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The novel peripherally active cannabinoid type 1 and serotonin type 3 receptor agonist AM9405 inhibits gastrointestinal motility and reduces abdominal pain in mouse models mimicking irritable bowel syndrome

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ABSTRACT

The endocannabinoid system (ECS) plays a crucial role in numerous physiological processes in the central and peripheral nervous systems. In the gastrointestinal (GI) tract, selective cannabinoid (CB) receptor agonists exert potent inhibitory actions on motility and pain signalling.

In the present study, we used mouse models of diarrhea, hypermotility, and abdominal pain to examine whether a novel synthetic CB_1 receptor agonist AM9405 [(2-(2,6-dihydroxy-4-(2-methyloctan-2-yl) phenyl) – 1,3-dimethyl-1H-benzo[*d*]imidazol-3-ium bromide); also known as GAT379] exhibits effects of potential therapeutic relevance.

AM9405 significantly slowed mouse intestinal motility in physiological conditions. Moreover, AM9405 reversed hypermotility and reduced pain in mouse models mimicking symptoms of functional GI disorders, such as stress-induced diarrhea and writhing test. Interestingly, some of the effects of AM9405 were blocked by a 5-HT_3 antagonist suggesting interaction with 5-HT_3 receptors.

In our study we show that combining CB_1 agonism with 5-HT_3 agonism may alter physiological functions and experimental pathophysiologies in a manner that make such compounds promising drugs for the future treatment of functional GI disorders.

1. Introduction

The endocannabinoid system (ECS) comprises of biochemical compounds known as endocannabinoids, which are synthesized from membrane-bound lipid precursors (Elia et al., 2015), cannabinoid (CB) receptors as well as enzymes responsible for synthesis (e.g. phospholipase D) and degradation (e.g. fatty acid amide hydrolase) of their ligands. Endocannabinoids include anandamide (AEA) and 2-arachidonoylglycerol (2-AG), along with naturally occurring acylethanolamides (AEs), such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) (Fichna et al., 2014; Ückert et al., 2017). The "classical" CB receptors, CB_1 and CB_2 are located in brain regions as well as in peripheral tissues, mainly on enteric neurons, nerve fibres and terminals in the enteric nervous system (ENS) (Duncan et al., 2005; Lalanne et al., 2017). The CB_1 receptors affect the neurotransmitter release in both central nervous system (CNS) and the ENS. Moreover, expression of CB_1 receptors has been reported in liver and adipose tissue. The CB_2 receptors have been found on immune cells, for example peripheral blood leukocytes (Berger et al., 2017; Kimball et al., 2010). The ECS plays an important role in many physiological and pathophysiological processes, including regulation of gut function, feeding behavior, pain processing, intestinal inflammation, immune function and neuroprotection (Chen et al., 2017; Elia et al., 2015).

There is plethora of evidence demonstrating the role that CBs play in the gastrointestinal (GI) tract including effects on motility, secretion, pain signalling and inflammation (Izzo and Sharkey, 2010). Stimulation of CB_1 receptors activates the G_i protein and triggers inhibition of acetylcholine release from enteric cholinergic neurons causing a decrease in smooth muscle contractility and reduction of peristalsis. Moreover, exogenous CB receptor ligands, for example plant-derived

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compound tetrahydrocannabinol (THC) decrease the production of gastric acid (Duncan et al., 2005; Elia et al., 2015). On this basis, CB ligands could be employed in the treatment of the GI disorders, such as irritable bowel syndrome (IBS) (Zgair et al., 2017). However, their clinical use is hampered by adverse events in the CNS, such as dependence and tolerance.

IBS is one of the most common bowel disorders with an unknown etiology, characterized by chronic, recurrent symptoms, such as abdominal pain, diarrhea and/or constipation. There are many factors contributing to the development of this disease including increased mucosal permeability, visceral hypersensitivity and/or disturbances in the brain-gut axis. IBS can be divided into four subtypes - IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed type IBS (IBS-M) and IBS unsubtyped (IBS-U). Even up to 18% of worldwide population suffer from IBS, mainly middle-aged women (Health, 2012; Jin et al., 2016). The treatment of IBS is very difficult, and therapeutic targets are still sought after.

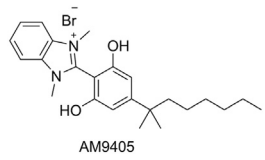
Several studies confirmed that endocannabinoids and CB receptors may be linked to the underlying physiological processes of IBS (Camilleri et al., 2013; Fichna et al., 2013b; Wong et al., 2012). For example, Wong et al. investigated the effect of non-selective CB receptor agonist dronabinol in patients with non-constipated IBS. Reduced fasting colonic motility was shown in participants suffering from IBS-D and IBS-M after administration of dronabinol (Wong et al., 2011). Moreover, CBs induce analgesic effect in various animal models of acute and chronic pain and the CB_1 receptor agonists reduce sensitivity to colorectal distension (Kimball et al., 2010; storr et al., 2008). Summarizing, the elements of ECS, mainly CB_1 receptors, can be targeted in order to achieve improvement in intestinal motility and visceral pain in patients with IBS.

In this study, we investigated the effect of AM9405 [(2-(2,6-dihydroxy-4-(2-methyloctan-2-yl)phenyl)-1,3-dimethyl-1H-benzo[*d*]imidazol-3-ium bromide); GAT379] (Fig. 1) on intestinal smooth muscle contractility, on motility of the gut and on visceral pain perception. Moreover, to characterize the mechanism of action of AM9405 we used murine knockout models as well as CB_1, CB_2 and serotonin receptor antagonists. We also employed different routes of administration to clarify whether the actions of AM9405 depend solely on receptors located in the periphery.

2. Materials and methods

2.1. Animals

For this study, we used male CD1 mice (Charles River, Canada), and CB_1 receptor knockout ($CB_1^{-/-}$) mice and their littermates on a C57Bl/6 background bred in the facility at the University of Calgary, Canada. The animals were housed at a constant temperature (22–23 °C) and maintained under a 12-h light/dark cycle in sawdust-lined transparent plastic cages with free access to chow pellets and tap water. All animal protocols were approved by the University of Calgary Animal Care Committee (protocol number M07102), and the experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care. All efforts were made to minimize animal suffering and to reduce the number of animals used.



AM9405

Fig. 1. The chemical structure of AM9405 molecule.

