BJP British Journal of Pharmacology

DOI:10.1111/bph.13069
www.brjpharmacol.org

RESEARCH PAPER

AM841, a covalent cannabinoid ligand, powerfully slows gastrointestinal motility in normal and stressed mice in a peripherally restricted manner

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Received
17 October 2014
Revised
19 December 2014
Accepted
2 January 2015

BACKGROUND AND PURPOSE

Cannabinoid (CB) ligands have been demonstrated to have utility as novel therapeutic agents for the treatment of pain, metabolic conditions and gastrointestinal (GI) disorders. However, many of these ligands are centrally active, which limits their usefulness. Here, we examine a unique novel covalent CB receptor ligand, AM841, to assess its potential for use in physiological and pathophysiological *in vivo* studies.

EXPERIMENTAL APPROACH

The covalent nature of AM841 was determined *in vitro* using electrophysiological and receptor internalization studies on isolated cultured hippocampal neurons. Mouse models were used for behavioural analysis of analgesia, hypothermia and hypolocomotion. The motility of the small and large intestine was assessed *in vivo* under normal conditions and after acute stress. The brain penetration of AM841 was also determined.

KEY RESULTS

AM841 behaved as an irreversible CB_1 receptor agonist *in vitro*. AM841 potently reduced GI motility through an action on CB_1 receptors in the small and large intestine under physiological conditions. AM841 was even more potent under conditions of acute stress and was shown to normalize accelerated GI motility under these conditions. This compound behaved as a peripherally restricted ligand, showing very little brain penetration and no characteristic centrally mediated CB_1 receptor-mediated effects (analgesia, hypothermia or hypolocomotion).

CONCLUSIONS AND IMPLICATIONS

AM841, a novel peripherally restricted covalent CB_1 receptor ligand that was shown to be remarkably potent, represents a new class of potential therapeutic agents for the treatment of functional GI disorders.

Abbreviations

CB, cannabinoid; DSE, depolarization-induced suppression of excitation; ENS, enteric nervous system; GI, gastrointestinal; IBS, irritable bowel syndrome

Tables of Links

TARGETS
CB_1 receptor
CB_2 receptor

LIGANDS			
Δ9-tetrahydrocannabinol	AM251	CRF	Oleoylethanolamide
ACh	AM630		WIN55,212-2

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

The endogenous cannabinoid (CB) system consists of CB_1 and CB_2 receptors, endogenous CB ligands and the biosynthetic and degradative enzymes for these endocannabinoids (Pertwee *et al.*, 2010; Di Marzo and De Petrocellis, 2012; Piomelli, 2014). The endocannabinoid system is importantly involved in the regulation of gastrointestinal (GI) motility (Aviello *et al.*, 2008; Izzo and Sharkey, 2010; Abalo *et al.*, 2012; Nasser *et al.*, 2014). CB_1 receptors are present within the enteric nervous system (ENS) and CB_1 receptor activation reduces the release of neurotransmitters, notably ACh, to limit excitatory neurotransmission in the ENS (Duncan *et al.*, 2005; Izzo and Sharkey, 2010; Hons *et al.*, 2012). Activation of the CB_1 receptor slows motility throughout the GI tract and reduces intestinal and colonic contractility in animal models (Aviello *et al.*, 2008; Izzo and Sharkey, 2010; Abalo *et al.*, 2012; Nasser *et al.*, 2014).

Only a few clinical studies have investigated the effects of CBs on GI motility. In healthy volunteers, orally administered Δ^9-tetrahydrocannabinol (dronabinol) slows gastric emptying and reduces colonic motility, but has limited effects on small intestinal motility (McCallum *et al.*, 1999; Esfandyari *et al.*, 2006; 2007). In patients with irritable bowel syndrome (IBS), Wong *et al.* reported that dronabinol had only rather modest effects on fasting colonic motility in patients with diarrhoea-predominant and alternating forms of IBS (Wong *et al.*, 2011), and in a second study, reported no effects on gastric, intestinal and colonic transit in patients with diarrhoea-predominant IBS (Wong *et al.*, 2012). Oral Δ^9-tetrahydrocannabinol is rapidly metabolized and its use as a therapeutic agent is severely limited by unwanted psychotropic and neurological 'side effects' (Borgelt *et al.*, 2013; Volkow *et al.*, 2014). Therefore, the opportunity to translate the potential of CBs for the treatment of GI motility disorders such as diarrhoea-predominant IBS, which affects millions around the world, have not been fully realized. The limitations of traditional CBs are not restricted to GI disorders, hence considerable efforts have been made to develop peripherally restricted CB ligands where the beneficial actions can be attained in the absence of central side effects (Kunos *et al.*, 2009; Chorvat, 2013). Many of these compounds have been found, in animal studies, to have very favourable activity profiles for the potential treatment of a range of disorders, including disturbed GI motility (Cluny *et al.*, 2010a,b; Yu *et al.*, 2010; Tam *et al.*, 2012).

A novel covalent CB agonist, AM841, has recently been developed (Picone *et al.*, 2005; Pei *et al.*, 2008; Hurst *et al.*, 2010; Szymanski *et al.*, 2011). AM841 interacts with the CB_1 receptor binding site through a structural motif shared by all CB ligands. It subsequently irreversibly attaches to a single specific amino acid within that site, to activate the CB_1 receptor. Because of its unique chemical properties and its demonstrated effect in reducing symptoms of experimental colitis in mice (Fichna *et al.*, 2014), we examined this compound to assess its potential as a new CB receptor ligand for use in physiological and pathophysiological *in vivo* studies.

Methods

In vitro *studies*

The total number of mice used in this study was 502. Details are provided in the results and figure legends. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Culture preparation. Procedures were approved by the Animal Care Committee of the Indiana University and conformed to the Guidelines of the National Institutes of Health on the Care and Use of Animals. Mouse (CD1 strain, Harlan Laboratories, Indianapolis, IN, USA) hippocampal neurons isolated from the CA1–CA3 region of four mice were cultured on microislands as described previously (Furshpan *et al.*, 1976; Bekkers and Stevens, 1991).

Electrophysiology. All experiments were performed on isolated autaptic neurons (Straiker and Mackie, 2005; Straiker

et al., 2012). Whole-cell voltage-clamp recordings from autaptic neurons were carried out at room temperature using an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA, USA). The extracellular solution contained (in mM) 119 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.5 $MgCl_2$, 30 glucose and 20 HEPES. A continuous flow of solution through the bath chamber (1–3 mL·min^{-1}) ensured rapid drug application and clearance. Drugs were prepared as stock, then diluted into extracellular solution at their final concentration and used on the same day. Drugs dissolved in DMSO were used at a final DMSO concentration of <0.1%. Recording pipettes of 1.8–3 MΩ were filled with (in mM) 121.5 K gluconate, 17.5 KCl, 9 NaCl, 1 $MgCl_2$, 10 HEPES, 0.2 EGTA, 2 MgATP and 0.5 LiGTP. Access resistance was monitored and only cells with stable access resistance were included for data analysis. Data were acquired at a sampling rate of 5 kHz. Stimulus protocols to elicit depolarization-induced suppression of excitation (DSE) were as described previously (Straiker and Mackie, 2005).

Trafficking studies. Cell surface receptors were measured as previously described (Daigle *et al.*, 2008). For internalization, cells were incubated with the indicated agonist for the indicated times, fixed and cell surface receptors were determined. For the recycling assay, cells were treated with agonist for 30 min and then treated with antagonist for the indicated times, before fixation and determination of cell surface receptors. For the recycling assays, cells were treated with 70 μM cycloheximide after the initial internalization to prevent the synthesis of new CB_1 receptors.

