Brain, Behavior, and Immunity 38 (2014) 38–52

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Src family kinases involved in CXCL12-induced loss of acute morphine analgesia ☆

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ARTICLE INFO

Article history:
Received 19 July 2013
Received in revised form 30 October 2013
Accepted 9 November 2013
Available online 18 November 2013

Keywords:
Opioid receptors
Chemokine
Analgesia
Src kinases
CXCR4
Spinal cord
Microglia

ABSTRACT

Functional interactions between the chemokine receptor CXCR4 and opioid receptors have been reported in the brain, leading to a decreased morphine analgesic activity. However the cellular mechanisms responsible for this loss of opioid analgesia are largely unknown. Here we examined whether Src family-kinases (SFK)-linked mechanisms induced by CXCR4 contributed to the loss of acute morphine analgesia and could represent a new physiological anti-opioid signaling pathway. In this way, we showed by immunohistochemistry and western blot that CXCL12 rapidly activated SFK phosphorylation *in vitro* in primary cultured lumbar rat dorsal root ganglia (DRG) but also *in vivo* in the DRG and the spinal cord. We showed that SFK activation occurred in a sub population of sensory neurons, in spinal microglia but also in spinal nerve terminals expressing mu-(MOR) and delta-opioid (DOR) receptor. In addition we described that CXCR4 is detected in MOR- and DOR-immunoreactive neurons in the DRG and spinal cord. *In vivo*, we demonstrated that an intrathecal administration of CXCL12 (1 μg) significantly attenuated the subcutaneous morphine (4 mg/kg) analgesia. Conversely, pretreatment with a potent CXCR4 antagonist (5 μg) significantly enhanced morphine analgesia. Similar effects were obtained after an intrathecal injection of a specific SFK inhibitor, PP2 (10 μg). Furthermore, PP2 abrogated CXCL12-induced decrease in morphine analgesia by suppressing SFK activation in the spinal cord. In conclusion, our data highlight that CXCL12-induced loss of acute morphine analgesia is linked to Src family kinases activation.

1. Introduction

Opioids remain the best treatment for managing acute, severe pain. However, their clinical efficacy is limited by the development of tolerance to their analgesic effects after chronic or acute administration (for review see Kissin et al., 1991; Larcher et al., 1998; Laulin et al., 2002; Ossipov et al., 2003; Williams et al., 2013). Different mechanisms have been implicated in the development of tolerance to opioid analgesic effect such as the activation of the glutamate N-methyl-D-aspartate (NMDA) receptor (Celerier et al., 1999), dynorphin (Vanderah et al., 2000) and the activation of the descending pain pathways (Vanderah et al., 2001) or morphological changes of opioid receptors (for review see Williams et al. (2013)). Several studies have identified the chemokines, a superfamily of small proteins with a crucial role in immune (Proudfoot, 2002) and neuromodulation functions (Rostene and Buckingham, 2007; Reaux-Le Goazigo et al., 2013), and their receptors as modulators of acute opioid analgesia (Szabo et al., 2002; Chen et al., 2007). *In vivo* studies report that administration of the chemokine CXC motif ligand 12 (CXCL12)/stromal cell-derived factor-1 (SDF-1) into the central gray matter *via* the activation of its cognate G protein-coupled receptor CXCR4 modulate the analgesia mediated by mu-opioid receptors (MOR) or delta-opioid receptors (DOR) (Szabo et al., 2002; Chen et al., 2007). Indeed, prior local treatment with the chemokine CXCL12 markedly reduces or completely blocks the antinociceptive (Szabo et al., 2002; Chen et al., 2007) or electrophysiological (Heinisch et al., 2011) effects of opioid receptor agonists such as morphine administered to the periaqueductal gray matter. However the intracellular mechanisms

Abbreviations: DOR, delta-opioid receptor; DRG, dorsal root ganglia; GPCR, G protein-coupled receptor; IR, immunoreactivity; i.t., intrathecal; MOR, mu-opioid receptor; PAG, periaqueductal gray matter; PBS, phosphate-buffered saline; s.c., subcutaneously; SFK, Src family kinases.

☆ Please see Brief Commentary by N. Eijkelkamp found on page 36 of this issue.

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http://dx.doi.org/10.1016/j.bbi.2013.11.010

triggered by the chemokine CXCL12 *via* CXCR4 that lead to opioid receptor desensitization characterized by a loss of analgesic effects of morphine have not been investigated.

In pain-sensing systems, the chemokine CXCL12 and its receptor CXCR4 are produced by sensory neurons and glial cells in dorsal root ganglia (DRG) and the dorsal horn spinal cord (Bhangoo et al., 2007; Luo et al., 2008; Dubovy et al., 2010; Knerlich-Lukoschus et al., 2011; Tysseling et al., 2011; Reaux-Le Goazigo et al., 2012). We recently demonstrated, in a study based on light and electron microscopy, the detection of CXCL12 immunoreactivity (IR) and CXCR4-IR in CGRP-containing sensory neurons, with both these molecules conveyed to central sensory nerve terminals in dorsal spinal cord (Reaux-Le Goazigo et al., 2012). In addition, the colocalization of MOR and CXCR4 on individual neurons in the periaqueducal gray matter (PAG) has recently been reported (Heinisch et al., 2011). We also showed that a single intrathecal (i.t.) CXCL12 injection in naive rats caused allodynia-like behavior that was maintained over three days (Reaux-Le Goazigo et al., 2012). Prior treatment with a CXCR4-neutralizing antibody significantly decreased this CXCL12-induced mechanical hypersensitivity for 24 h, implicating CXCR4 receptors in the effects of CXCL12.

