MATERIAL TABLE

CHAPITRE III CONCLUSION

LIST OF ABBREVIATIONS

CCR2	**Chemokine chemoattractant receptor 2**
MCP-1	**Monocyte chemoattractant protein-1**
BMDM	**Bone marrow-derived microglia**
BrdU	**Bromodeoxyuridine**
GFAP	**Glial fibrillary acidic protein**
GFP	**Green fluorescent protein**
Iba-1	**Ionized calcium-binding adaptor molecule**
DH	**Dorsal horn**
VH	**Ventral horn**
P2X4	**Purinergic receptor-4**
TLR4	**Toll like receptor-4**

Chapter I

GENERAL INTRODUCTION

1.1 Neuropathic pain

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage (Merskey H and Bogduk N, 1994). Pain pathways begin with sensory neurons of the dorsal root ganglia (DRG) and trigeminal ganglion, which constitute the first relay. Primary sensory neurons send their axons peripherally to the body surface, muscles, and viscera, and centrally to enter the ascending pathways via pain transmission neurons in the spinal cord or brainstem trigeminal nuclei that carry information to the brain.

Neuropathic pain is a complex and chronic pain state that usually is accompanied by damage or dysfunction of peripheral and/or central neuronal pathways after injury, cancer, diabetes, or infection. It is characterized by spontaneous and/or abnormal stimulus-evoked pain, such as allodynia (pain sensation evoked by normally innocuous stimuli) and/or hyperalgesia (increased pain intensity evoked by normally painful stimuli). The pathophysiological processes underlying the etiology of neuropathic pain involve molecular and cellular changes in neuronal plasticity and anatomical reorganization at various levels of the peripheral and central nervous systems (Campbell and Meyer, 2006). Recent findings have highlighted the active involvement of glial cells in the pathogenesis of nerve injury-induced neuropathic

pain and uncover new targets for potential painkilling drugs (Marchand et al, 2005; Tsuda et al, 2005)

Neuropathic pain, caused by various central and peripheral nervous system disorders, is especially problematic because of its severity, chronicity and resistance to simple analgesics. Millions of people worldwide suffer from this disorder. Unfortunately, many forms of neuropathic pain cannot be adequately treated using conventional analgesics (Sindrup and Jensen, 1999); these can only be managed partially with antidepressants and antiepileptics with various levels of success (Watson, 2000). The field of neuropathic pain research and drug discovery is still in the early stages of development, with many unmet goals. In the coming years, several advances are expected in the basic and clinical sciences of neuropathic pain, which will provide new and improved therapies for patients who continue to experience this disabling condition.

1.2 Macrophage and microglia

Macrophages are cells within tissues that originate from monocytes. Macrophages are versatile cells that play many roles. As scavengers, they rid the body of worn-out cells and other debris. After digesting a pathogen, macrophages will present antigens of the pathogen to T cells, a crucial event in the initiation of immune responses. As secretory cells, monocytes and macrophages are vital to the regulation of immune responses and the development of inflammation; they produce

an amazing array of powerful chemical substances including enzymes, complement proteins, and regulatory factors such as interleukin (IL)-1. Immediately after nerve injury, resident macrophages and monocytes from the peripheral blood rush to the lesion site and massively infiltrate damaged nerves and the DRGs on the ipsilateral side, eventually surrounding cell bodies from injured sensory neurons. The immune response in the periphery is driven by invasion of immune cells and orchestrated with local satellite and Schwann's cells through cytokines/chemokines network (Scholz and Woolf, 2007). Pro-inflammatory cytokines, including IL-1β, TNF-α, and IL-6 can directly modulate neuronal activity and elicit spontaneous action potential discharges (Wolf et al., 2006).

Microglia are considered as being the resident macrophages of the brain and spinal cord. They make up 5-15% of the total glial population in the CNS. They become activated at early stage in response to injury, infections, ischemia, brain tumors and neurodegeneration. Activated microglia are characterized by a specific morphology, proliferation, increased expression of cell surface markers or receptors and changes in functional activities, such as migration to areas of damage, phagocytosis, and production/release of pro-inflammatory substances (Gehrmann et al., 1995). There are many different microglial activation states, with various components expressed with different time-courses and intensities that are dependent on the stimulus that triggers activation. Functional and morphological changes are not time-locked, one can be detected in the absence of the other (Raivich et al., 1999).
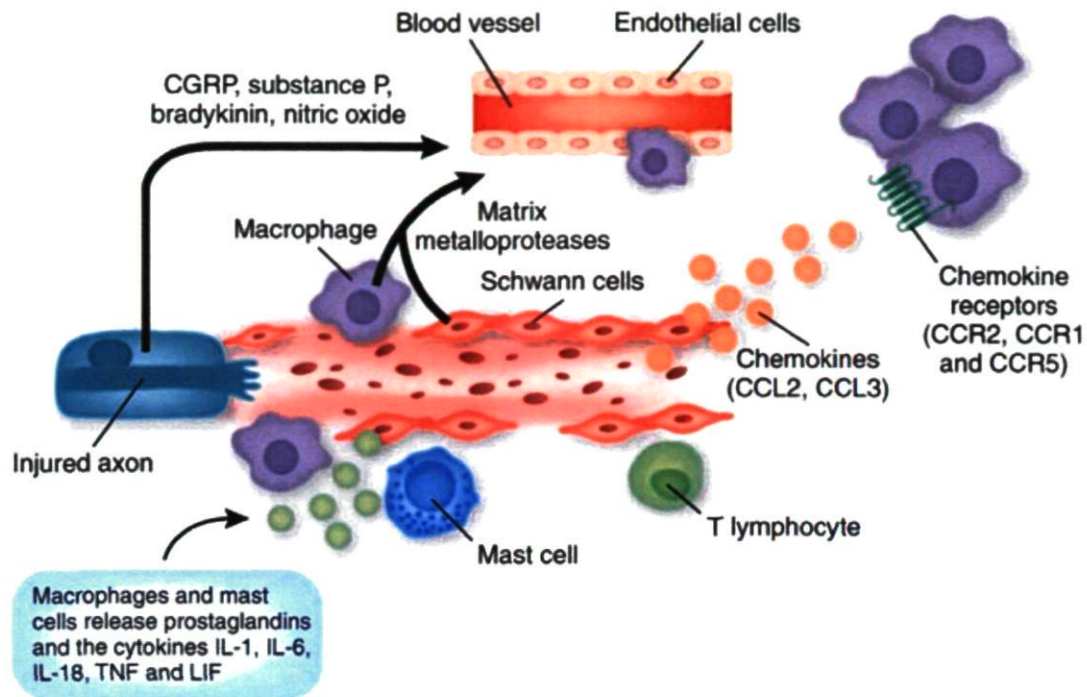
Figure 1.2-(A) *Macrophages and Schwann cells produce matrix metalloproteases that degrade the blood-nerve barrier. Macrophages and mast cells release prostaglandins and the cytokines IL-1β, IL-6, IL-18, TNF and LIF. TNF has an autocrine effect on macrophages that is mediated through TNFR1 activation and enhances cytokine synthesis and release. TNF also promotes further macrophage infiltration.*

Different from other glial cells, microglia originate from monocytic mesodermal cells and also derive from the infiltration of hematopoietic cells during the early development of the CNS. The normal CNS is characterized by two major monocyte-related populations: highly ramified, resting microglia inside the CNS parenchyma and perivascular macrophages on the luminal side of brain vessel-associated basal membranes. During development and shortly after birth, monocyte precursor cells populate the brain and thereby give rise to microglia. The renewal of microglia in the

adult CNS is traditionally thought to occur exclusively via proliferation of resident differentiated cells.   However, recent evidence suggests that, under certain circumstances, microglia can also come from a second population (macrophages). These macrophages are elongated and located around blood vessels. Perivascular macrophages are gradually replenished by circulating monocytes.   Thus, severe inflammation could lead monocytes to infiltrate the brain through the blood-brain barrier. Many of the hematognous monocytes invading CNS parenchyma will later ramify and gradually transform into microglia-like cells. Inflammatory stimuli may also lead to rapid activation of resident microglia, which may transform into phagocytic macrophages in the presence of cellular debris. Thus, both populations of macrophages of(macrophages-resident microglia and hematogenous macrophages-monocytes) may contribute to the removal of debris.

Microglia act as the first and main form of active immune defense in the CNS. Microglia are distributed throughout the brain and spinal cord and are constantly moving and analyzing the CNS for damaged neurons, plaques, and infectious agents(Nimmerjahn, science,2005). The brain and spinal cord are considered "immune privileged" organs in the sense that they are separated from the rest of the body by a series of endothelial cells, interconnected by complex tight junction, known as the blood-brain barrier (BBB) and blood-spinal cord barrier(BSB), respectively. One of the roles of the BBB/BSB is to prevent most infections microbes from reaching the vulnerable nervous tissue. In the case in which infectious agents are directly introduced into the CNS, microglia must react quickly by initiating

inflammation and destroying infectious agents before they cause damage to neural tissue. Microglias are extremely sensitive to even small pathological changes occurring in the CNS. Microglia activation could be neuroprotective or neurotoxic, which depends partially on the microenvironment.

1.3   Activated Microglia and Neuropathic Pain

Until very recently, chronic pain, such as neuropathic pain, was thought to be mediated only through the dysfunction of neurons. However, recent studies have shown that neuroimmune alterations may also contribute to pain after injury to the nervous system. The fact that peripheral nerve injury can induce spinal microglial/astrocytic activation has been demonstrated in several chronic neuropathic pain models (Colburn et al., 1999; Zhang et al ., 2003; Fu et al., 1999). Activated microglia release pain-enhancing substances such as pro-inflammatory cytokines, nitric oxide (NO), prostaglandins (PGs), and excitatory amino acids (EAA) (Hashizume et al., 2000), that excite spinal pain responsive neurons either directly or indirectly, and promote the release of other transmitters that can act on nociceptive neurons (Watkins et al., 2003). Several drugs that disrupt glia signaling by targeting glial activation (Milligan et al., 2003; Raghavendra et al., 2003), inhibiting the synthesis of cytokines, blocking pro-inflammatory cytokine receptors (Sweitzer et al., 2001), or disrupting pro-inflammatory cytokine signaling pathway (Sweitzer et al.,

2004) have successfully controlled enhanced nociceptive states in animal models. All these data strongly suggest that spinal cord glia are important pain modulators.

At the junction between incoming central neurons from DRG sensory reunions and cell bodies from secondary ascending neurons, microglia patrol the spinal cord environment and are ready to intervene if necessary, like following an injury.