2.2. In vitro organ bath studies

All experiments were performed according to the methodology described earlier (Sałaga et al., 2014). Mice were killed by cervical dislocation. Full-thickness pieces (0.5–1 cm) of the ileum and distal colon were isolated and kept in the ice-cold oxygenated Krebs-Ringer solution (NaCl 115 mM, KCl 8.0 mM, KH_2PO_4 2.0 mM, $NaHCO_3$ 25 mM, $MgCl_2$ 2.4 mM, $CaCl_2$ 1.3 mM, and glucose 10 mM). The contents of the intestines were gently flushed. Each piece of tissue was used for a single experiment only. One end of each intestinal segment was attached to the bottom of the individual organ bath, whereas the other end to a FT03 isometric force displacement transducer (Grass Technologies, West Warwick, RI, USA) using a silk thread. Each piece was located between two platinum electrodes in organ baths containing Krebs (25 ml) equilibrated with 95% O_2 and 5% CO_2 at 37 °C. A tension of 0.5 g was applied, and the preparations were left for 30 min to equilibrate. The changes in tension were amplified by a P11T amplifier (Grass Technologies) and recorded on a personal computer using the POLYVIEW software (Polybytes Inc., Cedar Rapids, IA). Electrical field stimulation was administered by a S88X stimulator (Grass Technologies, EFS, 8 Hz, 60 V, pulse duration 0.5 ms, train duration 10 s), and supplied through electrodes located around the tissue. The longitudinal contractile responses were characterized in the presence of increasing concentrations of WIN 55,212-2 and AM9405 (10^{-12}-10^{-6}), with a contact time for each concentration of 10 min. Before the addition of drug, the mean amplitude of four twitch contractions was expressed, and treated as an internal control. Changes in contractions were showed as the percentage of the internal control. The effect of the vehicle was tested in control experiment. To characterize the involvement of CB receptors and 5-HT_3 receptor, selective antagonists were appended into the organ baths 15 min earlier to AM9405: AM251 (CB_1 receptor antagonist, 10^{-6} M), AM630 (CB_2 receptor antagonist, 10^{-6} M) and ondansetron (5-HT_3 receptor antagonist, 10^{-6} M). Tissues obtained from $CB_1^{-/-}$ were used to ensure the selectivity of the test compounds.

2.3. Whole gastrointestinal transit

Whole GI transit time test estimates the time of passage of non-absorbable color dye (150 µl of liquid, consisting of 5% Evans blue and 5% Arabic gum) through the GI tract. Vehicle, AM9405, or WIN 55,212-2 (0.1, 1 mg/kg each) was injected i.p. 15 min prior dye administration. The CB_1 receptor antagonist AM251 (1 mg/kg) used in the experiment was injected intraperitoneally (i.p.) 15 min before the administration of only AM9405. Directly after dye administration, mice were moved to individual cages, which were located on a white piece of paper facilitating recognition of colored stool pellets. The whole GI transit time was estimated from administration of the dye until excretion of the first colored pellet.

2.4. Colonic bead expulsion test

Distal colonic expulsion was determined as described previously (Sałaga et al., 2015, 2014). Shortly, animals were fasted overnight. Subsequently, AM9405 or WIN 55,212-2 were administered i.p. (at the doses ranging from 0.01 to 1 mg/kg) or p.o., intracolonically (i.c.) (both 6 mg/kg) and 15 min later (15–75 min in the time-course experiments), a prewarmed (37 °C) glass bead (2 mm) was loaded into the distal colon 2 cm proximally to the anus using a silicone catheter. After load of the bead, mice were placed to individual cages, and the time to bead expulsion was measured. The selective CB_1 (AM251; 1 mg/kg) or CB_2 (AM630; 1 mg/kg) antagonists were injected i.p. or i.c.v. 15 min before AM9405 or WIN 55,212-2 administration. The time to bead expulsion was measured also in the presence of selective 5-HT_3 receptor antagonist, ondansetron, which was injected i.p. (1 mg/kg) before AM9405. Mice that did not excrete the bead within 30 min were euthanized to verify the presence of the bead in the lumen of the colon. $CB_1^{-/-}$ mice

were used to ensure the selectivity of the test compounds.

2.5. Mouse model of castor oil-induced diarrhea

Mice were fasted overnight before the experiment. On the day of the experiment, AM9405 (doses ranging from 0.01 to 1 mg/kg) or loperamide (LOP; 0.1 mg/kg) were administered i.p. 15 min before the p.o. administration of the castor oil (0.2 ml/mouse), which induced diarrhea. Moreover, AM9405 was injected p.o. (1 and 10 mg/kg) 15 min before castor oil to test its bioavailability. Selective antagonists of CB_1 and CB_2 receptors were injected i.p. at the dose of 1 mg/kg each. Immediately after administration of castor oil mice were transferred into individual cages, which were located on the clean, white paper. Time between injection of castor oil and the occurrence of liquid feces was determined and compared between groups.

2.6. Mouse model of 5-hydroxytryptophan-induced diarrhea

The experiment was conducted according to the methodology described earlier (Hedge et al., 1994; Pascual et al., 2002) with minor modifications. Briefly, fecal mass output was assessed in non-fasted mice. Animals were administered i.p. with 5-hydroxytryptopan (5-HTP) at the dose of 1 mg/kg to induce diarrhea and were put in a single cage for 1 h. Then animals were removed from the cages and fecal pellets were collected and weighed. AM9405 (0.1 and 1 mg/kg) was injected i.p. 15 min before serotonin. CB_1 and 5-HT_3 receptor antagonists were administered 15 min before AM9405.

2.7. Mouse model of stress-induced hypermotility

The fecal pellet output was assessed in non-fasted mice (Sobczak et al., 2014). In physiological conditions, mice were separated into individual cages for habituation one day before experiment. In stress conditions (new environment) mice were separated 15 min after vehicle or AM9405 (0,1 or 1 mg/kg, i.p.) injection. Then animals were located on a metal grid and the mass and number of fecal pellets excreted over a 60 min was counted as a measure of GI tract motility. Selective antagonists of CB_1 and 5-HT_3 receptors were injected i.p. at the dose of 1 mg/kg each.

2.8. Behavioral pain responses to visceral pain

In this model mustard oil was used (MO, allyl isothiocyanate) to induce visceral pain. Behavioral pain responses to i.c. injection of MO were assessed as described earlier (Sałaga et al., 2014; Sobczak et al., 2014). Briefly, vaseline petroleum jelly was applied to the perianal area to rule out the stimulation of somatic areas. Then 50 µl of MO (1% in 70% ethanol) was injected i.c. under isoflurane anesthesia and animals were separated into transparent plastic boxes. After 5 min of recovery time, spontaneous behaviors were observed and counted for 20 min. The behaviors included squashing of lower abdomen against the floor, licking of the abdomen, abdominal retractions, stretching the abdomen (each behavior counted as 1). AM9405 or WIN 55,212-2 were injected i.p. (doses ranging from 0.01 to 0.3 mg/kg) 15 min before the MO instillation in the wild-type or $CB_1^{-/-}$ mice. Additionally, AM251, AM630 and ondansetron (1 mg/kg each) were administrated i.p. 15 min before AM9405. Moreover, AM251 (1 mg/kg) was administrated i.c.v. 15 min before AM9405 and WIN 55,212-2.

