Research Paper

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Acute visceral pain relief mediated by A_3AR agonists in rats: involvement of N-type voltage-gated calcium channels

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Abstract

Pharmacological tools for chronic visceral pain management are still limited and inadequate. A_3 adenosine receptor (A_3AR) agonists are effective in different models of persistent pain. Recently, their activity has been related to the block of N-type voltage-gated Ca^{2+} channels ($Ca_v2.2$) in dorsal root ganglia (DRG) neurons. The present work aimed to evaluate the efficacy of A_3AR agonists in reducing postinflammatory visceral hypersensitivity in both male and female rats. Colitis was induced by the intracolonic instillation of 2,4-dinitrobenzenesulfonic acid (DNBS; 30 mg in 0.25 mL 50% EtOH). Visceral hypersensitivity was assessed by measuring the visceromotor response and the abdominal withdrawal reflex to colorectal distension. The effects of A_3AR agonists (MRS5980 and Cl-IB-MECA) were evaluated over time after DNBS injection and compared to that of the selective $Ca_v2.2$ blocker PD173212, and the clinically used drug linaclotide. A_3AR agonists significantly reduced DNBS-evoked visceral pain both in the postinflammatory (14 and 21 days after DNBS injection) and persistence (28 and 35 days after DNBS) phases. Efficacy was comparable to effects induced by linaclotide. PD173212 fully reduced abdominal hypersensitivity to control values, highlighting the role of $Ca_v2.2$. The effects of MRS5980 and Cl-IB-MECA were completely abolished by the selective A_3AR antagonist MRS1523. Furthermore, patch-clamp recordings showed that A_3AR agonists inhibited $Ca_v2.2$ in dorsal root ganglia neurons isolated from either control or DNBS-treated rats. The effect on Ca^{2+} current was PD173212-sensitive and prevented by MRS1523. A_3AR agonists are effective in relieving visceral hypersensitivity induced by DNBS, suggesting a potential therapeutic role against abdominal pain.

Keywords: A3 adenosine receptor, DRG, Abdominal pain, IBS, IBD

1. Introduction

Visceral pain management is a major clinical problem, because of the lack of effective and safe drugs.[11] Abdominal pain is the most common form of visceral hypersensitivity and is often the result of inflammatory bowel diseases (IBDs).[14,51,84,85] The poor correlations between reported abdominal pain intensity and IBD activity raise close similarities with the irritable bowel syndrome (IBS), a symptom-based clinical condition defined by the presence of persistent abdominal pain and altered bowel habits, in the absence of any other disease able to account for these symptoms.[24,35,51,58,84,85] Frequently, IBS results from a previous intestinal damage caused by severe infections or prolonged inflammatory processes.[24,83] In the inflamed gut, there is breakdown of intestinal barrier function, abnormal secretion, changes in the patterns of motility, and altered visceral sensation, which altogether contribute to generation of symptoms (diarrhoea, cramping, and pain). Pain, in particular, persists beyond the relief of inflammation, thus revealing a peculiar kind of chronic hypersensitivity thought to be due to inflammatory, immune, and neuropathic mechanisms.[9,26,40,53,86] Currently, the most efficacious therapies against visceral hypersensitivity are mainly directed toward treating bowel dysfunction, whereas drugs able to directly target the associated pain are still unsatisfactory.[11]

The neuromodulator adenosine exerts potent and long-lasting pain suppression in preclinical models as well as in human studies.[105] Moreover, adenosinergic signalling is known to modulate intestinal functionality.[2–4] For decades, it was thought that the analgesic effects of adenosine were mediated by A_1 adenosine receptor (A_1AR) activation,[8,54,65,77,105] but research efforts over the past several years have also implicated a key role for the A_3AR subtype.[43,75] For example, A_3AR agonists are able to block the development of trauma- and chemotherapeutic-

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induced neuropathic pain[17,42] and are also effective in reducing inflammatory and cancer-related pain.[50,93,100,101] Recently, we found that selective A_3AR stimulation inhibits the opening of N-type voltage-gated Ca^{2+} channels ($Ca_v2.2$) and decreases the electrically evoked excitation of isolated rat dorsal root ganglia (DRG) neurons. This mechanism could explain the antihyperalgesic effect of A_3AR agonists across different models.[19] Therapeutic targeting of A_1AR has been limited to severe cardiovascular side effects; by contrast, A_3AR agonists are already in advanced clinical trials for different indications with a good safety profile.[29,80]

The aims of this study were to: (1) evaluate the efficacy of selective A_3AR agonists in postinflammatory visceral hypersensitivity induced in male and female rats by the intrarectal administration of 2,4-dinitrobenzenesulfonic acid (DNBS); (2) investigate the electrophysiological effects of selective A_3AR stimulation on DRG neurons isolated from control and DNBS-treated animals.

2. Materials and methods

An extensive description of materials and methods has been reported in the supplemental digital content (available at http://links.lww.com/PAIN/B10).

2.1. Animals

We used male and female Sprague-Dawley rats (Envigo, Varese, Italy) weighing approximately 220 to 250 g at the beginning of the experimental procedure. All animal manipulations were conducted according to the Directive 2010/63/EU of the European Parliament and of the European Union Council (September 22, 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the described experiments was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines.[57] Gastrointestinal motility assays (GI) were conducted using Sprague-Dawley male rats (Envigo, 250-350 g) in accordance with the University of Arizona Institutional Animal Care and Use Committee (approval 06-110). Experiments were performed on male rats when not otherwise stated.

2.2. Determination of female estrous cycle phases

Estrous cycle was assessed in rats by analysing rat vaginal smears as previously described.[39] This screening was performed before the behavioral test; tests were selectively performed in female rats in estrous/proestrous phase to avoid sensitive difference related to the estrous cycle. Moreover, in most studies, this phase is reported to be related to the highest pain sensitivity,[45,46,59,60,74] therefore suitable for studying pain-relieving compounds.

2.3. Induction of colitis

Colitis was induced using the method described previously by Fornai et al.[30] with minor changes. In brief, during a short period of anaesthesia with isoflurane (2%), 30 mg of DNBS in 0.25 mL of 50% ethanol was administered intrarectally through a polyethylene PE-60 catheter inserted 8 cm proximal to the anus. Control rats received 0.25 mL of saline solution. Subsequent experimental procedures began 14 days after DNBS administration to allow for recovery from the initial colonic inflammation.

2.4. Assessment of visceral sensitivity by visceromotor response and abdominal withdrawal reflex

The visceromotor response (VMR) to colorectal distension (CRD) was used as an objective measure of visceral sensitivity. Visceromotor response assessment was conducted in animals under light anaesthesia (2% isoflurane) 14 and 21 days after DNBS administration. The amplitude of the abdominal contraction consequent to colorectal stimulation (a balloon inflated with 0.5, 1, 2, and 3 mL referred to as distension volume) was quantified by electromyography recordings as reported by Parisio et al.[64] Behavioural responses to CRD (0.5, 1, 2, and 3 mL referred to as distension volume) were assessed through abdominal withdrawal reflex (AWR) measurement in conscious animals by using a semiquantitative score (0-4) as described previously.[16] The measurements were conducted 14, 21, 28, and 35 days after DNBS administration.

