

RESEARCH PAPER

Cannabidiolic acid methyl ester, a stable synthetic analogue of cannabidiolic acid, can produce 5-HT_{1A} receptor-mediated suppression of nausea and anxiety in rats

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BACKGROUND AND PURPOSE

The aim of this study was to compare the abilities of cannabidiolic acid methyl ester (HU-580) and cannabidiolic acid (CBDA) to enhance 5-HT_{1A} receptor activation *in vitro* and produce 5-HT_{1A}-mediated reductions in nausea and anxiety *in vivo*.

EXPERIMENTAL APPROACH

We investigated the effects of HU-580 and CBDA on (i) activation by 8-hydroxy-2-(di-*n*-propylamino)tetralin of human 5-HT_{1A} receptors in CHO cell membranes, using [³⁵S]-GTPγS binding assays, (ii) gaping by rats in acute and anticipatory nausea models, and (iii) stress-induced anxiety-like behaviour, as indicated by exit time from the light compartment of a light–dark box of rats subjected 24 h earlier to six tone-paired foot shocks.

KEY RESULTS

HU-580 and CBDA increased the E_{max} of 8-hydroxy-2-(di-*n*-propylamino) tetralin *in vitro* at 0.01–10 and 0.1–10 nM, respectively, and reduced signs of (i) acute nausea at 0.1 and 1 μg·kg^{−1} i.p. and at 1 μg·kg^{−1} i.p., respectively, and (ii) anticipatory nausea at 0.01 and 0.1 μg·kg^{−1}, and at 0.1 μg·kg^{−1} i.p. respectively. At 0.01 μg·kg^{−1}, HU-580, but not CBDA, increased the time foot-shocked rats spent in the light compartment of a light–dark box. The anti-nausea and anti-anxiety effects of 0.01 or 0.1 μg·kg^{−1} HU-580 were opposed by the 5-HT_{1A} antagonist, WAY100635 (0.1 mg·kg^{−1} i.p.).

CONCLUSIONS AND IMPLICATIONS

HU-580 is more potent than CBDA at enhancing 5-HT_{1A} receptor activation, and inhibiting signs of acute and anticipatory nausea, and anxiety. Consequently, HU-580 is a potential medicine for treating some nausea and anxiety disorders and possibly other disorders ameliorated by enhancement of 5-HT_{1A} receptor activation.

Abbreviations

8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; CBD, cannabidiol; CBDA, cannabidiolic acid; FS, foot shock; HU-580, cannabidiolic acid methyl ester; LiCl, lithium chloride; No FS, no foot shock; WAY100635, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate salt

Introduction

Cannabidiolic acid (CBDA) is a major constituent of *Cannabis sativa*. It was first isolated in 1955 (Krejčí and Šantavý, 1955), and its structure (Figure 1) was elucidated in 1965 by analysis of the physical properties of its methyl ester (Mechoulam and Gaoni, 1965). Its synthesis from **cannabidiol** was subsequently reported (Mechoulam and Ben-Zvi, 1969). CBDA gradually decarboxylates, while still in the plant, to cannabidiol (Figure 1) (Mechoulam, 1973), a process that is speeded up by heat. Whereas cannabidiol has been the topic of a large number of publications and its biological/therapeutic properties have now been reasonably well identified (Mechoulam *et al.*, 2002; Zhornitsky and Potvin, 2012; Cascio and Pertwee, 2014), our knowledge of the pharmacology of CBDA is much more limited. However, even the limited amount of information on this phytocannabinoid that has been published suggests that it may have a wide variety of actions and effects. Thus, it has been shown to inhibit breast cancer cell migration (Takeda *et al.*, 2017) and to cause a down-regulation of COX-2 (Takeda *et al.*, 2014).

Considerable recent evidence suggests that CBDA (at a dose as low as 1 µg·kg⁻¹ i.p.) can induce potent **5-HT_{1A} receptor**-mediated anti-nausea effects as indicated by its apparent ability to prevent both vomiting in *Suncus murinus* and acute nausea-induced behaviour of conditioned gaping in rats (Grill and Norgren, 1978) by enhancing 5-HT_{1A} receptor activation (Bolognini *et al.*, 2013; Rock and Parker, 2013; Rock and Parker, 2015). As well as reducing acute nausea, CBDA has the potential to reduce anticipatory (conditioned) nausea, an effect experienced by chemotherapy patients upon returning to the clinic in which they received their nauseating treatment (Rock *et al.*, 2014; 2015; 2016). There are currently no effective selective treatments for anticipatory nausea once it develops in these patients. It is noteworthy, therefore, that Rock *et al.* (2014; 2015; 2016) have demonstrated that CBDA reduces contextually elicited conditioned gaping (a model of anticipatory nausea), also by a 5-HT_{1A}-dependent mechanism of action. Finally, like cannabidiol, CBDA has also been shown to produce anxiolytic-like effects under conditions of high stress at doses as low as 0.1 µg·kg⁻¹, i.p (Rock *et al.*, 2017).

The instability of CBDA (Crombie and Crombie, 1977), especially when subjected to heat, weakens the case for developing it as a medicine. Hence, we decided to search for a more stable analogue with a similar biological profile. We eventually decided to compare CBDA with its methyl ester (HU-580, Figure 1), since decarboxylation of phenolic acids is known to proceed through the carboxyl anion (RCOO⁻) of the acid

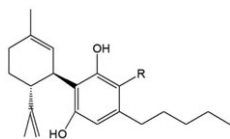


Figure 1

Structure of cannabidiol (R = H), CBDA (R = COOH) and HU-580 (R = COOCH₃).

(Norman and Coxon, 1993) which is not readily formed by the methyl ester, and indeed found that when kept at 4°C for 21 days, HU-580 remained unchanged, contrary to CBDA which partly decomposed. Here, we present evidence that HU-580 is even more potent than CBDA at producing signs of 5-HT_{1A} receptor-mediated suppression of nausea. Our initial experiments explored the possibility that HU-580 can enhance the ability of the selective 5-HT_{1A} agonist, 8-hydroxy-2-(di-*n*-propylamino)tetralin (**8-OH-DPAT**), to stimulate [³⁵S]-GTPγS binding to membranes obtained from human 5-HT_{1A} receptor-expressing CHO cells, with a potency equal to or even greater than that shown previously to be displayed by CBDA (Bolognini *et al.*, 2013). Since these experiments yielded positive results, we went on to evaluate the relative potency of CBDA and HU-580 at inducing apparent 5-HT_{1A} receptor-mediated suppression of acute and anticipatory nausea-induced behaviour in the rat gaping models. Furthermore, we evaluated the relative abilities of extremely low doses of HU-580 and CBDA to produce anxiolytic-like behaviour in a model of stress-induced anxiogenic responding.

Methods

In vitro procedures

CHO cells. CHO cells stably transfected with cDNA encoding human 5-HT_{1A} receptors (a generous gift from Dr Keith Parker) were maintained at 37°C and 5% CO₂ in Gibco™ Ham's F-12 Nutrient Mix supplied by Fisher Scientific UK Ltd that was supplemented both with 2 mM L-glutamine, 10% FBS and 0.6% penicillin-streptomycin, all also supplied by Fisher Scientific UK Ltd, and with the disulphate salt of G418 [(2R,3S,4R,5R,6S)-5-amino-6-[[[(1R,2S,3S,4R,6S)-4,6-diamino-3-[[[(2R,3R,4R,5R)-3,5-dihydroxy-5-methyl-4-(methylamino)oxan-2-yl]oxy]-2-hydroxy cyclohexyl]oxy]-2-[(1R)-1-hydroxyethyl]oxane-3,4-diol; 600 mg·mL⁻¹] supplied by Sigma-Aldrich UK.