2.9. The writhing test

This test was conducted as described before by Fichna et al. (2013a). Animals were administrated i.p. (0.01–1 mg/kg) with AM9405 or WIN 55,212-2 15 min before the i.p. injection of acetic acid solution (10 ml/kg of 0.5% vol/vol, in 0.9% NaCl). Subsequently, animals were located in individual cages, and allowed for 5 min recovery. Then, the total number of writhes was measured for 15 min. Elongation of the body and the development of tension in the abdominal muscles and hind paws were regarded as symptoms of the writhing response.

2.10. Drugs

All drugs and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (Poznan, Poland). AM9405 was synthesized by Ritesh Tichkule and Ganesh Thakur at Northeastern University, Boston. WIN 55–212-2, ondansetron dihydrochloride, loperamide, AM251 ([*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide) and AM630 ([6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone) were purchased from Tocris Bioscience (Ellisville, MO). In the in vitro experiments (isolated smooth muscle strips), all drugs were dissolved in dimethylsulfoxide. In the in vivo tests, drugs were dissolved in 5% dimethylsulfoxide in saline, which was used as vehicle in control experiments. The vehicles in the used concentrations had no effects on the observed parameters.

2.11. Statistics

The data are presented as mean ± S.E.M. In the in vitro experiments n designates the number of individual tissues. Statistical analyses were performed using PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Student's *t*-test was used to compare single treatment means with control means. Analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test was used for analysis of multiple treatment means. Differences with a probability value of $P < 0.05$ were considered statistically significant.

3. Results

3.1. AM9405 inhibits smooth muscle contractility in vitro

We first compared the effect of AM9405 and a non-selective CB agonist used as a reference, WIN 55,212-2 on isolated mouse ileum and colon contractility. Both compounds (10^{-12}- 10^{-6} M) inhibited the EFS-induced twitch contraction of the ileum and the colon in a concentration-dependent manner and higher potency was observed in the colon than in the ileum (for the colon $IC_{50\ AM9405}$ = 0.076 ± 0.0054 nmol/l and $IC_{50\ WIN\ 55,212\text{-}2}$ = 0.28 ± 0.06 nmol/l, for the ileum $IC_{50\ AM9405}$ = 45.71 ± 7.59 nmol/l and $IC_{50\ WIN\ 55,212\text{-}2}$ = 44.67 ± 7.76 nmol/l; Fig. 2 A and B). No effect was observed in the tissues obtained from $CB_1^{-/-}$ mice.

As shown in Fig. 2C and D, the effect of AM9405 in both ileum and colon was partially blocked by the CB_1 antagonist AM251 (10^{-6} mol/l), but not CB_2 antagonist AM630 (10^{-6} mol/l). Selective 5-HT_3 receptor antagonist ondansetron did not block the effect of AM9405 in the ileum but partially reversed it in the colon (Fig. 2E and F).

3.2. AM9405 prolongs the time of the whole gastrointestinal transit in vivo

To test the effect of AM9405 on mouse GI motility in vivo we used the whole GI transit time test. AM9405 administered i.p. at the doses of 0.1 and 1 mg/kg produced a potent, dose-dependent inhibitory effect on whole GI motility, which was blocked by pre-treatment with AM251 (1 mg/kg i.p.) (Fig. 3). The reference compound WIN 55,212-2 exhibited similar effect on the whole GI transit time.

3.3. AM9405 inhibits colonic motility in vivo after systemic administration

AM9405 produced a dose dependent inhibitory effect on the colonic expulsion at the doses ranging from 0.01 to 1 mg/kg i.p. (Fig. 4A). Furthermore, we examined the time course of this effect demonstrating that it lasts up to 30 min after i.p. administration of AM9405 (Fig. 4B).