2.5. Upper gastrointestinal transit treatment and harvesting

All experiments were performed as previously described.[66,76,95] Before experiment, rats were fasted for 12 hours, maintaining the access to water ad libitum. After 15 minutes had elapsed, rats were then given an oral gavage with fluorescein isothiocyanate dextran (300 μL of 5 mg/mL 70 kDa FITC-dextran; Sigma, Milan, Italy). Forty min after completing the gavage, rats were anaesthetized with isoflurane and whole blood harvested through cardiac puncture. After blood harvest, the upper portion of the gastrointestinal tract was carefully removed, being cut proximally at the pyloric sphincter and distally at the cecum. The intestine was sectioned into 10 equal lengths, which were homogenized in 1 mL 1X PBS and then centrifugated. Fluorescence was determined in the supernatants collected from intestinal lining (10 μL) and content diluted into 90 μL 1X PBS; serum fluorescence was determined after dilution of 10 μL serum into 40 μL 1X PBS using a ClarioStar (BMG Labtech, Milan, Italy) at 483 to 530 nm. Readings were then collected and prepared for statistical analyses.

2.6. Drug administration

The selective A_3 AR agonist, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide (Cl-IB-MECA; Tocris Bioscience, Bristol, United Kingdom), and the selective A_3 receptor antagonist, 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate (MRS1523; Sigma-Aldrich, Milan, Italy), were dissolved in 5% DMSO and 5% TWEEN 20 saline solution for in vivo administration. The new, highly selective (10,000-fold vs each of the other 3 receptor subtypes) A_3AR agonist, MRS5980, (1S,2R,3S,4R,5S)-4-(2-((5-chlorothiophen-2-yl)ethynyl)-6-(methylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide[89] was dissolved in 5% DMSO saline solution for in vivo administration. As already reported,[50] the antiallodynic effects of the A_3AR agonists used in this work were lost in the A_3AR knockout mouse and in the presence of the A_3AR antagonist MRS1523.[49] N-[[4-(1,1-dimethylethyl)phenyl]methyl]-N-methyl-L-leucyl-N-(1,1-dimethylethyl)-O-(phenylmethyl)-L-tyrosinamide (PD173232) was purchased from Alomone Labs (Jerusalem, Israel) and dissolved in 5% DMSO and 5% TWEEN 20 saline solution for

in vivo administration. Both the vehicle and the DNBS control groups received an i.p. injection of the vehicle used for the administration of the respective molecules. The acute injection of the vehicles (5% DMSO or 5% DMSO + 5% TWEEN 20) did not alter visceral pain threshold and did not induce side effects in the animals. The guanylate cyclase-C (GC-C) agonist linaclotide (Allergan, Buckinghamshire, United Kingdom) was dissolved in water and orally administered 1 hour before starting the behavioural tests.[25] Cl-IB-MECA, MRS5980, and PD173232 were administered i.p. 15 minutes before the test. MRS1523 was administered i.p. 15 minutes before Cl-IB-MECA and MRS5980. Gastrointestinal transit study: the compound MRS5980 was dissolved in 5% DMSO, and then suspended in saline. MRS5980 and vehicle were administered through i.p. injection to all animals in study at 2.4 μmol·kg^{-1} body weight (1 mL/kg injection volume). Seventy kDa FITC-dextran (Sigma) was dosed by oral gavage at a concentration of 5 mg/mL, 300 μL per rat. Additional pharmacological characteristics of A_3 AR agonists are shown in the Supplemental material (available at http://links.lww.com/PAIN/B10).

2.7. Cell cultures

Primary DRG neurons (related to colon-sensitive innervations: T12, T13, L4, L5, L6, S1, S2) were isolated from vehicle- or DNBS-treated (at 14th day) rats and cultured for 1 to 2 days before being used for experiments as described.[19]

2.8. Electrophysiology

Whole-cell patch-clamp recordings were performed as previously described.[19] Passive membrane properties of DRG neurons isolated from control or DNBS-treated rats were investigated under physiological-like conditions by using the following K-gluconate- based pipette solution (mM): KGlu 130; NaCl 4.8; KCl 10; $MgCl_2$ 2; $CaCl_2$ 1; Na_2-ATP 2; Na_2-GTP 0.3; EGTA 3; HEPES 10 (pH 7.4 with KOH). Resting membrane potential (Vm) was measured immediately after seal breakthrough by switching the amplifier to the current-clamp mode. The calculated liquid junction potential for K-gluconate pipettes in our experimental conditions was 15.0 mV, and Vm values reported in the present research have been corrected accordingly.

Voltage-dependent Ca^{2+} currents (VDCCs) were recorded by using a Cs^+-based pipette solution having the following composition (mM): CsCl (130); NaCl (4.8); KCl (10); $MgCl_2$ (2); $CaCl_2$ (1); Na_2-ATP (2); Na_2-GTP (0.3); EGTA (3); and HEPES (10—pH 7.4 with CsOH). The extracellular solution was (in mM): NaCl (147); CsCl (4); $MgCl_2$ (1); and $CaCl_2$ (5); HEPES (10); D-glucose (10); pH 7.4 with NaOH. Tetrodotoxin (TTX; 1 μM) and 5-(4-butoxy-3-chlorophenyl)-N-[[2-(4-morpholinyl)-3-pyridinyl]methyl]-3-pyridinecarboxamide (A887826; 200 nM) were added to the extracellular solution to block TTX-sensitive Na_v1.1, 1.2, 1.3, 1.4, 1.6, 1.7 channels and TTX-resistant Na_v1.8, respectively. VDCC currents were evoked by a 0 mV step depolarization (200 ms) once every 30 seconds to minimize Ca^{2+} current run down. Peak Ca^{2+} current (I_{Ca} peak) was measured as the peak current amplitude reached during the first 50 ms of voltage step. Steady-state Ca^{2+} current (I_{Ca} st-state) was measured as the averaged current amplitude measured between 160 and 190 ms of voltage step. The current-to-voltage relationship (I-V plot) of Ca^{2+} currents was obtained by eliciting 10 depolarizing voltage steps (200-ms duration, 10-mV increments, 5-second interval) from −50 to +50 mV starting from a holding potential (Vh) of −65 mV.

Data were acquired with an Axopatch 200B amplifier (Axon Instruments, San Jose, CA), low-pass filtered at 10 kHz, and stored and analysed with pClamp 9.2 software (Axon Instruments). Membrane resistance (Rm) and membrane capacitance (Cm) were routinely measured by fast hyperpolarizing voltage pulses (from −60 to −70 mV, 40-ms duration). Averaged currents were normalized to cell capacitance and expressed as pA/pF.

