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CCL5 and cytokine expression in the rat brain: differential modulation by chronic morphine and morphine withdrawal

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Abstract

Opioids have been shown to influence the immune system and to promote the expression of pro-inflammatory cytokines in the central nervous system. However, recent data have shown that activation of opioid receptors increases the expression and release of the neuroprotective chemokine CCL5 from astrocytes *in vitro*. To further define the interaction between CCL5 and inflammation in response to opioids, we have examined the effect of chronic morphine and morphine withdrawal on the *in vivo* expression of CCL5 as well as of pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor- (TNF-). Rats undergoing a chronic morphine paradigm (10 mg/kg increasing to 30 mg/kg, twice a day for 5 days) showed a two-fold increase of CCL5 protein and mRNA within the cortex and striatum. No changes were observed in the levels of IL-1 and TNF-. Naltrexone blocked the effect of morphine. A chronic morphine paradigm with no escalating doses (10 mg/kg, twice a day) did not alter CCL5 levels compared to saline-treated animals. On the contrary, rats undergoing spontaneous morphine withdrawal exhibited lower levels of CCL5 within the cortex as well as increased levels of pro-inflammatory cytokines and Iba-1 positive cells than saline-treated rats. Overall, these data suggest that morphine withdrawal may promote cytokines and other inflammatory responses that have the potential of exacerbating neuronal damage.

Keywords

CCL5; frontal cortex; IL-1; microglia; morphine dependence; naltrexone; striatum; morphine withdrawal

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Introduction

The adverse effects of opioids such as heroin and morphine on the adaptive and innate immune systems have been recognized [reviewed in (Hutchinson et al., 2011; Roy et al., 2011)]. Less is known about opioid-induced changes in central nervous system (CNS) immune responses. Morphine has been shown to promote the expression of pro-inflammatory cytokines and chemokines in the CNS (Johnston et al., 2004; Schwarz et al., 2013). Cytokines and chemokines are soluble polypeptides that were discovered for their ability to modulate immune responses as well as cell migration. In the CNS, these polypeptides are synthesized and released mostly by glial cells, which are considered the immune competent cells of the CNS as well as crucial components of synaptic plasticity (Huang et al., 2000; Ransohoff and Brown, 2012). Therefore, opioids, through cytokines and chemokines, could contribute to pathological states or participate in the maintenance of healthy neurons. On the other hand, morphine-mediated increase of cytokines/chemokines appears to be more pronounced in brain areas that participate in the neuronal network involved in opiate abuse/reward, including the nucleus accumbens (Schwarz et al., 2011). Moreover, in recent years, several lines of independent investigations have shown that the immune system may play a role in the behavior outcome following opioid administration [reviewed in (Coller and Hutchinson, 2012)]. Thus, re-evaluating whether a given cytokine or chemokine is enhanced in response to an inflammatory pathological state or to activation of a brain circuitry involved with drug abuse is crucial for a better understanding of opioid action and immune responses.

There are a number of cytokines and chemokines in the brain (Cartier et al., 2005). Chemokines and their receptors are often involved with the progression of neurodegenerative diseases. However, some of these molecules exhibit a profile similar to that of neuromodulators (Adler and Rogers, 2005; Rostene et al., 2007) or behave like neurotrophic factors. For instance the chemokine CCL5, formerly known as regulated on activation normal T-cell expressed and secreted (RANTES), was discovered for its ability to attract and activate mononuclear phagocytes, as well as several other leukocyte types, to sites of inflammation/injury. However, CCL5's main receptor CCR5 is expressed in the CNS (Avdoshina et al., 2011; Westmoreland et al., 2002). CCL5 exerts neurotrophic (Bolin et al., 1998) and neuroprotective activity against various neurotoxins including glutamate, beta-amyloid, HIV and nitric oxide (Bruno et al., 2000; Kaul et al., 2007; Tripathy et al., 2010). Injection of CCL5 into the periaqueductal grey blocks the antinociceptive effect of opioid agonists (Chen et al., 2007). Moreover, chronic morphine promotes the expression and release of CCL5 from primary cortical astrocytes (Avdoshina et al., 2010) or astrocytes from the nucleus accumbens and ventral tegmental area in morphine-dependent rats (Hutchinson et al., 2009). Thus, this chemokine could participate in modulation of pain in response to opioids or play a role in opioid dependence.

