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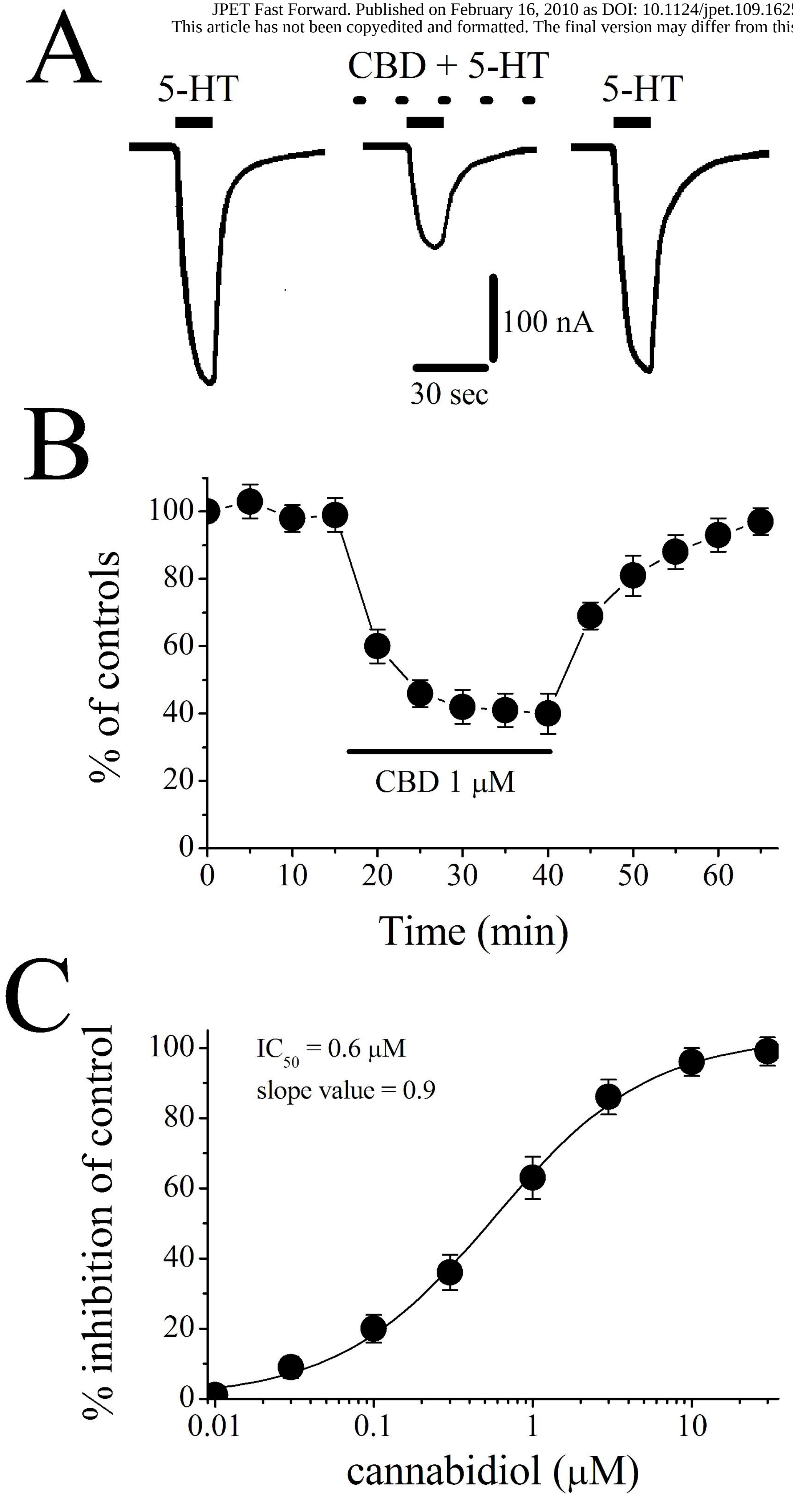
Figure 1