Cell capacitance was used to estimate neuronal diameter by assuming an approximated spherical cell shape according to the calculated Cm for all biological membranes of 1 μF/cm^2 and to the equation of the sphere surface: $A = 4\pi r^2$.

The in vitro concentrations were chosen on the base of our previous work: the A_3AR agonists Cl-IB-MECA and MRS5980 were applied at 30 nM, a concentration able to produce maximal inhibition of Ca^{2+} currents in rat DRG neurons.[19]

2.9. Statistical analysis

Behavioural measurements were performed on 6 animals for each treatment. All the experimental procedures were performed by a researcher blind to the treatment. Results were expressed as mean ± SEM. The analysis of variance (ANOVA) of behavioural data was performed by one-way ANOVA with Bonferroni's significant difference procedure used for post hoc comparisons. Statistics of electrophysiological data was performed by Student paired or unpaired *t* test or by one-way ANOVA followed by Bonferroni's post hoc test, as appropriate. *P* values of less than 0.05 were considered significant. Data were analyzed using the "Origin 9" software (OriginLab, Northampton, MA). Data from gastrointestinal transit measures were analyzed in GraphPad Prism 7. Standard curves were fit using a nonlinear, hyperbolic equation for individual plates. A two-way ANOVA with Tukey post hoc test was performed using the variables of GI segment and treatment. Serum samples were compared using an unpaired *t* test. Data were considered statistically significant when $P < 0.05$ to detect 20% difference with 80% power (necessary n-values determined using G.Power3.1).

3. Results

3.1. A_3AR agonists reduce colitis-induced visceral hypersensitivity in rats

The visceromotor response and the abdominal withdrawal reflex (VMR and AWR) to CRD were measured by using progressively increasing balloon volumes as pressuring stimuli on the colon (highest volume: 3 mL, to avoid tissue damage). Fourteen and 21 days after DNBS injection, both VMR and AWR were significantly higher in comparison to controls (vehicle + vehicle) as shown, respectively, in **Figures 1A–B** (day 14) and **1C–D** (day 21). On day 14, the acute administration of MRS5980 (0.3, 1.2, and 2.4 μmol·kg^{-1}; i.p.) dose-dependently reduced the postinflammatory visceral hypersensitivity induced by DNBS; the magnitude of the reduction was similar for VMR and AWR. The highest dose (2.4 μmol·kg^{-1}) completely reversed the sensitivity alteration back to the value of control rats. MRS5980 1.2 μmol·kg^{-1} partially but significantly reduced the response of the animals to CRD. The lowest dose of MRS5980 (0.3 μmol·kg^{-1}) was ineffective in both tests (**Figs. 1A and B**). On day 21, the effect of MRS5980 (0.3, 1.2, and 2.4 μmol·kg^{-1}) was confirmed (VMR and AWR to CRD, **Figs. 1C and D**, respectively).

Figure 2A shows the result of the pretreatment with the selective A_3AR antagonist MRS1523[49] on the antihyperalgesic effect of MRS5980 on day 14. MRS1523 (5 μmol kg^{-1}) completely

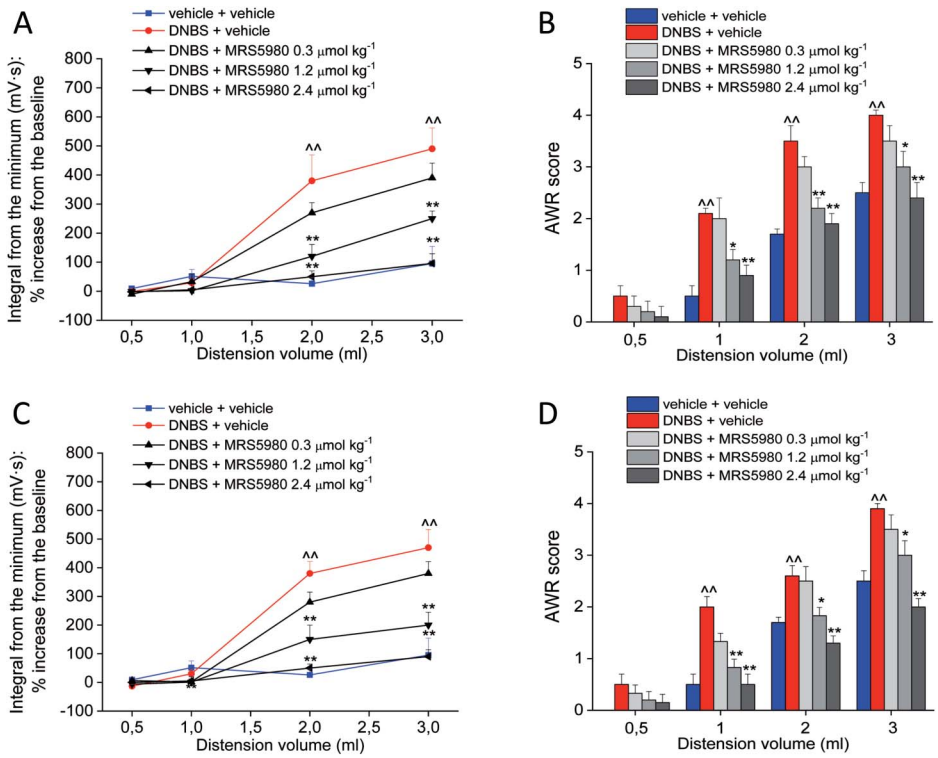

Figure 1. Effect of MRS5980 on postinflammatory visceral pain induced by DNBS. Effect of MRS5980 (0.3, 1.2, and 2.4 μmol·kg^{-1}; i.p.) on visceromotor response (VMR) to CRD (left column) and abdominal withdrawal reflex (right column) on day 14 (A and B) and day 21 (C and D) after DNBS-induced colonic inflammation. Each value is the mean ± SEM of 6 rats per group. ^^$P < 0.01$ vs vehicle-treated normal controls (blue). *$P < 0.05$ and **$P < 0.01$ vs DNBS + vehicle-treated group (red). CRD, colorectal distension.

abolished the pain-relieving effect of MRS5980 (2.4 μmol kg^{-1}) confirming a A_3AR mechanism of action. Furthermore, the pain-relieving effect evoked by A_3AR stimulation was confirmed by using another selective A_3AR agonist, Cl-IB-MECA. On day 14, the acute administration of Cl-IB-MECA dose-dependently relieved visceral pain in DNBS-treated animals, reducing their VMR to the value of controls (dosed at 1.2 μmol·kg^{-1}, **Fig. 2B**). The lowest dose of 0.3 μmol·kg^{-1} evoked a weaker effect only with the 2 mL stimulus. The Cl-IB-MECA (1.2 μmol·kg^{-1}) effect was blocked by MRS1523 (5 μmol·kg^{-1}, **Fig. 2B**).