Cessation of morphine use leads to withdrawal. It has been established that withdrawal can be detrimental to immunosuppression leading to susceptibility to outside pathogens. For example, opioid withdrawal can reduce the levels of circulating lymphocytes in morphine dependent macaques (Weed and Steward, 2005). Moreover, opioid withdrawal can affect cytokine production in blood cells (Das et al., 2011; Desjardins et al., 2008) or in brain (Liu et al., 2011). Thus, the profile of chemokine/cytokine expression in rats that are morphine-dependent or undergoing withdrawal could be different. This study aimed to elucidate the *in vivo* effects of chronic morphine treatment, in comparison to morphine withdrawal on the expression of CCL5 and pro-inflammatory cytokines that could be involved in the mechanisms of neurotoxicity or drug addiction.

Materials and Methods

Animals

Three month old male Sprague-Dawley rats (Charles River, Germantown, MD) were acclimated a minimum of one week prior to conducting experiments. Homozygous CCL5 (CCL5^{-/-}) mice (B6.129P2-Ccl5^{tm1Hso/J}) and wild type C57BL/6J mice were purchased from the Jackson Laboratory (Famington, CT). Mice were viable, normal in size and did not display any gross physical or behavioral abnormalities. Animals were housed under standard conditions, two per cage, with food and water available *ad libitum*, and were maintained on a 12-h light/dark cycle for the duration of the treatment protocols. All studies were carried out following the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and approved by Georgetown University Animal Care and Use Committee.

Animal treatments

Morphine sulfate was received from the National Institutes of Drug Abuse, (NIH, Division of Neuroscience & Behavioral Research, Research Triangle Park, NC). (-)- Naltrexone hydrochloride (NTX) was purchased from Sigma, St Louis, MO. All drugs were diluted in isotonic and filtered sterilized saline. Drugs doses are reported as free base. To cause morphine tolerance and dependence rats were treated s.c. with escalating doses of morphine from 10 mg/kg to 30 mg/kg b.i.d over the course of five days (Table 1). Rats were sacrificed 2 h after the last injection. A group of rats received escalating doses of morphine concomitantly to NTX (10 mg/kg, s.c.) to block the analgesic effect of morphine. The efficacy of chronic NTX in preventing morphine induced analgesia was determined by the radiant tail-flick method according to an established protocol (D'Amour and Smith, 1941). In brief, animals were placed on the tail-flick apparatus (Socrel DS20, Milan, Italy) with their tails smoothed into the tail groove. The radiant heat intensity was calibrated to obtain average latency values of 4 seconds. A cut off time of 10 seconds was used to avoid tissue damage. Baseline was established for all animals before treatment paradigms to reduce the novelty effect of the behavioral task.

To cause morphine withdrawal, animals were treated with increasing dosages of morphine (10 mg/kg to 30mg/kg) b.i.d. over the course of five days as described above, after which, morphine treatment was interrupted and spontaneous withdrawal observed for 60 h. Withdrawal symptoms included diarrhea, weight loss, piloerection and irritability, compared to control animals.

After the treatments, animals were anesthetized with a mixture of ketamine/xylazine (80 mg/kg and 20 mg/kg, respectively) and intracardially perfused with ice cold phosphate buffered saline (PBS). Whole brains were quickly removed for biochemical or immunohistochemical analyses. For biochemical analyses, frontal cortex, striatum, hippocampus and cerebellum were dissected on ice. For immunohistochemistry, whole brain were removed and post-fixed in 4% paraformaldehyde (PFA) overnight followed by 30% sucrose.

Enzyme-Linked Immunosorbant Assay (ELISA)

Brain lysates were prepared as previously described (Bachis et al., 2010). CCL5, IL-1, and TNF- α levels were determined using the DuoSet ELISA Development System Kits (R&D, Minneapolis, MN), according to the manufacturer's instructions. The content of total proteins in each sample was determined by bicinchoninic acid assay (Thermo Scientific Inc., Rockford, IL).

