FEBS Letters 580 (2006) 4306–4310

Fractalkine and minocycline alter neuronal activity in the spinal cord dorsal horn

Samuel A. Owolabi, Carl Y. Saab*

Department of Surgery, Rhode Island Hospital, Brown University School of Medicine, Providence, RI 02903, USA

Received 20 April 2006; revised 27 June 2006; accepted 28 June 2006

Available online 7 July 2006

Edited by Ned Mantei

Abstract Fractalkine (FKN) evokes nociceptive behavior in naïve rats, whereas minocycline attenuates pain acutely after neuronal injury. We show that, in naïve rats, FKN causes hyperresponsiveness of lumbar wide dynamic range neurons to brush, pressure and pinch applied to the hindpaw. One day after spinal nerve ligation (SNL), minocycline attenuates after-discharge and responses to brush and pressure. In contrast, minocycline does not alter evoked neuronal responses 10 days after SNL or sciatic constriction, but increases spontaneous discharge. We speculate that microglia rapidly alter sensory neuronal activity in naïve and neuropathic rats acutely, but not chronically, after injury.

Keywords: Pain; Neuropathic; Microglia; Neuron; Fractalkine; Minocycline

1. Introduction

Evidence has recently emerged demonstrating the contribution of glia (mainly astrocytes and microglia) to neuropathic pain [7,22,30,32–34] and attenuation of opioid analgesia [6,17]. Nevertheless, the essential link tying microglial function to neuronal processing of nociceptive information is missing.

Fractalkine (FKN) and minocycline are known modulators of microglial function. Fractalkine is the only CX3C ligand-1 chemokine belonging to the CX3C family [28]. Intrathecal administration of FKN evokes dose-dependent mechanical allodynia and thermal hyperalgesia in naïve rats [24], whereas FKN-induced pain behavior is reversed by minocycline, an inhibitor of microglia [25]. According to many in vivo and in vitro studies, minocycline inhibits microglial activation under various pathological conditions without affecting neurons, astroglia, or oligodendroglial progenitors [26,27]. In the spinal cord, FKN receptor (CX3CR1) expression is up-regulated after chronic constriction injury (CCI) of the sciatic nerve concomitantly with microglial activation [31]. Intrathecal administration of a neutralizing antibody against CX3CR1 delays or reduces neuropathic behavior [24]. In contrast, intrathecal minocycline attenuates neuropathic behavior one day, but not one week, following neuropathic injury [20].

Accordingly, we hypothesized that FKN induces neuronal hyperresponsiveness to natural stimuli in naïve rats, whereas minocycline reverses these aberrant changes in rats acutely, but not chronically, after peripheral neuropathic injury.

2. Materials and methods

Adult male Sprague-Dawley rats (200–250 g) were used. For spinal nerve ligation (SNL), rats were anesthetized with isoflurane (1.5%) and lumbar 5–6 spinal nerves tightly ligated with 6–0 silk thread [19]. A slightly modified CCI was induced by placing three (instead of four) loose chromic gut (4–0) ligatures around the sciatic nerve [1]. Mechanical and thermal nociceptive thresholds were determined as previously described [2,8,9].

Rats were anesthetized with sodium pentobarbital (i.e., 60 mg/kg) for extracellular single unit recording using Spike2. Neuronal units were isolated based on their responses to natural stimuli within receptive fields strictly mapped on the ipsitaleral hindpaw (only units at depth <250 μm corresponding to laminae I–II were studied). Five mechanical stimuli were applied in the following order: Brush, by a cotton applicator to the skin; 3 von Frey filaments of non-nociceptive (0.6 g) and nociceptive (8 and 15 g) strengths, respectively; pressure or pinch by attaching arterial clip (144 or 583 g/mm^2). Only multireceptive wide dynamic range (WDR) neurons were selected. Background discharge was recorded for 20 s, and stimuli were applied serially for 20 s. Receptive fields were clearly marked by coloring the dermatome to monitor changes in size that could be caused by drug or vehicle application. Activities of 2–3 U/rat were recorded from 3 to 4 rats/group (6–12 U/group).

A cotton pledget soaked with FKN solution (30 ng in 10 μl aCSF, R&D systems) or vehicle was placed on the spinal cord proximal to the site of electrode penetration. Activity was recorded from single units 10–20 min before or 20–50 min following FKN or vehicle application. Similarly, minocycline hydrochloride (Sigma) or vehicle was applied to the spinal cord (100 μg in 10 μl aCSF). Significance was computed using Student's *t*-test and Fisher's exact test.

