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In vitro and *in vivo* characterization of the bifunctional μ- and δ- opioid receptors ligand MCRT on mouse gastrointestinal motility

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ABSTRACT

Background: Chimeric opioid MCRT was a novel multi-target ligand based on morphiceptin and PFRTic-NH_2, and produced potent analgesia (ED_{50} = 0.03 nmol/mouse) with less upper gastrointestinal dysmotility. In this study, we sought to perform the tests to evaluate the pharmacological effects of MCRT on distal colon motility and defecation function. Moreover, opioid receptor antagonists and neuropeptide FF (NPFF) receptor antagonists were utilized to explore the mechanisms.

Methods: Isolated mouse colon bioassay and colonic bead expulsion were to characterize MCRT-induced inhibition of colonic motility *in vitro* and *in vivo*, respectively. Fecal pellet output was to evaluate the defecation function.

Results: (1) *In vitro*, MCRT increased colonic contraction *via* μ- and δ- opioid receptors (MOR and DOR). (2) *In vivo*, MCRT delayed colonic bead expulsion (ED_{50} = 1.1 nmol/mouse) independent of opioid and NPFF receptors. (3) *In vivo*, MCRT inhibited fecal number (ED_{50} = 1.43 nmol/mouse) and dry weight (ED_{50} = 1.63 nmol/mouse), which was mediated by DOR partially but not MOR.

Conclusions: (1) Data indicated that MCRT was less prone to induce gastrointestinal dysmotility at analgesic doses, and provided a possibility for safer opioid analgesic. (2) Based on the mechanism explorations, we speculated on the existence of such an opioid receptor subtype or MOR/DOR heterodimer, which was involved in the central analgesia and the *in vitro* colonic contractions but not the central colonic dysmotility.

1. Introduction

While highly efficacious at alleviating pain, clinical applications of opioid were restricted to a broad array of unwanted effects, including tolerance, addiction, constipation, and respiratory depression (Ricardo Buenaventura et al., 2008; Chou et al., 2015). Among these side-effects, the morbidity rate of constipation was reported as much as 40–95% after opioid therapy, and constipation was considered as a major issue (Imam et al., 2018). In preclinical trials, the subcutaneous analgesic ED_{50} value for morphine was 2.27-fold and 1.52-fold greater than that in inhibition of upper gastrointestinal and colorectal transit, respectively (Mori et al., 2013). Endomorphins (EMs), the typical endogenous μ-opioid receptor (MOR) peptides, also had remarkable inhibitory effects on the gastrointestinal motility at their analgesic ED_{50} dose for EM-1 and EM-2 (Tseng et al., 2000; Wang et al., 2014). Separation of analgesia from adverse events has always been our pursuit of opioid research.

On the basis of MOR ligand morphiceptin (YPFP-NH_2) and neuropeptide FF (NPFF) derivative (PFRTic-NH_2), we designed and synthesized the chimeric heptapeptide MCRT (YPFPFRTic-NH_2) *via* an overlapping proline (Li et al., 2012; Li et al., 2013; He et al., 2017). In our previous report, MCRT proved to be a bifunctional ligand of MOR and δ-opioid receptor (DOR), and produced potent and dose-dependent antinociception when given intracerebroventricularly (ED_{50} = 0.03 nmol/mouse, i.c.v.) (Li et al., 2012). It was noteworthy that MCRT at the dose of 1 nmol/mouse, far higher than its analgesic ED_{50}, failed to delay gastric emptying and small intestine transit, indicating the separation of analgesia from gastrointestinal dysmotility (Li et al., 2012; He et al., 2017). To a certain extent, our previous data gave the evidence that MCRT possessed a promising intrinsic analgesic

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property and deserved further research.

Constipation is a result of the combination of various factors, like decreased peristalsis, increased non-propulsive contractions and so on (Ross et al., 2008; Beckett et al., 2018). Apart from the upper gastrointestinal tract, colonic organ also played pivotal roles in opioid-induced constipation as well as chronic idiopathic constipation (Camilleri, 2011; Shahid et al., 2012; Mori et al., 2013). In the present study, we sought to characterize the effects of MCRT on colonic non-propulsive contraction *in vitro* (isolated colon bioassay) and propulsive motility *in vivo* (colonic bead expulsion). Moreover, in fecal pellet output test, fecal number and dry weight were recorded to evaluate the MCRT-induced constipation. To explore the underlying mechanism, naloxone (the non-selective opioid receptors antagonist), cyprodime (the selective MOR antagonist), naltrindole (the selective DOR antagonist), BIBP3226 (the mixed antagonist of NPY Y1 and NPFF receptors) and RF9 (the selective antagonist of NPFF receptor) were utilized.