Western blot analysis

Brain areas were homogenized in RIPA buffer (Millipore, Billerica, MA) containing protease-phosphatase inhibitors (Roche Applied Science, Indianapolis, IN) by sonication and protein elution at 4°C. Proteins were separated in a NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and transferred to a PVDF membrane using the iBlot device (Invitrogen). Membranes were blocked with 5% milk in PBS and 0.05% Tween and probed with rabbit polyclonal anti-CCR5 antibody (1 µg/ml, Abcam, Cambridge, MA). Membranes were stripped and reprobed with mouse monoclonal anti- β -actin antibody (1:10000, Sigma-Aldrich, St. Louis, MO) as a loading control. Immune complexes were detected by the corresponding secondary antibody and chemiluminescence reagent (Thermo Fisher Scientific Inc.). The intensity of immunoreactive bands was quantified using Quantity One® 1-D (Bio-Rad Laboratories, Inc., Hercules, CA) and expressed in arbitrary units (A.U.) after normalization to β -actin immunoreactivity.

Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction (qPCR)

Total mRNA was extracted using the RNeasy Plus Mini Kit according to manufacturer's specifications (Qiagen, Valencia, CA). qPCR for CCL5 was performed using primers that displayed linear amplification as previously described (Avdoshina et al., 2010). Ribosomal protein L13 (RPL13) and TATA box binding protein were used as housekeeping genes to normalize for total mRNA in each sample. Primers for housekeeping gene were obtained from RealTimePrimers.com. For RPL13 were 5-GTGAGGGCATCAACATTCT-3, 5-CATCCGCTTTTCTTGTCAT-3 (forward, reverse); For TATA box, 5-CGATAACCCAGAAAGTCGAA-3, 5-AGATGGGAATTCCAGGAGTCTTTTCCAGCAAGTCAATCC-3, (forward, reverse). The specificity of the qPCR was determined by the analysis of the dissociation curves. The 7900HT Fast Real-Time PCR System (Life Technologies Corp, Carlsbad, CA) was used. Data were analyzed using SDS v2.3 software (Life Technologies).

Immunohistochemistry

PFA post-fixed brains were sectioned at 40 µm by a sliding microtome (Microm International, Heidelberg, Germany). Sections were blocked in PBS-Blocking Buffer (1% bovine serum albumin, 0.2% milk, 0.3% Triton) for 30 minutes prior to ionized calcium binding adaptor molecule 1 (Iba-1) primary antibody (1 µg/ml, Wako, Japan). Sections were incubated overnight at 4°C. Corresponding secondary antibody was used to detect Iba-1 staining. After washing with PBS, sections were mounted on microscope slides using ProLong® gold antifade reagent (Invitrogen). Immunofluorescence was analyzed using a FV300 laser confocal scanning system attached to an Olympus IX-70 upright microscope. Cells were counted using a 20X objective and analyzed by ImageJ (Schneider et al., 2012) in a series of 20X digitized microscope images. A total of 5 fields per section (6 sections per animal) throughout the frontal cortex and rostral striatum were used (N=6 rats per group). The number of Iba-1 positive cells for each group was then averaged and expressed as cells per section.

Statistical Analysis

Data were evaluated by one-way ANOVA followed by multiple comparisons by Tukey's test (Sigma plot®, Systat Software Inc., Chicago, IL).

Results

Brain expresses CCL5

Expression of CCL5 in rodent brain has been mostly demonstrated after injury or inflammation (Brown et al., 2010; Denes et al., 2010; Ghirnikar et al., 1996; Lee et al., 2005). However, relatively little is known about expression of CCL5 in different brain areas of naïve animals. Therefore, we determined the expression of CCL5 (protein and mRNA) by ELISA and qPCR, respectively in various brain areas of adult male rats. Spleen and thymus were used as controls. Rats were perfused with saline prior to decapitation to flush out blood that could contain CCL5. CCL5 was observed in several brain areas such as the cortex, striatum, hippocampus and cerebellum. CCL5 was more abundant in the cerebellum than the other areas (Fig. 1A). However, the levels of CCL5 protein in these brain areas were at least four-fold lower than that observed in the thymus or spleen (Fig. 1A). Spleen exhibited a higher amount of CCL5 than thymus. In addition, we verified expression of CCL5 in various brain areas by qPCR. Indeed, CCL5 mRNA levels in the brain were at least four-fold lower than those in thymus and spleen (Fig. 1B). Authenticity of CCL5 expression in the brain was also determined by ELISA of lysates from various brain areas of wild type and CCL5^{-/-} mice. No CCL5 was detected by ELISA in CCL5^{-/-} mice (data not shown).