3. Results

Repetitive natural stimulation and application of vehicle did not affect spontaneous or evoked neuronal activity. Behavioral testing of rats with peripheral nerve injury on the same day prior to recording confirmed decreases in mechanical and thermal thresholds associated with neuropathic pain (Fig. 1).

In naïve rats, neuronal activity increased after FKN application in all units. In particular, mean responses to brush, pressure and pinch were significantly ($P < 0.05$) increased from 6 ± 0.8, 7.4 ± 3.0 and 10.6 ± 2.1 to 16.4 ± 3.5, 15.5 ± 4.3 and 20 ± 3.4, respectively (Fig. 2A, $n = 11$ U/group, mean responses to other stimuli were not significantly different). In contrast, minocycline did not change neuronal activity in naïve rats (Fig. 2B, $n = 6$–11 U/group).

*Corresponding author. Fax: +1 401 444 8052.
E-mail address: carl_saab@brown.edu (C.Y. Saab).

0014-5793/$32.00
doi:10.1016/j.febslet.2006.06.087

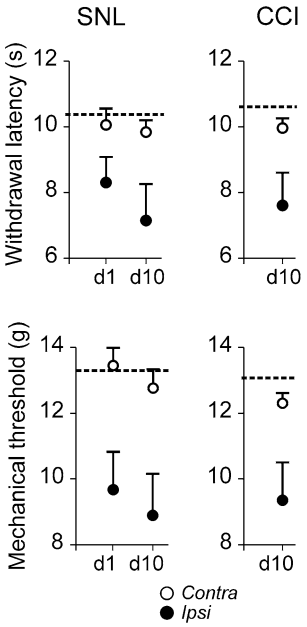

Fig. 1. Mechanical thresholds and thermal withdrawal latencies are shown for hindpaws ipsilateral and contralateral to injury by SNL or CCI (n = 5 rats/group, ±S.E.M, dotted line represents baseline values).

In rats one day after SNL, a decrease in neuronal activity was observed after minocycline application in all units recorded. Particularly, mean responses to brush and pressure stimuli were significantly ($P < 0.05$) decreased from 10.6 ± 3.1 and 9.4 ± 2.1 to 4.6 ± 1.4 and 5.1 ± 2.1, respectively (Fig. 3A, n = 9–10 U/group, mean responses to other stimuli were not significantly different).

In rats 10 days after SNL, no change in evoked responses was observed in any unit after minocycline application (n = 8–9 U/group, data not shown). However, an increase in spontaneous discharge emerged during periods of un-evoked activity from 2.4 ± 0.6 to 5.7 ± 2.1 (Fig. 3B, n = 8–9 U/group). To further corroborate these results, neuronal activity was similarly recorded in rats (n = 4) 10 days after CCI; in these rats, spontaneous discharge increased from 2.1 ± 1.3 to 4 ± 0.8 following minocycline (Fig. 3B, n = 9–10 U/group), whereas evoked responses were not changed.

Generally, neuronal modulation by FKN or minocycline reached maximal levels within 30 min (Fig. 4), with a tendency for reversal 50 min following minocycline in rats one day after SNL (Fig. 4A). In naïve rats, however, hyperresponsiveness remained elevated 50 min after FKN application (Fig. 4A and B).

After-discharge observed following noxious pinch stimuli (defined as continuous discharge after stimulus removal) was studied in all units (Fig. 5). In naïve rats, FKN increased the number of units exhibiting after-discharge from 12.5% to 72.7% (n = 8–11 U/group, $P < 0.05$). In rats one day after SNL, after-discharge was only observed in 11.1% of units after minocycline application compared to 50.0% (n = 9–10 U/group, $P < 0.05$); ten days after SNL, however, no significant change was observed after minocycline (75.0% compared to