In vivo *studies*

Animals for behavioural and GI studies. Five to 7 weeks-old male CD1 mice (26–40 g) as well as 9–24 weeks-old male $CB_1^{-/-}$ and $CB_2^{-/-}$ mice (23–46 g) on a CB57BL/6 background and 6–15 weeks-old male C57BL/6 wild-type mice ($CB_1^{+/+}$; 18–34 g) were used. The C57BL/6 and the CD1 mice were obtained from Charles River (Montreal, Quebec, Canada) and the $CB_1^{-/-}$, $CB_2^{-/-}$ and $CB_1^{+/+}$ were bred at the University of Calgary mouse breeding facility as previously described (Cluny *et al.*, 2009). Mice were housed at a constant temperature of 22°C and kept at a constant photoperiod (12:12 h light-dark cycle) in plastic cages on sawdust with access to standard laboratory chow and tap water *ad libitum*. The mice were allowed to acclimatize for at least 1 week. All drugs for the *in vivo* studies were dissolved in Tween 80 (1%) and DMSO (2%) and then further diluted in 0.9% saline up to the final concentration. These experiments were approved by the University of Calgary Animal Care Committee and were performed in accordance with guidelines established by the Canadian Council of Animal Care.

Spontaneous locomotor activity. Locomotor activity was measured using an infrared beam activity monitor (Columbus Instruments, Columbus, OH, USA) as previously described (Cluny *et al.*, 2010a). C57BL/6 mice were individually placed in the apparatus (at the same location) and the counts were recorded for 10 min. The apparatus was cleaned with 70% EtOH between subjects. Mice underwent a trial in the morning (between 9:00 and 12:00 h) to acclimatize them to the locomotor activity box and the experiment was conducted in the afternoon (between 13:00 and 16:00 h) of the same day. Mice were injected i.p. with either AM841 (0.1 or 1.0 mg·kg^{-1}), WIN55,212-2 (1 mg·kg^{-1}) or vehicle (2% DMSO, 1% Tween 80 in physiological saline) 20 min before being placed in the locomotor activity box.

Thermoregulation. Body temperature was measured using silicone-coated data loggers (SubCue, Calgary, Canada) that were implanted surgically into the abdomen of $CB_1^{-/-}$ and $CB_1^{+/+}$ mice under halothane anaesthesia as previously described (Duncan *et al.*, 2013). We used $CB_1^{-/-}$ in this assay to further validate the specificity of AM841 *in vivo*. Mice were allowed to recover for 1 week, injected with AM841 (0.1 mg·kg^{-1}; i.p.) and killed 24 h later. The data logger was removed and the data were retrieved and analysed using SubCue Analyzer software. Core body temperature was taken every 5 min from 100 min to 400 min post-agonist injection. The maximum decrease in body temperature from baseline (–100 to 0 min) was determined. Because the effects of CBs on core body temperature, including WIN55,212-2, have been well documented, additional 'positive controls' were not performed (Dalle Carbonare *et al.*, 2008; Wallace *et al.*, 2009; Wiley *et al.*, 2014).

Analgesia. Mice were treated with vehicle in the morning and 20 min later were assessed for pain (nociceptive) threshold using a thermal analgesia meter (Columbus Instruments) set at 55°C as previously described (Duncan *et al.*, 2013). Following a 2 h recovery, mice were either injected with vehicle, AM841 (0.1 mg·kg^{-1}) or WIN55,212-2 (1.0 mg·kg^{-1}) and reassessed for pain threshold 20 min later. The time in s for the mice to respond to the heated plate by either licking or flicking of the hind paws or jumping was defined as the pain threshold. A cut-off time of 30 s was implemented. The hot plate was cleaned with 70% EtOH between mice. The difference between the responses at both time points was determined and the differences in hot plate latency were compared by paired *t*-test.

Upper GI transit. Upper GI transit experiments were performed as described in detail previously (Cluny *et al.*, 2009). Briefly, 20 min after i.p. administration of drugs (or vehicle), 0.2 mL of 5% Evans blue suspension in 5% gum Arabic was given by gastric gavage. Fifteen minutes later, animals were killed by cervical dislocation and the total intestine was immediately removed. The distance travelled by the coloured marker was measured and expressed as percentage of the total length of the small intestine from the pylorus to the caecum. In separate experiments, the effect of antagonists [AM251 (0.5–5 mg·kg^{-1}) and AM630 (1–5 mg·kg^{-1})] were tested on the actions of AM841 (0.1 mg·kg^{-1}). Mice received antagonists 20 min before AM841. The effect of AM841 (1 mg·kg^{-1}) was additionally tested in $CB_1^{-/-}$ and $CB_2^{-/-}$ mice and their wild-type littermates.

Colonic propulsion. Distal colonic propulsion was measured using the method first reported by Raffa *et al.* (1987). Briefly, 20, 120 and 220 min after i.p. administration of drugs (or vehicle), a plastic bead (2.5 mm) was inserted 3 cm into the distal colon of each mouse using a silicone pusher. The time to expulsion of the bead was determined for each animal three times (at 100 min intervals) and then the mean of these three time points was calculated. A higher mean expulsion

time indicates a stronger delay of colonic propulsion. In separate experiments, the effect of AM251 (2 mg·kg^{-1}) and AM630 (1 mg·kg^{-1}) was tested on AM841 (0.1 mg·kg^{-1}). The effect of AM841 (1 mg·kg^{-1}) was additionally tested in $CB_1^{-/-}$ and $CB_2^{-/-}$ mice and their wild-type littermates.

Stress enhanced upper GI transit. Previous experiments from our laboratory have shown that moving group-housed mice from their home cage individually into a transparent plastic cage without a sawdust floor or bedding, termed 'novel environment', accelerated upper GI transit time (Cluny *et al.*, 2009). Mice were injected with AM841 (0.0001–10 mg·kg^{-1}, i.p.) or vehicle and placed individually into the novel environment described earlier. Other mice received an i.p. vehicle injection and were returned to their home cage, with their cage mates, and served as controls for baseline upper GI transit. Twenty minutes later, mice were gavaged with 0.1 mL of a charcoal marker (10% charcoal suspension in 5% gum Arabic) and upper GI transit was measured 15 min after the gavage as detailed earlier.

Stress-enhanced colonic transit. Mice were injected with AM841 (0.01 mg·kg^{-1}, i.p.) or vehicle and 20 min later were transferred from their home cage (group housed three to four per cage) into individual transparent plastic cages (novel environment) without bedding or a wire lid. Mice did not receive food or water during the 1 h of monitoring. Fecal pellets were removed from the cage every 15 min for 1 h. Pellet number was recorded. Some mice were trained by mock injecting them (abdominal needle poke) and then 20 min later placing them into the novel environment for 1 h each day for 3 days before the experiment. The mice that did not receive training are referred to as 'stressed' while those that were exposed to the novel environment repeatedly are referred to as 'trained'.

Brain penetration studies

Animals and dosing protocol. Procedures were approved by the Animal Care Committee of Northeastern University and conformed to the Guidelines of the National Institutes of Health on the Care and Use of Animals. Male CD1 mice (Charles River Laboratories, Willmington, MA, USA) were acclimatized to vivarium conditions for 1 week before the experiments; water and food were provided *ad libitum*. On the experimental day, the mice (25–30 g) were administered AM841 (1 mg·kg^{-1} i.p. in a dosing volume of 0.2 mL emulphor : ethanol : saline (1:1:18) with 3% DMSO).

Collection and storage of biological samples. Samples were taken at various time points: 2 min to 8 h after i.p. administration. Animals were killed by cervical dislocation followed by decapitation. Plasma and brain tissue were flash frozen in liquid nitrogen and stored at –80°C until analysis (Rahn *et al.*, 2011).