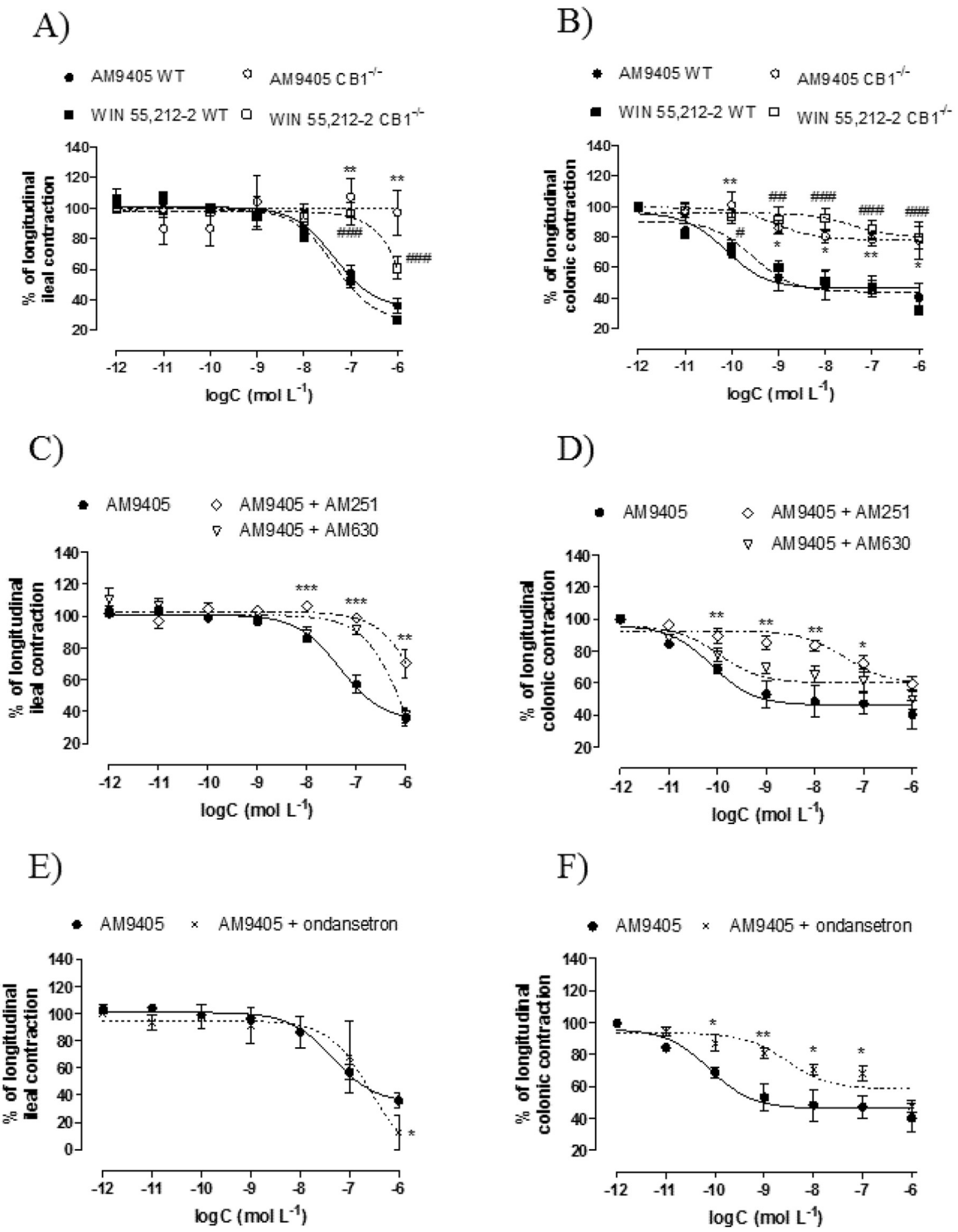


Fig. 2. Concentration–response curves showing the inhibitory effect of AM9405 on the longitudinal smooth muscle contraction in isolated mouse ileum and colon. Effect of increasing concentrations of AM9405 and the synthetic $CB_{1/2}$ agonist WIN 55,212-2 on the contractility of WT and $CB_1^{-/-}$ mouse ileum (A) and colon (B). Effect of AM9405 alone and in the presence of CB_1 antagonist AM 251 (10^{-6} mol/l) or CB_2 antagonist AM 630 (10^{-6} mol/l) on the contractility of mouse ileum (C) and colon (D). Effect of AM9405 alone and in the presence of the 5-HT_3 receptor antagonist ondansetron (10^{-6} mol/l) on the contractility of mouse ileum (E) and colon (F). Data represent mean ± S.E.M of n = 6–10. *P < 0.05, **P < 0.01, ***P < 0.001, as compared with AM9405 alone. #P < 0.05, ##P < 0.01, ###P < 0.001, as compared with WIN 55,212-2 alone.

To determine the bioavailability of AM9405 we also examined its action after p.o. and i.c. administration. At the dose of 6 mg/kg p.o. our test compound significantly prolonged the time to bead expulsion 15 min after injection (Fig. 4C). This effect was no longer observed 45 min after administration, nor was seen after the i.c. injection (6 mg/kg) (Fig. 4D).

To examine the selectivity of AM9405 we used mouse knockout model ($CB_1^{-/-}$) and pharmacological blockade of $CB_{1/2}$ and 5-HT_3 receptors. The effect of AM9405 and WIN 55,212-2 on the colonic motility was not present in the $CB_1^{-/-}$ mice (Fig. 5A). Pharmacological blockade of CB_1 but not CB_2 receptors with AM251 i.p. and AM630 i.p.,

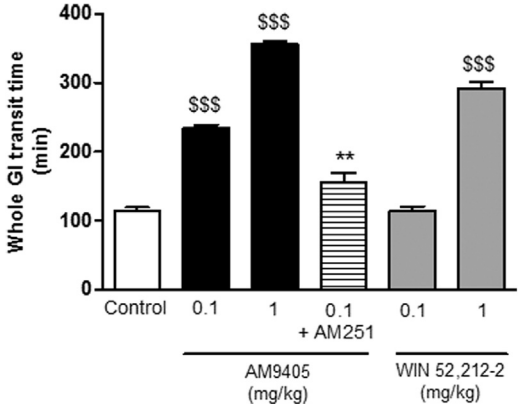


Fig. 3. The effects of AM9405 on GI motility in physiological conditions. Effect of i.p. administration of AM9405 (0.1 and 1 mg/kg) alone and in the presence of a CB_1 antagonist AM 251 (1 mg/kg, i.p.), as well as the effect of non-selective CB receptor agonist WIN 55,212-2 (0.1 and 1 mg/kg i.p.) on the whole GI transit time. Data represent mean ± S.E.M of n = 6–10 animals per group. $$ $P < 0.001, as compared with the control group. **P < 0.01, as compared with the AM9405 at the dose of 0.1 mg/kg.

respectively, reversed the changes in the colonic motility induced by both AM9405 and WIN 55,212-2 (Fig. 5 B). Of note, AM251 administered i.c.v. did not block the effect of AM9405 i.p., while it reduced the effect of WIN 55,212-2 i.p. (Fig. 5C).

Pre-treatment of mice with 5-HT_3 selective antagonist ondansetron (1 mg/kg i.p.) blocked the effect of AM9405 (1 mg/kg, i.p.) on the colonic bead expulsion. No effect was seen after administration of ondansetron alone (Fig. 5D).

3.4. Antidiarrheal and anti-hypermotility action of AM9405 in vivo

To investigate the antidiarrheal activity of AM9405, we used a mouse models of castor oil- and 5-HTP-induced diarrhea. Intragastric administration of castor oil caused accumulation of water and electrolytes in the mouse intestine, what resulted in acute diarrhea in the control animals. AM9405 produced a dose dependent anti-diarrheal effect at the doses ranging from 0.01 to 1 mg/kg i.p. (Fig. 6A). A well known anti-diarrheal drug LOP (0.1 mg/kg, i.p.), which was used as a reference compound in this experiment significantly prolonged the time to diarrhea occurrence in mice. The effect of AM9405 (0.1 mg/kg, i.p.) was significantly reduced in mice pretreated with AM251 but not AM630 (both antagonists at the dose of 1 mg/kg i.p.) (Fig. 6B). AM9405 remained active after p.o. administration at the doses of 1 and 10 mg/kg (Fig. 6C).

Treatment with 5-HTP caused significant increase in the fecal mass excreted within 60 min after the injection. AM9405 (0.1 and 1 mg/kg, i.p.) significantly reduced the total fecal mass excreted by animals and this effect was blocked by pre-treatment with i.p. AM251 but not i.p. ondansetron (Fig. 6D).

To further characterize the influence of AM9405 on the GI tract in pathophysiological conditions, mouse model of GI hypermotility was used in the study. In the stress-induced mouse model of hypermotility, the number of fecal pellets excreted by animals was measured for 60 min placing non-treated animals in a novel environment significantly increased fecal output compared with non-stressed control animals (Fig. 6E). AM9405 administered i.p. at the dose of 1 mg/kg inhibited GI hypermotility in stressed mice (Fig. 6E).

3.5. AM9405 is a potent analgesic in mouse models of abdominal pain

In order to assess the analgesic activity of AM9405, two mouse models of abdominal pain were used. In the model elicited by the i.c. administration of MO, i.p. administration of AM9405 (0.1 mg/kg) and WIN 55,212-2 (0.3 mg/kg) resulted in a significant decrease in the number of pain-related behaviors in the WT but not $CB_1^{-/-}$ mice (Fig. 7A). The action of AM9405 was dose dependent and reversed by CB_1 but not CB_2 antagonist (Fig. 7B). The i.c.v. administration of CB_1 antagonist AM251 at the dose of 1 mg/kg did not block the analgesic effect of AM9405 (0.1 mg/kg, i.p.) but significantly reversed the antinociceptive effect induced by i.p. WIN 55,212-2 (0.3 mg/kg i.p.) (Fig. 7C). Of note, the antinociceptive effect produced by AM9405 was not blocked by pre-treatment with ondansetron (Fig. 7D).

