Potential role of CXCL10/CXCR3 signaling in the development of morphine tolerance in periaqueductal gray

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Abstract

Tolerance to morphine antinociception hinders its long-term use in clinical practice.

Interaction between neuron and microglia has been proved to play critical role in the mechanism of morphine tolerance, while CXCL10/CXCR3 signaling has been implicated in neuron-glia signaling and morphine analgesia. This study aims to investigate whether CXCL10/CXCR3 signaling in periaqueductal gray (PAG) contributes to the development of morphine tolerance by modulating neuron-microglia interaction. The results showed that the expressions of CXCR3 and CXCL10 were gradually increased in parallel with repeated morphine administration and activation of microglia. CXCR3 was co-localized with neuronal marker NeuN, while CXCL10 was derived from microglia. Microglia inhibitor minocycline significantly attenuated the expression of CXCL10, besides, both minocycline and CXCR3 inhibitor alleviated the development of morphine tolerance. Taken together, our study provided the evidence that CXCL10/CXCR3 signaling in PAG is involved in the development of morphine analgesic tolerance *via* neuron-microglia interaction.

Keywords: Morphine tolerance; Chemokine; CXCL10; CXCR3

Abbreviations

CXCL9, C-X-C motif chemokine 9; CXCL10, C-X-C motif chemokine 10; CXCL11, C-X-C motif chemokine 11; CXCR3, C-X-C motif chemokine receptor 3; rmCXCL10, recombinant mouse CXCL10 protein; CaMKII, Ca^{2+}/calmodulin dependent protein kinase II; CREB, cAMP response element binding protein; GABA, gamma-aminobutyric acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial

fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; IL-1β, interleukin-1 beta; NeuN, neuronal nuclei; NFκB, nuclear factor kappa B; PAG, periaqueductal gray; TNFα, tumor necrosis factor alpha

1. Introduction

Tolerance to morphine-induced antinociceptive effect hinders its prolonged usage in the clinic. The roles of neuronal intracellular cascades including desensitization of opioid receptors, endocytosis of opioid receptor and functional changes of glutamate receptors in the mechanisms of morphine tolerance have been well investigated (Martini and Whistler, 2007; Tai *et al.*, 2007; Williams *et al.*, 2013). Accumulating evidences suggest that microglia may play an essential role in the development of morphine tolerance (Horvath *et al.*, 2010; Wang *et al.*, 2010b; Eidson and Murphy, 2013a). Microglia could be activated in response to morphine-induced neuronal changes, while microglia-derived proinflammatory factors including cytokines and chemokines, in turn, promote the neuronal sensitization (Horvath *et al.*, 2010; Wang *et al.*, 2010b). These studies indicate the importance of the interaction between neuron and microglia in the mechanism of morphine tolerance.

Periaqueductal gray (PAG) and its descending projections to rostral ventromedial medulla and spinal cord comprise an essential neural circuit for opioid-mediated analgesia (Basbaum and Fields, 1978). Recent studies demonstrated that opioid

tolerance is accompanied by activation of microglia in PAG (Eidson and Murphy, 2013b), and inhibition of microglia activities could attenuate the development of morphine tolerance (Eidson and Murphy, 2013a). Although the contribution of microglia activation in PAG to morphine tolerance has been reported, little is known about the underlying mechanism of neuron-microglia interaction (Cui *et al.*, 2006).

Chemokines, a family of small cytokines, could directly induce chemotaxis of responsive cells. Several studies indicate that chemokine receptors, such as CXCR2, are co-expressed by opioid-containing leukocytes. Inhibiting some chemokines (CXCL1 and CXCL2/3) could substantially result in the decreased number of opioid-containing immune cells in inflammatory tissue and in consequence abolish the endogenous peripheral opioid analgesia (Brack et al., 2004; Machelska, 2007), whereas some chemokines (e.g. CCL5, CXCL12, and CX3CL1) are described to be able to induce pain or decrease central analgesic effects of opioid receptor agonists in animals without inflammation (Chen et al., 2007; Oh et al., 2001). Chemokines could also emerge as potential modulators of neuron-microglia interaction in opioid tolerance-related neuroinflammation and chronic neuropathic pain (Biber *et al.*, 2008; Old and Malcangio, 2012). The chemokine receptor CXCR3 could be activated by its ligands including CXCL9, CXCL10 and CXCL11 (Loetscher *et al.*, 1998). Activation of CXCR3 is involved in NMDA-induced hippocampal cell death (van Weering *et al.*, 2011), entorhinal cortex lesion (Rappert *et al.*, 2004) and brain ischemia (Biber *et al.*,

2001) by inducing neuron-microglia interaction. Previous study has reported that CXCL10 was upregulated in nervous system with neuroinflammatory pain (Müller *et al*., 2010). Recently, our studies have shown that single morphine administration promoted CXCL10 expression in spinal neurons, while blocking the function of CXCL10 could enhance the effect of morphine analgesia in cancer pain animal (Ye *et al.*, 2014; Bu *et al.*, 2014). In addition, CXCR3 was co-localized with neuron, astrocyte and microglia in bone cancer pain model (Guan *et al*., 2015), suggesting CXCL10/CXCR3-related neuron-microglia interaction may play a critical role in the formation of bone cancer pain and morphine analgesic effect. Activation of microglia could be responsible to neuronal changes and aggravate the development of morphine tolerance by releasing pro-nociceptive factors such as chemokines (Horvath *et al.*, 2010; Wang *et al.*, 2010b). However, the role of CXCL10/CXCR3 in the morphine tolerance in PAG remains unclear. Thus, in the present study, we hypothesized that activation of CXCL10/CXCR3 signaling might participate in the mechanism of morphine tolerance by modulating neuron-microglia interaction.

