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RESEARCH PAPER

Effect of Mas-related gene (Mrg) receptors on hyperalgesia in rats with CFA-induced inflammation via direct and indirect mechanisms

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BACKGROUND AND PURPOSE

Mas oncogene-related gene (Mrg) receptors are exclusively distributed in small-sized neurons in trigeminal and dorsal root ganglia (DRG). We investigated the effects of MrgC receptor activation on inflammatory hyperalgesia and its mechanisms.

EXPERIMENTAL APPROACH

A selective MrgC receptor agonist, bovine adrenal medulla peptide 8-22 (BAM8-22) or melanocyte-stimulating hormone (MSH) or the μ-opioid receptor (MOR) antagonist CTAP was administered intrathecally (i.t.) in rats injected with complete Freund's adjuvant (CFA) in one hindpaw. Thermal and mechanical nociceptive responses were assessed. Neurochemicals were measured by immunocytochemistry, Western blot, ELISA and RT-PCR.

KEY RESULTS

CFA injection increased mRNA for MrgC receptors in lumbar DRG. BAM8-22 or MSH, given i.t., generated instant short and delayed long-lasting attenuations of CFA-induced thermal hyperalgesia, but not mechanical allodynia. These effects were associated with decreased up-regulation of neuronal NOS (nNOS), CGRP and c-Fos expression in the spinal dorsal horn and/or DRG. However, i.t. administration of CTAP blocked the induction by BAM8-22 of delayed anti-hyperalgesia and inhibition of nNOS and CGRP expression in DRG. BAM8-22 also increased mRNA for MORs and pro-opiomelanocortin, along with β-endorphin content in the lumbar spinal cord and/or DRG. MrgC receptors and nNOS were co-localized in DRG neurons.

CONCLUSIONS AND IMPLICATIONS

Activation of MrgC receptors suppressed up-regulation of pronociceptive mediators and consequently inhibited inflammatory pain, because of the activation of up-regulated MrgC receptors and subsequent endogenous activity at MORs. The uniquely distributed MrgC receptors could be a novel target for relieving inflammatory pain.

Abbreviations

BAM8-22, bovine adrenal medulla 8-22; CFA, complete Freund's adjuvant; DRG, dorsal root ganglia; i.pl., intraplantar; i.t., intrathecal; MOR, μ-opioid receptor; Mrg, Mas oncogene-related gene; MSH, melanocyte-stimulating hormone; nNOS, neuronal NOS; POMC, pro-opiomelanocortin; SNSR, sensory neuron-specific receptors

Introduction

Mas oncogene-related gene (Mrg) receptors (Dong *et al.*, 2001), also known as sensory neuron-specific receptors (SNSR; Lembo *et al.*, 2002), are a large family of GPCRs (receptor nomenclature follows Alexander *et al.*, 2011). This type of receptor has been identified in mice (Dong *et al.*, 2001), rats (Lembo *et al.*, 2002; Zylka *et al.*, 2003), humans (Dong *et al.*, 2001; Lembo *et al.*, 2002), gerbils (Zylka *et al.*, 2003), rhesus monkeys (Burstein *et al.*, 2006) and macaques (Zhang *et al.*, 2005). The Mrg receptor family comprises many members, divided into four groups (MrgA–MrgD) in rodents (Dong *et al.*, 2001), but seven groups (MrgX1–MrgX7) in humans (Dong *et al.*, 2001; Choi and Lahn, 2003). Importantly, most members of the Mrg receptor family are exclusively distributed in small diameter neurons of trigeminal and dorsal root ganglia (DRG; Dong *et al.*, 2001; Lembo *et al.*, 2002), implying their involvement in nociceptive transmission. This highly restricted distribution of Mrg receptors may allow nociception to be processed or modulated in a highly selective manner and provides a drug target which should have minimal side effects in the CNS (Simonin and Kieffer, 2002).

Surprisingly, the functional roles of Mrg receptors in various types of pain have not yet been fully described. Attention has been drawn to MrgC receptors, partly because of the availability of the specific agonists, bovine adrenal medulla peptide 8-22 (BAM8-22; Lembo *et al.*, 2002; Guan *et al.*, 2010) and (Tyr^6)-γ2-MSH-6-12 [melanocyte-stimulating hormone (MSH)] (Han *et al.*, 2002; Lembo *et al.*, 2002). MrgC receptors are not involved in pain processing under normal conditions (physiological pain) as the deletion of MrgC genes (Ndong *et al.*, 2009; Guan *et al.*, 2010) and pharmacological activation of the receptors (Cai *et al.*, 2007b; Guan *et al.*, 2010) did not alter nociceptive thresholds, but these receptors can modulate pathological pain. For example, spinal application of BAM8-22 attenuates mechanical allodynia induced by peripheral nerve injury (Guan *et al.*, 2010). However, there is a disagreement regarding the role of MrgC receptors in inflammatory pain. Rats with SNSR1 (corresponding to MrgC receptors) knocked-down by small interfering RNA (siRNA) show a reduction in thermal hyperalgesia in the complete Freund's adjuvant (CFA) model, suggesting a role of MrgC receptors in the induction of inflammatory pain (Ndong *et al.*, 2009). Data from mice indicate that MrgC receptors display protective actions in inflammatory pain as animals with MrgC cluster knock-out showed enhanced wind-up responding to C fibre inputs and/or increased pain responses following an intraplantar (i.pl.) injection of formalin (Guan *et al.*, 2010), CFA or carrageenan (Liu *et al.*, 2009).

As MrgC receptors possess high therapeutic potential, it is important to clarify the effects of MrgC receptor activation on inflammatory pain. The present study examined the effects of intrathecal (i.t.) administration of BAM8-22 and MSH on inflammatory pain, up-regulation of neuronal NOS (nNOS) and expression of CGRP and c-Fos, which are believed to be ascribed to or indicate hypersensitivity, in the rat model of CFA-induced inflammation. We also investigated the possibility that the stimulation of MrgC receptors increased the activation of μ-opioid receptors (MORs) by endogenous agonists. Some of these results have been presented in abstract form (Hong *et al.*, 2012).

Methods

Animals

All animal care and experimental treatments complied with the guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983) and were approved by the Animal Care Committee of Fujian Normal University. Efforts were made to minimize animal suffering and the number of animals used in our experiments. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 196 animals were used in the experiments described here. Male Sprague–Dawley rats (250–320 g; Animal Center of Fujian Medical University, Fuzhou, China) were housed individually at 22°C with 50% humidity under a 12 h light/dark cycle and given free access to food and water. Inflammation was induced by s.c. injection of 150 μL of 1 mg·mL^{-1} CFA into the hindpaw. Control rats received an injection of saline (150 μL).

Intrathecal catheter implantation

Drugs were delivered spinally via i.t. catheter (described below) or percutaneous lumbar puncture (see Supporting Information Appendix S1).

Animals were implanted with chronic indwelling catheters with some modification of the previously described technique (Pogatzki *et al.*, 2000). Briefly, rats were injected with i.p. pentobarbitone (50 mg·kg^{-1}) and shaved along the occiput and neck. An incision was made overlying the atlanto-occipital junction, and the dura mater was exposed by blunt dissection. An incision was made in the dura, and a polyethylene catheter (PE-10; Stoelting, Wood Dale, IL, USA), with a loose knot cemented with dental acrylic 8.0 cm from the end, was threaded caudally to position its tip at the L4-5 segments of the spinal cord. The external end of the catheter was externalized at the back of the neck. The catheter was then flushed with 10 μL of saline and plugged. The rats were housed individually after surgery and allowed to recover for approximately 7 days before being used for behavioural testing. Only the animals with no evidence of neurological deficits after catheter placement were used for subsequent experimentation.

The MrgC receptor agonist, BAM8-22 or MSH, was administered i.t. at 0 and 24 h. To investigate the possible involvement of MORs, some rats also received i.t. injection of the selective MOR antagonist CTAP (Pelton *et al.*, 1985).

Assessment of nociceptive behaviour

A radiant heat stimulus from a Plantar Test Meter (IITC Life Science Inc., Woodland Hills, CA, USA) was applied by aiming a light beam through a hole in the light box through the glass plate to the middle of the plantar surface of the rat's hindpaw. The heat intensity was adjusted to obtain average paw withdrawal latency (PWL) of 7–9 s, and the cut-off time was set at 20 s to prevent tissue damage. PWL at any test time point was measured three times at 1.5 min interval, and the mean value of these measurements was taken (Zeng *et al.*, 2006; Boettger *et al.*, 2007).