3.2. N-type voltage-gated Ca^{2+} channel block relieves colitis-induced visceral pain in rats

A_3AR agonists have been recently reported to inhibit $Ca_v2.2$-mediated currents in DRG neurons, thus suggesting a possible mechanism for pain relief.[19] To verify this hypothesis, we evaluated the effect of acute administration of the selective N-type $Ca_v2.2$ blocker PD173212[38] in our visceral pain model. The test (VMR assessment) was performed on day 14 after DNBS injection. As shown in **Figure 3C**, PD173212 (0.0017-1.7 μmol·kg^{-1}, i.p.) dose-dependently reduced the visceral hypersensitivity induced by DNBS. The compound started to be effective at a dose of 0.017 μmol·kg^{-1} and completely relieved abdominal pain when administered at a ten-fold higher dose (**Fig. 2C**).

3.3. Comparison between the effects of MRS5980 and linaclotide in male and female rats

The effects of MRS5980 against the postinflammatory hypersensitivity induced by DNBS were evaluated in both male and female rats and compared with the effects of linaclotide, a guanylate cyclase-C (GC-C) agonist that represents the reference drug in the management of pain in patients affected by IBS with predominant constipation (IBS-C).[18,97] On day 14, 0.2 and 0.066 nmol kg^{-1} linaclotide strongly reduced the VMR to CRD in male DNBS-treated animals, whereas the ten-fold lower dose was ineffective (**Fig. 2D**).

Similar to males, 14 days after DNBS injection in female rats, both VMR and AWR to colorectal distension were significantly higher in comparison to controls (**Figs. 3A and B**). The increase of visceral sensitivity was even more pronounced in females than in males as DNBS-treated females showed a significantly increased response also when stimulated with the 0.5 mL volume of distension in both the test performed (**Figs. 3A and B**). The acute i.p. administration of MRS5980 dose-dependently reduced visceral hyperalgesia induced by DNBS in female rats. In particular, the highest doses (1.2 and 2.4 μmol·kg^{-1}) reversed visceral sensitivity alteration back to the value of controls in both VMR and AWR tests (**Figs. 3A and B**), showing similar efficacy to linaclotide (0.2 nmol·kg^{-1}, **Fig. 3A**). The lowest dose of MRS5980 (0.3 μmol·kg^{-1}) was ineffective in females like in males (**Figs. 3A and B**). To note, tests were performed in female animals synchronized for the estrous cycle choosing the proestrous/estrous phases to eliminate any possible confounding factor in the evaluation of A_3 receptor agonists' activity.

3.4. MRS5980 reduces persistent visceral pain

This type of pain has the peculiar tendency to become chronic in patients remitting from an intestinal damage.[84,85] Unlike VMR, the

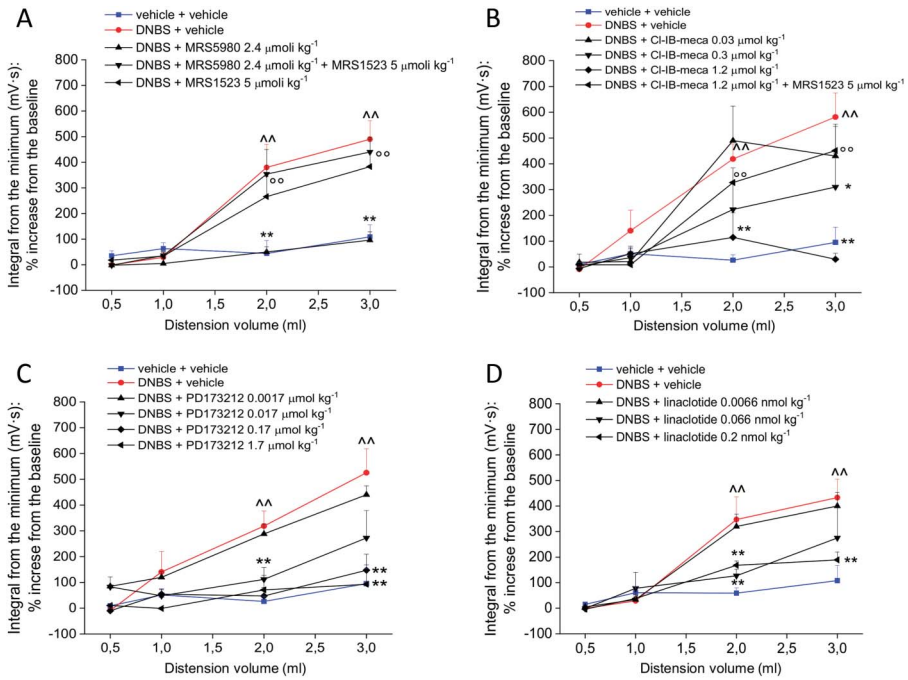

Figure 2. Evaluation of the role of adenosine A_3ARs and N-type voltage-gated calcium channels in visceral pain relief and comparison with the effect of reference drugs. Tests were performed 14 days after DNBS treatment by measuring the visceromotor response (VMR) to colorectal distension (CRD) after compound administration. (A) Effect of pretreatment with the selective A_3 antagonist MRS1523 (5 μmol·kg^{-1} i.p.) on the visceral pain-relieving effect of the highly selective A_3AR agonist, MRS5980 (5 μmol kg^{-1} i.p.). (B) Effect of the selective A_3 agonist, Cl-IB-MECA (0.03, 0.3, and 2.4 μmol·kg^{-1}; i.p.). (C) Effect of the selective N-type voltage-gated calcium channel ($Ca_v2.2$) blocker, PD173212 (0.0017-1.7 μmol·kg^{-1} i.p.). (D) Effect of linaclotide (0.0066-0.2 nmol·kg^{-1} p.o.). Each value is the mean ± SEM of 6 rats per group. ^^$P < 0.01$ vs vehicle-treated normal controls (blue). *$P < 0.05$ and **$P < 0.01$ vs DNBS + vehicle-treated group (red). °°$P < 0.01$ vs DNBS + MRS5980 (2.4 μmol·kg^{-1}, black triangles in A) or Cl-IB-MECA (1.2 μmol kg^{-1}, black triangles in B).

assessment of AWR allows the evaluation of DNBS-induced hypersensitivity for a long time permitting the measurement of persistent pain. The behavioural response was still altered 28 and 35 days after the colonic irritation (**Figs. 4A and B**, respectively). Hence, we used this test to evaluate the effect of MRS5980 on visceral pain in a more delayed phase after the initial insult. On Day 28 and 35, the pain-relieving effect of MRS5980 (0.3, 1.2, and 2.4 μmol·kg^{-1}) was as potent as that seen earlier. The effect on chronic hypersensitivity was dose-dependent, and the highest dose (2.4 μmol·kg^{-1}) was again able to reverse the pain threshold of DNBS-treated animals to the value of controls (**Figs. 4A and B**).