Sample extraction and LC-MS/MS analysis. The tissue sample extraction procedure was a modified version of the Folch extraction (Folch *et al.*, 1957; Rahn *et al.*, 2011). Calibration curves were constructed from the ratios of the peak areas of the analytes versus the internal standard in extracted standards made in homogenized purchased mouse brain or plasma (Bioreclmationivt.com).

An Agilent 1100 series HPLC (Agilent Technologies, Wilmington, DE, USA) was the front end for a Thermo Finnigan Quantum Ultra triple quad mass spectrometer (ThermoElectron, San Jose, CA, USA) equipped with a Phenomenex Gemini C18 column (Phenomenex, Torrance, CA, USA; 2 × 50 mm, 5 µm). The mobile phases used were 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) in a 9 min gradient. AM841 was ionized in APCI+ and detected in selected reaction monitoring mode. Non-compartmental analysis was conducted on the plasma and brain samples via the 'sparse data model' using WinNonlin software (Pharsight Corp, Mountain View, CA, USA).

CB_1 receptor mRNA expression

CB_1 receptor mRNA expression in ileal and an hypothalamic samples of non-stressed and novel environment-stressed CD1 mice was compared using real-time PCR as described previously (Bashashati *et al.*, 2012). RNA was extracted using the QIAGEN RNeasy Plus Mini Kit (Qiagen, Mississauga, ON, Canada) and a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used for generating cDNA. The rodent GAPDH probe (VIC), as the internal reference, and TaqMan Gene Expression assay kit for CB_1 receptors (Mm00432621_s1), both from Applied Biosystems, were used for real-time PCR performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems). Results were analysed by ABI Prism 7000 SDS (Applied Biosystems) and presented as relative quantification values.

Statistics. Prism 6 for Windows (version 6.03, GraphPad Software, La Jolla, CA, USA) was used for analysis of the data. *T*-tests were performed between two groups and one- or two-way ANOVA followed by appropriate *post hoc* tests were used for multiple groups. P values <0.05 were considered statistically significant. Data are presented as mean ± SEM.

Drugs

AM251, AM630 and WIN 55,212-2 were obtained from Tocris (Ellisville, MO, USA). SR141716 was obtained from NIDA drug supply (Bethesda, MD, USA). AM 841 was synthesized in the laboratory of Alexandros Makriyannis (Center for Drug Discovery, Northeastern University, Boston, MA, USA) (Picone *et al.*, 2005) and was prepared following the procedures used for synthesis of hexahydrocannabinols described previously (Busch-Petersen *et al.*, 1996) (Figure 1). All drugs were dissolved in Tween 80 (1%) and DMSO (2%) and then

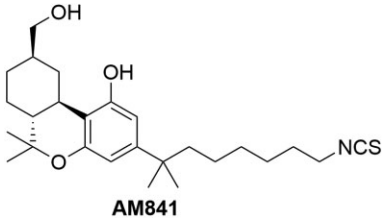

Figure 1
The structure of (-)-7′-isothiocyanato-11-hydroxy-1′,1′-dimethylheptylhexahydrocannabinol – AM841.

further diluted in 0.9% saline up to the final concentration. Evans blue and gum Arabic were obtained from Sigma-Aldrich (Oakville, ON, Canada). Throughout the manuscript, the nomenclature used for drug and molecular targets follows the guidelines of the British Journal of Pharmacology's Concise Guide to Pharmacology (Alexander *et al.*, 2013).

Results

AM841 (Figure 1) was originally described as an irreversible covalent CB ligand (Picone *et al.*, 2005). We first assessed if AM841 behaves as a covalent ligand using two-model biological assay systems *in vitro*. We then compared AM841 to the commonly used CB receptor ligand WIN55,212-2 in *in vivo* studies.

AM841 irreversibly inhibits synaptic transmission and occludes DSE

We assessed whether AM841 behaved as an irreversible CB ligand using a well-characterized *in vitro* model system that possesses endogenous CB signalling, autaptic cultures of hippocampal neurons (Sullivan, 1999; Straiker and Mackie, 2005). In autaptic cultures of hippocampal neurons, treatment with AM841 (100 nM) substantially and potently inhibited EPSCs (Figure 2A and B), with an EC_{50} of 6.8 nM. This inhibition was not reversed by treatment with the CB_1 receptor antagonist SR141716 [Figure 2A–C; relative EPSC charge (1.0 = baseline): AM841 (100 nM) 0.56 ± 0.09, $n = 5$; SR141716 (200 nM) 0.60 ± 0.14, $P > 0.05$, paired *t*-test]. In contrast, SR141716 treatment readily reversed inhibition by the classical CB agonist WIN55,212-2 (Figure 2D–F, control WIN inhibition 0.56 ± 0.06; SR141716 (100–200 nM): 1.05 ± 0.03, $P < 0.005$ paired *t*-test). As predicted by its irreversible inhibition of EPSCs, in same-cell experiments, AM841 occluded endogenous CB signalling (Figure 2C; control DSE inhibition: 0.42 ± 0.04; DSE after AM841: 0.90 ± 0.01; $P < 0.01$, paired *t*-test).

Incubation of CB_1 receptor-expressing HEK cells with increasing concentrations of AM841 for increasing times demonstrated a dose-dependent loss of cell surface CB_1 receptor immunoreactivity (Figure 2G). In the presence of AM841 (100 nM) or WIN55,212-2 (100 nM), CB_1 receptors were rapidly internalized (Figure 2H). However, while CB_1 receptors in the WIN55,212-2-treated cells rapidly recycled to the cell surface after treatment with SR141716 (100 nM), no recycling was evident in the AM841-treated cells, even after 120 min, by which time most of the CB_1 receptors in WIN55,212-2-treated cells had returned to the cell surface (Figure 2H). These results suggest that AM841 remains bound to CB_1 receptors even in the presence of SR141716, preventing recycling of the receptors to the cell surface.

AM841 behaves as a peripherally restricted CB_1 receptor agonist in vivo

When administered to mice, AM841 did not produce the obvious behavioural effect typically associated with CB_1 receptor agonists, reduced spontaneous locomotor activity. We therefore systematically assessed three parameters of the CB tetrad test in detail (Little *et al.*, 1988): (i) spontaneous locomotor activity (hypomotility), (ii) analgesia and (iii) hypothermia. We first assessed spontaneous locomotor activity in mice treated with AM841 (0.1 or 1 mg·kg^{-1}) and WIN55,212-2 (1 mg·kg^{-1}). These doses were chosen based on preliminary studies. AM841 produced no significant reduction in spontaneous locomotor activity, whereas WIN55,212-2 significantly reduced locomotor activity (Figure 3A). We next assessed the latency on a hot plate to paw withdrawal after injection of the test compound, compared with the time taken to withdrawal after vehicle injection. Analgesia, observed as an increase in latency, was clearly observed after treatment with WIN55,212-2 (1 mg·kg^{-1}), but not with AM841 (0.1 mg·kg^{-1}) (Figure 3B). Finally, we assessed the thermoregulatory effects of AM841 in $CB_1^{+/+}$ and $CB_1^{-/-}$ mice over a 6–7 h time frame (Figure 3C). AM841 caused a transient stress-induced hyperthermia, but had no lasting effect on core body temperature in either group of animals. Taken together, these data suggest that AM841 is not centrally active when acutely administered *in vivo*.

The effects of i.p. AM841 are restricted to the periphery

These results led us to assess the brain penetration of AM841 after an i.p. injection. Animals were administered AM841 (1 mg·kg^{-1}, i.p.) and the brain and plasma levels were measured over 8 h. The AM841 standard curve was linear with a regression value of >0.996 and the extraction efficiencies were greater than 85% (plasma) and 83% (brain homogenate). The plasma area under the curve for AM841 was 3.75 ± 0.50 min·μg·mL^{-1} and the brain 0.20 ± 0.02 min·μg·mL^{-1}, giving a ratio of 0.05. By comparison, brain-penetrant CBs have ratios of 1 or more (Dyson *et al.*, 2005; Rahn *et al.*, 2011; Adam *et al.*, 2012).