Mechanical threshold was measured in the hindpaw using an automated von Frey type system (Dynamic Plantar Anesthesiometer 37400; Ugo Basile, Comerio VA, Italy). The

stimulator unit was placed beneath the selected hindpaw with the filament below the plantar surface of the rat. A paw withdrawal response was elicited by applying an increasing force (measured in grams) using a stainless steel filament (0.5 mm diameter). The force was increased at a rate of 2.5 $g \cdot s^{-1}$ until the rat moved its paw. A force of 50 g for 30 s was used as a cut-off point to preclude possible damage to the paw. The force was measured three times at 1.5 min interval to generate mean values.

Immunohistochemistry

Rats were deeply anaesthetized with sodium pentobarbitone (60 $mg \cdot kg^{-1}$ i.p.). The animals were perfused intracardially with cold 0.01 M PBS and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The L4-5 segments of the spinal cord and DRG were removed and post-fixed in the same fixative overnight. Tissues were then transferred into 30% sucrose in PB for cryoprotection. Spinal cord sections (40 µm thick) and DRG sections (10 µm thick) were cut on a cryostat (MICROM GmbH, HM550; Walldorf, Germany). Immunocytochemistry was performed at room temperature on free-floating sections (spinal cord) or slides (DRG) using an avidin–biotin complex technique as described previously (Cai *et al.*, 2007a). To permit comparisons across treatment groups, sections from different groups were processed simultaneously. After pretreatment with 0.3% H_2O_2 and 10% normal goat serum, sections were incubated at 4°C with polyclonal rabbit anti-nNOS (1:2500; Biosciences Pharmingen, San Diego, CA, USA) or anti-CGRP [1:10 000; Santa Cruz Biotechnology (Shanghai) Co., Ltd., Shanghai, China] for 24 h. The tissues were then transferred to biotinylated secondary IgG complex (1:200 in 10% goat serum in PBS) for 2 h followed by exposure to avidin–biotin HRP complex (1:100; Vector Laboratories, Burlingame, CA, USA) for 1 h. The chromogen was developed with 0.01% H_2O_2 and 0.05% diaminobenzidine. After being thoroughly rinsed with PBS, spinal sections were mounted on gelatin-coated slides, air dried, dehydrated in a series of graded ethanol, cleared in xylene and coverslipped. Using different sections ($n = 2$), the primary antibody against either nNOS or CGRP was omitted in the immunocytochemical process, which resulted in the absence of staining. The quantification of immunoreactivity (IR) for CGRP and nNOS is described in the Supporting Information Appendix S1.

For double immunostaining of nNOS with MrgC receptors, DRG sections were first incubated in 10% normal donkey serum and next in a mixture of goat polyclonal antibody against nNOS (1:100; Abcam, Cambridge, MA, USA) with rabbit antisera against MrgC receptors (1:100; Phoenix Pharmaceuticals, Burlingame, CA, USA) for 24 h at 4°C. Sections were then incubated in a mixture of donkey anti-goat IgG conjugated with FITC (1:200; Abcam) and donkey anti-goat IgG conjugated with rhodamine (1:100) for 2 h at room temperature. nNOS-IR appeared green, whereas MrgC receptor-IR appeared red. Images were captured using a confocal microscopy system (C1-Si; Nikon, Tokyo, Japan). For control, omission of the primary antibody resulted in negative staining in all tested sections. Sections of DRG were incubated with MrgC receptor antiserum that was preabsorbed with MrgC receptor protein [1 µM, Phoenix Biotech (Beijing) Co., Ltd., Beijing, China]. These procedures resulted in the complete loss of staining.

Western blot, quantitative real-time PCR and ELISA

Methods for these procedures are described fully in Supporting Information Appendix S1.

Data analysis

CFA-induced responses, such as thermal supersensitivity (Malcangio and Bowery, 1994), chemical release (Cabot *et al.*, 2001) in the hindpaw and expression of nociceptive or antinociceptive molecules in DRG (Galeazza *et al.*, 1995; Puehler *et al.*, 2004) and spinal dorsal horn (Nahin *et al.*, 1989), are elicited only on the ipsilateral side. Therefore, the expression of MrgC mRNA and opioid molecules on the side contralateral to CFA injection were used as control values.

Data are expressed as mean ± SEM. Statistical significance between groups was examined using ANOVA followed by Tukey's test for multiple comparisons. To detect changes over the time between two groups (treatment group × time), data were analysed using a two-way ANOVA. A *P*-value of less than 0.05 was considered statistically significant.

Materials. Sodium pentobarbitone was obtained from Shengong, Shanghai, China. CFA and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BAM8-22, MSH and BAM8-18 were synthesized in Huadatianyuan Co. (Shanghai, China; see Supporting Information Appendix S1).

Results

Activation of MrgC receptors produces instant and delayed attenuations of CFA-induced heat hyperalgesia but does not change CFA-induced mechanical allodynia

As a pilot study showed that i.t. administration of BAM8-22 inhibited, rather than enhanced, CFA-evoked hyperalgesia in rats (as observed in mice by Guan *et al.*, 2010), the antinociceptive effects of activating MrgC receptors were measured. CFA was injected in one hindpaw on day 0 and BAM8-22 (3.3, 10 and 30 nmol), or saline was administered i.t. on days 0 and 1. The CFA injection combined with saline i.t., produced hyperalgesia indicated by a marked decrease in PWL on days 1 and 2 (Figure 1A). However, the hyperalgesic response was attenuated in the group given BAM8-22 at 30 nmol on day 1 and was reduced even further on day 2. These changes were significantly different from corresponding control ($P < 0.5$ or 0.001). I.t. BAM8-22 at a dose of 10 nmol also attenuated hyperalgesia on day 2 ($P < 0.001$). Moreover, the highest dose of BAM8-22 administered produced a rapidly developing (by 20 min) but short lasting (<60min) increase in PWL, compared with the saline-treated group. This rapid response is illustrated in Figure 1B, where the BAM8-22-induced changes have been expressed as a percentage of the pre-BAM8-22 value and shows that 30 nmol BAM8-22 induced 120–125% PWL ($P < 0.001$) for 40 min.

To confirm the effect of MrgC receptor activation on CFA-induced hyperalgesia, we used another MrgC receptor agonist, MSH, with a structure different from that of