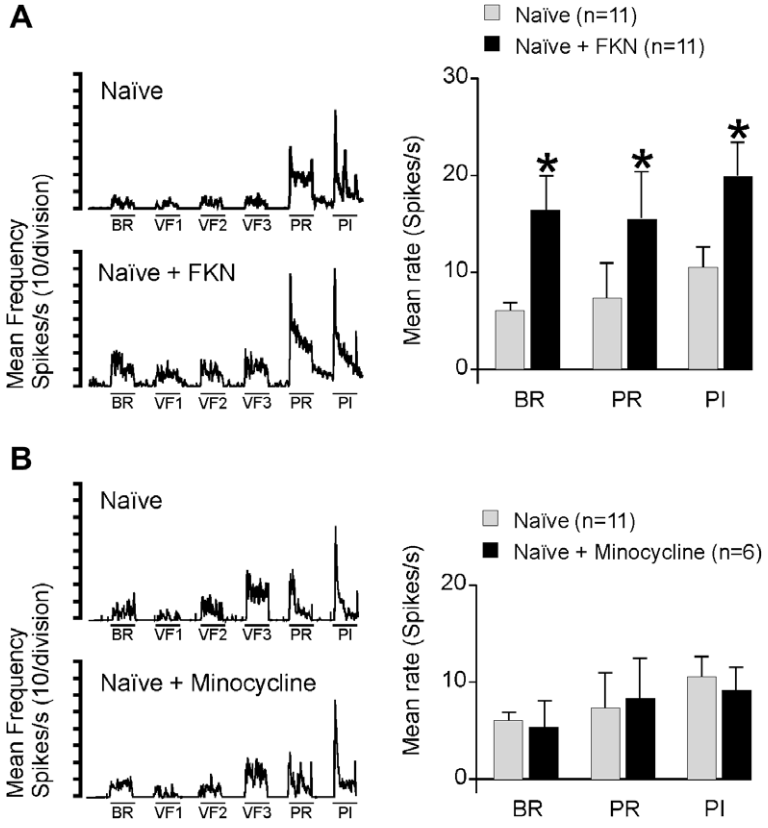

Fig. 2. Traces in left panels illustrate neuronal activity in naïve rats before and 20 min after FKN (A) or minocycline (B). Upper traces in A and B show baseline activity before drug application; lower traces show activity of the same unit respectively 20 min after drug application. Right panels in A show significant ($^{*}P < 0.05$) increases in the mean responses to brush (BR), pressure (PR) and pinch (PI) of units recorded before (naïve) or after FKN. No change was observed after minocycline (B).

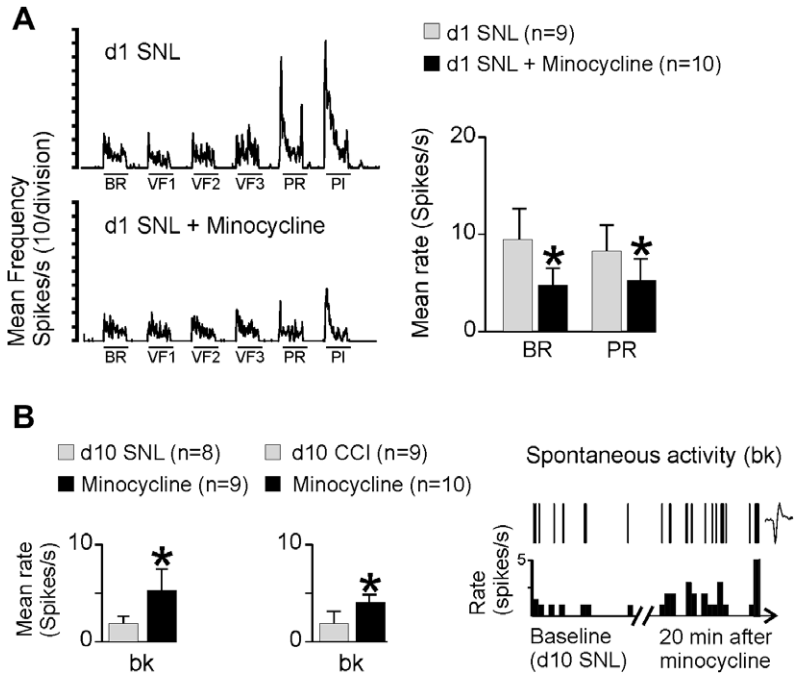

Fig. 3. Panels in A were constructed similarly to those in Fig. 2; note significant decreases ($^{*}P < 0.05$) in mean responses to brush and pressure stimuli after minocycline application in rats d1 after SNL. However, significant ($^{*}P < 0.05$) increase in spontaneous activity (bk, 20 s preceding BR stimulus) was evident when mean values were computed at d10 after SNL (B example from one unit is shown in right panel). Similar results were found for rats 10 days after CCI.