In the writhing test, the i.p. administration of AM9405 and WIN 55,212–2 at the doses ranging from 0.01 to 1 mg/kg resulted in a significant reduction of the number of writhes (Fig. 7E).

4. Discussion

It has been known for centuries that the symptoms of several GI disorders may be alleviated by plant-based remedies derived from *Cannabis* sp. They can be used to treat functional motility disorders, as well as emesis and abdominal pain. When first cannabinoids had been isolated and CB receptors had been cloned, molecular studies led to the identification of the underlying mechanisms that involve activation of CB_1 receptors. CB_1-dependent decrease in intracellular cAMP leads to the inhibition of intestinal motility, secretion and perception of pain. Ever since, attempts were undertaken to develop synthetic drugs targeting CB_1 receptors in the gut. In the present study, we show that a newly synthesized, orally available CB_1 agonist, AM9405, is a strong regulator of intestinal motility and pain signalling in physiological and pathophysiological conditions. These findings may open up opportunities for the design of novel therapeutics for functional GI ailments, including diarrhea-predominant IBS.

Major symptoms of IBS include disruption of intestinal motility, secretion and pain perception. To address the potential inhibitory effect of AM9405 on intestinal motility we measured the contractility of smooth muscle ex vivo. These experiments showed a substantial reduction of both ileal and colonic contractility, which is in line with a number of previous reports on CB_1 ligands (Fichna et al., 2014; Izzo and Sharkey, 2010; Vera et al., 2017). Interestingly, the effect of AM9405 was much higher in the colon compared to the ileum. Such difference may arise from varying distribution and/or density of the target receptors for this compound in different segments of the GI tract. The effect of AM9405 was dependent on CB_1 but not CB_2 receptors as proved both pharmacologically and with the use of $CB_1^{-/-}$ animals. In vivo studies on the GI motility of the whole as well as lower GI tract in physiological conditions confirmed the observations made in the ex vivo conditions. These experiments also revealed that AM9405 retains its activity in the colon after oral administration while the i.c. administration was not effective, suggesting that our compound may not cross the colonic epithelium-blood barrier or might undergo rapid degradation by colonic microbiota.

Notably, both the effect on the isolated colonic smooth muscle strips and colonic motility in vivo were blocked by the selective 5-HT_3 antagonist ondansetron suggesting the involvement of this class of receptors in the mechanism of AM9405 action. This phenomenon is particularly interesting since it points to an agonist activity of AM9405 on 5-HT_3 receptors. Most likely, modifications introduced to the parent molecule changed the mechanism of the action of the compound and shifted the equilibrium towards 5-HT_3 receptors. Such information is crucial for further structure-activity relationship studies.

There are several examples of downstream pathways linking CB_1 and 5-HT_3 receptors that may play a role in observed physiological changes. Ondansetron could potentially affect downstream CB_1 receptor-mediated effects, such as inhibition of adenylyl cyclase through G_i protein and subsequent drop in the cAMP. On the other hand, administration of ondansetron could act upstream CB_1 and 5-HT_3 receptors, by stimulating the production of endocannabinoids that compete with AM9405 for binding with CB_1 sites (Feng et al., 2014). A less

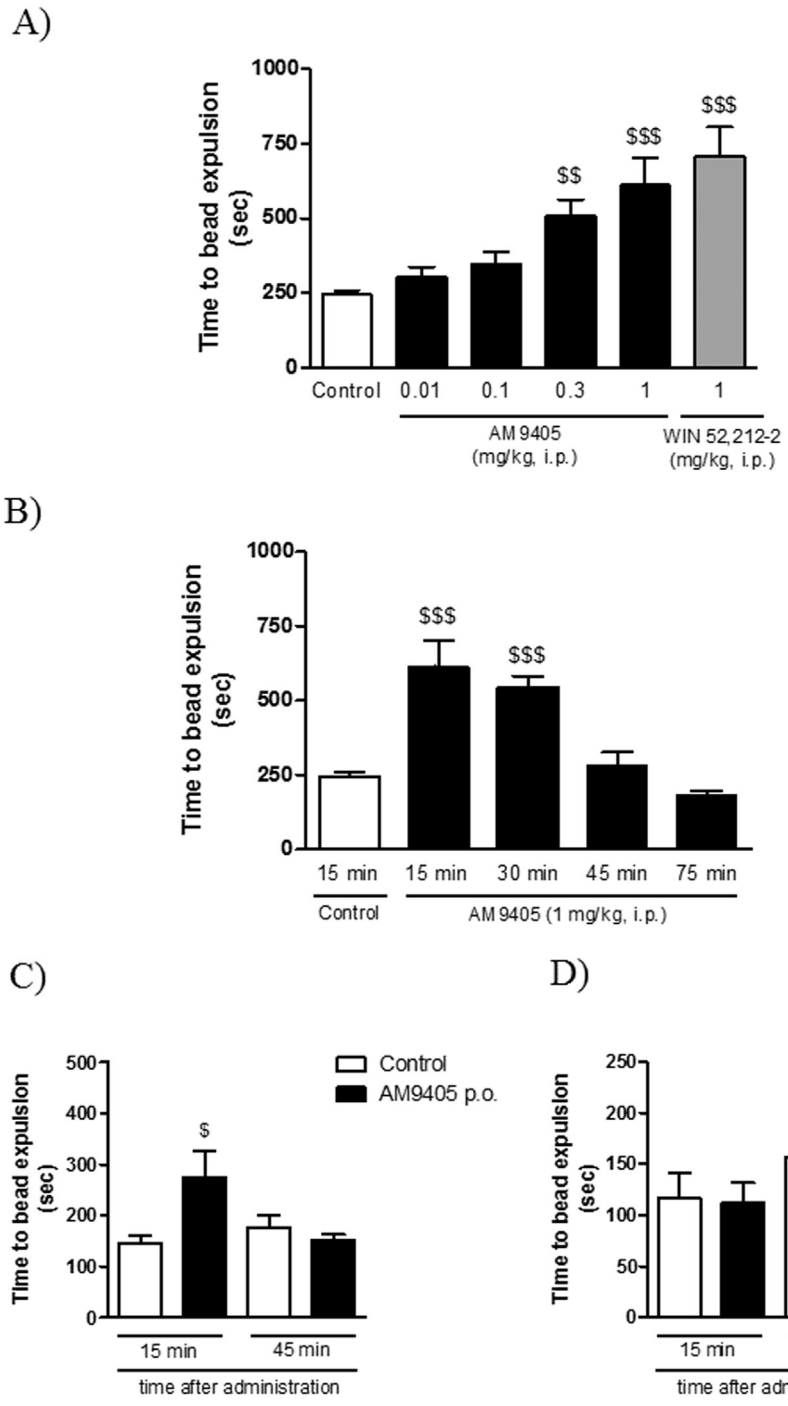


Fig. 4. In vivo effect of AM9405 on colonic bead expulsion time in mice. The dose-response experiment showing the inhibitory effect of AM9405 on the colonic motility at the doses ranging from 0.01 to 1 mg/kg i.p. Non-selective CB receptor agonist WIN 55,212-2 (1 mg/kg i.p.) was used as a reference compound (A). The time course of the changes of bead expulsion time after i.p. administration of AM9405 (1 mg/kg) (B). Changes in the bead expulsion time after p.o. (C) and i.c. (D) administration of AM9405 (6 mg/kg). Data represent mean ± S.E.M of n = 8–10 mice for each experimental group. $ $P < 0.01, $$$P < 0.001, as compared with the control group.

