First in Vivo Evidence for a Functional Interaction between Chemokine and Cannabinoid Systems in the Brain

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ABSTRACT

Growing evidence supports the idea that in addition to their well established role in the immune system, chemokines might play a role in both normal and pathological brain function, and the chemokine network could interact with other neuromodulators. The chemokine stromal cell-derived growth factor (SDF)-1α/CXCL12, a member of the CXC chemokine family, was tested for its possible effect on the analgesic responses of the cannabinoid receptor agonist aminoalkylindole 4,5-dihydro-2-methyl-4-(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6H-pyrrolo[3,2,1ij]quinolin-6-one (WIN55,212-2, hereafter WIN 55,212-2) at the level of the periaqueductal gray (PAG), a brain region critical to the processing of pain signals, and others, which show that the activation of SDF-1α/CXCL12 receptors in the brain, showing that the activation of SDF-1α/CXCL12 receptors (CXCR4) in the PAG interferes with the analgesic effects of WIN 55212-2.

Pain is one of the most widespread and intractable of human complaints, as well as being one of the most difficult syndromes to treat successfully with drugs and surgery. The pathogenesis of pain states is immensely complex, involving structural, physiological, and pharmacological changes throughout the neuroaxis. Multiple pharmacological agents have been used to treat diverse pathological pain states, including opiates, nonsteroidal, anti-inflammatory drugs, anticonvulsants, antidepressants, and others (Guindon et al., 2007).

In recent years, the analgesic effects of cannabinoids and cannabinoid receptor stimulation has been reported (Martin et al., 1996; Welch et al., 1998; Scott et al., 2004). The development of synthetic cannabinoid agonists has provided remarkable advances in cannabis research. One such ligand is the aminoalkylindole (+)-WIN 55,212-2 (hereafter WIN 55,212-2) that binds to CB1 and CB2 cannabinoid receptors (Felder et al., 1995). Previous studies have demonstrated that WIN 55,212-2 is highly potent and that it is pharmacologically active in vivo. WIN 55212-2 prevents intravenous cocaine self-administration, and it induces hypoesthesia in rats (Compton et al., 1992; Martin and Lichtman, 1998; Rawls et al., 2002). We have recently shown that cannabinoids can also act as antipyretic agents (Benamar et al., 2007). With respect to antinociception, WIN 55,212-2 has been demonstrated to produce antinociception in the paw withdrawal, radiant heat tail-flick, and formalin tests (Martin et al., 1996; Tsou et al., 1996; Meng et al., 1998).

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ABBREVIATIONS: (+)-WIN 55,212-2, 4,5-dihydro-2-methyl-4-(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6H-pyrrolo[3,2,1ij]quinolin-6-one (WIN55,212-2); CB, cannabinoid; SDF, stromal cell-derived growth factor; CXCR, CXC chemokine receptor; PAG, periaqueductal gray; SR 141716A, (N-piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride SR 1417222; 1–10 μg given into the PAG blocked the WIN 55,212-2-induced antinociception. In contrast, the selective CB2 antagonist N-[15S]-endo-1,3,3-trimethyl bicyclo heptan-2-yl]-5-(4-chloro-3-methyl-phenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528; 10 μg) did not alter the WIN 55,212-2-induced antinociception. Pretreatment with SDF-1α/CXCL12 (100 ng) caused a reduction in antinociceptive responses of WIN 55,212-2. The inhibitory effect of SDF-1α/CXCL12 on WIN 55,212-2-induced antinociception was reversed by 1,1'-[1,4-phenylenebis(methylene)]bis [1,4,8,11-tetraazacyclotetradecane] octahydrobromide dihydrate (AMD 3100) (10–50 ng), an antagonist of the SDF-1α/CXCL12, acting at its receptor, CXCR4. This study reports the first in vivo evidence of a functional interaction between chemokine and cannabinoid systems in the brain, showing that the activation of SDF-1α/CXCL12 receptors (CXCR4) in the PAG interferes with the analgesic effects of WIN 55212-2.
Chemokines are a family of small (8–12 kDa) proteins involved in cellular migration and intercellular communication. One of the chemokines thought to have important roles in the brain is SDF-1α/CXCL12. SDF-1α/CXCL12 binds mainly to only one receptor, CXCR4, for which it is the sole ligand (Bleul et al., 1996). Deletion of either the SDF-1α/CXCL12 or CXCR4 gene results in abnormal cerebellar and hippocampal development, suggesting a role of this chemokinergic system in neurogenesis (Zou et al., 1998; Lu et al., 2002). Besides the regulation of homeostatic processes, increasing evidence implicates the SDF-1α/CXCL12 signaling system in the pathogenesis of tumors, infections, and inflammatory processes in several diseases. CXCR4 is up-regulated in human immunodeficiency virus and simian immunodeficiency virus encephalitis, experimental allergic encephalitis, and brain tumors, where its expression is increased in astrocytes, infiltrating leukocytes, and/or endothelial cells on neovessels (Jiang et al., 1998; Sanders et al., 1998; Vallat et al., 1998; Westmoreland et al., 1998; McManus et al., 2000; Rempel et al., 2000). In vivo studies have shown a direct role of SDF-1α/CXCL12 in nociception. When injected intradermally into the rat paw, this chemokine induces pain (Zhang et al., 2004).