Morphine increases CCL5 expression

Previous data have shown that activation of μ opioid receptors increases CCL5 levels *in vitro* (Avdoshina et al., 2010; Wetzel et al., 2000). We then determined whether morphine changes CCL5 protein and mRNA levels *in vivo* by comparing the effects of various paradigms of acute and chronic administrations of morphine versus saline. Rats received escalating doses of morphine twice a day over the course of five days (as described in Materials and Methods) along with a saline injected cohort as a comparison. On day five, rats received a final injection of morphine (30 mg/kg, s.c.) and were sacrificed 2 h later. In morphine-treated animals, there was a significant increase of CCL5 levels in the frontal cortex and striatum but not in the hippocampus (Fig. 2A). To confirm that morphine changes CCL5 expression, we determined the levels of CCL5 mRNA in the cortex. The hippocampus was used as a negative control. Morphine elicited a ~3 fold increase in CCL5 mRNA only in the cortex (Fig. 2B), confirming that morphine increases CCL5 expression.

The chronic paradigm used to increase CCL5 elicits tolerance to the analgesic effect of 10 mg/kg morphine as measured by tail flick (data not shown). The opioid receptor antagonists NTX have been used to block the analgesic effect of morphine. To examine whether the increase in expression of CCL5 by morphine is opioid receptor-mediated, rats received NTX (10 mg/kg, s.c.) concomitantly to morphine. This dose of NTX was selected because in preliminary studies we were able to block completely the analgesic effect of morphine as determined by tail flick assay ($p < 0.01$ morphine vs saline; $p < 0.01$ morphine vs NTX). NTX inhibited the increase in CCL5 evoked by morphine (Fig. 2A).

To test whether the up-regulation of CCL5 expression requires a chronic treatment that induces tolerance and dependence, we employed two additional treatment schedules. Rats received a single acute dose of morphine (10 mg/kg, s.c.) and were sacrificed 2 or 18 h later. Another group of rats received non-escalating doses of morphine (10 mg/kg, s.c.) twice a day for 5 days (to mimic a recreational or maintenance-like paradigm) and were sacrificed 2 h after the last injection. Neither treatment changed CCL5 expression (Figs. 2C and D) in any region examined. Thus, it appears that morphine causes an up-regulation of CCL5 only when given at increasing concentrations.

Morphine does not change CCR5 levels

Our data show that chronic morphine increases CCL5. This chemokine binds three receptors, CCR1, CCR3 and CCR5. CCR5 appears to be down-regulated by its ligands (Escola et al., 2010; Signoret et al., 2000). In addition, activation of μ opioid receptors has been shown to cause heterologous desensitization of CCR5 in chinese hamster ovary cells (Chen et al., 2004). Conversely, activation of CCR5 by CCL5 can desensitize opioid receptors in the periaqueductal gray matter (Szabo et al., 2002). Therefore, we examined whether the increase in CCL5 by chronic morphine alters the levels of CCR5. CCR5 immunoreactivity was determined by Western blot analysis using specific antibodies. We failed to observe changes in CCR5 levels between saline, morphine alone, NTX alone or morphine + NTX-treated rats in any region examined (Figs. 3A and B).

Chronic morphine does not promote pro-inflammatory responses

Opioids have been shown to modulate immunoresponses in the periphery [reviewed in (Hutchinson et al., 2011)] as well as to alter the release of pro-inflammatory cytokines such as IL-1 (Bessler et al., 1990) or IL-6 (El-Hage et al., 2008b) from glia or endothelial cells (Wang et al., 2012). Thus, morphine-mediated increases in CCL5 could represent an inflammatory response. To assess the specificity of the effect of morphine, we measured the levels of IL-1 and TNF- α , two pro-inflammatory cytokines that promote the proliferation of immune cells. We found a distinct effect of morphine depending upon the treatment paradigm used. In fact, chronic escalating doses of morphine elicited a decrease in IL-1 in the cerebral cortex but produced no changes in other areas (Fig. 4A). This effect was blocked by NTX (Fig. 4A). Chronic morphine failed to change TNF- α in any region examined (Fig. 4B). However, the acute morphine treatment increased the levels of IL-1 at 2 h in the cerebral cortex and hippocampus and at 18 h in the other regions (Fig. 4C). Acute morphine changes TNF- α levels by 18 h only in the cortex and cerebellum (Fig. 4D). Thus, it appears that chronic morphine exhibits an opposite inflammatory profile from the acute treatment.