likely explanation of this phenomenon may involve a crosstalk between 5-HT and CB systems. Such interactions were previously reported by Feng et al. (Feng et al., 2014) however the exact mechanism remains unexplored. CB sites were shown to form functional homo- and heterodimers with other types of receptors, such as opioid, orexin, dopamine and to be co-localized with 5-HT_3 receptors on the vagal nerve (Ellis et al., 2006; Izzo and Sharkey, 2010; Kearn, 2005; Rios et al., 2006). However, it seems unlikely that CB and 5-HT_3 receptors form such structures given their distant origin and structure - CB sites belong to G protein-coupled receptors, while 5-HT_3 receptors are ligand-gated ion channels.

Encouraged by these results we investigated the anti-diarrheal activity of our test compound. Diarrhea is one of the most common problems of patients with IBS since IBS-D accounts for approximately 80% of all cases of IBS (Pimentel, 2018). AM9405 exhibited strong anti-diarrheal activity through CB_1 but not CB_2 or 5-HT_3 receptors and, importantly for possible future human use, the effect was also present after oral administration of the compound. Of note, both secretory diarrhea and stress-induced hypermotility were alleviated by AM9405 suggesting a potent action independent of the stimulus that causes the impairment of gut function.

To fully characterize the pharmacological effect of AM9405 in the GI tract, we also examined its potential antinociceptive activity in two well established mouse models of abdominal pain. The MO-induced model of abdominal pain is used to study the neurogenic and inflammatory visceral nociception caused by stimulation of transient receptor potential cation channels (TRPs). This is of particular importance since it has been proposed that low-grade inflammation is involved in

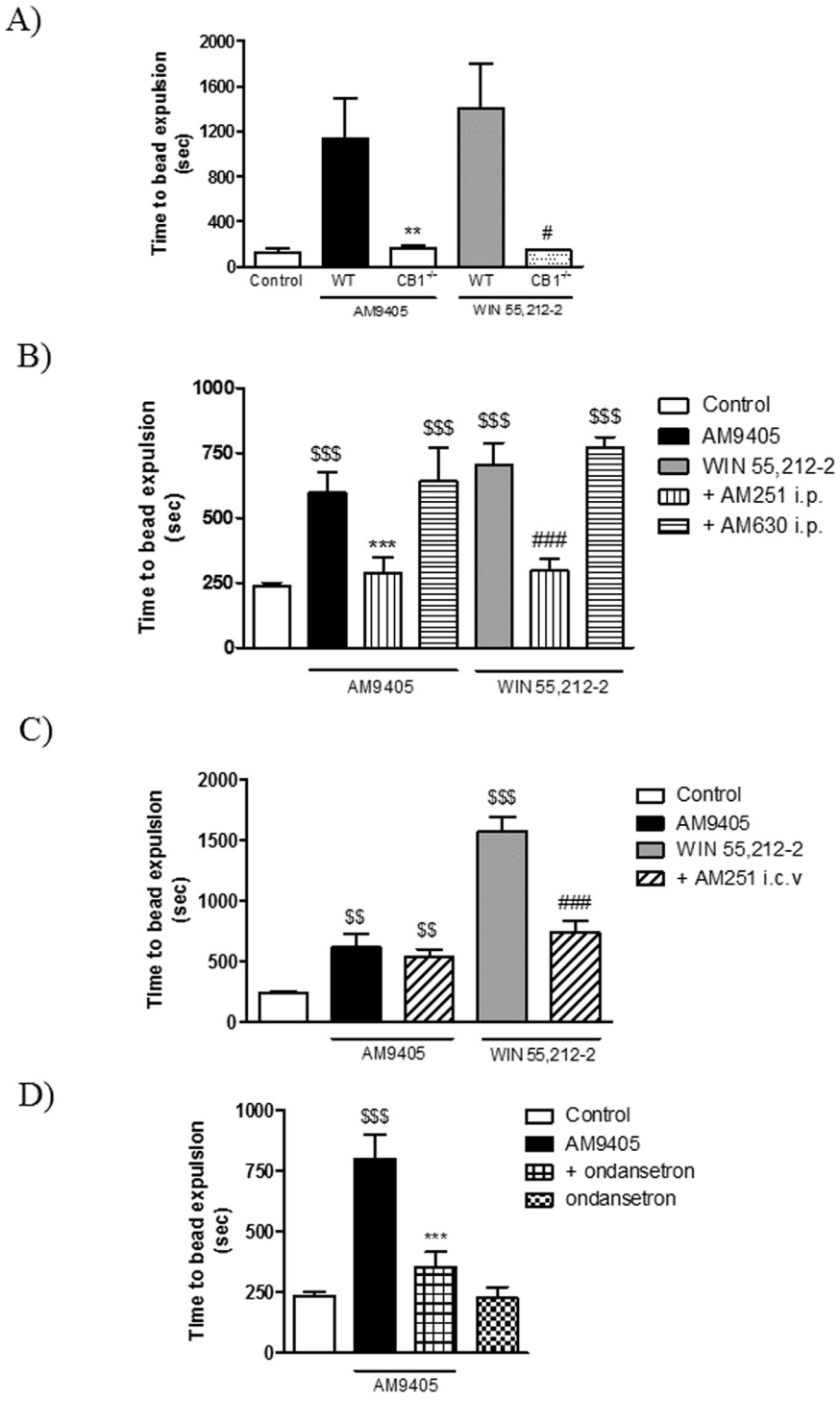


Fig. 5. The effect of AM9405 on the colonic motility in mice is dependent on CB_1 and 5-HT_3 but not CB_2 receptors. Effect of AM9405 and WIN 55,212-2 (both compounds at the dose of 1 mg/kg, i.p.) on the bead expulsion time in WT and $CB_1^{-/-}$ mice (A). The effect on the bead expulsion time of AM9405 and WIN 55,212-2 (both compounds at the dose of 1 mg/kg, i.p.) alone or in the presence of selective CB_1 (AM 251; 1 mg/kg i.p.) or CB_2 (AM 630; 1 mg/kg i.p.) antagonists (B). The effect on the bead expulsion time of AM9405 and WIN 55,212-2 (both compounds at the dose of 1 mg/kg, i.p.) alone or in the presence of selective CB_1 antagonist AM 251 (1 mg/kg) administered i.c.v. (C). The effect on the bead expulsion time of AM9405 (1 mg/kg, i.p.) alone or in the presence of selective 5-HT_3 receptor antagonist ondansetron (1 mg/kg i.p.) (D). Data represent mean ± S.E.M of n = 6–8 mice for each experimental group. $$P < 0.01, $$$P < 0.001, as compared with the control group. **P < 0.01, ***P < 0.001, as compared with AM9405 alone. #P < 0.05, ###P < 0.001, as compared with WIN 55,212-2 alone.