Chemokines play a role in both normal (Bajetto et al., 2001) and pathological brain function (Hesselgesser and Horuk, 1999; Mennicken et al., 1999). Recent in vivo studies have revealed that the chemokine system also could interact with other neuromodulatory systems in the brain. For example, our laboratory has demonstrated that chemokines in the PAG interact with the opioid system (Szabo and Rogers, 2001; Chen et al., 2007). The present study attempted to determine whether SDF-1α/CXCL12 in brain interacts with the cannabinoid system, by investigating the ability of this chemokine to alter the analgesic function of the cannabinoid agonist WIN 55212-2 in the PAG.

**Materials and Methods**

**Animals**

All animal use procedures were conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and they were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Zivic-Miller), weighing 250 to 300 g, were used in this study. They were housed three per cage for at least 1 week before surgery, and they were fed laboratory chow and water ad libitum. Ambient temperature was 21 ± 0.3°C and a 12-h light/12-h dark cycle was used.

**Surgical Procedures**

Rats were anesthetized with an i.p. injection of a mixture of ketamine hydrochloride (80 mg/kg) and acepromazine maleate (0.2 mg/kg). A sterilized stainless steel C313G cannula guide (22-gauge; Plastics One Inc., Roanoke, VA) was implanted in the PAG according to standard procedures (Benamar et al., 2002, 2004). The stereotaxic coordinates were as follows: 7.8 mm posterior to bregma, 0.5 mm from the midline, and 3.5 mm ventral to the dura mater (Paxinos and Watson, 1998). A C313DC cannula dummy (Plastics One Inc.) of identical length was inserted into the guide tube to prevent its occlusion. The animals were returned to individual cages in the environmental room.

**Microinjection**

After a 7-day recovery period, rats were allowed to habituate to test chambers for 1 h before testing. Either vehicle or drug was microinjected into the PAG in a volume of 1 μl via a C313I internal cannula (28-gauge; Plastics One Inc.). The C313I internal cannula was connected by polyethylene tubing to a 10-μl Hamilton syringe. A volume of 1 μl of drug or vehicle was delivered at a rate of 1 μl/min (manually), and the internal cannula was left in place an additional 90 s to allow diffusion. Immediately thereafter, a cannula dummy (C313DC) was inserted into the cannula guide to prevent any contamination.

**Drugs**

The cannabinoid agonist WIN 55212-2 and its inactive enantiomer WIN 55212-3, SR 141716, and SR144528 were obtained from Sigma-Aldrich (St. Louis, MO). These drugs were dissolved in cremophor, dimethyl sulfoxide, and saline (1:1:18). AMD 3100 was obtained from Sigma-Aldrich, and it was dissolved in pyrogen-free saline. Reombinant rat SDF-1α/CXCL12 was purchased from R&D Systems (Minneapolis, MN), and it was reconstituted in sterilized artificial fluid (CMA/Microdialysis, Stockholm, Sweden).

**Statistical and Histological Analysis**

All results were expressed as mean ± S.E.M. Statistical analysis of differences between groups was determined by analysis of variance followed by Dunnett’s post test. A value of P less than 0.05 was considered statistically significant. At the conclusion of the experiments, each rat received an injection of 1 μl of cresyl violet, and then it was anesthetized and perfused transcardially with 0.9% isotonic saline, followed by phosphate-buffered saline, 4% paraformaldehyde, pH 7.4. The brain was removed, stored in the same fixative for 4 h, kept in 20% sucrose overnight, and cut into 20-μm sections on a freezing microtome. Each coronal section was mounted according to standard histological procedures (Benamar et al., 2004), and the site of injection was verified by locating the dye and then mapping on anatomical reconstructions in the coronal planes. Only data from animals in which the site of injection was clearly located within the PAG were included in the studies. We used 156 rats to conduct the experiments, and only eight rats were eliminated because the site of microinjection was located outside the PAG.