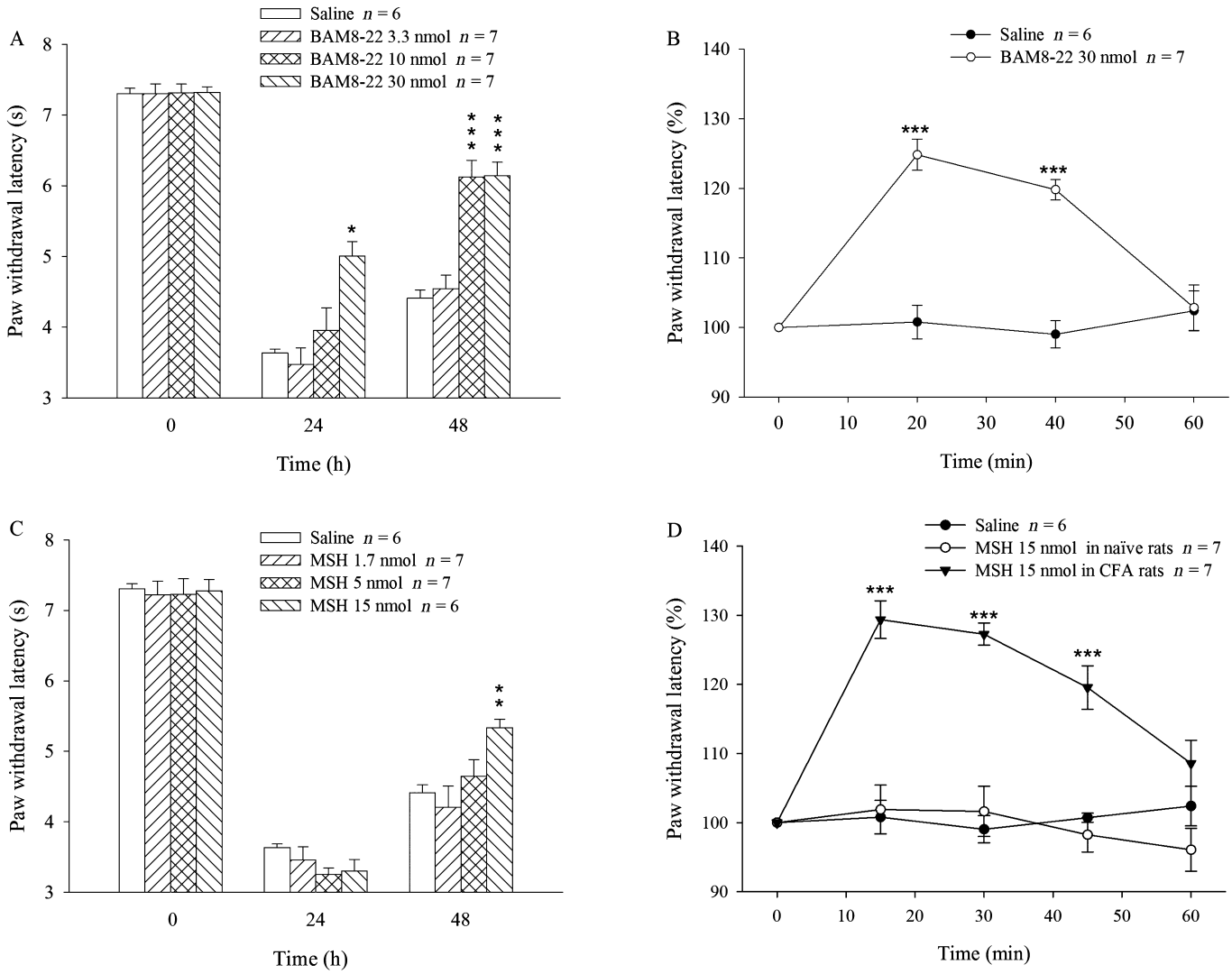

Figure 1

PWL in response to radiant heat. Animals were treated with i.pl. CFA (150 μL) on day 0, and i.t. BAM8-22 (3.3, 10.0 and 30.0 nmol) or MSH (1.7, 5.0 and 15.0 nmol) on days 0 and 1. PWL was measured on days 0, 1 and 2 prior to any injections in (A) and (C). PWL was also measured on day 1 after the injection of BAM8-22 (B) or MSH (D). In (B) and (D), PWL was calculated as percentage of pretreatment baseline PWL (100%). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$: compared with saline group (A and C) or pretreatment baseline (B and D).

BAM8-22. As shown in Figure 1C, i.t. administration of MSH on day 0 did not change CFA-induced hyperalgesia on day 1. However, PWL was significantly increased on day 2 in the group treated with 15 nmol MSH compared with the CFA/saline group ($P < 0.01$). MSH (15 nmol) administered on day 1 also produced the rapid increase in PWL compared with the pre-treated level, an increase that lasted for 45 min ($P < 0.001$; Figure 1D). In contrast, MSH (15 nmol) given i.t. to naïve animals failed to alter thermal pain threshold (Figure 1D).

The effect of BAM8-22 on CFA-induced mechanical sensitivity was also assessed. CFA was injected at 0 h and saline or BAM8-22 (30 nmol) was given i.t. at 0, 24, 48 and 72 h. Baseline mechanical threshold in the hindpaw was 34.2 ± 0.4 g in CFA + saline group. The threshold was decreased to 16.2 ± 0.6 g on day 1 and maintained throughout the experiment (20–23 g on days 2–4; $n = 8$, data not shown). However, the daily administration of BAM8-22 for 4 days did not change CFA-induced hypersensitivity compared with CFA + saline group ($n = 9$, data not shown).

Activation of MrgC receptors attenuates CFA-induced expression of CGRP, nNOS and c-Fos in DRG or spinal dorsal horn

Because CGRP plays a pivotal role in pathogenesis of inflammatory hyperalgesia (Seybold *et al.*, 1995; Fehrenbacher *et al.*, 2003), we examined the effect of i.t. BAM8-22 on CFA-induced CGRP expression. A number of small to medium sized neurons exhibited staining for CGRP in DRG. On the side contralateral to CFA injection, the proportion of CGRP-IR positive small or medium neurons (Figure 2A and E) was lower than the corresponding proportions on the ipsilateral side ($P < 0.01$; Figure 2B and E), illustrating the unilateral

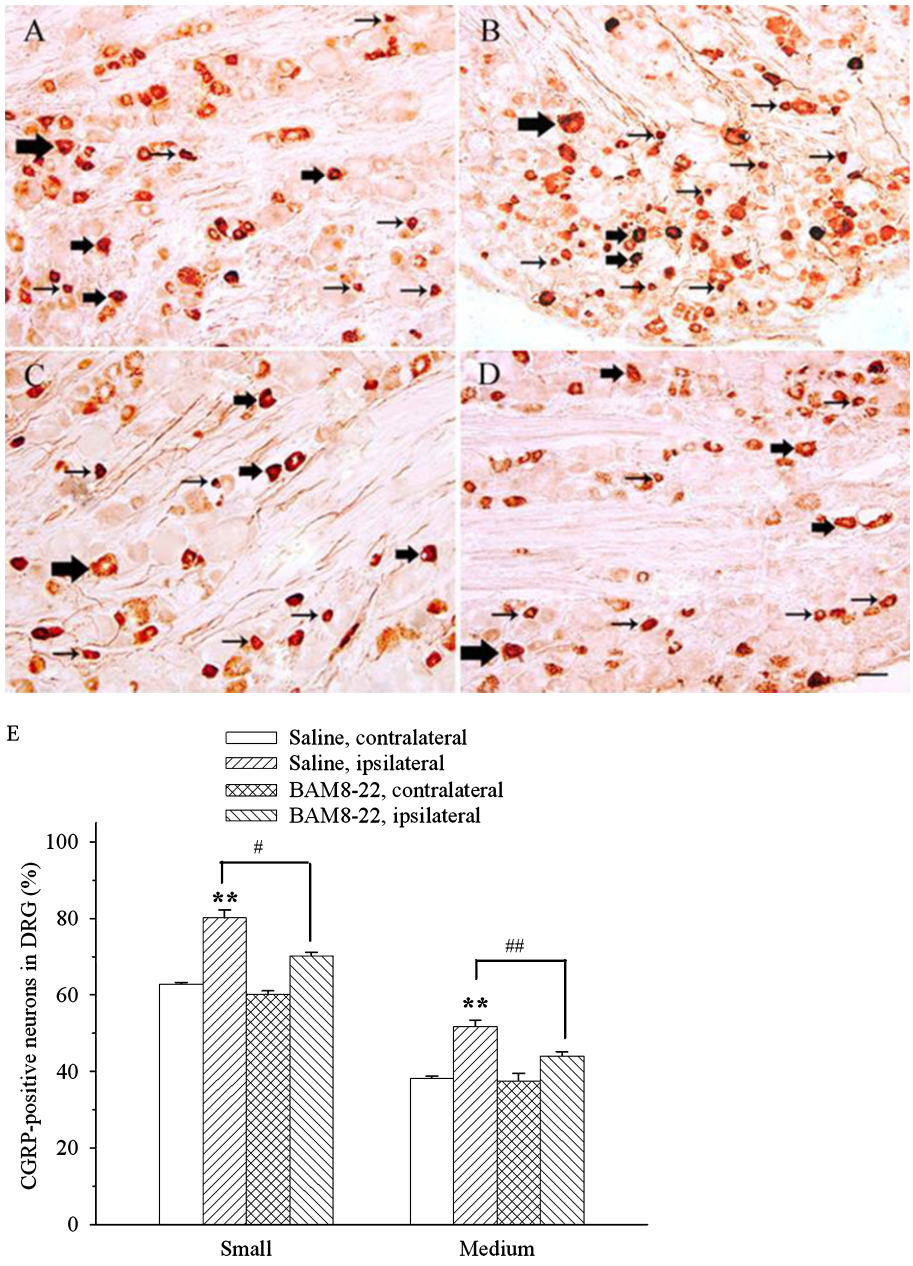

Figure 2

Effect of i.t. administration of BAM8-22 on CFA-evoked expression of CGRP in DRG. CFA was injected in the right hindpaw on day 0, and BAM8-22 (30 nmol) was administered i.t. on days 0 and 1. The L4-6 DRG were collected on day 2. Photomicrographs of transverse sections of DRG show CGRP-IR in small (small arrows), medium (medium arrows) and large (large arrows) neurons on the contralateral (A and C) and ipsilateral (B and D) sides in CFA/saline (A and B) and CFA/BAM8-22 (C and D) groups. Histograms show means ± SEM of CGRP-IR-positive neurons in DRG (E). $^{**}P < 0.01$ compared with the contralateral side. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; compared with CFA/saline group. $n = 4$ each. Scale bar = 50 μm.

induction of CGRP by CFA. Following i.t. administration of BAM8-22, the proportion of CGRP-positive neurons in small and medium subpopulations on the ipsilateral side was reduced, to values significantly different from the CFA/saline group ($P < 0.05$–0.01; Figure 2C and E).

