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ORIGINAL ARTICLE

Involvement of central opioid receptors in protective effects of methadone on experimental colitis in rats

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Abstract

Purpose There are several lines of evidence on the protective roles of opioids in gastrointestinal inflammatory conditions. This study aims to distinguish the central and peripheral roles of methadone, a non-selective opioid receptor agonist, in an acute model of ulcerative colitis in male rats.

Methods Ulcerative colitis was induced by intrarectal administration of acetic acid 4%. Methadone was injected subcutaneously (s.c.), 5 and 10 mg/kg, and intracerebroventricular (i.c.v.), 50 and 300 ng/rat. Opioid antagonists were employed. Methylnaltrexone (MNTX; 5 mg/kg, i.p.), a peripherally acting opioid receptor antagonist, and naltrexone (NTX; 5 mg/kg, i.p. and 10 ng/rat, i.c.v.), a peripherally and centrally acting opioid receptor antagonist were injected before methadone (10 mg/kg, s.c. and or 300 ng/rat, i.c.v.) administration. NTX (5 mg/kg, i.p. and 10 ng/rat, i.c.v.) were administered 30 min prior to administration of methadone (10 mg/kg, s.c. and 300 ng/rat, i.c.v.), respectively. MNTX (5 mg/kg, i.p.) was injected 30 min prior to methadone (10 mg/kg, s.c.). Seventy-two hours following colitis induction, macroscopic and microscopic mucosal lesions, and the colonic levels of tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) were determined.

Results Methadone (300 ng/rat, i.c.v.) and Methadone (5 and 10 mg/kg, s.c.) improved the macroscopic and microscopic scores through opioid receptors. Also, a significant reduction in TNF-α and IL-1β was observed. Peripherally and centrally injected NTX significantly reversed methadone 10 mg/kg s.c. anti-inflammatory effects while MNTX could not completely reverse this effect. Moreover, centrally administered methadone (300 ng/rat) showed the anti-inflammatory effect which was reversed by central administration of NTX (10 ng/rat).

Conclusions The opioid receptors mainly the central opioid receptors may mediate the protective actions of methadone on the experimental model of inflammatory bowel disease in rat.

Keywords Ulcerative colitis · Methadone · Acetic acid · Opioid receptors · Brain–gut axis · Rat

Nahid Fakhraei and Nina Javadian contributed equally in the study.

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Introduction

Inflammatory bowel disease (IBD) is characterized by a chronic inflammatory process in the gut due to leukocyte infiltration and excessive generation of pro-inflammatory cytokines (Fakhraei et al. 2014). Colitis triggers activation of the neuroendocrine system via hypothalamic corticotropin-releasing factor (CRF) pathways in the paraventricular nucleus of the hypothalamus (PVN) involved in the autonomic, behavioral, and neuroendocrine response to inflammation (Porcher et al. 2004; Greenwood-Van Meerveld et al. 2006).

The main therapeutic goals in IBD are control of intestinal inflammation and treatment of the most common clinical symptoms (Baumgart and Sandborn 2007). Diarrhea is considered one of the main symptoms in IBD and its mechanisms are complex and multifactorial. The mainstays of conventional pharmacotherapy for IBD are aminosalicylates and corticosteroids. Immunosuppressive agents and biological response modifiers are alternative therapies. Nevertheless, available drugs are not universally effective and cause considerable adverse effects. As a result, there is a demand for research that leads to the development of new therapeutic approaches (Mousavizadeh et al. 2009).

Opioids are recognized for their anti-nociceptive and anti-diarrheal roles in the gastrointestinal (GI) tract. Opioid receptors and their ligands play important roles in the GI secretory and motor functions. Activation of opioid receptors may modulate several inflammatory mechanisms like mitogen-induced immune mononuclear cells (Eisenstein and Hilburger 1998), splenocyte proliferation (Shahabi et al. 1991), and production of inflammatory and immunomodulatory cytokines (Peterson et al. 1998). Moreover, immune cells have been shown to express different opioid receptors; kappa (κ), mu (μ), and delta (δ) which bind both agonists and antagonists (McCarthy et al. 2001; Janecka et al. 2004). Opioid receptors, which are expressed in immune cells are often the same as or similar to neuronal-type opioid receptors, particularly κ- and δ. Studies also indicated expression of opioid receptors or binding sites on lymphocytes that are selective for morphine (Bidlack 2000). It has been shown that chronic administration of narcotic analgesics significantly reduced immune cell function (Bryant et al. 1987) and opiates inhibited chemokine-induced chemotaxis (Grimm et al. 1998).

In this context, using opioids as therapeutic options in IBD has been widely discussed (Philippe et al. 2003; Zagon and McLaughlin 2011). Surprisingly, anti-inflammatory roles of peripheral opioid agonists in experimental colitis models in mice were demonstrated. Notably, the opioids exert their anti-inflammatory effects mainly through a peripheral route (Philippe et al. 2003). In fact, opioids might modify the inflammatory process through their effects on the synthesis and secretion of pro- and anti-inflammatory cytokines. Increased expression of κ-opioid receptors was reported in the inflamed rat colon induced by trinitrobenzenesulfonic acid (TNBS) (Sengupta et al. 1999) and acetic acid 5% (Burton and Gebhart 1998). In addition, expression of μ- and δ-opioid receptors increased in intestinal inflammation induced by croton oil in mice (Pol et al. 1994).

Interestingly, psycho-immune modulation through the brain–gut axis might have a pivotal role in the pathogenesis of IBD. Several comorbidities, including psychiatric disorders like depression and anxiety have been reported in IBD patients (Bonaz and Bernstein 2013). In this context, nitric oxide (NO)-mediated neuroinflammation might be responsible for the behavioral despair associated with a mouse model of Crohn's disease (Heydarpour et al. 2016). On the other hand, the central nervous system (CNS) regulates innate immune responses. For example, the neuroendocrine stress response and the sympathetic and parasympathetic nervous systems inhibited innate immune responses (Sternberg 2006).

Evidence for involvement of the cholinergic anti-inflammatory pathway in IBD pathogenesis is well established (Seyedabadi et al. 2018). Acetylcholine (ACh), the main neurotransmitter in the vagus nerve, decreases the production of pro-inflammatory cytokines (Khalifeh et al. 2015; Seyedabadi et al. 2018). All these findings indicate that new strategies for IBD treatment should focus on interventions that both ameliorate IBD psychological comorbidities and potentiate central anti-inflammatory mechanisms. Opioids could be among the potential candidates. They elicit potent anti-inflammatory properties in experimental peripheral and central inflammatory diseases.

Methadone is a synthetic opioid agonist that has a greater penetration through the blood–brain barrier (BBB) rather than other opioids such as morphine (Kafami et al. 2013). Among opioids, the immunomodulatory effect of methadone has been reported in many immune diseases, for example, human immunodeficiency virus (HIV) and diabetes mellitus type 1 (Al-Hashimi et al. 2013). The immunomodulatory effects of methadone have been addressed in neurodegenerative diseases and peripheral inflammatory conditions (Amirshahrokhi et al. 2008; Kafami et al. 2013). Interestingly, in vitro studies demonstrated that methadone activates nicotinic ACh receptors (Pakkanen et al. 2005) and this effect might potentiate CNS-induced anti-inflammatory cascades in the gut.

Accordingly, this study aims to distinguish the effects of central and peripheral administration opioid receptor agonist, methadone on colonic inflammation in acetic acid-induced colitis in rats.

Materials and methods

Animals

Male Wistar rats (6–7 weeks old) weighing 200–250 g were kept for a week prior to study so as to be adapted to the animal room conditions. The animal room was maintained at 22–24 °C with a lighting regimen of 12-hour light/12-hour dark. Rats had free access to standard pelleted chew and water. All the experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) with theapproval of Research and Medical Ethics Committees of Tehran University of Medical Science.