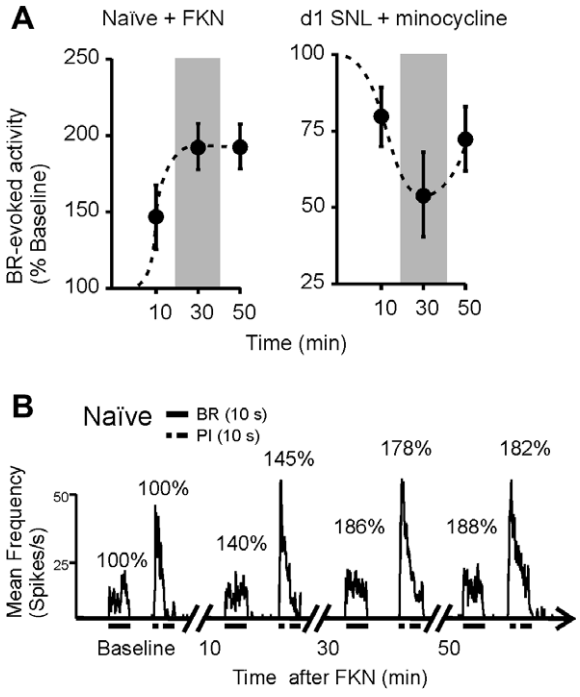

Fig. 4. Time course of neuronal modulation by FKN or minocycline was computed in 3 U/group as percent change in brush-evoked responses compared to baseline at 10, 30 and 50 min after drug application (A); example from one unit in B shows percent increases in brush and pinch-evoked responses after FKN.

55.5%, $n = 8$–9 U/group). In addition, receptive field sizes remained constant for each unit recorded before and after FKN or minocycline application.

4. Discussion

In this study, we tested our hypothesis that activity of WDR neurons in the superficial dorsal horn of the spinal cord is altered by FKN or minocycline known to influence sensory behavior and to activate or inhibit microglia, respectively. Fractalkine caused neuronal hyperresponsiveness to brush, pressure and noxious pinch in naïve rats, in addition to an increase in the number of cells with after-discharge typically associated with spontaneous pain behavior and 'wind-up' [23]. These results are in agreement with neuropathic behavior following intrathecal administration of FKN at a similar concentration [24]. In rats with peripheral nerve injury, reversal of aberrant neuronal responses with minocycline one day, but not ten days after SNL (except for an increase in spontaneous discharge), strongly supports behavioral data describing attenuation of neuropathic pain using minocycline at a similar concentration acutely, but not chronically following neuronal injury [13,20]. Furthermore, the increased spontaneous activity observed in this study following minocycline indicates a potential neuroprotective role for microglia chronically after injury, regardless of injury incongruity by SNL or CCI. Interestingly, nociceptive behavior in rodents is usually tested based on withdrawal reflexes evoked in response to natural stimuli, while spontaneous pain behavior is generally overlooked.

Although our results suggest that FKN may cause neuronal sensitization (hyperresponsiveness and burst firing, data not shown), it does not however enlarge the receptive field area, a phenomenon often associated with central sensitization. In addition, minocycline did not reduce receptive field sizes in rats with peripheral nerve injury, suggesting that FKN and minocycline do not fully contribute to sensitization of WDR neurons. Our findings are inconsistent, however, with the

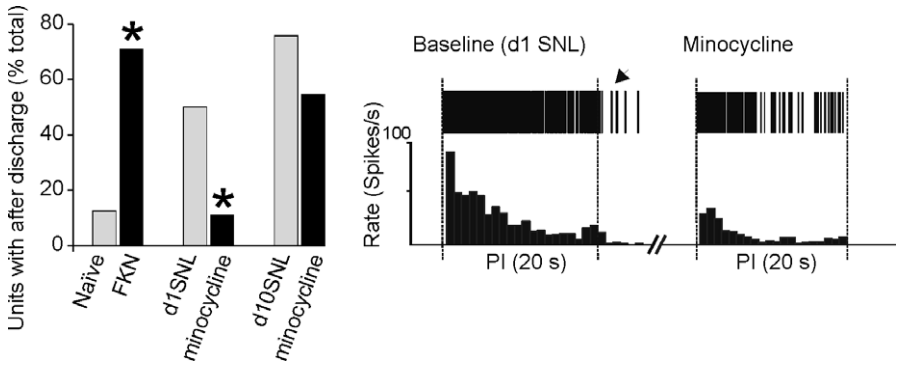

Fig. 5. Bar histograms show percent number of units with after-discharge in each group ($^{*}P < 0.05$). One example (right panel) shows action potentials (upper traces) in a rat d1 after SNL derived from corresponding rate histograms (lower traces) of the same unit with maintained discharge after stimulus removal (arrow, pinch applied within period indicated by dashed vertical lines); note absence of after-discharge activity 20 min following minocycline application.