Chronic morphine and microglia

Chronic morphine has been shown to alter microglia activation in specific brain nuclei (Hutchinson et al., 2009). Microglia, the primary immunocompetent cells of the CNS, express CCL5 (Avdoshina et al., 2010; Kremlev et al., 2004; Rezaie et al., 2002). Thus, morphine-mediated increase in CCL5 could be associated with an increase in microglia activation. Therefore, we determined whether chronic escalating doses of morphine with or without NTX change the number and/or morphology of microglia in the cortex and striatum. In the frontal cortex we did not observe a significant change in the number of microglia between saline- (Figs. 5A and I), morphine- (Figs. 5B and I), NTX- and morphine + NTX-treated rats (Fig. I). Moreover, microglia in all experimental groups exhibited a similar 'ramified' morphology (Figs. 5C and D), which is a typical characteristic of non-phagocytotic microglia. However, the striatum exhibited a different profile. In fact, the number of Iba-1 positive cells was decreased in the striatum of morphine-treated rats (Figs. 5F and I) when compared to saline-treated rats (Figs. 5E and I). In addition, the effect of morphine was blocked by NTX (Fig. 5I). There was no apparent change in the morphology of Iba-1 positive cells between experimental groups (Figs. 5G and H). Thus, it appears that the accumulation of CCL5 observed in our studies does not coincide with an increase in microglia proliferation.

Morphine withdrawal reduces CCL5 in the frontal cortex

The morphine-mediated increase in CCL5 expression observed *in vitro* and *in vivo* requires prolonged exposure to morphine. Nevertheless, it is unclear whether this increase occurs as a

response to morphine dependence/tolerance or withdrawal, two distinct physiological effects that are associated with the chronic use of morphine. To determine the effect of withdrawal, rats received the escalating doses of morphine and were allowed to undergo spontaneous withdrawal for 60 h. CCL5 levels were then measured in several brain areas. We observed a reduction of CCL5 in the frontal cortex and no changes in other areas (Fig. 6A). Intriguingly, withdrawal evoked an increase in IL-1 in several brain areas (Fig. 6B), and TNF- in the striatum (Fig. 6C).

We next examined the effect of morphine withdrawal on CCR5 levels. Morphine withdrawal elicited a decrease in CCR5 in the frontal cortex and hippocampus (Figs. 7A and B). In addition, rats undergoing withdrawal exhibited higher levels of Iba-1 positive cells than saline-treated animals in the striatum (Figs. 8E, F and I) but not in the frontal cortex (Figs. 8A, B and I). No apparent changes in microglia morphology were observed in all experimental groups (Figs. 8C, D, G and H). Thus, it appears that morphine withdrawal causes an up-regulation of inflammatory cytokines and microglia proliferation.

Discussion

There is little known about the expression of CCL5 in adult brain. Our data show that CCL5 is expressed in several brain areas of adult naïve rats although at levels lower than those observed in spleen and thymus. The cerebellum exhibits the highest levels of CCL5 expression among the brain areas examined. The uneven expression/distribution of CCL5 in the uninjured brain could be consistent with the suggested role that certain chemokines could act as neuropeptides rather than chemoattractants or modulator of inflammation (Adler and Rogers, 2005; Rostene et al., 2007). This notion is supported by our findings that chronic morphine augments the expression of CCL5 only in certain brain areas and is consistent with previous data showing that chronic morphine increases CCL5 expression *in vitro* (Avdoshina et al., 2010). In this study we did not investigate the cellular specificity of CCL5 induction by morphine; however, our previous data have shown that morphine increases CCL5 in astrocytes but not in neurons or microglia (Avdoshina et al., 2010). Because astrocytes display significant regional heterogeneity (Zhang and Barres, 2010) it might be inferred that the effect of morphine might be an astrocytic response. More studies are required to prove this hypothesis.

The morphine-mediated increase of CCL5 is blocked by NTX, suggesting that morphine may affect CCL5 expression through opioid receptors. This suggestion is supported by *in vitro* data showing that morphine, and the μ opioid receptor agonist DAMGO, increase CCL5 expression (Avdoshina et al., 2010). On the other hand, evidence has shown that morphine can also activate toll-like receptor 4 (TLR4) to mediate pro-inflammatory responses (Wang et al., 2012), analgesia (Hutchinson et al., 2010) as well as opioid drug reward (Hutchinson et al., 2012). In addition, our data have shown that the endotoxin lipopolysaccharide (LPS) that recognizes TLR4 increases CCL5 in primary cultures of microglia (Avdoshina et al., 2010). Thus, we cannot rule out that CCL5 expression can also be modulated by TLR4. Additional experiments are needed to confirm or rule out the involvement of TLR4 activation in morphine-mediated CCL5 expression.