Chemicals

Methadone, naltrexone, methylnaltrexone, and dexamethasone were purchased from Merck co. (Darmstadt, Germany). Xylazine and ketamine were bought from Alfasan co. (Woerden Holland). Acetic acid, formalin solution, and diethyl ether oxide were obtained from Dr. Mojallali Chemical Laboratories (Iran).

Experimental groups

The study period was 72 h and the drugs were started 1 h following the induction of colitis (day one) and continued in two successive days. The rats were divided into 18 groups of 7–9. Two control groups received intrarectal (i.r.) acetic acid (one got s.c. saline and another i.c.v. saline). Two sham groups were administered distilled water intrarectally (one got s.c. saline and another i.c.v. saline). Further, a standard treatment group received dexamethasone (1 mg/kg, i.p.) (Antonioli et al. 2007). Moreover, two IBD groups for methadone (5 and 10 mg/kg, s.c.) and two IBD groups for methadone (50 and 300 ng/rat, i.c.v.) administrations were assigned. To assess which route of the opioid administration is the primary action site of methadone, the most effective dose for each route was determined and their combinations with the antagonists assessed. In this regard, nine groups were randomly selected. Non-selective opioid receptor antagonists, methylnaltrexone (MNTX; 5 mg/kg, ip) and naltrexone (NTX; 5 and 10 mg/kg, i.p. and 10 ng/rat, i.c.v.) were injected individually 30 min before administration of the most effective dose of methadone (10 mg/kg, s.c. and 300 ng/rat, i.c.v.). NTX (10 ng/rat, i.c.v.) was administered before the most effective dose of methadone 300 ng/rat, three distinct IBD groups received the antagonists. Finally, to prove that peripheral administration of methadone exerts its protective effect against colitis by a central mechanism, three groups received intrarectal (i.r.) acetic acid; one of them received NTX (10 ng/rat, i.c.v.) + methadone (10 mg/kg, s.c.), one group received saline (i.c.v.) + methadone (10 mg/kg, s.c.) and another received saline (i.c.v.) + saline (s.c.) (Table 1).

Induction of colitis

Pathological profile of the acute colitis induced by acetic acid might vary from those of the chronic disease. Acetic acid induces a non-specific inflammation similar to chronic models such as trinitrobenzenesulphonic acid. However, this model might have limitations for addressing the cascades that initiate inflammation in humans. On the other hand, excessive oxidative stress, prolonged infiltration of neutrophils and elevated levels of inflammatory mediators (that also occur in acute model), factors with significant pathological roles in human IBD including, allow using acetic acid-induced colitis, a reliable model for screening agents

Table 1 Different treatments in the study groups, according to methadone administration routes

Groups	Subcutaneous route	Intracerebroventricular route
Sham	Distilled water (i.r.), saline (s.c.)	Distilled water (i.r.), saline (i.c.v.)
Control	Acetic acid (i.r.), saline (s.c.)	Acetic acid (i.r.), saline (i.c.v.)
Dexamethasone	Acetic acid (i.r.), dexamethasone (1 mg/kg, i.p.)	–
Methadone	Acetic acid (i.r.), methadone (5 or 10 mg/kg, s.c.)	Acetic acid (i.r.), methadone (50 and 300 ng/rat, i.c.v.)
Antagonists	Acetic acid (i.r.), NTX (5 mg/kg, i.p.), saline (s.c.)	Acetic acid (i.r.), NTX (10 ng/rat, i.c.v.), saline (i.c.v.)
	Acetic acid (i.r.), NTX (5 mg/kg, i.p.), methadone (10 mg/kg, s.c.)	Acetic acid (i.r.), NTX (10 ng/rat, i.c.v.), methadone (300 ng/rat, i.c.v.)
	Acetic acid (i.r.), NTX (10 ng/rat, i.c.v.), methadone (10 mg/kg, s.c.)	–
	Acetic acid (i.r.), saline (i.c.v.), methadone (10 mg/kg, s.c.)	–
	Acetic acid (i.r.), saline (i.c.v.), saline (s.c.)	–
	Acetic acid (i.r.), MNTX (5 mg/kg, i.p.), saline (s.c.)	–
	Acetic acid (i.r.), MNTX (5 mg/kg, i.p.), methadone (10 mg/kg, s.c.)	–

with potential benefits in IBD (Yamada et al. 1991; Elson et al. 1995; Medany et al. 2005; Mahgoub et al. 2005; Perez-Navarro et al. 2005).

The rats fasted for 24 h prior to any intra-colonic studies; however, they always had access to water. Briefly, the rats were anesthetized with a mixture (1:1 v/v; 1 ml/kg body weight) of xylazine 2% (10 mg/kg i.p.) and ketamine 10% (50 mg/kg) (Alfasan Woerden Holland). Colitis was then induced according to the previous methods (Fakhraei et al. 2014; Rahimian et al. 2016). Briefly, a medical-grade polyurethane cannula for enteral feeding (external diameter 2 mm) was inserted into the anus, and the tip advanced 7 cm proximal to the anus verge and 1 ml 4% acetic acid (V/V) (Merck, Darmstadt, Germany) introduced into the colon.

Intracerebroventricular (i.c.v.) injections

Rats were anesthetized by an i.p. injection of a mixture (1:1 v/v; 1 ml/kg body weight) of xylazine 2% (10 mg/kg) and ketamine 10% (50 mg/kg). The rats were placed individually in a stereotaxic apparatus. A midline incision of the scalp was made and the skull carefully cleared from the skin and muscles. After that, a hole was drilled into the skull above the right lateral brain ventricle, according to coordinates obtained from Paxinos and Watson (2007), from the bregma: anterior–posterior = − 0.8 mm, lateral − 1.5 mm and depth − 3.5 mm. Drugs were injected directly into the right lateral ventricle using a 30-gauge needle (Plastic One Inc.), connected to a 10-µl Hamilton syringe by a PF-50 catheter (Intramedic Polyethylene Tubing, Clay Adams, Sparks, MD) filled with saline. A small air bubble (1 µl) was drawn at the distal end of the PE-50 tubing for visual monitoring of the i.c.v. injection. Drugs were administered at a fixed volume of 5 µl (at a constant rate of 2 µl/min) into the right lateral ventricle (Hashemizadeh et al. 2014; Chen et al. 2018).

Macroscopic and histopathologic colon damages

Seventy-two hours following the colitis induction, the rats were euthanized. In an ice bath, the distal colons were cut open and cleansed gently with normal saline. Subsequently, the colons were cut into two similar pieces, one for histopathologic assessment (kept in 5 ml of formalin 10% w/w) and another for analysis of biochemical markers.

After cutting open and cleaning the colon samples, high-quality photos were taken for the macroscopic evaluation. The macroscopic scoring was performed by an independent observer (Morris and Moore 1989; Fakhraei et al. 2014) (Table 2).

For evaluation based on microscopical (histologic) characters, the tissues were fixed in phosphate-buffered formaldehyde, embedded in paraffin and 4-µm sections were prepared. The tissues were stained with hematoxylin and eosin (H&E) and were evaluated by light microscopy and scored in a blinded manner by an expert pathologist using a Zeiss® microscope equipped with an Olympus® color video camera for digital imaging as indicated in Table 3. Each section was then scored for each feature separately by establishing the product of the grade for that feature and the percentage involvement (in a range from 0 to 12 for inflammation and for extent, and in a range from 0 to 16 for regeneration and for crypt damage) (Dieleman et al. 1998).