[³⁵S]-GTPγS binding assay. Each assay was carried out with human 5-HT_{1A} CHO cell membranes (50 µg protein per well), **GTPγS**-binding buffer (50 mM Tris-HCl; 50 mM Tris-Base; 5 mM MgCl₂; 1 mM EDTA; 100 mM NaCl; 1 mM DTT and 0.1% BSA), 0.1 nM [³⁵S]-GTPγS and 30 µM GDP, in a final volume of 500 µL (Cascio *et al.*, 2010). Binding was initiated by the addition of [³⁵S]-GTPγS to the wells. Non-specific binding was measured in the presence of 30 µM GTPγS. Assays were performed at 30°C for 60 min (Cascio *et al.*, 2010). The reaction was terminated by a rapid vacuum filtration method using Tris-binding buffer as described previously by Cascio *et al.* (2010), and the radioactivity was quantified by liquid scintillation spectrometry. In all the [³⁵S]-GTPγS-binding assays, we used 0.1 nM [³⁵S]-GTPγS, 30 mM GDP and a protein concentration of 5 µg per well. CBDA, HU-580, 8-OH-DPAT and **WAY100635** were stored at -20°C as 10 mM stock solutions dissolved in DMSO.

In vivo procedures

Animals. Animal procedures complied with the Canadian Council on Animal Care, and the protocols were approved

by the Institutional Animal Care Committee at University of Guelph. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). A total of 200 naïve male Sprague–Dawley rats, obtained from Charles River Laboratories (St Constant, Quebec), were used for all *in vivo* studies. Rats were individually housed (for acute nausea studies) or pair-housed [for anticipatory nausea and light–dark emergence studies] in home cages made of opaque white plastic (48 × 26 × 20 cm), containing bed-o-cob bedding from Harlan Laboratories, Inc. (Mississauga, Ontario), a brown paper towel, and Crink-I’Nest™ from The Andersons, Inc. (Maumee, Ohio). Additionally, in the home cage, rats were provided with a soft white paper container that was 14 cm long and 12 cm in diameter. All rats were subjected to an ambient temperature of 21°C and a 12/12 h light–dark schedule (lights off at 07:00 h) and maintained on food (Highland Rat Chow [8640]) and water *ad libitum*. For the acute and anticipatory nausea studies, their body weights ranged from 263 to 329 g on the day of conditioning. For the light–dark emergence studies, their body weights ranged from 320 to 387 g on the day of test.

Apparatus. For the studies of acute nausea (*in vivo* experiment 1), rats were placed in taste reactivity (Grill and Norgren, 1978) chambers with their cannula attached to an infusion pump (Model KDS100, KD Scientific, Holliston, MA, USA) for fluid delivery. The taste reactivity chambers were made of clear Plexiglas (22.5 × 26 × 20 cm) that sat on a table with a clear glass top. A mirror beneath the chamber at a 45° angle facilitated viewing of the ventral surface of the rat to observe orofacial responses. The conditioning chamber was in a dark room next to a 25 W light source. A video camera (Sony DCR-HC48, Henry’s Cameras, Waterloo, ON, Canada) fire-wired into a computer was focused on the mirror and used to record each rat’s orofacial reactions during the 2 min taste reactivity test. The video tapes were later scored using ‘The Observer’ (Noldus Information Technology Inc., Leesburg, VA, USA) software.

For *in vivo* experiment 2, contextually elicited conditioned gaping (a model of anticipatory nausea) was measured using a distinctive conditioning chamber made of opaque black Plexiglass (22.5 × 26 × 20 cm) with an opaque lid that sat on a table with a clear glass top. A mirror beneath the chamber at a 45° angle facilitated viewing of the ventral surface of the rat to observe orofacial responses. The conditioning chamber was in a dark room next to a 25 W light source. A video camera that was fire-wired into a computer was focused on the mirror to record each rat’s orofacial reactions during the 5 min test trial. The video tapes were later scored using ‘The Observer’ software. To assess activity, an activity chamber made of white Plexiglas (60 × 25 × 25 cm) was used, illuminated by a red light found in a different room that the contextual chamber was used to create a different context from the AN chamber. The activity of each rat was captured by video camera and sent to the Ethovision software programme (Noldus, Inc., NL) to measure distance (cm) travelled.

For the *in vivo* experiment 3, anxiolytic-like responding was evaluated using the light–dark emergence apparatus, which consisted of an opaque white plastic rectangular box

that was divided into two compartments: a small (25 cm wide × 20.5 cm long × 20.5 cm high) enclosed dark box built of opaque black plastic with a door (8 cm wide × 10 cm high) leading to a larger (39.5 cm long × 25 cm wide) open lit box. The open lit box was illuminated by one lamp (with a 60 W bulb, 180 lux in the light chamber) positioned 115 cm above the centre of the lit box. A video camera was mounted over the top of the light–dark box, and the video tapes were analysed by the Ethovision software (Noldus Information Technology, Leesburg, VA, USA) for the duration of time spent in the light box for the 5 min test. For the foot shock (FS) session, the rats were placed in sound attenuating MED Associates fear conditioning chambers (St. Albans, VT, USA). The 6 min FS session consisted of six 0.8 mA foot shocks delivered 1 min apart. Each 0.5 s shock was preceded by a 30 s auditory tone (90 Db, 5000 Hz) as described by Bluett *et al.* (2014).

In vivo procedures

In vivo experiment 1: dose-related effects of CBDA and HU-580 on acute nausea and 5-HT_{1A} receptor mediation of HU-580 effects. All rats were surgically implanted with an intraoral cannula according to the procedures described by Limebeer *et al.* (2010). On the day of surgery, the rats were injected with an antibiotic (Derapin: 100 mg·kg^{−1} s.c.; Pfizer Animal Health, Pfizer Canada Inc, Kirkland, Quebec, Canada) 30 min prior to being anaesthetized with isoflurane (4–5% induction, 1.5% maintenance in O₂). Surgical plane anaesthesia, as indicated by absence of the hind limb withdrawal reflex and defined by the Canadian Council of Animal Care, was induced before any surgery began, and was adjusted as necessary. Once sufficient anaesthesia had been induced, a 2 cm² section of skin was shaved at the back of the neck at the level of the scapula. The skin was prepared by cleaning with soap (Bactistat; Ecolab, St. Paul, MN, USA) and wiping with 70% isopropyl alcohol followed by 7% Betadine solution (Purdue Products L.P., Stamford, CT, USA). Each rat was then administered a 5 mg·kg^{−1} injection (i.p.) of the anti-inflammatory/analgesic drug carprofen (Rimadyl; Pfizer Canada Inc., Kirkland, Quebec, Canada). A thin-walled 15-gauge stainless steel needle was inserted into the shaved area on the neck, directed subcutaneously around the ear and brought out behind the first molar inside the mouth. A 10 cm length of Intra Medic PE90 tubing (Clay Adams Brand; Becton Dickinson and Co., Sparks, MD, USA) with an inner diameter of 0.86 mm and an outer diameter of 1.27 mm was then inserted through the needle after which the needle was removed. Betadine (10%) was applied to the puncture site and three elastic discs (2 cm²) were placed over the exposed end of the tubing and drawn to the skin at the back of the neck for the purpose of stabilizing the cannula. The cannula was held secure in the oral cavity by a 6 mm disc of polypropylene mesh (297 micron; Small Parts Inc., Miramar, FL, USA) secured behind the heat flanged intraoral opening. The rats were then returned to their home cage and monitored daily for 3 days. For 3 days following surgery the rats were weighed and their cannulae were flushed with an antiseptic mouth wash. During this time, the rats were also monitored for activity, vocalization, dehydration, rigidity, and presence of porphyrin staining around the eyes. On the first post-

surgical day, the rats were also given an analgesic/anti-inflammatory injection of Rimadyl (5 mg·kg⁻¹ i.p.).