Nerve injury induced-stereotypic microglial activation is characterized by a striking increase of Iba-1 ir on the ipsilateral side DH and VH. Iba-1 (ionized binding adaptor protein-1) is a marker specific for microglia (Fig. 1.2). On the contralateral side, the resting microglia have long, ramified processes, and they are well spaced from one another. On the ipsilateral side, the affected microglia show an increase in the size of the cell body, a thickening of proximal processes and a decrease in the ramification of distal branches; they appear to be more densely packed together. Temporally, microglia respond to nerve injury very early. The increase of OX-42 ir (another marker for microglia) started both in VH and DH at day 3 post-injury, increased significantly at day 7, then peaked at day 14, a time at which activated microglia were condensed around sensory nerve terminals in the dorsal horn and around motoneuron cell bodies in the ventral horn. From day 22, the intensity of OX-42-ir declined. On day 150, intensity of OX-42ir was similar to that on the contralateral side, although some activated microglia were also present on the contralateral side (Zhang and DeKoninck, 2006). Microglial activation is a graded process of acquisition of many functions: not only the morphological transformation

from ramified to rod like or ameboid shape, Microglial activation also results in temporal changes in gene expression. The acquired functions could include cell proliferation, migration, phagocytosis, regulation of antigen-presenting cell capabilities, up-regulation of cell surface receptors, and secretion of pro-inflammatory mediators: cytokines, chemokines, PGE2, secretion of anti-inflammatory cytokines.
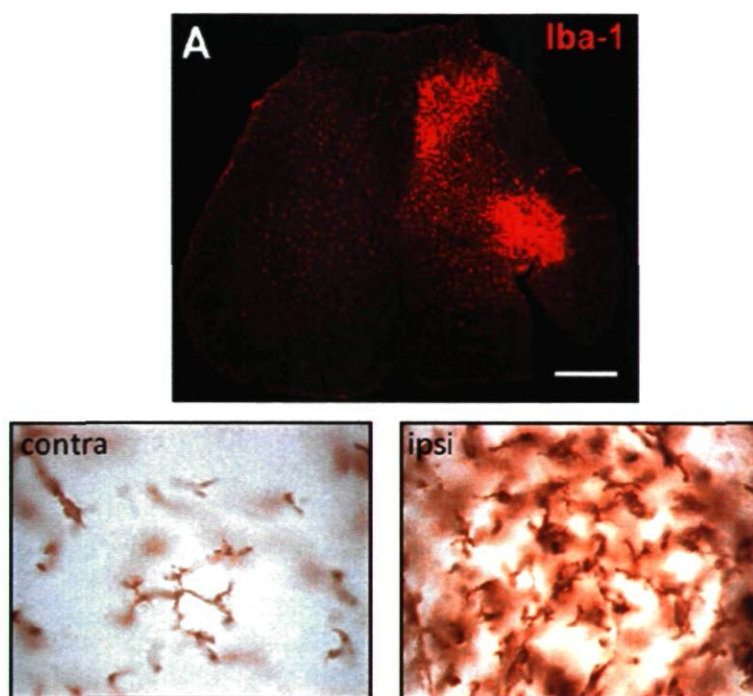

Figure 1.3 Two week after sciatic nerve injury, dense clusters of microglial cells occur in the ventral horn of the spinal cord, surrounding the cell bodies of motor neurons. Massive microglial activation is also found in the dorsal horn, in the projection territories of the central terminals of injured primary afferent fibers.

However, it remains unknown what is or are the trigger(s) that induce(s) spinal glial activation following peripheral nerve injury.

1.4 MCP-1/CCR2

Chemokines are a family of small proteins (8-10 kDa), initially recognized as chemoattractant cytokines controlling immune cell trafficking, a process important in host immune surveillance. According to the number and spacing of the conserved N-terminal cysteines, chemokines are classified into four classes (C, CC, CXC, CX3C) and act through specific or shared receptors that all belong to the superfamily of G-protein-coupled receptors (GPCR). Chemokines are extracellular signaling proteins that produce endocrine, paracrine and autocrine effects. In contrast to circulating endocrine hormones, they can exert their effects on nearby cells over short extracellular distances at low concentration. It is well established that chemokines play an important role in the pathogenesis of neuroinflammatory disease states, such as multiple sclerosis (Bajetto et al., 2001).

Monocyte chemoattractant protein-1 (MCP-1) is a member of the CC chemokine family that specifically attracts and activates monocytes to sites of inflammation (Leonard et al., 1991). Recent studies have suggested a role for MCP-1 and its receptor CCR2 in nociception. Both intraplantar and intrathecal injections of this chemokine have been shown to induce mechanical allodynia (Tanaka et al., 2004). Mutant mice lacking the MCP-1 receptor CCR2 (CCR2$^{-/-}$) do not develop tactile allodynia after nerve injury (Abbadie et al., 2003). Expressed normally by peripheral immune cells, MCP-1 can be induced in neurons by nerve injury (Flugel et al., 2001; Schreiber et al., 2001; Zhang and De Kononck, 2006). Following sciatic nerve injury, MCP-1 mRNA and protein expression has been identified on DRG sensory neurons

on the side ipsilateral to the nerve injury. The induction of MCP-1 in damaged neurons started as early as day 1 post-injury, peaked around 1-2 weeks and then reduced progressively afterwards. In addition, induced MCP-1 protein in DRG was transported to the ipsilateral spinal cord dorsal horn (Zhang and De Koninck, 2006). CCR2, the receptor for MCP-1 is expressed selectively on cells of monocyte/macrophage lineage in the periphery (Rebenko-Moll et al., 2006) and can be induced in spinal microglia by peripheral nerve injury (Abbadie et al., 2003). It has also been demonstrated that both spatially and temporally, MCP-1 induction is closely correlated with the subsequent surrounding microglial activation (Zhang and De Koninck, 2006). All these data strongly suggest that the induced neuronal MCP-1 could be the signaling molecule that activates resident microglial activation and and/or attracts peripheral macrophages into the spinal cord, and consequently contributes to the development of mechanical allodynia (Figure. 1.4).
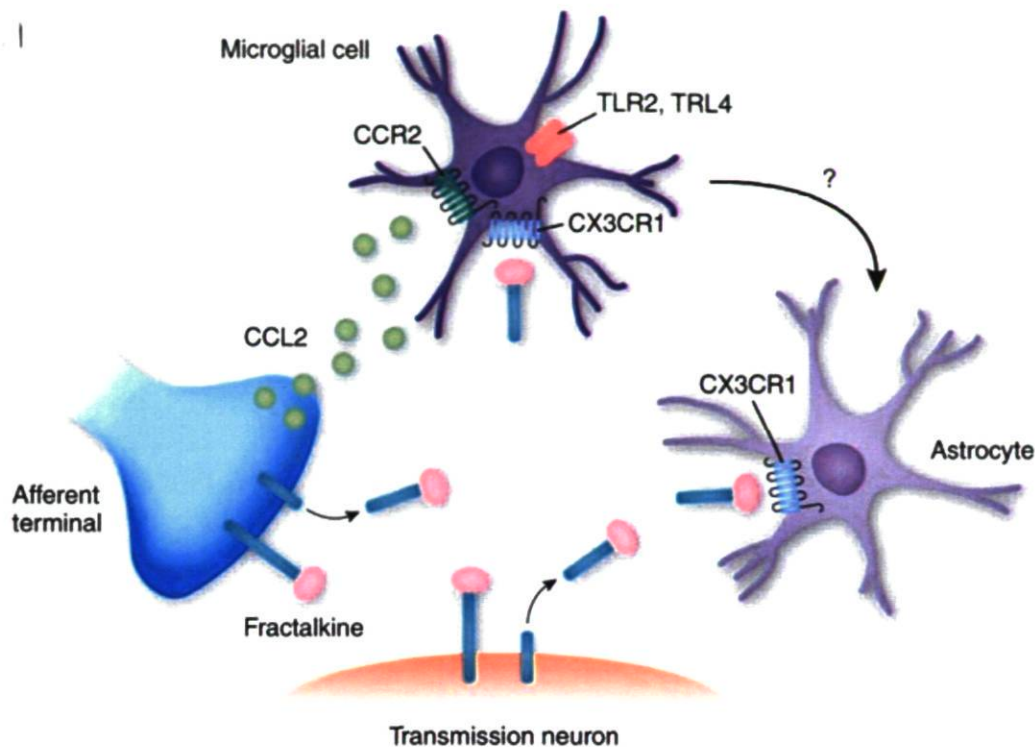
Figure 1.4 *Microglial recruitment depends on signaling pathways involving chemokine CCL2 (MCP-1) and its receptor CCR2.*

1.5 Research problem

The mechanisms underlying neuropathic pain after peripheral nerve injury are still under investigation, especially the involvement of glial cells in the pathogenesis. Neuropathic pain is generally chronic, severe, and resistant to treatment with counter analgesics. The advanced research activities could unravel new targets for an effective treatment. During my graduate studies, I tried to answer the following questions:

1. What are the origins of these activated microglia?

Are they only CNS resident microglia or do they also include some newly generated microglia?

Are they only CNS resident microglia or do some of them are derived from infiltrating monocytes?

2. What triggers quiescent spinal cord glia to become activated in response to nerve damage?

1.6 Animal model and behavioral testing

1.6.1 Why we chose the partial sciatic nerve ligation (PSNL) model

The PSNL model (Seltzer et al., 1990*) is widely used in the study of neuropathic pain. Perhaps even more importantly, partial nerve injury is the main cause of causalgiform pain disorders in humans. Thus, this model could similarly mimic injuries observed in patients suffering from neuropathic pain.

1.6.2 How to make PSNL and the main characteristics of the model

In mice, like in rats, we unilaterally ligated about half of the sciatic nerve high in the thigh. Within a few hours after the operation, and for several months thereafter, mice developed guarding behavior of the ipsilateral hind paw and licked it often, suggesting the possibility of spontaneous pain. The plantar surface of the foot was evenly hyperesthetic to non-noxious and noxious stimuli. None of the mice autotomized. There was a sharp decrease in the withdrawal thresholds ipsilaterally in

response to up-down Von Frey hair stimulation at the plantar side. Heating also elicited aversive responses, suggesting thermal hyperalgesia. Those companion reports suggest that this preparation could serve as a model for syndromes of the causalgiform variety that are triggered by partial nerve injury and maintained by sympathetic activity.

1.6.3 How to perform behavioral testing for allodynia and hyperalgesia.

The mice were habituated to handling and testing equipment at least 2 hours per day during 2 or 3 days before experiments and at least one hour before each test after surgery. Threshold for tactile allodynia was measured with a series of von Frey filaments (Semmes-Weinstein monofilaments). The mice stood on a metal mesh covered with a plastic dome. The four plantars should be in touch with the mesh and mice should be in good testing condition (i.e., resting status and no glooming, no sleeping, no moving, no other activity). To determine the testing status of the mice is one of the most important points to obtain the right results. Another important point is that all tests should be done in the same period of the daytime, since the animal's bioactivity differs in function of the time of the day.

The plantar surface of the hind paws was touched with different von Frey filaments having bending force from 0.16 to 1.4 g until the threshold that induced paw withdrawal was found. We used up-down withdraw threshold method and recorded 5 stimuli after the first reverse reaction. Unresponsive mice received a maximal score of

2g. The variability in the withdrawal threshold was observed between the left and the right hind paws after the PSNL. In this study, we focused on the Von Frey test to analyze the development of neuropathic pain.

1.7 Presentation

The mode of presentation adopted for this master's thesis is the one of the series articles. The research article included constitutes the second chapter and was published in *The Journal of Neuroscience, Nov 7, 2007, 27(45):12396-12406; doi:10.1523/ JNEUROSCI. 3016-07.2007.* I note that I was responsible for the majority of the experiments that were included in this manuscript. I conducted nerve injury surgeries on mice with different genetic background and carried out all pain behavioral testing before and after nerve injury. I performed immunohistochemical studies, including double labeling, as well as qualitative and quantitative analyse of images. I also participated in the preparation of the manuscript.