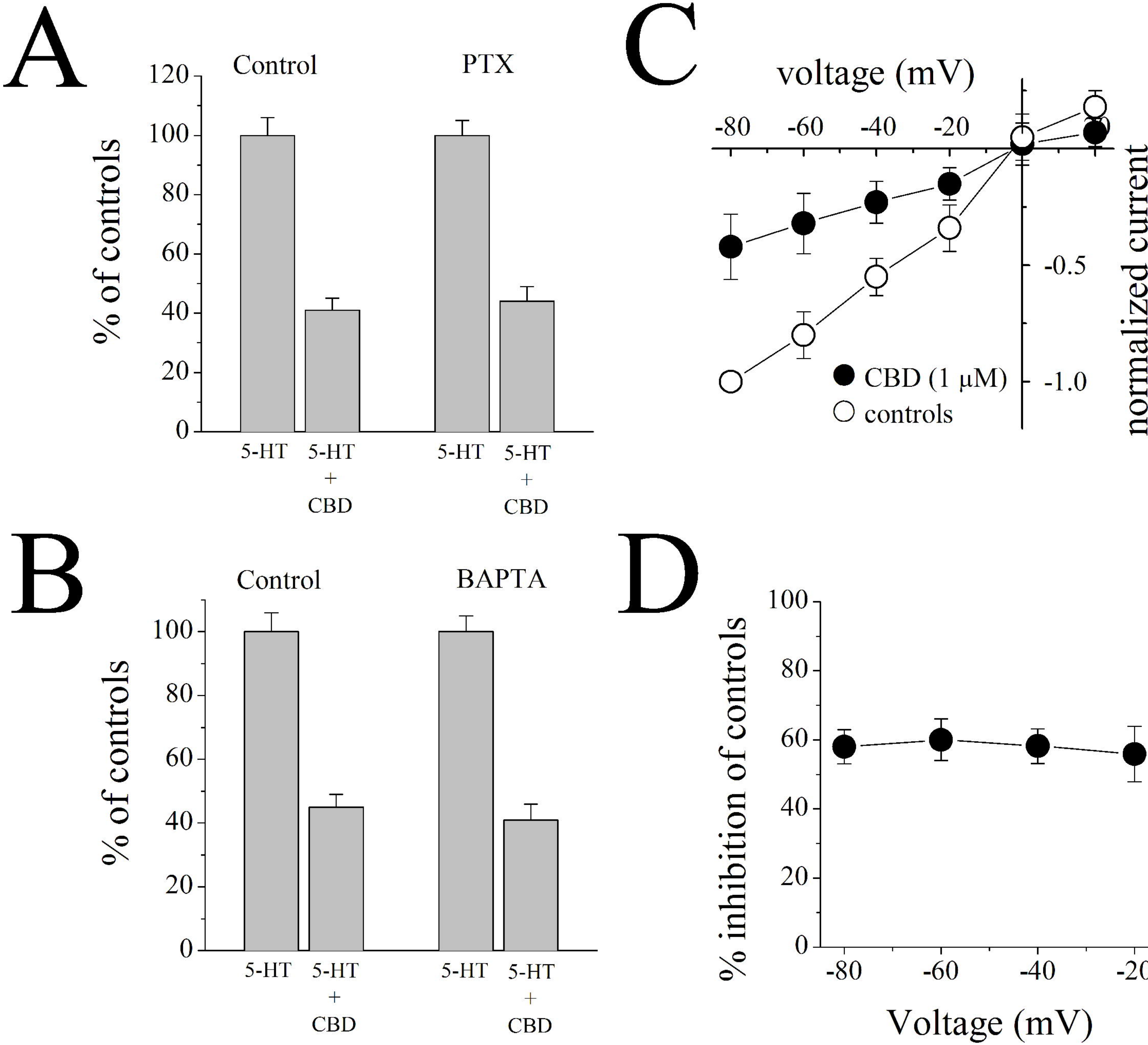

A
Control
PTX
% of controls
0
20
40
60
80
100
120
5-HT
5-HT + CBD
B
Control
BAPTA
% of controls
0
20
40
60
80
100
5-HT
5-HT + CBD
C
voltage (mV)
-80
-60
-40
-20
20
-0.5
-1.0
normalized current
CBD (1 μM)
controls
D
% inhibition of controls
0
20
40
60
80
100
-80
-60
-40
-20
Voltage (mV)