Following post-surgical monitoring, the rats received an adaptation trial in which they were placed in the taste reactivity chamber with each rat's cannula attached to the infusion pump. During adaptation, water was infused into their intraoral cannulae for 2 min at a rate of 1 mL·min⁻¹. On the day following the adaptation trial, the rats received a conditioning trial in which they were administered a pretreatment injection of vehicle (VEH) ($n = 8$), CBDA (0.01, 0.1, 1 µg·kg⁻¹; $n = 8$ per group) or HU-580 (0.01, 0.1, 1 µg·kg⁻¹; $n = 8$ per group). Forty-five minutes after the pretreatment injection, the rats were individually placed in the chamber and infused, p.o., with 0.1% saccharin solution for 2 min at the rate of 1 mL·min⁻¹. Immediately after the saccharin infusion, all rats were injected with 20 mL·kg⁻¹ of 0.15 M LiCl and returned to their home cage. Seventy-two hours later, rats were tested drug-free. Rats were again infused p.o. with 0.1% saccharin solution for 2 min at the rate of 1 mL·min⁻¹ while the orofacial responses were video recorded from a mirror at a 45° angle beneath the chambers. Rats were then returned to their home cages. Two additional groups were added to determine the mechanism of action. These rats were injected with WAY100635 (0.1 mg·kg⁻¹) 15 min prior to an injection of either vehicle ($n = 8$) or 0.1 µg·kg⁻¹ HU-580 ($n = 6$). The video tapes were later scored by an observer blind to the experimental conditions using 'The Observer' for the behaviours of gaping (large openings of the mouth and jaw, with lower incisors exposed).

In vivo experiment 2: effect of CBDA and HU-580 on anticipatory nausea and 5-HT_{1A} receptor mediation of HU-580 effects. To compare the potential of HU-580 and CBDA to reduce anticipatory nausea, the contextually elicited conditioned gaping paradigm was used (e.g. Limebeer *et al.*, 2010; Rock *et al.*, 2014; see also Figure 2B). Rats underwent four conditioning trials during which the distinctive context was paired with 127 mg·kg⁻¹ LiCl. On each trial, rats were injected with LiCl and then immediately placed in the conditioning chamber for 30 min. This procedure was repeated four times with a 48 h interval between conditioning trials. For the test trial, rats were randomly assigned to one of five treatment groups ($n = 6$ per group): VEH, 0.1 µg·kg⁻¹ CBDA, 0.1 µg·kg⁻¹ HU-580, 0.01 µg·kg⁻¹ CBDA, 0.01 µg·kg⁻¹ HU-580. Pretreatments were injected 45 min before the rats were given an saline injection (20 mL·kg⁻¹ i.p.) and individually placed in the conditioning (contextual) chamber for 5 min, and orofacial responses were video recorded. To investigate the mechanism of action of HU-580, two additional groups of rats were administered 0.1 mg·kg⁻¹ WAY-VEH ($n = 8$), 0.1 mg·kg⁻¹ WAY-0.1 µg·kg⁻¹ HU-580 ($n = 8$). VEH or WAY100635 were administered 15 min before HU-308 or VEH. The video tapes from the test trial were scored by an observer blind to the experimental conditions using 'The Observer' for the behaviours of gaping (large openings of the mouth and jaw, with lower incisors exposed).

Immediately following the test trial, rats were put in the activity chamber (white Plexiglas, 60 × 25 × 25 cm, illuminated by a red light) for 15 min, and locomotor activity was captured by a video camera and sent to a computer using

EthoVision software (Noldus, Inc, NL) to measure distance (cm) travelled.

In vivo experiment 3: effect of CBDA and HU-580 on anxiety-like responding and 5-HT_{1A} receptor mediation of HU-580 effects. The effect of CBDA and HU-580 on anxiety-like responding was evaluated using the light-dark box emergence test following either foot shock stress or no foot shock (No FS) stress (Figure 2C). Bluett *et al.* (2014) have demonstrated that anxiety-like responding in this test is greatly enhanced 24 h following foot shock stress. Also, Rock *et al.* (2017) have shown that CBDA (at doses as low as 0.1 µg·kg⁻¹ i.p.) prevents the enhanced anxiety-like responding following foot shock stress, by a 5-HT_{1A}-dependent mechanism of action. Therefore, we compared the relative effectiveness of an even lower dose (0.01 µg·kg⁻¹, i.p.) of CBDA and HU-580 in this paradigm. Since we found that HU-580 was anxiolytic at this low dose, we subsequently evaluated the ability of the 5HT_{1A} receptor antagonist, WAY100635, to reverse the suppression of anxiety-like responding by HU-580.

All rats were acclimatized to the facility for 13 days prior to experimental manipulations, with weighing and handling occurring for eight of these days. After this acclimatization, the rats received a single FS stress session or No FS stress session 24 h before the light-dark emergence test (Bluett *et al.*, 2014). For the FS group, the rats were placed in sound-attenuating MED Associates fear conditioning chambers (St. Albans, VT, USA). The 6 min FS session consisted of six 0.8 mA FSs delivered 1 min apart. Each 0.5 s shock was preceded by a 30 s auditory tone (90 Db, 5000 Hz) as described by Bluett *et al.* (2014). The No FS stress group remained in their home cage during this session.

Twenty-four hours later, the rats were subjected to the light-dark emergence test. Rats in the FS group and the No FS group were pretreated with VEH, 0.01 µg·kg⁻¹ CBDA or 0.01 µg·kg⁻¹ HU-580. Forty-five minutes later, they were placed in the dark chamber of the light-dark box, and their movement was tracked for a 5 min test. To investigate the possibility that the effect of HU-580 was 5-HT_{1A} receptor-mediated, additional groups were injected with WAY100635, 15 min prior to VEH or 0.01 µg·kg⁻¹ HU-580. The number of seconds spent in the light box was measured. Groups were as follows: No FS-VEH ($n = 9$), FS-VEH ($n = 12$), No FS-0.01 µg·kg⁻¹ CBDA ($n = 8$), FS-0.01 µg·kg⁻¹ CBDA ($n = 8$), No FS-0.01 HU-580 ($n = 8$), FS-0.01 HU-580 ($n = 8$), No FS-0.1 µg·kg⁻¹ WAY-VEH ($n = 8$), FS-0.1 µg·kg⁻¹ WAY-VEH ($n = 7$), No FS-0.1 µg·kg⁻¹ WAY-0.01 µg·kg⁻¹ HU580 ($n = 8$), FS-0.1 µg·kg⁻¹ WAY-0.01 µg·kg⁻¹ HU-580 ($n = 8$).

In vitro and in vivo data analysis

Net agonist-stimulated [³⁵S]-GTPγS binding values were calculated by subtracting basal binding values (obtained in the absence of agonist) from agonist-stimulated values (obtained in the presence of agonist) (Cascio *et al.*, 2010). Values are expressed as means and variability as SEM or as 95% confidence limits. Mean EC₅₀ and mean maximal effect (E_{max}) values, and SEM or 95% confidence limits of these values, have been calculated by nonlinear regression analysis using the equation for a sigmoid concentration–response curve (GraphPad Prism). *P* values <0.05 were considered