**Experimental Protocol**

**Cold-Water Tail-Flick Test.** The latency to flick the tail in cold water was used as the antinociceptive index, according to a standard procedure in our laboratory (Benamar et al., 2002). A 1:1 mix of ethylene glycol/water was maintained at −3°C with a circulating water bath (model 9500; Fisher Scientific, Pittsburgh, PA). The nociceptive threshold was taken as the latency until the rat removed or flicked its tail. For each animal, the first reading was discarded to minimize variability, and the remaining two readings were averaged to determine the baseline latency. The baseline of CWT latency before drug injection was 12 ± 2 s. Rats whose baseline values fell within a range of 10 to 20 s were used in the experiments. A cut-off limit of 60 s was set to avoid damage to the tail. Latencies to tail-flick after injection were expressed as percentage change from baseline. The percentage maximal possible antinociception (٪MPA) for each animal at each time was calculated using the formula ٪MPA = [(test latency – baseline latency)/(60 – baseline latency)] × 100.

**Effect of Intra-PAG Injection of WIN 55212-2.** After a 60-min baseline interval, WIN 55,212-2 (100–400 ng), WIN 55,212-3 (400 ng), or vehicle was injected into the PAG at time 0, and nociception was measured for 60 min using the CWT. To assess whether the WIN 55,212-2-induced antinociception was via CB1 or CB2 receptors, either SR 141716A or SR144528, respectively, was administered directly into the PAG before WIN 55,212-2. After a 60-min baseline interval, SR 141716A (1–10 μg) or SR144528 (10 μg) was injected. WIN 55,212-2 was injected into the PAG 30 min later. The nociception was measured for 60 min.

**Effect of SDF-1α/CXCL12 on WIN 55,212-2-Induced Antinociception.** After a 60-min baseline interval, SDF-1α/CXCL12 (25–100 ng), or vehicle was injected into the PAG. Thirty minutes later, WIN 55,212-2 was injected into PAG, and nociception was measured...
for 60 min. In separate experiments, it was determined whether intra-PAG CXCR4 mediates the inhibitory effect of SDF-1α/CXCL12 on WIN 55,212-2-induced antinociception. After a 60-min baseline interval, AMD 3100 (10–100 ng) was injected into the PAG. Thirty minutes later, SDF-1α/CXCL12 was injected into the PAG followed 30 min later by WIN 55,212-2. The nociception was measured for 60 min.

**Results**

**Intra-PAG Injection of WIN 55,212-2 Produced Antinociception in a Dose-Dependent Manner.** The present experiments were carried out with the aim of demonstrating that WIN 55,212-2 microinjected directly into the PAG is able to evoke an antinociceptive response in the CWT test. As expected, the administration of WIN 55,212-2 into the PAG induced antinociception in a dose-dependent manner. Doses of 100 to 400 ng/μl produced significant antinociceptive effects relative to vehicle. The onset was rapid, with an effect observed 15 min after injection ($F_{4,21} = 2.84; P < 0.05$) (Fig. 1). The dose of 100 ng produced a marked antinociception in the CWT, reaching a peak level (35.5 ± 6.9% MPA) at 30 min. With a dose of 200 ng, the peak was 60 ± 17.9% MPA, at 30 min after injection. The maximum antinociceptive effect was reached at a dose of 400 ng, and the peak was 100% MPA at 30 min after injection. To show that this effect is not due to a nonspecific interaction with hydrophobic regions of functional proteins or their lipid surroundings in the cell membrane and that the cannabinoid receptor has stereoselectivity, we tested WIN 55,212-3, an inactive enantiomer, on pain threshold. WIN 55,212-3 (400 ng) had no antinociceptive or hyperalgesic effect compared with vehicle ($P > 0.05$) (Fig. 1).

**CB1 Receptors Mediate the Antinociception Induced by the Intra-PAG Injection of WIN 55,212-2.** To determine whether CB1 receptors in the PAG mediated WIN 55,212-2-induced antinociception, SR 141716A (1–10 μg) was injected into the PAG 30 min before the intra-PAG injection of WIN 55,212-2. SR 141716A dose-dependently attenuated the antinociceptive effect induced by WIN 55,212-2 ($F_{3,25} = 3.07; P < 0.05$) (Fig. 2). In contrast to SR 141716A, SR144528 (10 μg) did not alter WIN 55,212-2-evoked antinociception ($F_{2,17} = 3.59; P > 0.05$) (Fig. 3). Neither SR 141716A nor SR144528 by itself had an effect on the CWT.