Because no antagonist of MrgC receptors is available, another MrgC receptor agonist, MSH, was used to confirm the receptor activation. As illustrated in Figure 3A, CFA injection increased the expression of nNOS in the spinal dorsal horn compared with saline treatment ($P < 0.05$). The i.t. administration of the non-active MrgC homologue BAM8-18 (30 nmol) did not change CFA-induced increase in nNOS expression ($P > 0.05$). However, after i.t. MSH (15 nmol), CFA injection induced a level of nNOS that was significantly lower than that in the saline group ($P < 0.01$; Figure 3A). Similarly, i.t. MSH, but not BAM8-18, inhibited the CFA-induced c-Fos protein expression in dorsal horn, compared with saline group ($P < 0.01$; Figure 3B).

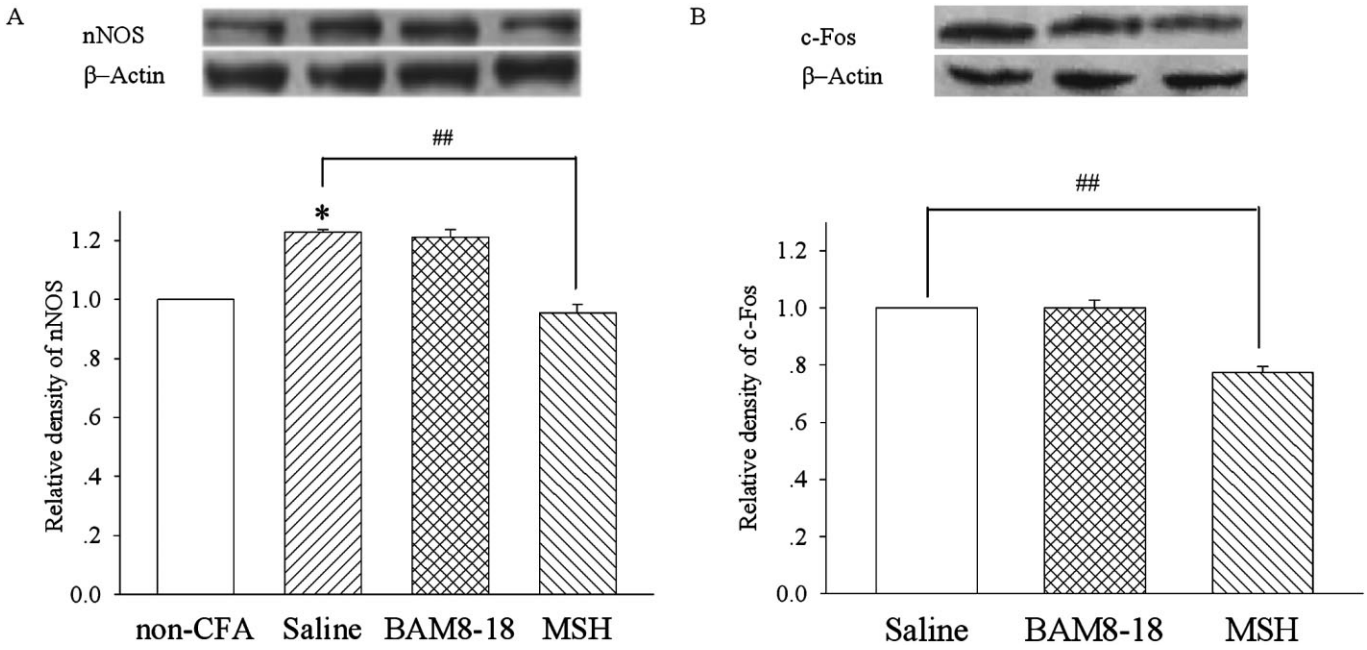

Figure 3

Effect of i.t. administration of MSH on CFA-evoked expression of nNOS and c-Fos proteins in the spinal dorsal horn. CFA was injected i.pl. in the right hindpaw on day 0, and MSH (15 nmol), saline or BAM8-18 (30 nmol) was administered i.t. on days 0 and 1. The lumbar spinal cord was collected on day 2. The expression of nNOS (A) and c-Fos (B) proteins in the spinal dorsal half was assayed by Western blot on the side ipsilateral to CFA injection. *$P < 0.05$ compared with non-CFA group. $^{\#\#}P < 0.01$ compared with CFA/saline group. $n = 4$ each.

CFA injection up-regulates the expression of MrgC receptor mRNA

We reported earlier that CFA-induced inflammation increased the expression of the endogenous MrgC receptor ligand BAM22 in the spinal dorsal horn and DRG (Cai *et al.*, 2007a). We therefore evaluated the effects of CFA injection on the expression of mRNA for MrgC receptors. As demonstrated in Figure 4, there was a basal level of MrgC mRNA expression in lumbar DRG in animals injected with saline in the hindpaw. Following CFA injection, MrgC receptor mRNA was increased over basal values on days 1 and 2 respectively ($P < 0.05$).

Activation of MrgC receptors leads to endogenous activation of MORs

The results showing that the short-acting compounds BAM8-22 and MSH (Grazzini *et al.*, 2004) produced long-lasting inhibition of CFA-induced hyperalgesia suggested a non-MrgC receptor-mediated mechanism. Therefore, the possible involvement of endogenous agonists of MORs was assessed. PWL was decreased at 4 h (on day 0) following CFA plus i.t. saline injections ($P < 0.001$). After CFA plus i.t. BAM8-22 (30 nmol) administered at 0 h, PWL was less decreased at 4 h (Figure 5A; $P < 0.001$ vs. CFA/saline group), showing that BAM8-22 attenuated CFA-induced hyperalgesia. In a separate group treated with CFA/BAM8-22, CTAP (10 nmol) given i.t. at 4 h reduced PWL in the next 60 min to a level that was significantly different from pre-CTAP level ($P < 0.05$–0.001), reversing the anti-nociceptive effects of the BAM8-22 treatment. However, saline administered i.t. at 4 h did not change the CFA/BAM8-22-induced response. Moreover, i.t. CTAP (10 nmol) did not change thermal nociceptive threshold in naïve rats ($n = 6$, data not shown). Similarly, when CTAP (10 nmol) was given i.t. at 48 h, the BAM8-22-induced increase in PWL was also abolished, compared with the pretreatment level or saline group ($P < 0.01$–0.001; Figure 5B).

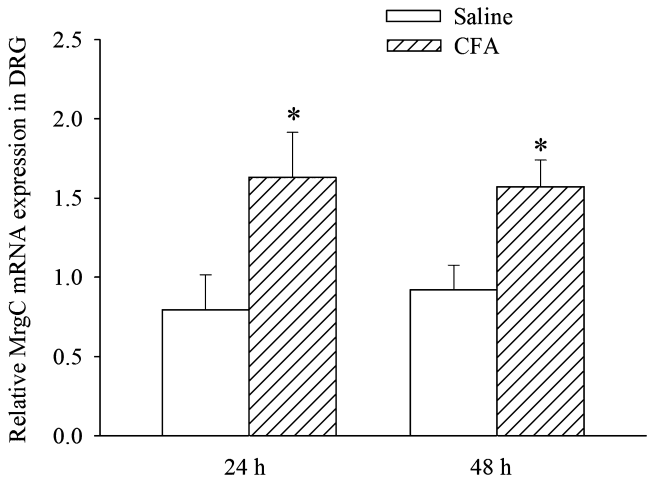

Figure 4

Levels of MrgC receptor mRNA in DRG. CFA (150 μL) or saline was injected i.pl. in the right hindpaw on day 0, and L4-6 DRG were collected on days 1 (24 h) and 2 (48 h). The levels are expressed relative to the level of MrgC receptor mRNA in saline group. Data are shown as mean ± SEM. *$P < 0.05$. $n = 5$.