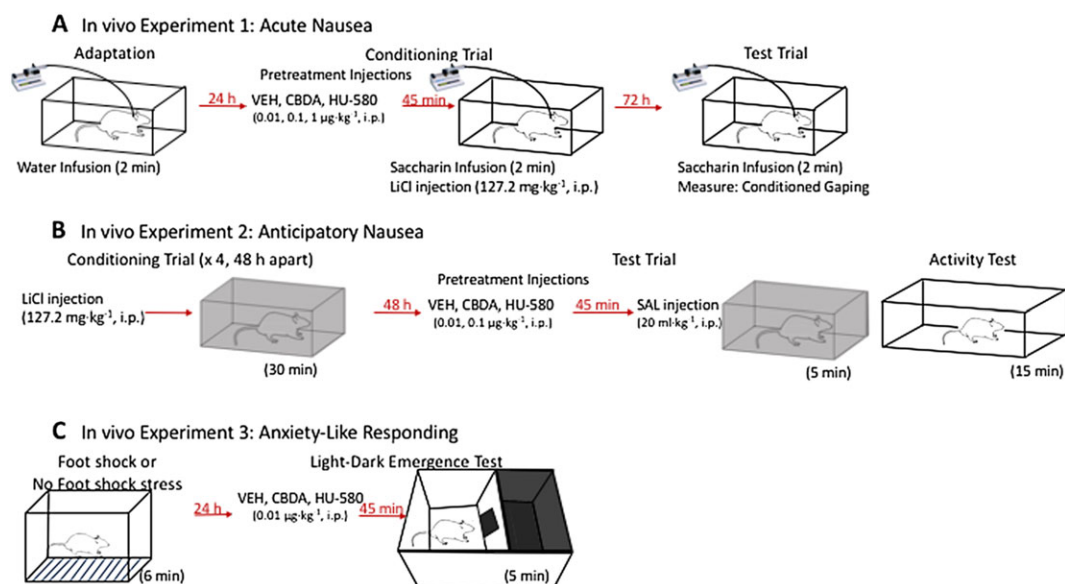


Figure 2

An illustration of the *in vivo* experimental procedures. (A) For the acute nausea model, rats undergo intra-oral cannulation surgery and after recovery receive an adaptation trial consisting of a 2 min water infusion in the taste reactivity chamber. Twenty-four hours later, they receive a single conditioning trial. Rats are pretreated with vehicle (VEH), CBDA or HU-580 and, 45 min later, are placed in the taste reactivity chamber where they are infused with 0.1% saccharin for 2 min. To investigate the mechanism of action of HU-580, additional groups of rats received WAY100635 15 min prior to HU-580 or VEH. Immediately after the saccharin infusion, rats are injected with LiCl and returned to the homecage. Seventy-two hours later, rats were subjected to a test trial during which they are returned to the taste reactivity chamber where they are infused with 0.1% saccharin for 2 min, and the number of gapes expressed are quantified. (B) For the anticipatory nausea model, rats receive four conditioning trials, 48 h apart, during which they are injected with LiCl and placed in the black conditioning chamber for 30 min, and then returned to the homecage. Forty-eight hours after the final conditioning trial, rats receive a test trial where they are pretreated with VEH, CBDA or HU-580 and, 45 min later, are injected with saline (SAL) and returned to the black conditioning chamber for 5 min while the number of gapes expressed are quantified. To investigate the mechanism of action of HU-580, additional groups of rats received WAY100635 15 min prior to HU-580 or VEH. Immediately after the anticipatory nausea test, rats are placed in a white activity chamber, and their distance travelled is tracked for 15 min. (C) For the anxiety-like responding model, rats receive either footshock (FS) or No FS (remain in home cage) stress. Those that receive footshock stress are placed in sound-attenuating MED Associates fear conditioning chambers. The 6 min FS session consists of six 0.8 mA foot shocks delivered 1 min apart. Each 0.5 s shock is preceded by a 30 s auditory tone (90 Db, 5000 Hz). Twenty-four hours later, rats are pretreated with VEH, CBDA or HU-580 45 min prior to placement in the dark chamber of the light-dark box, and time spent in the light box is tracked. To investigate the mechanism of action of HU-580, additional groups of rats received WAY100635 15 min prior to HU-580 or VEH.

significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

For analysis of data from the acute nausea experiment (*In vivo* experiment 1), a single factor ANOVA was conducted for the mean number of gapes in the 2 min test, and subsequent pairwise comparisons were assessed with least significant difference (LSD) *post hoc* tests.

For analysis of data from the anticipatory nausea (AN) experiment (*In vivo* experiment 2), a single factor ANOVA was conducted for the number of gapes in the 5 min AN test and for the total distance travelled in the activity test, and subsequent pairwise comparisons were assessed with LSD *post hoc* tests.

For analysis of data from the anxiety-like responding experiment (*In vivo* experiment 3), the amount of time spent in the light box during the light-dark emergence test was entered into a 2×5 between factors ANOVA with the factors of FS stress/No FS stress and each pretreatment and $\mu\text{g}\cdot\text{kg}^{-1}$ i.p. dose condition (VEH, 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ CBDA, 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580, WAY-VEH or WAY-HU-580). Subsequent

independent *t*-tests were conducted to explore the interaction. Significance levels were set at $P < 0.05$.

Drugs and materials used in vitro. 8-OH-DPAT and WAY100635 were supplied by Bio-Techne (Abingdon, UK). [^{35}S]-GTP γ S (1250 Ci mmol^{-1}) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA), and GTP γ S, GDP and DMSO from Sigma-Aldrich UK. CBDA and its methyl ester (HU-580) were provided by Raphael Mechoulam.

Drugs used in vivo. Lithium chloride (LiCl; Sigma Aldrich) was prepared in a 0.15 M solution with sterile water and was administered i.p. at a volume of 20 $\text{mL}\cdot\text{kg}^{-1}$ (127.2 $\text{mg}\cdot\text{kg}^{-1}$ dose). CBDA and its methyl ester (HU-580), both provided by Raphael Mechoulam, were dissolved in a glass graduated tube in 1 mL ethanol with 1 mL Tween80 (Sigma) added to the solution, and the ethanol was evaporated off with a nitrogen stream, after which 9 mL of saline was added (final Tween80:saline ratio = 1:9). CBDA or HU-580 were administered to rats i.p. at a dose of 0.01, 0.1 or 1.0 $\mu\text{g}\cdot\text{kg}^{-1}$, in a volume of 1 $\text{mL}\cdot\text{kg}^{-1}$, using a stock solution containing

one or other of these compounds at a concentration of 0.01, 0.1 or 1.0 $\mu\text{g}\cdot\text{mL}^{-1}$ respectively. WAY100635 (Sigma, St Louis, MO, USA) was dissolved in saline at a concentration of 0.1 $\text{mg}\cdot\text{mL}^{-1}$ and administered to rats i.p. at a dose of 0.1 $\text{mg}\cdot\text{kg}^{-1}$ (1 $\text{mL}\cdot\text{kg}^{-1}$).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Results

CBDA and HU-580 enhance the ability of a 5-HT_{1A} receptor agonist to stimulate [³⁵S]GTP γ S binding to human 5-HT_{1A} receptors *in vitro*

As found previously in [³⁵S]-GTP γ S binding experiments performed with rat brainstem membranes (Bolognini *et al.*, 2013), CBDA enhanced the stimulation of [³⁵S]-GTP γ S binding induced by the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT, to membranes obtained from CHO cells stably transfected with human 5-HT_{1A} receptors (Figure 3 and Table 1). Concentrations of CBDA in the sub-micromolar range, producing significant increases in the mean E_{max} of 8-OH-DPAT at 0.1, 1.0 and 10 nM, but not at 0.01 or 100 nM. None of these increases in mean E_{max} was accompanied by any significant change in the mean EC_{50} of 8-OH-DPAT ($P > 0.05$; Table 1). The methyl ester of CBDA, HU-580, was even more potent than CBDA at enhancing 8-OH-DPAT-induced stimulation of [³⁵S]-GTP γ S binding to human 5-HT_{1A} receptor-expressing CHO cell membranes (Figure 4 and Table 2). Thus, it produced a significant increase in the mean E_{max} of 8-OH-DPAT not only at 0.1, 1.0 and 10 nM (like CBDA) but also at 0.01 nM (unlike CBDA). HU-580 did not increase the mean E_{max} of 8-OH-DPAT either at 100 nM (like CBDA) or at 0.001 nM and did not significantly affect the mean EC_{50} of 8-OH-DPAT at any of the concentrations investigated (Table 2). When administered by itself, at concentrations of 0.01, 0.1, 1, 10 or 100 nM, HU-580 did not behave as a 5-HT_{1A} receptor agonist or inverse agonist as indicated by the lack of a detectable effect of any of these concentrations on [³⁵S]-GTP γ S binding to membranes obtained from human 5-HT_{1A} receptor-transfected CHO cells ($n = 6$; data not shown).