Table 2 Macroscopic scores evaluating extent of observable colon changes

Macroscopic scores	Description
0	Intact epithelium with no damage
1	Localized hyperemia but no ulcer
2	Linear ulcer with no significant inflammation
3	Linear ulcer with inflammation at one site
4	Two or more sites of ulcer and inflammation
5	Two or more sites of ulcer and inflammation extending over 1 cm

Table 3 Histopathologic scores evaluating inflammation severity, inflammation extent, and crypt damage

Feature graded	Grade	Description
Inflammation severity	0	None
	1	Slight
	2	Moderate
	3	Severe
Inflammation extent	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Tissue regeneration	4	No tissue repair
	3	Surface epithelium not intact
	2	Regeneration with crypt depletion
	1	Almost complete regeneration
	0	Complete regeneration or normal tissue
Crypt damage	0	None
	1	Basal 1/3 damaged
	2	Basal 2/3 damaged
	3	Only surface epithelium intact
	4	Entire crypt and epithelium lost
Tissue involvement (%)	1	1–25%
	2	26–50%
	3	51–75%
	4	76–100%

Cytokine measurements

The colonic levels of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) were determined with an enzyme-linked immunosorbent assay (ELISA kit) (Enzo Life Sciences, Lorrach/Germany). The colon was dissected out and homogenized in 50-mmol/L ice-cold potassium phosphate buffer (pH 6.0) containing 0.5% of hexadecyltrimethylammonium bromide. Afterward, the homogenate was centrifuged at 4000 rpm for 20 min at 4 °C and the supernatant was separated and kept at − 80 °C until analysis. Briefly, the wells were pre-coated with a monoclonal antibody serving to trap cytokine molecules in the homogenated specimen. Eventually, the results were expressed as pg/mg of the wet tissue (Fakhraei et al. 2014).

Statistical analysis

All the values are expressed as mean ± standard error using SPSS (version 19.0, Chicago, USA). One-way analysis of variance was employed for analyzing the data, followed by Tukey's post hoc test for multiple comparisons. Significance was ascribed when $P < 0.05$.

Results

Mortality rates

The mortality rates are shown in Table 4. As can be observed, sham and methadone (300 ng/rat, i.c.v.) groups did not have any mortality. The mortality rate for control, methadone (10 mg/kg, s.c.); and NTX (5 mg/kg, i.p.) was 40–45%. However, mortality rate of methadone (5 mg/kg, s.c.) and MNTX (5 mg/kg, i.p.) was 33.3%. NTX (5 mg/kg, i.p.) + met (10 mg/kg, s.c.) group had 50% mortality which represented the highest mortality in the animals.

Comparing the effective doses of methadone against colitis and experimental pain: in a study, the analgesic ED50, the effective dose for 50 percent of the group, for methadone s.c. was found to be 2.04 mg/kg (95% confident limit, 1.58–2.63) in the rat (Liu et al. 1983). On the other hand, the median lethal dose (MLD) for methadone was 22.5 (19.3:24.1) mg/kg in the rat (Borron et al. 2002). Besides, NTX was not toxic in any species (rat, dog, and monkey) at the dose of at least 20 mg/kg, which is 20 times higher than recommended clinically (1 mg/kg) (Willette and Barnett 1981).

Macroscopic data

Effect of methadone on acetic acid-induced colitis is illustrated in Fig. 1, according to the macroscopic scores. Control group has higher scores which are very highly significant compared to sham group $P < 0.001$. In addition, methadone group (50 ng/rat, i.c.v.) is very highly significant compared to the sham group and has a higher score, $P < 0.001$. Moreover, methadone groups (5 and 10 mg/kg, s.c.), dexamethasone group (1 mg/kg, i.p.) and methadone (300 ng, i.c.v.) show very highly significant differences compared to the control group and have lower scores, $P < 0.001$.

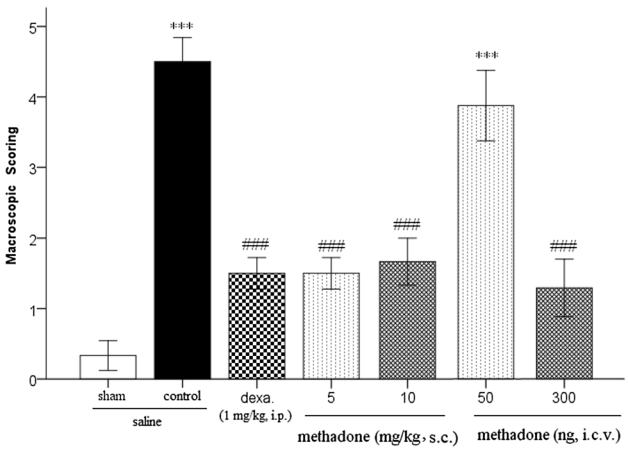

Fig. 1 Macroscopic scores showing effects of methadone on acetic acid-induced colitis. Methadone (5 and 10 mg/kg, s.c.) and (50 and 300 ng/rat, i.c.v.) administrations ($n = 6$–8). ***$P < 0.001$ significantly different from sham group. ###$P < 0.001$ significantly different from colitis group

Table 4 Mortality rate percentages and deaths per total number in the group, according to the administration routes in IBD rats

Groups	Mortality rate % (deaths/total no.)	Groups	Mortality rate % (deaths/total no.)
Sham	0	IBD + methadone (10 mg/kg, s.c.) + MNTX (5 mg/kg, i.p.)	28.3 (2/7)
Control	40 (4/9)	IBD + methadone (10 mg/kg, s.c.) + NTX (5 mg/kg, i.p.)	50 (4/8)
IBD + dexa. (1 mg/kg, i.p.)	20 (2/9)	IBD + methadone (50 ng/rat, i.c.v.)	33.3 (3/9)
IBD + methadone (5 mg/kg, s.c.)	33.3 (3/9)	IBD + methadone (300 ng/rat, i.c.v.)	0
IBD + methadone (10 mg/kg, s.c.)	45 (3/7)	IBD + NTX (10 ng/rat, i.c.v.)	45 (3/7)
IBD + MNTX (5 mg/kg, i.p.)	33.3 (3/9)	IBD + methadone (300 ng/rat, i.c.v.) + NTX (10 ng/rat)	33.3 (3/9)
IBD + NTX (5 mg/kg, i.p.)	40 (4/9)		