3.5. MRS5980 does not impair gastrointestinal transit

To determine whether the A_3AR agonist MRS5980 influenced gut motility, we assessed total gastrointestinal transit. Groups of 6 rats were treated with either vehicle (5% DMSO) or MRS5980 (2.4 μmol·kg^{-1}). Fifteen min later, animals received an oral gavage of 70 kDa FITC dextran (5 mg/mL, 300 mL per rat). Samples were harvested 40 min after 70 kDa FITC-dextran administration. Total fluorescence of 70 kDa FITC-dextran was determined for each of the 10 segments (**Fig. 5A**). 70 kDa FITC was detected in vehicle-treated samples in segments 5 (8.9 ± 2.5 μg/mL), 6 (3.0 ± 2.2 μg/mL), and 8 (6.3 ± 3.2 μg/mL). MRS5980 treatment showed a similar pattern of GI transit of 70 kDa FITC; segment 5:11.17 ± 5.0 μg/mL,

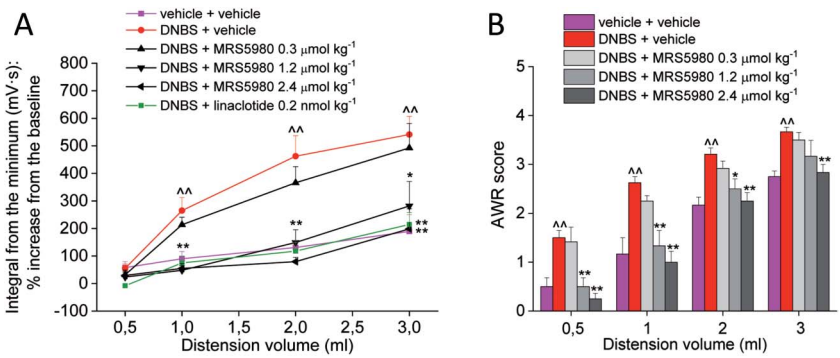

Figure 3. Effect of MRS5980 and linaclotide on visceral pain induced by DNBS in female rats. Effect of MRS5980 (0.3, 1.2, and 2.4 μmol·kg^{-1}; i.p.) and linaclotide (0.2 nmol·kg^{-1} p.o.) on visceromotor response (VMR, A) to CRD on day 14 after DNBS injection. Effect of MRS5980 (0.3, 1.2, and 2.4 μmol·kg^{-1}; i.p.) on abdominal withdrawal reflex (AWR, B) to CRD on day 14 after DNBS injection. Each value is the mean ± SEM of 6 rats per group. ^^$P < 0.01$ vs vehicle-treated normal controls (violet). *$P < 0.05$ and **$P < 0.01$ vs DNBS + vehicle-treated group (red). CRD, colorectal distension.

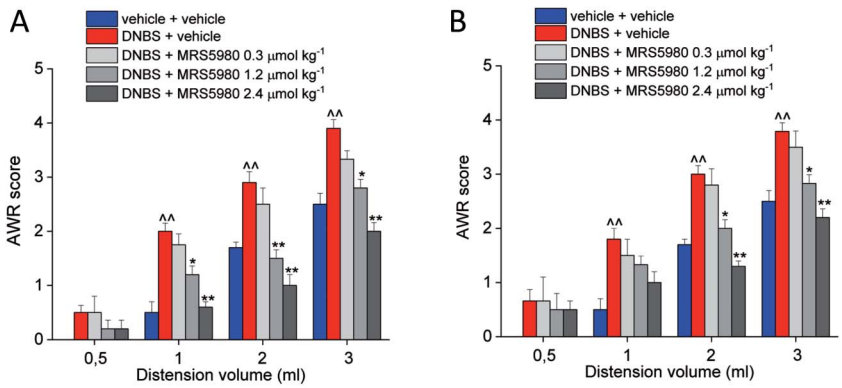

Figure 4. Effect of MRS5980 on persistent visceral pain induced by DNBS injection. Effect of MRS5980 (0.3, 1.2, and 2.4 μmol kg^{-1}; i.p.) was observed 28 days (A) and 35 days (B) after DNBS treatment. The compound was administered 15 minutes before the first colorectal distension (CRD). Visceral pain was assessed by measuring the animal abdominal withdrawal reflex (AWR) to CRD. Each value is the mean ± SEM of 6 rats per group. ^^$P < 0.01$ vs vehicle-treated normal controls (blue). *$P < 0.05$ and **$P < 0.01$ vs DNBS + vehicle-treated group (red).

segment 6: 11.2 ± 6.3 μg/mL, and segment 8:15.1 ± 14.3 μg/mL. Detection of 70 kDa FITC-dextran in all other segments was <4 μg/mL (2.1-3.7 μg/mL). No significant difference between treatments ($P = 0.453$) or the interaction of treatment and GI segment (F(9,92) = $P = 0.8676$) was found. FITC levels were significantly different between the GI segments ($P = 0.0010$) indicative of intestinal movement as anticipated. These data indicate that A_3AR agonism with MRS5980 does not statistically reduce GI transit as compared to vehicle.

To determine whether MRS5980 influenced paracellular uptake of 70 kDa from the GI tract, we measured fluorescence in serum collected from the same animals (**Fig. 5B**). Serum levels of 70 kDa FITC-dextran were minimal (vehicle:0.82 ± 0.27; MRS5980:1.38 ± 0.57) and not significantly different ($P = 0.3955$). These data suggest that MRS5980 does not influence the paracellular integrity of GI epithelium alone as compared to vehicle.

3.6. A_3R agonists inhibit N-type voltage-gated Ca^{2+} channels in dorsal root ganglia neurons isolated from control or DNBS-treated rats

To gain insight into mechanisms underlying the antihyperalgesic role of A_3ARs, we explored electrophysiological properties and Cl-IB-MECA effects in DRG primary sensory neurons isolated from vehicle-treated (control group) or DNBS-treated rats (14 days after treatment). Concerning passive membrane properties (**Fig. 6A**), DNBS neurons presented a more depolarized Vm in comparison to those from the vehicle-treated animals (**Fig. 6A**, left panel: from −61.4 ± 2.2 mV in vehicle-treated to −52.0 ± 2.6 mV in DNBS group, n = 23 and n = 33, respectively, $P = 0.0143$, unpaired Student *t* test), whereas no obvious differences were found in Rm or Cm (**Fig. 6A**, central and right panels). Cm was, on average, 27.6 ± 4.1 pF in vehicle-treated animals and 28.3 ± 2.7 pF in DNBS-treated animals (**Fig. 6A**, right panel), corresponding to a cell diameter of 29.9 and 30.7 μm, respectively, thus confirming that present data were collected from small–medium-sized DRG neurons. Of note, VDCCs, activated by a depolarizing voltage step protocol (from −50 to +50 mV, 200 ms: see insert in **Fig. 6B**), showed some differences in DNBS-treated vs vehicle-treated rats. Although if total peak currents were unchanged, either in amplitude (**Fig. 6C**) or time to peak (**Fig. 6D**), Ca^{2+} currents measured at the steady state in DNBS neurons were significantly smaller in amplitude (**Fig. 6E** right panel: from −21.5 ± 8.1 pA/pF in vehicle-treated to −1.4 ± 4.0 pA/pF in DNBS group, n = 15 and n = 14, respectively, $P = 0.0393$, unpaired Student *t* test).

