NORTHWSTERN UNIVERSITY

Mechanisms Underlying Chemokine Signaling-Mediated Hypersensitization of Primary Sensory Neurons in Association with Neuropathic Pain

A DISSERTATION

SUBMITTED TO THE GRADUTE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

for the degree

DOCTOR OF PHILOSOPHY

Field of NEUROSCIENCE

By

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EVANSTON, ILLINOIS

December 2008

ABSTRACT

Mechanisms Underlying Chemokine Signaling-Mediated Hypersensitization of Primary Sensory Neurons in Association with Neuropathic Pain

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Pain normally subserves a vital role in the survival of the organism by prompting the avoidance of situations associated with tissue damage. However, the sensation of pain can become dissociated from its normal physiological role when the pain-sensing nervous system becomes hypersensitive, a condition known as neuropathic pain. Currently available treatment options for neuropathic pain are generally ineffective, but the development of novel therapies is hampered by our incomplete understanding of the underlying mechanisms. Despite its complex pathophysiology, it is clear that neuropathic pain is associated with changes in the excitability of the primary sensory neurons of dorsal root ganglia (DRG), which are caused by long-term changes in gene expression in these neurons.

Chemokines are a family of small secreted proteins originally identified by their chemoattractant activity in the immune system. Recent evidence has suggested that upregulated expression of one chemokine, monocyte chemoattractant protein-1 (MCP1, also known as CCL2), and its receptor, CCR2, in DRG neurons may be an important step in generating neuropathic pain. In this thesis, the molecular mechanisms underlying i) neuronal sensitization by activation of CCR2-mediated signaling, and ii) upregulation of MCP1 and CCR2 gene expression in DRG neurons were

investigated. To this end, novel transgenic reporter mice were generated in which MCP1 and CCR2 can be simultaneously visualized *in vivo*. These mice allowed examination of the dynamic changes in cellular localization of active MCP1-CCR2 signaling in neuropathic pain as well as in other neuropathological conditions. The results of this study will provide valuable insights into identification of novel therapeutic targets to treat neuropathic pain.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Professor Richard J. Miller whom I sincerely respect as a scientist, a teacher, and a person.

I would also like to express my sincere gratitude to the members of my thesis committee, Professors Fletcher A. White, Warren G. Tourtellotte, and Jaime García-Añoveros for their scientific guidance as well as emotional support throughout the course of this study.

I thank the current and former members of the Miller laboratory: Dr. Peter Toth, Dr. Bula Bhattacharyya, Dr. Abdelhak Belmadani, Dr. Ghazal Banisadr, Dr. Lori Tran, Dr. Sonia Bhangoo, Dr. Dongjun Ren, Dr. Wenan Qiang, Dr. Huan Ngo, Divakar Mithal, Caroline Freitag, and Sarah Kay Welch. In particular, I thank Dr. Toth for his help in imaging experiments, Dr. Qiang for his advice on the NFκB inhibitor experiments, Dr. Bhangoo for her help in the LPC experiments, Dr. Banisadr for her help in the EAE experiments, Ms. Welch for her help in genotyping experiments, and Ms. Freitag for her thorough editing of this thesis.

I would also like to express my earnest gratitude to Professor Kyungjin Kim who trained and encouraged me to become a scientist.

I thank my parents and my sister whose support enabled me to continue this study.

Finally, I thank my wife who makes what I do more meaningful.

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CHAPTER 1

Introduction

Chemokines are a family of chemotaxic cytokines originally identified by their roles in the orchestration of leukocyte trafficking. Chemokines contain an N-terminal signal peptide (19-21 amino acids) which is cleaved during maturation, and mature chemokines are relatively small ranging from 8 to10 kDa in molecular weight. More than 50 chemokines have been identified so far.

They can be classified into four families according to the geometry of two conserved cysteine residues at their N termini: CXC (alpha), CC (beta), XC (gamma) and CX3C (delta) families (Table 1). For example, the first two cysteines of alpha chemokines are separated by a single amino acid, and therefore they are called CXC chemokines. Their cognate receptors are called CXC chemokine receptors or CXCRs (R for receptor). The first two cysteines of beta chemokines are adjacent, and therefore they are called CC chemokines. Their receptors are CC chemokine receptors or CCRs. Gamma, or XC, chemokines have only one cysteine, and their receptors are called XCRs. There is only one member (fractalkine) of the delta chemokine family, and its first two cysteines are separated by three amino acids. Therefore, it is called a CX3C chemokine (CX3CL1, L for ligand) and its receptor is CX3CR1.

Each chemokine has not only a systematic name (e.g. CX3CL1) but also a conventional name (e.g. fractalkine). More than 20 chemokine receptors have been identified thus far, and all belong to the G protein-coupled receptor (GPCR) family. All known functions of chemokines are exerted through these receptors. As there are fewer receptors than ligands, ligand-receptor interactions, at least those studied *in vitro*, are often promiscuous. However, many chemokine receptors have a major ligand to which they bind most *in vitro* and/or preferentially *in vivo*.

Receptor	Chemokines: systematic name (conventional name)
CC chemokine receptors	
CCR1	CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL13 (MCP-4)
CCR2	CCL2 (MCP-1), CCL7 (MCP-3), CCL8 (MCP-2), CCL12 (MCP-5), CCL13 (MCP-4)
CCR3	CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL11 (Eotaxin), CCL13 (MCP-4), CCL15 (MIP-5), CCL24 (Eotaxin-2), CCL26 (Eotaxin-3)
CCR4	CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CCL17 (TARC), CCL22 (MDC)
CCR5	CCL3 (MIP-1α), CCL5 (RANTES), CCL8 (MCP-2), CCL14 (MIP-1β)
CCR6	CCL20 (Exodus-1/LARC/MIP-3α)
CCR7	CCL19 (Exodus-3/ELC/MIP-3β), CCL21 (Exodus-2/SLC/6Ckine)
CCR8	CCL1 (I-309), CCL17 (TARC), CCL19 (Exodus-3/ELC/MIP-3β)
CCR9	CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CCL8 (MCP-2), CCL11 (Eotaxin), CCL13 (MCP-4), CCL14 (MIP-1β), CCL25 (TECK)
CCR10	CCL27 (CTACK), CCL28 (MEC)
CCR11	CCL2 (MCP-1), CCL19 (Exodus-3/ELC/MIP-3β), CCL21 (Exodus-2/SLC/6Ckine), CCL25 (TECK)
CXC chemokine receptors	
CXCR1	CXCL6 (GCP-2), CXCL8 (IL-8)
CXCR2	CXCL1 (GRO-α) CXCL2 (GRO-β), CXCL3 (GRO-γ), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL7 (NAP-2), CXCL8 (IL-8)
CXCR3	CXCL10 (IP-10), CXCL9 (MIG), CXCL11 (ITAC)
CXCR4	CXCL12 (SDF-1)
CXCR5	CXCL13 (BLC)
CXCR6	CXCL16
CXCR7	CXCL11 (ITAC), CXCL12 (SDF-1)
CX3C chemokine receptor	
CX3CR1	CX3CL1 (Fractalkine)
XC chemokine receptor	
XCR1	XCL1 (Lymphotactin), XCL2 (SCM-1b)

Table 1. Chemokine receptor selectivity

Chemokine Signaling and the Development of the Nervous System

Although the functions of chemokines and their receptors were well studied in the immune system, their roles in the nervous system were not initially appreciated. However, studies during the last two decades have made it clear that chemokine signaling plays various roles in the nervous system. The first observation indicating that chemokines might have effects in the nervous system came from investigations of human immunodeficiency virus-1 (HIV-1) neuropathogenesis. Gp120, the coat protein of HIV-1 which binds CD4 in combination with either chemokine receptor CCR5 or CXCR4, was found to produce apoptosis of cultured neurons (Brenneman et al. 1988). This study suggested the presence of 'gp120 receptors' on neurons although the true identity of these receptors was unknown.

In fact, the expression of chemokine receptors by neurons was not generally appreciated until the late 1990's when several independent groups reported their expression in neuronal cell lines (Hesselgesser et al. 1997), cultured neurons (Meucci et al. 1998; Ohtani et al. 1998), and brain slices (Horuk et al. 1997; Xia et al. 1997). After further studies, chemokine signaling began to emerge as a key regulator of neuronal development (Zou et al. 1998) as well as neuronal survival (Meucci et al. 1998) and neural communication (Giovannelli et al. 1998).

The concept of chemokine signaling functioning beyond the control of leukocyte trafficking is perhaps best understood from an evolutionary point of view. The more than 50 identified chemokines exert all of their functions via the activation of GPCRs (Tran and Miller 2003). During the course of evolution, the expansion of the chemokine family to over 50 members

paralleled the development of complex immune systems in higher vertebrates (Huising et al. 2003). However, the advent of the chemokine family antedates the emergence of the immune system. Indeed, homologues of a chemokine, stromal cell-derived factor -1 (SDF1, also known as CXCL12), and its receptor, CXCR4, are found in animals that do not possess an immune system (Huising et al. 2003).

The conserved functions of CXCR4 signaling are well documented from the studies of CXCR4 (Tachibana et al. 1998; Zou et al. 1998) and SDF1 (Ma et al. 1998; Nagasawa et al. 1996) knockout mice and null mutant zebrafish (Knaut et al. 2005). The phenotypes of SDF1 and CXCR4 knockout mice are very similar. This is consistent with the generally accepted idea that CXCR4 is the only receptor for SDF1. However, it should be noted that a recently discovered chemokine receptor, CXCR7, does bind to SDF1, suggesting that it may mediate unknown functions of SDF1 (Miao et al. 2007; Schonemeier et al. 2008; Sierro et al. 2007; Valentin, Haas, and Gilmour 2007).

The ancient evolutionary origins of CXCR4 suggest vital roles for CXCR4-mediated signaling. Indeed, knockout mice of CXCR4 or SDF1 show severe defects in the development of diverse tissues (Zou et al. 1998; Nagasawa et al. 1996; Tachibana et al. 1998). Further research has shown that SDF1 and CXCR4 are expressed in a temporally and spatially controlled manner in these tissues. In the nervous system, CXCR4 is expressed by neural progenitor cells destined to develop into various parts of the central and peripheral nervous system. CXCR4-mediated signaling not only guides them to sources of SDF1 but also controls their proliferation (Tran and Miller 2003). Accordingly, disruption of SDF1 or CXCR4 results in the abnormal formation of

many structures including the cerebellum, the dentate gyrus of the hippocampus, and the dorsal root ganglia (Lu, Grove, and Miller 2002; Zou et al. 1998; Belmadani et al. 2005; Bagri et al. 2002; Knaut et al. 2005).

Chemokine Signaling in the Adult Nervous System

Chemokine receptors are not only expressed by neural progenitors during development but also by cells of the adult nervous system, suggesting that their roles may extend beyond the control of neural progenitor cell migration (Tran and Miller 2003). Generally speaking, chemokine receptors can be expressed by neural progenitor cells in the neurogenic regions of the adult brain and by mature neurons and glia of the central and peripheral nervous systems.

For example, CXCR4 receptors are expressed in the subventricular zone of the lateral ventricle, the olfactory bulbs, and the subgranular zone of the hippocampal dentate gyrus (DG) – all neurogenic regions of the adult brain (Tran et al. 2007; Berger et al. 2007; Banisadr et al. 2003; Stumm et al. 2002), suggesting that they play roles in the control of adult neurogenesis. This idea was recently confirmed in studies of DG neurogenesis (Bhattacharyya et al. 2008; Kolodziej et al. 2008). In the adult DG, CXCR4 is expressed by immature progenitor cells that give rise to neurons in the granular layer, and also by the immediate progeny of these progenitor cells including rapidly amplifying cells, neuroblasts, and immature granule neurons. CXCR4's ligand, SDF1, is also expressed by neurons in the vicinity (Banisadr et al. 2003; Stumm et al. 2002). In the adult DG, it is thought that the ongoing level of neurogenesis must be coordinated to the level of neural activity such that the number of granule cells changes according to ongoing need – a

process termed ‘excitation-neurogenesis coupling’ Bhattacharyya et al.’s research also showed that the action of GABA is critical to this process.

GABA generally produces hyperpolarization and inhibitory responses in mature neurons by activating a Cl channel (GABA-A receptor). In immature neurons and progenitor, however, GABA produces depolarization and an excitatory effect because these cells have a high concentration of intracellular Cl due to differentiation stage-specific expression of a Na-K-2Cl transporter, NKCC1 (a Cl importer) (Ge et al. 2006; Tozuka et al. 2005). In the neurogenic area of the DG, tonic release of GABA from basket cells induces tonic activation of GABA-A receptors in the progenitor cells promotes their differentiation into mature granule cells. Many factors which are know to modulate the rate of DG neurogenesis affect GABAergic transmission to progenitor cells.

Indeed, SDF1-CXCR4 signaling promotes DG neurogenesis by positively modulating the GABAergic inputs to these progenitor cells. Recent studies have demonstrated that SDF1 is stored in vesicles in the nerve terminals of DG neurons including GABAergic basket cells, and that SDF1 cooperates with GABA to regulate the differentiation of neural progenitors to granule neurons (Bhattacharyya et al. 2008).

An important implication of these studies is that SDF1 can act as a neurotransmitter, and this idea can be extended to other chemokines expressed by mature neurons. Unlike SDF1 which is constitutively expressed and released by basket cells, most chemokines are not expressed by cells of the nervous system at high levels under normal conditions. Rather, expression of most

chemokines is upregulated in association with the activation of the inflammatory response. Under circumstances where they are upregulated by mature neurons in response to various insults (Fryer et al. 2006; Schreiber et al. 2001), controlled release of chemokines by neurons is also observed. These include the expression of CCL21 in cortical neurons following injury (de Jong et al. 2005), and the expression of MCP1 by sensory neurons in association with neuropathic pain (Jung et al. 2008), which is the subject of this thesis.

As will be discussed in the next chapter, chemokines synthesized and released in these neurons appear to play a neuromodulatory role. Chemokine receptors can be expressed by neurons or microglia, suggesting that chemokine signaling may be important in communication between neurons (Jung et al. 2008) or between injured neurons and microglia (de Jong et al. 2005). In addition, because chemokines can also be upregulated by astrocytes and endothelia cells in injured tissues and chemokine receptors are expressed by neural progenitor cells and circulating leukocytes. Therefore, chemokine signaling is in a unique position to coordinate neuroinflammatory responses (de Jong et al. 2005) and subsequent repair processes (Belmadani et al. 2006).

The Perception of Pain

Primary sensory neurons are responsible for processing somatosensory information including touch, proprioception, temperature, and pain. They are a type of pseudounipolar neuron (i.e. possessing only axons but no dendrites) whose cell bodies are located in dorsal root ganglia (DRG) outside the central nervous system. A subpopulation of DRG neurons, known as primary

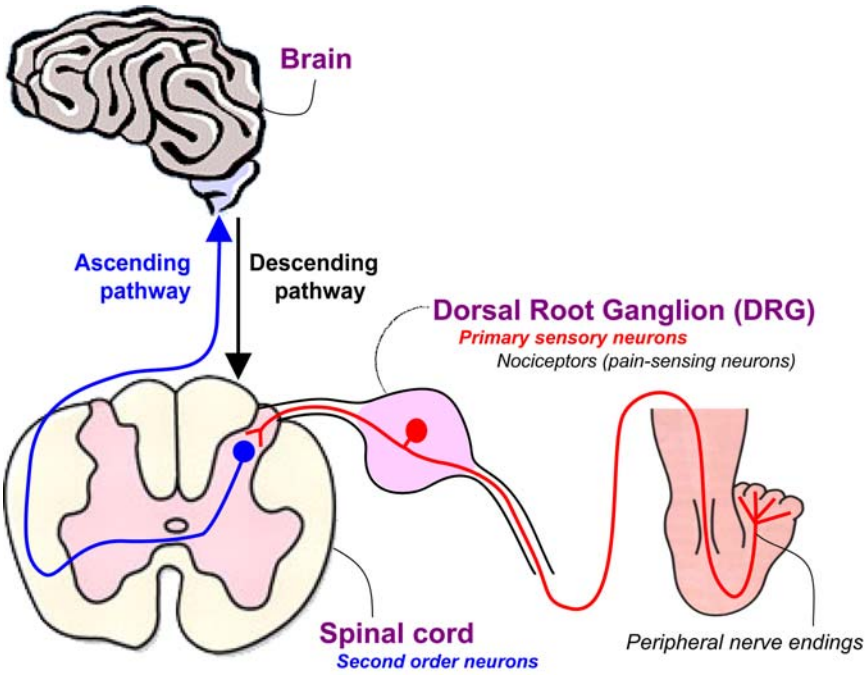


Figure 1.1. The pain pathway. The neural pathway of nociception begins with primary afferent neurons which project to the superficial lamina of the dorsal horn of the spinal cord. Second-order neurons in the dorsal horn convey noxious signals to the brainstem, midbrain, and thalamus. Finally, third-order neurons relay these electrical signals to the somatosensory/cingulate cortex and limbic system. Descending modulatory influences arrive in the spinal cord dorsal horn and are derived from the midbrain periaqueductal gray, the locus ceruleus, and the rostral ventromedial medulla.

nociceptors (*noci-* is derived from the Latin for "hurt"), are dedicated to detection of noxious stimuli (Figure 1.1). Primary nociceptors include fast-conducting, lightly myelinated Aδ fibers, which convey the initial stimulus of nociception (mechanosensitive or mechanothermal) and slow-conducting, unmyelinated C-fibers that transmit a less intense nociceptive sensation.

Primary nociceptors have both a peripheral process innervating receptive fields including skin and muscles, and a centrally projecting axon that conveys nociceptive information to second order neurons in the dorsal horn of the spinal cord. Information is then relayed through the thalamus to higher sensory and emotional centers in the brain. Primary nociceptors also give

input to spinal cord interneurons which then initiate the nociceptive withdrawal reflex prompting avoidance of noxious stimuli. Descending pathways originating in the cortex and the midbrain provide negative modulatory feedback signals at the level of the spinal cord. Finally, impulses generated in primary nociceptors can travel back along the peripheral axon to distal endings resulting in local release of neuropeptides in the injury environment. This neuropeptide release produces vasodilatation, venule permeability, plasma extravasation, edema, and leukocyte influx -a process termed 'neurogenic inflammation'.

Neuropathic Pain

Typically, the local response to various types of injury or infection involves the release of peripheral chemical mediators. These injury-associated factors have two functions. One role is to attract leukocytes to the point of injury as part of the inflammatory response (Charo and Ransohoff 2006). The second role is to sensitize the pain pathway. This sensitization occurs first in the periphery, by enhancing the response of primary nociceptors to painful stimuli (Zimmermann 2001). Then, the increased excitatory activity of these primary nociceptors results in greater transmitter release in the spinal cord, thus enhancing neuronal activity in pain pathways in the central nervous system, which is known as spinal or central sensitization (Woolf 1983).

Although pain clearly plays an important survival role in safeguarding the individual from potential sources of tissue damage, the perception of pain can also be the result of a dysfunctional nervous system. Under some circumstances , nociceptor-driven electrical activity

becomes divorced from normal injury-related pathophysiology, so that pain is produced in the absence of any appropriate stimulus (Campbell et al. 1988; Torebjork, Lundberg, and LaMotte 1992). This *hyper*sensitization is now known as pathological or "neuropathic" pain.

The causes of neuropathic pain vary. Traumatic injury to the nervous system, diseases (e.g. diabetes), cancer, viral infection (e.g. HIV-1), and the toxic side effects of diverse drug regimens (e.g. anti-cancer chemotherapy) are some well-known examples. The resulting symptoms range from mildly increased sensitivity to touch or temperature changes, to excruciating pain. Heightened pain sensitivity under these circumstances is often classified into two types: allodynia (pain evoked by a normally innocuous stimulus) and hyperalgesia (enhanced pain evoked by a noxious stimulus). Exactly what changes are responsible for pain hypersensitivity under these conditions is not well understood.

From a therapeutic point of view, neuropathic pain is an intractable problem. For example, cancer patients undergoing chemotherapy are sometimes forced to quit treatment after developing unbearable and untreatable pain in association with their therapy. Once established, neuropathic pain is not readily responsive to traditional anti-pain drugs such as non-steroidal anti-inflammatory drugs (NSAIDS). Moreover, although opiates may be used for acute or for chronic pain states (e.g., terminal cancer), alleviation of neuropathic pain is more problematic because high doses are often required, thus narrowing the therapeutic index (Hempenstall et al. 2005). The remaining available drugs used to treat these syndromes (tricyclic antidepressants, antiepileptics) are not particularly effective and are also associated with a number of negative side effects (Watson 2000).

It is estimated that about 7% of the population is affected by neuropathic pain and more than 70% patients describe their pain intensity as severe (Bouhassira et al. 2008). Novel therapies for neuropathic pain represent a great medical need. Therefore, it is imperative that researchers identify novel therapeutic targets by understanding the detailed molecular mechanisms underlying neuropathic pain syndromes.