the pathophysiology of sustained nociceptor activity in IBS. The writhing test is a model of peritoneal pain combining visceral and somatic mechanisms that lacks pharmacological specificity. This model generates brief acute reactions that cannot be extrapolated directly to relevant visceral pain observed in humans (González-Cano et al., 2017). In both tests, we observed a potent, dose dependent analgesic effect after systemic (i.p.) administration, which was mediated by CB_1 but not CB_2 or 5-HT_3 receptors. The antinociceptive effect was not present in $CB_1^{-/-}$ animals. Interestingly, the pro-nociceptive effect induced by MO was not alleviated by ondansetron alone, which is a well-known drug used in the treatment of IBS. In line, a recent meta-analysis of several randomized controlled clinical trials by Zheng et al. (Zheng et al., 2017) has shown that ondansetron improves bowel habits and stool consistency rather than abdominal discomfort and pain in IBS patients as compared to controls. On the other hand, the lack of anti-nociceptive effect of ondansetron in this model may be solely due to its mechanism of action. Probably, blockade of 5-HT_3 ion channel is not sufficient to counteract the influx of ions to the sensory neurons through TRPA1 and TRPV1 activated by MO.

A major issue accompanying the development of novel CB ligands is their potential effect on the CNS. Hence, one of our most prominent goals was to find a novel CB_1 agonist deprived of the CNS-related side effects typical for other cannabinoids. CB_1 receptors, when activated in the brain, produce hallucinations, dependence and may negatively

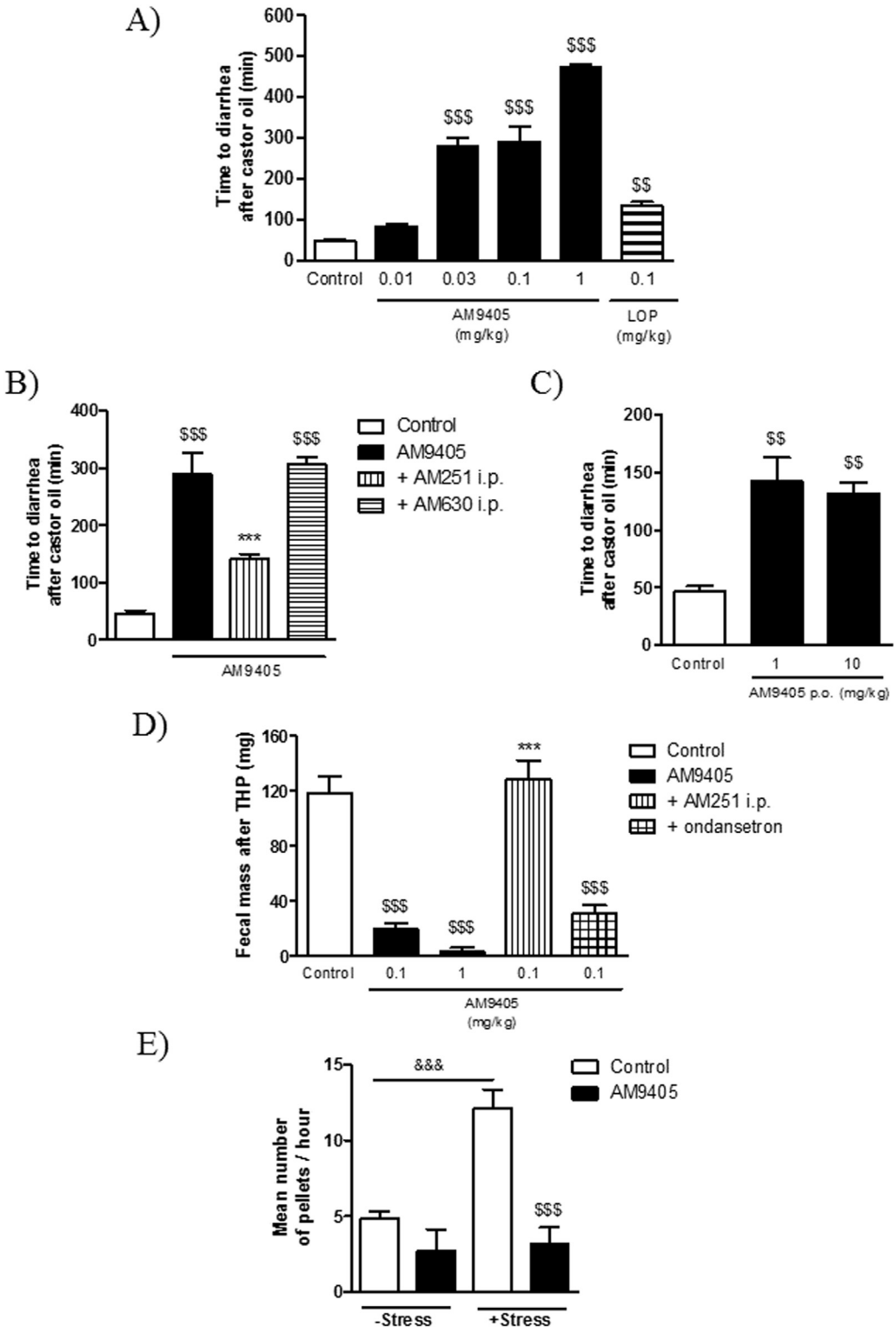


Fig. 6. Antidiarrheal activity of AM9405. The dose-response experiment, after i.p. administration of AM9405 (0.01–1 mg/kg) with LOP (0.1 mg/kg i.p.) as a reference drug, on the delay of the emergence of castor oil-induced diarrhea (A). The antidiarrheal effect of AM9405 alone and in the presence of selective CB_1 (AM 251; 1 mg/kg i.p.) or CB_2 (AM 630; 1 mg/kg; i.p.) antagonists (B). The antidiarrheal activity of AM9405 after p.o. administration (C). The effect of AM9405 (0.1 and 1 mg/kg i.p.) alone and in the presence of selective CB_1 antagonist AM 251 (1 mg/kg, i.p) or 5-HT_3 antagonist ondansetron (1 mg/kg i.p.) on the 5-HT induced hypermotility in mice (D). The effect of i.p. administration of AM9405 (1 mg/kg) on the defecation pattern in stressed and non-stressed mice (E). Data represent mean ± S.E.M of n = 6–8 mice for each experimental group. $$P < 0.01, $$$P < 0.001, as compared with the control group. * **P < 0.001, as compared with AM9405 alone. &&& P < 0.001, non-stressed control vs. stressed control.