Chapter II

EXPRESSION OF CCR2 IN BOTH RESIDENT AND BONE MARROW-DERIVED MICROGLIA PLAYS A CRITICAL ROLE IN NEUROPATHIC PAIN

2.1 ABSTRACT

Neuropathic pain resulting from damage to or dysfunction of peripheral nerves is not well understood and difficult to treat. Although CNS hyperexcitability is a critical component, recent findings challenge the neuron-centric view of neuropathic pain etiology and pathology. Indeed, glial cells were shown to play an active role in the initiation and maintenance of pain hypersensitivity. However, the origins of these cells and the triggers that induce their activation have yet to be elucidated. Here we show that, after peripheral nerve injury induced by a partial ligation on the sciatic nerve, in addition to activation of microglia resident to the CNS, hematogenous macrophage/monocyte infiltrate the spinal cord, proliferate, and differentiate into microglia. Signaling from chemokine monocyte chemoattractant protein-1 (MCP-1, CCL2) to its receptor CCR2 is critical in the spinal microglial activation. Indeed, intrathecal injection of MCP-1 caused activation of microglia in wild-type but not in CCR2-deficient mice. Furthermore, treatment with an MCP-1 neutralizing antibody prevented bone marrow-derived microglia (BMDM) infiltration into the spinal cord after nerve injury. In addition, using selective knock-out of CCR2 in resident microglia or BMDM, we found that, although total CCR2 knock-out mice did not

develop microglial activation or mechanical allodynia, CCR2 expression in either resident microglia or BMDM is sufficient for the development of mechanical allodynia. Thus, to effectively relieve neuropathic pain, both CNS resident microglia and blood-borne macrophages need to be targeted. These findings also open the door for a novel therapeutic strategy: to take advantage of the natural ability of bone marrow-derived cells to infiltrate selectively affected CNS regions by using these cells as vehicle for targeted drug delivery to inhibit hypersensitivity and chronic pain.

2.2 INTRODUCTION

The pathophysiological processes underlying the etiology of neuropathic pain involve molecular and cellular changes in neuronal plasticity and anatomical reorganization at various levels of the peripheral nervous system and CNS (Marx, 2004◆; Baron, 2006◆; Campbell and Meyer, 2006◆). Recent findings have highlighted the active involvement of glial cells in the pathogenesis of nerve injury-induced neuropathic pain and uncover new targets for potential painkilling drugs (Marchand et al., 2005◆; Tsuda et al., 2005◆).

Peripheral nerve injury induces activation of spinal microglial cells (Coyle, 1998◆; Colburn et al., 1999◆; Fu et al., 1999◆; Zhang et al., 2003◆). Activated microglia contribute to neuropathic pain symptomology through the release of molecules that act as direct modulators of neuronal excitability (Tsuda et al., 2003◆; Coull et al., 2005◆).

A major question remains unanswered: where do these activated microglial cells come from and is there a specific population involved in pain? The normal CNS is characterized by two major monocyte-related populations: highly ramified CNS resident microglia and hematopoietic perivascular macrophages (Raivich and Banati, 2004). The renewal of microglia in adulthood occurs not only through the proliferation of preexisting cells but also through the recruitment of precursors that derive from bone marrow (BM), because the perivascular macrophages replenished by circulating monocyte could migrate through basal membrane into the CNS parenchyma, a process enhanced in different forms of inflammatory neuropathology (Streit et al., 1989; Lawson et al., 1992; Priller et al., 2001; Sweitzer et al., 2002). The relative contribution of resident and invading microglia to the pathogenesis may vary depending on the setting and severity of the injury, as is evidenced by the different dynamics of BM-derived cell accumulation (Furuya et al., 2003; Priller et al., 2006; Solomon et al., 2006; Denker et al., 2007). An understanding of the distinct contribution of cells of the monocytic lineage in injury-induced neuropathic pain is important for directing the search for novel therapeutic targets.

Whenever neurons are injured, microglia become activated, both at the primary lesion sites and remote from primary damage, at sites where the damaged neurons project (Kreutzberg, 1996). Thus, microglial activation is likely to be controlled by endangered neurons. The identity of the molecules involved in neuron–microglia signaling in different injury conditions remains an active subject of investigation. Chemokines and their receptors constitute an elaborate signaling system that plays an

important role in cell-to-cell communication not only in the peripheral immune system but also in the CNS (Ransohoff and Tani, 1998◆; Ambrosini and Aloisi, 2004◆; Moser et al., 2004◆; Rot and von Andrian, 2004◆). Monocyte chemoattractant protein-1 (MCP-1), also named CCL2, is a member of the CC family chemokine that specifically attracts and activates monocytes to the sites of inflammation (Leonard et al., 1991◆). Absent in normal CNS, MCP-1 was found to be induced in facial nucleus neurons by facial nerve transection (Flugel et al., 2001◆), in sympathetic ganglion neurons after postganglionic axotomy (Schreiber et al., 2001◆), and in DRG sensory neurons and spinal cord motor neurons by chronic constriction of the sciatic nerve (Tanaka et al., 2004◆; Zhang and De Koninck, 2006◆). CCR2, the receptor for MCP-1, is expressed selectively on cells of monocyte/macrophage lineage in periphery (Rebenko-Moll et al., 2006◆) and can be induced in spinal microglia by peripheral nerve injury (Abbadie et al., 2003◆). We also demonstrated that, both spatially and temporally, MCP-1 induction is closely correlated with the subsequent surrounding microglial activation (Zhang and De Koninck, 2006◆). We predicted that the induced neuronal MCP-1 could be the signaling molecule that activates resident spinal microglial and/or attracts peripheral macrophages into the spinal cord. Also, it could contribute to peripheral sensitization by attracting macrophages to the injured nerve and DRG. It has been demonstrated that mice lacking the CCR2 [CCR2 knock-out (KO)] had impaired nociceptive response typically associated with neuropathy (Abbadie et al., 2003◆), but the exact contribution of CCR2 in resident and bone marrow-derived microglia has yet to be clearly defined.

In the present study, we identified the origins of activated microglia by using chimeric mice in which their bone marrow was replaced by one that expresses green fluorescent protein (GFP). We show that, after peripheral nerve injury, in addition to activation of microglia resident to the spinal cord, blood-borne macrophages have the ability to infiltrate the spinal cord, proliferate, and differentiate into activated microglia. We also showed that infiltration of peripheral macrophages into the spinal cord after nerve injury involves direct MCP-1/CCR2 signaling from the CNS to the periphery. The fact that both resident microglia and bone marrow-derived macrophages participate in the modulation of central sensitization in neuropathic pain indicates that inhibition of either resident microglia or of peripheral macrophages may not be an efficient approach to relieve neuropathic pain. Both need to be targeted.

2.3 MATERIELS AND METHODS

Animals

Adult (7- to 12-week-old) male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Hemizygous transgenic mice expressing GFP under the control of the chicken ß-actin promoter and cytomegalovirus enhancer and CCR2 knock-out mice were initially obtained from the same vendor. Local colonies of GFP and CCR2KO mice were then established and maintained on a C57BL/6 background, respectively. Mice were housed four per cage after weaning in a temperature- and

humidity-controlled vivarium, on a 14/10 h light/dark cycle (lights on at 6:00 A.M. and off at 8:00 P.M.), with access to rodent chow and water *ad libitum*. Behavioral experiments were conducted from 8:00 A.M. to 4:00 P.M. All protocols were conducted according to the Canadian Council on Animal Care guidelines, as administrated by the Laval University Animal Welfare Committee.

Generation of bone marrow-chimeric mice

Recipient mice were exposed to 10-gray total-body irradiation using a cobalt-60 source (Theratron-780 model; MDS Nordion, Ottawa, Ontario, Canada). A few hours later, the animals were injected via tail vein with ~5 x 10^6 bone marrow cells freshly collected from donor mice. The cells were aseptically harvested by flushing femurs with Dulbecco's PBS (DPBS) containing 2% fetal bovine serum. The samples were combined, filtered through a 40 μm nylon mesh, centrifuged, and passed through a 25 gauge needle. Recovered cells were resuspended in DPBS at a concentration of 5 x 10^6 vial nucleated cells per 200 μl. Irradiated mice transplanted with this suspension were housed in autoclaved cages and treated with antibiotics (0.2 mg of trimethoprim and 1 mg of sulfamethoxazole per milliliter of drinking water given for 7 d before and 2 weeks after irradiation). Animals were subjected to partial sciatic nerve ligation 3–5 months after transplantation.

GFP chimeric mice.

GFP-positive (GFP$^+$) transgenic mice were used as BM donors. C57BL/6 mice were irradiated and transplanted with GFP$^+$ cells via the tail vein.

Central CCR2KO chimeric mice. CCR2KO mice were used as BM recipients. GFP$^+$ transgenic BM cells were transplanted into irradiated CCR2KO mice.

Peripheral CCR2KO chimeric mice. GFP$^+$ transgenic mice were used as BM recipients. Bone marrow cells collected from CCR2KO mice were transplanted into irradiated GFP$^+$ transgenic mice.

The presence of GFP$^+$ donor-derived cells in the peripheral circulation of transplant recipients in each chimeric group was analyzed 8 weeks after transplantation by fluorescence-activated cell sorting. The GFP chimeric mice and central CCR2KO chimeric mice used in the protocol had all >80% (83.6 ± 5.03%; $n = 50$) of GFP$^+$ peripheral blood leukocytes, and peripheral CCR2KO chimeric mice had only 1.53 ± 0.03% ($n = 10$) GFP$^+$ cells in the blood.

Irradiation bone marrow chimeric mouse generation is currently widely used to distinguish blood-derived and CNS resident microglia. To exclude the possibility that the cell recruitment is an artifact of irradiation or bone marrow transplantation, some additional approaches, such as intrasplenic injection of 6-carboxylfluorescein diacetate, a long-lasting intracellular fluorescent tracer, and using unirradiated parabionts with surgically anatomosed vasculature have been reported. As seen in GFP bone marrow chimeras, monitoring invasion of blood-derived cells in the absence of previous irradiation and bone marrow transplantation clearly revealed that recruitment of leukocytes across the blood–brain barrier contributes to the accumulation of ionizing calcium-binding adaptor molecule-positive (Iba-1$^+$) cells

within the CNS parenchyma in different pathological conditions (Bechmann et al., 2005✦; Massengale et al., 2005✦). More importantly, irradiation does not affect the ability of resident cells to proliferate after spinal cord injury (Horky et al., 2006✦).

Nerve injury model and behavioral studies

Partial sciatic nerve ligation was conducted according to the method of Seltzer et al. (1990)✦ as adapted to mice (Malmberg and Basbaum, 1998✦). Briefly, under isoflurane anesthesia and aseptic conditions, the left sciatic nerve was exposed at high-thigh level. The dorsum of the nerve was carefully freed from surrounding connective tissues at a site near the trochanter. A 8-0 suture was inserted into the nerve with a 3/8 curved, reversed-cutting mini-needle (Tyco Health Care, Mississauga, Ontario, Canada) and tightly ligated so that the dorsal one-third to one-half of the nerve thickness was trapped in the ligature. The wound was then closed with two muscle sutures (4-0) and two to three skin sutures (4-0). In sham-operated mice, the nerve was exposed and left intact. The wound was closed as in injured mice.