AM841 is a potent and efficacious CB_1 receptor agonist in the GI tract in vivo

We next examined the effects of AM841 compared with WIN55,212-2 at inhibiting upper GI transit in the mouse. In vehicle-treated animals, the marker used to determine intestinal transit travelled 84.2 ± 2.4% of the intestinal length in 15 min. AM841 potently inhibited transit to a maximum of about 70% of the level seen in vehicle-treated animals (Figure 4A). The EC_{50} of this response was 0.004 mg·kg^{-1}. WIN55,212-2 was far less potent than AM841 (Figure 4A). The effects of AM841 (1 mg·kg^{-1}) were abolished in $CB_1^{-/-}$ mice and virtually identical in $CB_2^{-/-}$ and wild-type mice (Figure 4B). They were also abolished by pretreatment with AM251 (5 mg·kg^{-1}) but were unaffected by AM630 (5 mg·kg^{-1}) (Figure 4C).

We then examined the magnitude and duration of the effects of AM841 and WIN55,212-2 at inhibiting colonic transit. AM841 potently inhibited colonic bead expulsion with an EC_{50} of 0.03 mg·kg^{-1} (Figure 5A). The maximum magnitude of the effects – a five- to sixfold slowing of colonic transit (0.1 mg·kg^{-1}) – was comparable with that observed with WIN55,212-2 (3 mg·kg^{-1}), 20 min after administration (Figure 5B). AM841 was more potent than WIN55,212-2 (Figure 5A and B). The effects of AM841 were completely abolished in $CB_1^{-/-}$ mice and virtually identical in $CB_2^{-/-}$ mice

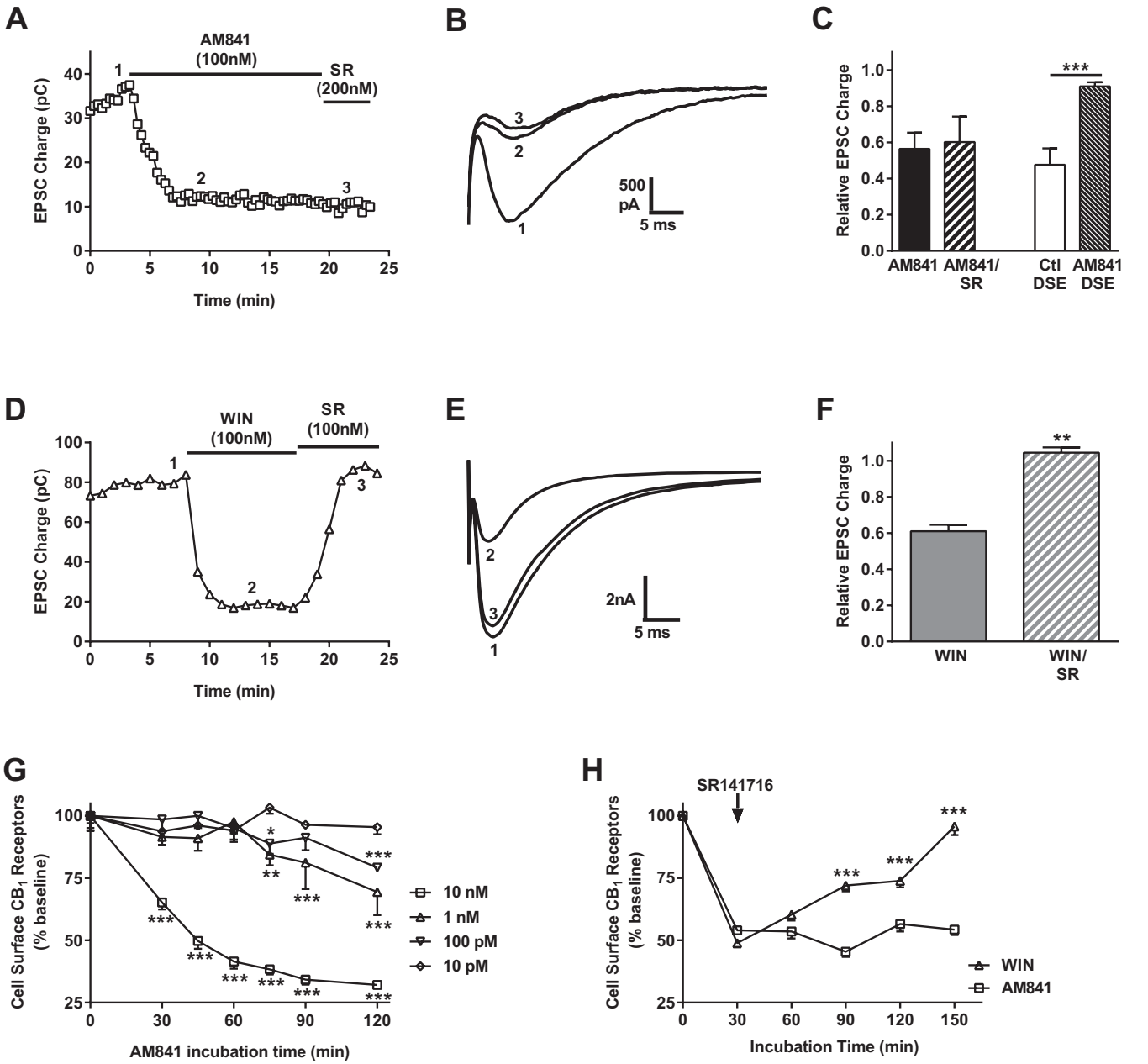

Figure 2

AM841 irreversibly inhibits EPSCs and internalizes CB_1 receptors. (A) AM841 (100 nM) robustly inhibited EPSCs in autaptic hippocampal neurons. Time course shows the integral of the EPSCs (charge, in pC) in response to AM841. The inhibition was not reversed by the CB_1 antagonist SR141716 (200 nM). (B) EPSCs at time points 1, 2 and 3 from (A). (C) Summary bar graph showing average EPSC inhibition for AM841 alone and with SR141716 ($P > 0.05$ paired *t*-test) as well as inhibition resulting from depolarization-induced suppression of excitation (3 s depolarization) before and after AM841 treatment. $n = 5$ per group. ***$P < 0.001$ – paired *t*-test. (D) SR141716 (100 nM) readily reversed inhibition by the CB_1 agonist WIN55212-2 (100 nM). (E) EPSCs at time points 1, 2 and 3 from (D). (F) Summary bar graph for data from (D) showing average EPSC inhibition for WIN55,212-2 alone and with SR141716. $n = 4$ per group. **$P < 0.01$ – paired *t*-test. (G) Surface CB_1 receptors were detected after incubation with the indicated concentration of AM841 for the indicated times. $n = 3$ per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with baseline levels of CB_1 receptors before incubation with AM841 – two-way ANOVA with Dunnett's test for multiple comparison. (H) Cell surface CB_1 receptors were measured as in (G). Thirty minutes treatment with 100 nM WIN55,212-2 (WIN-2) or 100 nM AM841 internalized ~50% of surface CB_1 receptors. SR141716 (100 nM) caused rapid recycling of CB_1 receptors to the cell surface in WIN55,212-2-treated cells but had no effect in AM841-treated cells. $n = 12$ per group. ***$P < 0.001$ compared with WIN55,212-2 – two-way ANOVA with Sidak's test for multiple comparison.