**Effect of Intra-PAG Injection of SDF-1α/CXCL12 (100 ng) on the Antinociceptive Effect Induced by WIN 55,212-2.** In agreement with previous studies (Szabo et al., 2002; Chen et al., 2007), SDF-1α/CXCL12 at doses ranging from 10 to 100 ng failed to exhibit any evidence of antinociception or hyperalgesia activity in the CWT test compared with the control group. However, a higher dose of SDF-1α/CXCL12 (>100 ng) showed a hyperalgesic effect (data not shown). Accordingly, we used the nonhyperalgesic dose of SDF-1α (25–100 ng) to allow a clear analysis of the effect of SDF-1α/CXCL12 on WIN 55,212-2-induced antinociception. Rats receiving an initial injection of artificial cerebrospinal fluid followed by WIN 55,212-2 manifested a significant increase in tail-flick latency (antinociception). However, pretreatment with SDF-1α/CXCL12 (100 ng) 30 min before WIN 55,212-2 administration caused a reduction in antinociceptive response from 15 to 45 min ($F_{3,17} = 3.20; P < 0.05$) (Fig. 4). In an attempt to examine whether the SDF-1α/CXCL12 effect was mediated through its receptor, we administered the SDF-1α/CXCL12 antagonist AMD 3100 into the PAG (10–100 ng/μl). The results showed that when the AMD 3100, at a dose of 50 or 100 ng, is given 30 min before the injection of SDF-1α/CXCL12 at 100 ng/μl, the attenuation of the WIN 55,212-2-induced antinociception by the chemokine is prevented ($F_{3,25} = 2.99; P < 0.05$) (Fig. 5). However, the lower dose, 10 ng did not alter the effects of SDF-1α on WIN 55,212-2-induced antinociception ($P > 0.05$) (Fig. 5). The intra-PAG injection of AMD 3100 by itself has no effect on nociceptive threshold (data not shown).

**Discussion**

In the present study, we provide the first in vivo evidence of a functional interaction between chemokine and cannabinoid systems in the brain, specifically the PAG. In the first

![Fig. 1. Effects of intra-PAG injection of WIN 55,212-2. WIN 55,212-2 or WIN 55,212-3 was given at time 0. Data are expressed as %MPA. N, number of rats. * P < 0.05 compared with vehicle.](image1.png)

![Fig. 2. Effect of SR 141716A (1–10 μg, –30 min) on WIN 55,212-2-induced antinociception. WIN 55,212-2 was injected at time 0. Data are expressed as %MPA. N, number of rats.](image2.png)
the nucleus reticularis gigantocellularis pars alpha or into the rostral ventromedial medulla blocked the antinociceptive effect of WIN 55,212-2 (Meng et al., 1998; Monhemius and Simpson, 2001). SR144528 did not affect WIN 55,212-2-induced antinociception, indicating that the antinociception is insensitive to CB2 receptor activation in the PAG.

In the second series of experiments, we determined whether SDF-1α/CXCL12 interacts with the cannabinoid system in the PAG, by investigating the ability of this chemokine to alter the analgesic function of WIN 55212-2. The pretreatment with SDF-1α/CXCL12 (100 ng) significantly attenuated the antinociceptive effect of the WIN 55212-2, suggesting that the chemokine SDF-1α/CXCL12 is able to interfere with the control of analgesia at the level of the PAG. PAG injection of SDF-1α/CXCL12, at doses from 10 to 100 ng, was ineffective on its own, and it did not result in either antinociception or hyperalgesia in the CWT test.

In an attempt to examine whether the SDF-1α/CXCL12 effect was mediated through its receptor, the CXCR4 antagonist, AMD 3100 was administered directly into the PAG. The results showed that when AMD 3100 is given 30 min before the injection of SDF-1α/CXCL12, the blockade of WIN 55212-2-induced antinociception by the chemokine is prevented, indicating that the inhibitory effect of SDF-1α/CXCL12 is mediated by CXCR4 in the PAG, and the activation of CXCR4 in the PAG results in a desensitization of the analgesic function of cannabinoid receptors and enhanced perception of pain.