2. Material and methods

2.1. Animals

Male Kunming strain mice (the Experimental Animal Center of Lanzhou University, China) were used. Animals were housed in the room maintained at 22 ± 1 °C under a 12-h light/dark cycle with food and water available *ad libitum*. The experimental protocol was designed in compliance with guidelines from the Ethics Committee of Animal Experiments at Lanzhou University (China) and the China Council on Animal Care (Zhou et al., 2015; Zheng et al., 2017).

2.2. Chemicals

MCRT was prepared by a standard Fmoc-based solid-phase synthetic method, and the crude peptide was purified and analyzed by reverse-phase high performance liquid chromatography. The structure assignment was characterized by electrospray ionization mass spectrum (He et al., 2017). Cyprodime hydrochloride, naltrindole hydrochloride, and BIBP3226 trifluoroacetate were from Tocris Bioscience (Xi'an, China). Naloxone hydrochloride was from J&K Scientific (Beijing, China). RF9 trifluoroacetate was from Santa Cruz Biotechnology (Shanghai, China). BIBP3226 was dissolved in 3% DMSO solutions and all other chemicals were dissolved in sterilized saline (0.9% NaCl).

2.3. In vitro organ bath study

Responses of *in vitro* preparations were recorded with BL-420F recorder system (Chengdu Techman Software Co., Ltd., Chengdu, China). Mice (25–30 g) were deprived of food for 12 h, and then the segments of ileum (5 cm proximal to the cecum) and distal colon (1.5 cm proximal to the anus) were dissected (Yu et al., 2007; Ono et al., 2014). Preparations were suspended along the axis of the muscle in the organ baths containing 10 ml Krebs' solution (37 °C, 95% O_2 and 5% CO_2) (Wang et al., 2010). Prior to drug addition, the tissues were equilibrated for about 60 min under 1 g tension with replacements of Krebs' solution every 15 min. Non-cumulative MCRT (10^{-7}–10^{-5} M) was gently added. The MCRT-elicited responses were recorded for at least 10 min and the induced tension changes within 60 s were averaged. At the end of each assay, carbachol (10^{-6} M) was added as the internal contractile control. Antagonists naloxone, cyprodime, naltrindole, BIBP3226 and RF9 were incubated 10 min prior to MCRT.

2.4. Drugs administration

Intracerebroventricular administration was performed *via* a pre-implant cannula according to our previous report (He et al., 2017; Jiang et al., 2018). Briefly, mice (21 ± 1 g) were anesthetized and operated on the stereotaxic apparatus. After the implantation and fixation of the cannula into the lateral ventricle (0.5 mm posterior to Bregma, 1.1 mm lateral to midline and 2.0 mm in depth), the postoperative mice were housed individually. Five days' recovery later, the mice weighting 25–30 g were utilized in the following experiments. Chemicals were i.c.v. administrated in a volume of 3 μl within 2 min.

In colonic bead expulsion test, naloxone (5 nmol), BIBP3226 (5 nmol) and RF9 (5 nmol) were i.c.v. pretreated 10 min prior to MCRT (3 nmol) (Fang et al., 2006; Wang et al., 2016). In fecal pellet output test, antagonists (naloxone, cyprodime, naltrindole and RF9) at the dose of 10 nmol were i.c.v. co-administered with 3 nmol MCRT to avoid the stress stimulation-induced defecation.

2.5. Colonic bead expulsion

Colonic bead expulsion was performed as described previously (DeWire et al., 2013; Zielińska et al., 2016). In order to obtain an accurate measurement, mice were fasted 4 h with water available. 5 min after i.c.v. administration, a pre-warmed glass bead (2 mm in diameter) was inserted 2 cm into the distal colon with a silicone pusher under lightly isoflurane anesthesia. Then, the mouse was individually placed in the cage equipped with camera monitor, and the time to bead expulsion was recorded as bead latency. The cutoff time was set to 30 min.

2.6. Fecal pellet output

5 min after central injection of MCRT, mice were placed in the individual cages with a metal grid-bottom and the total excretions were collected on a sheet of paper below for a 30 min period. Thereafter, all the fecal pellets were desiccated at 50 °C until the mass was no longer changed. Fecal number and dry weight were determined in this manner in vehicle- and MCRT-treated animals.