2. Material and methods

2.1. Animals

Adult male Swiss Webster mice, weighing 20-25 g, were purchased from Animal Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Animals were housed under a 12-hour light/dark cycle at room temperature of 22 ± 1 ℃ and relative humidity 40-60 % with food and water available *ad libitum*. The experimental protocols were reviewed and approved by Institutional Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science & Technology. All experimental protocols and animal handling procedures were carried out in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals.

2.2. Drug administration

Animals were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneally). In order to facilitate injection, stainless cannula guides (0.60 mm external and 0.35 mm internal diameters) was implanted unilaterally into dorsal part of PAG (-4.6 mm posterior to bregma, +/- 0 mm lateral to the midline and -2 mm ventral to the dorsal surface of the skull) according to the previous study (Masse *et al.*, 2008). A metallic cannula dummy was placed in the cannula guides after surgery to avoid blood clots. Animals were allowed a 7-day recovery period before the following experiments.

The following drugs were micro-injected into PAG 30 min before morphine administration, respectively: CXCR3 inhibitor AMG487 (10 or 20 μg, 0.25 μL, diluted in 20 % 2-hydroxypropyl-β-cyclodextrin, once daily; Sigma, St. Louis, MO, USA), microglia inhibitor minocycline (10 pmol, 0.25 μL, diluted in saline, once daily; Sigma, St. Louis, MO, USA) (Wei *et al.*, 2008; Eidson and Murphy, 2013a), recombinant

mouse CXCL10 protein (rmCXCL10; 20 μg, 0.25 μL, diluted in saline, once daily; ProSpec-Tany TechnoGene, Rehovot, Israel).

2.3. Chronic morphine tolerance

To induce chronic morphine tolerance, mice were repeatedly administrated with morphine subcutaneously (10 mg/kg, twice daily with 12 h intervals), from day 1 (D1) to day 7 (D7) (Zhou *et al.*, 2010; Ferrini *et al.*, 2013).

2.4. Mechanical nociceptive tests

Nociceptive thresholds of mice were assessed by measuring paw withdrawal thresholds *via* von Frey filaments as described previously (Liu *et al.*, 2012; Zhou et al., 2013). Briefly, behavioral tests were performed on day 0 (D0) and 30 min after morphine administration from day 1 to day 7. Mice were tested individually in a deep rectangular stainless-steel tank and allowed 15 minutes for habituation before tests. The region between foot pads in the plantar aspect of right hind paw was stimulated by a series of von Frey hairs with logarithmically incrementing forces (0.04, 0.07, 0.16, 0.4. 0.6, 1, 2, 4, 6, 8, 10, 15 and 26 g). Abrupt paw withdrawal, licking, or shaking was considered as positive responses. Once a withdrawal response was elicited, the test would be repeated starting with the next descending filament until no response occurred. An interval of 10 seconds was applied between the stimulations of filaments. The lowest amount of force that elicits a positive response was recorded. Three trials were performed on each animal with a time interval of 10 minutes, and the average value was considered as the

paw withdrawal thresholds, represented in grams (g). All behavioral tests were conducted under blind conditions.

2.5. Real-time Polymerase Chain Reaction

Total RNA was isolated from PAG with Trizol (Invitrogen, Carlsbad, CA). RNA was used to synthesize cDNA with SuperScript Reverse Transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR reaction was performed with StepOne Real-time PCR System (Applied Biosystems) according to the manufacturer's instructions. Specific primers for mouse CXCR3 and endogenous control mouse GAPDH were obtained from PrimerDepot (Table 1). Relative quantification of mRNA was determined by using $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Data were presented as fold changes normalized to control group.

2.6. Western blot analysis

Animals were sacrificed and total proteins of PAG tissues were extracted immediately. The tissues were homogenized in a radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Wuhan, China) containing 1 % Phenylmethanesulfonyl fluoride. The protein concentration was determined by BCA assay (Boster, Wuhan, China). After denatured by boiling in a sample buffer, 50 μg proteins from each sample were separated on SDS polyacrylamide gel and then transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) by electrophoresis. The membranes were blocked with 5 % non-fat milk in TBST (0.1 % Tween 20 in TBS) for 0.5 hour at room

temperature and then incubated with specific primary antibodies (Table 2) overnight at 4 °C followed by HRP-conjugated goat anti-rabbit IgG (1: 5000, Boster, Wuhan, China) or HRP-conjugated goat anti-mouse IgG (1: 1000, Bioyeartech, Wuhan, China) for 2 hours at room temperature in TBS containing 0.05 % Tween-20. Labeled proteins were then detected by ChemiDocXRS+ chemiluminescence imaging system (Bio-Rad, Hercules, CA, USA). The protein levels were presented as density relative to that of β-actin.

2.7. Immunofluorescence staining

Mice were anesthetized with pentobarbital sodium and perfused through aortic cannula with 20 mL of saline, followed with 20 mL of 4 % paraformaldehyde for 20 minutes. The fixed brains were removed from cranial cavity and post-fixed in the same fixative solution overnight at 4 °C. The tissues were then embedded in paraffin and sections containing PAG were mounted on slides. Immunofluorescence staining was performed as previously described (Kong *et al.*, 2013). Briefly, slides were blocked with goat serum for 60 min, followed by incubation with specific primary antibodies (Table 2) overnight at 4 °C. Then the sections were incubated with DyLight 488-conjugated goat anti-mouse IgG (1: 100, Abbkine, Inc., Redlands, CA, USA), Cyanine 3 (Cy3)-conjugated goat anti-mouse IgG (1: 200, Abbkine, Inc., Redlands, CA, USA) or Cyanine 3 (Cy3)-conjugated goat anti-rabbit IgG (1: 200, Abbkine, Inc., Redlands, CA, USA) for 1 hour at room temperature. Sections were washed with PBST for 10 min and

mounted in 50% gelvatol (diluted with PBS) for microscopic imaging. Fluorescent images were captured using a fluorescence microscope (Leica, German).