Immunohistochemistry studies have shown that the SDF-1α/CXCL12 protein is constitutively and regionally expressed in specific neuronal populations throughout adult rat brain (Banisadr et al., 2003), particularly in the mesencephalon (such as the PAG). An accumulating body of evidence suggests that CB1 cannabinoid receptors are located in the PAG (Cristino et al., 2006). Because chemokines and cannabinoids both are found in the PAG and their receptors are members of the G protein-coupled receptor family, one possible explanation of the attenuation effect of WIN 55212-2 by

**Fig. 3.** Effect of SR144528 (10 µg, −30 min) on WIN 55212-2-induced antinociception. WIN 55212-2 was injected at time 0. Data are expressed as %MPA. N, number of rats.

- aCSF (PAG) + WIN 55212-2 (400 ng, PAG) N=8
- AMD 3100 (10 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng, PAG) N=8
- AMD 3100 (50 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng, PAG) N=8
- AMD 3100 (100 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng, PAG) N=8
- AMD 3100 (100 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng, PAG) N=8
- AMD 3100 (100 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng, PAG) N=8

series of experiments, we investigated whether WIN 55212-2 microinjected into the PAG was able to induce an antinociceptive response in the CWT. As expected, the administration of WIN 55212-2 induced antinociception in a dose-dependent manner. This effect is not due to a nonspecific interaction with hydrophobic regions of functional proteins or their lipid surroundings in the cell membrane, because WIN 55212-3, an enantiomer of WIN 55212-2, did not affect the pain threshold, indicating that the effect of WIN 55212-2 is stereoselective.

SR 141716A prevented the WIN 55212-2-induced antinociception, indicating that the activation of CB1 receptors in the PAG mediates cannabinoid-induced antinociception. The antagonism by SR 141716A of cannabinoid-induced antinociception in the present study is consistent with previous reports. For example, the administration of SR 141716A into

**Fig. 4.** Effect of PAG pretreatment with SDF-1α/CXCL12 (25–100 ng, −30 min) on WIN 55212-2-induced antinociception. WIN 55212-2 was injected at time 0. Data are expressed as %MPA.

- Saline + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng) N=8
- AMD 3100 (10 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng) N=8
- AMD 3100 (50 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng) N=8
- AMD 3100 (100 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng) N=8
- AMD 3100 (100 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng) N=8
- AMD 3100 (100 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng) N=8
- AMD 3100 (100 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng) N=8
- AMD 3100 (100 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng) N=8
- AMD 3100 (100 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng) N=8
- AMD 3100 (100 ng) + SDF-1alpha (100 ng) + WIN 55212-2 (400 ng) N=8

**Fig. 5.** Antagonism of SDF-1α/CXCL12 effect on WIN 55212-2-induced antinociception by AMD 3100. Rats were treated intra-PAG with SDF-1α/CXCL12 30 min before WIN 55212-2. AMD 3100 was administered to rats at a dose of 100 ng 1 h before WIN 55212-2 (at 0 min). Data are expressed as %MPA. * P < 0.05 compared with SDF-1α/WIN 55212-2 group.
SDF-1α/CXCL12 in the brain is a heterologous desensitization that may occur at the G protein-coupled receptor level. Indeed, such an effect has been found in vitro and in vivo between SDF-1α/CXCL12 and opioid receptors in the PAG using the CWT test (Szabo et al., 2002; Chen et al., 2007).

In summary, our findings show that analgesic activity of the cannabinoid agonist WIN 55,212-2 in the brain can be overcome in situations in which there are elevated levels of chemokines, which frequently occurs during neuroinflammatory conditions. A number of recent studies have shown that, in addition to neurotransmitter and neuropeptide systems, the chemokine system in the brain also plays a role in normal brain function and brain diseases (Shen et al., 2000; Szabo et al., 2002; Adler et al., 2005). This topic is of growing significance, and it is critical that experiments be conducted to add to the body of information to establish this role in the brain. Our present findings support this idea, and they indicate that SDF-1 plays a role when the brain homeostasis is perturbed (in this case, under the influence of cannabinoids). In addition, the in vivo finding that the activation of CXCR4 depressed the analgesic function of chemokines and enhanced perception of pain has great implications. In situations where there is an elevation of chemokine levels in the brain (including most neuroinflammatory diseases, such as multiple sclerosis and human immunodeficiency virus encephalitis), a resulting loss of cannabinoid receptor function could lead to greater sensitivity to painful stimuli.

References


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