Peripheral Nerve Injury and Inflammation

One important development in our understanding of the cellular and molecular processes that produce neuropathic pain concerns the role of the immune system. Immunity can be divided into two different phases –acquired and innate. Acquired immunity involves the phenomenon of immunological memory and includes antibody and lymphocyte responses to specific antigens. The forerunner, however, to acquired immunity is the innate immune response. This more basic type of immunity involves a generalized immune cell response, namely leukocyte recruitment, to a variety of toxic or pathological intrusions into physiological homeostasis. Leukocytes, as well as Schwann cells, neurons, astrocytes, and microglia, express molecules such as Toll-like receptors (TLRs), Nod-like receptors (NLRs) and RIG-like receptors (RLRs). These cellular receptors can recognize shared molecular patterns expressed by infectious agents, cell debris, or other cellular detritus and, in response, initiate a cascade of cytokine synthesis that goes on to orchestrate a general cellular response to these potential problems (Kim et al. 2007; Tanga, Nutile-McMenemy, and DeLeo 2005; Watkins et al. 2007; Tawfik et al. 2007; Creagh and O'Neill 2006).

The activation of innate immune inflammatory responses is also frequently linked to the development of pathology. In the present context, it is believed that the innate immune response to injury, extending beyond its role in promoting the influx and activation of leukocytes, plays a prominent role in the establishment of chronic pain states. Although inflammatory and neuropathic pain syndromes are often considered distinct entities, emerging evidence suggests that proinflammatory cytokines produced in association with the innate immune response are clearly implicated in the actual development and maintenance of neuropathic pain and are a necessary prelude to its development. As such, both neuroinflammatory and associated immune responses following nerve damage may contribute as much to the development and maintenance of neuropathic pain as to the initial nerve damage itself.

Animal Models of Neuropathic Pain

Well-controlled human studies have provided us important mechanistic insights as well as results immediately applicable to clinics. Like in many other diseases, however, patients with neuropathic pain normally have multiple causes or no obvious causes. Also, complex variables such as age, sex, and health history complicate the interpretation of many experiments. Genetic variances further complicate the matter even in well-designed experiments.

On the other hand, animal models are easier to control and are very reproducible, although their clinical relevance is less clear. Many animal models that mimic the causes and clinical features of neuropathic pain in humans have been developed. In particular, several rodent models have

been invaluable tools in elucidating molecular and cellular mechanisms underlying the genesis of neuropathic pain. In general, a simple surgical or pharmacological manipulation is applied to rats or mice to induce stereotypical pain hypersensitivity, which then can be measured by standardized behavioral tests. The current understanding of neuropathic pain is primarily obtained from studies using animal models. These models include sciatic nerve transsection (Subang and Richardson 2001; Taskinen and Roytta 2000), partial ligation of the sciatic nerve (Abbadie et al. 2003; Tanaka et al. 2004; Lindia et al. 2005), chronic constriction injury of the sciatic nerve (Milligan et al. 2004; Zhang and De Koninck 2006; Kleinschnitz et al. 2005), chronic compression of the L_4L_5 DRG (CCD, a rodent model of spinal stenosis) (White et al. 2005; Sun et al. 2006), lysophosphatidylcholine-induced focal nerve demyelination (Jung et al. 2008; S. Bhangoo et al. 2007), bone cancer pain (Vit et al. 2006; Khasabova et al. 2007), zymosan-induced inflammatory pain (Milligan et al. 2004; Xie et al. 2006; Verge et al. 2004), and the injection of an anti-retroviral drug (S.K. Bhangoo et al. 2007).

Chemokine Signaling and Pain: The Traditional View

The traditional view on the involvement of chemokine signaling in pain has been that it plays a role in the initial stage of tissue damage by activating a cascade of signaling events that leads to inflammatory pain. In response to various types of injury or infection, activation of the inflammatory response induces local synthesis and release of many molecules (e.g. chemokines) which constitute the local inflammatory milieu. These chemical mediators attract leukocytes (e.g. monocytes, neurotrophils, and mast cells) that express corresponding receptors (e.g. chemokine receptors) into the site of tissue injury. Recruited leukocytes, together with local cells at the

injury site, secrete various chemical mediators of pain (e.g. bradykinin) which sensitize primary afferent fibers innervating the site of injury. The sensitized nerve endings then produce enhanced pain responses known as inflammatory pain (Zimmermann 2001). However, a more recent study showed that in some circumstances primary sensory neurons in DRG can also express functional chemokine receptors. Moreover, activation of chemokine receptors in these neurons was excitatory suggesting that chemokines may have a direct effect on the excitability of DRG neurons, as well as an indirect effect through recruitment of leukocytes (Oh et al. 2001).

Neuropathic Pain and Chemokine Signaling

Despite its complex pathophysiology, it is clear that neuropathic pain is the result of the prolonged abnormal activity of the nociceptive pathway. This activity generally results from hypersensitization of primary nociceptors (i.e. peripheral sensitization), increased synaptic activity at the spinal cord (i.e. central sensitization), or both. In any case, it is generally believed that sensitization results from long-term changes in the neuronal expression of molecules that mediate the generation and transmission of action potentials (e.g. ion channels and receptors). But, how different genes are regulated under different circumstances is not well understood and is the subject of active research.

Accumulating evidence suggests that upregulation of chemokine signaling in DRG neurons is a common mechanism that initiates peripheral sensitization. In many animal models, one or more chemokine receptors were upregulated by DRG neurons associated with, or in close proximity to, the injury (Subang and Richardson 2001; Sun et al. 2006; Taskinen and Roytta 2000; Abbadie et

al. 2003; Tanaka et al. 2004; Lindia et al. 2005; Milligan et al. 2004; Zhang and De Koninck 2006; Kleinschnitz et al. 2005; Jung and Miller 2008; S. Bhangoo et al. 2007; Vit et al. 2006; Khasabova et al. 2007; Verge et al. 2004; Xie et al. 2006; S.K. Bhangoo et al. 2007; White et al. 2005). Moreover, in several instances it has also been demonstrated that sensory neurons will actually upregulate the synthesis of chemokines in addition to their cognate receptors (Sun et al. 2006; White et al. 2005; S. Bhangoo et al. 2007; Jung et al. 2008; Jung et al. 2007). In particular, the chemokine, monocyte chemoattractant protein-1 (MCP1, also known as CCL2), and its receptor, CCR2, have been identified as the most frequently upregulated genes in association with neuropathic pain (White, Bhangoo, and Miller 2005; White, Jung, and Miller 2007; Thacker et al. 2008; Jung and Miller 2008; Abbadie et al. 2003; S. Bhangoo et al. 2007; Sun et al. 2006). Some indications as to how MCP1-CCR2 signaling contributes to the development of pain hypersensitivity were recently presented by White *et al.* (2005) using the CCD model, an animal model of spinal stenosis (White et al. 2005). Under normal conditions, DRG neurons express neither MCP1 nor CCR2 at a high level. Their expression is, however, significantly increased in DRG neurons as animals develop neuropathic pain (S. Bhangoo et al. 2007; White et al. 2005). DRG neurons of animals with pain hypersensitivity, and which therefore have an increased level of CCR2 expression, can be depolarized or show an increase in intracellular Ca in response to MCP1. This indicates that the activation of CCR2 receptors in these neurons is excitatory. Taken together, these data suggested that in states of neuropathic pain the activation of CCR2 receptors in DRG neurons may induce sensitization of ion channels via Ca-mediated signaling pathways. Further confirming the importance of CCR2 signaling in this phenomenon, genetic deletion of the CCR2 receptor inhibits development of neuropathic pain (Abbadie et al. 2003).

These studies clearly demonstrate that the upregulation of MCP1 and CCR2 and the consequent activation of CCR2 signaling in DRG neurons are important events in the genesis of neuropathic pain. However, the molecular mechanisms underlying these phenomena are not well understood. This thesis aims to answer the following questions: (1) How is the activation of CCR2-mediated signaling translated into neuronal sensitization? More specifically, (a) what are the intracellular signaling pathways that connect CCR2 receptors to ion channels? (b) What are those ion channels? (c) How do neurons regulate the release of MCP1? (2) What signals induce the upregulation of MCP1 and CCR2 in DRG neurons? (3) In addition to the DRG, where is MCP1-CCR2 signaling activated in states of neuropathic pain?

In order to answer these questions at an animal level, novel transgenic mice were generated which enabled visualization of cellular and subcellular localization chemokines and chemokine receptors *in vivo*. Using these mice, dynamic changes in cellular localization of active MCP1-CCR2 signaling were examined *in vivo* in the context of neuropathic pain as well as other related neuropathologies

Focal demyelination by lysophosphatidylcholine (LPC) was, after some deliberation, chosen as the animal model for neuropathic pain in this research (S. Bhangoo et al. 2007; Jung et al. 2008; Wallace et al. 2003). The initial observation of DRG upregulation of MCP1 and CCR2 was made in rat experiments using the CCD model. However, the CCD model, which employs the implantation of a metal rod to compress the DRG, is very difficult to apply to mice. In contrast, the LPC model involves a simple surgery to implant a detergent-soaked sponge which locally demyelinates the sciatic nerve. Importantly, animals with LPC focal nerve demyelination have

shown upregulation of MCP1 and CCR2 in DRG neurons as well as consistent development of pain hypersensitivity. Finally, behavioral manifestations of neuropathic pain in this model are reversed by a specific receptor antagonist to CCR2 even after hyperalgesia is fully developed (S. Bhangoo et al. 2007). This further supports the validity of using this model to study the role of chemokines in neuropathic pain.

However, there are limitations to any animal model, because such tools do not have perfect clinical relevance to the human conditions they mimic. The LPC model was developed to imitate a number of human PNS and CNS inflammatory demyelinating diseases including Guillain-Barre syndrome (GBS), Charcot-Marie Tooth disease types 1 and 4, and multiple sclerosis (Boukhris et al. 2007; Carter et al. 1998). LPC models not only the demyelination characteristic of these conditions, but also the neuropathic pain which often accompanies the human disease state. Epidemiological studies suggest that chronic pain syndromes afflict 50 to 80% of patients with MS and 70 to 90% of individuals with GBS (Moulin 1998). It is important to study the contribution of chemokines to LPC in animals because research suggests that cytokines play a role in the human pathology. Disease-related components possibly central to neuropathic pain symptomology, including axonal and Wallerian degeneration (Bruck 2005), may act as triggers for cytokine cascades that result in the upregulation and chronic expression of chemokines and their cognate receptors (Mahad, Howell, and Woodroofe 2002; Charo and Ransohoff 2006). Therefore, the results of animal-level studies such as those described in this thesis, although encouraging, should be parsimoniously applied to human chronic pain syndromes resulting from demyelination.

CHAPTER 2

Molecular Mechanisms Underlying MCP1-CCR2 Signaling-Mediated Sensitization of Dorsal Root Ganglion Neurons

This chapter has been published (Jung et al. 2008).

ABSTRACT

It has been previously observed that expression of the chemokine, monocyte chemoattractant protein-1 (MCP1), and its receptor, CCR2, is upregulated by dorsal root ganglion (DRG) neurons in association with rodent models of neuropathic pain. MCP1 increases the excitability of nociceptive neurons after a peripheral nerve injury, while disruption of MCP1-CCR2 signaling blocks the development of neuropathic pain. These data suggest that MCP1-CCR2 signaling is responsible for heightened pain sensitivity.

In order to define the mechanisms of MCP1 signaling in DRG, intracellular processing, release, and receptor-mediated signaling of MCP1 in DRG neurons were studied. It was found that in a focal demyelination model of neuropathic pain both MCP1 and CCR2 were upregulated by the same neuronal population, which included TRPV1-expressing nociceptors. MCP1 expressed by DRG neurons was packaged into large dense-core vesicles (LDCVs) whose release could be induced from the soma by depolarization in a Ca-dependent manner. Activation of CCR2 by MCP1 sensitized nociceptors via transactivation of transient receptor potential (TRP) channels. These results suggest that MCP1 and CCR2, upregulated by sensory neurons following peripheral nerve injury, might participate in signaling events that contribute to the sustained excitability of primary afferent neurons.

INTRODUCTION

Chemokines (CHEMOtaxic cytoKINEs) are a family of small proteins which play a prominent role in the trafficking of immune cells and in the orchestration of inflammatory responses. However, chemokines and their receptors are not only expressed in the immune system, but are also widely expressed in the nervous system during development and in adulthood. Indeed, chemokines appear to play several important roles in neuronal development and in the function of the mature nervous system (Tran and Miller 2003). Chemokine receptors are expressed by diverse populations of neurons and glia. For example, cultured DRG neurons express numerous functional chemokine receptors. Activation of these receptors excites DRG neurons and produces pain hypersensitivity (Oh et al. 2001).

Recent reports have sought to define how such responses may contribute to states of chronic pain. It has been suggested that the chemokine, MCP1, and its receptor, CCR2, may play a particularly important role in the genesis of neuropathic pain, as both molecules are not normally expressed in the DRG but are expressed in association with nerve injury (White et al. 2005). MCP1-CCR2 signaling may contribute to the neuronal hyperexcitability associated with neuropathic pain, as suggested by that fact that application of MCP1 to CCR2-expressing neurons strongly depolarizes them (Sun et al. 2006; White et al. 2005). Furthermore, CCR2 knockout mice show impaired development and maintenance of neuropathic pain (Abbadie et al. 2003). Although these findings suggest that MCP1-CCR2 signaling is important to the genesis and/or

maintenance of neuropathic pain, the molecular mechanism by which MCP1 produces these effects *in vivo* is unclear.

This research reveals that MCP1 expressed in DRG neurons acts as a novel neuromodulator, which exhibits localization to neurotransmitter-containing secretory vesicles, Ca- and voltage-dependent secretion, and a postsynaptic mechanism of action. These data support the hypothesis that MCP1 signaling in the DRG contributes to the maintenance of nociceptor hyperexcitability in neuropathic pain.

RESULTS

Mouse DRG neurons express both MCP1 and CCR2 in association with cutaneous hyperalgesia

The expression of MCP1 and CCR2 is upregulated in the DRG in several rodent models of neuropathic pain (S. Bhangoo et al. 2007; Sun et al. 2006; White et al. 2005; Xie et al. 2006). However, the relative location of MCP1- and CCR2-expressing neurons has not been determined. In order to address this question, a state of cutaneous hyperalgesia was induced in CCR2-EGFP BAC reporter mice (see Materials and Methods) using lysophosphatidylcholine (LPC)-mediated focal demyelination of the sciatic nerve (S. Bhangoo et al. 2007; Wallace et al. 2003). Fourteen days after focal demyelination, the associated lumbar DRG were isolated, cryosectioned, and stained for MCP1 using a polyclonal antibody. In agreement with previous results seen in rats, both MCP1 and CCR2 were upregulated in comparison to sham-operated control animals in which neither molecule was expressed by DRG neurons (S. Bhangoo et al. 2007) (Figure 2.1A-

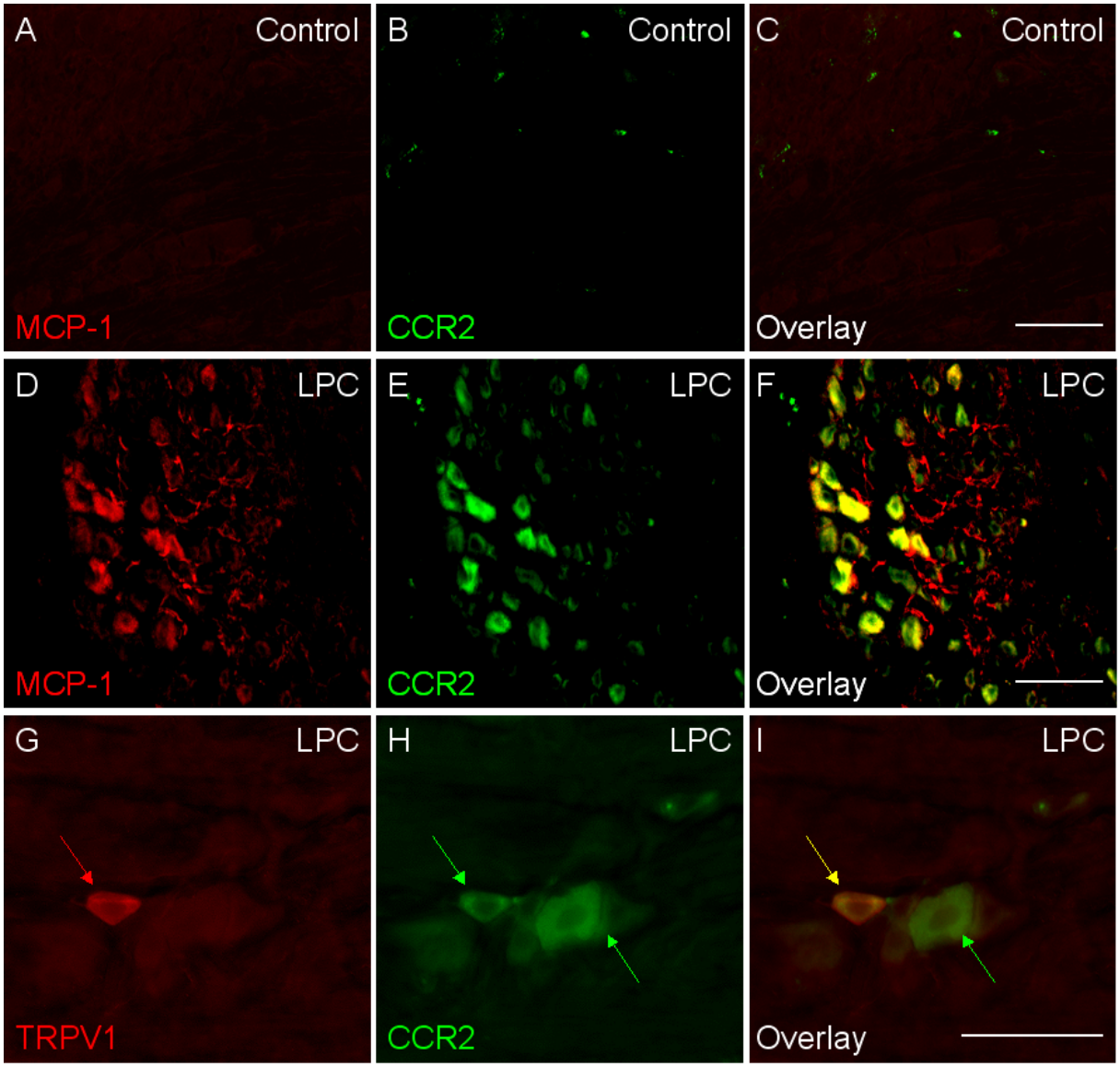


Figure 2.1. DRG neurons express MCP1 and CCR2 in association with peripheral neuropathy. Sciatic nerve demyelination was induced by lysophosphatidylcholine (LPC) in CCR2-EGFP BAC transgenic mice. DRG were isolated at post operative day (POD) 14, cryosectioned, and subjected to immunohistochemistry using a polyclonal anti-MCP1 antibody. (A-C) Sham-operated control. (D-F) LPC-treated group. Note that many neuronal cell bodies express both MCP1 and CCR2. In addition, MCP1 is also observed in numerous axon processes throughout the ganglion. (G-I) TRPV1 expressing nociceptors (red arrows) upregulated CCR2 expression (yellow arrow). Some of larger neurons that do not express TRPV1 also expressed CCR2 (green arrow). Scale bars, 100 μm.

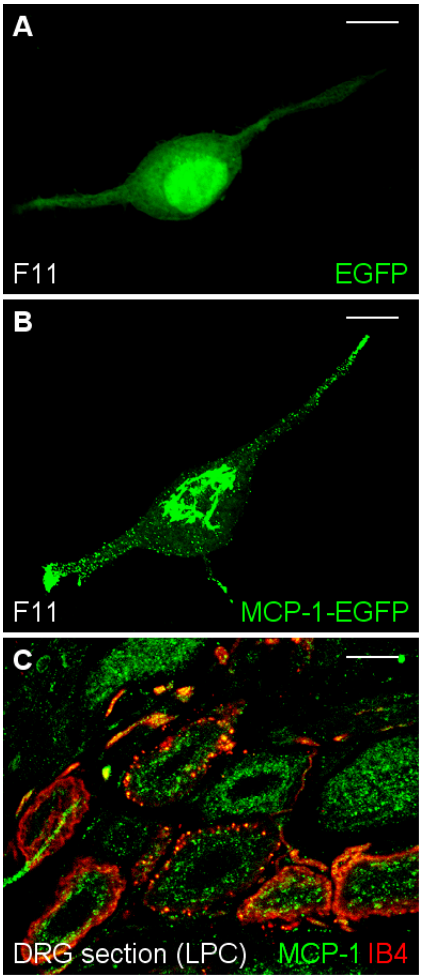


Figure 2.2. MCP1-EGFP is localized to vesicle-like structures in F11 cells. (A-B) F11 cells were transfected with either an MCP1-EGFP fusion protein expressing vector or empty EGFP vector and induced to differentiate for 3 days. To make the fusion construct, the entire coding sequence of MCP1 including the pro-domain was used. (A) EGFP localized primarily in the nucleus and diffusively in the cytoplasm. (B) The MCP1-EGFP fusion protein was absent from the nucleus and exhibited a punctate expression pattern. It was concentrated at the perinuclear region and the terminals of neurites. (C) MCP1 immunofluorescence in the DRG of an LPC-treated mouse. MCP1 staining was perinuclear with a punctate distribution. Isolectin B4 (IB4) binding is a marker for a population of small nociceptive neurons. Scale bars, 20 μm.