Consistent with our previous work[19] and with the above in vivo results (**Fig. 2**), we confirmed that Cl-IB-MECA (30 nM) inhibited Ca^{2+} currents in DRG neurons isolated either from vehicle- or DNBS-treated rats (**Figs. 7A and B**) and the effect was prevented

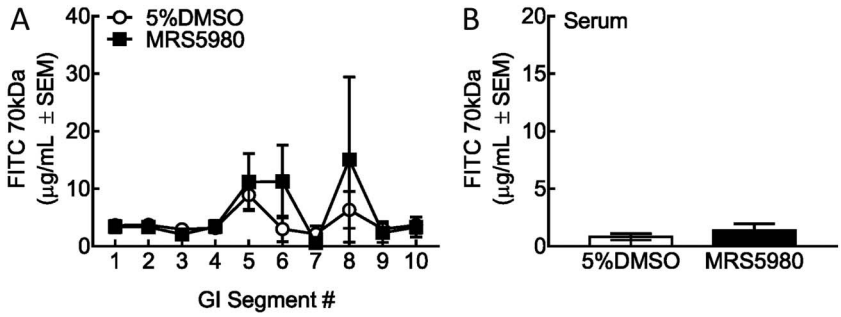

Figure 5. Gastrointestinal transit is not reduced by acute A_3AR agonism with MRS5980. (A) Amount of 70 kDA FITC-dextran in small intestine segments 15 minutes after MRS5980 or vehicle dosing and 40 minutes after oral gavage of the marker. Two-way ANOVA, interaction: F(9,92) = 0.8676; $P = 0.9553$; GI Section: $P = 0.0010$; Treatment: $P = 0.4584$ (B) Amount of 70,000 kDa FITC-dextran in serum samples of 15 minutes after MRS5980 or vehicle dosing and 40 minutes after oral gavage of the marker (unpaired *t* test, $P = 0.3955$ MRS5980 compared to vehicle.) Each value is the mean ± S.E.M. of 6 rats. ANOVA, analysis of variance.

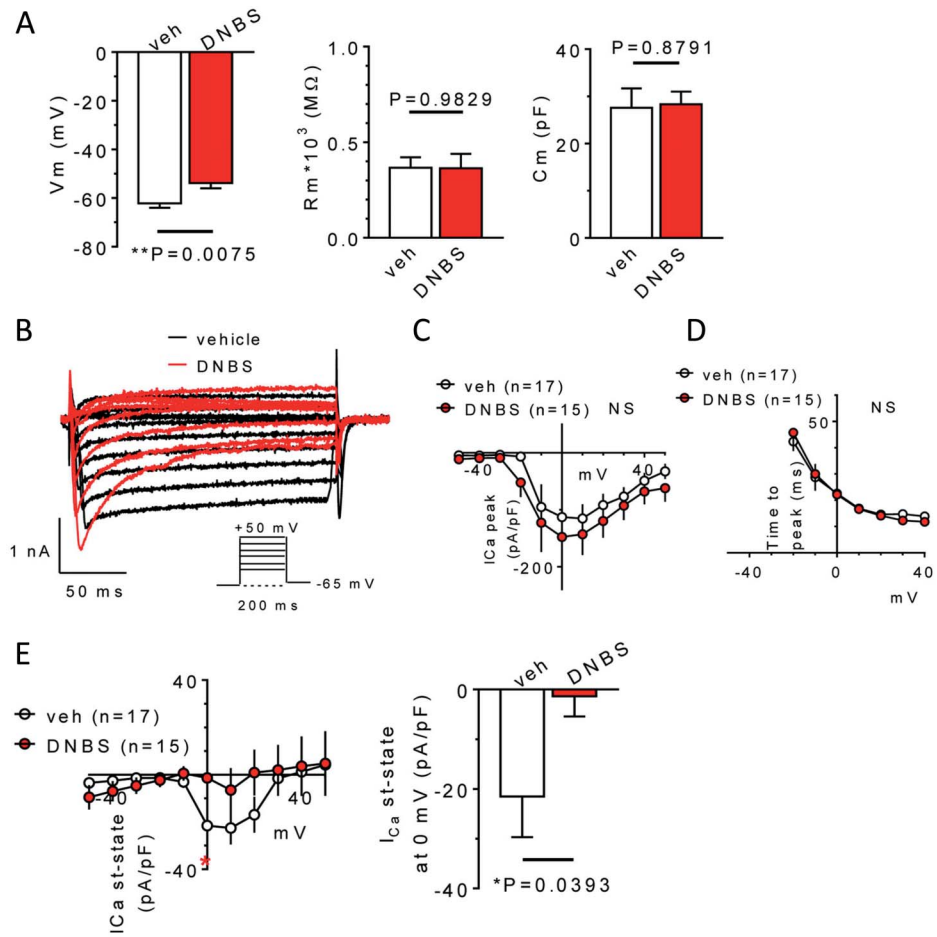

Figure 6. Electrophysiological properties of DRG neurons isolated from vehicle- or DNBS-treated rats. (A) Pooled data (mean ± SEM) of passive membrane properties (resting membrane potential: Vm, left panel; membrane resistance: Rm, central panel, and cell capacitance: Cm, right panel) measured in DRG neurons isolated from vehicle- (veh) or DBNS-treated rats (DNBS). Unpaired Student *t* test, n = 23 and n = 33, respectively. (B) Original Ca^{2+} current traces elicited in representative DRG neurons isolated from a vehicle- (black traces) or DNBS-treated (red traces) rat by applying a depolarizing voltage step protocol (from −50 to +50 mV, 200 ms duration, Vh = −65 mV: see inset). Averaged I-V plots of peak Ca^{2+} currents (C), and pooled data of time to peak of Ca^{2+} currents (D), measured at different step potentials in 17 cells isolated from vehicle-treated rats and 15 cells isolated from DNBS-treated animals. No significant differences were found between groups at any step potential, unpaired Student *t* test. (E) Left panel: averaged I-V plot of steady-state Ca^{2+} currents measured in 17 cells isolated from vehicle-treated rats and 15 cells isolated from DNBS-treated animals. Right panel: pooled data of the same currents measured at the 0 mV step. (A) significant reduction of steady-state Ca^{2+} currents was found in the DNBS-treated group, $P = 0.0393$, unpaired Student *t* test. DRG, dorsal root ganglia.

by the A_3AR antagonist MRS1523 (100 nM: **Fig. 7B**) and by the N-type Ca^{2+} channel blocker PD173232 (1 μM: **Figs. 7C–E**). No differences were found in the percentage of Cl-IB-MECA-inhibited Ca^{2+} current in neurons isolated from vehicle- or DNBS-treated rats (20.0 ± 4.1% and 22.5 ± 6.1% of I_{Ca} inhibition in Cl-IB-MECA, respectively, n = 7 in both groups; $P = 0.7332$ unpaired Student *t* test; **Fig. 7C**) nor in the percentage of PD173232-blocked currents (72.3 ± 4.8% and 77.7 ± 8.2%, of I_{Ca} inhibition in PD173232, respectively; n = 4 in both groups; $P = 0.5867$ unpaired Student *t* test, data not shown). Similar results to those obtained with the prototypical A_3AR agonist Cl-IB-MECA were recorded with MRS5980 (30 nM): the compound inhibited, to a similar extent, Ca^{2+} currents either in neurons isolated from vehicle- or DNBS-treated rats and the effect was prevented by MRS1523 and by the N-type channel blocker PD173232 (1 μM: **Figs. 7C–E**).