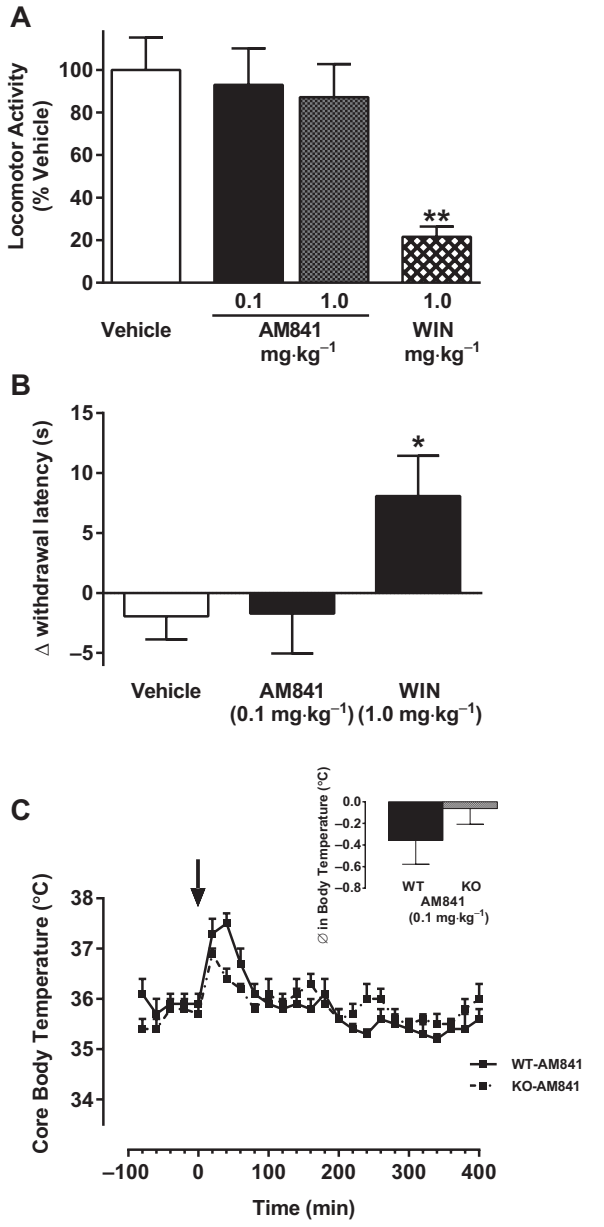

Figure 3

The effects of AM841 on locomotion (A), analgesia (B) and thermoregulation (C). (A) AM841 (0.1 and 1 mg·kg^{-1}) produced no significant reduction in locomotion at the doses tested. In contrast, WIN55,212-2 (1 mg·kg^{-1}) significantly reduced locomotor activity compared with the vehicle-treated mice. n = 5–7 per group. **P < 0.01 – one-way ANOVA with Dunnett's test for multiple comparison. (B) AM841 (0.1 mg·kg^{-1}) had no effect on paw withdrawal latency compared with vehicle controls whereas WIN55,212-2 (1 mg·kg^{-1}) significantly increased the time to withdrawal, indicative of analgesia. n = 6–13 per group. *P < 0.05 – one-way ANOVA with Dunnett's multiple comparison. (C) The thermoregulatory effects of AM841 (0.1 mg·kg^{-1}) were tested in wild-type C57Bl/6 mice and $CB_1^{-/-}$ mice. There was a rapid increase in body temperature on initial injection because of the acute stress of injection; this was seen in both groups of mice. AM841 had virtually no effect on core body temperature over ~7 h. Inset in (C) is the maximum change in body temperature from baseline (100 min before injection) over the time monitored post-injection (400 min). n = 7–8 per group, P > 0.05 t-test.

compared with wild-type controls (Figure 5C). They were abolished by AM251 (2 mg·kg^{-1}) but unaffected by AM630 (1 mg·kg^{-1}) (Figure 5D).

AM841 is more potent in vivo *in acutely stressed mice and reduces stress-enhanced intestinal and colonic motility*

Finally, because of the properties of AM841, we examined whether we could use this compound in an assay of dysmotility: to correct acute stress-induced enhancement of intestinal and colonic transit.

Upper GI transit was significantly increased in the stressed, compared with the non-stressed mice (non-stressed: 49.5 ± 2.6%; stressed: 56.2 ± 2.6% of intestinal length; P < 0.05, n = 12). AM841 (0.0001–10.0 mg·kg^{-1}) dose-dependently slowed upper GI transit in stressed and non-stressed animals (Figure 6A). Interestingly, the EC_{50} was 0.001 mg·kg^{-1} in the stressed animals, which is slightly lower than that observed in non-stressed animals. A dose of AM841 that slightly slowed upper GI transit in normal mice (0.001 mg·kg^{-1}) significantly slowed transit in the stressed animals (Figure 6B). The effects of AM841 on upper GI transit were abolished by AM251 (5 mg·kg^{-1}) but not by AM630 (5 mg·kg^{-1}) (Figure 6B). Stressed rodents showed an enhanced output of faecal pellets (Martinez *et al.*, 1997; Million *et al.*, 2007). The cumulative faecal pellet output at 15, 30, 45 and 60 min was 1.1 ± 0.4, 1.8 ± 0.6, 2.8 ± 0.9 and 3.1 ± 0.9, respectively, in trained mice versus 3.0 ± 0.5, 3.7 ± 0.7, 4.8 ± 0.9 and 6.0 ± 0.9, respectively, in stressed mice (n = 10–11), with the peak response occurring in the first 15 min of the observation period. AM841 (0.01 mg·kg^{-1}) completely normalized the enhanced faecal pellet output observed in the acutely stressed mice (Figure 6C).

Acute stress does not change CB_1 mRNA expression in the GI tract

Because AM841 appeared to have a greater potency in stressed mice, we examined CB_1 mRNA levels measured by real-time PCR. CB_1 mRNA levels were not significantly different in the ileum (or hypothalamus) of novel environment-stressed mice compared with non-stressed animals (data not shown).

Discussion

Here, we describe the actions of a novel covalent CB agonist AM841 (Picone *et al.*, 2005). *In vitro*, AM841 behaved as an irreversible CB_1 receptor ligand, as expected from its chemical properties. AM841 potently reduced GI motility through an action on CB_1 receptors in the small and large intestine under physiological conditions. It appears that AM841 is even more potent under conditions of acute stress and was shown to normalize accelerated GI motility under these conditions. This compound behaved as a peripherally-restricted ligand, showing very little brain penetration and no characteristic CB_1 receptor-mediated effects in the CNS. These findings extend the observations demonstrating that AM841 is effective in suppressing experimentally-induced colitis in mice (Fichna *et al.*, 2014).