Opioids have been shown to interfere with immune responses by reducing macrophage survival (Bhat et al., 2004), inhibiting lymphocyte proliferation (Wang et al., 2002), as well as increasing synthesis of pro-inflammatory cytokines and chemokines (Wetzel et al., 2000) in peripheral blood mononuclear cells. In addition, intrathecal morphine induces cytokines in the CNS which, in turn, appears to modulate tolerance to morphine and therefore to be involved in morphine-mediated hyperalgesia (Hutchinson et al., 2008; Johnston et al., 2004). The data presented in this study show that different paradigms of morphine treatment

promote a diverse profile of activation of chemokines and cytokines in the brain. Chronic morphine induces the expression of the chemokine CCL5 in the cortex and striatum, without a concomitant increase in IL-1 and TNF-. These two cytokines are typically induced under inflammatory conditions such as those observed in response to head trauma, infections, and other pathological brain insult (Tonelli et al., 2008; Wang and Shuaib, 2002). Acute morphine increases IL-1 but not CCL5, thus supporting data that acute opioid administration alters the production of the pro-inflammatory cytokines and chemokines in the rodent brain (Schwarz et al., 2011). Withdrawal also elicits an increase in both pro-inflammatory cytokines but decreases CCL5. Thus, it appears that acute morphine or withdrawal and not chronic morphine may promote the expression of pro-inflammatory cytokines that are known to play a major role in inflammation (Pribiag and Stellwagen, 2013).

The functional relationship between opioids and expression of CCL5 is still speculative. CCL5 belongs to the superfamily of chemokines that promotes leukocyte trafficking into inflammatory sites and induces the proliferation of certain killer cells. This chemokine is also a natural HIV suppressive factor when secreted by activated immune cells (Cocchi et al., 1995). Thus, in the immune system, CCL5 plays a key role in the innate and adaptive immune responses. Less is known about the function of CCL5 in brain. CCL5 has been shown to down-regulate the analgesic property of morphine (Szabo et al., 2002). In addition, CCL5 has been shown to promote growth and survival of astrocytes *in vitro* (Bakhiet et al., 2001). This property may be relevant because morphine and opioid signaling have been shown to promote proliferation of astroglia in the postnatal brain (Sargeant et al., 2008) and to modulate neurogenesis. Conversely, loss of CCR5 signaling, the main receptor for CCL5, impairs the ability of morphine to activate glia (El-Hage et al., 2008a). Moreover, the ability of morphine to induce proliferation is attributed to the activation of the mitogen-activated protein kinase pathway which has also been activated by CCL5 (Roscic-Mrkic et al., 2003). Thus, CCL5 may be involved in the ability of opioids to modulate glia proliferation/activation and could be a critical component of behavior sensitization induced by opioids that also lead to abuse and dependence. These changes result in a modified homeostatic state such that reaction to various stimuli including drug exposure is altered. Indeed, it has been suggested that activation of microglia in the nucleus accumbens is necessary for the reinstatement of morphine-seeking behavior (Schwarz et al., 2011). Nonetheless, the contribution of CCL5 in promoting microglia proliferation or activation remains to be further explored as we did not find a significant change in the morphology or number of microglia in those brain areas in which morphine up-regulates CCL5 expression. However, our study shows that CCL5, a chemokine with multiple functions, is increased by chronic morphine treatment in brain areas implicated in drug abuse, such as frontal cortex and striatum. This is consistent with other results showing morphine-mediated CCL5 expression in areas involved in opioid reward such as the nucleus accumbens (Hutchinson et al., 2009). Thus, CCL5 may participate in the initiation and maintenance of compulsive opioid use as suggested for other immune responses (Hutchinson et al., 2012). Further studies are needed to characterize the role (if any) of CCL5 in opioid abuse.