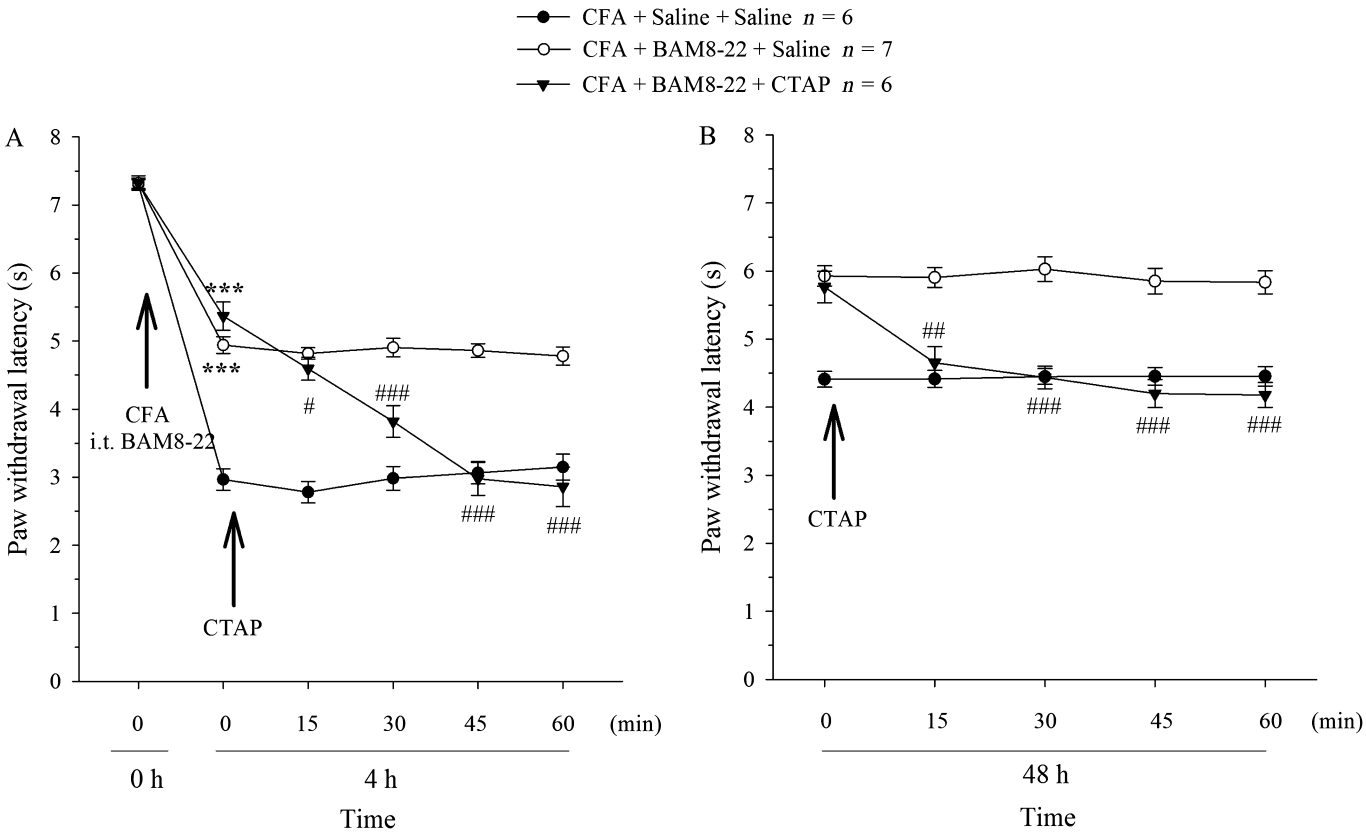

Figure 5

Effect of CTAP on BAM8-22-induced anti-nociceptive responses. CFA was injected i.p.l. on day 0 and i.t. BAM8-22 (30 nmol), or saline was administered i.t. on days 0 and 1. CTAP (10 nmol) or saline was given i.t. at 4 h (A) or 48 h (B). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with pre-CTAP value. $^{***}P < 0.001$ compared with CFA + BAM8-22 + CTAP group.

The involvement of MORs was further evaluated by measuring CGRP mRNA levels. To match the same protocol performed in the behavioural study (see Figure 1), CFA (i.p.l.) was given at 0 h and BAM8-22 (60 nmol) was administered i.t. via lumbar puncture at 0 and 24 h. In a separate group, the same protocol was repeated except that CTAP (20 nmol) was injected i.t. at 24 and 48 h. I.pl. injection of saline or i.pl. CFA plus i.t. saline was used as control. The L4-6 DRG were harvested at 48.5 h. As illustrated in Figure 6, treatment with CFA + i.t. saline increased CGRP mRNA levels by twofold compared with i.pl. saline ($P < 0.001$), and this increase was abolished by i.t. BAM8-22 ($P < 0.001$ vs. CFA + i.t. saline). However, in the presence of CTAP, the treatment with CFA + BAM8-22 still increased CGRP mRNA levels by twofold ($P < 0.001$ vs. CFA + i.t. BAM8-22).

To strengthen the evidence for the activation of MORs initiated by MrgC receptor agonists, mRNA expression of MOR as well as pro-opiomelanocortin (POMC) and β-endorphin content in the lumbar spinal cord and DRG were assayed. CFA was injected i.pl. on day 0, and BAM8-22 (30 nmol) or saline was administered i.t. on days 0 and 1. Tissue samples were harvested on day 2. As illustrated in Figure 7A–D, the expression of MOR mRNA and POMC mRNA in the spinal cord and DRG on the ipsilateral side was not different from the contralateral side following treatment with CFA. However, i.t. BAM8-22 significantly increased the expression of MOR mRNA and POMC mRNA in the spinal cord and DRG on the ipsilateral side ($P < 0.05$). The β-endorphin content of spinal dorsal horn on the ipsilateral side was similar to that on the contralateral side, in the CFA/saline group. Following treatments with CFA and i.t. BAM8-22, the β-endorphin content on the ipsilateral side was increased significantly above values from the contralateral side or in the CFA/saline group ($P < 0.01$; Figure 7E).

MrgC receptors attenuate CFA-induced nNOS expression and co-localize with nNOS in DRG neurons

To explore the mechanisms underlying the activity of MrgC receptors, the effect of i.t. BAM8-22 on CFA-induced nNOS expression in DRG was examined. To match the behavioural changes, the experimental protocol was similar to the behavioural study. CFA (i.p.l.) was given at 0 h, and BAM8-22 (30 nmol) was administered i.t. at 0 and 24 h. Saline or CTAP (10 nmol) was injected i.t. at 24 and 48 h. Animals treated with i.pl. saline or i.pl. CFA at 0 h were used as controls. DRG at L4-6 were harvested at 48.5 h. In accordance with previous studies, a number of small- to medium-sized neurons exhibited staining for nNOS in DRG. On the side ipsilateral to i.pl. saline injection, relatively few (15–20%) small or medium

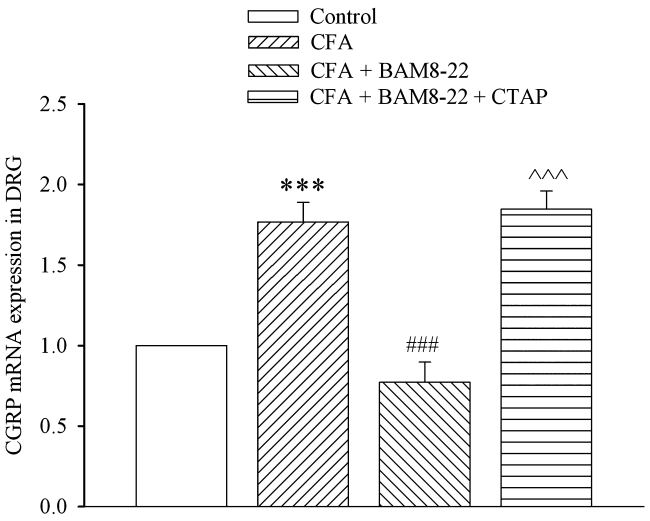

Figure 6

Effect of i.t. administration of BAM8-22 on CFA-evoked CGRP mRNA levels in DRG in the presence or absence of CTAP. CFA was injected i.pl. at 0 h and i.t. BAM8-22 (60 nmol) or saline was administered by lumbar puncture at 0 and 24 h. CTAP (20 nmol) or saline was given by lumbar puncture at 24 and 48 h. Saline was injected i.pl. at 0 h as control. The L4-6 DRG were harvested at 48.5 h. ***$P < 0.001$ compared with saline group. ###$P < 0.001$ compared with CFA + i.t. saline group. ^^^$P < 0.001$ compared with CFA + i.t. BAM8-22 group. $n = 4–5$.

neurons were nNOS-IR positive (Figure 8A and E), but i.pl. CFA markedly increased the expression of nNOS-IR in both populations of neurons ($P < 0.001$ compared with saline group; Figure 8B and E). Following i.t. administration of BAM8-22, the proportion of CFA-induced nNOS-IR neurons in small and medium subpopulations was clearly reduced, and the reductions were significant compared with CFA group ($P < 0.01–0.001$; Figure 8C and E). However, in the presence of CTAP, this inhibitory effect of BAM8-22 on CFA-induced nNOS expression was completely reversed in both small and medium neurons ($P < 0.01–0.001$, compared with CFA + BAM8-22 (no CTAP) group; Figure 8D and E).