2.7. Statistical analysis

Data were presented as means ± standard error of the mean (S.E.M.) of 8–10 mice. One-way ANOVA was employed, followed by Dunnett's post-hoc test for multiple comparisons. $P < .05$ was considered to be statistically significant.

3. Results

3.1. In vitro actions of MCRT on the colonic contractions

In vitro experiments, ileum and colon were typically utilized to compare the non-propulsive effects of chemicals on the small and large intestines. For this reason, non-cumulative MCRT (10^{-7}–10^{-5} M) was added into the ileum and colon organ bath. As Fig. 1A revealed, no contractile response was detected in the ileum bath after the addition of MCRT even up to 10^{-5} M. By comparison, MCRT elicited significant contractions of distal colon muscle stripe in a dose-dependent manner. The threshold dose of MCRT was 10^{-6} M with tension changes of 0.18 ± 0.02 g, and MCRT at 10^{-5} M showed the maximal tension changes (Fig. 1B and Table 1).

Functional assay indicated that MCRT was a mixed MOR/DOR ligand (Li et al., 2012). The opioid receptors antagonists were examined to explore the mechanisms of the MCRT-induced colonic contractions. As was expected, pre-treated naloxone (10^{-6} M), the non-selective opioid receptors antagonist, fully blocked the contractile responses induced by MCRT (10^{-5} M), suggesting an involvement of opioid receptors (Fig. 2A). Then, MOR antagonist cyprodime and DOR antagonist naltrindole were used to confirm the receptors more accurately. Fig. 2B and C demonstrated that cyprodime (3×10^{-6} M) or naltrindole (10^{-6} M) almost completely abolished the MCRT-induced contractile responses.

Besides, considering the fact that the C-terminal pharmacophore of

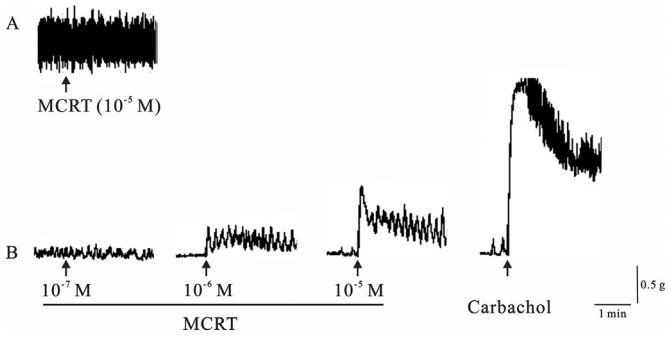

Fig. 1. Effects of MCRT on ileum and colon preparations. (A) MCRT (10^{-5} M) failed to influence the mouse ileum contraction. (B) Representative tracings of distal colonic contractile responses to MCRT (10^{-7}–10^{-5} M). Arrows indicate the time of chemicals treatments. Carbachol (10^{-6} M) was added as the internal contractile control. Tracings demonstrated were from one colon muscle stripe.

Table 1
MCRT (10^{-7}–10^{-5} M) induced the mouse distal colonic contractions.

Index dose of MCRT	Tension changes (g)	Carbachol responses (%)
10^{-7} M	0.008 ± 0.006	0.32 ± 0.31
10^{-6} M	0.18 ± 0.02	7.58 ± 1.23
10^{-5} M	0.4 ± 0.09	14.41 ± 2.53

MCRT was a NPFF derivative, effects of MCRT on the distal colon in the absence or presence of BIBP3226 (the mixed antagonist of NPY Y1 and NPFF receptors) and RF9 (the selective antagonist of NPFF receptor) were measured. However, neither BIBP3226 nor RF9 failed to influence the MCRT-induced contractions (Fig. 2D and E).

