Supplemental material and methods

Pharmacological characteristics of A_3 agonists. Cl-IB-MECA has been established as moderately A_3-receptor selective in human, rat and mouse [5]. A reference to the human and mouse A_3-receptor selectivity of MRS5980 is: Tosh et al. [16]. Although the affinity was not measured specifically in the rat, this chemical series of (N)-methanocarba nucleosides, including representative agonist MRS5698, has shown in vivo potency and efficacy at the rat A_3 adenosine receptor [8; 15]
The C_{max} of MRS5980 following oral administration (1 mg/kg, p.o.) in the rat is 29 ng/mL (equal to 63 nM) [17]. We also know that MRS5980 is completely protective (100% reversal of mechano-allodynia) in the CCI model of chronic neuropathic pain in the mouse at a dose of 3 μmol/kg, p.o., which is equal to 1.37 mg/kg [16]. Here, an i.p. dose of MRS5980 of 2.4 μmol kg^{-1} (equal to 1.1 mg/kg) was effective in the rat (Figure 2A). This dose completely reversed the sensitivity alteration back to the value of control rats. This dose is roughly comparable to the fully efficacious dose in the mouse (3 μmol/kg, p.o.), and in the rat is expected to achieve a similar or greater (because it is administered i.p. rather than p.o.) peak plasma concentration as seen in the pharmacokinetic experiment.

Animals. We used male and female Sprague-Dawley rats (Envigo, Varese, Italy) weighing approximately 220-250 g at the beginning of the experimental procedure. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least 1 week after their arrival. Four rats were housed per cage (size 26 × 41 cm). They were fed a standard laboratory diet and tap water ad libitum, and kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European Parliament and of the European Union Council (22 September 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 [9]. All efforts were made to minimize animal suffering and to reduce the number of animals used. Gastrointestinal motility assays (GI) were conducted using twelve, Sprague-Dawley, male rats (Envigo, 250-350g) 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.

Determination of female estrous cycle phases. Estrous cycle was assessed in rats by analysing rat vaginal smears. Each animal was anaesthetized with isofluorane (4%) to collect the vaginal smears. A pipette tip was filled with approximately 0.2 ml of sterile water and inserted approximately 3/5 mm into the rat's vagina. Sterile water was quickly released from the pipette, then immediately drawn back into it. The sample containing cells was placed on an untreated glass microscope slide and viewed while still wet under a light microscope at 100 magnification, for determining the stage of estrus [4]. 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 the majority of studies this phase is reported to be related to the highest pain sensitivity [6; 7; 10; 11; 13], therefore suitable for studying pain relieving compounds.

Assessment of visceral sensitivity by Viscero-Motor Response. The viscero-motor response (VMR) to colorectal balloon distension was used as an objective measure of visceral sensitivity. Two EMG electrodes were sutured into the external oblique abdominal muscle under deep anaesthesia and exteriorised dorsally [2]. VMR assessment was carried out under light anaesthesia (2% isoflurane). A lubricated latex balloon (length: 4.5 cm) was attached on an embolectomy catheter and connected to a water-filled syringe used to perform colorectal distension (CRD). The syringe was used to fill the balloon placed into the colon with various volumes of water (0.5, 1, 2, 3 ml, referred to as distension volume). The EMG signal consequent to colorectal stimulation was recorded, amplified and filtered (Animal Bio Amp, ADInstruments, Colorado Springs, CO, USA), digitised (PowerLab 4/35, ADInstruments), and analysed and quantified using LabChart 8 (ADInstruments). To quantify the VMR magnitude at each distension volume, the area under the curve (AUC) immediately before the distension (30 s) was subtracted from the AUC during the balloon distension (30 s) and responses were expressed as percentage increase from the baseline. The time elapsed between two consecutive distensions was 5 min, and the entire protocol lasted 45 min. The measurements were carried out 14 and 21 days after DNBS administration.

Assessment of visceral sensitivity by Abdominal Withdrawal Reflex. Behavioral responses to CRD were assessed via Abdominal Withdrawal Reflex (AWR) measurement in conscious animals using a semi-quantitative score as described previously [1]. Briefly, rats were anesthetized with isoflurane, and a lubricated latex balloon (length: 4.5 cm), attached to polyethylene tubing, assembled to an embolectomy catheter and connected to a syringe filled with water was inserted through the anus into the rectum and descending colon of adult rats. The tubing was taped to the tail to hold the balloon in place. Then rats were allowed to recover from the anaesthesia for 30 min.