Our findings that i.t. administration of BAM8-22 inhibited CFA-induced up-regulation of nNOS suggested a modulating effect of MrgC receptors on nNOS expression. Accordingly, the possible co-localization of MrgC receptors with nNOS was examined. As illustrated in Figure 9A, nNOS was localized in the soma of small- and medium-sized DRG neurons. Staining for MrgC receptors was diffusely distributed throughout the cytoplasm but was excluded from the nucleus (Figure 9B). Figure 9C shows the co-localization of MrgC receptors with nNOS. The specificity of the MrgC staining was assessed by rabbit normal serum (not shown) and preabsorption controls (Figure 9D); all these conditions showed no MrgC immunoreactivity.

Discussion

The present study demonstrated an increase in the expression of MrgC receptor mRNA in L4-6 DRG following the injection of CFA in a hindpaw. The i.t. administration of two MrgC receptor agonists with different structures produced both immediate and delayed attenuations of CFA-evoked thermal hyperalgesia. These effects were associated with the inhibition of up-regulation of nNOS and CGRP as well as c-Fos expression in the spinal dorsal horn or DRG. Furthermore, the delayed anti-hyperalgesia and inhibition of nNOS and CGRP expressions induced by BAM8-22 were abolished by the MOR antagonist CTAP. Treatment with i.t. BAM8-22 also increased the expression of the mRNAs coding for MOR and POMC as well as β-endorphin content in the spinal cord and/or DRG. In addition, MrgC receptors were co-localized with nNOS in DRG neurons. These results suggest that the activation of MrgC receptors can modulate inflammatory pain by suppressing the up-regulation of pronociceptive mediators and dorsal horn neuronal activation, which was partially attributed to the activation of MORs by endogenous agonists, following MrgC receptor activation.

The intriguing feature of MrgC receptors is their unique distribution in primary nociceptive neurons (Dong *et al.*, 2001; Lembo *et al.*, 2002). The study showing that, of the CNS tissues, the spinal cord is the only one displaying specific binding for MSH (Grazzini *et al.*, 2004) implies the localization of MrgC receptors in the central terminal of primary afferents. These receptors do not play a role in pain processing under normal conditions because neither the deletion of MrgC gene (Ndong *et al.*, 2009; Guan *et al.*, 2010) nor the activation of MrgC receptors by i.t. BAM8-22 (Cai *et al.*, 2007b; Guan *et al.*, 2010) or MSH (present study) altered basal nociceptive thresholds. However, MrgC receptors do reduce pain intensity under inflammatory conditions (Liu *et al.*, 2009; Guan *et al.*, 2010). In accordance with previous reports (Galeazza *et al.*, 1995; Safieh-Garabedian *et al.*, 1995), the present study showed that thermal hyperalgesia developed at 4 h and was maintained throughout the experiment following CFA injection. The activation of MrgC receptors by i.t. administration of the highly specific MrgC receptor agonists BAM8-22 (Lembo *et al.*, 2002; Guan *et al.*, 2010) and MSH (Han *et al.*, 2002; Lembo *et al.*, 2002) both produced a rapid (20-40 min) inhibition of CFA-evoked hyperalgesia. These results are in contrast to those from a recent study reporting that MrgC receptor knock-down using siRNA, reduced CFA-induced heat hyperalgesia (Ndong *et al.*, 2009). However, our results are consistent with other earlier studies showing that i.t. administration of BAM8-22 or MSH inhibits the wind-up response (Guan *et al.*, 2010), formalin-induced pain and dorsal horn neuronal activation (Chen *et al.*, 2006; Guan *et al.*, 2010), and NMDA- (Chen *et al.*, 2008) as well as CFA-evoked hypersensitivity (Guan *et al.*, 2010). Moreover, our behavioural observations were supported by the biochemical results (see below). However, the present study showed that MrgC receptor activation did not alter CFA-induced mechanical allodynia. This result may indicate that the interactions of MrgC receptors with inflammation-associated hypersensitivity to mechanical stimuli differs from their interactions with thermal stimuli. Such differences between the inhibition of thermal and mechanical hypersensitivity have been reported earlier (Boettger *et al.*, 2007; Schepers *et al.*, 2008).

The inhibitory effect of MrgC receptors is not seen under normal conditions. Our study showed that peripheral inflammation up-regulated the expression of mRNA coding for

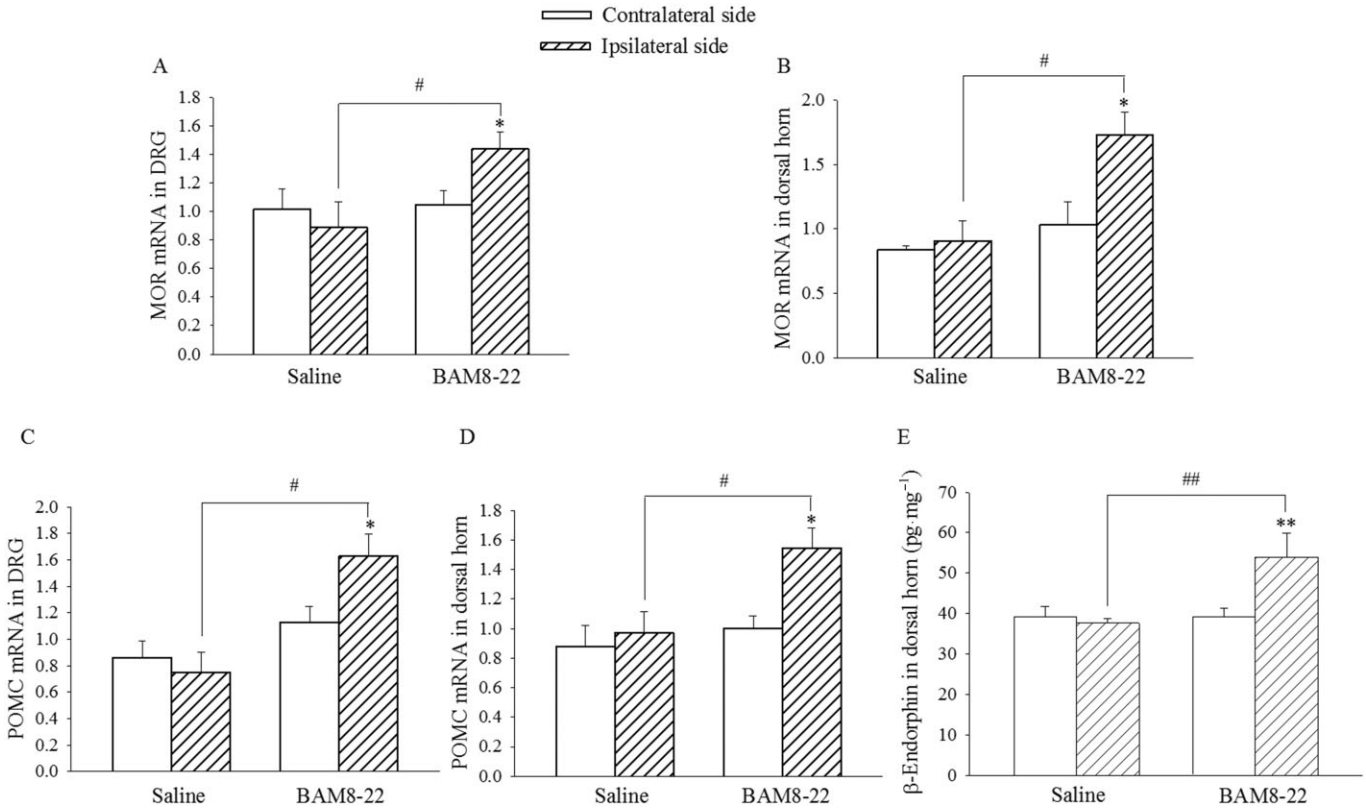

Figure 7

Influence of i.t. BAM8-22 on mRNA levels of MOR as well as POMC and β-endorphin content in the spinal dorsal horn and/or DRG. CFA was injected i.pl. in the right hindpaw on day 0, and BAM8-22 (30 nmol) or saline was administered i.t. on days 0 and 1. The lumbar spinal dorsal horn and L4-6 DRG were collected on day 2. mRNA levels of MOR (A and B) and POMC (C and D) and β-endorphin content (E) in the spinal cord and/or DRG were assayed. The data represent normalized averages derived from the threshold cycles in quantitative PCR. $^{*}P < 0.05$ compared with the contralateral side. $^{\#}P < 0.05$, $^{\#\#}P < 0.0$; compared with saline group. $n = 5$.