In vivo experiment 1: dose-related effects of CBDA and HU-580 on acute nausea and 5-HT_{1A} receptor mediation of HU-580 effects

At a dose of 0.1 $\mu\text{g}\cdot\text{kg}^{-1}$, but not at 0.01 or 1 $\mu\text{g}\cdot\text{kg}^{-1}$, HU-580 was more effective than CBDA in reducing acute nausea as assessed by the rat gaping model. HU-580's suppressive effect on acute nausea (0.1 $\mu\text{g}\cdot\text{kg}^{-1}$) was blocked by WAY100635. A single factor ANOVA revealed a significant group effect $F(8, 61) = 3.9$; $P < 0.05$. Figure 5 presents the

mean number of gapes displayed by the various pretreatment groups. Subsequent LSD *post hoc* comparison tests revealed that both compounds reduced LiCl-induced gaping responses relative to vehicle at a dose of 1 $\mu\text{g}\cdot\text{kg}^{-1}$ ($P < 0.05$), replicating our previous findings (Limebeer *et al.*, 2010; Rock and Parker, 2013). However, at the even lower dose of 0.1 $\mu\text{g}\cdot\text{kg}^{-1}$, that is, subthreshold for a CBDA-induced reduction of nausea-like behaviour, HU-580 reduced LiCl-induced conditioned gaping behaviour relative to vehicle ($P < 0.05$). Rats pretreated with HU-580 (0.1 $\mu\text{g}\cdot\text{kg}^{-1}$) also gaped significantly less than group WAY-0.1 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580 ($P < 0.05$), indicating a 5-HT_{1A} receptor-mediated effect.

In vivo experiment 2: effect of CBDA and HU-580 on anticipatory nausea and 5-HT_{1A} receptor mediation of HU-580 effects

At an extremely low dose of 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$, but not at 0.1 $\mu\text{g}\cdot\text{kg}^{-1}$, HU-580 was more effective than CBDA in reducing anticipatory nausea as assessed by the contextually elicited conditioned gaping model. The suppressive effect of HU-580 (0.1 $\mu\text{g}\cdot\text{kg}^{-1}$) was blocked by pretreatment with WAY100635. A single factor ANOVA revealed a significant group effect $F(6, 39) = 8.7$; $P < 0.05$. Figure 6A presents the mean number of gapes displayed. Subsequent LSD *post hoc* comparisons revealed that compared to VEH controls, at a dose of 0.1 $\mu\text{g}\cdot\text{kg}^{-1}$, both CBDA and HU-580 reduced conditioned gaping (P values < 0.05); however, the groups did differ at a dose of 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$, with group HU-580 gaping significantly less than VEH controls ($P < 0.05$) and group 0.01 CBDA ($P = 0.05$). Rats pretreated with HU-580 (0.1 $\mu\text{g}\cdot\text{kg}^{-1}$) also gaped significantly less than group WAY-0.1 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580 ($P < 0.05$), indicating a 5-HT_{1A} receptor-mediated effect.

A single factor ANOVA for the locomotor activity test (Figure 6B) revealed no significant effect on distance moved, $F(6, 39) = 0.9$, $P > 0.05$.

In vivo experiment 3: anxiolytic effects of CBDA and HU-580

Figure 7 presents the mean number of seconds spent by rats in the light box for each of the various pretreatment groups that received FS or No FS 24 h prior the light-dark test. As can be seen, FS stress greatly enhanced the anxiety-like responding of decreased time spent in the light box. At a low dose of 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$, HU-580, but not CBDA, reversed the effect of FS on the anxiety-like responding of decreased time spent in the light box. The 2×5 ANOVA for the number of seconds spent in the light box revealed a significant main effect of FS stress, $F(1, 84) = 25.6$; $P < 0.05$, and a FS stress \times pretreatment interaction, $F(4, 84) = 3.2$; $P, 0.05$. To analyse the interaction, subsequent independent *t*-tests revealed that rats pretreated with VEH ($P < 0.05$), 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ CBDA ($P < 0.05$), WAY-VEH ($P < 0.05$) or WAY-0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580 ($P = 0.05$) spent less time in the light box following FS stress than following No FS stress, but rats pretreated with 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580 did not display this anxiogenic-like response. Furthermore, subsequent single factor ANOVAs of the time spent in the light box revealed a significant pretreatment effect among the FS groups, $F(4, 38) = 4.6$; $P < 0.05$, but not among the No FS groups. Among the FS groups,

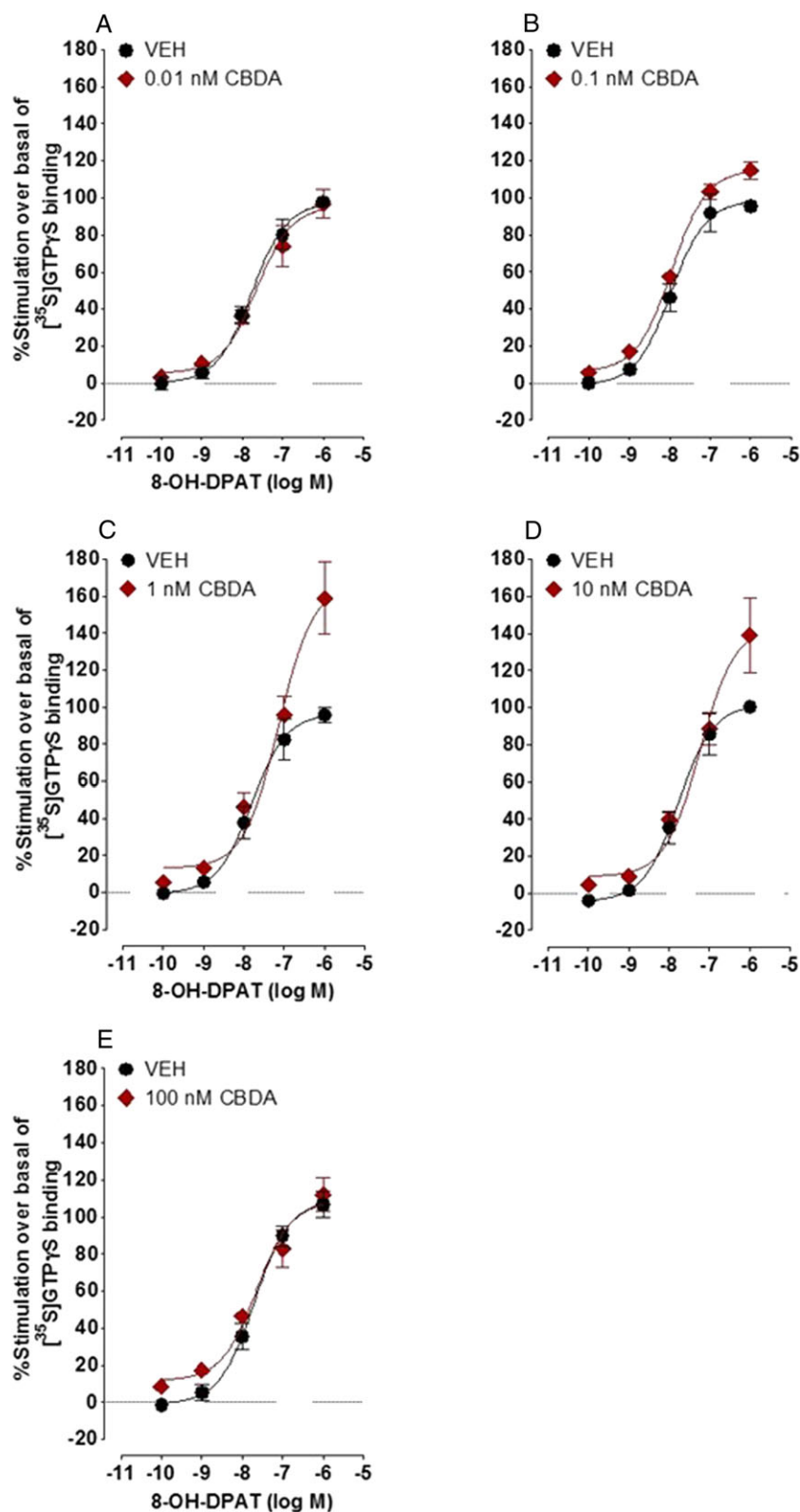


Figure 3

Effect of CBDA (0.01, 0.1, 1.0, 10 or 100 nM) on 8-OH-DPAT-induced stimulation of $[^{35}\text{S}]\text{-GTP}\gamma\text{S}$ binding to membranes obtained from CHO cells stably transfected with human 5-HT_{1A} receptors. Symbols represent mean values \pm SEM ($n=6$). Mean E_{max} and EC_{50} values for 8-OH-DPAT determined in the presence of CBDA or just of its vehicle (VEH), DMSO, together with the 95% confidence limits of these values, are listed in Table 1.