2.8. Statistical analysis

All data were presented as mean ± SEM. Multiple comparisons were analyzed using one-way ANOVA followed by Bonferroni's multiple comparison tests. Data collected from paw withdrawal thresholds tests were analyzed by using a repeated measures two-way ANOVA followed by Bonferroni's *post-hoc* test. $P < 0.05$ was considered to be statistically significant difference.

3. Results

3.1. CXCR3 expression in PAG was gradually increased during the development of morphine tolerance

We first examined whether morphine tolerance was successfully established after repeated morphine administration (10 mg/kg, twice daily, s.c.). The results of behavioral tests showed that paw withdrawal thresholds of morphine-treated mice decreased dramatically on day 4 as shown in Fig. 1A ($F_{(1,72)} = 35.53$, $P < 0.05$). We then sought to investigate the time course of CXCR3 expression in PAG during the development of morphine tolerance. As shown in Fig. 1B and 1C, both the expressions of CXCR3 mRNA and protein were gradually increased along with repeated morphine administration when compared with baseline ($P < 0.01$). CXCR3 immunoreactivity in

PAG in morphine-treated mouse was co-localized with neuronal marker NeuN, but not with microglial marker Iba-1 or astrocytic marker GFAP (Fig. 1D), indicating that CXCR3 expressions are increased in neurons in PAG.

3.2. Repeated administration of morphine increased the expression of CXCL10 derived from microglia

CXCL10 is one of the ligands of CXCR3 and spinal CXCL10 has been shown to be associated with morphine antinociceptive effect (Harris *et al.*, 2012), therefore, we detected the expression of CXCL10 in PAG after repeated morphine administration. The results showed that levels of CXCL10 were increased on day 3 and day 7 in morphine-treated mice when compared with those in saline-treated mice (Fig. 2A). CXCL10 immunoreactivities were predominantly co-localized with microglia marker Iba-1 in morphine-treated mice (Fig. 2B), indicating that microglia may be the major cellular source of CXCL10 in PAG. The densities of Iba-1 positive microglia in morphine-treated mice were also increased on day 5 and day 7 when compared with baseline ($P < 0.01$) (Fig. 2C and 2D).

3.3. The development of morphine tolerance was attenuated by inhibition of CXCR3 activation and promoted by exogenous CXCL10

To determine whether CXCR3 is involved in the development of morphine tolerance

directly, the CXCR3 inhibitor AMG487 or rmCXCL10 was micro-injected into PAG 30 min before morphine administration, respectively. As shown in Fig. 3A, a single dose of AMG487 (10 or 20 μg) did not affect the antinociceptive effect of morphine on the 1st day of morphine administration ($P > 0.05$), but consecutive treatments with AMG487 attenuated the development of morphine tolerance ($F_{(2,72)} = 34.65$ for AMG487 10 ug/day; $F_{(2,72)} = 26.13$ for AMG487 20 ug/day, $P < 0.05$), indicating that activation of CXCR3 is required for the development of drug tolerance. Administration of rmCXCL10 (20 μg) could weaken morphine analgesic effect and promote the formation of morphine tolerance ($F_{(2,72)} = 103.1$, $P < 0.05$), which could be prevented by co-administration with AMG487 ($F_{(3,144)} = 64.64$, $P < 0.05$) (Fig. 3B). These findings demonstrate that CXCL10 and CXCR3 are involved in the development of morphine tolerance.

3.4. Inhibition of microglia attenuated the expression of CXCL10 and the development of morphine tolerance

To verify that microglia-derived CXCL10 is involved in the development of morphine tolerance, microglia inhibitor minocycline (Tikka *et al.*, 2001), was intra-PAG injected to inhibit the activation of microglia (Eidson and Murphy, 2013a). The results showed that pre-treatment with minocycline (10 pmol) could prevent the development of morphine tolerance ($F_{(2,72)} = 42.38$, $P < 0.05$) and co-administration with rmCXCL10

could reverse the effect of minocycline ($F_{(3,144)}$ = 28.17, P < 0.05) (Fig. 4A). Minocycline could down-regulate the expression of CXCL10 induced by repeated morphine treatment (P < 0.01) (Fig. 4B). Proliferation of microglia was observed in PAG during the development of morphine tolerance, however, it was inhibited by pre-treatment of minocycline, but not affected by AMG487 or rmCXCL10 (P < 0.01) (Fig. 4C and 4D). Increased expression of CXCR3 which was induced by morphine was attenuated by AMG487 (P < 0.01) but not by minocycline (P > 0.05) (Fig. 4E). These results provide promising evidences that activation of microglia in PAG contributes to the development of morphine tolerance may partially through increasing CXCL10 expression, which might be directly correlated with the up-regulation of microglia activity during the development of morphine tolerance, however microglia-derived CXCL10 does not affect the activity of microglia and the expression of CXCR3.