3.2. Central MCRT delayed colonic bead expulsion

Correspondingly, colonic bead expulsion was measured to evaluate the central effects of MCRT on colonic motility *in vivo* (Fig. 3). Compared with the basic colonic expulsion time of saline group (404.15 ± 11.69 s), central injection of MCRT (0.5–4 nmol/mouse) delayed colonic bead expulsion in a dose-dependent manner

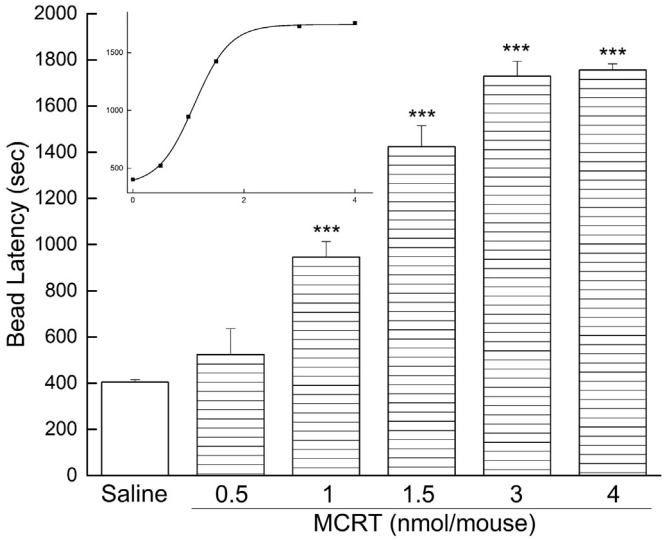

Fig. 3. Supraspinal effects of MCRT on colonic bead expulsion. Intracerebroventricular administration of MCRT (0.5–4 nmol/mouse, i.c.v.) dose-dependently inhibited colonic bead expulsion, and the fitting dose-response curve was present at the top left corner. Data represent means ± S.E.M. for 8–10 mice. ***$P < .001$, MCRT *vs.* Saline.

(ED_{50} = 1.1 ± 0.02 nmol/mouse). MCRT at 0.5 nmol/mouse produced a relatively increased bead latency but without statistical significance (523.75 ± 112.34 s, $p > .05$), and MCRT at 3 nmol/mouse almost reached the assay maximum of 30 min (1729.33 ± 64.29 s, $p < .001$).

To investigate the possible involvement of opioid and NPFF receptors, naloxone, BIBP3226 and RF9 were 10 min i.c.v. pre-injected prior to MCRT. None of the three antagonists, by themselves, showed any influence to the expulsion time in mice (Fig. 4B). Unexpectedly, totally differently from the mechanisms *in vitro*, MCRT-induced abnormal colonic propulsive motility *in vivo* was completely insensitive to opioid and NPFF receptors blockade (Fig. 4A).

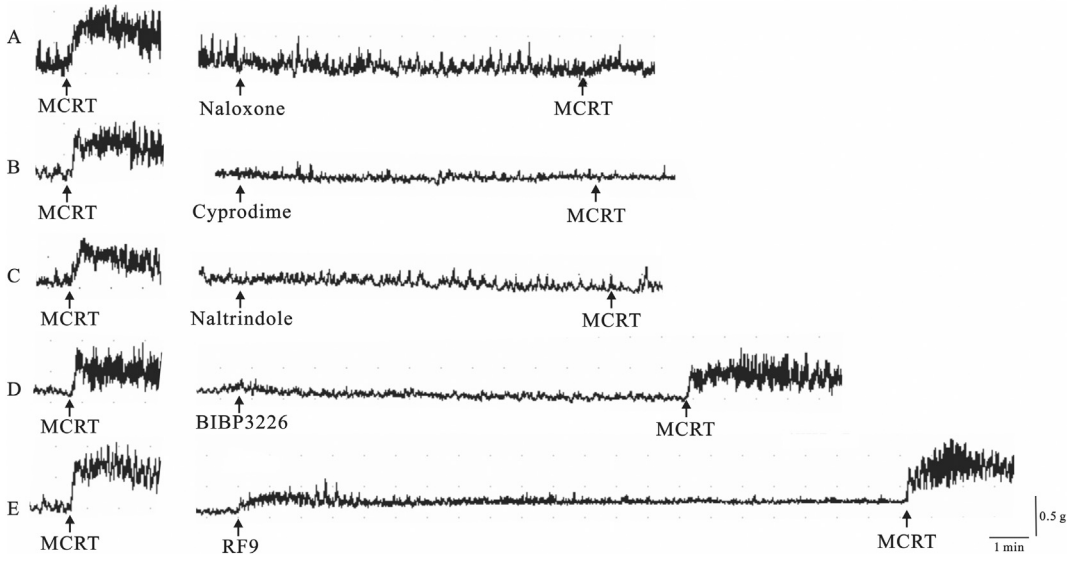

Fig. 2. Influence of MCRT on colon preparation in the absence or presence of antagonists. The first addition of MCRT (10^{-5} M) was used to verify the contracting activity. Then, (A) naloxone (non-selective antagonist of opioid receptor, 10^{-6} M), (B) cyprodime (μ-selective antagonist of opioid receptor, 3×10^{-6} M), (C) naltrindole (δ-selective antagonist of opioid receptor, 10^{-6} M), (D) BIBP3226 (mixed antagonist of NPY Y1 and NPFF receptors, 3×10^{-5} M) and (E) RF9 (selective antagonist of NPFF receptor, 3×10^{-5} M) were incubated 10 min prior to the second addition of MCRT. Arrows indicate the time of chemicals treatments. Tracings demonstrated were from one colon muscle stripe.