Transmigration of leukocytes across the blood-brain barrier is among one of the neuroinflammatory responses mediated by CCL5 (Ubogu et al., 2006). Moreover, human genetic studies on CCL5 polymorphisms have suggested an association of this chemokine with multiple sclerosis (Gade-Andavolu et al., 2004). Thus, the up-regulation of CCL5 by morphine could represent an inflammatory response. The data presented in our work support a different role for CCL5. In fact, we did not find evidence of an increase in other classic pro-inflammatory cytokines such as IL-1 and TNF-, with a chronic morphine treatment that up-regulated CCL5. Thus, we could speculate that a morphine-mediated increase in CCL5 could represent an anti-inflammatory and pro-survival effect. Evidence that certain

chemokines are able to promote neuronal survival is overwhelming. This is particularly true for CCL5 and its receptor CCR5, which have demonstrated survival-promoting activities. For instance, CCL5 prevents N-methyl-D-aspartate and amyloid toxicity (Bruno et al., 2000) and promote neuronal survival in Alzheimer's disease (Tripathy et al., 2010). CCL5 has also been shown to prevent neuronal apoptosis evoked by the M-tropic HIV protein gp120 *in vitro* (Avdoshina et al., 2010; Kaul et al., 2007). Thus, it appears that this chemokine can inhibit neuronal death produced by various pro-apoptotic stimuli.

Chronic morphine treatment that elicits tolerance and dependence causes contrasting responses compared to withdrawal. Whereas morphine increased CCL5 levels, withdrawal decreased it. The opposite was seen for levels of IL-1 and TNF-. These cytokines can dramatically alter neuronal or glial signaling, and consequently, their survival. The increase in pro-inflammatory cytokines may explain why withdrawal causes caspase-3 dependent apoptosis (Emeterio et al., 2006). In addition, withdrawal augmented the number of Iba-1 positive cells in the striatum, suggesting an increase in microglia proliferation. A similar effect has been described in other brain areas (Hutchinson et al., 2009). These cells are necessary for normal brain function. However, activated microglia have also been shown to play a role in neurotoxicity. For instance, they eliminate synapses during development (Paolicelli et al., 2010) and are the main source of pro-inflammatory cytokines including IL-1 and TNF- (Block et al., 2007). Thus, withdrawal could play a role in several metabolic processes that ultimately can lead to a decline in synaptic plasticity and neuronal survival.

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Highlights

Our study shows that a differential effect of morphine withdrawal and dependence on CCL5, microglia and inflammatory cytokines.

Figure 1. Expression of CCL5 in adult rat brain

Adult male rats were perfused with saline prior to the removal of their brains and peripheral tissues. Levels of CCL5 protein and CCL5 mRNA were determined by ELISA (A) and qPCR (B) as described in Materials and Methods. CTX=frontal cortex; STR=striatum; HIP=hippocampus; CB=cerebellum; THY=thymus; SPL=spleen. Data are the mean $\pm$ SEM of 5 rats per group.

Figure 2. Chronic morphine increases CCL5 expression in the frontal cortex and striatum
Rats received saline or escalating doses of morphine (Mor., from 10 mg/kg to 30 mg/kg) over the course of five days, and were sacrificed 2 h after the last injection. A group of animals received NTX alone or concomitantly to morphine. CCL5 protein (A) and CCL5 mRNA (B) were determined in the indicated brain areas by ELISA and qPCR, respectively, as described in Materials and Methods. C. Rats received saline or a single injection (acute) of morphine (Mor., 10 mg/kg, s.c.) and were sacrificed 2 and 18 h later. D. Rats received saline or a daily injection of morphine (10 mg/kg, s.c.) twice a day (Maintenance) over the course of five days. CCL5 levels were determined in the indicated brain areas. Data are the mean $\pm$ SEM of 6 rats per group; * p <0.01 vs saline; # p <0.01 vs chronic morphine.

Figure 3. Chronic morphine does not alter CCR5 levels

Rats received chronic morphine treatment and NTX as described in figure 2. Indicated brain areas were dissected and analyzed for CCR5 immunoreactivity. **A.** Representative Western blots of cortical lysates (1= saline, 2=NTX, 3=morphine, 4=morphine + NTX) using CCR5 antibodies. Blots were stripped and reprobbed with a β -actin antibody. **B.** Densitometric analysis of CCR5 immunoreactivity. Data, expressed in A.U. (see Materials and Methods), are the mean $\pm$ SEM of 4 rats per group.