Table 1

Effects of CBDA on the mean EC₅₀ and E_{max} values of 8-OH-DPAT for its stimulation of [³⁵S]-GTPγS binding to membranes obtained from CHO cells stably transfected with human 5-HT_{1A} receptors

Pretreatment	Mean EC ₅₀ (nM)	95% confidence limits (nM)	Mean E _{max} (%)	95% confidence limits (%)	n
Vehicle	18	10 and 30	98	89 and 107	6
0.01 nM CBDA	23	10 and 50	96	83 and 108	6
Vehicle	11	6 and 20	99	88 and 109	6
0.1 nM CBDA	11	8 and 15	115*	110 and 121	6
Vehicle	16	8 and 32	97	85 and 109	6
1.0 nM CBDA	73	32 and 166	167*	138 and 196	6
Vehicle	17	9 and 33	102	90 and 114	6
10 nM CBDA	57	24 and 140	144*	118 and 169	6
Vehicle	20	12 and 35	109	98 and 119	6
100 nM CBDA	23	11 and 49	109	96 and 122	6

See also Figure 3.

*Each asterisk indicates a significant difference (**P* < 0.05) between a mean E_{max} value of 8-OH-DPAT determined in the presence of a particular concentration of CBDA and the mean E_{max} value of 8-OH-DPAT displayed in the previous row that was determined in the same experiment in the presence of vehicle (DMSO) instead of CBDA. Significant differences are indicated by non-overlapping 95% confidence limits.

subsequent Bonferroni tests revealed that only group 0.01 µg·kg⁻¹ HU-580 spent significantly more time in the light box than group VEH (*P* < 0.05).

Discussion

The results obtained in this investigation confirm our previous findings (Bolognini *et al.*, 2013) that CBDA displays significant potency both at producing an apparent enhancement of the activation of 5-HT_{1A} receptors, by the direct 5-HT_{1A} receptor agonist, 8-OH-DPAT, *in vitro*, and at producing a 5-HT_{1A} receptor-mediated reduction of both acute and anticipatory nausea in rats, *in vivo* (Bolognini *et al.*, 2013; Rock *et al.*, 2014, 2015).

The new *in vitro* data we have now obtained suggest, first, that CBDA can enhance the activation not only of rat brain stem 5-HT_{1A} receptors, as shown previously (Bolognini *et al.*, 2013), but also of human 5-HT_{1A} receptors (this paper) and, second, that at both rat brain stem (Bolognini *et al.*, 2013) and human 5-HT_{1A} receptors (this paper), CBDA induces such enhancement with a bell-shaped concentration–response curve in the sub-micromolar range.

The *in vitro* data described in this paper also reveal an important similarity between the pharmacological effects of CBDA and its methyl ester, HU-580. More specifically, these data have provided convincing evidence that HU-580 shares the ability of CBDA to produce an apparent enhancement of the activation of human 5-HT_{1A} receptors by 8-OH-DPAT in the [³⁵S]-GTPγS binding assay. Importantly, HU-580 produced such enhancement both with greater potency and with an even broader bell-shaped concentration–response curve than CBDA. Thus, significant enhancement was induced by HU-580 at concentrations of 0.01 to 10 nM (Table 2) and by CBDA at concentrations of 0.1 to 10 nM (Table 1). Whereas, at concentrations of 1, 10 and 100 nM, HU-580 produced slightly less enhancement of 8-OH-DPAT-induced 5-HT_{1A} receptor activation

than CBDA, HU-580 produced slightly greater enhancement of this activation than CBDA, at concentrations of 0.01 and 0.1 nM (Tables 1 and 2).

It is noteworthy that none of the concentrations of CBDA and HU-580 that significantly increased E_{max} values of 8-OH-DPAT for its stimulation of [³⁵S]-GTPγS binding to 5-HT_{1A} receptors produced any significant change in the EC₅₀ of 8-OH-DPAT (Tables 1 and 2). This finding suggests that CBDA and HU-580 may have been acting as positive allosteric modulators of the activation of these receptors by 8-OH-DPAT, there being evidence that some positive allosteric modulators do indeed increase the E_{max} values but not the potencies of agonists at certain receptors (Christopoulos *et al.*, 2014). The possibility that CBDA and HU-580 target an allosteric site on the 5-HT_{1A} receptor, as positive allosteric modulators, merits further investigation. It is also noteworthy that the positive *in vitro* data for CBDA and HU-580 we obtained in this investigation all came from experiments performed with CHO cells transfected with human 5-HT_{1A} receptors. Consequently, it will be of interest to establish, in a follow-up investigation, whether similar results would be obtained in experiments performed with a human-derived cell line that expresses human HT_{1A} receptors constitutively.

Moving on to our new *in vivo* data, these too reveal similarities between the pharmacological effects of HU-580 and CBDA. Thus, these data show that the ability of CBDA to reduce acute and anticipatory nausea in rats extends to HU-580. Importantly, as also found in our *in vitro* experiments, HU-580 displayed even greater potency than CBDA. More specifically, effective suppression of acute nausea-induced conditioned gaping was induced by HU-580 at a dose as low as 0.1 µg·kg⁻¹ i.p., whereas the lowest effective dose of CBDA for the production of such suppression was 1 µg·kg⁻¹ i.p. (Figure 5). Indeed, we found that at a dose as low as 0.01 µg·kg⁻¹ i.p., HU-580, but not CBDA, suppressed contextually elicited conditioned gaping. We