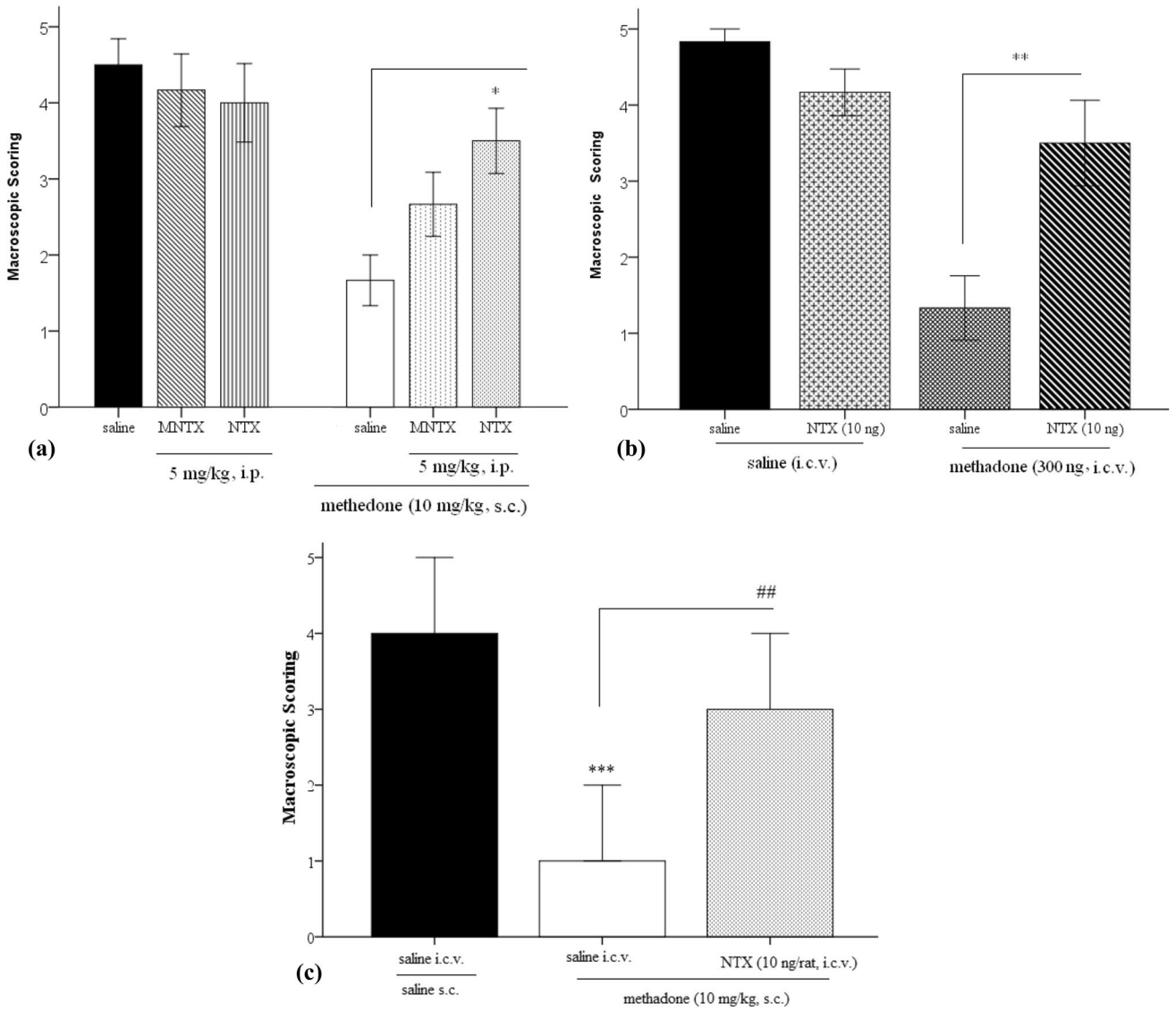

Fig. 2 Effect of opioid antagonists on methadone doses (10 mg/kg, s.c. and 300 ng/rat, i.c.v.) in acetic acid-induced colitis according to macroscopic scores. **a** Methadone sc and antagonists i.p. administrations. **b** i.c.v. methadone and antagonists administrations ($n=6$–8). $^{*}P<0.05$ and ** $P<0.01$ significantly different from methadone groups. **c** NTX (10 ng/rat, i.c.v.) and methadone (10 mg/kg, s.c.) administrations. $^{***}P<0.001$ significantly different from control group. $^{\#\#}P<0.01$ significantly different from methadone group (10 mg/kg, s.c.)

Figure 2 illustrates the effect of the opioid antagonists on methadone effect in acetic acid-induced colitis, according to macroscopic scores. Figure 2a shows the administration of methadone (10 mg/kg, s.c.) alone or 30 min after administration of the opioid antagonists (MNTX and NTX 5 mg/kg, i.p.). NTX (5 mg/kg, i.p.) significantly reverses methadone 10 mg/kg, s.c. effects ($P<0.05$). Figure 2b shows administration of NTX (10 ng/rat, i.c.v.), alone and or 30 min before methadone (300 ng/rat i.c.v.). NTX (10 ng/rat, i.c.v.) highly significantly reverses methadone (300 ng/rat, i.c.v.) effects ($P<0.01$). Figure 2c shows administration of NTX (10 ng/rat, i.c.v.) 30 min before methadone (10 mg/kg, s.c.). As can be seen, methadone (10 mg/kg s.c.) has lower macroscopic score which are very highly significant compared with the control group ($P<0.001$). Conversely, NTX (10 ng/rat, i.c.v.), highly significantly ($P<0.01$) reverses the effect of methadone (10 mg/kg, s.c.).

Figure 3 represents the effects of methadone on the amount of TNF-α. Control group is very highly significant from the sham group and has a higher TNF-α level, $P<0.001$. Dexamethasone (1 mg/kg, i.p.), methadone (10 mg/kg, s.c.) and methadone (50 and 300 ng/rat, s.c.)

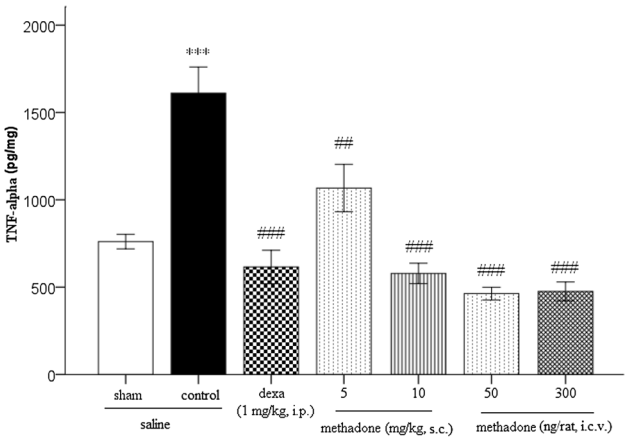

Fig. 3 Effect of methadone on amount of TNF-α in IBD rats. Methadone (5 and 10 mg/kg, s.c.) and (50 and 300 ng/rat, i.c.v.) administrations, ($n=6$–8). $^{***}P<0.001$ significantly different from sham group. $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ significantly different from control group

groups are very highly significant from the control group ($P<0.001$) and have lower levels of TNF-α. Methadone (5 mg/kg, s.c.) is also highly significant from the control group ($P<0.01$) and has a lower TNF-α level.

In Fig. 4, the impact of the opioid antagonists on the methadone doses (10 mg/kg and 300 ng/rat) in acetic acid-induced colitis, according to TNF-α levels ($n=6$–8) is demonstrated. Figure 4a represents s.c. methadone administrations. NTX and MNTX (5 mg/kg, i.p.) 30 min. before methadone (10 mg/kg, s.c.) increase TNF-α levels compared to methadone 10 mg/kg alone, the differences were highly significant $P<0.01$ and very highly significant $P<0.01$, respectively. Figure 4b represents i.c.v. methadone administrations. NTX 30 min before methadone 10 ng/rat increases TNF-α level very highly significantly $P<0.001$ compared to methadone 10 ng/rat.

Effect of methadone on the amount of IL-1β on acetic acid-induced colitis is represented in Fig. 5 ($n=6$–8). Control group is very highly significant from the sham group and has a higher IL-1β level, $P<0.001$. In addition, methadone (50 ng/rat, i.c.v.) group is very highly significant from the sham group and has a higher IL-1β level ($P<0.001$). Dexamethasone (1 mg/kg, i.p.), methadone (10 mg/kg, s.c.) and methadone (300 ng/rat, i.c.v.) groups are very highly significant from the control group $P<0.001$ and have a lower level of IL-1β. Methadone (5 mg/kg, s.c.) also is highly significant from the control group ($P<0.01$) and has lower level of IL-1β.

Figure 6 represents impact of opioid antagonists on the optimum methadone doses (10 mg/kg and 300 ng/rat) in acetic acid-induced colitis according to IL-1β levels ($n=6$–8). Figure 6a shows s.c. methadone administrations. NTX (5 mg/kg, i.p.) 30 min before methadone (10 mg/kg, s.c.) very highly significantly increases IL-1β level in IBD rats compared to methadone 10 mg/kg, $P<0.001$. Figure 6b represents i.c.v. methadone administrations. NTX (10 ng/rat, i.c.v.) 30 min before methadone (300 ng/rat, i.c.v.) very