All animals were assessed for mechanical sensitivity before surgery and from days 2–3 after injury until they were killed for histological studies. The investigator was totally blinded to the treatments the mice received. Paw-withdrawal threshold was measured with calibrated von Frey fibers using the up–down method (Chaplan et al., 1994✦), as described previously (Mogil et al., 1999✦). Mice were placed on a metal mesh floor with small Plexiglas cubicles (9 x 5 x 5 cm high), and a set of eight calibrated von Frey fibers (ranging from 0.008 to 1.40 g of force) were applied to the

plantar surface of the hindpaw until they bent. The threshold force required to elicit withdrawal of the paw (median 50% paw withdrawal) was determined on two tests separated by at least 1 h. All animals were habituated for at least 2 h to their individual Plexiglas observation chamber before testing. Baseline data (day 0) was obtained by averaging measurements made 1–2 d before surgery.

Intrathecal injections

In a subset of animals, recombinant mouse (rm) MCP-1 (R & D Systems, Minneapolis, MN) or neutralizing antibody against mouse MCP-1 (R & D Systems) were injected by intrathecal punctions at the level of L5–L6 under isoflurane anesthesia. The rmMCP-1 was delivered every 2 d (2 µg in 10 µl of saline per injection) in adult naive CCR2KO and wild-type (C57BL/6) mice, and the animals were killed at day 6 after the first injection and processed for immunohistochemistry as described below. The MCP-1 neutralizing antibody was delivered in adult GFP chimeric mice with nerve injury. Starting from the day of surgery, mice received an injection of the antibody every 2 d until day 13 after injury (4 µg in 10 µl of saline per injection). Animals were then perfused for visualization of GFP cell infiltration at day 14 after injury. Mice in the control groups received intrathecal injections of an equal volume of saline.

Immunohistochemistry

In wild-type (C57BL/6) and GFP chimeric mice, bromodeoxyuridine (BrdU) (50

mg/kg; Sigma, St. Louis, MO) was injected intraperitoneally at day 3 after injury, and animals were killed 2 h, 4 d, 11 d, and 27 d after injection. To collect the spinal cord tissues of all animals used in the current study, mice were deeply anesthetized via an intraperitoneal injection of a mixture of ketamine hydrochloride and xylazine and then rapidly perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in sodium phosphate buffer. Lumbar spinal cords were removed and postfixed overnight. Lumbar spinal cord (L4–L5 segments) were cut into 30 μm sections, then collected in a cold cryoprotectant solution (0.05 M sodium phosphate buffer, pH 7.3, 30% ethylene glycol, and 20% glycerol), and stored at –20°C.

To allow the detection for BrdU-labeled cells, free-floating sections were pretreated with 50% formamide in 2x SSC for 2 h at 65°C, followed by 15 min in 2x SSC at room temperature, 30 min in 2N HCl at 37°C, 10 min in 0.1M borate buffer at room temperature. Nonspecific labeling was blocked with TBS plus 0.25% Triton X-100, 1% BSA, and 3% normal goat serum for 1 h. A polyclonal goat anti-rat antibody against BrdU (1:250; Accurate Chemicals, Westbury, NY) was incubated with tissue sections for 48 h at 4°C. After primary antibody incubation, sections were rinsed in TBS and incubated in Alexa 488-conjugated goat anti-rat IgG (in TBS containing 0.25% Triton X-100, 1% BSA, and 3% normal goat serum, 1:250; Invitrogen, Carlsbad, CA) for 1 h. After rinses in TBS, sections were mounted onto slides and coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Regular immunofluorescent staining was performed to identify the phenotypes of infiltrated BM cells and spinal microglia reaction to the peripheral nerve injury. Free-floating sections were first treated in TBS containing 3% normal serum, 1% BSA, and 0.25 Triton X-100 for 1 h at room temperature and then 30 μm spinal cord sections were incubated overnight at 4°C with antibodies listed below: mouse anti-neuron-specific nuclear protein (NeuN) monoclonal antibody (for neurons, 1:1000; Chemicon, Temecula, CA), rabbit anti-Iba-1 polyclonal antibody (for microglia and macrophages, 1:1000; Wako Chemicals, Richmond, VA), rabbit anti-glial fibrillary acid protein (GFAP) polyclonal antibody (for astrocytes, 1:1000; DakoCytomation, Carpinteria, CA), rabbit anti-NG2 polyclonal antibody (chondroitin sulfate proteoglycan, for oligodendrocyte progenitors, 1:250; Chemicon), and monoclonal rat anti-CD31 (for endothelial cells, 1:1000; BD Biosciences PharMingen, San Diego, CA), respectively, followed by a 60 min incubation at room temperature in fluorochrome-conjugated goat secondary antibody. The sections were then mounted onto SuperFrost slides (Fisher Scientific, Nepean, Ontario, Canada) and coverslipped with Vectashield mounting medium (Vector Laboratories). In some cases, to better identify the anatomical distribution of infiltrated cells, additional immunostaining was performed using a polyclonal antibody against GFP (1:1000; Invitrogen), revealed by a DAB-based enzymatic method; the tissue was then counterstained with thionin to identify the parenchyma.

Image analysis

Images were acquired either using an Olympus Optical (Tokyo, Japan) microscope (AX-70) equipped with a Spot Camera or a Zeiss (Oberkochen, Germany) LSM 510 confocal laser-scanning microscope. Colocalization was ensured with confocal Z stacks at 1 μm intervals and visualization in three-dimensional orthogonal planes. Quantitative analysis of the immunofluorescence intensity was performed on images digitized using a constant set of parameters (exposure time, gain, and post-image processing) with special care to avoid signal saturation. We measured the intensity of Iba-1 immunofluorescence as the average pixel intensity within a rectangle (197 x 533 pixels) on the dorsal horn (DH) (lamina I–IV) and a rectangle (224 x 294 pixels) on the ventral horn (VH) (lamina IX), on both sides relative to the side of injury (MetaMorph, version 6.2r6; Universal Imaging, Downingtown, PA). GFP$^+$ cells, BrdU$^+$ cells, and Iba-1$^+$ microglial cells were counted by two independent investigators in four different regions of interest [ipsilateral DH (DHi), contralateral DH (DHc), VHi, and VHc]. Only ramified GFP$^+$/Iba-1$^+$ cells within parenchymal gray matter were included.

Statistics

All data are presented as means ± SEM. Statistical analysis was based on the following: (1) repeated-measures ANOVA followed by Dunnett's case-comparison *post hoc* test for behavioral analyses; (2) paired *t* test for the difference in intensity of Iba-1 signal between ipsilateral and contralateral side in the DH and VH, respectively; (3) unpaired *t* test for the difference in intensity of Iba-1 signal between groups

(peripheral/central CCR2KO DHi vs GFP chimera DHi; peripheral/central CCR2KO VHi vs GFP chimera VHi).

2.4 RESULTS

Infiltration of bone marrow-derived cells into spinal cord after peripheral nerve injury

To identify the origins of the activated microglia observed in the spinal cord after peripheral nerve injury, we transplanted GFP-expressing bone marrow stem cells into irradiated C57BL/6 mice (GFP chimeric mice). We found that, in naive animals, GFP$^+$ cells were virtually absent in the spinal cord parenchyma, and the few GFP$^+$ cells found in the spinal cord had an elongated shape and were restricted to blood vessels (Fig. 1*A*). We then subjected the mice to either a sham surgery of the thigh or a partial sciatic nerve ligation injury. In sham-operated mice, the number of GFP cells was slightly higher in the spinal cord, but there was no significant difference between the ipsilateral and contralateral sides (Fig. 1*A*). In contrast, many ramified GFP-expressing cells were present in the DHi and VHi of the L4–L5 spinal cord after nerve injury (Fig. 1*A*). The different morphologies of GFP$^+$ cells are depicted in Figure 1*B–D*. The results were confirmed by immunolabeling with a polyclonal antibody against GFP for infiltrated cells. Counterstaining with thionin helped better identify the anatomical localization of GFP$^+$ cells (Fig. 1*E–H*).
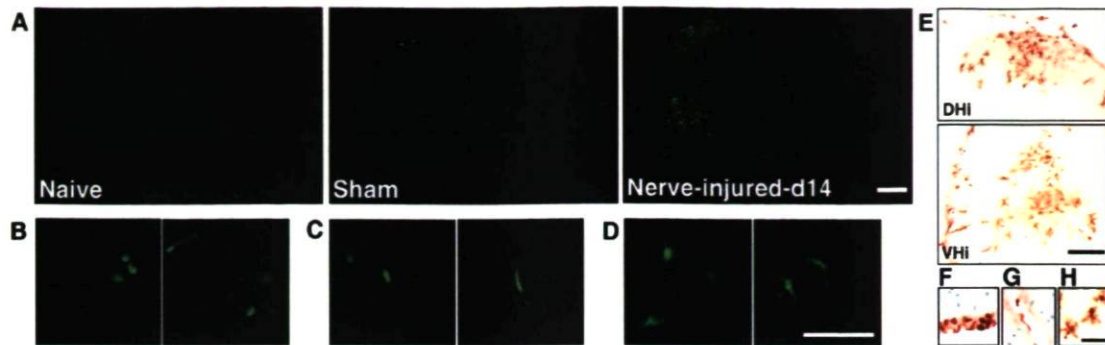
Figure 1. *Infiltration of bone marrow-derived cells into the spinal cord after sciatic nerve injury. A, BM-derived cells in the lumbar spinal cord of naive, sham-operated and sciatic nerve-injured mice. No ramified GFP⁺ cells were found in the spinal parenchyma of non-injured animals. In contrast, GFP⁺ cell infiltration was remarkable in injured animals, which was restricted in the ipsilateral side of the injury (scale bar, 1 mm). B–D, Meningeal GFP⁺ cells had a round shape (B), they were elongated when associated with blood vessels (C), and ramified within the parenchyma (D) (scale bar, 100 μm). E–H, Immunolabeling of infiltrated cells with a polyclonal antibody against GFP, counterstained by thionin, revealed their anatomical localization in DHi and VHi (day 14 after injury) (E) and different cellular shapes depending on the distribution in the meninges (F), within vessels (G), and parenchyma (H) (scale bar, 50 μm).*