Figure 2

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Figure 3

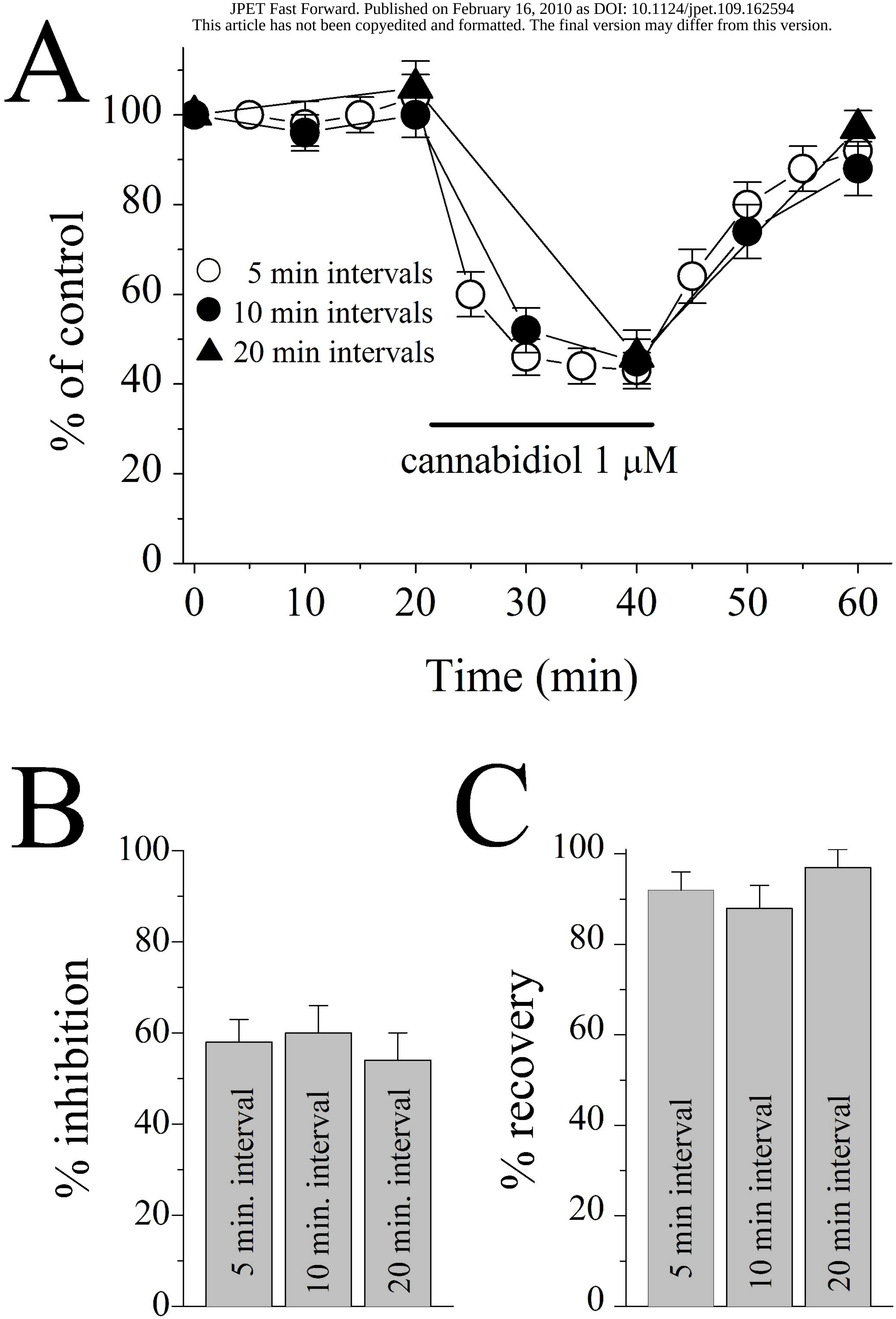

Figure 4

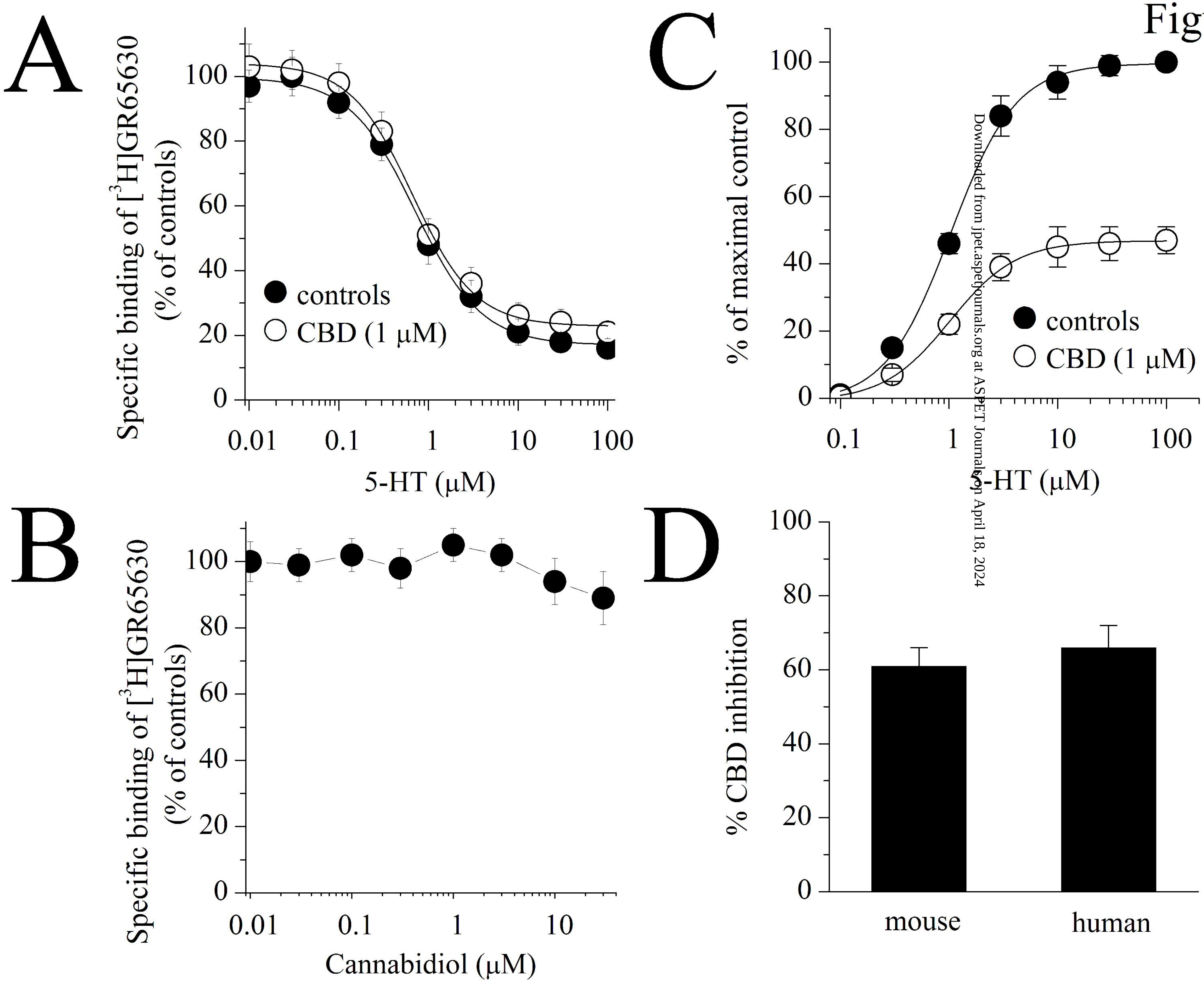
A
Specific binding of [3H]GR65630 (% of controls)
controls
CBD (1 μM)
5-HT (μM)
0.01
0.1
1
10
100
0
20
40
60
80
100
B
Specific binding of [3H]GR65630 (% of controls)
Cannabidiol (μM)
C
% of maximal control
controls
CBD (1 μM)
5-HT (μM)
D
% CBD inhibition
mouse
human