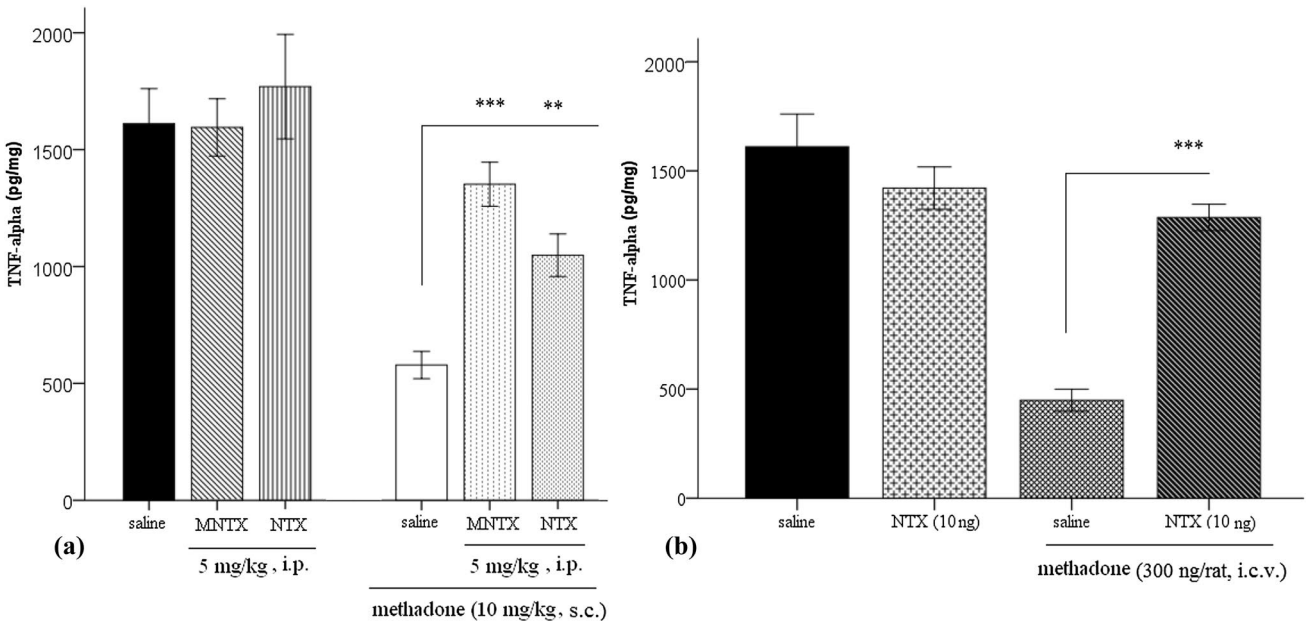

Fig. 4 Effect of opioid antagonists on the optimum methadone doses (10 mg/kg, s.c. and 300 ng/rat, i.c.v.) in acetic acid-induced colitis according to TNF-α levels. **a** s.c. administrations. **b** i.c.v. administrations ($n=6$–8). $^{**}P<0.01$ and $^{***}P<0.001$ significantly different from methadone groups

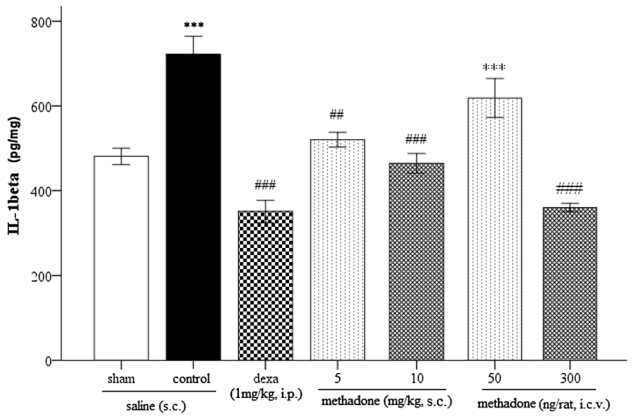

Fig. 5 Effect of methadone on amount of IL-1β in IBD rats. Methadone (5 and 10 mg/kg, s.c.) and (50 and 300 ng/rat, i.c.v.) administrations, ($n=6$–8). $^{***}P<0.001$ significantly different from sham group. $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ significantly different from control group

highly significantly increases IL-1β level compared to methadone (300 ng/rat) $P<0.001$.

Figure 7 illustrates photographic images of rat colitis colons. Macroscopic examination reveals: mucosal erythema with ulcer and exudate in vehicle-treated group (a), mild erythema without ulcer in dexamethasone 1 mg/kg (b), methadone 10 mg/kg (c), methadone 300 ng/rat (d) groups; erythema with patchy erosions in (MNTX 5 mg/kg + methadone 10 mg/kg) group (e); erythema with ulcer and exudate in (NTX 5 mg/kg + methadone 10 mg/kg) (f) and (NTX 10 ng/rat + methadone 300 ng) (g) groups. Besides, mucosal erythema with ulcer and exudate in (NTX 10 ng/rat, i.c.v. + methadone 10 mg/kg, s.c.) (h) and mild erythema with a restricted small ulcer in (saline i.c.v. + methadone 10 mg/kg, s.c.) (i).

Cross-sections of H&E stained sections of acetic acid-treated colon tissues are illustrated in Fig. 8. A, F, and G show severe (grade 3) transmural inflammation (grade 3) with entire crypt damage (grade 4) and desquamated epithelium in most parts of the specimen (grade 4). There is no evidence of tissue regeneration (grade 4). B, C, and D: moderate inflammation (grade 2) in mucosa and submucosa (grade 2) without crypt damage (grade 0) and almost complete regeneration (grade 1) are seen. E: Moderate mucosal and submucosal inflammations (grade 2) with crypt damage in 2/3 of the basal part (grade 2) are seen. Features of regeneration with crypt depletion (grade 2) are also evident.

Microscopic (histophatologic) features

Table 5 demonstrates the pathologic results of the s.c. methadone route. IBD group has higher inflammation severity and extent which are very highly significant compared to the sham group, $P<0.001$. Moreover, crypt damage and percentage of involvement are very highly significantly increased as well, $P<0.001$. Regeneration and total index are also raised highly significantly $P<0.01$ and very highly significantly $P<0.001$, respectively. In comparison to the IBD group, dexamethasone shows highly significantly lower

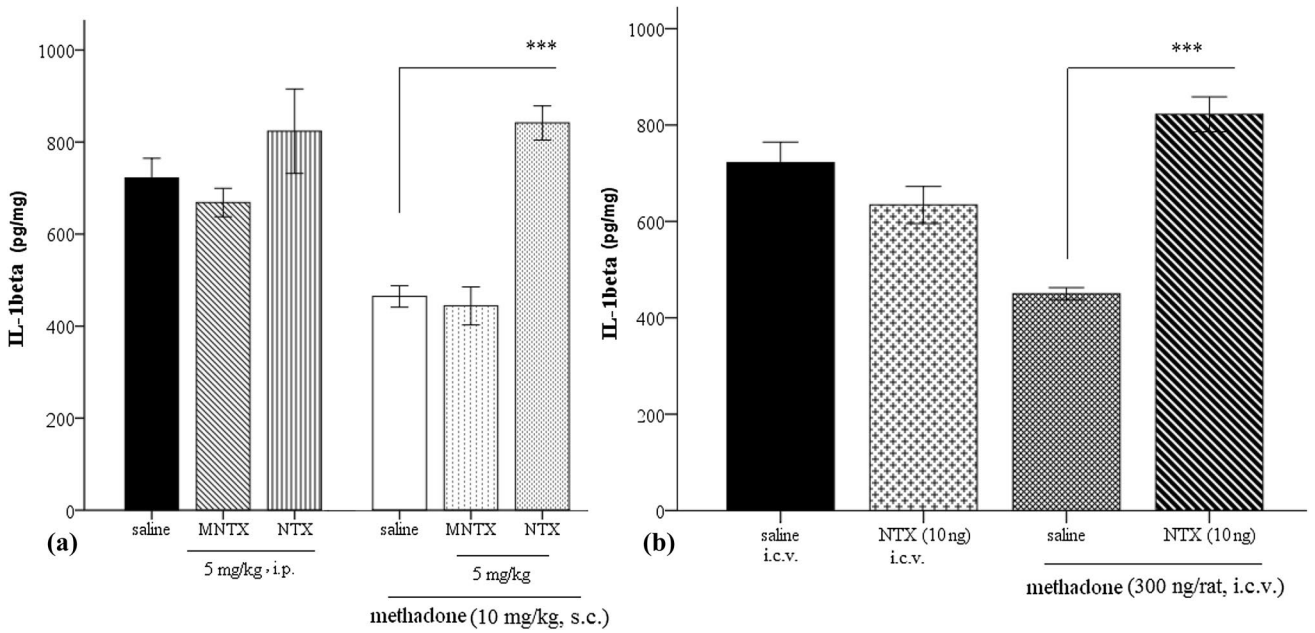

Fig. 6 Effect of opioid antagonists on the optimum methadone doses (10 mg/kg and 300 ng/rat) in acetic acid-induced colitis according to IL-1β levels. **a** s.c. and **b** i.c.v. administrations ($n=6$–8). $^{***}P<0.001$ significantly different from methadone groups