To identify the phenotype of these ramified GFP⁺ donor cells, we used an antibody directed against Iba-1 to label microglia. Confocal microscope analysis on *x*, *y*, and *z* orthogonal planes provided direct evidence that virtually all GFP⁺ cells present within the spinal cord parenchyma were Iba-1-positive ramified cells (Fig. 2A–C). Double

immunolabeling of GFP with other cellular markers, NeuN for neurons, GFAP for

astrocytes, NG2 for oligodendrocyte progenitors, and CD31 for endothelial cells, were

also conducted. No evidence of GFP colocalization with the above markers has been
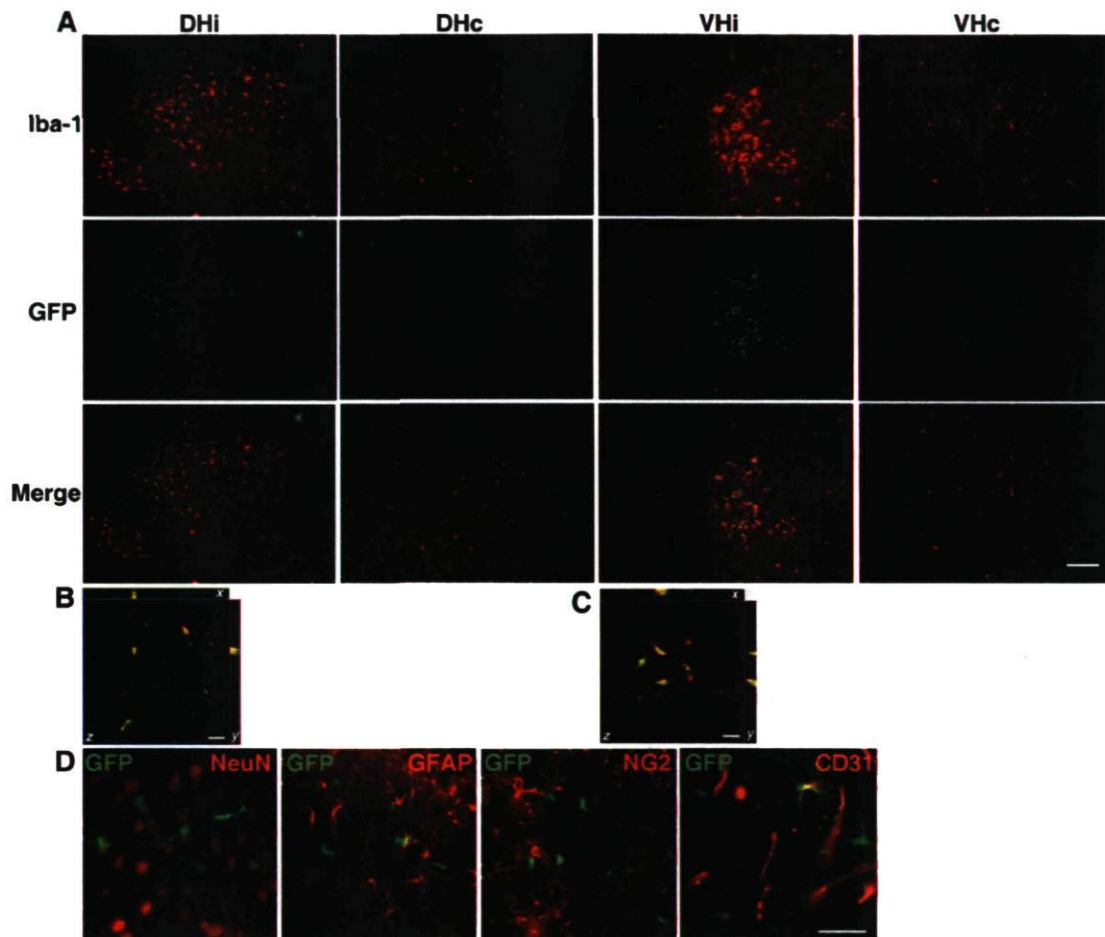
observed (Fig. 2D).

Figure 2. *Phenotype identification of infiltrated BM-derived cells in the lumbar spinal*

cord. A, Intense Iba-1 labeling was found in activated microglia in the ipsilateral side

spinal cord (DH/VH), whereas Iba-1 immunofluorescence was weak (red) in the

contralateral DH/VH. Ramified GFP$^+$ cells (green) overlapped with the Iba-1

immunoreactive signal in the ipsilateral DH/VH. Almost all ramified GFP$^+$ cells

within the parenchyma were Iba-1$^+$ (merge) (scale bar, 200 μm), which was

confirmed by confocal microscopic analysis in the x, y, and z orthogonal planes in

*DH (**B**) and VH (**C**) (scale bar, 10 µm). **D**, No colocalization of GFP with other cellular markers (NeuN, GFAP, NG2, and CD31) was observed in the lumbar spinal cord dorsal horn 14 d after injury (scale bar, 100 µm).*

To determine the temporal profile of BM-derived cell infiltration and its relationship with CNS resident microglia, we quantified the number of ramified GFP-expressing cells among all Iba-1 immunoreactive microglia in four different regions of interest: DHi, DHc, VHi, and VHc. Three days after nerve lesion, an average of 9% of microglia in the DHi and 22% in the VHi expressed GFP (DHi, 3.6 ± 0.6 GFP$^+$ cells; VHi, 8.0 ± 2.3 GFP$^+$ cells). At days 7–14 after injury, 25–27 and 41–42% of microglia in the DHi and the VHi were found to be GFP$^+$, respectively. One month after the injury, the number of GFP$^+$ cells decreased to 6 and 16% in the DHi and the VHi, respectively (Fig. 3). The time course of the increase in GFP$^+$ cell number in the lumbar spinal cord paralleled that of microglial activation (Fig. 3).
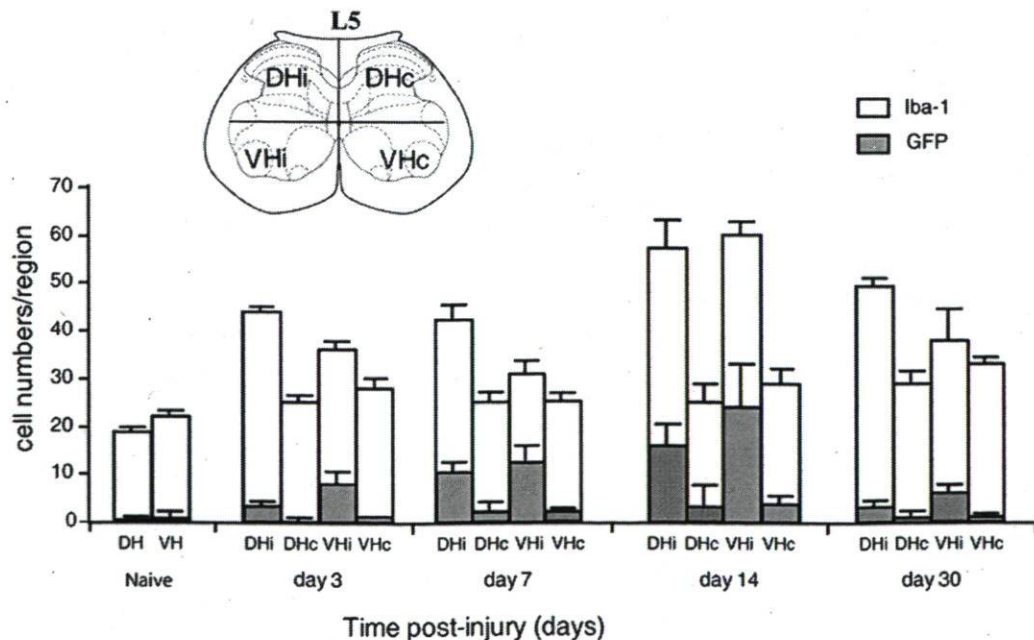
Figure 3. Temporal profile of BM-derived cell infiltration in the spinal cord parenchyma. The numbers of Iba-1$^+$ microglia (white bars) and GFP$^+$/Iba-1$^+$ BM-derived microglia (gray bars) were determined in fours regions of the spinal cord (4–6 sections per mouse, 6 mice per group). Note the significant increase of BM-derived microglia in the ipsilateral side DH/VH starting from day 3 and peaking at day 14. Correspondingly, the number of microglia in the ipsilateral side, including BM-derived microglia increased also from day 3 to day 14 (data are expressed as mean ± SEM).

Proliferation and differentiation of bone marrow-derived cells within the spinal cord parenchyma

We then assessed the plasticity of these infiltrating cells by determining their capacity to proliferate and differentiate into microglia. Animals were injected with BrdU 3 d after injury and perfused at different time points afterward (days 3–30). Immunofluorescence staining of incorporated BrdU revealed that peripheral nerve injury induced cell proliferation in the spinal cord, ipsilateral to the side of nerve injury, from days 3 to 14 (Fig. 4A). Nerve injury increased the number of BrdU$^+$ cells in both irradiated GFP chimeric mice and non-irradiated control C57BL/6 mice equally (data not shown). Double immunolabeling of BrdU with GFP demonstrated

that both resident cells (red arrow) and BM-derived hematopoietic cells (yellow arrow) proliferated within the spinal cord parenchyma (Fig. 4*B*), in which 19.7 and 22.3% of BrdU$^+$ cells derived from peripheral macrophages in the DHi and the VHi, respectively (Fig. 4*C*).

A BrdU

ipsilateral contralateral

B BrdU GFP Merge **D** on the meninges

1μm

2μm in the parenchyma -d3

3μm

4μm in the parenchyma -d7

5μm in the parenchyma -d14

6μm

7μm in the parenchyma -d30

C

DHi 80.3% 19.7%

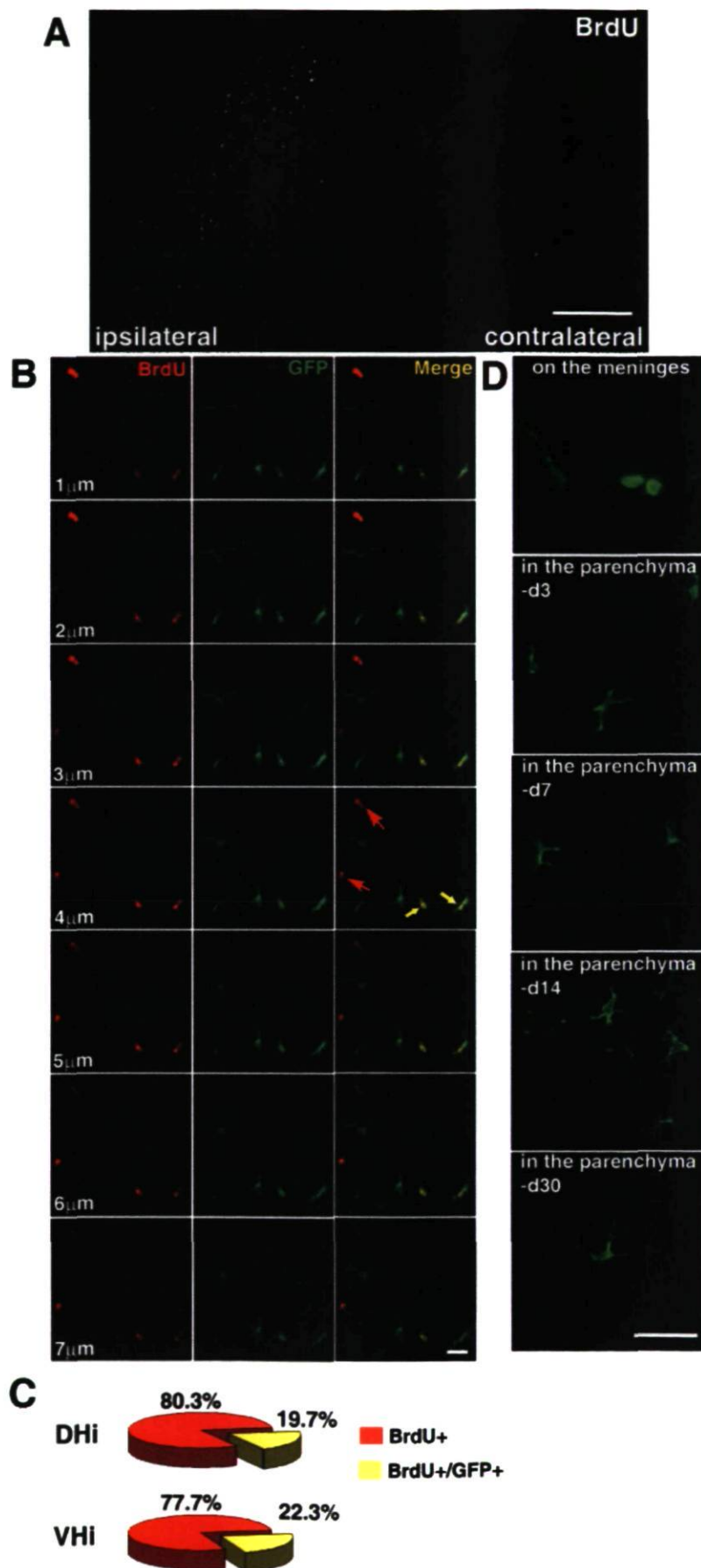■ BrdU+
□ BrdU+/GFP+

VHi 77.7% 22.3%

Figure 4. *Proliferation and differentiation of infiltrated bone marrow-derived cells.*