4. Discussion

In the present work, we studied the role of A_3AR as a possible new pharmacological target for relieving visceral pain. The novel highly selective second-generation A_3AR agonist, MRS5980, as well as the first-generation A_3AR agonist, Cl-IB-MECA, were effective against postinflammatory visceral hypersensitivity in rats. The intracolonic injection of DNBS in rodents induces a long-lasting visceral hypersensitivity,[1,32,33] which can be measured with high reproducibility as a lowered sensory threshold to CRD.[52,61,82] DNBS-induced visceral hypersensitivity persists after the resolution of the acute inflammatory phase,[1,40,53] making this model suitable to investigate visceral pain related not only related to IBD but also to IBS, a chronic disease characterized by a marked abdominal pain in the absence of histopathological explanations.[84,90]

The administration of A_3AR agonists in the model of post-inflammatory visceral pain showed an efficacy equivalent to that of linaclotide, an approved treatment for reducing abdominal pain (and also abdominal bloating and bowel symptoms) in adult patients suffering from IBS with predominant constipation (IBS-C).[13,18]

The efficacy of MRS5980 and linaclotide was confirmed in female rats. These data have a clinical significance because IBS affects more women than men.[12] Abundant evidence from

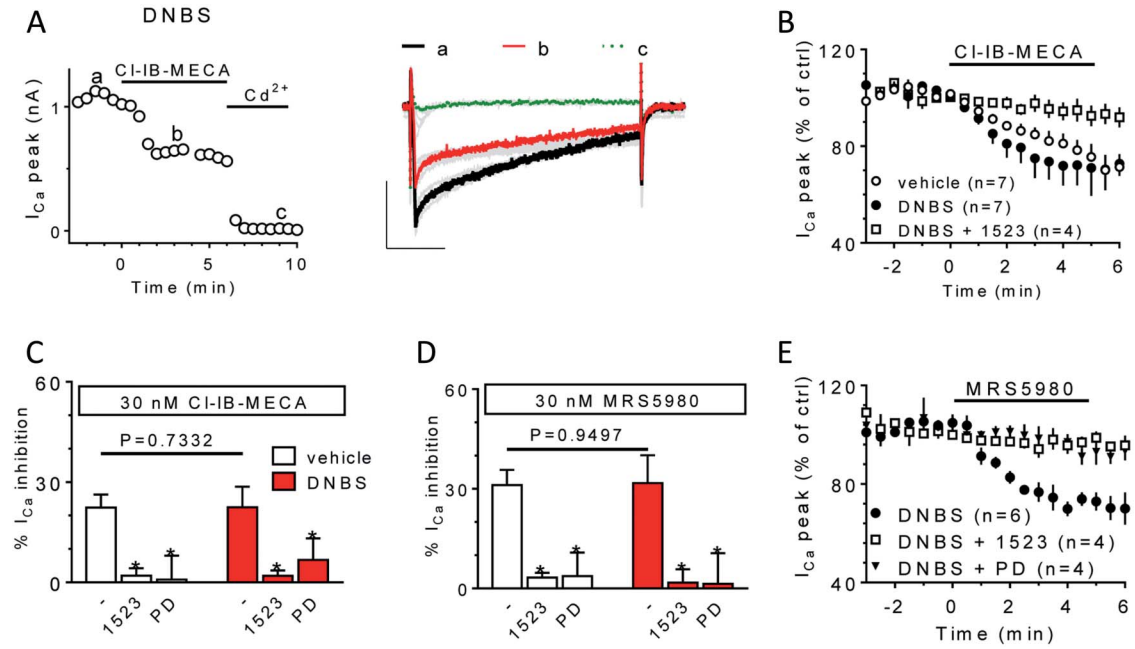

Figure 7. Effects of the adenosine A_3R agonists CI-IB-MECA and MRS5950 on voltage-gated Ca^{2+} currents in isolated DRG neurons. (A) Time course (left panel) and representative Ca^{2+} current traces (right panel) measured in a representative DRG neuron isolated from a DNBS-treated rat (DNBS) before and after the application of the A_3R agonist CI-IB-MECA (30 nM) or the Ca^{2+} channel blocker Cd^{2+} (100 μM). (B) Averaged time courses of peak Ca^{2+} currents during CI-IB-MECA (30 nM) superfusion in vehicle-treated or DNBS-treated animals in the absence or presence of the A_3AR antagonist, MRS1523 (1523: 100 nM). Pooled data (mean ± SEM) of % Ca^{2+} current inhibition induced by CI-IB-MECA (C) or MRS5980 (D) applied alone or in combination with MRS1523 or PD173232 (PD; 1 μM) in vehicle-treated (white columns) or DNBS-treated (red columns) animals. In all panels: *P <0.05 vs respective control, paired Student t test. (E) Averaged time course of peak Ca^{2+} currents before or after MRS5980 (30 nM) superfusion DNBS rats in the presence of the A_3AR antagonist MRS1523 or the Ca^{2+} channel blocker PD173232 (1 μM). DRG, dorsal root ganglia.

epidemiologic studies clearly demonstrates that women are at substantially greater risk for many clinical pain conditions.[5] In accordance, the present results showed a higher visceral hypersensitivity in female animals than in males. This phenomenon is not limited to abdominal/pelvic pain (such as IBS, painful bladder syndrome, and dyspareunia), but also includes conditions associated with neuropathic pain, musculoskeletal pain, orofacial pain, and headache/migraine.[27] Women are therefore more likely than men to pursue a variety of treatments for many painful conditions.