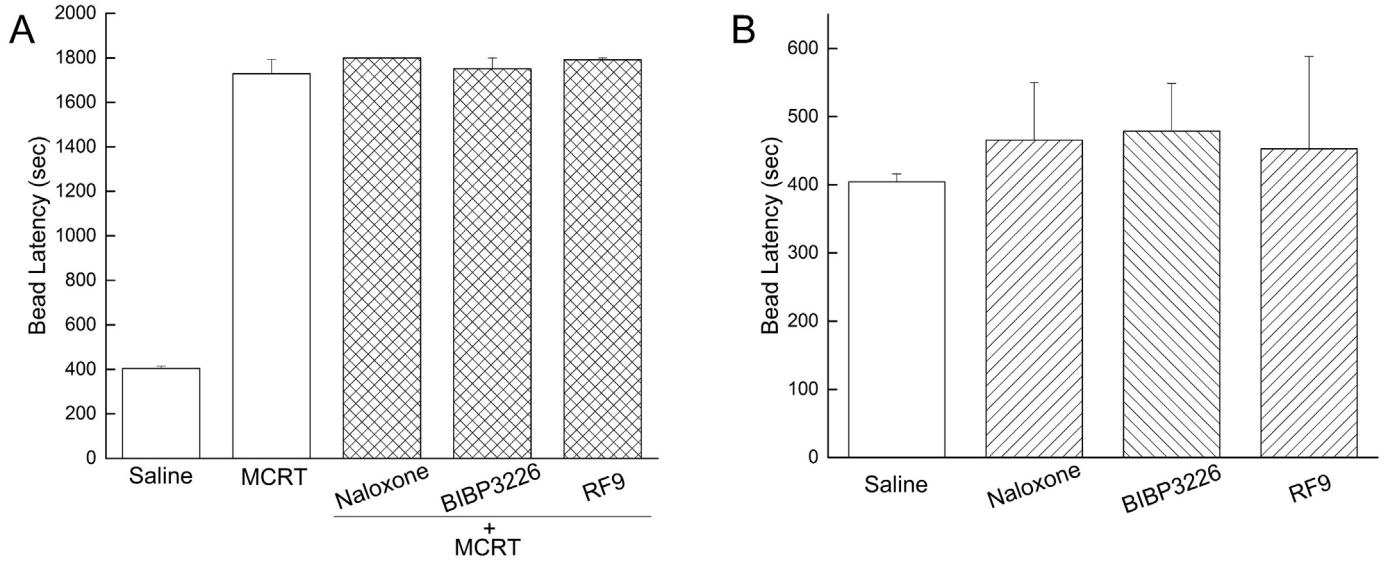

Fig. 4. Influence of pre-injected antagonists on MCRT-induced colonic dysmotility. (A) Effects of naloxone (5 nmol/mouse, i.c.v.), BIBP3226 (5 nmol/mouse, i.c.v.) and RF9 (5 nmol/mouse, i.c.v.) on reduction of colonic bead latency induced by MCRT (3 nmol/mouse, i.c.v.). (B) Effects of the antagonists by themselves on colonic bead expulsion in mice. All data were present as means ± S.E.M. for 8–10 mice.

3.3. Central MCRT reduced fecal pellet output

Fecal pellet output was considered as an intuitive representation of opioid-induced constipation, so the number of fecal pellets and the fecal dry weight were recorded to evaluate the MCRT-induced defecation dysfunction. As shown in Fig. 5, central injection of MCRT inhibited fecal pellet output significantly and dose-dependently with the ED_{50} of 1.43 nmol/mouse for fecal number and 1.63 nmol/mouse for fecal dry weight. Comparing with the saline-treated mice, the fecal pellet output was not modified by MCRT at 1 nmol/mouse. And the MCRT-induced constipation reached a significant and maximal level at the dose of 3 nmol/mouse, the fecal number and the fecal dry weight of which were 0.29 ± 0.18 (Fig. 5A, $P < .001$) and 4.57 ± 3.08 mg (Fig. 5B, $P < .001$), respectively.