Figure 4. Acute and chronic morphine evoke a differential expression of cytokines in the brain
 A group of rats (A and B) received chronic escalating (chronic) doses of morphine (Mor.) alone or in combination with NTX, and were sacrificed 2 h later. Saline-treated rats were used as controls. Another group of rats (C and D) received an acute (single) injection of morphine and were sacrificed 2 and 18 h later. The levels of IL-1 β (A and C) and TNF- α (B and D) were determined in the indicated areas by ELISA. Data are the mean $\pm$ SEM of 4 rats per group. * p <0.05, ** p <0.01 vs saline. # p <0.01 vs chronic morphine.

Figure 5. Chronic morphine decreases the number of Iba-1 positive cells in the striatum
Rats received a chronic morphine treatment as described in figure 2 and were sacrificed 2 h after the last injection. Brains were prepared for immunohistochemistry and sectioned as described in Materials and Methods. Serial sections were stained for Iba-1 and analyzed using a confocal microscope. Images are representative sections from cortex of saline (**A**) and morphine (**B**)-treated rats, or from the striatum of saline (**E**) and morphine (**F**)-treated rats. **C** and **D**= higher magnification of the area (indicated by a square) included in **A** and **B**, respectively. **G** and **H** = higher magnification of the area (indicated by a square) included in **E** and **F**, respectively. Bar in **A**= 100 μ m, bar in **C**=20 μ m. **I**. Semiquantitative analysis of

Iba-1 positive cells. Cells were counted using ImageJ software in serial sections from the indicated brain areas. A total of 5 randomly selected fields per section (5 sections each animal, 1 section every 80 μm) were used (N=4 rats per group). * $p<0.05$ versus saline, # $p<0.05$ vs chronic morphine.

Figure 6. Morphine withdrawal affects chemokines and cytokines in different brain areas

Rats were treated with saline or increasing doses of morphine (10 mg/kg to 30mg/kg) over the course of five days, after which morphine treatment was interrupted to cause spontaneous withdrawal. Animals were sacrificed 60 h after the last injection. CCL5 (A), IL-1 β (B) and TNF- α (C) levels were measured in the indicated brain areas by ELISA. Data are the mean $\pm$ SEM of 4 rats per group. *p<0.05 vs saline.

Figure 7. Withdrawal changes CCR5 levels

Rats were treated as described in Fig. 6. **A.** Representative Western blot analysis of cortical lysates using CCR5 antibodies. Blots were stripped and re-probed with a β -actin antibody. **(B)** CCR5 immunoreactivity was determined in several brain areas as described in Materials and Methods. Data are the mean $\pm$ SEM of 4 rats per group. * $p < 0.05$ vs saline.

Figure 8. Morphine withdrawal increases the number of Iba-1 positive cells in the striatum
The brains of rats undergoing spontaneous withdrawal were analyzed with Iba-1 antibody using a confocal microscope as described in Materials and Methods. Representative cortical (A and B) or striatal (E and F) sections of saline-treated rats (A and E) and rats undergoing withdrawal (B and F) stained with Iba-1 antibody. C and D = higher magnification of the area (indicated by a square) included in A and B, respectively. G and H = higher magnification of the area (indicated by a square) included in E and F, respectively. Bar in A = 100 μ m, bar in C = 20 μ m. I. Semiquantitative analysis of Iba-1 positive cells using ImageJ software in serial sections from the indicated brain areas. A total of 5 randomly

selected fields per section (5 sections per animal, 1 section every 80 μm) were used (N=4 rats per group). * $p<0.05$ versus saline.

Table 1

Morphine treatment paradigms

Paradigm	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Chronic Morphine	AM	10mg/kg	20mg/kg	20mg/kg	30mg/kg (sac 2 h)	**	**	**
	PM	10mg/kg	20mg/kg	30mg/kg	**	**	**	**
Morphine Maintenance	AM	10mg/kg	10mg/kg	10mg/kg	10mg/kg (sac 2 h)	**	**	**
	PM	10mg/kg	10mg/kg	10mg/kg	**	**	**	**
Morphine Withdrawal	AM	10mg/kg	20mg/kg	20mg/kg	30mg/kg	No treatment	No treatment	sac60 h
	PM	10mg/kg	20mg/kg	30mg/kg	No treatment	No treatment	No treatment	

Morphine injections were administered subcutaneously (s.c.) 12h apart over the course of 5 days. Animals were sacrificed (sac) 2h after the last injection for chronic morphine and morphine maintenance paradigms, and 60h after the last injection for morphine withdrawal. To block the effect of morphine throughout the time-course, naltrexone was administered 30 minutes prior to morphine injections.