MrgC receptors in DRG. This finding is in accordance with the enhanced bioactivity of MrgC receptors in inflammation, as shown by the stronger wind-up or enhanced pain responses after i.pl. injection of formalin (Guan *et al.*, 2010), CFA or carrageenan (Liu *et al.*, 2009) in MrgC cluster knockout mice. The increase in mRNA for MrgC receptors following the induction of inflammation may explain the observations that i.t. injection of BAM8-22 or MSH inhibits inflammatory pain (Chen *et al.*, 2006; Guan *et al.*, 2010), but not physiological pain (Cai *et al.*, 2007b; Guan *et al.*, 2010). The up-regulation of MrgC receptor mRNA may parallel the enhanced expression of BAM22, a putative endogenous ligand of MrgC receptors (Lembo *et al.*, 2002), in the spinal cord and DRG in CFA-induced inflammation (Cai *et al.*, 2007a). However, plastic changes that develop in pathological pain involve not only pro-nociceptive mediators but also anti-nociceptive molecules. For example, inflammation has been shown to enhance the expression of opioid receptors in peripheral nerve terminals (Stein and Zollner, 2009) and cannabinoid receptors in DRG and in hindpaws (Hsieh *et al.*, 2011). Apparently, the significance of enhanced expression of MrgC receptors is an attempt to reduce pain severity (Liu *et al.*, 2009; Guan *et al.*, 2010), similar to other anti-nociceptive molecules (Rittner *et al.*, 2008). Up-regulation of MrgC receptors may allow MrgC receptor agonists exert greater biological activity. Therefore, in our experiments, the inhibition of inflammatory pain by MrgC receptor agonists could be partially attributed to the up-regulation of MrgC receptors, following CFA injection.

In agreement with behavioural responses, i.t. BAM8-22 or MSH inhibited CFA-induced increase in the pro-nociceptive mediators, CGRP and nNOS, in DRG and/or the spinal cord. The induction of inflammatory pain has been attributed to the up-regulation of CGRP (Miletic and Tan, 1988; Ryu *et al.*, 1988) and nNOS (Meller *et al.*, 1994; Boettger *et al.*, 2007). CGRP is synthesized in DRG (Nahin and Byers, 1994; Galeazza *et al.*, 1995) and released from primary sensory nerve terminals (Kuraishi *et al.*, 1988) during CFA-induced inflammation, leading to the sensitization of nociceptive neurons in the spinal cord (Miletic and Tan, 1988; Ryu *et al.*, 1988) and DRG (Ryu *et al.*, 1988). Accordingly, blockade of CGRP receptors by antagonists (Kawamura *et al.*, 1989; Neugebauer *et al.*, 1996; Sun *et al.*, 2003) relieves inflammatory pain.

The production of NO by nNOS in neurons (Snyder, 1992) also plays a pivotal role in the induction of inflammatory hyperalgesia. Expression of nNOS is increased in the spinal cord of the CFA model of inflammatory hyperalgesia (Chu

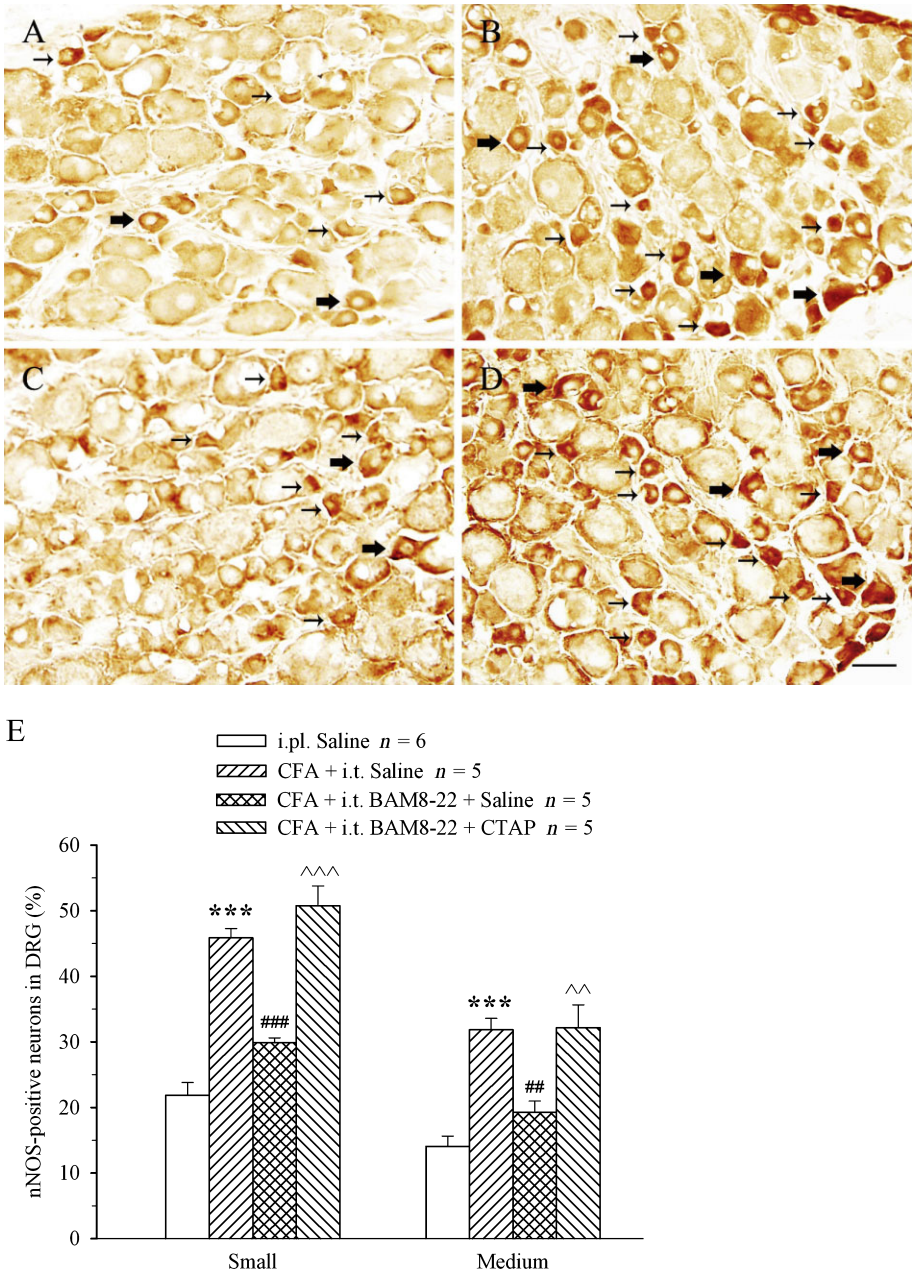

Figure 8

Effect of i.t. administration of BAM8-22 on CFA-evoked expression of nNOS in DRG in the presence or absence of CTAP. Saline (A) or CFA (B, C and D) was i.pl. at 0 h. BAM8-22 (30 nmol; B, C and D) and saline (C) or CTAP (10 nmol; D) were administered i.t. at 0/24 and 24/48 h respectively. The L4-6 DRG were collected at 48.5 h. Photomicrographs of transverse sections of DRG show nNOS-IR expressions in small (small arrows) and medium (large arrows) neurons. Histograms show means ± SEM of nNOS-IR-positive neurons (E). ***$P < 0.001$ compared with saline group. $^{###}P < 0.001$ compared with CFA + i.t. saline group. $^{\wedge\wedge\wedge}P < 0.001$ compared with CFA + i.t. BAM8-22 group. Scale bar = 50 μm.