A, Representative BrdU staining in the lumbar spinal cord of GFP chimeric mice (14

d after injury, 11 d after BrdU injection). Note the increase of BrdU-positive cells in

the lumbar spinal cord, ipsilateral to the injury (scale bar, 1 mm). ***B,*** *Z-sectioned scan*

with confocal microscope (Zeiss LSM 510) through the extent of BrdU-positive

nucleus to verify the double labeling with GFP$^+$ cells. BrdU and GFP colocalization

showing cell proliferation within BM-derived microglia (yellow arrow); BrdU single

labeling showing cell proliferation in resident cells (red arrowhead) (scale bar, 20

µm). ***C,*** *Quantitative analysis of the number of GFP$^+$/BrdU$^+$ cells over total BrdU$^+$*

cells (14 d after injury, n = 3, 3–4 sections per animal, total of 277 BrdU$^+$ cells in DH

and 201 BrdU$^+$ cells in VH were counted) indicating that, 14 d after injury, when

microglial activation on the ipsilateral side reached its peak, ~20% of proliferating

cells derived from peripheral macrophages (data are presented as mean ± SEM). ***D,***

Photomicrographs showing the morphological plasticity of BM-derived cells over

time. Until they were recruited into the parenchyma, they were round/oval shaped on

the endothelium; shortly after their penetration (day 3 after injury), BM-derived

GFP$^+$ cells displayed few short branches with a large cell body; during days 7–14,

they developed into ramified cells; 30 d after injury, these infiltrated BM-derived cells

differentiated into highly ramified microglia (scale bar, 100 µm).

We next analyzed morphological changes in these infiltrated GFP$^+$ cells. Although

they had a round shape when lining the meninges, they developed broad and short

processes once they infiltrated the parenchyma (day 3). At later time points (days 7–30), most GFP$^+$ cells were highly ramified microglial cells with relatively small cell bodies, resembling their resident counterparts (Fig. 4C). Thus, newly recruited hematogenous macrophages invaded the spinal cord parenchyma proliferated and differentiated gradually into highly ramified microglia.

Role of CCR2 in mediating microglial chemotaxis in the spinal cord

To test the hypothesis that CCR2 is critical in resident microglial activation and peripheral macrophage infiltration, we first compared Iba-1 immunofluorescence in sections of L4–L5 spinal cord taken from wild-type (C57BL/6) and CCR2-deficient (CCR2KO) mice. Although nerve injury induced a striking increase of Iba-1 immunoreactivity in the DHi and VHi of wild-type (C57BL/6) mice at day 14 after nerve injury, such an increase was almost completely abolished in CCR2KO mice (Fig. 5A,B). This finding suggests that CCR2 expression is necessary for both activation of resident microglia and chemotaxis of BM-derived cells after peripheral nerve injury.

Chemotaxis of BM-derived cells may, however, occur secondarily to activation of resident microglia expressing CCR2. To test for this possibility, we next generated two other groups of chimeric mice by transplanting BM cells collected from GFP transgenic mice into irradiated CCR2KO mice (central CCR2KO chimera) and by

transplanting CCR2KO bone marrow cells into irradiated GFP transgenic mice (peripheral CCR2KO chimera). The expression pattern of Iba-1 was similar in wild-type non-irradiated mice and GFP chimeric mice, showing that irradiation and bone marrow cell transplantation did not modify the ability of resident microglia and BM-derived cells to respond to nerve injury (Fig. 5A). We quantified the mean intensity of Iba-1 immunoreactive signal in defined regions of the dorsal and ventral horns, in which microglial activation was considered to be the most prominent. All groups of chimeric mice exhibited significant differences in Iba-1 staining between ipsilateral and contralateral sides after injury (Fig. 5B). In addition, the Iba-1 signal was significantly lower on the side ipsilateral to the injury in both groups of CCR2KO chimeric mice when compared with the ipsilateral side in GFP chimeric mice (Fig. 5A,B).
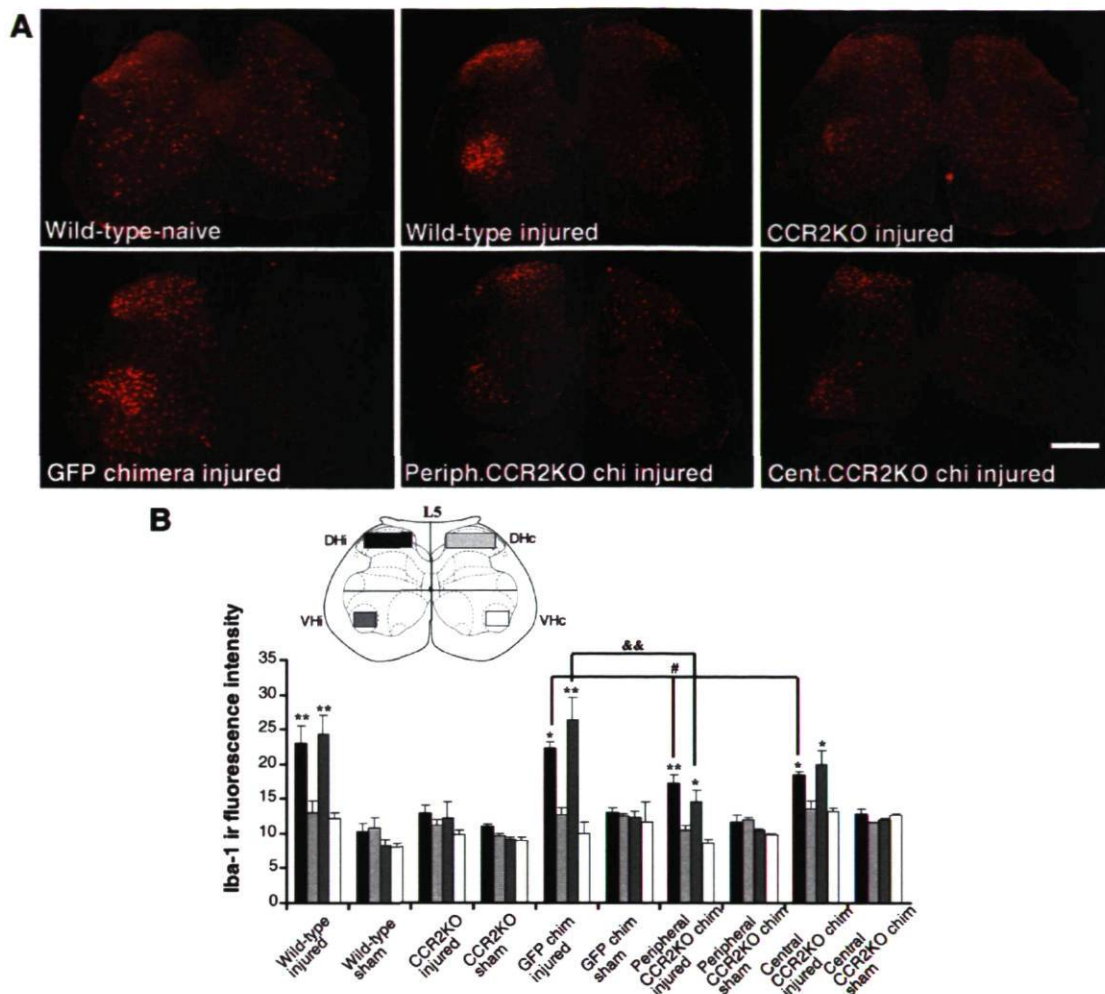
Figure 5. *Role of CCR2 in mediating microglial chemotaxis in the spinal cord.* ***A,***
Photomicrographs depicting representative Iba-1-positive cells in mouse L5 regions
14 d after the sham operation or injury. Partial sciatic nerve ligation induced a
striking increase of Iba-1 immunoreactivity (ir) in the ipsilateral side DH/VH in wild-
type C57BL/6 mice; this increase in Iba-1 immunoreactive signal was almost
completely abolished in CCR2KO mice, whereas both groups of CCR2KO chimeric
mice exhibited partial attenuation when compared with GFP chimeric mice after
sciatic nerve ligation (scale bar, 1 mm). ***B,*** *Intensity of Iba-1 signal determined as the*
average pixel intensity on specific regions of interest on L5 sections at 14 d after

injury (4–6 sections per mouse, 4 mice per group; data are expressed as mean ±

SEM; *p < 0.05, **p < 0.01, ipsilateral vs contralateral; #p < 0.05,

peripheral/central CCR2KO DHi vs GFP DHi; &&p < 0.01, peripheral CCR2KO VHi

vs GFP VHi).

The same result was obtained by counting Iba-1^{+} cells (Table 1). When CCR2 was

absent in the periphery, the decrease in Iba-1^{+} cell number (Table 1, *A* minus *C*)

corresponded to that of infiltrated cells in the GFP chimeric mice (Table 1, *B*).

Similarly, when CCR2 was absent in the CNS, the difference in Iba-1^{+} cell number

between central CCR2 KO and total CCR2KO (Table 1, *D* minus *E*) corresponded to

the number of infiltrated cells in the GFP chimeric mice (Table 1, *B*). The loss in Iba-

1 staining in each condition thus reflected the contribution of activated resident

microglia and bone marrow-derived infiltrated macrophages, respectively. The results

thus indicate that CCR2 expression is not only necessary for the activation of resident

microglia but is also directly responsible for recruitment of BM-derived cells to the

CNS.