Figure 5

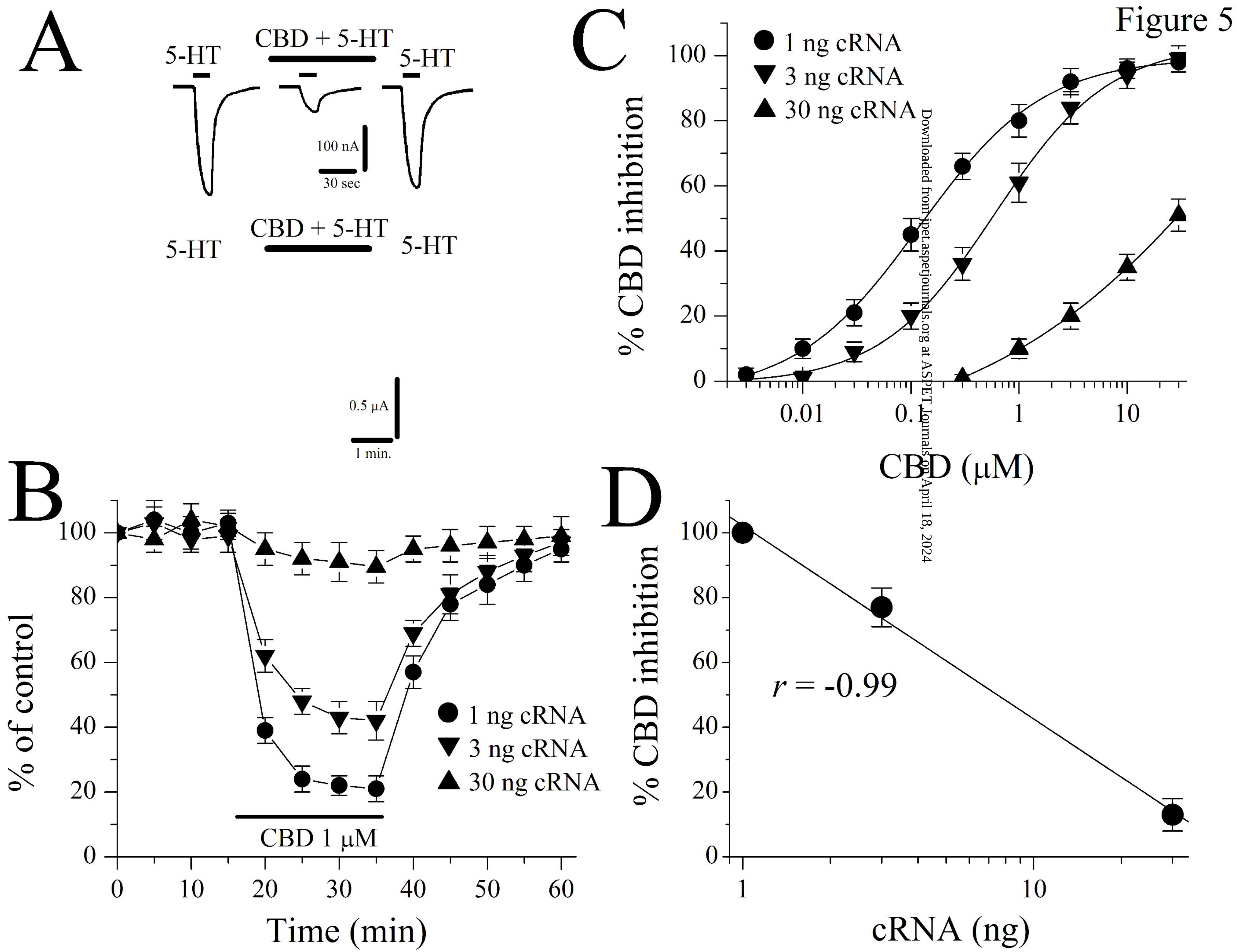
A
5-HT
CBD + 5-HT
5-HT
100 nA
30 sec
5-HT
CBD + 5-HT
5-HT
0.5 μA
1 min.
B
% of control
Time (min)
CBD 1 μM
1 ng cRNA
3 ng cRNA
30 ng cRNA
C
% CBD inhibition
CBD (μM)
1 ng cRNA
3 ng cRNA
30 ng cRNA
D
% CBD inhibition
cRNA (ng)
r = -0.99

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Figure 6