et al., 2005; Boettger *et al.*, 2007), whereas administration of the nNOS inhibitor (Pozza *et al.*, 1998) or the targeted disruption of the nNOS gene (Chu *et al.*, 2005) markedly attenuates CFA-induced hyperalgesia. Moreover, nNOS is linked to the up-regulation and release of CGRP to induce inflammatory hyperalgesia because the nNOS inhibitors, L-NAME and 7-nitroindazole, reduced inflammation-associated CGRP release from spinal dorsal horn (Garry *et al.*, 2000), and i.pl. injection of CFA failed to increase CGRP expression in DRG neurons in nNOS knock-out mice (Boettger *et al.*, 2007). As reported before (Pozza *et al.*, 1998; Chu *et al.*, 2005; Hong *et al.*, 2009), the present study showed that CFA injection increased the expression of nNOS and CGRP in the spinal dorsal horn or DRG. Importantly, these biochemical alterations were abolished following treatment with BAM8-22 or MSH. The present study demonstrated that nNOS was con-

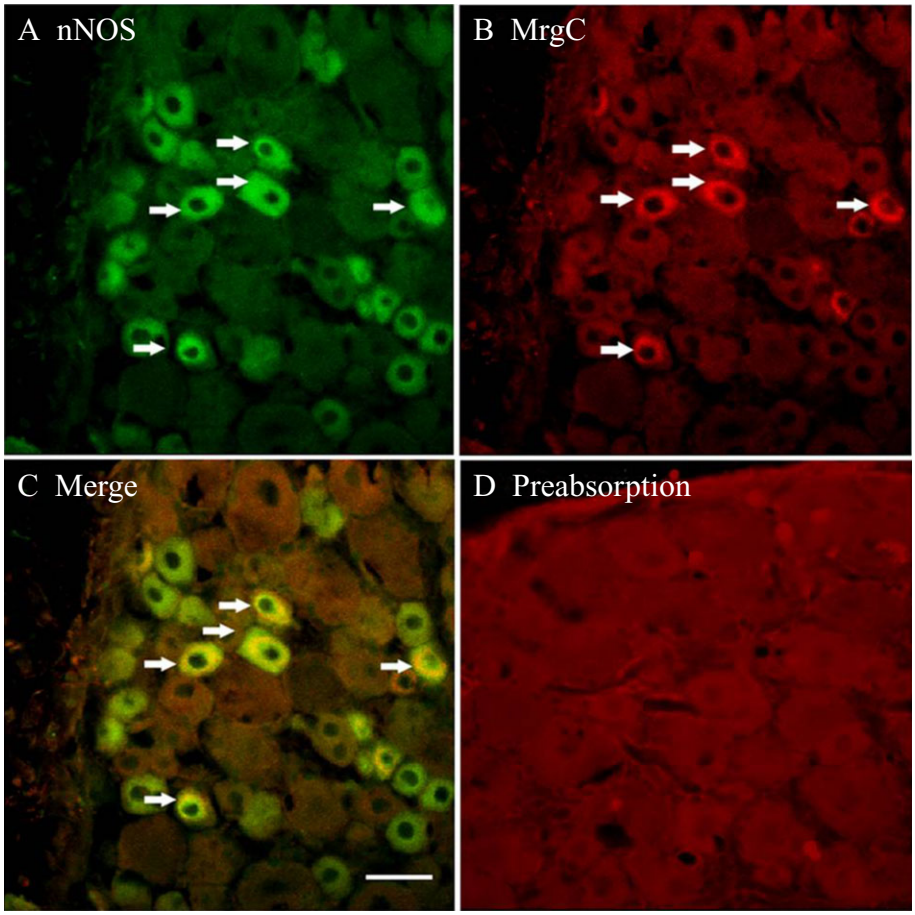

Figure 9

Confocal images showing the localization of nNOS-IR and MrgC receptor IR in DRG neurons. nNOS-IR neurons are identified by FITC fluorescence (A, arrows, green) whereas MrgC receptor-IR-positive neurons by rhodamine (B, arrows, red). nNOS-IR is extensively co-localized with MrgC receptor-IR in DRG neurons (C, arrows, yellow). Incubation of DRG section with anti-MrgC receptor antiserum preabsorbed with 1 μM MrgC receptor protein resulted in the complete absence of staining (D). Scale bar = 50 μm.

tained in MrgC receptor-expressing neurons in DRG. This might be the histological substrate for the modulation of CFA-induced nNOS expression by MrgC receptors. Based on the data discussed above, the suppression of nNOS by MrgC receptor activation is likely to result in inhibition of CGRP up-regulation. Consistent with the inhibition of nNOS and CGRP expression in DRG, c-Fos expression in the spinal cord was also abolished, reflecting the overall inhibition of spinal neuronal activation (Harris, 1998). Taken together, these results suggest that the mechanism underlying the effect of BAM8-22 or MSH on hypersensitivity is linked to the inhibition of CFA-induced up-regulation of nNOS and CGRP and spinal neuronal activation.

As demonstrated before (Chen *et al.*, 2006; 2008; Guan *et al.*, 2010), the anti-nociceptive effect of BAM8-22 and MSH was exerted immediately (within 20min) after their administration. Interestingly, attenuation of hyperalgesia was also observed at 4, 24 and 48 h following the administration of BAM8-22 or MSH at 0 and 24 h. This delayed long-lasting effect is more likely to be due to downstream mechanisms activated by MrgC receptors because BAM8-22 and MSH are degraded within 10–30 min in the spinal cord (Grazzini *et al.*, 2004). This possibility was supported by the results showing that the selective MOR antagonist CTAP (Pelton *et al.*, 1985), administered at 4 and 48 h, abolished the anti-nociceptive effects produced by i.t. administration of BAM8-22 at 0 and 24 h, respectively, suggesting that MOR-mediated mechanism(s) was involved in the delayed anti-hyperalgesia induced by BAM8-22. Consistent with these results, treatment with CTAP also abolished BAM8-22-induced inhibition of nNOS and CGRP (mRNA) expressions in DRG. As both BAM8-22 and MSH do not demonstrate any affinity with μ-, δ- or κ-opioid receptors (Lembo *et al.*, 2002), we explored next the potential production of endogenous opioid peptides. In accordance with previous studies (Puehler *et al.*, 2004; Shaqura *et al.*, 2004; Obara *et al.*, 2009), the present study showed no changes in the expression of MORs in both the spinal cord and DRG following CFA injection. Importantly, treatment with i.t. BAM8-22 increased MOR mRNA levels. Similarly, the CFA injection did not alter POMC mRNA levels in the spinal dorsal horn and DRG (Obara *et al.*, 2009). However, POMC mRNA level and β-endorphin content in the spinal dorsal horn and/or DRG were increased following the i.t. administration of BAM8-22. Many non-opioid receptors

produce anti-nociceptive effects partly via interactions with the endogenous opioid system. Responses to activation of oxytocin (Russo *et al.*, 2012), sphingosine-1-phosphate (Welch *et al.*, 2012) and CB_2 (Negrete *et al.*, 2011) receptors can be abolished by MOR antagonists. Activation of CB_2 receptors also increased the synthesis of POMC as well as β-endorphin (Su *et al.*, 2011) and stimulated the release of β-endorphin (Katsuyama *et al.*, 2013). Particularly relevant in this context are the reciprocal interactions involving the release of opioid peptides by cannabinoids or of endocannabinoids by opioids (Parolaro *et al.*, 2010). The data in the present study suggest that the activation of MrgC receptors in CFA-induced inflammation can up-regulate the expression of both MOR and POMC mRNAs and β-endorphin content in the spinal cord and/or DRG, resulting in delayed but long-lasting anti-nociception. Further experimentation is, however, needed to elucidate the mechanisms underlying this recruitment by MrgC receptor agonists of endogenous activation of MORs.

The treatment of various types of chronic pain still constitutes a real challenge as most analgesics display serious side effects that are often associated with unwanted actions in the CNS (Benyamin *et al.*, 2008). One of the main reasons is that receptors targeted by these analgesics are widely distributed in the CNS. Besides anti-nociception, the inhibition or activation of these receptors also alters other brain functions. Such unwanted CNS effects should be minimized following activation of MrgC receptors because of their highly restricted distribution in DRG. Endogenous μ-opioid agonists at the spinal level recruited by MrgC receptor activation should only exhibit analgesic activity and not the adverse effects of exogenously applied opiates (Viet and Schmidt, 2012). Therefore, the present study suggests that targeting MrgC receptors should be considered as a novel approach for the treatment of inflammatory pain.

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Conflict of interest

The authors have no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12326

Appendix S1 Methods for percutaneous lumbar puncture, quantification of IR for CGRP and nNOS in DRG, Western blots for nNOS and c-Fos in spinal dorsal horn, RT-PCR for mRNAs of CGRP, MrgC receptors, MOR and POMC in DRG and/or spinal dorsal horn and ELISA for β-endorphin in spinal dorsal horn.