F). Interestingly, following LPC treatment, neurons in close apposition co-expressed MCP1 and CCR2 in their cell bodies, suggesting a type of autocrine or paracrine regulatory role for MCP1-CCR2 signaling within the DRG.

MCP1-EGFP is localized to secretory vesicles

MCP1, like other chemokines, is a small secreted protein. In order to examine how it is processed once expressed by DRG neurons, an MCP1-EGFP fusion protein was expressed in

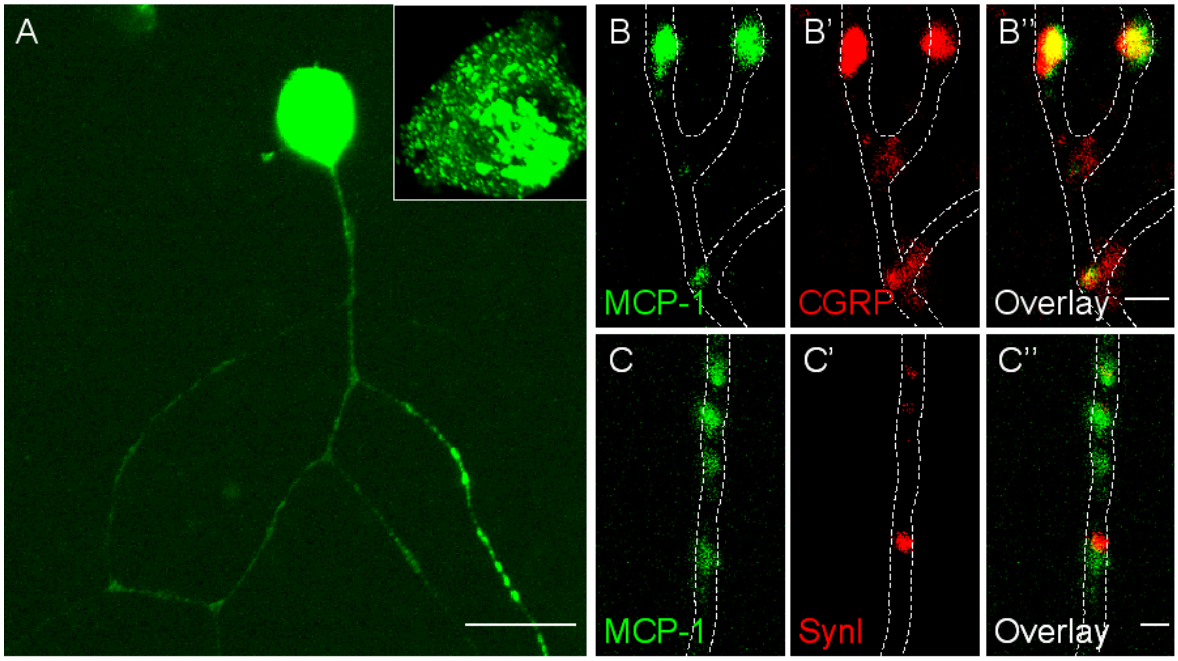


Figure 2.3. MCP1-EGFP localizes to large dense-core vesicles (LDCVs) in DRG neurons. Cultured DRG neurons were infected with an MCP1-EGFP expressing adenovirus and stained for CGRP and SynI. (A) MCP1-EGFP was concentrated in the perinuclear area, and localized in a punctate pattern in the soma and along axons. Signals in the soma were saturated to allow appreciation of the punctate localization along axons. An unsaturated image of a soma is magnified in the inset. (B-C) Magnified view of axonal localization of MCP1-EGFP. (B-B'') MCP1-EGFP co-localized with CGRP. (C-C'') MCP1-EGFP did not co-localize with SynI. Scale bars: A, 20 μm; B'', C'', 1 μm.

cultured DRG neurons and sensory neuron-derived F11 cells (Francel et al. 1987). Unlike EGFP, which diffusively localized throughout cells including in the nucleus (Figure 2.2), MCP1-EGFP exhibited a distinct punctate cytoplasmic localization with a concentrated perinuclear pattern (Figure 2.2B and 2.3A). The perinuclear signal co-localized with TGN38, a marker for the trans-Golgi network (Figure 2.4A-C), indicating that the MCP1-EGFP fusion protein was sorted into the secretory pathway in sensory neurons. Punctate signals in the cell body and axonal processes cytoplasm co-localized with the neuropeptide, calcitonin gene-related peptide (CGRP), suggesting that MCP1-EGFP was packaged into large dense-core vesicles (LDCVs) (Figure 2.3B and 2.4D-F). Accordingly, MCP1-EGFP did not generally co-localize with Synaptophysin I

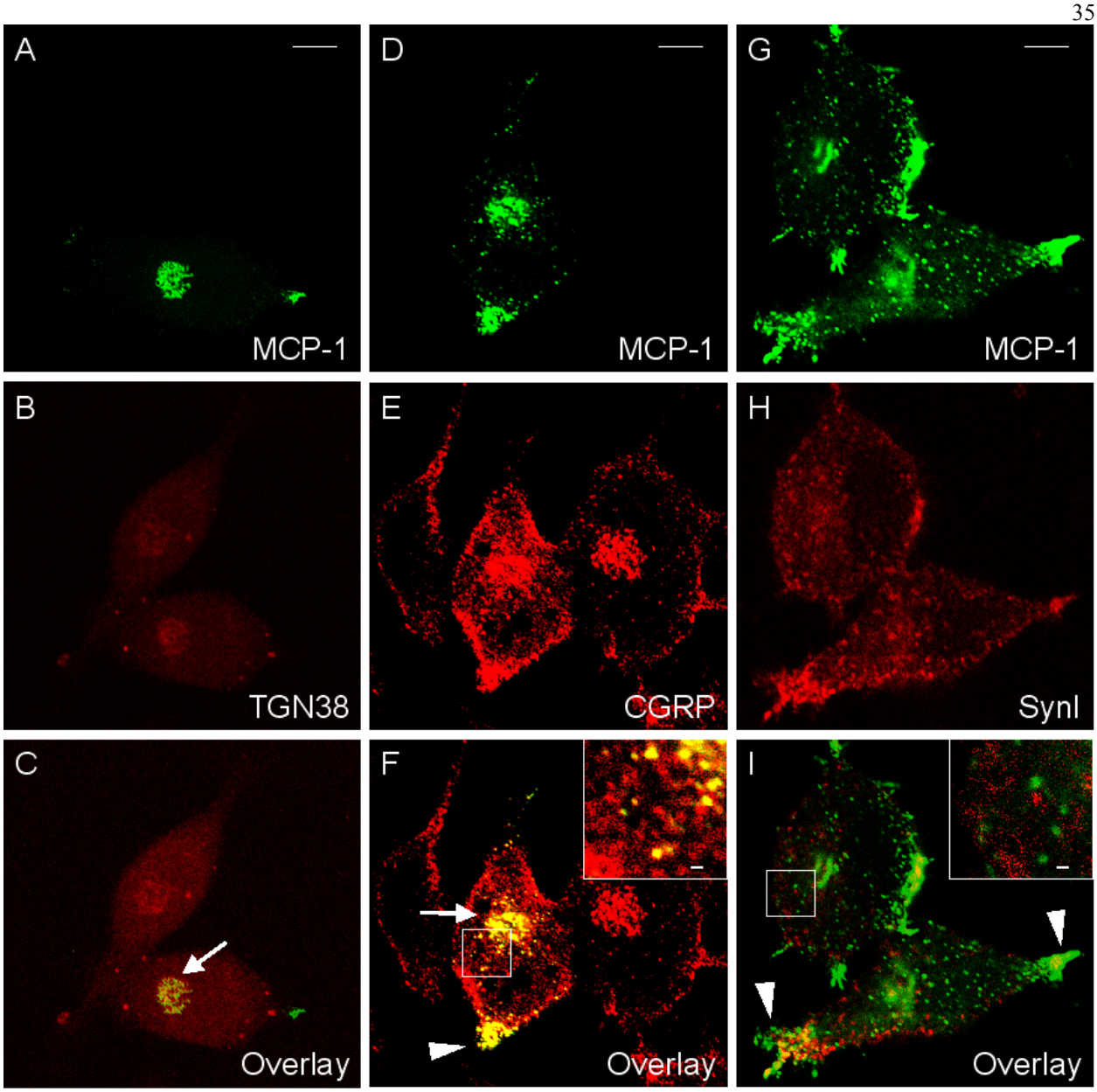


Figure 2.4. MCP1-EGFP is processed along the secretory pathway in F11 cells. F11 cells transfected with MCP1-EGFP were analyzed by immunocytochemistry. (A-C) Perinuclear MCP1-EGFP co-localized with TGN38, a marker for the trans-Golgi network (arrow). (D-F) MCP1-EGFP co-localized with Calcitonin gene-related peptide (CGRP), a peptide neurotransmitter secreted from DRG neurons. The co-localization was evident in the perinuclear area (arrow), neurite terminals (arrow head), and in the cytoplasm (inset). The boxed area is magnified in the inset. (G-I) MCP1-EGFP did not generally co-localize with Synaptophysin I (SynI), a marker for small synaptic vesicles (SVs) (inset), although occasional co-localization was observed in neurite terminals (arrowhead). Scale bars: A, D, G, 20 μm; F, I, 1 um.

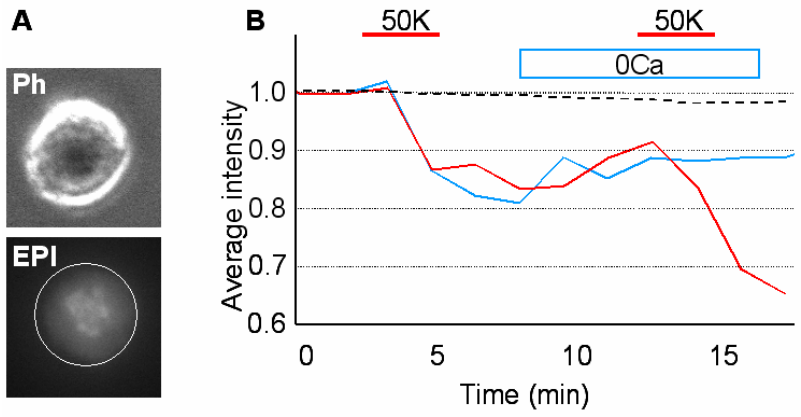


Figure 2.5. MCP1-EGFP is released from the somata of DRG neurons. DRG neurons infected with an MCP1-EGFP expressing adenovirus were analyzed using a fluorescence release assay. (A) Representative phase contrast (ph) and fluorescence (EPI) images of a DRG neuronal soma are shown. DRG neurons were identified by their round and phase-bright somata. The entire soma was selected as a region of interest (ROI: white circle). (B) Depolarization by high potassium (50K) induced the release of MCP1-EGFP (red trace) (n=8). When neurons were not stimulated, fluorescence intensity was not significantly changed for the duration of recording (dashed trace). No release was detected when extracellular Ca was absent (0Ca)

(SynI), a marker for small synaptic vesicles (SSVs) (Figure 2.3C and 2.4G-I). The number and distribution of CGRP-positive and SynI-positive vesicles were the same in transfected neurons were compared to untransfected, indicating that adenovirus-mediated expression of MCP1-EGFP did not significantly alter the secretory pathways in DRG neurons (data not shown). Further, immunofluorescent labeling of endogenous MCP1, which was upregulated in LPC-treated animal DRG, also exhibited a punctate localization pattern, suggesting that MCP1 is also processed and stored in vesicles in DRG neurons *in vivo* (Figure 2.2C).

MCP1-EGFP is released upon depolarization

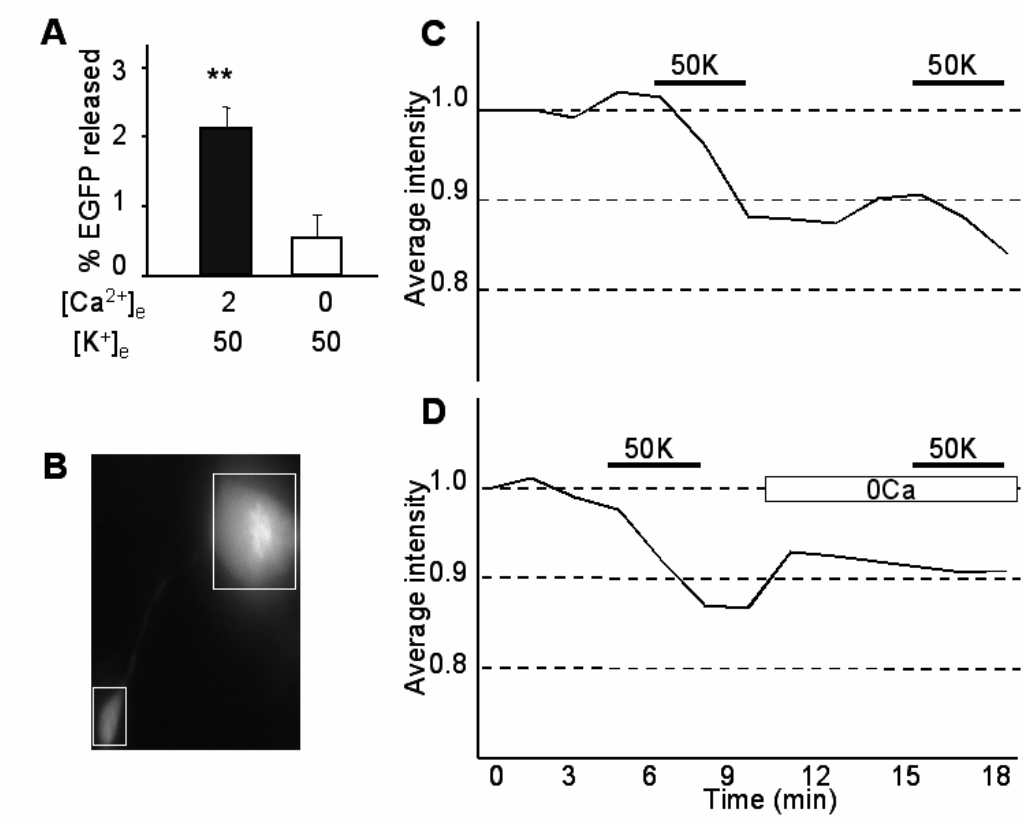


Figure 2.6. MCP1-EGFP is released from the soma and neurite terminals of F11 cells by depolarization in a Ca-dependent manner. F11 cells were transfected with MCP1-EGFP and allowed to differentiate for 3 days. Release of MCP1-EGFP was analyzed by live-cell fluorescence imaging and ELISA. (A) ELISA was performed to detect released MCP1-EGFP. To induce release, cells were incubated with BSS containing varying concentrations of extracellular K and Ca ([K]e and [Ca]e, respectively) for 30 min at room temperature. ELISA was performed with the supernatants using a GFP antibody. Significant release was detected by high K induced depolarization, but only when extracellular Ca was present (**$p<0.01$ vs. unstimulated control). (B) A representative image of the fluorescence release assay is shown. Images were taken using a 40X water immersion objective lens. Somata and neurite terminals where the signal was concentrated were taken as regions of interest (ROIs: white boxes). Fluorescence intensity was monitored in ROIs while cells were continuously perfused with BSS. The initial intensity was set to 1, and all values were normalized to this. (C) Depolarization by high potassium (50K) induced a steep decrease in fluorescence intensity (n=11 for both somata and neurite termini). (D) No decrease in intensity was observed when extracellular Ca was absent (0Ca) (n=8 for both somata and neurite termini).

Neuropeptides expressed by DRG neurons such as substance P and CGRP are secreted from cell bodies by Ca-dependent exocytosis (Huang and Neher 1996; Ouyang et al. 2005). In order to investigate whether the secretion of MCP1 from DRG neurons could also be induced in the same manner, MCP1-EGFP fusion protein release was imaged in real time using a fluorescence release assay. After recording, the changes in fluorescence signal intensity were analyzed using MetaMorph software. In order to selectively record release from cell bodies, neurons were re-plated prior to the recording to eliminate axonal processes (Figure 2.5). Any decrease in fluorescence intensity was regarded as release after determining that photobleaching of EGFP during the recording was negligible (dashed trace in Figure 2.5B).

The fluorescence intensity of the soma declined steeply following depolarization by a high concentration of extracellular potassium (50K), indicating that MCP1-EGFP release from the soma could be induced by depolarization (Figure 2.5B: red trace). However, release was not induced in the absence of extracellular Ca, indicating the involvement of voltage-dependent Ca influx in this process (Figure 2.5B: blue trace; Figure 2.6C-D). The Ca-dependency of MCP1-EGFP release was also confirmed by an enzyme-linked immunosorbent assay (ELISA), where release of MCP1-EGFP was observed only when cells were depolarized in the presence of extracellular Ca (Figure 2.6A). These results suggest that MCP1, upregulated in DRG neurons and packaged into vesicles, can be released from the soma when DRG neurons are excited by stimuli that increase $[Ca]_i$.

It should be noted that release of some other chemokines was differently regulated (Figure 4.2M). For example, SDF1 was localized to vesicles that were clearly different from the ones that store MCP1 as seen when both chemokines were expressed in the same cell (Figure 4.2I-K). Unlike

MCP1 which was mainly released through the regulated pathway (Figure 2.5-6 and 4.2.H), the release of SDF1 was mostly constitutive, that is, it was not increased by neuronal depolarization (Figure 4.2M). The identity of the SDF1-containing vesicles in DRG neurons was not studied. Several different chemokines including SDF1 and MCP1 are expressed by DRG neurons in different models of neuropathic pain (White, Bhangoo, and Miller 2005; White, Jung, and Miller 2007), therefore it is important to examine how these molecules are sorted and which mechanisms govern their release.

CCR2 activation hypersensitizes nociceptors

Many of the neurons which upregulate expression of MCP1 and CCR2 under pathological conditions also express TRP channels, a family of ion channels known to respond to noxious stimuli. It was hypothesized that MCP1-CCR2 signaling in LPC model DRG may mediate the nociceptor hypersensitivity underlying neuropathic pain by interacting with TRP channels. In the DRG, the majority of TRPV1-expressing nociceptors co-expressed CCR2 (82 ± 9% co-localization; n=4; expressed as mean ± SEM), although CCR2 upregulation was not limited to these cells. TRPV1 is a major TRP channel involved in temperature sensation and nociception (Tominaga and Caterina 2004). It is activated by painful stimuli such as heat, capsaicin, and protons. A number of inflammatory mediators including prostaglandins, adenosine, serotonin, nerve growth factor (NGF), and bradykinin can sensitize TRPV1 via the activation of phospholipase C (PLC), PI3 kinase, and protein kinase C ε (PKCε) (Zhang, Huang, and McNaughton 2005; Bhave et al. 2003; H.-h. Chuang et al. 2001). Another TRP channel that is critical to nociception, TRPA1, contributes to cold, mechanical, and chemical pain sensation.