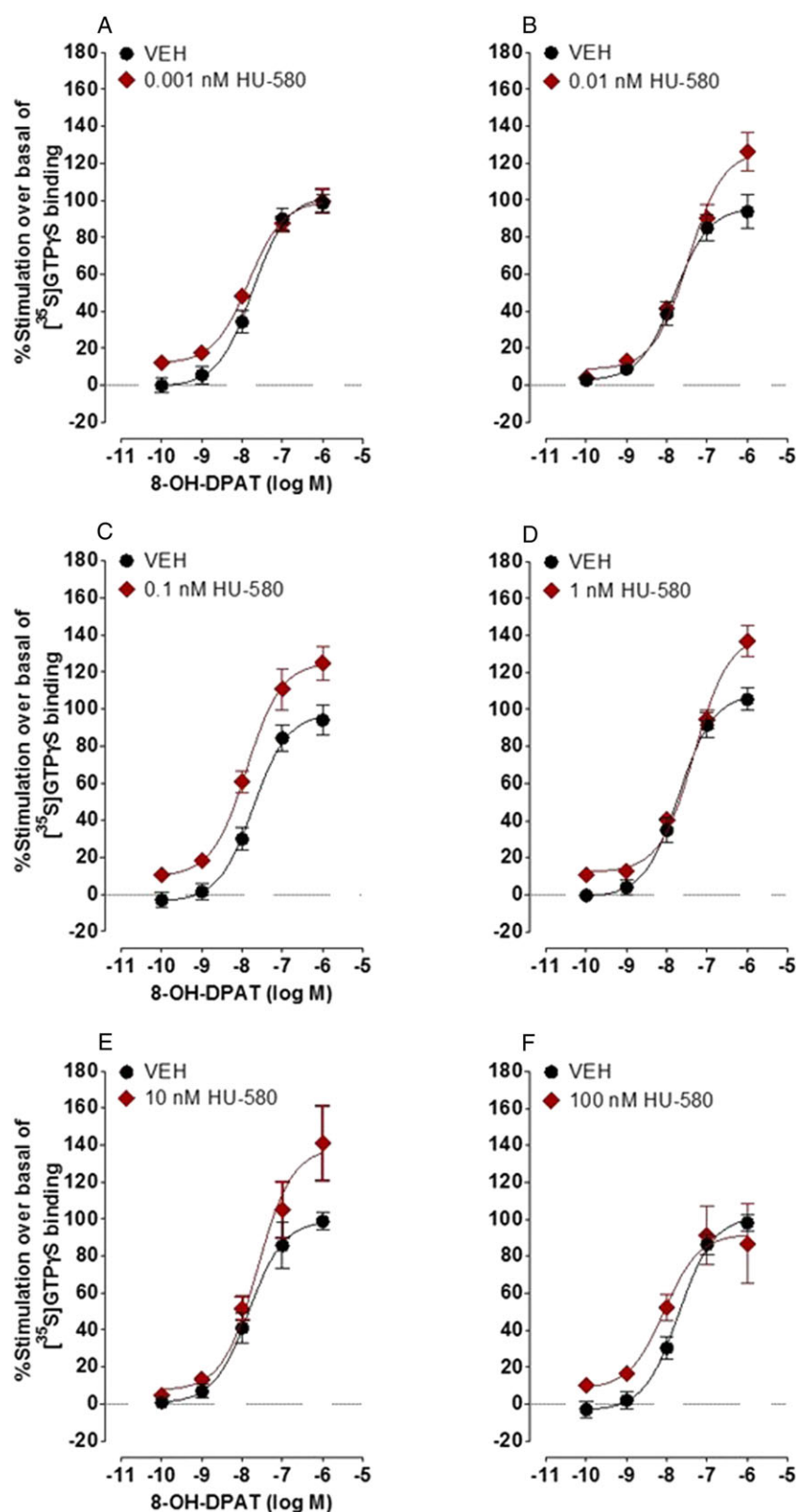


Figure 4

Effect of HU-580 (0.001, 0.01, 0.1, 1.0, 10 or 100 nM) on 8-OH-DPAT-induced stimulation of $[^{35}\text{S}]\text{-GTP}\gamma\text{S}$ binding to membranes obtained from CHO cells stably transfected with human 5-HT_{1A} receptors. Symbols represent mean values \pm SEM ($n = 6$). Mean E_{max} and EC_{50} values for 8-OH-DPAT determined in the presence of HU-580 or just of its vehicle (VEH), DMSO, together with the 95% confidence limits of these values, are listed in Table 2.

Table 2

Effects of HU-580 on the mean EC₅₀ and E_{max} values of 8-OH-DPAT for its stimulation of [³⁵S]-GTPγS binding to membranes obtained from CHO cells stably transfected with human 5-HT_{1A} receptors

Pretreatment	Mean EC ₅₀ (nM)	95% confidence limits (nM)	Mean E _{max} (%)	95% confidence limits (%)	n
Vehicle	18	11 and 30	102	93 and 112	6
0.001 nM HU-580	14	9 and 22	100	98 and 106	6
Vehicle	16	8 and 31	96	85 and 107	6
0.01 nM HU-580	34	19 and 63	127*	114 and 140	6
Vehicle	19	10 and 36	98	86 and 109	6
0.1 nM HU-580	13	7 and 24	125*	113 and 138	6
Vehicle	20	11 and 34	108	98 and 119	6
1.0 nM HU-580	48	31 and 74	141*	129 and 152	6
Vehicle	15	7 and 31	99	87 and 112	6
10 nM HU-580	24	9 and 65	139*	116 and 162	6
Vehicle	20	12 and 34	102	92 and 111	6
100 nM HU-580	9	2 and 39	92	72 and 112	6

See also Figure 4.

*Each asterisk indicates a significant difference (**P* < 0.05) between a mean E_{max} value of 8-OH-DPAT determined in the presence of a particular concentration of HU-580 and the mean E_{max} value of 8-OH-DPAT displayed in the previous row that was determined in the same experiment in the presence of vehicle (DMSO) instead of HU-580. Significant differences are indicated by non-overlapping 95% confidence limits.

have also shown that, as demonstrated previously in experiments with CBDA (Bolognini *et al.*, 2013; Rock and Parker, 2013), and indeed with cannabidiol (Rock *et al.*, 2012), suppression of LiCl-induced gaping and contextually elicited conditioned gaping by HU-580 can be completely prevented by the 5-HT_{1A} receptor-selective antagonist, WAY100635. Finally, although CBDA has recently been found to reduce FS enhancement of anxiogenic-like behaviour in the light-dark box emergence test at doses of 0.1, 1 and 100 µg·kg⁻¹ i.p. (Rock *et al.*, 2017), in the present investigation, we found that it did not share the ability of HU-580 to reduce FS enhancement of anxiogenic-like behaviour in the light-dark emergence test at the lower dose of 0.01 µg·kg⁻¹ i.p., suggesting that HU-580 may be even more potent than CBDA in reducing stress-induced anxiety. Furthermore, we also obtained convincing evidence that the ability of HU-580 to reduce FS enhancement of anxiogenic-like behaviour is 5-HT_{1A} receptor-mediated, evidence similar to that already obtained from experiments with CBDA (Rock *et al.*, 2017) and cannabidiol (Campos and Guimarães, 2008). Future studies should extend this finding to other measures of anxiogenic-like behaviour and examine other potential sites of action of CBDA and HU580 in their anti-nausea and anti-anxiety-like effects, such as antagonism of the allosteric site of the CB₁ receptor (Laprairie *et al.*, 2015). It will also be important to establish whether, as found in our *in vitro* experiments, HU-580 is effective over a broader range of doses than CBDA. Thus, such a finding, together with our discoveries that HU-580 is both more stable than CBDA, and more potent than CBDA, at least versus signs of acute and anticipatory nausea, would strengthen the hypothesis that HU-580 has markedly greater potential than CBDA as a new medicine for the management of unwanted symptoms such as nausea.

Ideally, drugs used as medicines should, when stored, display stability over a reasonable period of time. Hence, since stored CBDA undergoes significant decomposition, even at 4°C, a major aim of this project was to develop a compound that produces no less potency than CBDA in the assays described in this paper but displays much greater stability over a reasonable length of time when stored at this temperature. It is noteworthy, therefore, that we found that HU-580 is, indeed, more stable than CBDA when stored at 4°C for 21 days. In addition, our finding that HU-580 seems to be more potent than CBDA both *in vitro* and *in vivo* supports the hypothesis that the pharmacological effects produced by HU-580 in our experiments did not depend on its decomposition or metabolism to CBDA. It will be of interest to establish in a follow-up investigation, both why HU-580 displayed greater potency than CBDA in our assays and whether it is HU-580 itself that produces the effects we observed in our experiments with this compound or whether HU-580 is a promising 'pro-drug' that was converted in our *in vitro* and/or *in vivo* assays to one or more compounds that are more active than HU-580 itself.

In conclusion, this investigation has provided evidence that the methyl ester of CBDA, HU-580, displays even greater potency than CBDA at suppressing signs both of acute and anticipatory nausea, and of stress-induced anxiety in rats, and that it produces these effects in a 5-HT_{1A} receptor-dependent manner. Further experiments are still needed to determine the extent to which this apparent potency difference occurred (i) because CBDA had undergone at least some conversion to one or more less potent enhancers of 5-HT_{1A} receptor activation, such as CBD (Bolognini *et al.*, 2013), or to one or more inactive compounds, during our experiments, and/or (ii) because CBDA

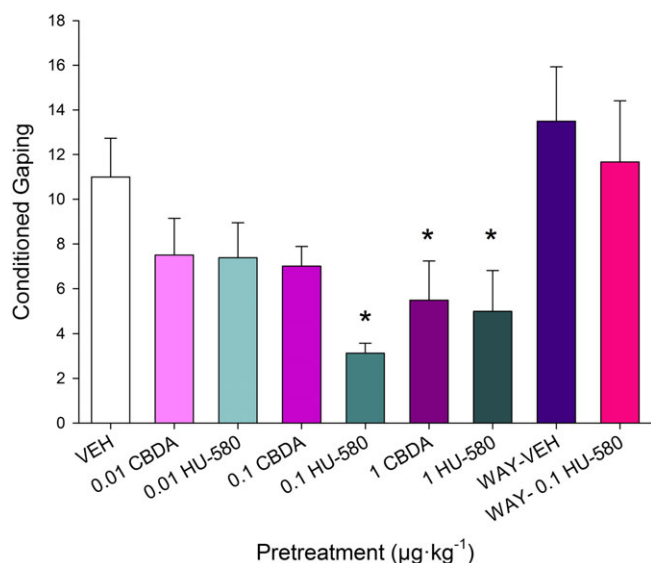


Figure 5

Mean number of conditioned gapes elicited by a LiCl-paired saccharin solution among the rats pretreated with various doses of CBDA ($n = 8$ per group) or HU-580 ($n = 8$ per group) or just with vehicle (VEH; $n = 8$). Additional groups were administered a pretreatment of WAY100635 ($0.1 \text{ mg} \cdot \text{kg}^{-1}$) 15 min prior to $0.1 \text{ mg} \cdot \text{kg}^{-1}$ HU-580 ($n = 6$) or VEH ($n = 8$). Results are presented as mean \pm SEM and $*P < 0.05$, depicts mean responses to CBDA or HU-580, which differed significantly from mean responses to VEH.