Table 1. Quantification of Iba-1$^+$ cell numbers in the lumber spinal cord of mice with different CCR2 genetic background 14 d after peripheral nerve injury

	GFP chimeric mice		Periph. CCR2KO mice	Cent. CCR2KO mice	CCR2KO mice
	A. Iba-1+ cells	B. GFP+ cells	C. Iba-1+ cells	D. Iba-1+ cells	E. Iba-1+ cells
DHi	59.7±2.75	16±4.72	41.65±2.09**	42.50±3.63**	27.72±2.1**
DHc	25.2±3.83	3±0.26	22.80±1.56	20.90±3.53	23.78±1.33
VHi	56.55±6.03	21.3±8.1	33.05±4.37**	42.20±2.42*	26.02±3.56**
VHc	28.65±2.95	4±1.67	26.80±0.43	25.40±2.92	26.94±0.98

The number of Iba-1$^+$ microglia was determined in four regions (DHi, DHc, VHi, and VHc) of the spinal cord (4–6 sections per mouse, 4 mice per group) in four groups (GFP chimeric, peripheral CCR2KO chimeric, central CCR2KO chimeric, and CCR2KO) of mice at 14 d after injury. Note that, on the ipsilateral side, the number of Iba-1$^+$ cells was significantly less in mice lacking peripherally, centrally, or totally CCR2 receptor than that in GFP chimeric mice (data are expressed as mean ± SEM; *p < 0.05, **p < 0.01, peripheral, central, and total CCR2KO DHi/VHi vs GFP DHi/VHi, respectively).

MCP-1 is the trigger for macrophage infiltration and activation of resident microglia via its cognate receptor CCR2

To identify the ligand that activated the CCR2 receptor, we injected intrathecally rmMCP-1 in wild-type and in CCR2KO mice. Exogenous MCP-1 induced an increase in the size of microglial cell bodies as shown by Iba-1 immunostaining in intact wild-type mice, and these changes were abolished in CCR2-deficient mice (Fig. 6A). In addition, we injected intrathecally an antibody against mouse MCP-1 in GFP chimeric mice having nerve injury to verify whether neutralization of MCP-1 could prevent peripheral macrophage infiltration. In four of seven animals, GFP$^+$ cell infiltration was completely abolished. Overall, the number of ramified GFP$^+$ cells was significantly

reduced in mice injected with the MCP-1 neutralizing antibody compared with mice that received control saline injection (Fig. 6B,C).
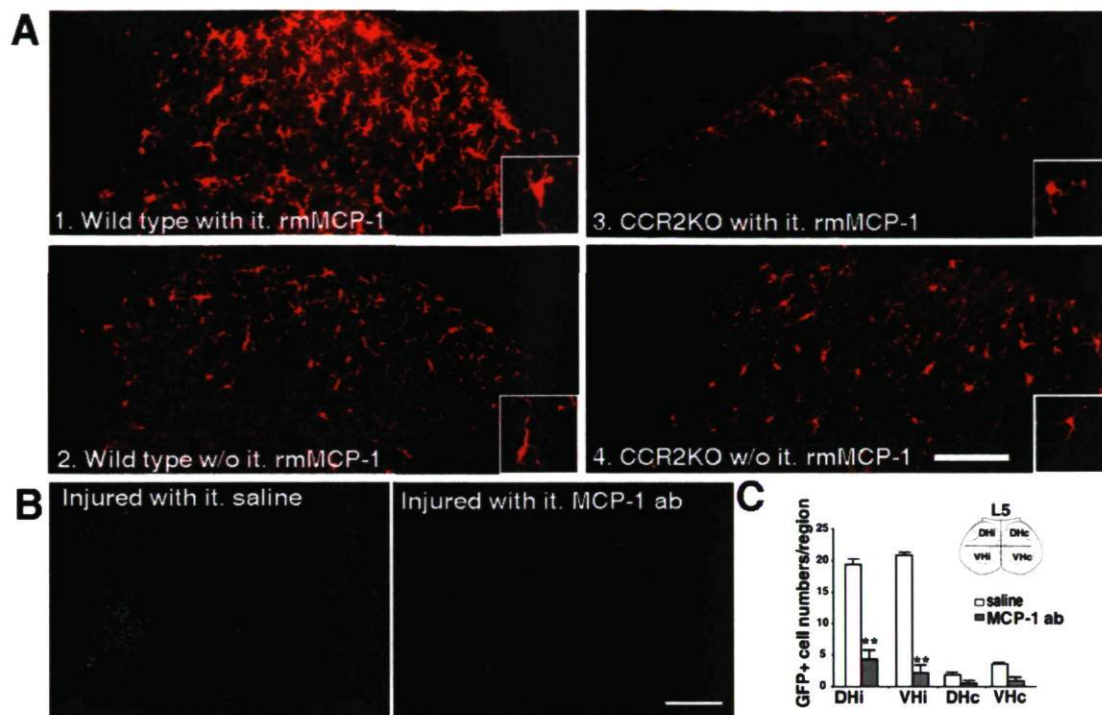

Figure 6. *Role of MCP-1 in stimulating resident microglial activation and peripheral macrophage infiltration via its cognate receptor CCR2. **A**, Intrathecal (it) delivery of rmMCP-1 (3 injections, 2 μg in 10 μl of saline per injection over 6 d) stimulated microglia in the spinal cord of intact wild-type mice as seen by the increase in size of their cell bodies (**A1**) when compared with that in mice which did not receive MCP-1 stimulation (**A2**). These changes were not observed in CCR2-deficient mice: microglial size and shape remained similar in CCR2KO mice either treated (**A3**) or not (**A4**) with MCP-1 (scale bar, 200 μm). Note the morphological changes highlighted in the insets. **B**, Infiltrated ramified GFP+ cells were no longer present in*

the ipsilateral side DH and VH of injured mice treated with MCP-1 antibody (ab).

Only few elongated and scattered GFP⁺ cells were found in blood vessels, although

the number of meningeal GFP⁺ cells significantly increased (scale bar, 1 mm). ***C***,

Quantitative analysis of GFP⁺ cells in the spinal cord of GFP chimeric mice after

sciatic nerve injury and MCP-1 neutralization (data are shown as mean ± SEM; n = 7

mice/group; **p < 0.01, mice treated with MCP-1 antibody vs mice treated with

saline). MCP-1 antibody significantly reduced the number of GFP⁺ cells within the

spinal cord parenchyma.

CCR2 in either CNS microglia or in bone marrow-derived macrophages is sufficient for the development of mechanical allodynia

Development of mechanical hypersensitivity (allodynia) is a clinically relevant characteristic of nerve injury. To address the relationship between mechanical allodynia and the chemotaxis of resident and BM-derived microglia, we measured paw-withdrawal threshold to mechanical stimuli in all animals before and after injury. Before nerve injury, the withdrawal threshold was not affected by the CCR2 gene deletion nor by the irradiation and bone marrow cell transplantation (Fig. 7). Wild-type C57BL/6 mice showed a robust decrease in withdrawal threshold from 0.35 ± 0.01 g before surgery to 0.09 ± 0.01 g ($p < 0.01$) at day 3 after surgery and maintained this hypersensitivity to the end of the testing period (day 14) (Fig. 7*A*). Mechanical allodynia was significantly attenuated in CCR2KO mice (Fig. 7*B*) and in GFP

chimeric mice treated with MCP-1 antibody (58 ± 3.8% reduction compared with saline treated mice at day 12 after injury; $n = 7$ per group), indicating that MCP-1/CCR2 signaling plays a critical role in the development of the hypersensitivity. In contrast, however, neither selective peripheral CCR2KO nor selective central CCR2 KO mice had their allodynia significantly attenuated (Fig. 7D,E). This result indicates that expression of CCR2 in either resident or BM-derived cells is sufficient for the development of mechanical allodynia after peripheral nerve injury.
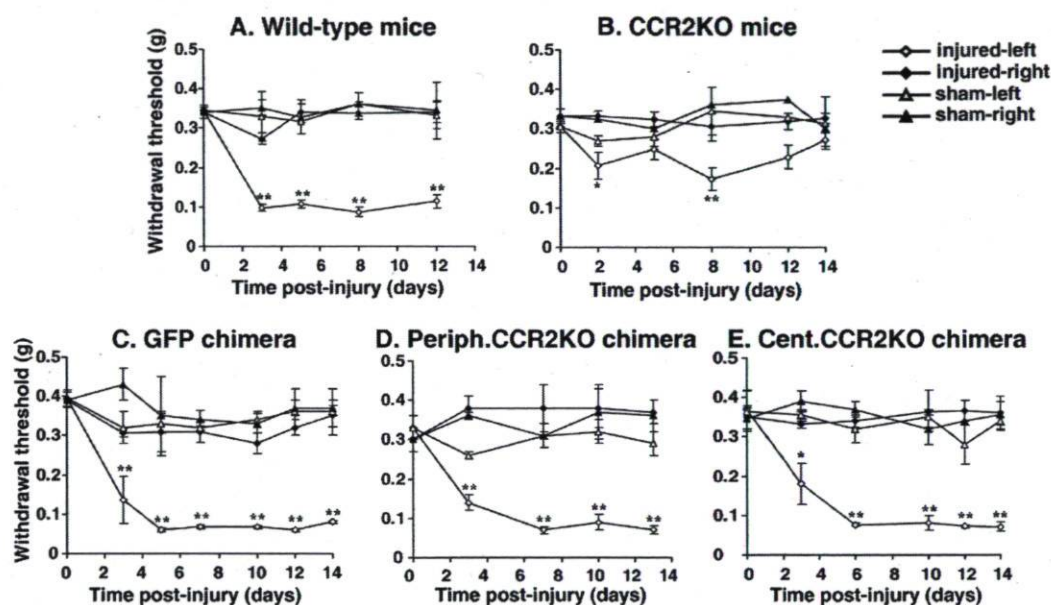

Figure 7. *Mechanical allodynia in response to partial sciatic nerve ligation. Injured paw-withdrawal thresholds decreased from baseline (~0.35 g) to below 0.1 g in all groups, except for CCR2KO mice. Significant decrease in withdrawal threshold occurred in all chimeric groups, indicating that CCR2 expression in either resident or bone marrow-derived microglia is sufficient to cause mechanical allodynia. Data are shown as mean ± SEM; *p < 0.05; **p < 0.01; n = 4–6 mice per group. Baseline data (day 0) was obtained by an average of two measurements, 1–2 d before surgery.*

2.5 DISCUSSION

Here we demonstrate that BM-derived macrophages have the ability to infiltrate the spinal parenchyma after peripheral nerve injury. Interestingly, in contrast to spinal cord injury, the blood–spinal cord barrier (BSCB) remains physically intact after peripheral nerve injury, yet our results show that chemotaxis occurs across the BSCB. These infiltrated macrophages proliferate and differentiate into microglia and, together with their resident counterparts, contribute to CNS microgliosis in response to peripheral nerve injury. We reported previously that MCP-1, the endogenous ligand for CCR2 receptors, is produced by injured neurons (Zhang and De Koninck, 2006◆). In the current study, we demonstrated that exogenous MCP-1 could induce spinal microglial activation and this activation is lost in CCR2KO mice. In addition, neutralization of MCP-1 prevented peripheral macrophage infiltration after nerve injury. Together, these findings imply a neuron-to-microglia and neuron-to-macrophage signaling mechanism underlying the central component of neuropathic pain pathogenesis. The fact that both resident and BM-derived microglia participate in the development of the pathology has direct clinical importance. Inhibiting either resident microglia or BM-derived macrophages may not be an effective approach to relieve neuropathic pain.