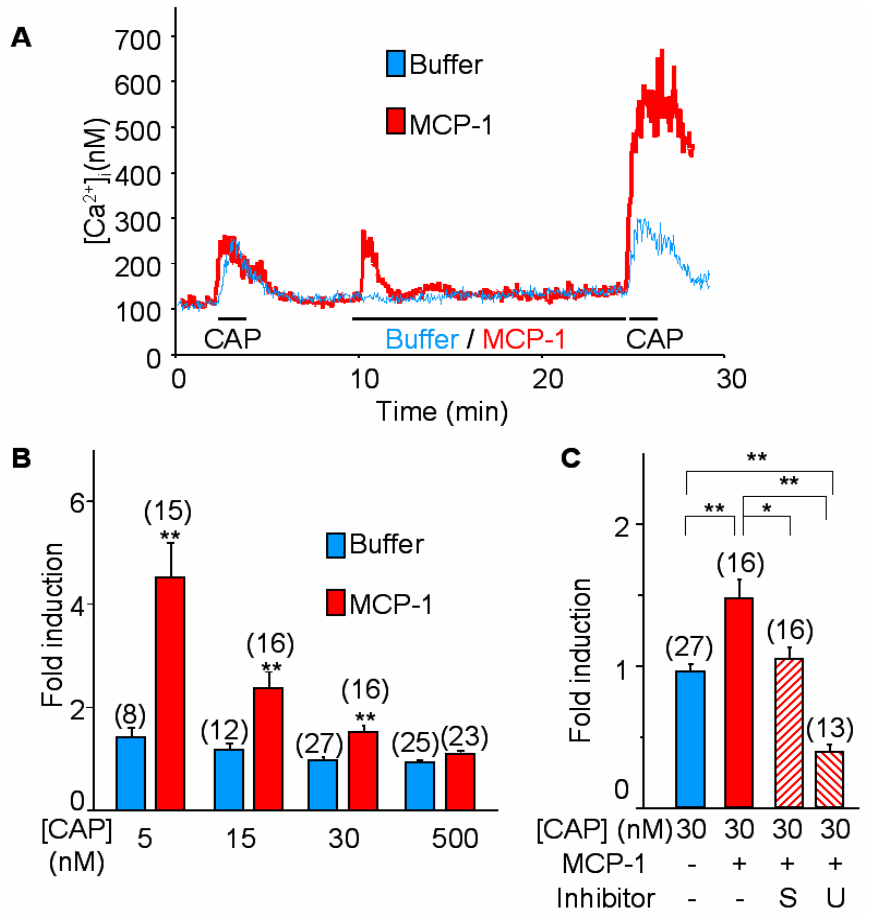


Figure 2.7. CCR2 activation sensitizes TRPV1. HEK293 cells were transfected with CCR2 and TRPV1 expressing vectors and the responses of TRPV1 to capsaicin (CAP) were measured by Ca imaging. Cells were treated with CAP for 1 min at the beginning and end of the recording (30 min). Between the two CAP treatments, cells were incubated with either 100 ng/ml MCP1 or buffer solution for 15 min. CAP responses were compared before and after the MCP1 treatment in each cell. Enhancement of the second response compared to the first represented sensitization of TRPV1 (A) Without MCP1, the amplitude of the two CAP responses was similar (blue trace), whereas the second CAP response increased after incubation with MCP1 (red trace). (B) Result of TRPV1 sensitization by MCP1 are summarized. Sensitization was measured with varying concentrations of CAP and was represented by a fold induction of the second CAP response compared to the first response in each cell. Sensitization was prominent at lower concentrations of CAP (**p<0.01 vs. mock application control of each concentration). (C) The mechanism of TRPV1 sensitization was examined with inhibitors of PLC (U; 10 μM of U73122) and PKC (S; 10 nM of staurosporine). The inhibitors were added 5 min before recording. Sensitization was completely blocked by both PKC and PLC inhibitors. Moreover, PLC inhibitors *desensitized* TRPV1 (*p<0.05 and **p<0.01). Numbers of recorded cells are noted in parentheses.

TRPA1 is a receptor-operated channel that can also be gated by the activation of the PLC pathway, and it is expressed by a subset of TRPV1-positive neurons in the DRG (Bautista et al. 2006; Kwan et al. 2006). Sensitization of these TRP channels by inflammatory mediators is believed to contribute to pain hypersensitivity (Huang, Zhang, and McNaughton 2006). CCR2 is a G protein-coupled receptor (GPCR) which can couple to Gαi or Gαq. In both cases, MCP1 binding leads to the activation of PLCβ (Kuang et al. 1996). Therefore, one of the effects of CCR2 activation in the DRG might be sensitization of TRP channels via MCP1 activation of PLC.

In order to examine this possibility, CCR2 and TRPV1 were co-expressed in HEK293 cells which were then treated twice with capsaicin (CAP). The CAP treatments were separated by a 20 min interval, during which MCP1 or buffer solution was applied to the bath. The amplitudes of the two CAP responses were measured using Ca imaging and compared for each cell. While the two responses to CAP were identical in the buffer-treated controls (Figure 2.7A: blue trace), MCP1 treatment significantly enhanced the second response to CAP indicating that CCR2 activation had sensitized TRPV1 channels in the cell (Figure 2.7A: red trace). This enhanced response was more prominent at lower concentrations of CAP, because the CAP responses became saturated at higher concentrations (Figure 2.7B and data not shown).

The mechanism of TRP channel sensitization was also studied using PLC-pathway inhibitors. MCP1-induced sensitization was almost completely blocked by staurosporine, a pan-PKC inhibitor, indicating that PKC activation is crucial in this process (Figure 2.7C). Moreover, the

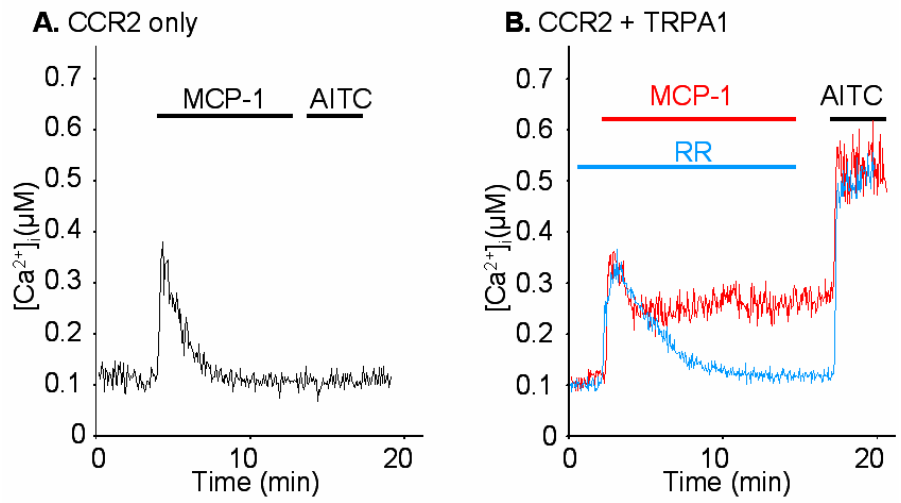


Figure 2.8. CCR2 activation sensitizes TRPA1. HEK293 cells were transfected with CCR2 and/or TRPA1 expressing vectors, and the responses to MCP1 (100 ng/ml) and allyl isothiocyanate (AITC) (10 μM), a TRPA1 agonist, were measured by Ca imaging experiments. Ruthenium red (RR; 30 μM) was added to block TRPA1 channels. (A) The response to MCP1 consisted of a transient component when MCP1 was expressed alone. (B) An additional sustained component appeared when MCP1 was co-expressed with TRPA1 (red trace). The sustained component was abolished by ruthenium red (blue trace). At the end of recording, cells were treated with AITC to confirm the expression of TRPA1.

inhibition of PLC by U73122 decreased the second CAP response to below basal levels (Figure 2.7C). PIP2 is known to inhibit TRPV1 (H.H. Chuang et al. 2001), therefore, it is hypothesized that the sustained inhibition of PLC may have desensitized TRPV1 by accumulation of PIP2, a PLC substrate.

The next experiment aimed to determine if CCR2 activation effected TRPA1 function. When only CCR2 receptors were expressed in HEK293 cells, MCP1 treatment induced a transient increase in $[Ca]_i$ which returned to baseline levels (Figure 2.8A). However, when TRPA1 was co-expressed with CCR2, the initial rise in $[Ca]_i$ reached a plateau level above the baseline which

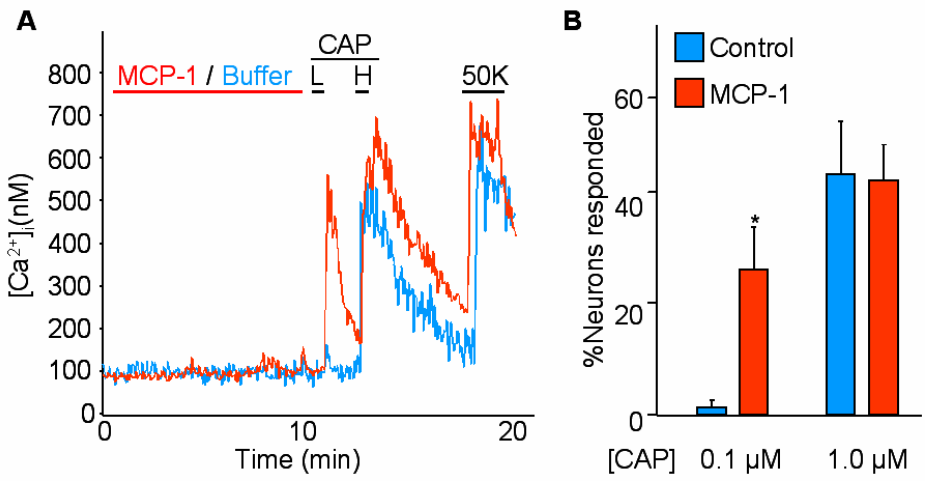


Figure 2.9. MCP1 sensitizes the capsaicin-responsiveness of cultured DRG neurons. Cultured DRG neurons transduced with a CCR2 expressing adenovirus were pre-treated with MCP1 or buffer solution for 10 min. Subsequently, neurons were treated with a low concentration (0.1 μM) of capsaicin (CAP) (L), a high concentration (1.0 μM) of CAP (H), and high potassium (50K). Neurons were identified by 50K-responsiveness. (A) In the control group, most neurons did not respond to 0.1 μM CAP (blue trace). In the MCP1-treated group, most neurons that responded to 1.0 μM CAP also responded to 0.1 μM CAP (red). (B) Results are summarized. There was no difference in the total number of CAP-responsive neurons. However, MCP1 pre-treatment increased neurons that were responsive to 0.1 μM CAP (pooled result of four different experiments. In the control group 27 out of 66 neurons responded to 1.0 μM CAP, of which only one responded to 0.1 μM CAP. In MCP1-treated group, 44 out of 89 responded to 1.0 μM CAP, of which 28 responded to 0.1 μM CAP).

persisted for the duration of the recording (Figure 2.8B: red trace). This effect was completely blocked by the TRPA1 antagonist, ruthenium red (RR), indicating that the activation of CCR2 had transactivated TRPA1 channels (Figure 2.8B: blue trace). Expression of TRPA1 by these cells was confirmed by their responses to TRPA1 agonist, allyl isothiocyanate (AITC), at the end of the recording.

These results suggest that DRG neurons that release MCP1 from their somata by depolarization may activate themselves (autocrine signaling) or neighboring TRPV1/A1-expressing neurons

(paracrine signaling). The final experiment was designed to test this hypothesis by using cultured DRG neurons overexpressing CCR2. Neurons were treated with MCP1 or buffer solutions for 10 min and then twice with increasing doses of CAP. At the end of recording, the high potassium solution, 50K, was added to identify neurons. In the control group, where CCR2 expressing neurons were not treated with MCP1, roughly 40% of neurons expressed TRPV1 (identified by responsiveness to either concentration of CAP), and these neurons only responded to the higher concentration of CAP (Figure 2.9A and B). The total number of TRPV1-expressing neurons did not change when they were pretreated with MCP1 (Figure 2.9B). However, most of these neurons now also responded to the lower concentration of CAP indicating that MCP1 had sensitized TRPV1 (Figure 2.9A and B).

DISCUSSION

The chemokine family originally garnered attention for their many roles as modulators of immune function, especially in the inflammatory response. Since their discovery, it has become clear that chemokines influence processes in areas beyond the immune system. In the nervous system, chemokines were first found to be important to the development of many structures. More recent research has made it clear that many *mature* neurons express chemokines and their receptors under a variety of circumstances (Horuk et al. 1997; Coughlan et al. 2000; van der Meer et al. 2000; Banisadr, Fontanges et al. 2002; Banisadr, Queraud-Lesaux et al. 2002; Gillard et al. 2002). This information has lead researchers to ask what functions chemokines might have in the adult nervous system.

Chemokine receptor expression by developing neurons is well documented; for example, cultured neonatal DRG neurons express multiple types of chemokine receptors (Oh et al. 2001). However, many of these receptors are not expressed constitutively by adult neurons *in vivo*. Yet, adult neuronal populations do upregulate their expression of chemokine receptors together with their ligands under conditions of peripheral nerve injury-induced neuropathic pain (Tanaka et al. 2004; S. Bhangoo et al. 2007; Sun et al. 2006; Zhang and De Koninck 2006; White et al. 2005). Several chemokines and their receptors have also been shown to be expressed by peripheral non-neuronal cells in the DRG and spinal cord during states of pain hypersensitivity (Abbadie et al. 2003; Milligan et al. 2004; Verge et al. 2004; White et al. 2005; S.K. Bhangoo et al. 2007).

Studies of chemokine expression have shown that MCP1 is one of the most frequently upregulated in neuropathology. For example, MCP1 and CCR2 upregulation was observed in peripheral sensory neurons in two different pain models (S. Bhangoo et al. 2007; White et al. 2005). Additional research on the role of MCP1 and CCR2 in pain has yielded two important findings that implicate MCP1 and CCR2 signaling in the generation of chronic pain hypersensitivity. First, DRG neurons isolated from animals exhibiting neuropathic pain behavior are strongly depolarized by MCP1, whereas neurons from naïve animals are not (S. Bhangoo et al. 2007; Sun et al. 2006; White et al. 2005). Second, experiments in CCR2 knockout mice showed that they failed to develop neuropathic pain in an animal injury model (Abbadie et al. 2003). Taken together, these data reveal that MCP1 can act as a neurotransmitter in DRG neurons and further point to a vital role for MCP1-CCR2 signaling in the establishment of chronic pain.

Many questions remain about MCP1 signaling including (1) how MCP1 secretion is regulated, (2) which cells MCP1 targets, and (3) how MCP1 signaling is transduced. Perhaps the most compelling question, however, asks if MCP1 functions as a neuromodulator in the DRG, as the data presented here suggests. If so, this research would support the growing hypothesis that chemokines, which are traditionally thought to act as immune response mediators, can also act as neurotransmitters.

The research presented in this chapter begins to answer these questions. The first experiments shed light on the MCP1 secretion process. In common with peptide neurotransmitters, MCP1 expressed in DRG neurons is processed into the secretory pathway through LDCVs and can be released by neuronal depolarization. It appears that MCP1 can be stored in the same vesicles as CGRP, an established neurotransmitter. Substance P and CGRP have also been shown to be released from the somata of DRG neurons by Ca-dependent regulated exocytosis (Ouyang et al. 2005; Huang and Neher 1996).

The next experiments addressed questions about MCP1's target cells and MCP1 signal transduction. MCP1 and CCR2 seem to be co-expressed in a population of closely juxtaposed neurons (Figure 2.1F), which suggests that MCP1-CCR2 signaling may participate in somatosomatic communication between DRG neurons. Furthermore, because chemokines such as MCP1 can also induce the release of CGRP (Qin, Wan, and Wang 2005), and CGRP has also been suggested as mediating somatosomatic signaling in the DRG (Knopp and Oxford 2006),

upregulated MCP1 expression and release could help to drive a coordinated state of hyperexcitability throughout the entire DRG.

It is now clear that chemokines are synthesized and released by many different types of cells. In most instances cells upregulate chemokines in the face of some prevailing pathology. A comparison of these results from adult DRG cultures with experiments in other cells types proposes that chemokines are differentially regulated in various cells. Previous research in leukocytes and endothelial cells, where chemokine functioning has been most widely studied, reveals that chemokines are also stored in secretory granules and their release can be regulated by different stimuli (Oynebraten et al. 2004; Oynebraten et al. 2005). Interestingly, single endothelial cells can express multiple types of chemokines and these are targeted to different populations of secretory vesicles that can be selectively released by different stimuli. In the present case it is also clear that DRG neurons can upregulate and store different chemokines (White, Bhangoo, and Miller 2005; White et al. 2005; S. Bhangoo et al. 2007; S.K. Bhangoo et al. 2007), and that these may also be targeted to diverse populations of secretory vesicles (Jung and Miller, unpublished data). Thus, the ultimate role of chemokine release in the context of neuropathic pain may be complex with different chemokines serving different roles.

Considering that many chemokines and their receptors are expressed in the nervous system, it is likely that chemokines participate widely in neural communication (Adler and Rogers 2005). The expression of chemokines and their receptors in the brain is often not uniform but instead region-specific (Banisadr, Fontanges et al. 2002; Banisadr, Queraud-Lesaux et al. 2002; Horuk et al. 1997; van der Meer et al. 2000). Therefore, it is compelling to study whether individual

chemokines have specific functions in different regions of the nervous system. Chemokines are not generally expressed by neurons at high concentrations (SDF1/CXCL12 is an important exception to this); as a result, they may be viewed as generally inducible neuromodulators that are particularly important under pathological circumstances. Thus, neuronally expressed chemokines may be thought of as a component of the innate immune response in the nervous system.

Other recent reports in the literature suggest that chemokines may be released from neurons in an activity-dependent manner. The chemokine CCL21 is induced by glutamate treatment in dissociated hippocampus and cortical neurons as well as in neurons in hippocampal slice culture (de Jong et al. 2005). CCL21-containing vesicles were observed in the trans-Golgi network, axons, and presynaptic structures of cultured neurons. The supernatant of cultured neurons treated with glutamate attracted microglia *in vitro* suggesting that CCL21 is released from vesicles by these neurons. Furthermore, Fryer et al. demonstrated the expression of eotaxin/CCL3 by parasympathetic neurons in a model of asthma, and they proposed that release of this chemokine from neurons played a role in the attraction of eosinophils (Fryer et al. 2006).

It is interesting to note that Gosselin et al. reported that spinal cord neurons constitutively express CCR2 under normal circumstances and MCP1 inhibits GABAergic transmission in these cells (Gosselin et al. 2005). This thesis did not investigate whether MCP1 is transported to the terminals of spinal afferents *in vivo*. However, it was observed that MCP1-containing vesicles were transported along the length of axons in cultured DRG neurons (Figure 2.2B and 2.3A), and

that MCP1 release could be induced from the neurite terminals of cultured sensory neurons (Figure 2.6B). Furthermore, immunohistochemical localization of MCP1 in DRG sections suggested its axonal localization *in vivo* (Figure 2.1B). Different neuropeptides have occasionally been found to be co-stored and co-released; thus, it is possible that MCP1 may also function together with other neuropeptides such as substance P and CGRP in modulating synaptic transmission in the dorsal horn of spinal cord. In addition, CCR2 expression can also be upregulated in microglial cells in the spinal cord providing yet another potentially important target for centrally released MCP1 (Abbadie et al. 2003).

If MCP1 acts as a neuromodulator, as suggested in this work, it remains to be understood how the activation of its receptor, CCR2, modulates neuronal communication. As was demonstrated, MCP1 and CCR2 are frequently expressed by neighboring neurons which suggests that MCP1 release may act in a paracrine manner. CCR2 was upregulated by the majority of TRPV1 expressing nociceptors. It was also demonstrated that the activation of CCR2 induced the sensitization of TRPV1 and TRPA1 cation channels that were expressed in nociceptive DRG neurons. The sensitization of TRPV1 by the CCR1 chemokine receptor and its ligand, MIP-1α, was also recently reported by Zhang et al. (2005). Thus, chemokines are part of an ever growing number of potential pain hypersensitivity mediators that transactivate TRP receptors expressed in DRG neurons. The circumstances under which this activation occurs, however, seem to be ligand specific. In the case of chemokines, their expression is specifically associated with states of neuropathic pain, suggesting that their role in generating DRG neuron excitability is particularly associated with this phenomenon.

In summary, it can be concluded that MCP1 may act as a neuromodulator in dorsal root ganglia based on the following findings: 1) MCP1 is expressed by DRG neurons; 2) MCP1 is packaged into secretory vesicles together with molecules such as CGRP that are known to act as neurotransmitters in the DRG; 3) MCP1 is released by neuronal excitation in a Ca-dependent manner; and 4) MCP1 can modulate the excitability of DRG neurons. MCP1-CCR2 signaling in the DRG is not present under normal circumstances but is induced under pathological conditions; therefore, the antagonism of MCP1-CCR2 signaling may constitute a novel therapeutic approach in treating neuropathic pain.

EXPERIMENTAL METHODS

Plasmid construction, adenovirus production, and materials

The MCP1-EGFP fusion protein construct (MCP1-EGFP) used in this study was made by cloning a PCR fragment of the MCP1 protein coding sequence into pEGFP-N1 (Clontech, Mountain View, CA, USA). The CCR2 expression vectors were made by a cloning PCR fragment of the CCR2 protein coding sequence into pEGFP-N1 and pIRES2-EGFP (Clontech). Sequence identity was confirmed by dideoxy-sequencing methods. The TRPV1 (transient receptor potential vanilloid receptor subtype 1) and TRPA1 (transient receptor potential ankyrin 1) expression vectors were kindly provided Drs. David Julius (University of California, San Francisco, CA, USA) and Jaime García-Añoveros (Northwestern University, Chicago, IL, USA), respectively. Adenoviruses were made by cloning the fragments containing the promoter, protein coding sequence, and poly-A signal into a pShuttle vector. The adenoviruses were then

generated according to the protocol in (He et al. 1998). All chemicals were purchased from Sigma (Grand Island, NY, USA) unless stated otherwise.