affect psychological health. A substantial number of publications reported disruption of cognitive and psychomotor effects after cannabis exposure (for review please see (Vindenes and Mørland, 2017)); effects of the acute as well as long-term exposure to THC include memory, learning and attention impairment and increased risk of paranoia. Notably, a synthetic inverse CB_1 agonist taranabant, meant to be used in the treatment of obesity had been withdrawn from phase III clinical studies due to the occurrence of depression, irritability, anxiety and suicidal thoughts in patients (Hernandez-Folgado, 2017). Consequently, the lack of in vivo brain CB_1 receptor occupancy is a desirable feature of all ECS-targeting compounds designed to treat GI disorders. To determine whether the effects of AM9405 on gut motility are mediated via CB_1 receptors located in the brain we applied local (i.c.v.) injection of CB_1 antagonist AM251. We observed that neither the effect on colonic propulsion, nor on visceral nociception was reversed suggesting that AM9405 does not reach brain CB_1 receptors after systemic administration what may be caused by low penetration of the blood brain barrier. Our observations thus suggest a favorable therapeutic profile of

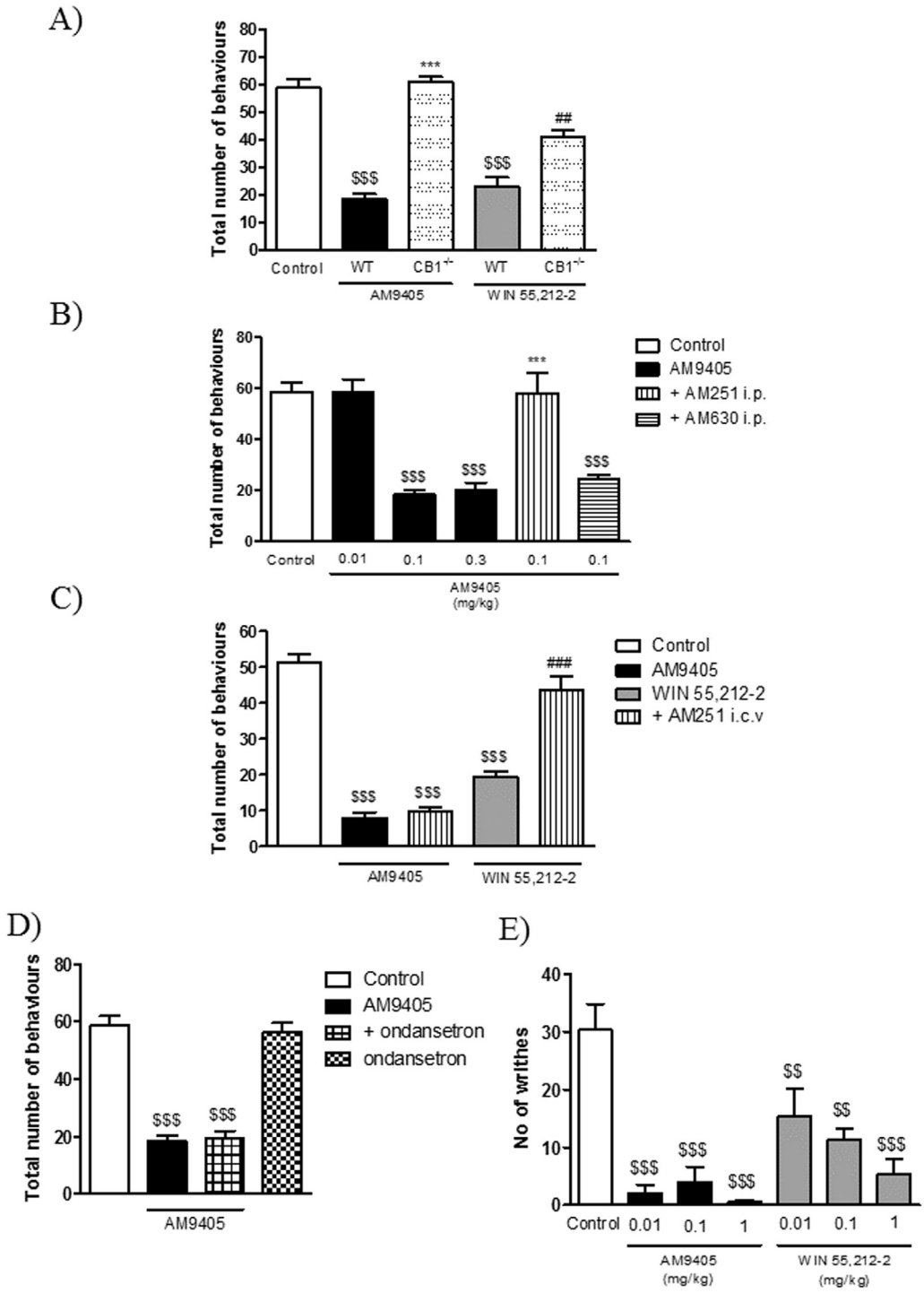


Fig. 7. Antinociceptive activity of AM9405. The effect of AM9405 (0.1 mg/kg, i.p.) and WIN 55,212-2 (0.3 mg/kg, i.p.) on the number of pain-related behaviors evoked by i.c. administration of mustard oil (MO) in the WT and $CB_1^{-/-}$ mice (A). The antinociceptive activity of AM9405 alone and in the presence of selective CB_1 (AM 251; 1 mg/kg i.p.) or CB_2 (AM 630; 1 mg/kg i.p.) antagonists (B). The effect of AM9405 and WIN 55,212-2 (both compounds at the dose of 0.3 mg/kg, i.p.) alone or in the presence of selective CB_1 antagonist AM 251 (1 mg/kg) administered i.c.v. (C). The antinociceptive effect of AM9405 (0.1 mg/kg, i.p.) alone or in the presence of selective 5-HT_3 receptor antagonist ondansetron (0.1 mg/kg i.p.) (D). The effect of i.p. administered AM9405 and WIN 55,212-2 (both compounds at the doses of 0.01, 0.1 and 1 mg/kg) on the number of writhes (E). Data represent mean ± S.E.M of 6–8 mice per group. $$P < 0.01; $$$P < 0.001, as compared with MO/acetic acid-treated animals. ***P < 0.001, as compared with AM9405 alone. ##P < 0.01; ###P < 0.001, as compared with WIN 55,212-2 alone.

AM9405.

5. Conclusion

The novel orally available compound AM9405, exhibits great potential for clinical use in functional GI disorders associated with pain and diarrhea. It seems likely that AM9405 will be further developed for its significant impact on the lower GI tract combined with good bioavailability and the lack of psychotropic side effects.

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Disclosures

The authors have nothing to disclose.

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