The more operation, the more stress. Undoubtedly, stress stimulation-induced defecation should not be recorded and those mice suffering stress should be excluded. Consequently, the co-administration of antagonists and MCRT was chose, and the dose of antagonists was 3.3-fold higher than MCRT. Fig. 6A and B illustrated that co-administration of naloxone but not RF9 blocked the decreases in fecal pellet output evoked by MCRT significantly and partially. Cyprodime (MOR antagonist) and naltrindole (DOR antagonist) were utilized to confirm the accurate receptor involved, and MCRT-induced constipation was sensitive to DOR but not to MOR blockade. None of the four antagonists, naloxone, cyprodime, naltrindole and RF9 showed any effects by themselves in fecal pellet output test (Fig. 6C and D).

4. Discussion

As a novel chimeric and bifunctional opioid peptide, MCRT produced potent analgesia in tail flick test (ED_{50} = 0.03 nmol/mouse) and delayed gastric emptying and intestinal transit at far higher dose than those needed for antinociception (Li et al., 2012; He et al., 2017). The separation of MCRT analgesia from upper gastrointestinal dysmotility elucidated its promising analgesic profile.

In vitro, MCRT induced significant naloxone-reversible effects on

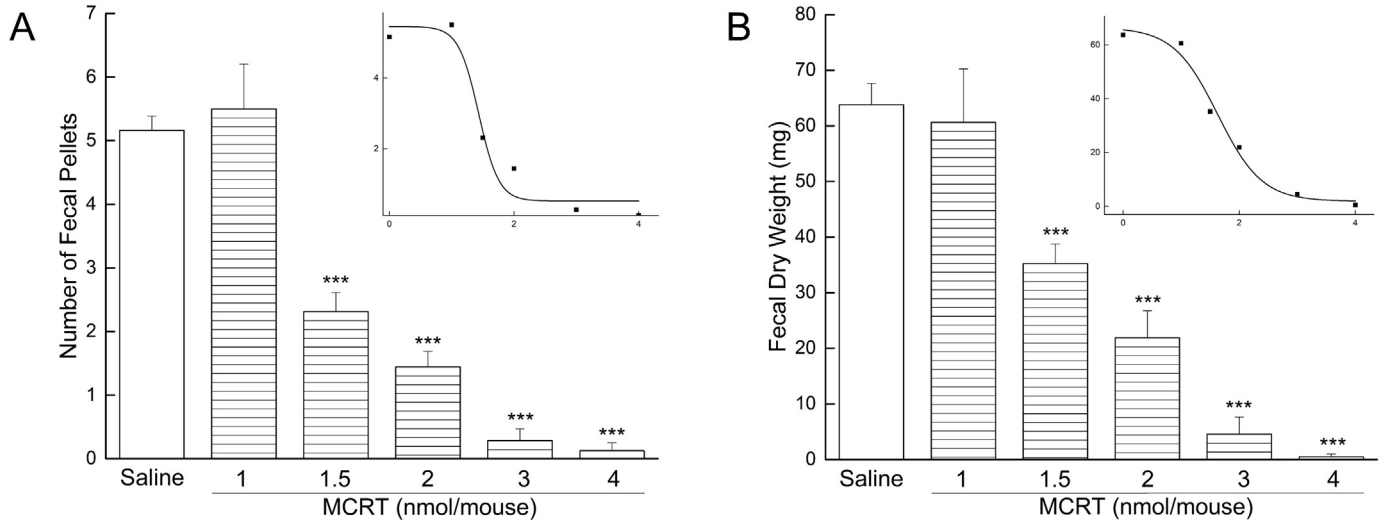

Fig. 5. Supraspinal effects of MCRT on fecal pellet output. Intracerebroventricular administration of MCRT (1–4 nmol/mouse, i.c.v.) dose-dependently delayed fecal number (A) and dry weight (B) in mice, and the fitting dose-response curve was present at the top right corner. Data represent means ± S.E.M. for 8–10 mice. ***$P < .001$, MCRT *vs.* Saline.