Cell culture and plasmid DNA transfection

The DRG neuronal cell line, F11, and human embryonic kidney cells (HEK293; tSA201 subclone) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 0.5% penicillin–streptomycin (P/S) at 37°C under 5% CO_2. One microgram of plasmid DNA was transfected using TransIT-LT1 (Mirus, Madison, WI, USA) according to the manufacturer's instructions. To induce differentiation of F11 cells, the culture medium was changed to Dulbecco's modified Eagle's medium supplemented with 0.5% FBS, 0.5% P/S, and 0.5 mM dibutyryl-cAMP 24 h after the transfection. Cells were incubated in this medium for 48–72 h to allow differentiation. Materials for cell culture were purchased from Invitrogen (Carlsbad, CA, USA) unless stated otherwise.

DRG culture and adenoviral infection

Mouse DRG neurons were prepared as described by Oh et al. (2001) with slight modifications. Cells were plated on poly-L-lysine- and laminin-coated coverslips (BD Biosciences, San Jose, CA, USA) and incubated in F12 medium supplemented with 0.5% FBS, 1% N_2, 50 ng/mL nerve growth factor (NGF) and 0.5% P/S. On the next day, 10 μM cytosine arabinoside was added to eliminate mitotic cells including ganglionic fibroblasts. Medium was replaced every 2–3 days, and cultures were maintained at 37°C with 5% CO_2. After 3–5 days in culture, DRG neurons were infected with an adenovirus expressing MCP1-EGFP at 50–100 multiplicity of infection. The infected cells were then used within 3-5 days.

Immunofluorescent labeling

To generate the tissue sections used for labeling, lumbar ganglia associated with the sciatic nerve ipsilateral to the focal nerve demyelination injury (n = 5) or sham treatment (n = 3) were immediately removed from mice transcardially perfused with 4% paraformaldehyde and post-fixed for 4 h. DRG were cut into 14 μm sections and stored at −20°C until use. Cultured cells plated on coverslips were fixed with 4% paraformaldehyde for 15 min. The antibodies used for labeling are as follows: anti-trans-Golgi network glycoprotein 38 (anti-TGN38) mouse monoclonal antibody (1 : 300; Affinity Bioreagents, Golden, CO, USA); anti-Synaptophysin I (anti-SynI) rabbit polyclonal antibody (1 : 300; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-calcitonin gene-related peptide (CGRP) goat polyclonal antibody (1 : 300, Santa Cruz Biotechnology); anti-MCP1 rabbit polyclonal antibody (1 : 1,000; Chemicon, Temecula, CA, USA); anti-TRPV1 rabbit polyclonal antibody (1 : 1000, Neuromics, Edina, MN, USA); and Texas red-conjugated secondary antibodies (1 : 500; Jackson Immunoresearch Laboratories, West Grove, PA, USA). Images were taken using a laser-scanning confocal microscope (Olympus, Melville, NY, USA) equipped with a 60× oil immersion objective lens (numerical aperture, 1.40) and using a Zeiss Axioplan II epifluorescence microscope (Carl Zeiss Microimaging GmbH, Göttingen, Germany). Percentages of immunopositive neurons were acquired using a previously described methodology (White et al. 2005).

Fluorescence-release assay

Cells plated onto a 35 mm culture dish were mounted on the stage of an upright fluorescence microscope (Zeiss Axioplan II, 40× water immersion objective lens) and perfused with a

balanced salt solution (in mM: 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and 10 glucose) at a rate of 1.5 mL/min by a gravity-fed system. To induce depolarization, a high concentration potassium solution (50 mM KCL, denoted as 50K hereafter) was prepared from BSS by adjusting the concentration of KCl from 5 to 50 mM and NaCl from 145 to 100 mM. Calcium-free solutions were prepared by replacing $CaCl_2$ with equimolar $MgCl_2$ and adding 0.5 mM EDTA. The EGFP signal was recorded every 10–40 s for 20 min, and signal intensity was calculated using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

Generation of CCR2-EGFP-BAC transgenic mice

Transgenic mice were created using the bacterial artificial chromosome (BAC) transgenic strategy, which involves transgenesis using a modified BAC clone containing a reporter gene inserted at the end of the gene of interest. BAC clones are large enough to accommodate a large amount of the sequence flanking the gene-of-interest, including many cis-regulatory elements. Therefore, reporter gene expression is expected to mimic the cells' endogenous expression of the gene-of-interest (Gong et al. 2003). The CCR2-containing BAC clone (MSM-529G05) was obtained from RIKEN DNA Bank (Ibaraki, Japan). The recombineering protocol (Lee et al. 2001) was used with slight modifications. Briefly, to generate the CCR2-BAC reporter vector, EGFP was inserted immediately downstream of the CCR2 coding sequence by λ-Red-mediated recombination. The EGFP-FRT-KAN-FRT targeting cassette was generated by self-ligation of the blunt-ended BglI/SmaI fragment of pIGCN21. The targeting cassette was amplified by PCR using the following chimeric primers where the 3' end was homologous to the targeting cassette and the 5' end was homologous to the last exon of CCR2: for upstream, 5'-

TGAGCTCTACATTCACTCCTTCCACTGGGGAGCAAGAGGTCTCGGTTGGGTTGGAT GATAATATGGCCACAACC-3'; for downstream: 5'-CTGTCTTTGAGGCTTGTTGCTATG-TACAAACTGCTCCCTCCTTCCCTGCTCTATTCCAGAAGTAGTGAGGA-3'. The primers were designed to target EGFP immediately downstream of the CCR2 coding sequence and upstream of the poly-A site. The stop codon of CCR2 was deleted to generate the CCR2-EGFP fusion construct. Transgenic mice were generated with the help of Center for Genetic Medicine, Northwestern University (Chicago, IL, USA).

Calcium imaging

The AM form of fura-2 (Invitrogen) was used as the fluorescent Ca indicator. All measurements were made at room temperature (21°C) as described previously (S. Bhangoo et al. 2007). The DRG cells loaded with fura-2AM (3 μM) were mounted onto the chamber (500 μL total volume) which was then placed onto the inverted microscope and perfused continuously by a balanced salt solution. Ratiometric images were monitored every 3 s and Ca concentration was calculated by a standard curve generated using a calcium calibration buffer kit (C-3721, Invitrogen). For MCP1 (R & D Systems, Minneapolis, MN, USA), capsaicin (CAP), and allyl isothiocyanate, 1 mL of solution was applied directly to the bath chamber after stopping the flow of BSS.

Statistics

Data from the ELISA and some Ca imaging experiments were presented as mean ± SEM. The ELISA experiment which measured the regulated release of MCP1-EGFP, and the experiment inhibiting TRPV1 sensitization were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests *post hoc*. The TRPV1 sensitization experiment using varying

concentrations of CAP was analyzed by unpaired Student's t-test. Statistical significance was set at $p < 0.05$.

CHAPTER 3

Molecular Mechanisms Underlying MCP1 and CCR2 Upregulation in Dorsal Root Ganglion Neurons

This chapter has been published except for the figure 3.10 (Jung and Miller 2008).

ABSTRACT

Upregulation of the chemokine, MCP1 and its receptor, CCR2, by dorsal root ganglion (DRG) neurons is an important process in the development and maintenance of neuropathic pain. However, the molecular mechanisms underlying neuronal upregulation of MCP1 and CCR2 have not been investigated. This research shows that MCP1 is upregulated by tumor necrosis factor-alpha (TNFα) through activation of the nuclear factor kappa B (NFκB) pathway. Research also revealed a conserved binding site for the nuclear factor of activated T-cells (NFAT) in the promoter region of the CCR2 gene. Experiments showed that the CCR2 promoter could be activated by a constitutively active form of calcineurin A and that a point mutation in the NFAT binding site abrogated this activation, proving that the NFAT element is functional. Activation of the NFAT pathway in the DRG neuronal cell line, F11, increased CCR2 promoter activity and induced CCR2 transcription. Moreover, depolarization of cultured DRG neurons induced *de novo* synthesis of CCR2 mRNA, which was blocked by the calcineurin inhibitors, cyclosporin A and FK506. These data indicate that CCR2 is a target of the NFAT pathway and suggest that tonic excitation of DRG neurons in association with chronic pain may lead to neuronal CCR2 upregulation via activation of the NFAT pathway.

INTRODUCTION

Long-term changes in the profile of gene transcription in dorsal root ganglion (DRG) cells and in the central nervous system (CNS) are believed to underlie the heightened pain sensitivity observed in neuropathic pain states. Many genes are differentially regulated by DRG neurons and glia in different animal models of chronic pain, suggesting multiple signaling pathways may contribute to this process (Mogil, Yu, and Basbaum 2000; Ji and Strichartz 2004; Basbaum and Woolf 1999). However, the mechanism by which such changes occur is poorly understood.

Under normal circumstances neither CCR2 nor its ligand, MCP1, are expressed by DRG neurons. However, expression of CCR2 has been found to be upregulated by DRG neurons in association with several animal models of neuropathic pain (S. Bhangoo et al. 2007; Sun et al. 2006; White et al. 2005). Importantly, DRG neurons from animals exhibiting neuropathic pain are strongly excited by the application of MCP1, whereas neurons from naïve animals are not (Sun et al. 2006; White et al. 2005). Moreover, CCR2 knockout mice do not develop neuropathic pain following spared nerve injury (Abbadie et al. 2003), suggesting that upregulation of CCR2 is a crucial step in the generation and/or maintenance of neuropathic pain. However, unlike MCP1 whose transcriptional regulation has been studied in non-neuronal cells, no studies have been done on the transcriptional regulation of CCR2 receptors. Consequently, it is of interest to researchers to define the signaling pathway responsible for the upregulation of these receptors in DRG neurons.

In addition to its diverse roles in the development and function of the immune system, it has recently been shown that the nuclear factor of activated T-cells (NFAT) family of transcription factors can play an important role in mediating long-term changes in neuronal excitability (Graef et al. 1999). NFAT cannot normally enter the nucleus until it is dephosphorylated, but can be activated by a Ca-dependent phophatase, calcineurin (CN). Thus, an increase in $[Ca]_i$ turns on the transcription of NFAT-dependent genes, and this is believed to link transient neuronal excitation to long-lasting transcriptional activation (Graef et al. 1999; Zanzouri et al. 2006).

This work reports that CCR2 is a target gene of the NFAT pathway in DRG neurons. The following experiments demonstrate that there is a functional and conserved NFAT binding element in the promoter region of the CCR2 gene. Activation of the NFAT pathway in DRG neurons and the in DRG neuronal cell line, F11, resulted in increased CCR2 promoter activity and initiated CCR2 transcription. These data suggest that repetitive excitation of DRG neurons under pathological circumstances may turn on activity-dependent transcription of CCR2 via NFAT signaling. Hence, antagonism of the NFAT pathway may be a novel point for therapeutic intervention in treating neuropathic pain.

RESULTS

MCP1 upregulation: involvement of the NFκB pathway.

The mechanisms underlying the inducible expression of MCP1 are relatively well understood in non-neuronal cells. Conserved binding elements for the transcription factor, nuclear factor kappa

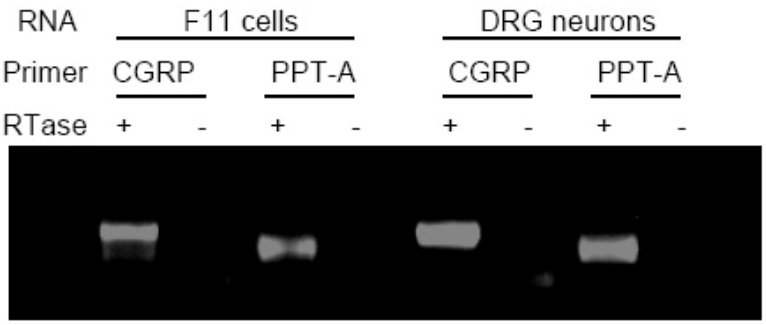


Figure 3.1. F11 cells express the neuropeptides of DRG neurons. Expression of CGRP and PPT-A, known neuropeptides expressed by DRG neurons, was examined in F11 DRG neurons by RT-PCR (RTase: reverse transcriptase). RTase-negative control reactions were performed for each group. F11 neurons expressed both CGRP and PPT-A.

B (NFκB), reside in the promoter region of the MCP1 gene. Activation of signaling pathways upstream of the NFκB pathway induces transcription of MCP1 in cultured fibroblasts (Ping, Jones, and Boss 1996). Like many pathological conditions with associated chronic inflammation, elevation of TNFα in serum is a common clinical feature of neuropathic pain (Empl et al. 2001). The activation of the receptors for TNFα, two of which are known (i.e. TNFRI and TNFRII), turns on a cascade of signaling events that ultimately leads to the activation of the transcriptional factor complex, NFκB. All neurons in DRG express TNFRI, whereas satellite glia surrounding neurons upregulate TNFα during the inflammatory response (Li et al. 2004). Therefore, the possibility that TNFα induces the expression of MCP1 in DRG neurons was examined.

A neuronal cell line derived from a DRG neuron (F11) was used as a cellular model for DRG neurons. F11 cells retain several features of mature sensory neurons including the expression of voltage-sensitive Ca channels (VSCCs), substance P-like peptides (Francel et al. 1987), opioid receptors (Fan et al. 1992), and transient receptor potential (TRP) channels (Jahnel et al. 2003). Moreover, they also express mRNA for calcitonin gene-related peptide (CGRP) and

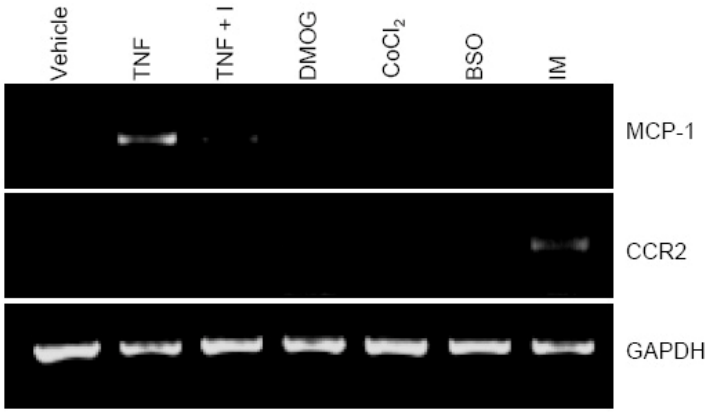


Figure 3.2. Differential regulation of MCP1 and CCR2 in F11 DRG neurons. F11 DRG neurons were stimulated with various chemicals (TNF: 20 ng/ml TNF-α; I: 30 nM NF-kB activation inhibitor; DMOG: 1 nM dimethyloxalylglycine; CoCl2: 1 nM CoCl2; BSO: 200 μM buthionine-Lsulfoximine; IM: 1 μM ionomycin), and RT-PCR was performed to measure MCP1 and CCR2 mRNA levels. Note that IM treatment only induced CCR2 expression and TNF-α only MCP1. TNF-α-mediated MCP1 induction was completely blocked by the NF-kB activation inhibitor.

preprotachykinin (PPT; a precursor for substance P) (Figure 3.1), known neuropeptides stored in primary nociceptors. In accordance with previous findings in mice and rats, F11 cells did not express MCP1 at a high level as revealed by RT-PCR. In contrast, treatment with TNFα significantly increased MCP1 mRNA, which could be completely blocked by an inhibitor of the NFκB pathway (NFκB activation inhibitor) (Figure 3.2). These results suggest that MCP1 is indeed a target gene of the NFκB pathway in DRG neurons, and that MCP1 can be upregulated by activation of TNFα receptors. However, CCR2 was not upregulated by TNFα suggesting that distinct mechanisms govern the gene expression of MCP1 and CCR2 in DRG neurons (Figure 3.2).

The structure of the mouse CCR2 gene

Unlike MCP1, the upregulation of CCR2 gene expression is not well understood. In order to gain insights into the gene regulatory mechanisms which underlie CCR2 upregulation, this study examined the genomic organization of the mouse CCR2 gene. It is composed of three exons with the entire protein coding region residing in the third exon (Figure 3.3A). The length of the 5'-untranslated region (5'-UTR) varies between the reported mRNA sequences (Figure 3.3A). The expected length of the first exon, deduced from the longest reported sequence (AK046579) known at the time, was 258 nucleotides (Figure 3.3A). In order to see if this sequence represented the transcription initiation site, a primer extension experiment was performed using a primer located 61 nucleotides downstream of the putative initiation site (Figure 3.3B). The RNA was isolated from a mouse monocytic cell line, WEHI265.1, from which the first mouse CCR2 cDNA was cloned (Kurihara and Bravo 1996). A specific band of the expected size was generated, indicating that the transcription initiation site lies near the expected region. To confirm this, RT-PCR was performed using a reverse primer located in exon 3 and a forward primer located in exon 1 or in exon 2. As expected, specific bands were only generated from the cDNA of WEHI265.1 cells but not from F11 cells, a DRG neuronal cell line, using both combinations of primer sets (Kurihara and Bravo 1996) (Figure 3.3C). However, a distinct band that was around 100 base pairs shorter than the expected size was also generated when the forward primer in exon 1 was used (Figure 3.3C: left diagrams). The two bands were cloned, and their sequence identities were verified. It was found that there is a complete set of splice donor and acceptor sites within exon 1, and that both mRNA species (complete exon 1 and deleted exon 1) co-exist in WEHI265.1 cells (Figure 3.3D).

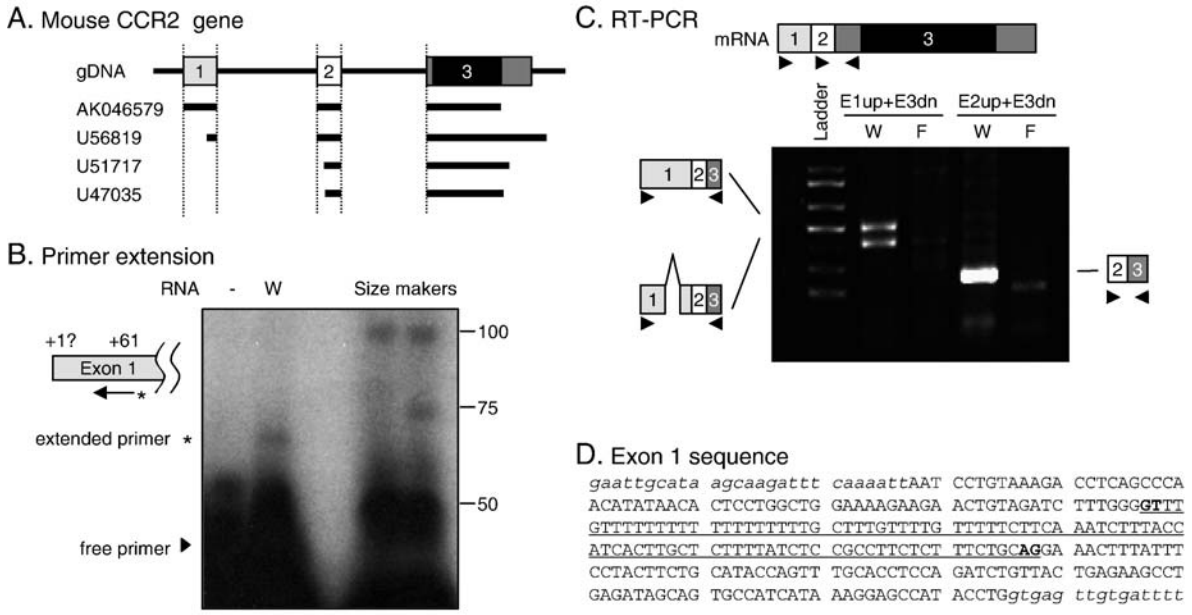


Figure 3.3. The genomic organization of the mouse CCR2 gene. (A) Reported mRNA species and their coverage on genomic DNA sequences (numbered boxes: exons; lines between exons: introns; a black box: protein coding region). (B) Primer extension experiment. The location of the primer is denoted in the diagram on the left (W: WEHI265.1 monocytic cell line). (C) RT-PCR experiments. Relative location of primers is denoted on the upper diagram (arrow heads: primers). Two sets of primers were used: E1up/E3dn and E2up/E3dn (left two and right two lanes, respectively). The structure of the PCR product is denoted in the left and right diagrams (W: WEHI265.1 monocytic cell line; F: F11 DRG neuronal cell line). (D) The sequence of the exon 1 (E1). Exonic sequence is in upper cases and non-exonic sequence in lower cases. An additional intronic sequence within the exon 1 is underlined. AG and GT consensus splice dinucleotides are in bold letters. An mRNA with this sequence spliced out corresponds to the shorter band in the leftmost lane in panel C.