4. Discussion

Our study demonstrated the potential role of microglial CXCL10-neuronal CXCR3 interaction in PAG in the development of morphine tolerance. On the one hand, repeated morphine treatment up-regulated the expression of CXCR3. Importantly, blocking CXCR3 slowed the development of morphine tolerance. On the other hand, morphine treatment was associated with microglia activation and subsequently increased expression of CXCL10. Inhibition of microglia activation down-regulated

CXCL10 expression and consequently prevented the development of morphine tolerance. Taken together, microglia-derived CXCL10 acts on neuronal CXCR3 receptor, which contributed to the development of morphine tolerance in PAG.

Chronic morphine administration induces the activation of microglia, which increases the glia-released cytokines including chemokine (Johnston *et al.*, 2004), tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β), resulting in the down-regulation of astrocytic glutamate transports proteins (GLT-1, GLAST) (Watkins *et al.*, 2009) and neuronal GABA (gamma-aminobutyric acid) receptors (Stellwagen *et al.*, 2005). Several studies have reported that morphine binds to neuronal mu opioid receptors in PAG which are primarily located in GABAergic neurons (Chieng & Christie, 1994; Commons *et al.*, 2000). These microglia-related cytokines could induce the intracellular changes, such as the activities of mu opioid receptors in GABAergic neurons, and effectively increase the neuronal excitability that may contribute to the development of morphine tolerance. CXCR3 signaling usually functions in chemotaxis and inflammatory responses in lymphocytes or inflammatory cells under various pathological conditions (Agostini *et al.*, 2001; Sorensen *et al.*, 2002; Wenzel *et al.*, 2007). Moreover, it was shown to mediate neuron-microglia interaction in acute brain injury and ischemia (Biber *et al.*, 2001; Rappert *et al.*, 2004; van Weering *et al.*, 2011). The expressions and functions of CXCR3 in neurons (Xia *et al.*, 2000), microglia (Rappert *et al.*, 2004) and astrocytes (Tanuma *et al.*, 2006) in various animal models

have been reported previously, but the cellular localization in PAG is still unknown. Our results found that the expression of CXCR3 in PAG was increased during the development of morphine tolerance, and CXCR3 was expressed in neurons, but not in glia. Moreover, blocking CXCR3 with its antagonist effectively prevented the development of morphine tolerance. These findings provide definitive evidences that neuronal chemokine receptor in PAG plays a critical role in the mechanism of morphine tolerance.

It has been reported that CXCL10 in spinal cord modulated morphine antinociceptive effect through CXCR3 signaling (Ye *et al.*, 2014). The function and expression of CXCL10 have been demonstrated in microglia (Shen *et al.*, 2006), neurons (Sui *et al.*, 2006) and astrocytes (Sanchez-Blazquez *et al.*, 2008) in *in vitro* studies. However, the types of neural cells which could express CXCL10 are various under different pathological conditions (Tanuma *et al.*, 2006; van Weering *et al.*, 2011). Our results showed that increased CXCL10 expression in PAG induced by morphine treatment was localized in microglia, but not in neurons or astrocytes. Interestingly, microglia chemotactic activation was found to be down-regulated by the activation of mu opioid receptors (Chao *et al.*, 1997), which means that the activity of microglia chemotaxis could be encouraged by desensitization of mu opioid receptor after repeated morphine stimulation. Consistent with this finding, the increased expression of CXCL10 was parallel with the development of morphine tolerance, and inhibiting microglia

activation with minocycline could not only down-regulate CXCL10 expression in microglia, but also prevent the development of morphine tolerance. These results suggested that activated microglia participate in the development of morphine tolerance by releasing CXCL10 which could activate CXCR3 and then might induce the cellular changes of signal transduction in neurons in PAG. Besides this study, most studies have identified that inhibiting microglia could attenuate the development of morphine tolerance (Wang *et al.*, 2010b; Wen *et al.*, 2011). However, a recent study reported that intra-PAG treatment with minocycline was not sufficient to attenuate morphine tolerance (Eidson and Murphy, 2013a). The reason for this discrepancy might be the different usage of minocycline in the experimental protocols. In the study of Edison LN et al., minocycline was only used in the early phase of the development of morphine tolerance (from day 1 to day 3 of morphine injection). Nevertheless, the inhibitory effect of minocycline on the formation of morphine tolerance was not observed until day 4 of morphine injection in our study, which suggests that microglia may play a role in the development of drug tolerance rather than analgesic effect of single administration of morphine. Taken together, our results indicate that CXCL10/CXCR3 signaling in PAG may contribute to the development of morphine tolerance by mediating neuron-microglia interaction.

When morphine binds to mu opioid receptor (MOR), multiple intracellular downstream pathways could be activated. The Gα and Gβγ subunits dissociate from one another,

which subsequently lead to the inhibition of cyclic-adenosine monophosphate (cAMP) formation and calcium conductance to produce the analgesic effect (Ingram and Williams, 1994; Schroeder *et al*, 1991). Previous study showed that inhibiting Gi protein could partially block the algesia induced by CXCL10, indicating that Gi protein is involved in the nociceptive signaling pathway related to CXCR3 (Ye *et al*, 2014). In the present study, our results suggested that CXCL10 and its receptor CXCR3 are involved in the development of morphine analgesic tolerance via neuron-microglia interaction in PAG. However, there is still lack of direct evidence to elucidate the mechanism of CXCL10/CXCR3 downstream signaling that may contribute to the development of morphine tolerance. Activation of several kinase transcription factor cascades may be required to mediate morphine tolerance, including calcium/calmodulin-dependent protein kinase II (CaMKII) and cAMP response element-binding protein (CREB) in neurons and p38 and nuclear factor kappa B (NFκB) in microglia because inhibitors of CaMKII and p38 pathways could reduce the increases of phosphorylated CREB and acetylated-NFκB levels and attenuate the development of tolerance (Ammon-Treiber and Hollt, 2005; He *et al.*, 2009; Sanchez-Blazquez *et al.*, 2008; Wang and Burns, 2009; Wang *et al.*, 2010a; Wang *et al.*, 2010b; Wang *et al.*, 2011). Chronic CXCL10 exposure could increase the phosphorylation of CREB in cultured hippocampal neurons (Bajova *et al.*, 2008), suggesting that the activation of neuronal CXCR3 induced by microglial CXCL10 may play a key role in the