Recruitment of circulating leukocytes into the CNS in normal physiological conditions and in pathological states supports the essential functions of

immunosurveillance and host defense. Although the molecular signals and detailed mechanisms responsible for the migration of specific inflammatory cells into the CNS compartment are not completely identified, accumulating evidence suggests that chemokines, in concert with adhesion molecules, are essential for the process (Charo and Ransohoff, 2006♦). MCP-1, identified originally as monocyte, memory T lymphocytes and NK cell-specific chemoattractant (Valente et al., 1988♦; Yoshimura et al., 1989♦) has been attributed a key role in regulating the infiltration of monocytes during inflammation. MCP-1 knock-out mice exhibited deficient monocyte recruitment in experimental autoimmune encephalomyelitis (Lu et al., 1998♦; Huang et al., 2001♦). Entorhinodentate axotomy induces leukocyte infiltration in the denervated hippocampus (Bechmann et al., 2005♦; Ladeby et al., 2005♦) in which induced MCP-1 expression by glial cells has been considered as critical in directing leukocytes to sites of axonal injury in the CNS (Babcock et al., 2003♦). It is interesting to note that, although numerous GFP cells were found in multiple regions of normal brain (the current study; data not shown) (Vallieres and Sawchenko, 2003♦; Simard and Rivest, 2004♦), such a process remains rare in the spinal cord of intact mice. However, bone marrow-derived cells infiltrated massively the affected regions ipsilateral to the peripheral nerve damage. This chemotaxis is dependent on MCP-1, because MCP-1 antibody treatment successfully reduced the number of infiltrated cells. This is also dependent on the MCP-1 receptor CCR2, because CCR2-deficient mice no longer exhibited such an accumulation of microglial cells. MCP-1 has the ability to alter expression of tight junction-associated proteins in endothelial cells of

the brain vascular system (Stamatovic et al., 2003*; Song and Pachter, 2004*), which results in a local and temporary increase of BSCB permeability (Gordh et al., 2006*). This may explain why MCP-1 and CCR2 play such a critical role in such a cell influx in the affected spinal cord. We then took advantage of this model to determine the respective contribution of BM-derived versus resident microglia in generating chimeric mice and found that both types of cells participate in this process. MCP-1 production by damaged neurons after peripheral nerve injury (Zhang and De Koninck, 2006*) may then trigger chemotaxis through its cognate receptor CCR2 expressed in resident and bone marrow-derived microglia.

In response to peripheral nerve injury, spinal glial cells, especially microglia, proliferate (Echeverry et al., 2007*). The results from the current study showed that both resident cells and blood-derived microglia retain their capacity to divide, in which 20% of proliferating cells derived from peripheral macrophages. We also observed that infiltrated blood-borne cells can differentiate into highly ramified Iba-1^{+} resident microglia but not in any other types of cells at all tested time points after injury (days 3–30). The potential plasticity of hematopoietic stem cells raised the questions on the trans-lineage differentiation. Several independent groups have provided evidence that bone marrow-derived cells participate in adult neurogenesis and angiogenesis by giving rise to neurons (Mezey et al., 2000*; Priller et al., 2001*), endothelial cells (Bailey et al., 2006*), and astrocytes (Kopen et al., 1999*). Consistent with some other studies (Simard and Rivest, 2004*; Massengale et al., 2005*), our findings indicated that bone marrow-derived stem cells and their progeny

maintain lineage fidelity within the spinal cord parenchyma in the pathology of neuropathic pain induced by peripheral nerve injury. The discrepancy may be explained by technical problems (specificity of cellular markers and sensitivity of histochemical methods) but most likely the difference may result from anatomical distributions, because the BM-derived cells exhibiting the characteristic morphology of cerebellar Purkinje neurons has been observed more frequently (Priller et al., 2001✦; Wright et al., 2001✦) and also from different pathophysiological conditions.

The contribution of glia and glia–neuron communication in enhancing nociceptive transmission has been well documented. Every animal model of nerve injury-induced exaggerated pain is associated with the activation of glia within the pain-responsive regions of the spinal cord (Tsuda et al., 2005✦). Such exaggerated pain states are mediated by glial activation, because they are blocked by drugs (e.g., fluorocitrate and minocycline) that block glial activation (Milligan et al., 2003✦; Raghavendra et al., 2003✦), by selective proinflammatory cytokine antagonists (Sweitzer et al., 2001✦), and by disrupting proinflammatory cytokine signaling pathway (Sweitzer et al., 2004✦). We revealed in this study that nerve injury induced microglial activation comprises the activation of preexisting resident microglia, as well as the recruitment of BM-derived peripheral macrophages. Of important impact is that both populations are involved in the central component of sensitization to enhance spinal neuronal excitability by dynamic glial modulators, such as ATP and BDNF (Tsuda et al., 2003✦; Coull et al., 2005✦). Either resident microglia (central sensitization) or peripheral macrophages (central sensitization by infiltration into the spinal cord and

peripheral sensitization by their activity at the injured site) is sufficient to cause the neuropathic pain. The fact that Iba-1 staining was significantly reduced in both central and peripheral CCR2KO chimeric mice (compared with GFP chimeric mice), while neuropathic pain behavior remained identical in all three lines of mice, suggests that there may be a floor effect on the behavior, i.e., that the amount of microglia activation in the wild type is supramaximal, in other words, more than enough to produce a full effect. Rutkowski et al. (2000)♦ demonstrated that mechanical allodynia was not altered by either deactivation of macrophages with CNI-1493 or by depletion of circulating macrophages using lisosome-encapsulated clodronate before peripheral nerve injury. They concluded that macrophages have limited role in generation of nerve injury-induced mechanical allodynia. It was also reported that macrophage depletion by intravenous injection of liposome-encapsulated clodronate reduced the number of macrophages in the injured nerve and alleviated slightly thermal hyperalgesia (Liu et al., 2000♦). The discrepancy with our results may stem from differences in the behavioral outcomes measured (mechanical allodynia vs thermal hyperalgesia). In light of our current findings, we suggested that peripheral macrophages have significant contribution not only in peripheral but also in central sensitization. However, blockade of only circulating macrophages is not enough to attenuate hypersensitivity, because the involvement of spinal cord resident microglia in the central component is not negligible.

Our results not only implicated MCP-1 as a necessary mediator for spinal microglial activation, they point to the chemokine as being a major player for the development of

mechanical allodynia. These data are consistent with another study showing an essential role of CCR2 in mediating neuropathic pain in mice (Abbadie et al., 2003♦). We further revealed the critical role of CCR2 in peripheral macrophages and resident microglia through neuron-to-macrophages and neuron-to-microglia interaction in the genesis of ongoing neuropathic pain. In addition, MCP-1 was reported to depolarize sensory neurons after chronic compression of the dorsal root ganglion (White et al., 2005♦), which implicates a direct neuron-to-neuron interaction of the ligand with its receptor. Some other mediators for neuron-to-glia communication that could lead to glial activation and consequent enhancement of pain have been suggested. ATP activates glia and the release of proinflammatory cytokines (Hide et al., 2000♦; Shigemoto-Mogami et al., 2001♦). Mice lacking either purinergic $P2X_4$ or $P2X_7$ receptors show an impaired ability to develop neuropathic pain (Tsuda et al., 2003♦; Chessell et al., 2005♦). The chemokine fractalkine has also been suggested to contribute to the hypersensitivity evoked by nerve injury through microglial activation (Verge et al., 2004♦). Mice that bear a mutation in the gene encoding toll-like receptor 4 (TLR4) have a reduced microglial activation and do not develop thermal and mechanical allodynia after peripheral nerve injury (Tanga et al., 2004♦). Heat shock protein 27 was proposed to be the potential endogenous ligand for TLR4 (Costigan et al., 1998♦). Although considerable redundancy may exist, microglial activation and recruitment through MCP-1/CCR2 seem a critical component for the pathogenesis of neuropathic pain.

The ability of macrophages to infiltrate and differentiate into fully functional microglia in the CNS parenchyma opens the door for new therapeutic strategies to treat neuropathic pain. Indeed, these cells are innately and specifically attracted to injured and diseased regions and may thus be used as effective and selective vehicles to deliver molecules that prevent neurodegeneration (Scholz et al., 2005♦), eliminate toxic proteins or pronociceptive signaling molecules (Shubayev and Myers, 2002♦; Coull et al., 2005♦), or deliver anti-inflammatory agents (Ledeboer et al., 2007♦). BM-derived cells will be able to follow the chemoattracting gradient provided by the distressed neurons (e.g., MCP-1) early after injury and specifically in the affected region and deliver the necessary molecules to limit microgliosis and neuronal hypersensitivity. A similar approach can be envisaged to treat neurodegenerative diseases, such as Alzheimer's disease (Simard et al., 2006♦), amyotrophic lateral sclerosis (Solomon et al., 2006♦), prion disease (Priller et al., 2006♦), and multiple sclerosis (Raivich and Banati, 2004♦) in which BM-derived cells massively infiltrate the affected regions.

2.6 Acknowledgements

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Chapter III

CONCLUSION AND FUTURE DIRECTIONS

Our results revealed that, after peripheral nerve injury, the activated spinal microglia is not only from the cells existed in the CNS, but also that of the population of cells from the bone marrow like macrophages in periphery. Those two populations of microglia (*i.e.*, resident microglia and peripheral macrophages) participate in the reaction of spinal microglia after partial ligation of the sciatic nerve. Bone marrow derived macrophages have the ability to infiltrate into the spinal cord parenchyma, proliferate and differentiate into fully functional microglia, like resident microglia. The expression of CCR2 in resident microglia and peripheral macrophages is necessary for the development of the neuropathic. The MCP-1/CCR2 system plays a critic role acting as a trigger for microglial activation and MCP-1 not only activates resident microglia, but also triggers the infiltration of circulating monocytes. To efficiently treat the illness like neuropathic pain, the resident microglia in the CNS and the macrophages derived from the bone marrow need to be targeted.

The blood-brain barrier (BBB) and the blood-spinal cord barrier (BSCB) strongly contribute to the immune privileged status of the CNS. In normal physiological conditions, only small, lipid-soluble molecules can freely diffuse through the capillary endothelial membrane connected between themselves by the tight junctions. Consequently, BBB/BSCB is an obstacle for CNS therapy and a serious bottleneck in drug development for CNS disease. Our study demonstrated that following peripheral nerve injury, not only resident microglia become activated,

under the guidance of the chemoattractant (e.g., MCP-1) gradient provided by the injured neurons, bone marrow derived-macrophages have the ability to infiltrate into the CNS parenchyma and differentiate into fully functional microglia, which could reach up to 30-40% of total microglia in affected regions. In the future, we will examine the possibility to use bone marrow-derived microglia/macrophages as cellular vehicles to deliver analgesic agents into affected spinal cord dorsal horn and around injured nerve to control neuropathic pain.

The expected results stem from the current study and some of our future experiments by using bone marrow derived macrophages as cellular vehicles to deliver anti-inflammatory agents directly into the spinal cord will provide a useful and exciting new drug delivery technology and also a new concept by targeting glial cells in the treatment of neuropathic pain. A similar approach can be envisaged to treat other neurodegenerative diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis, prion disease, and multiple sclerosis in which BM-derived cells massively infiltrate the affected regions.

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