Identification of the minimal promoter for CCR2

CCR2 is known to be constitutively expressed by several types of immune cells including monocytes, B-cells, activated T-cells, NK cells, and immature dendritic cells. However, as shown in Chapter 2, CCR2 is expressed by DRG neurons only under pathological conditions. As this information predicts, the DRG neuronal cell line, F11, did not express detectible levels of

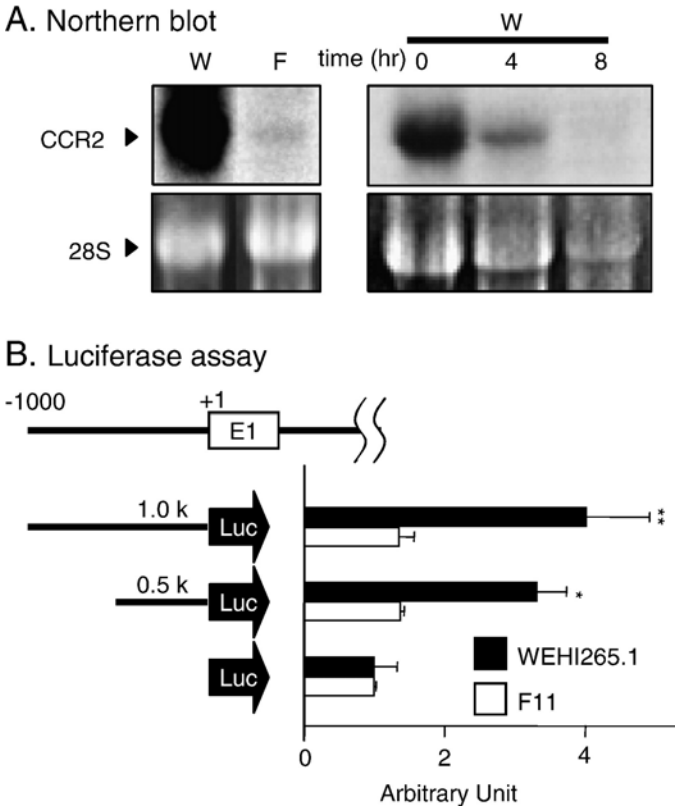


Figure 3.4. Cloning of a functional CCR2 promoter. (A) Cell type-specific CCR2 transcriptional activity. CCR2 was expressed by the WEHI265.1 (W) monocytic cell line but not by the F11 DRG neuronal cell line (F) under basal condition (left panels). The half-life of CCR2 mRNA was examined in WEHI265.1 (right panels). After the addition of a transcriptional inhibitor actinomycin D (ActD: 1 μg/ml), the CCR2 mRNA level rapidly decreased. 28S rRNA was used as an internal control. (B) Cell type specific CCR2 promoter activity. 1.0 kb and 0.5 kb fragments upstream of exon 1 (E1) were cloned into a luciferase reporter vector (Luc). Promoter activity was high in WEHI265.1 cells but very low in F11 cells ($p < 0.01$ and $p < 0.05$ between WEHI265.1 and F11, unpaired t-test).

CCR2, although WEHI265.1, the monocytic cell line, expressed high levels of CCR2 mRNA (Figure 3.3D and Figure 3.4A: left panels). The high expression of CCR2 mRNA in WEHI265.1 was due to active ongoing transcription as evidenced by the short half-life of CCR2 mRNA in these cells (Figure 3.4A: right panels). In order to define the minimal promoter region responsible for cell type-specific CCR2 expression, a 1 kilobase pair fragment upstream of the

transcription initiation site was cloned into a luciferase reporter vector, and the relative promoter activity was examined in these two cell lines. The activity of the CCR2 promoter recapitulated the transcriptional activity and this was maintained even with a 0.5 kb proximal fragment, suggesting that important regulatory cis-elements lie in this relatively short fragment of the promoter (Figure 3.4B).

Identification of a conserved binding element for the nuclear factor of activated T cells (NFAT)

In order to define the signaling pathways whose activation might lead to the upregulation of neuronal CCR2 receptors, putative transcription binding elements in the shorter fragment of the CCR2 promoter were sought using standard programs (MatInspector v6.0, Genomatix Software GmbH; MacVector, Accelrys). Several programs identified an NFAT binding element conserved between human and mouse in the proximity of the transcription initiation site (Figure 3.5A). To examine if the NFAT site was functional, an electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts from WEHI265.1 cells. Specific binding complexes were formed (Figure 3.5B: filled arrowheads), which could be competed for by unlabeled self-competitors but not by mutated competitors (GGAAAA → GGTTTT). The presence of NFAT in these binding complexes was confirmed by 'supershift' assays. The formation of specific binding complexes (Figure 3.5B: filled arrowheads) but not nonspecific complexes (Figure 3.5B: empty arrowheads) was inhibited by the addition of a pan-specific antibody to NFATc (which recognizes NFATc1 to c4). Neither complex was inhibited by an isotype-matched control antibody. Next, the CCR2 promoter was co-transfected with a

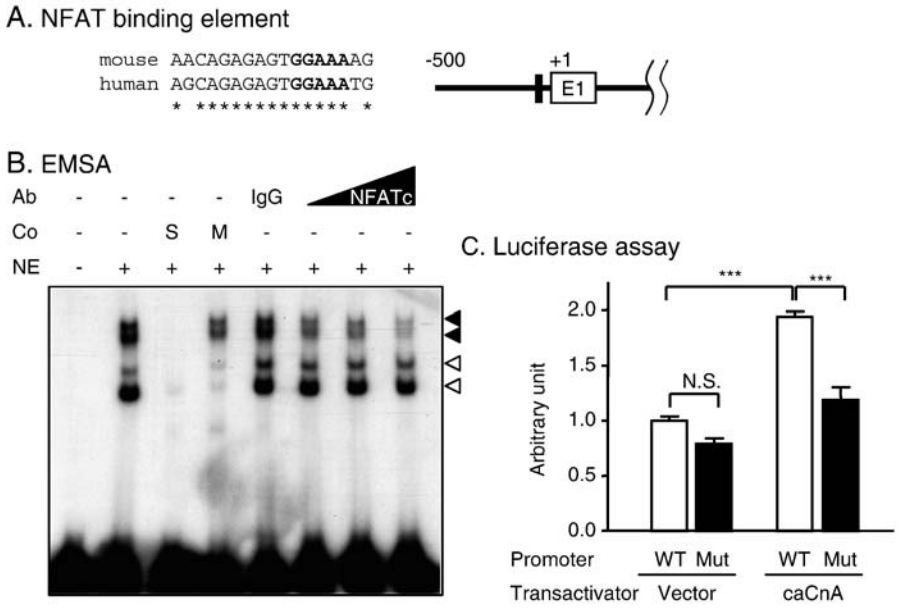


Figure 3.5. An NFAT element in the promoter. (A) The NFAT binding element near the transcription initiation site is conserved (*) between mouse and human (E1: exon 1). The core binding sequences are in bold. (B) A specific NFAT binding activity in the nuclear extract of WEHI265.1 cells (Co: cold competitors; S: self-competitors; M: GGAAA → GGTTT mutated competitors; NE: nuclear extract from WEH265.1 cells; a filled arrowhead: specific NFAT binding activity; an empty arrowhead: binding activity that is not specific to the core NFAT sequence; Ab: antibody; IgG: an isotype-matched control antibody; αNFATc: a pan-specific antibody to NFATc). (C) The NFAT binding element in the CCR2 promoter is functional. The CCR2 promoter (WT) or the promoter with a point mutation in the NFAT binding site (Mut: GGAAA → GGTTT) was co-transfected with a constitutively active form of calcineurin A (caCnA) or an empty vector ($p < 0.001$; N.S.: not significant, a post hoc Bonferroni's multiple comparison test following one-way ANOVA).

constitutively active form of calcineurin A (a catalytic subunit) (caCnA) to examine if the CCR2 promoter could be activated by the NFAT pathway. CCR2 promoter activity was increased by caCnA, and this was blocked by a point mutation in the NFAT binding sequence, finally indicating that the NFAT site in the CCR2 promoter is functional (Figure 3.5C).

Activation of the NFAT pathway upregulates CCR2.

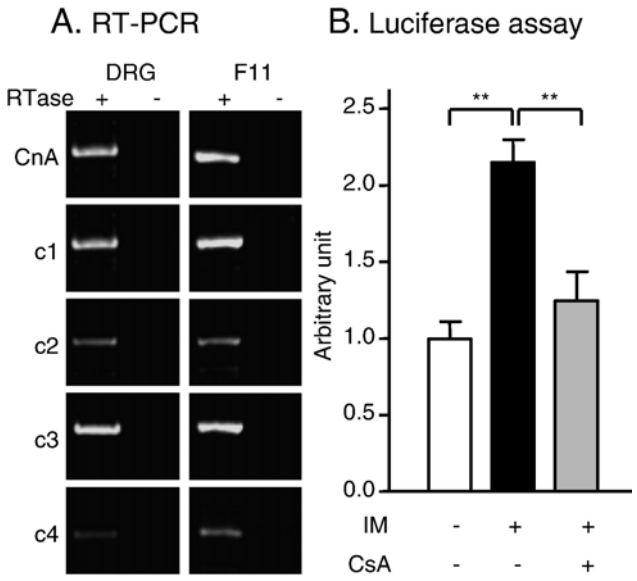


Figure 3.6. The components of the NFAT pathway in DRG neurons and F11 cells. Calcineurin A (CnA) and NFATc1–4 were expressed by cultured DRG neurons and F11 DRG neurons (RTase: reverse transcriptase). RTase-negative control reaction was performed for each group. (B) CCR2 promoter is activated by calcium ionophore ionomycin (IM: 1 μM) in F11 DRG neurons in a calcineurin inhibitor cyclosporin A (CsA: 1 μM)-sensitive way ($p < 0.01$, a post hoc Bonferroni's multiple comparison test following one-way ANOVA).

This experiment sought to determine if NFAT activation in DRG neurons or F11 cells leads to upregulation of CCR2. First, RT-PCR was performed to determine whether DRG neurons and F11 cells express components of the NFAT pathway. CnA and all isoforms of NFAT were expressed in both DRG and F11 neurons (Figure 3.6A). The second experiment asked if NFAT activation increases CCR2 promoter activity in F11 cells. F11 cells transfected with a CCR2 promoter-luciferase construct were treated with the Ca ionophore, ionomycin (IM). IM treatment increased CCR2 promoter activity which was blocked by co-treatment with a CN inhibitor, cyclosporin A (CsA), indicating that the IM-mediated increase in CCR2 promoter activity was due to NFAT activation (Figure 3.6B). Next, the study examined if IM treatment could also increase CCR2 mRNA. It was found that IM treatment increased CCR2 mRNA (Figure 3.7A),

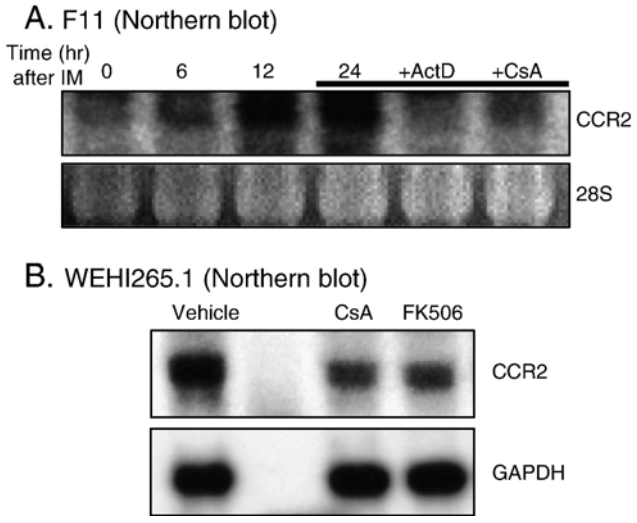


Figure 3.7. NFAT-dependent transcription of CCR2 in F11 and WEHI265.1 cells. (A) NFAT-dependent de novo transcription of CCR2 in F11 DRG neurons. Ionomycin (IM: 1 μM) treatment induced CCR2 mRNA which was blocked by a transcriptional inhibitor actinomycin D (ActD: 1 μg/ml). The induction was significantly inhibited by a calcineurin inhibitor cyclosporin A (CsA: 1 μM). 28S rRNA was used as an internal control. (B) NFAT-dependent transcription contributes to cell type-specific CCR2 expression. Calcineurin inhibitors CsA or FK506 (0.2 μM each) decreased baseline levels of CCR2 in WEHI265.1 monocytic cell line. GAPDH was used as an internal control.

and it was determined that enhanced CCR2 mRNA production was due to *de novo* synthesis, because the increase was completely blocked by the transcriptional inhibitor, actinomycin D (ActD). Moreover, this increase was mediated by activation of the NFAT pathway because it was inhibited by CsA (Figure 3.7A). It was reasoned that if the NFAT pathway is involved in the inducible regulation of CCR2 gene expression, it may also participate in the normal constitutive cell type-specific expression of CCR2. Indeed, experiments showed that CCR2 mRNA levels were significantly decreased in WEHI265.1 by FK506 or CsA (Figure 3.7B).

Activity-dependent transcription of CCR2 is mediated by the NFAT pathway in DRG neurons.

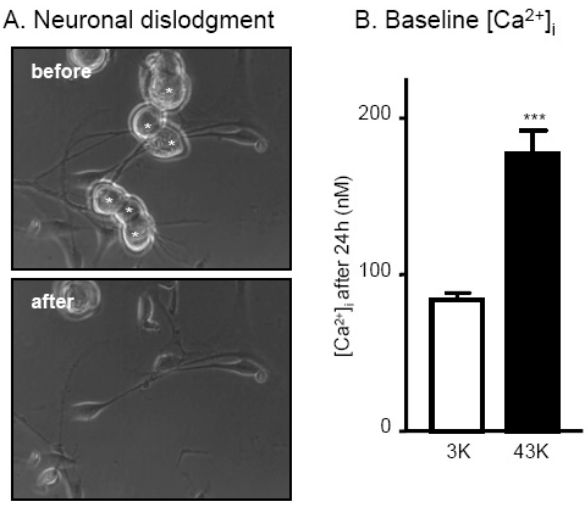


Figure 3.8. Semi-acute culture of DRG neurons. A) DRG isolated from 5-6 CD1 pups (postnatal day 3-5) were dissociated and plated on an uncoated and tissue culture treated dish for 2 hr in F12 medium supplemented with 0.5% FBS, 1% N2, 50 ng/ml NGF and 0.5% P/S. After cell attached, 0.25 volume of 200 mM KCl or 200 mM NaCl solution was added to culture media (final concentrations: for high K medium, 101 mM NaCl and 43 mM KCl; for control medium, 141 mM NaCl and 3 mM KCl). Calcineurin inhibitors (0.2 μM each) were added 30 min prior to the addition of the salt solutions. After 24 hr, neurons which are loosely attached to the dish (the upper panel: asterisk) were dislodged by gentle pipetting (the lower panel). Isolated neurons were subjected to RT-PCR. B) High K culture increased baseline levels of [Ca]i. After 24 hr culture in high potassium (43K) or control (3K) media, intracellular calcium level was examined by Fura-2 based Ca imaging as previously described (Bhangoo et al., 2007) (***$p<0.001$ vs. 3K, unpaired t-test).

The final experiments were designed to test the hypothesis that activation of NFAT could lead to the CCR2 upregulation in DRG neurons. A semi-acute DRG culture system was developed to measure mRNA selectively from neurons while minimizing contamination from non-neuronal cells (Figure 3.8A). Dissociated DRG cells were allowed to attach to an uncoated tissue culture dish for several hours, and then neurons were depolarized using high potassium (high K) medium. Twenty four hours later, loosely attached neurons were dislodged by gentle pipetting, total RNA was isolated, and RT-PCR was performed to measure CCR2 mRNA. Neurons incubated in high K media had an elevated level of basal $[Ca]_i$ due to activation of voltage-

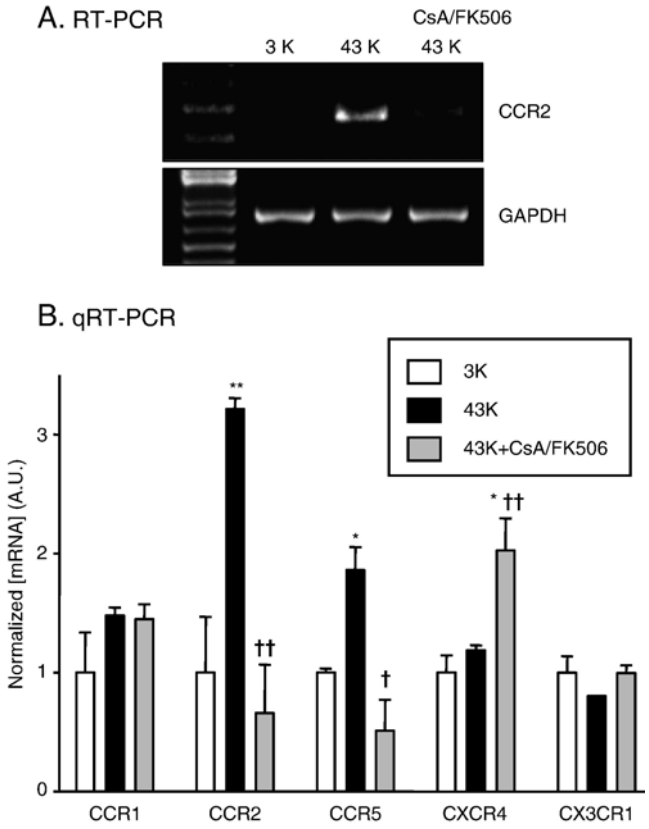


Figure 3.9. Depolarization-induced transcription of CCR2 in DRG neurons. (A) NFAT-dependent transcription of CCR2 is induced in DRG neurons by depolarization. Acutely isolated DRG neurons were depolarized by high external potassium (43K: 43 mM) for 24 h with or without calcineurin inhibitors (CsA/FK506: 0.2 μM each), which were added 30 min prior to depolarization. Control culture medium contained 3 mM of potassium (3K). Osmorality of different media was identical. Total RNA was isolated from neurons (see Supplementary Fig. 1) and subjected to RT-PCR. (B) Real-time quantitative RT-PCR was used to quantify mRNA levels of different chemokine receptors in conditions described in A (n = 3–5 independent DRG cultures) ($p < 0.01$, $p < 0.05$ vs. 3K and ††$p < 0.01$, †$p < 0.05$ vs. 43K, a post hoc Bonferroni's multiple comparison test following one-way ANOVA).

dependent Ca channels (Figure 3.8B). CCR2 mRNA was undetectable in the unstimulated group, in which neurons were incubated in a medium containing a low concentration of potassium while overall osmolarity was maintained (Figure 3.9A). However, high K stimulation increased CCR2 mRNA a phenomenon which was completely blocked by CsA/FK506, indicating that a

depolarization-induced increase in CCR2 mRNA was mediated by activation of the NFAT pathway (Figure 3.9A and B).

Expression of several other chemokine receptors has also been closely associated with the genesis of neuropathic pain (S. Bhangoo et al. 2007; S.K. Bhangoo et al. 2007). Therefore, DRG neurons were examined to determine whether additional chemokine receptors are targets of NFAT activation (Figure 3.9B). Several chemokine receptors (CCR1, CCR2, CCR5, CXCR4 and CX3CR1) were studied, and the increased expression of CCR2 and CCR5 was induced by high K depolarization in a CN inhibitor-sensitive manner (Figure 3.9B). Importantly, this data suggests that a group of chemokine receptor genes could be induced synchronously using the same mechanism. Expression of CCR1 and CX3CR1 remained unchanged; however, CXCR4 expression was increased by CN inhibitors indicating that the same pathway might have opposite effects on different genes. Overall, these data suggest that the transcription of different groups of chemokine receptors is differentially regulated at the transcriptional level in DRG neurons.

DISCUSSION

The sustained pain hypersensitivity and neuronal hyperexcitability associated with neuropathic pain is believed to result from long-term changes in gene expression in DRG and central dorsal horn neurons. Many genes have been identified whose expression is changed in association with the development of neuropathic pain using several animal models. However, the molecular mechanisms responsible for such changes are largely unknown (Mogil, Yu, and Basbaum 2000). In particular, the CCR2 chemokine receptor appears to play an important role in the genesis of

neuronal hyperexcitability in states of chronic pain (Abbadie et al. 2003; White, Bhangoo, and Miller 2005; White et al. 2005). This receptor is not expressed by DRG neurons under normal circumstances, but its expression is induced under pathological conditions. Accordingly, CCR2 expression was undetectable in cultured DRG neurons or F11 DRG neuronal cells. By examination of the promoter sequence of CCR2, it was found that expression in sensory neurons can be induced by activation of the NFAT pathway.

Transcriptional regulation of chemokine receptors in neurons has not been extensively studied. Most research on this topic has instead been focused on immune cells. Previous reports using human monocytic cells have suggested that tissue-specific expression of CCR2 was attributable to the differential activity of the transcription factors, Oct1 and C/EBPβ (Yamamoto et al. 1999). This study found that, in the mouse promoter, Oct1 and C/EBPβ binding sites are also conserved. As this information would suggest, it has been observed that co-transfection of C/EBPβ also activated the mouse CCR2 promoter. As this chapter has shown, the NFAT pathway can also activate the mouse CCR2 promoter in parallel with C/EBPβ (Jung and Miller, unpublished data). This suggests that multiple transcriptional pathways may control the upregulation of CCR2 gene expression under different circumstances. However, in addition to these transcription factors, constitutive activity of NFAT signaling may also contribute to tissue-specific expression because it was found that inhibition of the NFAT pathway by FK506 and CsA decreased constitutive levels of CCR2 mRNA in WEHI265.1 monocyte cells (Figure 3.8B).