attenuation of tactile allodynia ten days after SNL by spinal inhibition of p38-mitogen activated protein kinase (p38MAPK) expressed in microglia, consequently inhibiting microglia [16]. It should be noted that the anti-allodynic effects observed by Jin and colleagues [16] occurred 3 h following microglial inhibition, whereas our analysis of neuronal activity was mostly restricted to approximately 30 min following minocycline application. Alternatively, our experimental design requires the use of anesthetics, which have been reported to influence glial function [21]. For more conclusive data interpretation, recording neuronal activity in non-anesthetized animals is recommended for future studies using chronically implanted microelectrodes. Nonetheless, we speculate that microglia's contribution to pathological pain might differ between the acute or the chronic phase when astrocytes relay the pro-nociceptive drive [26].

Many types of peripheral nerve injuries trigger nociceptive behavior associated with microglial activation [5,10–12,14–16,29]. Although little is known about signaling between peripheral neurons and glia, Colburn et al. [3] speculated that microglial activation depended on dorsal root mediated signals (also refer to [4] identifying BDNF as a potent microglia-to-neuron signal). For example, FKN in the spinal cord is constitutively expressed by neurons and tethered to the membrane surface of primary afferents, whereas its receptor (CX3CR1) is predominantly expressed by microglia [31]. Following peripheral nerve injury, CX3CL1 is cleaved in the spinal cord and couple to CX3CR1, thus activating microglia [34,35]. Upon activation, microglia secrete proinflammatory mediators such as prostaglandins, proteases, cytokines (TNF-α, IL-1β, IL-6) and excitatory amino acids [18] whose receptors are expressed on dorsal horn neurons. In addition, microglia generate reactive oxygen species and nitrogen intermediates of adverse consequences regarding neuronal physiology.

In light of these observations, attenuation of neuronal sensitization by minocycline could be interpreted as resulting from a transient interruption in microglial release of proinflammatory mediators. We therefore conclude that the modulation of neuropathic behavior induced by FKN or minocycline may be explained in part by microglia-to-neuron signaling in the spinal cord dorsal horn.

Acknowledgement: Supported by funds from Rhode Island Foundation.

References

[1] Bennett, G.J. and Xie, Y.K. (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33, 87–107.
[2] Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M. and Yaksh, T.L. (1994) Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Meth. 53, 55–63.
[3] Colburn, R.W., Rickman, A.J. and DeLeo, J.A. (1999) The effect of site and type of nerve injury on spinal glial activation and neuropathic pain behavior. Exp. Neurol. 157, 289–304.
[4] Coull, J.A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., Gravel, C., Salter, M.W. and De Koninck, Y. (2005) BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 15, 1017–1021, 438(7070).
[5] Coyle, D.E. (1998) Partial peripheral nerve injury leads to activation of astroglia and microglia which parallels the development of allodynic behavior. Glia 23, 75–83.
[6] DeLeo, J.A., Tanga, F.Y. and Tawfik, V.L. (2005) Neuroimmune activation and neuroinflammation in chronic pain and opioid tolerance/hyperalgesia. Neuroscientist 10, 40–52.
[7] DeLeo, J.A. and Yezierski, R.P. (2001) The role of neuroinflammation and neuroimmune activation in persistent pain. Pain 90, 1–6.
[8] Dirig, D.M., Salami, A., Rathbun, M.L., Ozaki, G.T. and Yaksh, T.L. (1997) Characterization of variables defining hindpaw withdrawal latency evoked by radiant thermal stimuli. J. Neurosci. Meth. 76, 183–191.
[9] Dixon, W.J. (1980) Efficient analysis of experimental observations. Ann. Rev. Pharmacol. Toxicol. 20, 441–462.
[10] Fu, K.Y., Light, A.R. and Maixner, W. (2000) Relationship between nociceptor activity, peripheral edema, spinal microglial activation and long-term hyperalgesia induced by formalin. Neuroscience 101, 1127–1135.
[11] Garrison, C.J., Dougherty, P.M., Kajander, K.C. and Carlton, S.M. (1991) Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. Brain Res. 565, 1–7.
[12] Graeber, M.B. and Kreutzberg, G.W. (1988) Delayed astrocyte reaction following facial nerve axotomy. J. Neurocytol. 17, 209–220.
[13] Hains, B.C. and Waxman, S.G. (2006) Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. J. Neurosci. 26, 4308–4317.
[14] Hall, L.L., Borke, R.C. and Anders, J.J. (1989) Transection or electrical stimulation of the hypoglossal nerve increases glial fibrillary acidic protein immunoreactivity in the hypoglossal nucleus. Brain Res. 490, 157–161.
[15] Herzberg, U. and Sagen, J. (2001) Peripheral nerve exposure to HIV viral envelope protein gp120 induces neuropathic pain and spinal gliosis. J. Neuroimmunol. 116, 29–39.
[16] Jin, S.X., Zhuang, Z.Y., Woolf, C.J. and Ji, R.R. (2003) p38 mitogen-activated protein kinase is activated after a spinal nerve

ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. J. Neurosci. 23, 4017–4022.
[17] 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. and 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.
[18] Kettenman, H. and Ransom, B. (2005) Neurolgia, 2nd edn, Oxford University Press, Oxford.
[19] Kim, S.H. and Chung, J.M. (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50, 355–363.
[20] Ledeboer, A., Sloane, E.M., Milligan, E.D., Frank, M.G., Mahony, J.H., Maier, S.F. and Watkins, L.R. (2005) Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. Pain 115, 71–83.
[21] Lockwood, L.L., Silbert, L.H., Laudenslager, M.L., Watkins, L.R. and Maier, S.F. (1993) Anesthesia-induced modulation of in vivo antibody levels: a study of pentobarbital, chloral hydrate, methoxyflurane, halothane, and ketamine/xylazine. Anesth. Analg. 77, 769–774.
[22] McMahon, S.B., Cafferty, W.B. and Marchand, F. (2005) Immune and glial cell factors as pain mediators and modulators. Exp. Neurol. 192, 444–462.
[23] Melzack, R., Coderre, T.J., Katz, J. and Vaccarino, A.L. (2001) Central neuroplasticity and pathological pain. Ann. NY Acad. Sci. 933, 157–174.
[24] Milligan, E.D., Zapata, V., Chacur, M., Schoeniger, D., Biedenkapp, J., O'Connor, K.A., Verge, G.M., Chapman, G., Green, P., Foster, A.C., Naeve, G.S., Maier, S.F. and Watkins, L.R. (2004) Evidence that exogenous and endogenous fractalkine can induce spinal nociceptive facilitation in rats. Eur. J. Neurosci. 20, 2294–2302.
[25] Milligan, E., Zapata, V., Schoeniger, D., Chacur, M., Green, P., Poole, S., Martin, D., Maier, S.F. and Watkins, L.R. (2005) An initial investigation of spinal mechanisms underlying pain enhancement induced by fractalkine, a neuronally released chemokine. Eur. J. Neurosci. 22, 2775–2782.
[26] Raghavendra, V., Tanga, F. and DeLeo, J.A. (2003) Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. J. Pharmacol. Exp. Ther. 306, 624–630.
[27] Raghavendra, V., Tanga, F.Y. and DeLeo, J.A. (2004) Complete Freunds adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. Eur. J. Neurosci. 20, 467–473.
[28] Stievano, L., Piovan, E. and Amadori, A. (2004) C and CX3C chemokines: cell sources and physiopathological implications. Crit. Rev. Immunol. 24, 205–228.
[29] Tetzlaff, W., Graeber, M.B., Bisby, M.A. and Kreutzberg, G.W. (1988) Increased glial fibrillary acidic protein synthesis in astrocytes during retrograde reaction of the rat facial nucleus. Glia 1, 90–95.
[30] Tsuda, M., Inoue, K. and Salter, M.W. (2005) Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. Trends Neurosci. 28 (2), 101–107.
[31] Verge, G.M., Milligan, E.D., Maier, S.F., Watkins, L.R., Naeve, G.S. and Foster, A.C. (2004) Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. Eur. J. Neurosci. 20, 1150–1160.
[32] Watkins, L.R. and Maier, S.F. (2002) Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. Physiol. Rev. 82, 981–1011.
[33] Watkins, L.R. and Maier, S.F. (2003) Glia: a novel drug discovery target for clinical pain. Nat. Rev. Drug Discov. 2, 973–985.
[34] Watkins, L.R., Milligan, E.D. and Maier, S.F. (2001) Glial activation: a driving force for pathological pain. Trends Neurosci. 24, 450–455.
[35] Watkins, L.R., Milligan, E.D. and Maier, S.F. (2001) Spinal cord glia: new players in pain. Pain 93, 201–205.