AWR measurement consisted of visual observation of animal responses to graded CRD (0.5, 1, 2, 3 mL) by blinded observers who assigned AWR scores: No behavioural response to colorectal distention (0); Immobile during colorectal distention and occasional head clinching at stimulus onset (1); Mild abdominal muscle contraction but the absence of abdomen lifting from the platform (2); Observed strong contraction of the abdominal muscles and lifting of the abdomen off the platform (3); Body arching and lifting of the pelvic structures (4). The measurements were carried out 14, 21, 28 and 35 days after DNBS administration.

Upper gastro-intestinal transit treatment and harvesting. All experiments were carried out during a diurnal period of day/night cycle and performed as described [12; 14; 18]. Twelve hours prior to experiment rats were fasted; access to water was allowed *ad libitum* for duration of fasting period. Rats were separated into two treatment groups; these consisted of 1) MRS5980 (n=6) or 2) 5% DMSO in saline vehicle (n=6). After administering drugs, rats were placed back into cages for 15 min. After 15 min had elapsed rats were then given an oral gavage with fluorescein isothiocyanate dextran (300 µl of 5 mg/ml 70kDa FITC-dextran; Sigma, Milan, Italy). Upon completion of gavage, animals were placed back into their respective cages for 40 min. Rats were anaesthetized with isoflurane and whole blood harvested via cardiac puncture and allowed to coagulate for 30 min at room temperature, after which serum was collected by centrifuging coagulated blood at 4° C at 10,000 RPM for 10 min. Following blood harvest, the upper portion of the gastro-intestinal tract was carefully removed, being cut proximally at the pyloric sphincter and distally at the cecum. This section of intestine was then removed, stretched out and measured (total length). Based upon this measurement, the intestine was sectioned into 10 equal lengths and placed in pre-weighed 15 ml centrifuge tubes containing 1 ml 1X PBS (prepared in house); tubes were re-weighed to obtain weight of each intestinal section. Samples were then placed on ice and homogenized for 45 s in 1 ml 1X PBS using an Ultra Turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany). Homogenized samples were then centrifuged for 15 min at 14,000 RPM with the supernatant decanted into a new identically labeled tube for fluorescence reading. A FITC-dextran standard was prepared (1250, 937.5, 625, 437.5, 312.5, 187.5, 62.5 and 0 µg/ml) in duplicate. Fluorescence was determined in samples derived from supernatant collected from intestinal lining (10 µl) and content diluted into 90 µl 1X PBS; serum fluorescence was determined following dilution of 10 µl serum into 40 µl 1X PBS using a ClarioStar (BMG Labtech, Milan, Italy) at 483-530 nm. Readings were then collected and prepared for statistical analyses.

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 as described [3]. Briefly, ganglia were bilaterally excised and enzymatically digested using 2 mg/ml of collagenase type 1A and 1 mg/ml of trypsin (both compounds from SIGMA-Aldrich) in Hank's Balanced Salt Solution (HBSS) (25–35 min at 37 °C). Cells were then pelleted and resuspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented by 10% heat-inactivated horse serum and 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine for mechanical digestion. After centrifugation (1200 x g, 5 min), neurons were suspended in the above medium enriched with 100 ng/ml mouse neuronal growth factor (NGF) and 2.5 mM cytosine-β-D-arabino-furanoside free base, and then plated on 13 mm glass coverslips coated with poly-L-lysine (8.3 mM) and laminin (5 mM). DRG neurons were cultured for 1–2 days before being used for experiments.

Electrophysiology. Whole-cell patch-clamp recordings were performed at room temperature (RT: 20-22°C) as previously described [3]. Briefly, cells were transferred to a 1 ml recording chamber mounted on an inverted microscope platform (Olympus CKX41, Milan, Italy) and superfused at a flow rate of 1.5 ml/min by a three-way perfusion valve controller (Harvard Apparatus, Holliston, MA, USA) by the following extracellular solution (mM): NaCl 147; KCl 4; $MgCl_2$ 1; $CaCl_2$ 5; HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 10; D-glucose 10 (pH 7.4 with NaOH).

Borosilicate glass electrodes (Harvard Apparatus) were pulled with a Sutter Instruments puller (model P-87) to a final tip resistance of 1.5–3 MΩ. 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 break-through 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 s 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 s 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, CA), low-pass filtered at 10 kHz, and stored and analysed with pClamp 9.2 software (Axon Instruments, CA). 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 $\mu F/cm^2$ 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 [3].

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