The NFAT pathway is a particularly pertinent candidate pathway for regulating activation of genes responsible for neuropathic pain, because it can be activated by neuronal depolarization in

a Ca-dependent manner (Graef et al. 1999). It was recently shown that repetitive action potentials in sympathetic neurons resulted in the nuclear translocation of NFAT (Hernandez-Ochoa et al. 2007). The ectopic discharge of action potentials in DRG neurons is a typical feature of neuropathic pain (Basbaum and Woolf 1999), therefore, tonic excitation of DRG neurons under pathological conditions might induce the activity- and Ca-dependent transcription of NFAT target genes, including CCR2 receptors. CCR2 signaling in DRG neurons is itself excitatory (Sun et al. 2006; White et al. 2005), so the net effect of receptor upregulation would be amplification of hyperexcitability and pain. It is interesting to note that other chemokine signaling pairs are also upregulated in the DRG in association with neuropathic pain including CCR5 and CXCR4 receptors and their ligands (S. Bhangoo et al. 2007; S.K. Bhangoo et al. 2007).

As researchers have observed (Figure 3.9B), transcription of several chemokine receptor genes in DRG neurons appears to involve diverse signaling pathways. For example, upregulation of CCR2 and CCR5 in response to depolarization appears to proceed in an NFAT-dependent manner, whereas, CXCR4 was upregulated by inhibition of the NFAT pathway. The promoters for CCR2 and other chemokine receptors contain binding sites for multiple transcription factors, thus, it is likely that they can be regulated by different signaling pathways under different circumstances including depolarization. For example, chemokine receptor upregulation in response to inflammatory cytokines such as TNFα may also play a role in reshaping the gene expression profile of DRG neurons in states of pain hypersensitivity and may function through pathways in addition to NFAT (White, Bhangoo, and Miller 2005).

Interestingly, this work has demonstrated that in ganglia from animals exhibiting nociceptive pain behavior both CCR2 and its ligand MCP1 are upregulated by the neighboring and closely juxtaposed neurons (Chapter 2). As was shown here, CCR2 can be upregulated by depolarization in a Ca/NFAT-dependent manner, but MCP1 is not upregulated in response to these stimuli. However, MCP1 can be upregulated in a TNFα/NFkB-dependent manner (Figure 3.2). Thus, the entire panoply of important gene transcriptional changes occurring in DRG neurons in association with states of chronic pain presumably result from a spectrum of transcriptional events.

It is known that NFAT-mediated transcription plays a role in neuronal development and synaptic plasticity. With respect to DRG neurons in particular, Thayer and colleagues reported that bradykinin, a pronociceptive peptide secreted from the site of injury, activates NFAT signaling in DRG neurons resulting in NFAT-dependent transcription of COX2 (Jackson, Usachev, and Thayer 2007). Seybold and colleagues reported that NGF and BDNF, pronociceptive neurotrophins, also activate NFAT-dependent transcription (Groth et al. 2007). From these studies it can be speculated that the NFAT pathway is not only be activated in the development phase of neuropathic pain as a result of the action of multiple proinflammatory molecules, but is also activated during the maintenance phase in which activity- and Ca-dependent transcription takes place. Activation of diverse transcriptional pathways, including NFAT may turn on the transcription of a cohort of genes responsible for the sustained hyperexcitability of DRG neurons in neuropathic pain. Therefore, the NFAT pathway may represent a novel point for therapeutic intervention in the treatment of neuropathic pain. Indeed, a single injection of a calcineurin inhibitor, FK506, ameliorated established hyperalgesia in the LPC-mediated focal demyelination

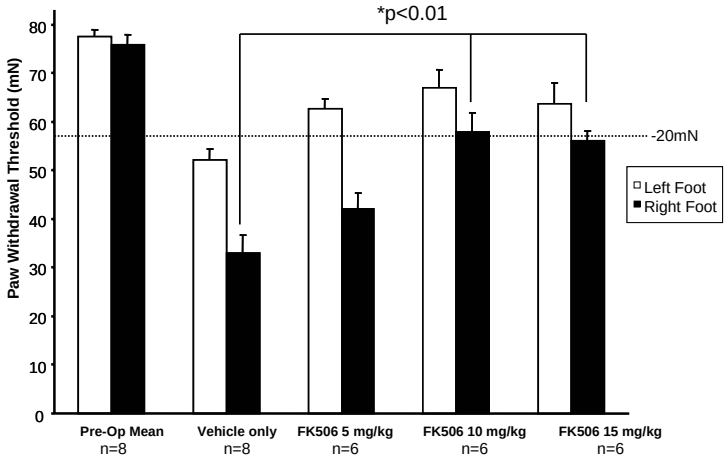


Figure 3.10. A calcineurin inhibotor, FK506, reverses pain hypersensitivity. Animals were subjected to a nerve demyelination injury on day 0 and nociceptive behavior was assessed on post-operative day (POD) 14. On POD14, animals received 5-15 mg/kg FK506 or saline control solution by intraperitoneal injection, and behavioral responses were tested 1 h later. Administration of 10 mg/kg or more FK506 to focal nerve demyelination injured rats resulted in a significant bilateral increase of mechanical threshold to elicit a paw withdrawal compared with vehicle-treated controls. Data represent means ± SE. (*$p < 0.01$). (Unpublished data by White *et al.*)

model of neuropathic pain. The reversal of pain hypersensitivity was incomplete and short-lived (around 4 h), but dose-dependent and statistically significant (Figure 3.10). Although the mechanism of action in this case is not clear, this promising result indicates that this question merits further consideration for uses in human therapy.

Inhibition of transcriptional pathways such as NFAT may regulate expression of several target genes, therefore, its inhibition may be more efficacious than individual receptor antagonists in the long-term reversal of chronic pain hypersensitivity. Considering the impressive effects of a single injection of FK506 after full establishment of pain hypersensitivity, the use of sustained NFAT antagonism as treatment for neuropathic pain appears promising. It may be particularly efficient in blocking the development of neuropathic pain, or in gradually reversing established

neuropathic pain. Long-term antagonism of the NFAT pathway should therefore be critically evaluated as a therapy for neuropathic pain.

EXPERIMENTAL METHODS

Plasmid constructions and materials

The fragments of the 5'-flanking region of CCR2 were amplified from mouse genomic DNA (from a CD-1 mouse) by polymerase chain reaction (PCR) and cloned into the pGL3-Basic (Promega). A point mutation in the NFAT binding site was introduced using the QuickChange site-directed mutagenesis kit (Stratagene). The constitutively active calcineurin A construct (caCnA) was a kind gift from Dr. Neil A. Clipstone (Loyola University Chicago, Maywood, IL) (Clipstone, Fiorentino, and Crabtree 1994). All PCR products were cloned into the pCR2.1 vector (Invitrogen) and sequence identities were confirmed by dideoxy-sequencing methods. Primers used in cloning are listed in Table 2.

Cell culture

F11 cells, a rat DRG neuron X mouse neuroblastoma hybrid, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.5% penicillin-streptomycin (P/S) at 37°C under 5% CO_2. WEHI265.1 mouse monocytic cells were grown under the same conditions except the medium was supplemented with 0.05 mM 2-mercaptoethanol. DRG neurons were isolated as described (Oh et al. 2001).

Northern blot analysis

Total cellular RNA was extracted with Trizol reagent (Life Technologies). RNA was dissolved in deionized water and denatured in 50% formamide, 6.2% formaldehyde, 20mM MOPS (3-[N-morpholino]propanesulfonic acid), 5 mM sodium acetate, and 1 mM EDTA at 60 °C for 5 min. Electrophoresis was performed at 100V for 1.5 hr in a 1.2% formaldehyde agarose gel. RNA was transferred to Nytran filter (Amersham) for 18 hr by capillary transfer. CCR2 and GAPDH probes labeled with ^{32}P-dCTP (Amersham) were prepared by random labeling of a mouse CCR2 cDNA 397 base pair fragment and a mouse GAPDH cDNA 800 base pair fragment cloned into pGEM-Teasy (Promega). Hybridization procedures and rehybridization with GAPDH cDNA were performed using the ExpressHyb hybridization solution (BD biosciences) according to the manufacturer's instructions. The primers used are listed in Table 2.

Reverse transcription-PCR (RT-PCR)

RNA samples (1 μg) were applied to the reverse transcription (RT) reaction containing 100 U of Superscript II, 0.5 μg of oligo(dT) primers, 20 U of RNase inhibitor, and 1 mM dNTPs in a buffer supplied by the manufacturer (Invitrogen). The RT reaction was carried out at 42°C for 50 min followed by inactivation at 70°C for 15 min. After this, RNase H was added and the reaction mixture was incubated at 37°C for 30 min. Subsequently, 1/50 of the reaction product was added to 20 μl of the PCR reaction mixture which contained 2.5 U of Taq DNA polymerase (Qiagen) in a buffer supplied by the manufacturer. Thirty-two to forty-two cycles of PCR amplification were carried out with the following conditions: denaturation at 95°C from 1 min, primer annealing at 55-60°C for 1 min, and primer extension at 72°C for 40 sec. Five microliter (5 μL) aliquots of PCR product were electrophoresed on a 1.2 – 2.0 % agarose gel in Tris-acetate-EDTA buffer, stained with ethidium bromide, and photographed under UV illumination.

For quantitative PCR, real-time PCR was performed using Taqman probe-based chemistry on a sequence detector (7900HT, Applied Biosystems). Taqman gene expression assays containing two primers and one Taqman probe for each gene were purchased from Applied Biosystems (listed in Table 2). Most assays (CCR2, CCR5, CXCR4, and CX3CR1) were designed to span an exon-exon boundary to exclude false positive amplification of contaminated genomic DNA. Also, experiments with the RTase-negative controls confirmed negligible levels of genomic DNA contamination in cDNA samples ($\Delta Ct \approx 10$). Relative quantification analyses were performed by a comparative Ct method using GAPDH as an internal control.

Primer extension

Twenty pmole of primer (Table 2) was end-labeled with ^{32}P-γ-dATP using T4 polynucleotide kinase (PNK). One hundred fmole of primer was hybridized to 10 μg of total RNA isolated from WEHI265.1 cells by incubating the sample at 58 °C for 20 min and slowly cooling to 10 °C, after which standard RT reaction mixture was added. Reaction products were resolved in a 5% polyacrylamide gel under denaturing conditions (7M urea and 1X Tris-borate-EDTA buffer).

Electrophoretic mobility shift assay (EMSA)

Four pmole of annealed oligonucleotides were labeled with 20 μCi of [α-^{32}P] dCTP (Amersham) using the Klenow fragment (Promega). Labeled probes were diluted to 40 fmole/μl. A total of 40 fmole of ^{32}P-probe was incubated with 2-5 μg of nuclear extracts (prepared with Nuclear/Cytosol Fractionation Kit, BioVision, Mountain View, CA) in gel shift assay buffer (20mM HEPES, pH 7.6, 0.1 mM KCl, 10% Glycerol, 0.1% Nonidet P-40, 2 mM DTT) containing 0.5 μg of polydeoxyinosinic-deoxycytidylic acid in a 20-μl reaction. Binding reactions were performed on

Primers for promoter cloning	
1.0 kb forward	5′-TTGGTACCTGCTTAGCTAGCATGTGT-3′
0.5 kb forward	5′-TTGGTACCAACTCACACAAAGGGATC-3′
Reverse	5′-AACTCGAGAATTTTGAAATCTTGCTTATGC-3′
mutNFAT forward	5′-GAAGAGAACAGAGAGTGGTTTAGCTATGAAAGACTTA-3′
mutNFAT reverse	5′-TAAGTCTTTCATAGCTAAACCACTCTCTGTTCTCTTC-3′
Primers for RT-PCR	
CCR2 exon 1 forward	5′-AATCCTGTAAAGACCTCAGCC-3′
CCR2 exon 2 forward	5′-TGCAAAGACCAGAAGAGG-3′
CCR2 exon 3 reverse	5′-CAGGATCCAAGCTCCAAT-3′
NFATc1 forward	5′-CAACGCCCTGACCACCGATAG-3′
NFATc1 reverse	5′-GGCTGCCTTCCGTCTCATAGT-3′
NFATc2 forward	5′-TGGCCCGCCACATCTACCCT-3′
NFATc2 reverse	5′-TGGTAGAAGGCGTGCGGCTT-3′
NFATc3 forward	5′-TGGATCTCAGTATCCTTTAA-3′
NFATc3 reverse	5′-CACACGAAATACAAGTCGGA-3′
NFATc4 forward	5′-CATTGGCACTGCAGATGAG-3′
NFATc4 reverse	5′-CGTAGCTCAATGTCTGAAT-3′
CnA forward	5′-CAGGGTGGTGAAAGCCGTTC-3′
CnA reverse	5′-GGATGTCCCCACAAACTGTG-3′
MCP-1 forward	5′-ATGCAGGTCCCTGTCATGCTT-3′
MCP-1 reverse	5′-GTTCACTGTCACACTGGTCA-3′
PPT-A forward	5′-TGCCAACGATGATCTAAATT-3′
PPT-A reverse	5′-CTTCTTTCGTAGTTCTGCAT-3′
CGRP forward	5′-AGATCCTGCAACACTGCCAC-3′
CGRP reverse	5′-CCACATTGGTGGGCACAAAGTTG-3′
GAPDH forward	5′-TTGACCTCAACTACATGG-3′
GAPDH reverse	5′-ATTGAGAGCAATGCCAGC-3′

Table 2.

Taqman gene expression assays for qRT-PCR	
CCR1	Mm00438260_s1
CCR2	Mm00438270_m1
CCR5	Mm01216171_m1
CXCR4	Mm99999055_m1
CX3CR1	Mm00438354_m1
GAPDH	Mm99999915_g1
Primers for the probes used in Northern blot	
CCR2 3′UTR forward	5′-GCAAGAGGTCTCGGTTGGGTTG-3′
CCR2 3′UTR reverse	5′-TCCTCCTTCTCACTCAGTCCTG-3′
Primer for the primer extension	
CCR2 exon 1 reverse	5′-TCTACAGTTCTTCTTTTCCAGCCAGG-3′
Oligomers for EMSA	
NFAT forward	5′-CTAGAGAGAGTGGAAAAGCTAT-3′
NFAT reverse	5′-CTAGATAGCTTTTCCACTCTCT-3′
Mutant NFAT forward	5′-CTAGAGAGAGTGGTTTAGCTAT-3′
Mutant NFAT reverse	5′-CTAGATAGCTAAACCACTCTCT-3′

Table 2 (Continued).

ice for 20 min and at 30 °C for 30 min. The reaction mixture was run on a 4 % native polyacrylamide (37.5:1) gel in 0.5x TGE (25 mM Trizma base, 20 mM Glycine, 1 mM EDTA) to resolve protein-DNA complexes. For competition assays, a 100-fold molar excess of

unlabeled double-stranded competitor DNA was added. For supershift assays, a pan-specific NFATc antibody which recognizes NFATc1 to c4 (sc-1149X) and an isotype-matched control antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were added at the beginning of the incubation at 30 °C. The oligomers used are listed in Table 2.

Transient transfection and luciferase assay

A luciferase assay was performed to measure CCR2 promoter activities. DNA was transfected using TranIT-LT1 (Mirus, WI) according to the manufacturer's instructions. One hundred ng of CCR2 promoter construct was co-transfected with 900 ng of a transactivator construct or an empty vector along with 10 ng of pRL-TK which served as an internal control. Renilla and firefly luciferase assays were performed in 24-48 hr after the transfection using commercial enzyme assay kits (Dual-Luciferase Reporter Assay System, Promega).

Von Frey test for mechanical allodynia

The von Frey test was performed on the area of the hind paws as previously described (S. Bhangoo et al. 2007).

CHAPTER 4

Generation of Transgenic Reporter Mice Using Modified Bacterial Artificial Chromosomes to Investigate Chemokine Signaling *in vivo*

ABSTRACT

Chemokines and chemokine receptors are widely expressed by cells of the nervous system as well as immune cells. Chemokine signaling plays a variety of roles in the nervous system including regulation of stem cell migration, axonal pathfinding and neurotransmission. Chemokine signaling is also of great importance in the regulation of neuroinflammatory responses. Therefore, the activation of chemokine receptors is an important indicator of various physiological and pathological processes. Chemokine receptors, which belong to the G protein-coupled receptor (GPCR) family, undergo extensive endocytosis upon binding of their cognate chemokine ligands, a phenomenon which can serve as a cellular marker for receptor activation.

In order to investigate chemokine signaling *in vivo*, transgenic reporter mice were generated in which the chemokine, MCP1, and its receptor, CCR2, are labeled by red and green fluorescent proteins, respectively. Reporter genes were inserted into bacterial artificial chromosomes (BACs), and their expression was driven by their endogenous regulatory elements which were included the flanking regions in the BAC. Using the mice, these experiments demonstrated that expression of MCP1 and CCR2, release of MCP1, and activation of CCR2, can be effectively visualized *in vivo.*

INTRODUCTION

Although MCP1-CCR2 signaling clearly plays important roles in orchestrating communication between the nervous and immune systems, cellular mechanisms of the signaling are poorly understood. Three important questions remain to be studied: (1) what types of cells express MCP1 constitutively and/or inducibly in various tissues, (2) how is MCP1 release regulated in different cells, and (3) how many CCR2 receptor expressing cells are activated under different circumstances. In order to answer these questions, it is necessary to be able to visualize endogenous MCP1 and CCR2. All known chemokine receptors belong to the family of G protein-couple receptors (GPCRs). CCR2, like many other GPCRs, undergoes extensive endocytosis upon binding of MCP1 (Figure 4.1F). Therefore, receptor endocytosis is a good indicator that a cell is undergoing or has undergone the activation of CCR2 receptors.

Using modified bacterial artificial chromosomes, transgenic reporter mice in which endogenous gene expression regulatory elements drive the expression of fluorescently tagged MCP1 (MCP1-mRFP1) and CCR2 (CCR2-EGFP) were generated. In these mice, the cis-elements that control expression of endogenous MCP1 and CCR2 at transcriptional and post-transcriptional levels drive the expression of MCP1-mRFP1 and CCR2-EGFP reporters. Therefore, subcellular as well as cellular localization of MCP1 and CCR2 could be faithfully reported by each reporter protein. Importantly, the interactions between MCP1 and CCR2 could also be observed, indicating that, in these mice, the activation of CCR2 receptors by MCP1 can be visualized *in vivo*.

RESULTS

Visualization of MCP1-CCR2 interactions *in vitro*

Labeling of GPCR and neuropeptide C-termini with fluorescent proteins has been widely used to track subcellular processes such as receptor trafficking and neuropeptide release (Levitan 2004; Kallal and Benovic 2000). Indeed, fusion of EGFP or mRFP1 to the C-terminus of CCR2 receptors did not interfere with either its intracellular processing (i.e. membrane localization) or its function (i.e. ligand binding-induced endocytosis and coupling to the increase in intracellular Ca) (Figure 4.1). Likewise, fusion of EGFP or mRFP1 to the C-terminus of two chemokines, MCP1 (Figure 4.2A-H) and SDF1 (Figure 4.2I-H), did not interfere with maturation, vesicular localization, or release. Therefore, one may be able to visualize the interactions between MCP1 and CCR2 using the ligand and the receptor labeled with different fluorophores (Figure 4.3A).