development of morphine tolerance through CaMKII/CREB signaling. In addition, p38MAPK has also been shown to participate in neuronal CREB phosphorylation (Freeland *et al.*, 2000; Ma *et al.*, 2001) and contribute to the development of morphine tolerance by facilitating microglia activation in spinal cord (Cui *et al.*, 2006; Cui *et al.*, 2008). Although detailed signaling pathways of CXCL10/CXCR3 associated with morphine analgesic tolerance have not yet been studied, some kinases related to other chemokine receptors, such as Src family-kinases which were identified to be involved in the mechanism of morphine analgesia (Rivat *et al*, 2014), should be considered in the further research to explore the intracellular mechanisms triggered by CXCR3 in morphine tolerance.

5. Conclusions

In summary, our study provides a novel insight into the roles of CXCL10/CXCR3 in the development of morphine tolerance in PAG and suggests the beneficial possibility of restoring morphine antinociceptive effect by inhibiting CXCR3 activation. These findings thus implicate a new clinical strategy for preventing morphine tolerance and may contribute to expanding the morphine usage in clinic.

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References:

Agostini, C., Calabrese, F., Rea, F., Facco, M., Tosoni, A., Loy, M., Binotto, G., Valente, M., Trentin, L., Semenzato, G., 2001. Cxcr3 and its ligand CXCL10 are expressed by inflammatory cells infiltrating lung allografts and mediate chemotaxis of T cells at sites of rejection. Am J Pathol. 158, 1703-1711.

Ammon-Treiber, S., Hollt, V., 2005. Morphine-induced changes of gene expression in the brain. Addict Biol. 10, 81-89.

Bajova, H., Nelson, T.E., Gruol, D.L., 2008. Chronic CXCL10 alters the level of activated ERK1/2 and transcriptional factors CREB and NF-kB in hippocampal neuronal cell culture. J Neuroimmunol. 195, 36-46.

Basbaum, A.I., Fields, H.L., 1978. Endogenous pain control mechanisms: review and hypothesis. Ann Neurol. 4, 451-462.

Biber, K., Sauter, A., Brouwer, N., Copray, S.C., Boddeke, H.W., 2001. Ischemia-induced neuronal expression of the microglia attracting chemokine Secondary Lymphoid-tissue Chemokine (SLC). Glia. 34, 121-133.

Biber, K., Vinet, J., Boddeke, H.W., 2008. Neuron-microglia signaling: chemokines as versatile messengers. J Neuroimmunol. 198, 69-74.

Brack, A., Rittner, H.L., Machelska, H., Leder, K., Mousa, S.A., Schafer, M., Stein, C., 2004. Control of inflammatory pain by chemokine-mediated recruitment of opioid-containing polymorphonuclear cells. Pain 112, 229–238.

Bu, H., Shu, B., Gao, F., Liu, C., Guan, X., Ke, C., Cao, F., Hinton, A.J., Xiang, H., Yang, H., Tian, X., Tian, Y., 2014. Spinal IFN-gamma-induced protein-10 (CXCL10) mediates metastatic breast cancer-induced bone pain by activation of microglia in rat models. Breast Cancer Res Treat. 143, 255-263.

Chao, C.C., Hu, S., Shark, K.B., Sheng, W.S., Gekker, G., Peterson, P.K., 1997. Activation of Mu Opioid Receptors Inhibits Microglial Cell Chemotaxis. J Pharmacol Exp Ther. 281, 998-1004.

Chen, X., Geller, E. B., Rogers, T. J., Adler, M. W., 2007. The chemokine CX3CL1/fractalkine interferes with the antinociceptive effect induced by opioid agonists in the periaqueductal grey of rats. Brain Res. 1153,52-57.

Chieng, B., Christie, M.J., 1994. Inhibition by opioids acting on mu-receptors of GABAergic and glutamatergic postsynaptic potentials in single rat periaqueductal gray neurones in vitro. Br J Pharmacol. 113, 303-309.

Commons, K.G., Aicher, S.A., Kow, L.-M., Pfaff, D.W., 2000. Presynaptic and postsynaptic relations of μ-opioid receptors to γ-aminobutyric acid-immunoreactive and medullary-projecting periaqueductal gray neurons. J Comp Neurol. 419, 532-542.

Cui, Y., Chen, Y., Zhi, J.L., Guo, R.X., Feng, J.Q., Chen, P.X., 2006. Activation of p38 mitogen-activated protein kinase in spinal microglia mediates morphine antinociceptive tolerance. Brain Res. 1069, 235-243.

Cui, Y., Liao, X., Liu, W., Guo, R., Wu, Z., Zhao, C., Chen, P., Feng, J., 2008. A novel role of minocycline: Attenuating morphine antinociceptive tolerance by inhibition of p38 MAPK in the activated spinal microglia. Brain Behav Immun. 22, 114-123.

Eidson, L.N., Murphy, A.Z., 2013a. Blockade of Toll-like receptor 4 attenuates morphine tolerance and facilitates the pain relieving properties of morphine. J Neurosci. 33, 15952-15963.