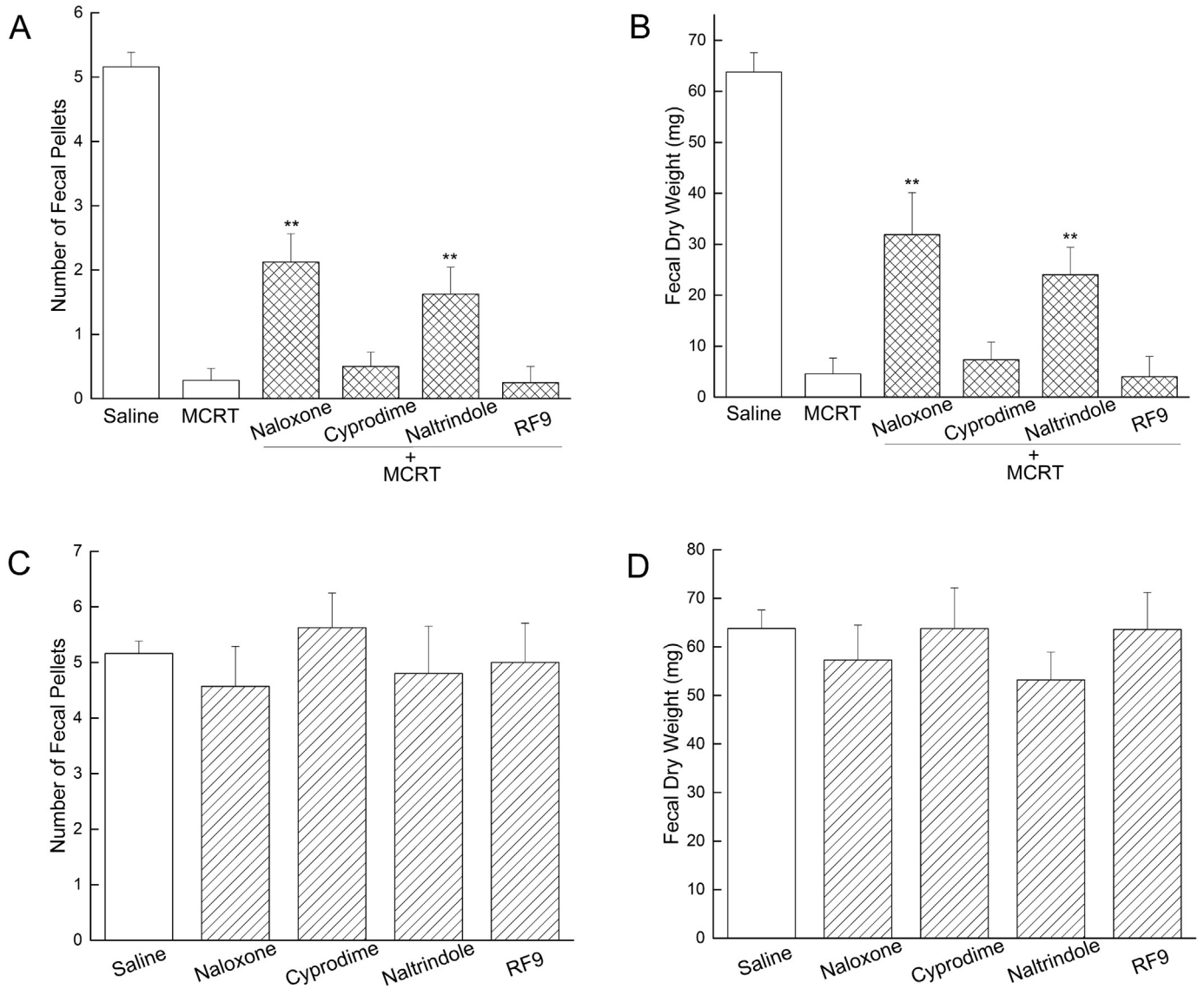

Fig. 6. Influence of MCRT on fecal pellet output in the absence or presence of antagonists. Effects of co-administration of MCRT and antagonists of opioid or NPFF receptors (10 nmol/mouse, i.c.v.) on MCRT-elicited inhibition on fecal number (A) and dry weight (B). Effects of the antagonists by themselves on fecal number (C) and dry weight (D). Data represent means ± S.E.M. for 8–10 mice. $^{**}P < .01$, MCRT + antagonist *vs.* MCRT.