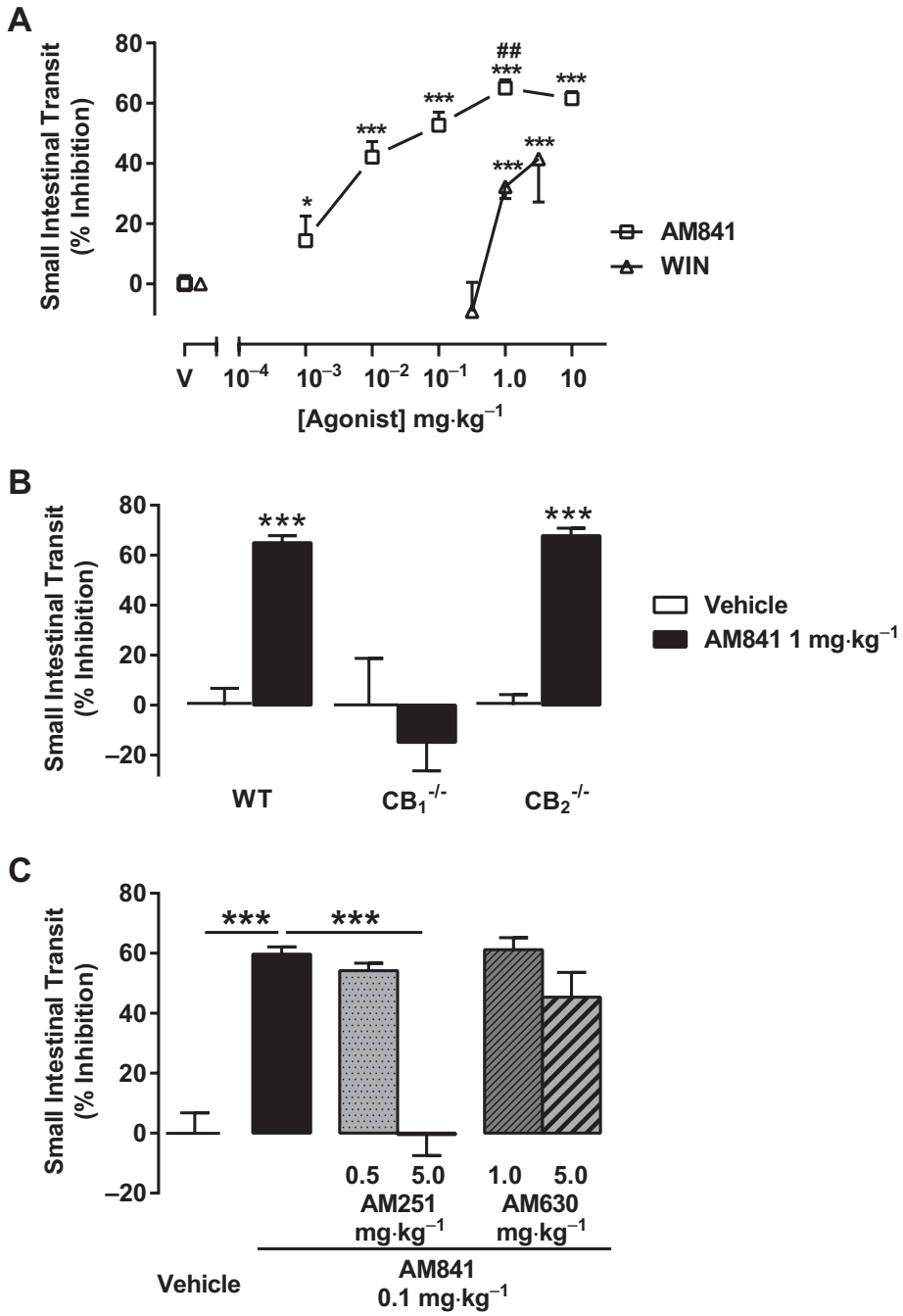

Figure 4

The effects of AM841 on small intestinal transit. (A) AM841 and WIN55,212-2 dose-dependently reduced small intestinal transit. Note that AM841 significantly slowed transit at doses as low as 0.001 mg·kg^{-1}. n = 4–21 per group. *P < 0.05,***P < 0.001 – one-way ANOVA with Sidak's multiple comparison, compared with vehicle-treated mice. At equimolar doses (1 mg·kg^{-1}), AM841 was more potent than WIN55,212-2 in slowing small intestinal transit. ##P < 0.01 – one-way ANOVA with Sidak's multiple comparison. (B) The effects of AM841 (1 mg·kg^{-1}) on small intestinal transit in wild-type (WT), $CB_1^{-/-}$ mice and $CB_2^{-/-}$ mice. Note that AM841 had no effect in $CB_1^{-/-}$ mice, whereas motility was reduced in both WT and $CB_2^{-/-}$ mice to the same extent. n = 6–12 per group. ***P < 0.001 – t-tests for each genotype of mice tested. (C) The effects of CB_1 (AM251) and CB_2 (AM630) receptor antagonists on the actions of AM841. Pretreatment with AM251 completely blocked the effects of 0.1 mg·kg^{-1} AM841 at 5 mg·kg^{-1}. n = 3–8 per group. ***P < 0.001 between treatment groups indicated. AM251 5 mg·kg^{-1}, but not at a 10-fold lower dose, reversed the effect of AM841 alone. AM630 had no significant effect on the action of AM841, one-way ANOVA with Sidak's multiple comparison.