Eidson, L.N., Murphy, A.Z., 2013b. Persistent peripheral inflammation attenuates morphine-induced periaqueductal gray glial cell activation and analgesic tolerance in the male rat. J Pain. 14, 393-404.

Ferrini, F., Trang, T., Mattioli, T.A., Laffray, S., Del'Guidice, T., Lorenzo, L.E., Castonguay, A., Doyon, N., Zhang, W., Godin, A.G., Mohr, D., Beggs, S., Vandal, K., Beaulieu, J.M., Cahill, C.M., Salter, M.W., De Koninck, Y., 2013. Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl(-) homeostasis. Nat Neurosci. 16, 183-192.

Freeland, K., Liu, Y.Z., Latchman, D.S., 2000. Distinct signaling pathways mediate the cAMP response element (CRE)-dependent activation of the calcitonin gene-related peptide gene promoter by cAMP and nerve growth factor. Biochem J. 345 Pt 2, 233-238.

Guan, X.H., Fu, Q.C., Shi, D., Bu, H.L., Song, Z.P., Xiong, B.R., Shu, B., Xiang, H.B., Xu, B., Manyande, A., Cao, F., Tian, Y.K., 2015. Activation of spinal chemokine receptor CXCR3 mediates bone cancer pain through an Akt-ERK crosstalk pathway in rats. Exp Neurol. 263, 39-49.

Harris, T.H., Banigan, E.J., Christian, D.A., Konradt, C., Tait, W.E., Norose, K., Wilson, E.H., John, B., Weninger, W., Luster, A.D., Liu, A.J., Hunter, C.A., 2012. Generalized Levy walks and the role of chemokines in migration of effector CD8+ T cells. Nature. 486, 545-548.

He, L., Kim, J.A., Whistler, J.L., 2009. Biomarkers of morphine tolerance and dependence are prevented by morphine-induced endocytosis of a mutant mu-opioid receptor. FASEB J. 23, 4327-4334.

Horvath, R.J., Romero-Sandoval, E.A., De Leo, J.A., 2010. Inhibition of microglial P2X4 receptors attenuates morphine tolerance, Iba1, GFAP and mu opioid receptor protein expression while enhancing perivascular microglial ED2. Pain. 150, 401-413.

Ingram, S.L., Williams, J.T., 1994. Opioid inhibition of Ih via adenylyl cyclase. Neuron. 13, 179-186.

Johnston, I.N., Milligan, E.D., Wieseler-Frank, J., Frank, M.G., Zapata, V., Campisi, J., Langer, S., Martin, D., Green, P., Fleshner, M., Leinwand, L., Maier, S.F., Watkins, L.R., 2004. A role for proinflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. J Neurosci. 24, 7353-7365.

Kong, F., Chen, S., Cheng, Y., Ma, L., Lu, H., Zhang, H., Hu, W., 2013. Minocycline attenuates cognitive impairment induced by isoflurane anesthesia in aged rats. PLoS One. 8, e61385.

Liu, X., Bu, H., Liu, C., Gao, F., Yang, H., Tian, X., Xu, A., Chen, Z., Cao, F., Tian, Y., 2012. Inhibition of glial activation in rostral ventromedial medulla attenuates mechanical allodynia in a rat model of cancer-induced bone pain. J Huazhong Univ Sci Technolog Med Sci. 32, 291-298.

Loetscher, M., Loetscher, P., Brass, N., Meese, E., Moser, B., 1998. Lymphocyte-specific chemokine receptor CXCR3: regulation, chemokine binding and gene localization. Eur J Immunol. 28, 3696-3705.

Ma, W., Zheng, W.H., Powell, K., Jhamandas, K., Quirion, R., 2001. Chronic morphine exposure increases the phosphorylation of MAP kinases and the transcription factor CREB in dorsal root ganglion neurons: an in vitro and in vivo study. Eur J Neurosci. 14, 1091-1104.

Machelska, H., 2007. Targeting of opioid-producing leukocytes for pain control. Neuropeptides, 41, 355-363.

Martini, L., Whistler, J.L., 2007. The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. Curr Opin Neurobiol. 17, 556-564.

Masse, F., Petit-Demouliere, B., Dubois, I., Hascoet, M., Bourin, M., 2008. Anxiolytic-like effects of DOI microinjections into the hippocampus (but not the amygdala nor the PAG) in the mice four plates test. Behav Brain Res. 188, 291-297.

Müller, M., Carter, S., Hofer, M.J., Campbell, I.L., 2010. Review: The chemokine receptor CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 in neuroimmunity – a tale of conflict and conundrum. Neuropathol Appl Neurobiol. 36, 368-387.

Oh, S.B., Tran, P.B., Gillard, S.E., Hurley, R.W., Hammond, D.L., Miller, R.J., 2001.

Chemokines and glycoprotein 120 produce pain hypersensitivity by directly exciting primary nociceptive neurons. J. Neurosci. 21, 5027–5035.

Old, E.A., Malcangio, M., 2012. Chemokine mediated neuron-glia communication and aberrant signaling in neuropathic pain states. Curr Opin Pharmacol. 12, 67-73.

Rappert, A., Bechmann, I., Pivneva, T., Mahlo, J., Biber, K., Nolte, C., Kovac, A.D., Gerard, C., Boddeke, H.W., Nitsch, R., Kettenmann, H., 2004. CXCR3-dependent microglial recruitment is essential for dendrite loss after brain lesion. J Neurosci. 24, 8500-8509.