spontaneous contractive activity of distal colon but not of ileum (Fig. 1). The region-specific contractile responses to MCRT was similar to that of EMs, which could be largely ascribed to the higher MOR expression level in colon than ileum (Yu et al., 2007; Ono et al., 2014). Results revealed that the threshold dose of MCRT to provoke colonic contractions was 10^{-6} M, which was as much as three orders of magnitude higher than that of EMs (10^{-9} M) (Yu et al., 2007). The complete inhibition of naloxone, cyprodime and naltrindole on MCRT-induced colonic contractions verified its MOR/DOR bifunctional property (Figs. 2A–C), which was consistent with our previous reports (Li et al., 2012). Overwhelming evidences proved that, rather than a direct myogenic effect, opioid-induced colonic contraction was mediated by myenteric neural pathways (Fontaine and Reuse, 1985; Yu et al., 2007; Maguma et al., 2012). From the facts, we deduced that the activity of MOR/DOR bifunctional MCRT was neurogenic as well. While neither BIBP3226 nor RF9 showed any influence on MCRT-induced contraction (Fig. 2D and E).

In tail flick test, the central analgesic ED_{50} values for EM-2 and morphine were about 5.51 nmol/mouse (Loh et al., 1998) and 1.85–5.85 nmol/mouse (Loh et al., 1998; Sato et al., 1999; Narita et al., 2002) after unit conversion, respectively. However, the threshold doses of both EM-2 and morphine in delaying colonic transit were < 0.5 nmol/mouse (Raffa et al., 1987; Wang et al., 2013), suggesting analgesic effects of the two classical opioids were unseparated from adverse events. By comparison, MCRT showed a promising separation of analgesia and gastrointestinal motor dysfunctions. Previous study reported that MCRT at the dose of 1 nmol/mouse had no effect on gastric empting and small intestine propulsion, and reached statistically significant inhibitions at 3–10 nmol/mouse (He et al., 2017). Moreover, in this article, ED_{50} values of MCRT were 1.1 nmol/mouse in colonic bead expulsion test, 1.43 nmol/mouse in fecal number test and 1.63 nmol/mouse in fecal dry weight test (Figs. 3 and 5). In summary, ED_{50} values for MCRT in tail flick test was 0.03 nmol/mouse far lower than that needed in gastrointestinal related tests (Li et al., 2012; He et al., 2017), and the separation of analgesia from gastrointestinal dysfunctions provided a path forward to produce safer opioid analgesic.

MCRT proved to be a MOR/DOR bifunctional ligand in functional assays on guinea-pig ileum and mouse vas deferens, and produced analgesia *via* MOR and DOR in tail flick test (Li et al., 2012). *In vitro* contractility study also verified its bifunctional profile (Figs. 2A–C).

Unexpectedly, entirely different from the regulatory mechanism of MCRT in tail flick test and *in vitro* contractility, the broad-spectrum opioid receptors antagonist naloxone failed to modify the MCRT-induced reduction in colonic bead expulsion (Fig. 4A). While in fecal pellet output test, MCRT was partially mediated by DOR antagonist naltrindole (Fig. 6A and B), which might be attributed to the involvement of DOR in MCRT-elicited upper gastrointestinal dysmotility (He et al., 2017). Besides, similar to the *in vitro* study, the NPFF receptor antagonists (BIBP3226 and RF9) failed to regulate MCRT in colonic bead expulsion test and fecal pellet output test (4A, 6A and 6B). In a word, MCRT was the first reported opioid that centrally mediated tail flick latency, upper gastrointestinal motility and colonic transit *via* the entirely distinct signaling pathways. Based on the mechanism explorations, we might speculate that there should be such an opioid receptor subtype or MOR/DOR heterodimer, which participated in the central analgesia and the *in vitro* colonic contractions but not the central colonic dysmotility.

Taken together, our results indicated that: (1) *in vitro*, MCRT elicited significant colonic contractions *via* MOR and DOR; (2) *in vivo*, central MCRT dose-dependently inhibited colonic bead propulsion and fecal pellet output with the required doses far higher than its analgesic dose, suggesting a separation of analgesia from gastrointestinal dysfunctions; (3) we speculated on the existence of such an opioid receptor subtype or MOR/DOR heterodimer, which participated in the central analgesia and the *in vitro* colonic contractions but not the central colonic dysmotility.

Author contributions

Conceived and designed the experiments: SD, CH. Performed the experiments: CH, HL. Analyzed the data: CH, HL, JZ. Wrote the paper: SD, CH. Provided the fund: LZ.

Conflicts of interest

The Authors declare no conflict of interest.

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