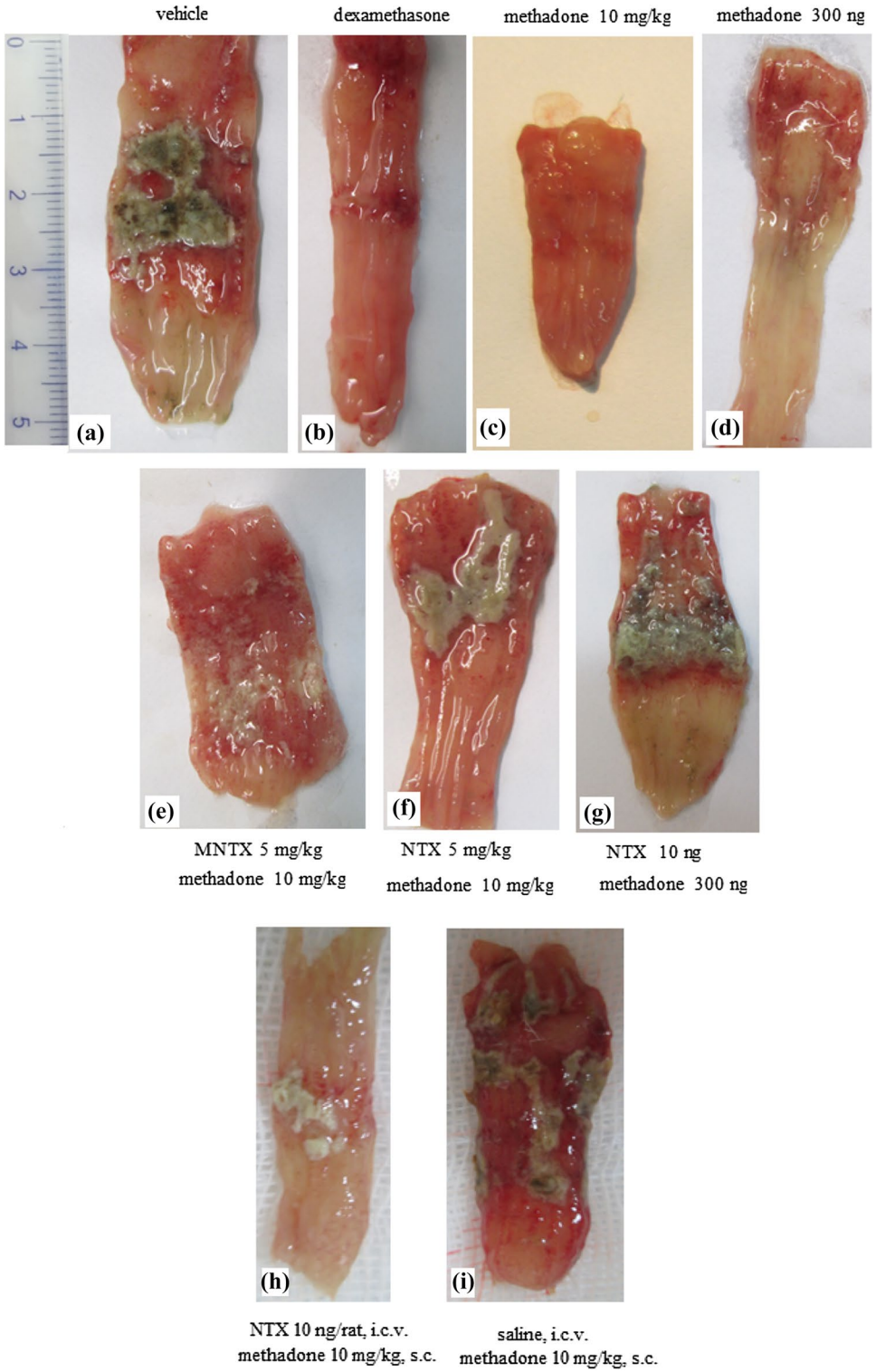

Fig. 7 Photographic images of rat colitis colons treated with **a** vehicle; **b** dexamethasone 1 mg/kg; **c** methadone 10 mg/kg; **d** methadone 300 ng; **e** MNTX 5 mg/kg + methadone 10 mg/kg; **f** NTX 5 mg/kg + methadone 10 mg/kg; **g** NTX 10 ng + methadone 300 ng; **h** NTX 10 ng + methadone 10 mg/kg; **i** saline i.c.v. ng + methadone 10 mg/kg

inflammation severity and significantly lower inflammation extent, $P < 0.01$ and $P < 0.05$, respectively. It also has significantly lower total index $P < 0.05$. Methadone 5 mg/kg shows significantly less crypt damage and percentage of involvement $P < 0.05$. In methadone 10 mg/kg group, inflammation severity decreases significantly and crypt damage decreases very highly significantly, $P < 0.05$ and $P < 0.001$, respectively. Moreover, it has a very highly significantly lower total index, $P < 0.001$. On the other hand, compared to methadone 10 mg/kg group, in methadone [10 mg/kg + MNTX 5 mg/kg] group crypt damage and percentage of involvement have a considerable increase, with a higher total index,