Rivat, C., Sebaihi, S., Van Steenwinckel, J., Fouquet, S., Kitabgi, P., Pohl, M., Melik Parsadaniantz, S., Reaux-Le Goazigo, A., 2014. Src family kinases involved in CXCL12-induced loss of acute morphine analgesia. Brain Behav Immun. 38, 38-52. doi: 10.1016/j.bbi.2013.11.010.

Sanchez-Blazquez, P., Rodriguez-Munoz, M., Montero, C., de la Torre-Madrid, E., Garzon, J., 2008. Calcium/calmodulin-dependent protein kinase II supports morphine antinociceptive tolerance by phosphorylation of glycosylated phosducin-like protein. Neuropharmacology. 54, 319-330.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 3, 1101-1108.

Schroeder, J.E., Fischbach, P.S., Zheng, D., McCleskey E.W., 1991. Activation of μ opioid receptors inhibits transient high- and low-threshold Ca 2+ currents, but spares a sustained current. Neuron. 6, 13-20.

Shen, Q., Zhang, R., Bhat, N.R., 2006. MAP kinase regulation of IP10/CXCL10 chemokine gene expression in microglial cells. Brain Res. 1086, 9-16.

Sorensen, T.L., Trebst, C., Kivisakk, P., Klaege, K.L., Majmudar, A., Ravid, R., Lassmann, H., Olsen, D.B., Strieter, R.M., Ransohoff, R.M., Sellebjerg, F., 2002. Multiple sclerosis: a study of CXCL10 and CXCR3 co-localization in the inflamed central nervous system. J Neuroimmunol. 127, 59-68.

Stellwagen, D., Beattie, E.C., Seo, J.Y., Malenka, R.C., 2005. Differential Regulation of AMPA Receptor and GABA Receptor Trafficking by Tumor Necrosis Factor-α. J Neurosci. 25, 3219.

Sui, Y., Stehno-Bittel, L., Li, S., Loganathan, R., Dhillon, N.K., Pinson, D., Nath, A., Kolson, D., Narayan, O., Buch, S., 2006. CXCL10-induced cell death in neurons: role of calcium dysregulation. Eur J Neurosci. 23, 957-964.

Tai, Y.H., Wang, Y.H., Tsai, R.Y., Wang, J.J., Tao, P.L., Liu, T.M., Wang, Y.C., Wong, C.S., 2007. Amitriptyline preserves morphine's antinociceptive effect by regulating the glutamate transporter GLAST and GLT-1 trafficking and excitatory amino acids concentration in morphine-tolerant rats. Pain. 129, 343-354.

Tanuma, N., Sakuma, H., Sasaki, A., Matsumoto, Y., 2006. Chemokine expression by astrocytes plays a role in microglia/macrophage activation and subsequent neurodegeneration in secondary progressive multiple sclerosis. Acta Neuropathol. 112, 195-204.

Tikka, T., Fiebich, B.L., Goldsteins, G., Keinanen, R., Koistinaho, J., 2001. Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. J Neurosci. 21, 2580-2588.

van Weering, H.R., Boddeke, H.W., Vinet, J., Brouwer, N., de Haas, A.H., van Rooijen, N., Thomsen, A.R., Biber, K.P., 2011. CXCL10/CXCR3 signaling in glia cells

differentially affects NMDA-induced cell death in CA and DG neurons of the mouse hippocampus. Hippocampus. 21, 220-232.

Wang, H.Y., Burns, L.H., 2009. Naloxone's pentapeptide binding site on filamin A blocks Mu opioid receptor-Gs coupling and CREB activation of acute morphine. PLoS One. 4, e4282.

Wang, Z., Ma, W., Chabot, JG., Quirion, R., 2010a. Calcitonin gene-related peptide as a regulator of neuronal CaMKII-CREB, microglial p38-NFκB and astroglial ERK-Stat1/3 cascades mediating the development of tolerance to morphine-induced analgesia. Pain. 151, 194-205. doi: 10.1016/j.pain.2010.07.006.

Wang, Z., Ma, W., Chabot, JG., Quirion, R., 2010b. Morphological evidence for the involvement of microglial p38 activation in CGRP-associated development of morphine antinociceptive tolerance. Peptides. 31, 2179-2184.

Wang, Z., Chabot, JG., Quirion, R., 2011. On the possible role of ERK, p38 and CaMKII in the regulation of CGRP expression in morphine-tolerant rats. Mol Pain. 7, 68. doi: 10.1186/1744-8069-7-68.

Watkins, L.R., Hutchinson, M.R., Rice, K.C., Maier, S.F., 2009. The "Toll" of Opioid-Induced Glial Activation: Improving the Clinical Efficacy of Opioids by Targeting Glia. Trends Pharmacol Sci. 30, 581-591.

Wei, F., Guo, W., Zou, S., Ren, K., Dubner, R., 2008. Supraspinal glial-neuronal interactions contribute to descending pain facilitation. J Neurosci. 28, 10482-10495.

Wen, Y., Tan, P., Cheng, J., Liu, Y., Ji, R., 2011. Role of microglia in neuropathic pain, postoperative pain, and morphine tolerance. J Formos Med Assoc. 110, 487-494.

Wenzel, J., Wiechert, A., Merkel, C., Bieber, T., Tuting, T., 2007. IP10/CXCL10 - CXCR3 interaction: a potential self-recruiting mechanism for cytotoxic lymphocytes in lichen sclerosus et atrophicus. Acta Derm Venereol. 87, 112-117.

Williams, J.T., Ingram, S.L., Henderson, G., Chavkin, C., von Zastrow, M., Schulz, S., Koch, T., Evans, C.J., Christie, M.J., 2013. Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. Pharmacol Rev. 65, 223-254.