The Src family kinases (SFKs) have recently been reported to modulate the activity, desensitization and internalization of G protein-coupled receptors (Luttrell and Luttrell, 2004; Gavi et al., 2006; Walwyn et al., 2007; Liu et al., 2008; Hong et al., 2009; Zhang et al., 2009). Recent data have indicated that activation of SFKs influence MOR and DOR trafficking and signaling *in vitro* (Kramer et al., 2000; Walwyn et al., 2007; Archer-Lahlou et al., 2009; Hong et al., 2009). The tyrosine phosphorylation of MOR at Tyr166 and Tyr336 were found to be dependent on the activation of Src. Such phosphorylations controlled the switch from inhibition to stimulation of adenylyl cyclase after prolonged morphine application in HEK cells and reduced agonist-induced G-protein activation in HEK cells expressing MOR-GFP (Zhang et al., 2009; Clayton et al., 2010). Activation of pathway involving Src kinase activation by CXCL12 has only been reported in mammalian cancer cells (Uchida et al., 2003; Cabioglu et al., 2005; Billadeau et al., 2006). In this context, we tested the original hypothesis that activation of CXCL12/CXCR4 axis blocked opioid receptor function by the activation of SFK downstream mechanisms in the spinal cord.

2. Methods

2.1. Animals

Adult male Sprague–Dawley rats (300 g; Janvier Labs, Le Genest Saint Isle) were maintained under controlled conditions (22 ± 1 °C, 60 ± 10% relative humidity, 12/12 h light/dark cycle, food and water *ad libitum*). All procedures were in strict in accordance with the guidelines for the care and use of experimental animals approved by the French National Institute of Medical Research (INSERM) and national and international law and with policies for the use of animals in neuroscience research (European Communities Council Directive 86/609/EEC, Authorization No. 75-1235 Granted to A. R-LG).

2.2. Drugs

Recombinant murine SDF-1α/CXCL12 peptide was purchased from PeproTech (Rocky Hill, NJ). Morphine chlorhydrate was obtained from Francopia (Gentilly, France). The selective inhibitor of the Src family of protein tyrosine kinases (PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) and a potent CXCR4 antagonist (1,3-Dicyclohexyl-2-(3-methyl-6,6-dimethyl-5,6-dihydroimidazo[1,2-b]thiazole)-2-thiopseudourea; IT1t dihydrochloride) were purchased from Merck Millipore. This antagonist acts as a selective and highly potent CXCR4 antagonist (IC_{50} = 1.1 nM in calcium mobilization assays) and effectively targets CXCR4-mediated binding of CXCL12 (IC_{50} = 11 nM in competitive binding assays using rat IR983F membrane). Selectivity of this small molecule IT1t for CXCR4 was further indicated by showing its interaction with the residue Asp97 (Wu et al., 2010), this residue being not conserved in CXCR7 (Yoshikawa et al., 2013).

2.3. Preparation of primary cultured rat DRG neurons for immunohistochemistry and Western blot

DRG neurons from adult rats were acutely isolated as described previously (Belkouch et al., 2011; Reaux-Le Goazigo et al., 2012). Bilateral lumbar DRGs were dissected out and enzymatically digested with collagenase A for 90 min and then in *Trypsin*/EDTA-*DNase* I solution for 15 min at 37 °C. (Sigma–Aldrich). Subsequently, DRG neurons were mechanically dissociated by repeated trituration using a fine polished Pasteur pipette in Neurobasal A medium (Gibco). Isolated neurons were centrifuged and the pellets were resuspended in Neurobasal A supplemented with 5% horse serum (Gibco), 2% B-27 supplement (Gibco), L-glutamine (0.1 mg/ml, Sigma–Aldrich), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were then filtered using a 70 μm filter and then cultured in 24- or 6 well clusters treated with poly-Dlysine/laminin-coated glass coverslips, and incubated at 37 °C in a humidified 95% air/5% CO2 atmosphere. DRG were allowed to differentiate and grow processes for 4 days. Then, cells were incubated with CXCL12 (225 ng in 250 μl corresponding to a concentration of 100 nM) for 30 min, alone or in presence of CXCR4 antagonist (100 ng in 250 μl corresponding to a concentration of 1 μM) incubated 30 min before CXCL12. DRG cultures were rinsed with 0.1 M phosphate buffer saline (PBS), fixed 10 min with 4% paraformaldehyde for immunohistochemistry or homogenized on ice in 100 μl radioimmunoprecipitation assay buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS] supplemented with proteases and phosphatase inhibitor cocktails (Sigma–Aldrich) for Western blot analysis. The DRG neurons were continuously exposed to culture media as a control.

2.4. Effect of the intrathecal injections of CXCL12 on Src family kinases activation in lumbar DRG and dorsal spinal cord

Rats were placed under isoflurane anesthesia and CXCL12 (1 μg), or vehicle (saline) were injected into the rats, via the intrathecal route (manual lumbar puncture over one minute), between the L5 and L6 vertebrae, in a total volume of 25 μl. Tissues were then collected 30 min later for protein phosphorylation analysis by immunohistochemistry and Western blot.

2.5. Immunohistochemical studies

2.5.1. Tissue preparation

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused with 100 ml of saline, followed by 4% paraformaldehyde in PBS, pH 7.4. Tissues were kept in immersion overnight in 4% paraformaldehyde. Sections (40 μm) of spinal cord were cut on a vibratome (Leica Microsystems, Germany). DRG were cryoprotected with solution of 30% (w/v) sucrose in PBS and frozen in isopentane. Cryostat sections (12 μm) were taken and mounted on Superfrost slides.

2.5.2. Immunofluorescence labeling

Single and double immunofluorescence staining were carried out for CXCR4 and p-SFK, with DOR and MOR. Sections were incubated in PBS containing 0.1% Triton X100 and 3% normal goat