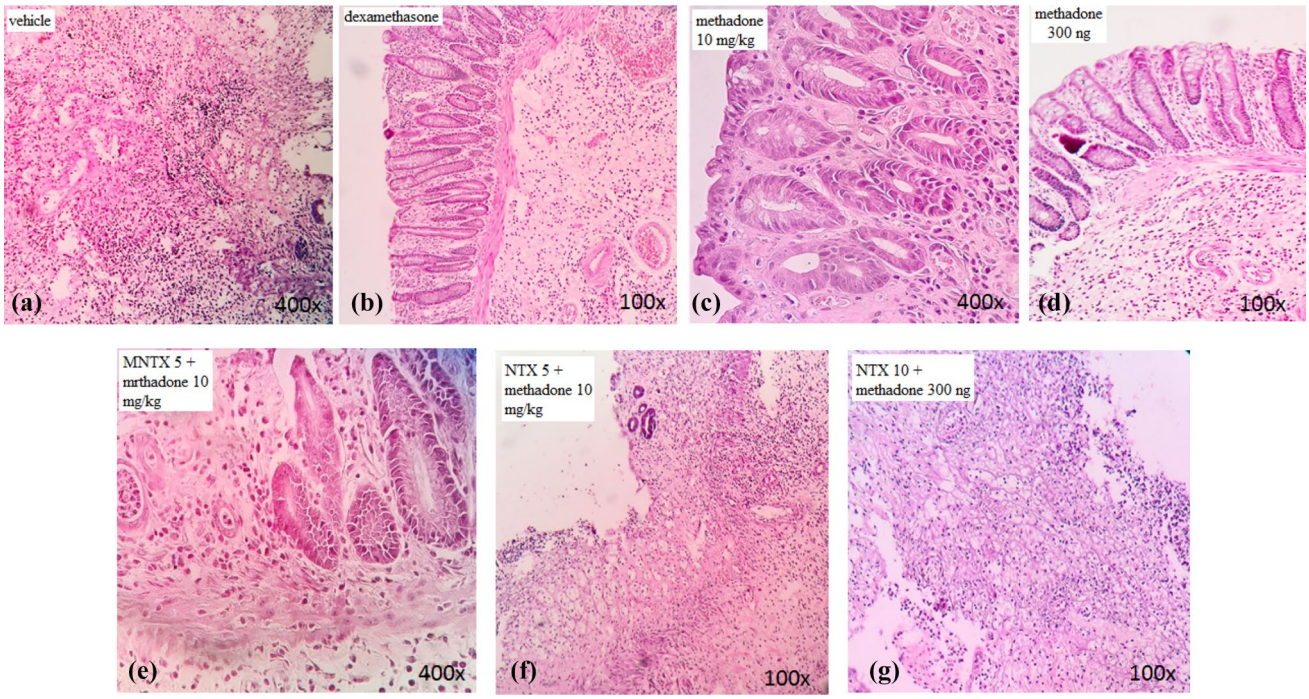

Fig. 8 H&E cross-sections of rat colitis colons treated with **a** vehicle; **b** dexamethasone 1 mg/kg **c** methadone 10 mg/kg; **d** methadone 300 ng; **e** MNTX 5 mg/kg + methadone 10 mg/kg; **f** NTX 5 mg/kg + methadone 10 mg/kg; **g** NTX 10 ng + methadone 300 ng

Table 5 Histopathologic parameters of the acetic acid-induced colitis, regarding the s.c. route of methadone injection

Groups	Inflammation severity (0–3)	Inflammation extent (0–3)	Crypt damage (0–4)	Involvement percentage (1–4)	Regeneration (0–4)	Total colitis index (1–18)
Sham	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	1 (1–1)	1 (1–1)
Control (IBD)	3 (3–3)^{a***}	3 (3–3)^{a***}	4 (4–4)^{a***}	2.83 (1–4)^{a***}	3 (2–4)^{a**}	15.83 (13–18)^{a***}
IBD + Dexa. (1 mg/kg)	1.5 (1–2)^{b**}	2 (2–2)^{b*}	3 (3–3)^{b}	1.5 (1–2)^{b}	2.5 (2–3)^{b}	10.5 (10–11)^{b*}
IBD + MNTX (5 mg/kg)	2.75 (2–3)^{b}	2.66 (2–3)^{b}	3 (2–4)^{b}	2.66 (1–4)^{b}	2.66 (1–4)^{b}	13.75 (8–18)^{b}
IBD + NTX (5 mg/kg)	2.66 (2–3)^{b}	3 (3–3)^{b}	3.66 (3–4)^{b}	3 (2–4)^{b}	2.83 (2–4)^{b}	15.16 (12–17)^{b}
IBD + Met. (5 mg/kg)	2.33 (2–3)^{b}	2.33 (2–3)^{b}	2.33 (1–4)^{b*}	2.33 (1–4)^{b*}	2.66 (2–3)^{b}	12 (9–15)^{b}
IBD + Met. (10 mg/kg)	1.83 (0–3)^{b*}	2.33 (1–3)^{b}	0.66 (0–2)^{b***}	0.66 (0–2)^{b*}	2.66 (1–4)^{b}	8.16 (4–13)^{b***}
IBD + Met. 10 mg/kg + MNTX 5 mg/kg	2.33 (2–3)^{c}	2.16 (1–3)^{c}	3.33 (2–4)^{c***}	3.33 (2–4)^{c***}	2.66 (2–4)^{c}	13.83 (9–16)^{c**}
IBD + Met. 10 mg/kg + NTX 5 mg/kg	2.66 (2–3)^{c}	2 (1–3)^{c}	3 (2–4)^{c**}	2.5 (2–4)^{c*}	2.5 (2–3)^{c}	12.83 (11–16)^{c*}
IBD + Saline (5 µl, i.c.v.) + Met. (10 mg/kg, s.c.)	2 (0–3)^{b*}	2.33 (1–3)^{b}	0.66 (0–2)^{b***}	0.66 (0–2)^{b*}	2.5 (2–3)^{b}	8.2 (4–13)^{b***}
IBD + NTX (10 ng/rat, i.c.v.) + Met. (10 mg/kg, s.c.)	3 (3–3)^{d**}	2.66 (2–3)^{d}	3.66 (3–4)^{d***}	2.5 (1–4)^{d*}	2.66 (2–3)^{d}	14.4 (11–18)^{d**}

Dexa. dexamethasone, *MNTX* methylnaltrexone, *NTX* naltrexone, *Met.* methadone

[a]Compared to sham

[b]Compared to control

[c]Compared to Met. 10 mg/kg

[d]Compared to saline 5 µl, i.c.v. + Met. 10 mg/kg, s.c.

*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

$P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively. Similarly, in the group [methadone 10 mg/kg + NTX 5 mg/kg], crypt damage and percentage of involvement have considerable increase, with a higher total index, $P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively.

In addition, the group (NTX 10 ng/rat, i.c.v. + methadone 10 mg/kg, s.c.) has significant increase in inflammation severity $P < 0.01$, crypt damage $P < 0.001$ and percentage of involvement $P < 0.05$ compared to the group (saline i.c.v. + methadone 10 mg/kg). Also, it has a higher total index $P < 0.001$.

Table 6 demonstrates the pathologic results of i.c.v. methadone route. Control group has very highly significantly higher inflammation severity and extent compared to the sham group, $P < 0.001$. Moreover, crypt damage, the percentage of involvement are increased very highly significantly as well, $P < 0.001$. Regeneration and total index are also raised very highly significantly $P < 0.001$. In comparison to the IBD group, dexamethasone shows considerably lower inflammation severity and extent, $P < 0.001$ and $P < 0.01$, respectively. It also has significantly lower crypt damage and total index, $P < 0.05$. In methadone 300 ng/rat group, the inflammation severity and extent decrease highly significantly $P < 0.01$. Moreover, it has significantly lower crypt damage, the percentage of involvement, regeneration, and total index, $P < 0.001$, $P < 0.05$, $P < 0.05$ and $P < 0.001$, respectively. On the other hand, compared to methadone 300 ng/rat group, in the group (methadone 300 ng/rat + NTX 10 ng/rat), crypt damage and the percentage of involvement have very highly significantly increases, $P < 0.001$ In addition, regeneration and total index rise significantly $P < 0.05$.

Discussion

The current investigation highlighted a crucial role for the brain–gut axis in the control of intestinal inflammation related to IBD in a rat model. Our results demonstrated that central and peripheral methadone administrations significantly reduced the severity of the ulcerative lesions induced by acid acetic and markedly improved macroscopic and microscopic scores via opioid receptor-dependent mechanisms. Nevertheless, using specific peripheral and central antagonists, our results showed that in the treatment of IBD, methadone acts mainly through the central route.

Virtually consistent with our experiment, two selective peripheral μ-opioid receptor agonists, named DALDA and DAMGO, significantly reduced inflammation in experimental colitis models in mice. They also stated that opioids exert their anti-inflammatory effects mainly through the peripheral route (Philippe et al. 2003). Showing physiologic anti-inflammatory effects of the peripheral opioid receptors in experimental colitis models, this experiment is approximately in line with our study except that the current experiment mostly highlights a role for central opioid receptors in control of a peripherally induced gut inflammation.