The trinitrobenzenesulfonic acid (TNBS)-induced model was used in preclinical studies to identify the potential clinical utility of linaclotide.[25] Our results with linaclotide in the DNBS-induced model support previous findings. The analgesic mechanism of linaclotide involves the activation of guanylate cyclase-C (GC-C) expressed on mucosal epithelial cells, resulting in the production and release of cyclic guanosine-3′,5′-monophosphate (cGMP). The extracellular cGMP acts on and inhibits nociceptors, thereby reducing nociception.[13] Although linaclotide is an efficacious, well-tolerated treatment option for improving both bowel symptoms and abdominal pain in IBS-C, it acts as a secretagogues and this implies that most patients reported episodes of diarrhea within the first 2 to 4 weeks of treatment.[18] This adverse effect also makes linaclotide unsuitable for patients affected by diarrhoea-predominant IBS or IBS with alternating constipation and diarrhoea. In healthy condition, adenosine modulated colonic cholinergic motility through activation of A_3 receptors in the myenteric plexus. Antonioli et al. demonstrated that A_3 receptor-mediated tonic inhibitory control by adenosine was impaired in inflamed bowel, despite the increased density of functioning and pharmacologically recruitable A_3 receptors found in the gut.[2] To verify a possible interaction of MRS5980 with gut motility, we performed focused experiments. Our findings using 70 kDa FITC-dextran revealed that MRS5980 does not impair gastrointestinal transit as compared to vehicle. Importantly, these results indicate that the antihyperalgesic efficacy of MRS5980 in visceral pain condition is not associated to motility alteration, suggesting a mechanism related to sensitivity modulation rather than spasmolytic activity. This profile is different, eg, from the opioid analgesic drugs, mainly used in patients affected by IBS-D, that in the same experiment reduce intestinal transit leading to constipation in humans.[10,47,66,76] Anyway, the impact of A_3 receptor agonists' repeated administrations on intestinal motility and sensitivity is an aspect that needs to be explored further in the perspective of developing these molecules as drugs and of establishing a therapeutic regime in patients. The lack of significant long-term effects on intestinal motility could privilege A_3 adenosine agonists over all the other drugs currently in use. Anyway, in clinical trials investigating the efficacy of A_3 receptor agonist on rheumatoid arthritis and psoriasis, no relevant gastrointestinal side effects were reported.[20,41,80]The efficacy of A_3AR agonists against visceral pain is consistent with data showing that the A_3AR is involved with multiple pain mechanisms at peripheral, spinal, and supraspinal levels.[43,50] There is evidence that A_3AR agonists are antihyperalgesic against neuropathic pain through inhibition of the astrocyte-associated neuroinflammatory response in the spinal cord,[42,44,96] a phenomenon strongly involved in pain persistence.[22,23] A_3AR activation has been reported to enhance the formation of anti-inflammatory cytokines[36,42,44,96] and the production of glial-derived neuroprotective substances.[99] Furthermore, in vitro and in vivo studies demonstrate that A_3AR produces its effects by inhibiting the p38 MAPK and NF-κB signaling pathways[42,55,92,94] and inflammasome activity.[96] All these mechanisms may contribute to the relief

of colitis-evoked visceral pain consistently with the reports that A_3AR activation reduced colitis-induced tissue injury by modulating the NF-κB signalling pathway and through inhibiting NLRP3 inflammasome activation and pyroptosis in human colonic epithelial cells.[70,71]

A_3ARs may limit excitatory neurotransmission, which is altered in chronic[34] as well as in visceral pain.[31,48,98] For example, the protective role of A_3AR in the first phase of ischemia seems to be at least partly related to a decrease in synaptic transmission.[67,68,72] Moreover, A_3AR activation protects against the neurotoxic intracellular Ca^{2+} rise mediated by P2X7 or NMDA receptors.[103,104] All the mechanisms described above could account for a therapeutic effect of repeated administrations of A_3 receptor agonists, which is another aspect interesting to study. Anyway, the acute efficacy showed by A_3 receptor agonists in reducing visceral hypersensitivity can be only partially explained by the mechanisms of action previously cited. This consideration has aroused our interest in elucidating further the pharmacodynamic mechanisms of A_3 receptor agonists on pain.

As recently demonstrated,[19] selective A_3AR stimulation inhibits N-type $Ca_v2.2$ opening in isolated rat DRG neurons. Here, we found that both Cl-IB-MECA and MRS5980 significantly inhibited $Ca_v2.2$ activation in DRG neurons of DNBS-treated rats. The effect was prevented by the selective A_3AR antagonist, MRS1523, and by the $Ca_v2.2$ blocker, PD173212, confirming the involvement of $Ca_v2.2$ in the A_3AR-mediated effect. We did not measure a significant difference between A_3AR-mediated inhibition in DRG neurons isolated from DNBS-treated vs vehicle-treated animals. However, alterations in passive membrane properties and steady-state Ca^{2+} currents were observed between the 2 groups. The resting membrane potential was significantly more depolarized in DRG neurons isolated from DNBS-treated animals, suggesting a hyperexcitable state. Interestingly, steady-state Ca^{2+} currents, but not peak Ca^{2+} currents, were markedly reduced in DRG neurons isolated from DNBS animals. In an attempt to explain the relationship between steady-state Ca^{2+} current reduction in DRG neurons and visceral hypersensitivity, we hypothesize that a decrease in sustained Ca^{2+} influx into the cell can lead to membrane potential instability and depolarization through a decrease of Ca^{2+}-activated K^+ channel (K_{Ca}) opening. Ca^{2+}-activated K^+ channel is known to stabilize the membrane potential and participate in repolarizing neurons after action potential firing, thus avoiding bursting activity.[37,63,102]

Moreover, a recent publication demonstrates that $Ca_v2.2$ induces a voltage-dependent, but Ca^{2+}-independent ATP secretion from the soma of DRG neurons.[15] ATP is a powerful mediator of pain, for example, ATP-induced P2X3 receptor activation is involved in visceral pain, as shown by the ability of P2X3 antagonist A-317491 to potently reduce hypersensitivity.[21]

The hypothesis that $Ca_v2.2$-mediated modulation contributes to A_3AR-mediated antihyperalgesia is supported by other evidence. Modulation of $Ca_v2.2$ channel activity is implied in the pharmacodynamics of pain-relieving compounds, such as other Gi-coupled receptor agonists, eg, opioids, cannabinoids, neuropeptide Y, and substance P.[7,78,81,87,91] Also, the neuropathic pain analgesics gabapentin and pregabalin[28] inhibit $Ca_v2.2$-mediated synaptic transmission.[6,88] Gabapentinoids are effective in reducing visceral pain and preventing spinal neuronal activation associated with CRD in animals[62,69,79] and have been suggested to be treatments for IBS.[11] A direct inhibitor of $Ca_v2.2$ channel activity, ziconotide, is currently used clinically for pain therapy, although it is limited to the intrathecal route.[56] In our hands, the i.p. administration of the specific $Ca_v2.2$ inhibitor PD173212[38,56,73] significantly decreased visceral hypersensitivity in DNBS-treated animals. In conclusion, A_3AR agonists seem to be a promising resource for visceral pain management, an unmet medical need that impairs quality of life.

Conflict of interest statement

K.A. Jacobson thanks the NIDDK Intramural Research Program (ZIA DK031117). D. Salvemini is a cofounder of BioIntervene, Inc, which licensed related intellectual property from Saint Louis University. The remaining authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B10.

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