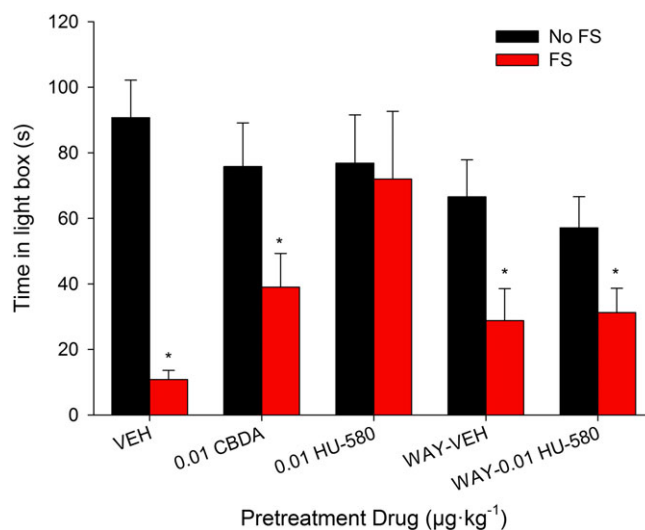


Figure 6

The mean time spent by rats in the light box, 24 h following exposure to no foot shocks (No-FS) or to FSs. All rats were injected i.p. with vehicle (VEH; $n = 9$ or 12), $0.01 \text{ µg} \cdot \text{kg}^{-1}$ CBDA ($n = 8$) or $0.01 \text{ µg} \cdot \text{kg}^{-1}$ HU-580 ($n = 8$), 45 min prior to a 5 min light-dark box emergence test. Additional groups were injected with $0.1 \text{ mg} \cdot \text{kg}^{-1}$ WAY100635 15 min prior to VEH ($n = 7$ or 8) or $0.01 \text{ µg} \cdot \text{kg}^{-1}$ HU-580 ($n = 8$). Each bar represents the mean \pm SEM. $*P < 0.05$, indicates a significant difference between FS and No FS stress groups.

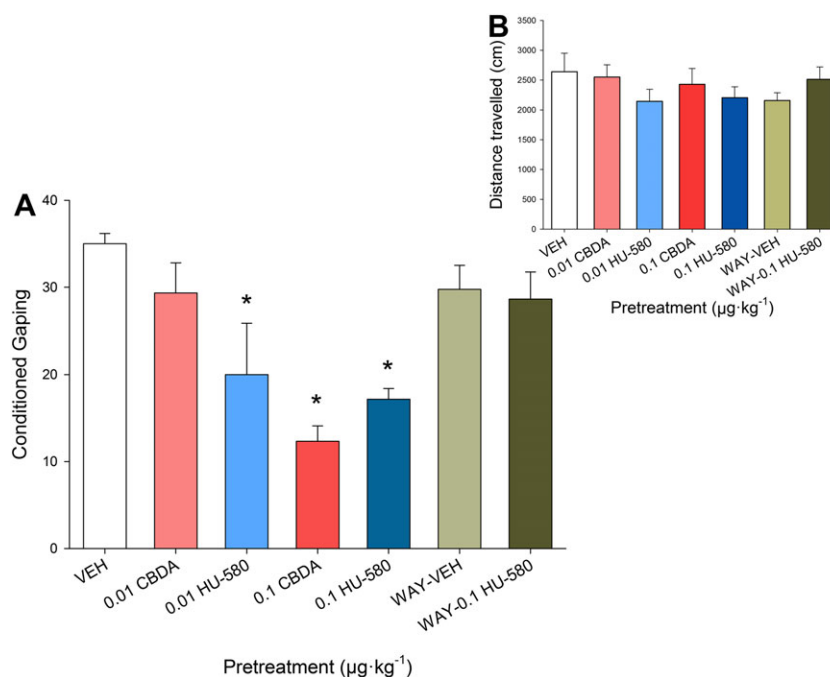


Figure 6

(A) Effect of CBDA or HU-580 ($0.01, 0.1 \text{ µg} \cdot \text{kg}^{-1}$) or vehicle (VEH) administered i.p. 45 min prior to the anticipatory nausea test ($n = 6$ per group). Additional groups were administered a pretreatment of WAY100635 ($0.1 \text{ mg} \cdot \text{kg}^{-1}$) 15 min prior to $0.1 \text{ mg} \cdot \text{kg}^{-1}$ HU-580 ($n = 8$) or VEH ($n = 8$). The mean number of conditioned gaping responses was measured during the anticipatory nausea test trial. Each bar represents the mean \pm SEM. $*P < 0.05$, significant difference from the VEH-treated control animals. (B) The mean distance (cm) travelled was measured in an activity test performed after the anticipatory nausea test. Each bar represents the mean \pm SEM.

itself really is less potent than HU-580 at enhancing 5-HT_{1A} receptor activation. In addition, since HU-580 is a relatively stable compound, our findings also prompt a need for human clinical research with this compound, especially since there is already evidence that CBD is anxiolytic in human subjects and that the ability of cannabidiol to decrease anxiety induced in healthy human volunteers by simulated public speaking is shared by the 5-HT_{1A} receptor agonist, ipsapirone (Zuardi *et al.*, 1993; Patel *et al.*, 2017). Such clinical research, performed with HU-580, and possibly subsequently with cannabidiol and CBDA as well, so that the therapeutic potential of these three cannabinoids can be compared, should be directed at establishing, firstly, whether HU-580 really does display therapeutic potential for the treatment of particular kinds of nausea and anxiety and, secondly, whether it could possibly be effective against any other disorders that might be ameliorated by enhancing the activation of 5-HT_{1A} receptors, for example, cerebral infarction, pain and depression (Bolognini *et al.*, 2013). The effectiveness of HU-580 versus anticipatory nausea will be particularly important to investigate as no specific therapy for this disorder is currently available. It will also be important both to identify the precise pharmacological action(s) through which HU-580, as well as CBDA and cannabidiol, appear to enhance agonist-induced activation of the 5-HT_{1A} receptor and to seek out any 5-HT_{1A} receptor-independent pharmacological actions of HU-580, particularly any which might affect its benefit-to-risk ratio in the clinic, for example, for its possible treatment of nausea or anxiety. Finally, in view of our findings that HU-580 seems to strengthen 5-HT_{1A} receptor activation and to produce 5-HT_{1A}-mediated amelioration of stress-induced anxiety in rats, the evidence that the 5-HT_{1A} receptor direct agonist, ipsapirone, is anxiolytic in human subjects (Zuardi *et al.*, 1993) prompts a need to investigate whether HU-580, or indeed CBDA or cannabidiol, interact synergistically with an exogenously administered 5-HT_{1A} receptor direct agonist to reduce signs of anxiety in rats or humans, more effectively, potentially and/or selectively than HU-580, CBDA, cannabidiol or a 5-HT_{1A} receptor direct agonist administered by itself.

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Author contributions

All authors contributed equally: R.G.P., L.A.P., E.M.R. and R.M. planned and designed the research; R.M., R.S. and C.H. designed and synthesized HU-580; L.A.S. performed the *in vitro* experiments; E.M.R., K.G. and C.L.L. performed the *in vivo* experiments; R.G.P., L.A.P. and E.M.R. analysed the data and the literature; R.G.P., L.A.P., E.M.R. and R.M. co-wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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