There is no doubt that opioid peptides are a link between the neuroendocrine and immune systems, and the immunomodulatory effect of enkephalins may play a significant clinical role in immune-mediated diseases through HPA axis and other parts of the limbic system in the brain (Collins and Verma-Gandhu 2006; Straub et al. 2008). Peripherally mediated immunosuppressive effects of opioids may play a significant role in opioid-induced immunosuppression (Wei et al. 2003). Accumulating evidence supported

Table 6 Pathological parameters of colitis induced by acetic acid, regarding the i.c.v. route of methadone injection

Groups	Inflammation severity (0–3)	Inflammation extent (0–3)	Crypt damage (0–4)	Involvement percent (1–4)	Regeneration (0–4)	Total colitis index (1–18)
Sham	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	1 (1–1)	1 (1–1)
Control (IBD)	3 (3–3)a***	3 (3–3)^{a***}	4 (4–4)^{a***}	2.83 (1–4)^{a***}	3 (2–4)^{a***}	15.83 (13–18)^{a***}
IBD + Dex.(1 mg/kg)	1.5 (1–2)^{b***}	2 (2–2)^{b**}	3 (3–3)^{b*}	1.5 (1–2)^{b}	2.5 (2–3)^{b}	10.5 (10–11)^{b*}
IBD + NTX 10 (ng/rat)	3 (3–3)^{b}	2.5 (2–3)^{b}	3.5 (3–4)^{b}	3.5 (3–4)^{b}	3 (2–4)^{b}	15.5 (13–18)^{b}
IBD + Met. 50 (ng/rat)	2.66 (1–3)^{b}	2.66 (1–3)^{b}	3.66 (3–4)^{b}	2.66 (1–4)^{b}	2.66 (2–3)^{b}	14.33 (9–16)^{b}
IBD + Met. 300 (ng/rat)	1.66 (1–2)^{b**}	2 (1–3)^{b**}	1.66 (0–3)^{b***}	1.16 (0–2)^{b*}	1.66 (1–2)^{b*}	8.16 (3–10)^{b***}
IBD + Met. 300 ng/rat + NTX 10 ng/rat	2.66 (2–3)^{c*}	2 (2–2)^{c}	3 (3–3)^{c***}	2.16 (1–3)^{c***}	2.66 (2–3)^{c*}	12.5 (10–14)^{c*}

Dex. dexamethasone, *NTX* naltrexone, *Met.* methadone

[a]Compared to sham

[b]Compared to control

[c]Compared to Met. 300 ng

*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

a role for endogenous opioid peptides such as enkephalins and endorphins in the development and/or perpetuation of inflammation (Rogers and Peterson 2003; Pol and Puig 2004). Chronic oral NTX promoted the mucosal healing in subjects with moderate to severe Crohn's disease (Smith et al. 2011). On the other hand, low doses of morphine were pro-inflammatory in adjuvant arthritis (Earl et al. 1994).

Opioid therapy is an exciting new development for treating inflammatory diseases such as arthritis, especially since they cause fewer side effects compared to molecules, which act outside the CNS. In this regard, κ-opioid drugs showed powerful anti-inflammatory effects, reducing arthritis severity by as much as 80%, attenuating the disease in a dose-dependent, stereo-selective and antagonist-reversible manner (Walker 2003). As another example, loperamide, a peripherally acting mu (μ)-opioid receptor agonist, commonly used as anti-diarrhea agent (Hanauer 2007). It showed analgesic and anti-inflammatory effects in a similar manner to peripheral endogenous opioids, in peripheral inflamed tissue in a rat model (Hua 2014). Moreover, loperamide showed both central and peripheral anti-nociceptive effects in the formalin test, an inflammatory pain model in rats (Shannon and Lutz 2002).

The vagus nerve (VN) (Bonaz 2007; Meregnani et al. 2011) and HPA axis (Mackner et al. 2011) have mainly been reported as a modulator of the neuro-intestinal inflammatory pathway (Pavlov et al. 2009). Moreover, impairments of the autonomic nervous system (ANS) and lower parasympathetic function have been reported as another etiological pathway in IBD (Pavlov et al. 2009). Neuromodulation as a therapeutic approach opens a new era in the treatment of IBD. Amongst the potential therapeutic neuronal pathways, the VN, based on its anti-inflammatory properties, is the most likely therapeutic target, in particular through VN stimulation. The VN is the longest nerve in the body innervating numerous organs including the GI tract (Bonaz et al. 2017). The VN is the main component of the parasympathetic nervous system and innervates the entire GI tract except for the rectum in the rat (Altschuler et al. 1993).

It is well established that dysregulation of the brain neurotransmitters can affect the peripheral inflammatory conditions such as IBD. Psycho-neuro-endocrine-immune modulation through the brain–gut axis likely has a key role in the pathogenesis of IBD (Taché and Bernstein 2009; Bonaz and Bernstein 2013). Chronic GI inflammation also induced anxiety and behavioral despair in mice which was associated with decreased hippocampal brain-derived neurotrophic factor (BDNF) messenger RNA and increased circulating TNF-α and interferon-α (Bercik et al. 2010). Similarly, the gut–brain communication was observed following induction of IBD in mice and the animals showed behavioral despair (Heydarpour et al. 2016).

In the current study, following central and peripheral administration of methadone, the notable reduction in elevated levels of the pro-inflammatory markers (TNF-α and IL-1β) was observed in IBD rats. During the course of experimental colitis, TNF-α releases and aggravates the tissue damage. Secretion of TNF-α by the epithelial cells may act as a pivotal factor in the pathogenesis of IBD. TNF-α, IL-1β and other inflammatory cytokines secreted by lymphocytes and macrophages in the inflamed intestine can profoundly affect the activation state of mesenchymal cells, thereby amplifying the inflammatory response and probably contributing to fibrosis, one of the most important complications of IBD (Daneshmand et al. 2009). In our investigation, both central and peripheral methadone administrations showed the protective effect on acetic acid-induced colitis. However, it should be noted that this effect of methadone was mediated mainly through the central opioid receptors. The i.c.v. route had more potency due to its significantly lower administered doses, which has shown to markedly diminish methadone drawbacks like cardiac and respiratory suppression (Ricardo Buenaventura et al. 2008; Chugh et al. 2008). This might also explain, at least partly, the difference in the effect of peripherally and centrally given methadone on intestinal lesions and mortality rate. While the previous one was reduced significantly by methadone peripheral injection, the mortality rate in IBD-rat was not affected. In contrast, 300 ng dose of methadone given centrally highly reduced both the mortality rate and the intestinal lesion. Although MNTX as a peripheral opioid antagonist could not significantly reverse the methadone anti-inflammatory effect, the contribution of peripheral mechanisms to the effect of methadone is also probable. Nevertheless, NTX reversed the protective effect of methadone both centrally and peripherally. In consistence with that, investigators have utilized MNTX, a quaternary form of NTX that does not cross the BBB, to separate central from peripheral effects. The compound was able to antagonize most of the immune alterations produced by systemic morphine injection when it was administered intracerebroventricularly, but failed to do the same while administered subcutaneously in rats (Lysle and Coussons-Read 1995; Fecho et al. 1996). In line with that, morphine inhibited carrageenan-induced paw swelling (Gyires et al. 1985) and near-toxic doses of morphine were able to attenuate adjuvant arthritis in rats (Levine et al. 1985; Walker et al. 1996). Moreover, a decrease in serum-free met-enkephalin value was observed in IBD patients (Owczarek et al. 2011). Following methadone treatment, the pathological parameters such as severity and extent of inflammation, crypt damages and, the tissue involvement reduced markedly and tissue regeneration improved. It is worth mentioning that though the scores were not significantly affected, MNTX reversed

the methadone-induced decreased level of TNF-α, consequently, the contribution of peripheral mechanisms to the effect of methadone cannot be excluded. Moreover, reversal of methadone's protective effect by NTX demonstrated that in the treatment of IBD, methadone may act mainly through the central route.

Conclusion

In this study, we showed for the first time that probably the central opioid receptors are mainly involved in anti-inflammatory aspects of methadone in acetic acid-induced colitis and the role of the brain–gut axis is highlighted. Because of diversity in opioid receptors, further studies are crucial to address which types of opioid receptors are involved in beneficial effects of central methadone. Our findings open a new platform to target gut inflammation in IBD-associated comorbidities like memory impairment, depression, and anxiety. Indeed, methadone would be considered as a pharmacophore to design central-acting therapeutic agents to treat IBD. Overall, our findings provide evidence that opioid receptors' agonists may have the potential to control the gut inflammation and might be new therapeutics.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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