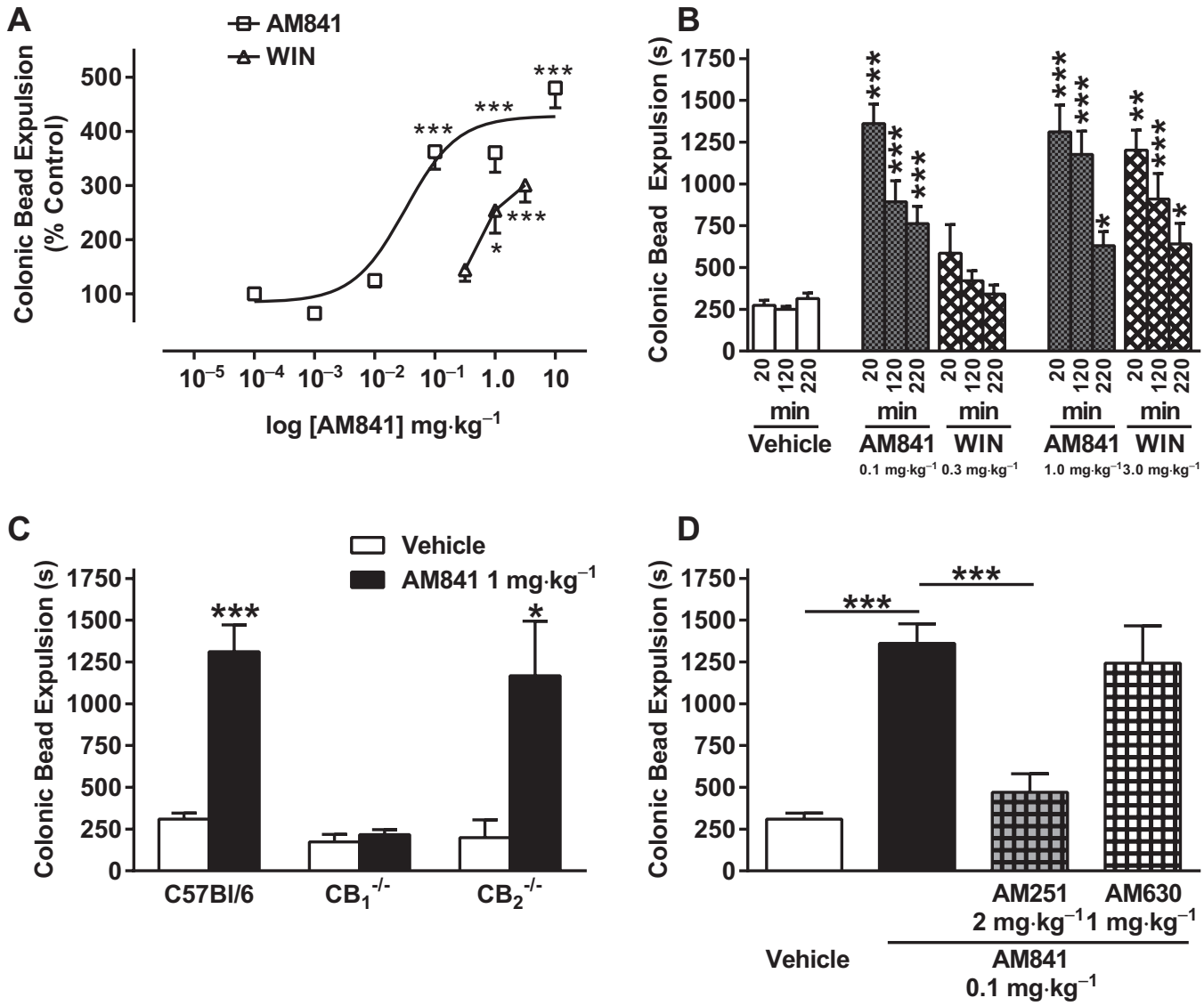

Figure 5

The effects of AM841 on colonic bead expulsion. (A) AM841 and WIN55,212-2 dose-dependently reduced colonic transit. Note that AM841 significantly slowed transit at doses as low as 0.1 mg·kg^{-1}. n = 6–23 per group. *P < 0.05, ***P < 0.001 compared with vehicle – one-way ANOVA with Sidak's multiple comparison. (B) The time-dependent effects of AM841 on colonic transit were compared with those of WIN55,212-2. Note that at the lower doses (0.1 mg·kg^{-1} AM841, 0.3 mg·kg^{-1} WIN55,212-2) WIN55,212-2 had no significant effect on colonic transit, whereas AM841 significantly reduced transit over nearly 4 h. n = 6–26 per group. *P < 0.05, **P < 0.01, ***P < 0.001 compared with vehicle at the same time point – one-way ANOVA with Dunnett's multiple comparison. (C) The effects of AM841 (1 mg·kg^{-1}) on colonic transit in wild-type (WT), $CB_1^{-/-}$ mice and $CB_2^{-/-}$ mice. Note that 1 mg·kg^{-1} AM841 had no effect in $CB_1^{-/-}$ mice, whereas motility was reduced in both WT and $CB_2^{-/-}$ mice to a similar extent. n = 5–11 per group. *P < 0.05, ***P < 0.001 compared with vehicle – t-test for each genotype of mice tested. (D) The effects of CB_1 (AM251) and CB_2 (AM630) receptor antagonists on the actions of AM841. Pretreatment with AM251 completely blocked the effects of 0.1 mg·kg^{-1} AM841 at 2 mg·kg^{-1}. n = 6–21 per group. ***P < 0.001 between groups indicated – one-way ANOVA with Sidak's multiple comparison. AM630 had no significant effect on the action of AM841.

AM841 is a classical CB analogue with an isothiocyanate group at the last carbon of its dimethylheptyl chain (Picone *et al.*, 2005). AM841 binds to the helix 6 of the CB_1 and CB_2 receptor, through different binding motifs (Picone *et al.*, 2005; Pei *et al.*, 2008; Szymanski *et al.*, 2011). For both receptors, the functional potencies of this ligand exceeded, by 20- to 50-fold, that of structurally related non-covalent analogues, and so AM841 was designated as a 'megagonist' (Szymanski *et al.*, 2011; Makriyannis, 2014).

Building on our understanding of the chemical properties of AM841, we first undertook some *in vitro* electrophysiological and receptor trafficking studies to confirm that it behaved as expected in these biological assay systems. Here, we found that AM841 behaved as a very potent (low nM) agonist which

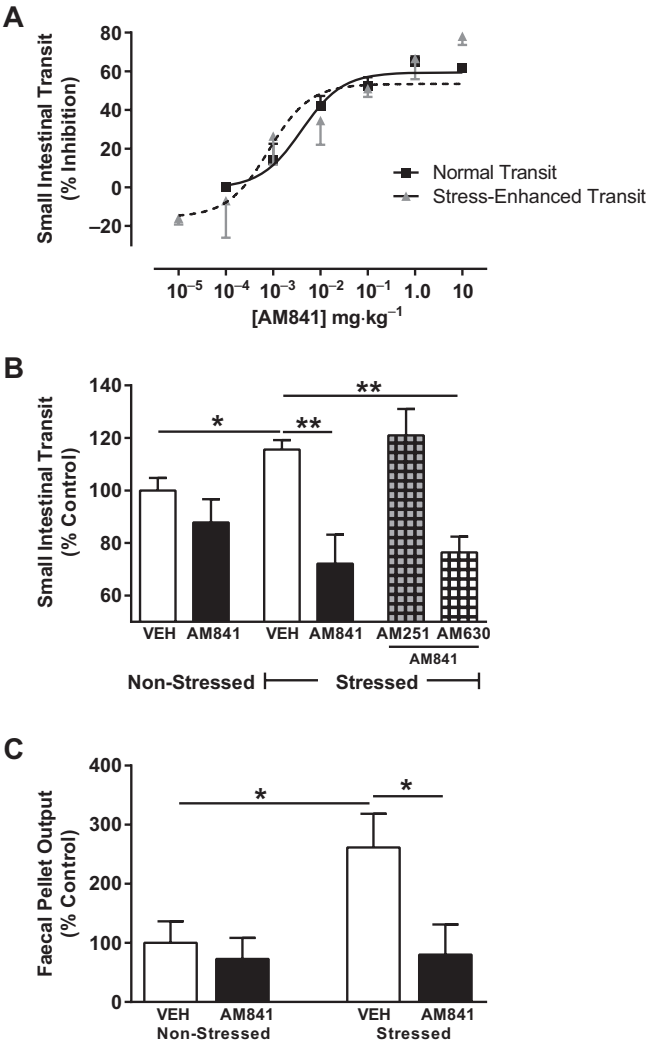

Figure 6

The effects of AM841 on small intestinal transit and colonic bead expulsion in acutely stressed mice. (A) AM841 dose-dependently reduced small intestinal transit in normal and stressed mice. There was a slight leftward shift of the dose-response curve in the stressed mice. n = 3–21 per group. (B) The effects of AM841 (0.001 mg·kg^{-1}) on small intestinal transit in non-stressed and stressed mice in the presence and absence of AM251 (5 mg·kg^{-1}) and AM630 (5 mg·kg^{-1}). Vehicle-treated stressed mice had a significantly increased small intestinal transit when compared with vehicle-treated non-stressed mice. n = 3–24 per group. *P < 0.05 – t-test. Note that at this dose, AM841 had no significant effect in non-stressed mice, but significantly reduced transit in stressed animals. **P < 0.01 compared with vehicle-treated stressed mice – two-way ANOVA with Sidak's multiple comparison. The effects of AM841 were blocked by AM251 but unaffected by AM630. **P < 0.01 compared to vehicle-treated stressed mice – one-way ANOVA with Sidak's multiple comparison. (C) The effects of AM841 on stress-enhanced faecal pellet output. Faecal pellet output was increased ~3-fold by placing the animals in a novel environment. Pretreatment with AM841 (0.01 mg·kg^{-1}) normalized the stress-enhanced faecal pellet output. n = 10–11 per group. *P < 0.05 between groups as indicated – two-way ANOVA with Sidak's multiple comparison.

was not displaced from the receptor after binding, using the inverse agonist SR141716, in contrast to the classic ligand WIN 55,212-2, which was readily displaced. Activation of CB_1 receptors by agonists often leads to CB_1 receptor internalization (Hsieh *et al.*, 1999). Incubation of CB_1 receptor-expressing HEK cells with AM841 caused a dose-dependent loss of cell surface CB_1 receptor immunoreactivity. If agonist application is brief and followed by antagonist, internalized CB_1 receptors can be recycled back to the cell surface (Hsieh *et al.*, 1999). Presumably, this occurs because after the antagonist displaces the agonist, the receptor is dephosphorylated and recycled back to the cell surface (Moore *et al.*, 2007). The absence of receptor recycling following antagonist treatment suggests that AM841 remained covalently bound to the CB_1 receptor, even after internalization.