Xia, M.Q., Bacskai, B.J., Knowles, R.B., Qin, S.X., Hyman, B.T., 2000. Expression of

the chemokine receptor CXCR3 on neurons and the elevated expression of its ligand IP-10 in reactive astrocytes: in vitro ERK1/2 activation and role in Alzheimer's disease. J Neuroimmunol. 108, 227-235.

Ye, D., Bu, H., Guo, G., Shu, B., Wang, W., Guan, X., Yang, H., Tian, X., Xiang, H., Gao, F., 2014. Activation of CXCL10/CXCR3 Signaling Attenuates Morphine Analgesia: Involvement of Gi Protein. J Mol Neurosci. 53, 571-579.

Zhou, D., Chen, M.L., Zhang, Y.Q., Zhao, Z.Q., 2010. Involvement of spinal microglial P2X7 receptor in generation of tolerance to morphine analgesia in rats. J Neurosci. 30, 8042-8047.

Zhou, Q., Wang, J., Zhang, X., Zeng, L., Wang, L., Jiang, W., 2013. Effect of metabotropic glutamate 5 receptor antagonists on morphine efficacy and tolerance in rats with neuropathic pain. Eur J Pharmacol. 718, 17-23.

Figure 1.

Enhanced expression of CXCR3 in PAG during the development of morphine tolerance. **A.** Pain thresholds of mice were assessed using paw withdrawal thresholds to mechanical pressure. The decreased paw withdrawal thresholds in morphine-treated

mice indicated the successful establishment of morphine tolerance. Repeated measurement two-way ANOVA followed by Bonferroni *post-hoc* test, * $P < 0.05$ vs. saline. n = 7 in each group. **B** and **C.** The mRNA and protein levels of CXCR3 were measured by real-time PCR and western blots, respectively. The expressions of CXCR3 were gradually increased along with repeated morphine administration. One-way ANOVA followed by Bonferroni *post-hoc* test, ** $P < 0.01$ vs. D0. n = 5 in each group. **D.** Double immunostaining of CXCR3 and cell-specific markers in PAG. The mergence showed that CXCR3 was localized in NeuN positive neurons (indicated by arrows). Scale bars: 100 μm.

Figure 2.

Repeated morphine treatment increased microglia-derived CXCL10 production in PAG. **A.** Western blot analysis showed that repeated morphine treatment increased the expression of CXCL10 on day 3 and day 7. One-way ANOVA followed by Bonferroni post-hoc test, * $P < 0.05$ vs. saline. ** $P < 0.01$ vs. saline. $n = 5$ in each group. **B.** Double immunostaining of CXCL10 and cell-specific markers in morphine-treated mice. CXCL10 was localized in Iba-1 positive microglia (indicated by arrows). Scale bars: 100 μm. **C** and **D.** Immunostaining of Iba-1 in morphine-treated mice. The densities of Iba-1 positive microglia significantly increased along with the development of morphine tolerance. Scale bars: 100 μm. One-way ANOVA followed by Bonferroni

post-hoc test, ** $P < 0.01$ vs. D0. $n = 5$ in each group.

Figure 3.

CXCL10 and CXCR3 participated in the development of morphine tolerance. **A.** The decreases of paw withdrawal thresholds in morphine-treated mice were attenuated by pre-treatment with AMG487 in a dose-related manner. Repeated measurement two-way ANOVA followed by Bonferroni *post-hoc* test, * $P < 0.05$ vs. sham. # $P < 0.05$ vs. morphine. $n = 7$ in each group. **B.** Pre-treatment with rmCXCL10 could decrease morphine antinociceptive effect from day 1 to day 3 and accelerate the development of morphine tolerance in mice, while co-administration of AMG487 could attenuate the effect of rmCXCL10. Repeated measurement ANOVA followed by Bonferroni *post-hoc* test, * $P < 0.05$ vs. sham. # $P < 0.05$ vs. morphine. $n = 7$ in each group.

Figure 4.

Inhibition of microglia activation attenuated CXCL10 expression and morphine tolerance. **A.** Intra-PAG treatment of minocycline (10 pmol) 30 min before morphine injection attenuated the development of morphine tolerance, whereas the effect of minocycline was inhibited by pre-treatment with rmCXCL10. Repeated measurement ANOVA followed by Bonferroni *post-hoc* test, * $P < 0.05$ vs. morphine. # $P < 0.05$ vs. Minocycline + Morphine. $n = 7$ in each group. **B.** Western blot analysis showed that

morphine treatment could increase CXCL10 expression. Pre-treatment with minocycline before morphine injection could down-regulate CXCL10 expression. One-way ANOVA followed by Bonferroni *post-hoc* test, * $P < 0.05$, ** $P < 0.01$ vs. saline. ## $P < 0.01$ vs. Morphine. $n = 5$ in each group. **C** and **D.** Immunostaining of Iba-1 in PAG. Minocycline could inhibit the expression of Iba-1 and rmCXCL10 could not alter the effect of minocycline. Scale bars: 100 μm. One-way ANOVA followed by Bonferroni *post-hoc* test, ** $P < 0.01$ vs. saline. ## $P < 0.01$ vs. Morphine. $n = 5$ in each group. **E.** Western blot analysis showed that morphine-induced CXCR3 expression could be attenuated by AMG487 but not by minocycline. One-way ANOVA followed by Bonferroni *post-hoc* test, ** $P < 0.01$ vs. saline. ## $P < 0.01$ vs. Morphine. $n = 5$ in each group.