When animals were injected with AM841, it was immediately apparent that their behaviour was similar to that of vehicle controls and no adverse events were observed following treatment. This finding led us to investigate the brain penetration of AM841 and to conduct behavioural tests to assess if this ligand displays any classical CB actions, namely, hypothermia, analgesia and/or hypomotility. In no case did we observe that AM841 exhibited the classical CNS actions of a CB_1 receptor agonist and this we attribute to its demonstrated inability to access the brain in significant quantities. One possible explanation for this observation is that the compound is a substrate for one or both of the multidrug resistance proteins (MRP1 and MRP2) as has been reported for the naturally occurring phenethyl isothiocyanate (Ji and Morris, 2005; Morris and Dave, 2014). Similar mechanisms have also recently been proposed for the exclusion of other peripherally restricted CB ligands (Pryce *et al.*, 2014). However, these authors noted that the strains of mice we used have polymorphic CB drug pumps that lack functionality (Pryce *et al.*, 2014). If this is the case, then other pumps or different exclusion mechanisms might exist for AM841. This has to be determined in future studies.

Having the properties of a peripherally restricted ligand makes AM841 a very interesting molecule for studies of the GI tract. The endocannabinoid system regulates GI motility, but investigations of the actions of CBs *in vivo* are frequently confounded by central effects of these drugs. Here, we have isolated the actions to the periphery and demonstrated that AM841 is an efficacious and highly potent agonist of CB_1 receptors. Indeed, these results demonstrate the remarkable ability of AM841 to slow down transit with an EC_{50} in the small intestine of around 4 µg·kg^{-1}. In the colon, the apparent potency was about 10-fold lower. In contrast, the potency of WIN55,212-2 did not seem to change much between these gut regions, although in both cases it was far less potent. It is not completely clear why this should be the case. In the colon, we were able to use a lower dose of AM251 to block the effects of AM841 than were required to block small intestinal transit (2 mg·kg^{-1} vs. 5 mg·kg^{-1}). These data suggest that in the colon, there is a lower effective receptor density on the enteric nerves than in the ileum. However, that has yet to be determined experimentally. Alternatively, the CB_1 receptor in the colon might be constitutively desensitized or in a state of inactivation to a greater extent than the ileum because of a higher endocannabinoid tone in that region of the gut. Tonically-released endocannabinoids are present throughout

the GI tract and CB 'tone' has been reported in both the small and large intestine (Pertwee, 2001; Izzo and Sharkey, 2010; Storr *et al.*, 2010), but the relative endocannabinoid tone between these regions of gut has never been compared. As mentioned earlier, CB_1 receptors are found on cholinergic nerves of the ENS and reduce the release of ACh (Duncan *et al.*, 2005; Izzo and Sharkey, 2010). The proportion of cholinergic and non-cholinergic nerves in the ileum and colon differs along the mouse GI tract, with a much higher proportion being non-cholinergic in the colon (De Man *et al.*, 2002; Mule *et al.*, 2007; Baldassano *et al.*, 2009; Bashashati *et al.*, 2012). Whether this also somehow influences the results of these *in vivo* transit studies remains to be determined. In the present study, we still observed an effect of AM841 on colonic propulsion 3 h after injection in control mice. Future investigations should examine the duration of action of covalent ligands, such as AM841, under both physiological and pathophysiological conditions and the potential consequences of irreversible agonism.

AM841 has been used previously as a high potency ligand to investigate the role of CB receptors in colitis (Fichna *et al.*, 2014). Here, it was given by the same route, i.p., and was found to block the development of colitis, albeit with a slightly lower potency than was observed for colonic transit in the current study. Interestingly, the effects of AM841 were found to be mediated by both CB_1 and CB_2 receptors in this pathophysiological condition (Fichna *et al.*, 2014), whereas for GI motility under the conditions of our studies, AM841 acted only on CB_1 receptors. In this study, AM841 was compared with another peripherally restricted compound CB13 (Dziadulewicz *et al.*, 2007), which was not able to block colitis following i.p. administration. CB13 [aka SAB378 (Dziadulewicz *et al.*, 2007; Cluny *et al.*, 2010a)] was shown by us to reduce motility via CB_1 receptors to a similar extent as seen for AM841 in the current study (Cluny *et al.*, 2010a). This suggests that the covalent nature or the potency of AM841 seems to confer some unique properties on AM841, as it is clearly peripherally-restricted and yet blocks colitis and inhibits motility.

Acute stress leads to an acceleration of small intestinal and colonic transit (Taché and Perdue, 2004). Under these conditions, we observed that AM841 was effective at slowing the accelerated transit and remarkably, a dose of AM841 without significant effect in normal animals now significantly slowed small intestinal transit and normalized colonic transit. These findings occurred without an alteration in receptor mRNA expression in the GI tract. Previous studies from our group showed in a similar paradigm that the endogenous lipid signalling molecule oleoylethanolamide was also able to reverse accelerated small intestinal motility with a higher potency than under physiological conditions (Cluny *et al.*, 2009). The receptor mediating this effect was not identified, but was not the CB_1 receptor. Nevertheless, it is tempting to speculate that stress leads to an alteration in lipid/CB signalling in the GI tract as an adaptive mechanism to slow small intestinal motility.

Our findings of enhanced faecal pellet output are very similar to those previously reported by Million *et al.* (2007). They showed that acute novel environment stress leads to enhanced faecal pellet output, the magnitude and duration of which are virtually identical to what we observed. This effect is mediated by corticotrophin-releasing factor (CRF) signalling pathways and cholinergic neurons of the myenteric plexus. Consistent with this model, we propose that AM841 acts on CB_1 receptors on myenteric cholinergic neurons to reduce transmission and slow the gut. From the results of our *in vivo* studies we cannot pinpoint the exact site of action, which could be through inhibition of presynaptic neurotransmitter release or prejunctional release or both.

Currently, there are very limited treatment options for functional GI disorders, in which motility is altered leading to severe symptoms (Camilleri, 2013). Cannabis is frequently used to relieve the symptoms of these disorders. However, the unwanted psychotropic effects limit its usefulness as a therapeutic agent. Here, we have discovered a class of molecule with potential beneficial actions on abnormally accelerated GI motility that lacks any central actions. Because of its high potency, efficacy and duration of action, it seems well suited to further development as a therapeutic agent.

In summary, AM841 is a novel covalent CB agonist that behaved as an irreversible CB_1 receptor ligand *in vitro*. AM841 showed little brain penetration and so behaved as a peripherally restricted CB ligand. AM841 potently reduced GI motility *in vivo* by acting at CB_1 receptors in the small and large intestine. When GI transit was accelerated under conditions of acute stress, AM841 was able to normalize it. These data suggest that this novel CB ligand represents a new class of potential therapeutic agents for the treatment of GI disorders.

Acknowledgements

Supported by the Canadian Institutes of Health Research (K. A. S.), National Institutes of Health (DA011322 and DA021696, K. M.; DA09158, DA7215 and DA3801, A. M.) and Deutsche Forschingsgemeinschaft (DFG STO645/9-1, M. A. S.). K. A. S. is the Crohn's and Colitis Foundation of Canada Chair in Inflammatory Bowel Disease Research.

Author contributions

C. M. K., M. A. S., G. A. T., J. T. W., J. W-M., A. S., M. R. E., S. P. N., M. B. and H. H. performed the research. C. M. K., M. A. S., K. M., A. M. and K. A. S. designed the research study. G. A. T., M. R. E. and S. P. N. contributed essential research tools. C. M. K., M. A. S., J. T. W., J. W-M., A. S., M. B., K. M. and K. A. S. analysed the data. C. M. K., M. A. S., G. A. T., J. T. W., A. S., S. P. N., M. B., K. M., A. M. and K. A. S. wrote the paper. All authors read, revised and approved the paper for publication. M. A. S., K. M., A. M. and K. A. S. obtained funding for these studies.

Conflicts of interest

The authors declare no conflicts of interest.

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