In order to investigate this possibility, cells transfected with either MCP1-mRFP1 or CCR2-EGFP were co-cultured. Unlike separate cultures, co-cultures with MCP1-mRFP1-expressing cells induced endocytosis of CCR2-EGFP which normally localized to the plasma membrane in the receptor-expressing cells (Figure 4.3B). Furthermore, it was clearly observed that vesicles containing both MCP1-mRFP and CCR2-EGFP existed inside the CCR2-EGFP transfected cells, suggesting that these may represent the vesicles which were endocytosed upon MCP1 binding (Figure 4.3B). Indeed, this process could be completely blocked by a specific CCR2 receptor antagonist (RA) (Figure 4.3C). Moreover, this process was found to be ligand-specific. Another chemokine, SDF1-mRFP1, did not induce CCR2-EGFP endocytosis, although it did induce endocytosis of its own cognate receptor, CXCR4-YFP, a phenomenon which was blocked by a

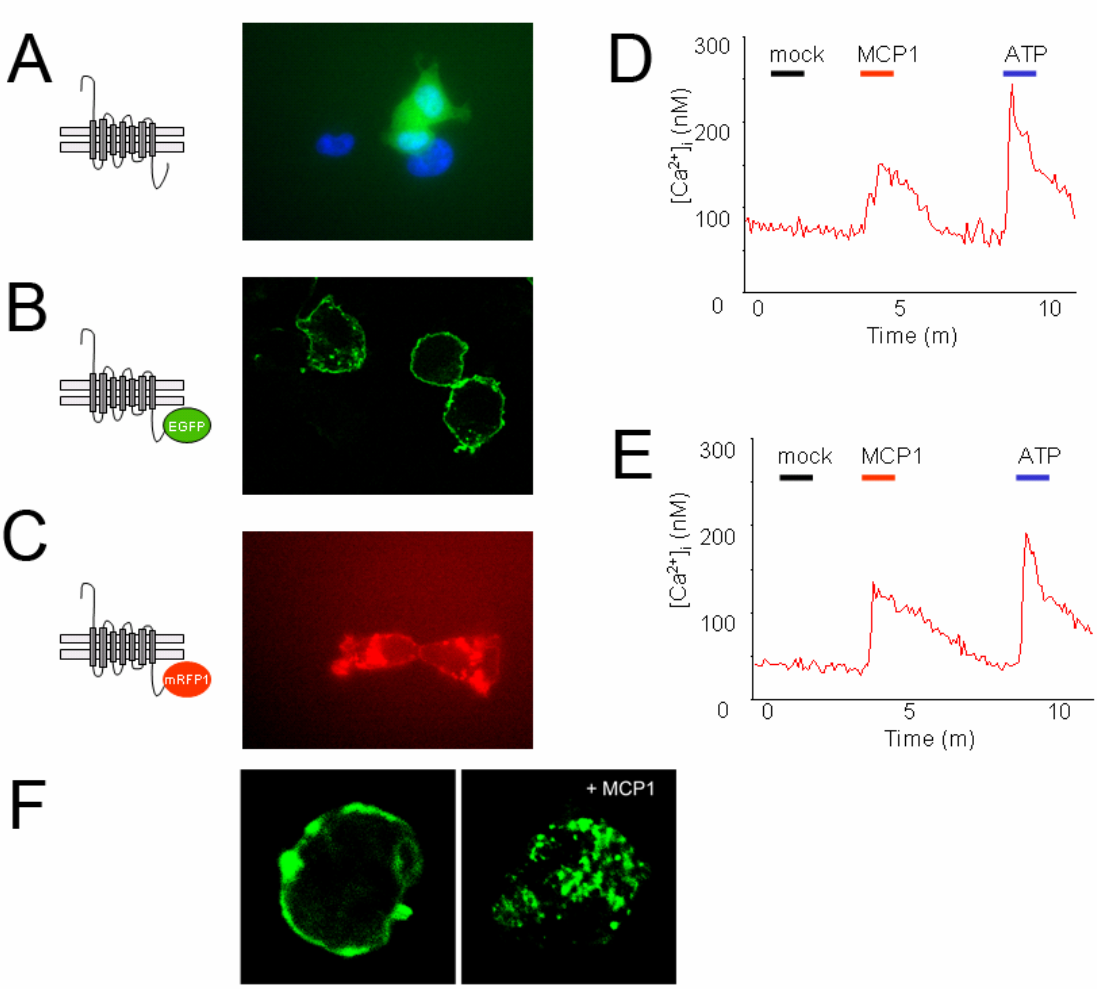


Figure 4.1. Fusion of EGFP or mRFP1 to the C-terminus of CCR2 receptors does not interfere with their membrane localization or functions. (A) CCR2-IRES-EGFP, (B) CCR2-EGFP, and (C) CCR2-mRFP1 were transfected separately into HEK293 cells. Unlike EGFP alone which filled entire cells (A), EGFP (B) and mRFP1 (C) when fused to CCR2 receptors localized primarily to the plasma membrane. The activation of both CCR2-EGFP (D) and CCR2-mRFP1 (E) in response to MCP1 increased the intracellular Ca concentration to the same degree as seen with unfused CCR2 receptors (data not shown). (F) CCR2-EGFP undergoes endocytosis upon binding of MCP1. 100 ng/ml MCP1 induced a decrease in cell surface localization and increased localization of CCR2-EGFP to intracellular vesicles within one hour (compare left and right panels).

specific CXCR4-RA, AMD3100 (Figure 4.3C, E, and F). The reverse was observed, namely MCP1-mRFP1 did not induce endocytosis of CXCR4-YFP (Figure 4.3D). Therefore, if an

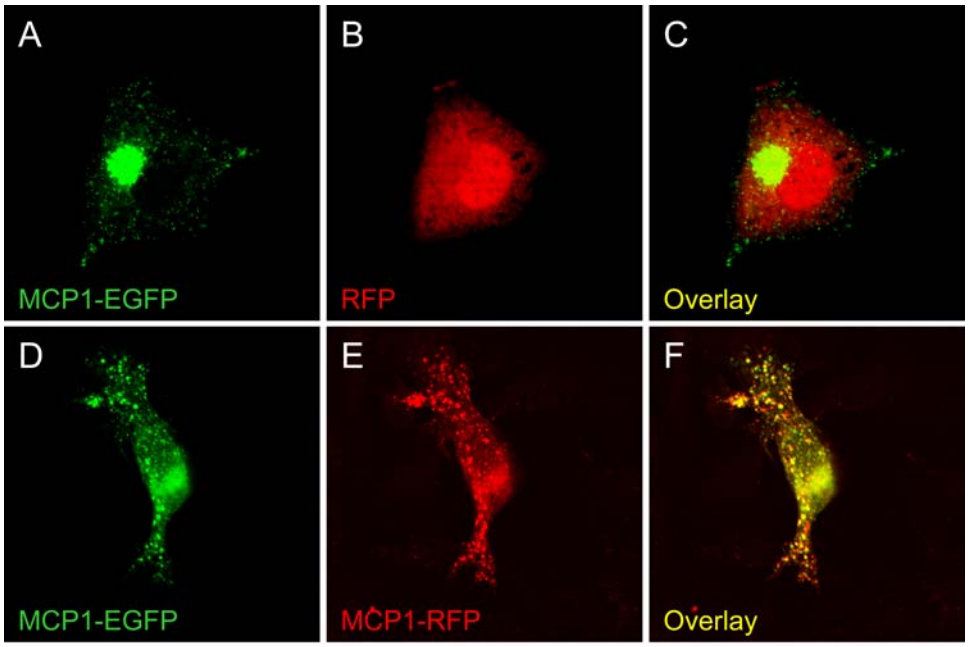


Figure 4.2. MCP1-EGFP and MCP1-mRFP1 fusion proteins are localized to secretory vesicles. (A-C) MCP1-EGFP and mRFP1 were co-transfected into HEK293 cells. Unlike mRFP1 alone which filled entire cells (B), MCP1-EGFP localized primarily to the *trans*-Golgi network and vesicles (A). (D-F) MCP1-EGFP and MCP1-mRFP1 were co-transfected into HEK293 cells and co-localized in most vesicles. (G) MCP1-mRFP1 and mRFP1 were transfected into HEK293 cells and analyzed by Western blot using an RFP antibody and an MCP1 antibody. A precursor form of MCP1-mRFP1 (***) was processed into mature forms (*, **). U, untransfected- ; R, mRFP1-; M, MCP1-mRFP1-transfected cells. ?, unidentified band. (H) F11 DRG neuronal cells transfected with MCP1-mRFP1 were differentiated and then depolarized by high K stimulation (50 mM: 50K), either with or without extracellular Ca (2 mM or 0 mM: 2Ca or 0Ca). The amount of MCP1-mRFP1 released into the culture medium was measured by sandwich ELISA using an RFP antibody and an MCP1 antibody. Ca-dependent regulated release as well as constitutive release of MCP1-mRFP1 was evident. (I-K) SDF1 is stored in vesicles distinct from the ones that store MCP1. SDF1-mRFP1 was localized to vesicular structures (J) but did not co-localize with MCP1-EGFP (K). (L) SDF1-mRFP1 (S) and mRFP1 (R) were transfected into HEK293 cells and analyzed by Western blot analysis using an RFP antibody and an MCP1 antibody. Pre-SDF1-mRFP1 (**) was cleaved into mature forms (*). U: untransfected cells. (M) The release of SDF1-mRFP1 was examined as in (H). In contrast to MCP1-mRFP1, the release of SDF1-mRFP1 was not increased by depolarization. The constitutive release was dependent on extracellular Ca.

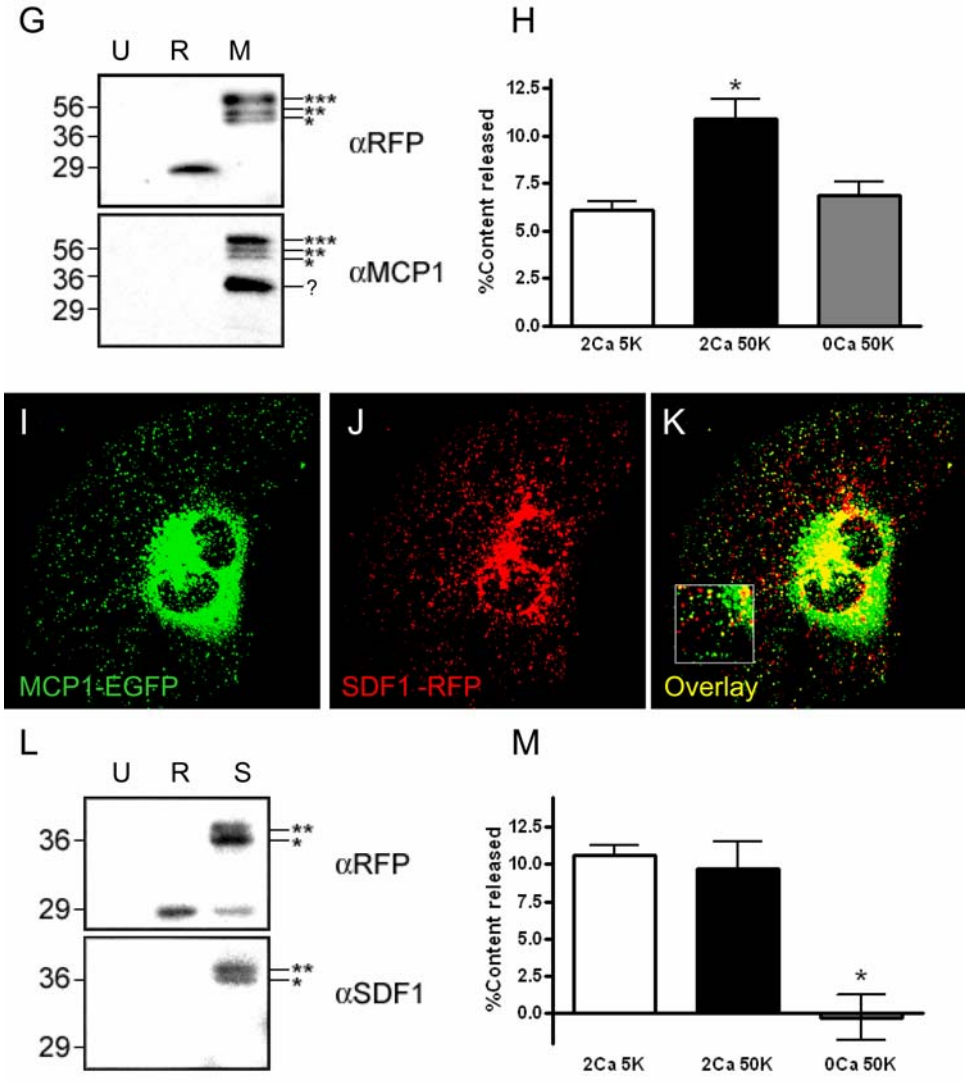


Figure 4.2 (Continued).

endogenous ligand and its cognate receptor could be labeled with different fluorescent proteins, it should also be possible to visualize the interactions of MCP1 and CCR2 *in vivo* (Figure 4.3A). This possibility was studied by generation of MCP1-mRFP1 and CCR2-EGFP bi-transgenic mice.

Generation of transgenic mice using modified bacterial artificial chromosomes (BACs)

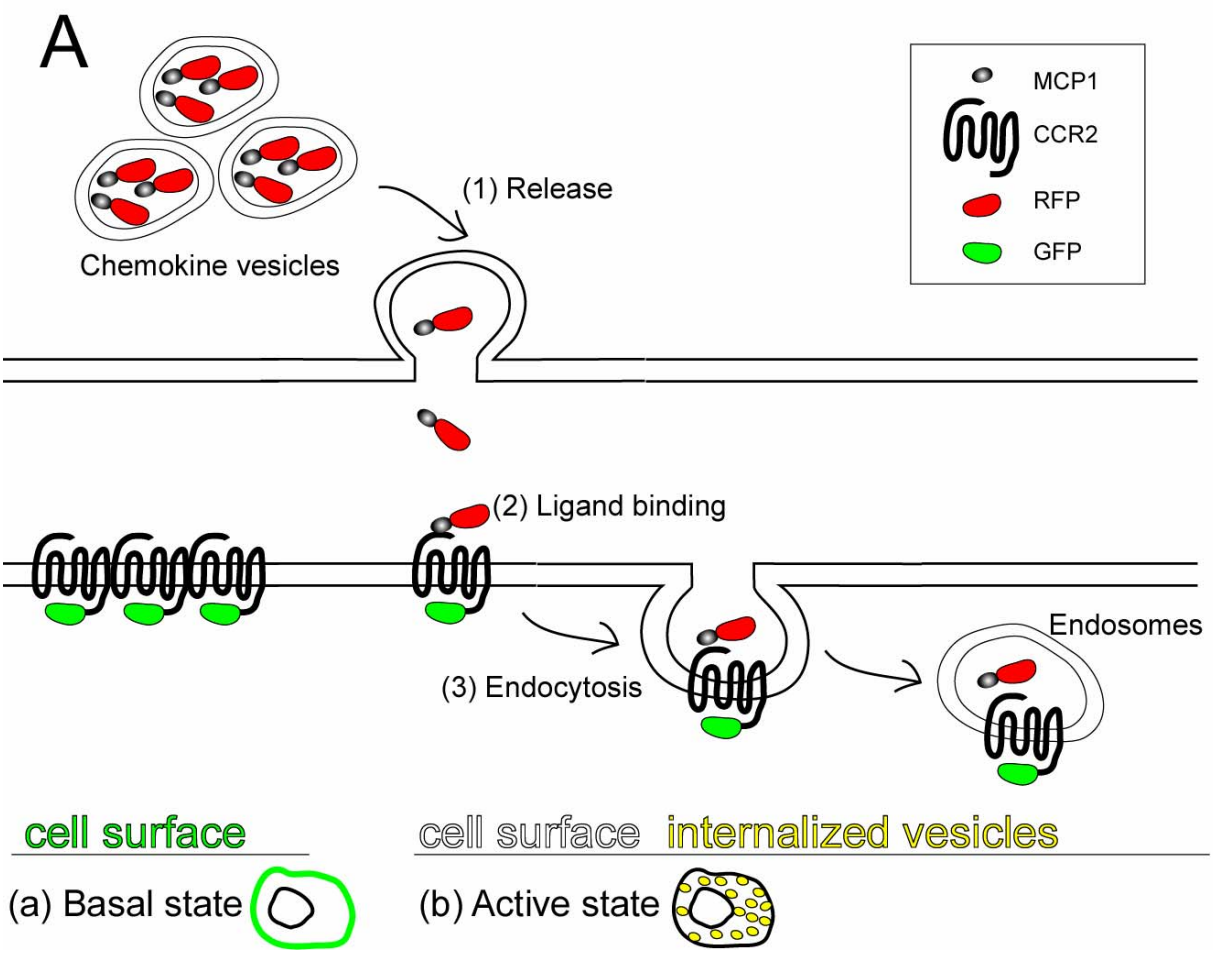
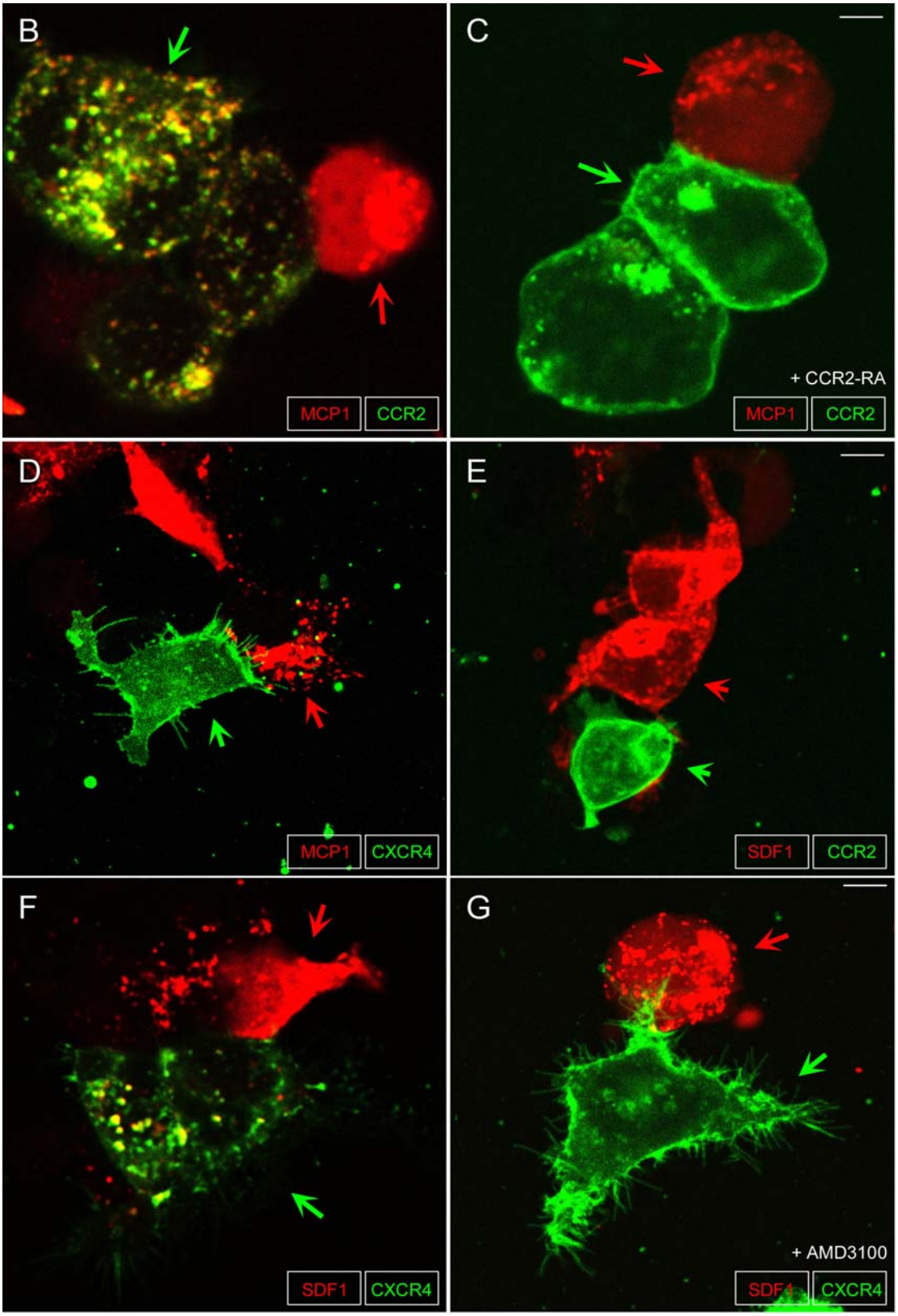


Figure 4.3. Visualization of MCP1-CCR2 interactions *in vitro*. (A) In a model of MCP1-CCR2 interactions, MCP1-RFP is localized to secretory vesicles (upper space) and CCR2-GFP to the place membrane (lower space) (a). When signaling occurs, released MCP1-RFP (1) binds to CCR2-GFP expressed on CCR2-expressing cells (2). Binding induces endocytosis of the MCP1-RFP/CCR2-GFP complexes (3). Indications of CCR2 activation include the loss of membrane-localized GFP signal and an increase in yellow vesicles containing both MCP1-RFP and CCR2-GFP (b). (B-G) In these experiments, HEK293 cells were transfected with either a chemokine ligand construct (MCP1-mRFP1 or SDF1-mRFP1) or a chemokine receptor construct (CCR2-EGFP or CXCR4-YFP). After 24 h, ligand-expressing cells were co-cultured with receptor-expressing cells. (B) Constitutively released MCP1-mRFP1 entered CCR2-EGFP-expressing cells, resulting in the formation of endocytic vesicles containing both MCP1-mRFP1 and CCR2-EGFP. (C) The interactions between MCP1-mRFP1- and CCR2-EGFP-expressing cells were completed blocked by a CCR2 receptor antagonist (CCR2-RA). MCP1-mRFP1 did not interact with CXCR4-YFP (D), and SDF1-mRFP1 did not interact with CCR2-EGFP (E). SDF1-mRFP1 did induce the endocytosis of CXCR4-YFP (F), and it was blocked by a specific CXCR4-RA (AMD3100) (G).



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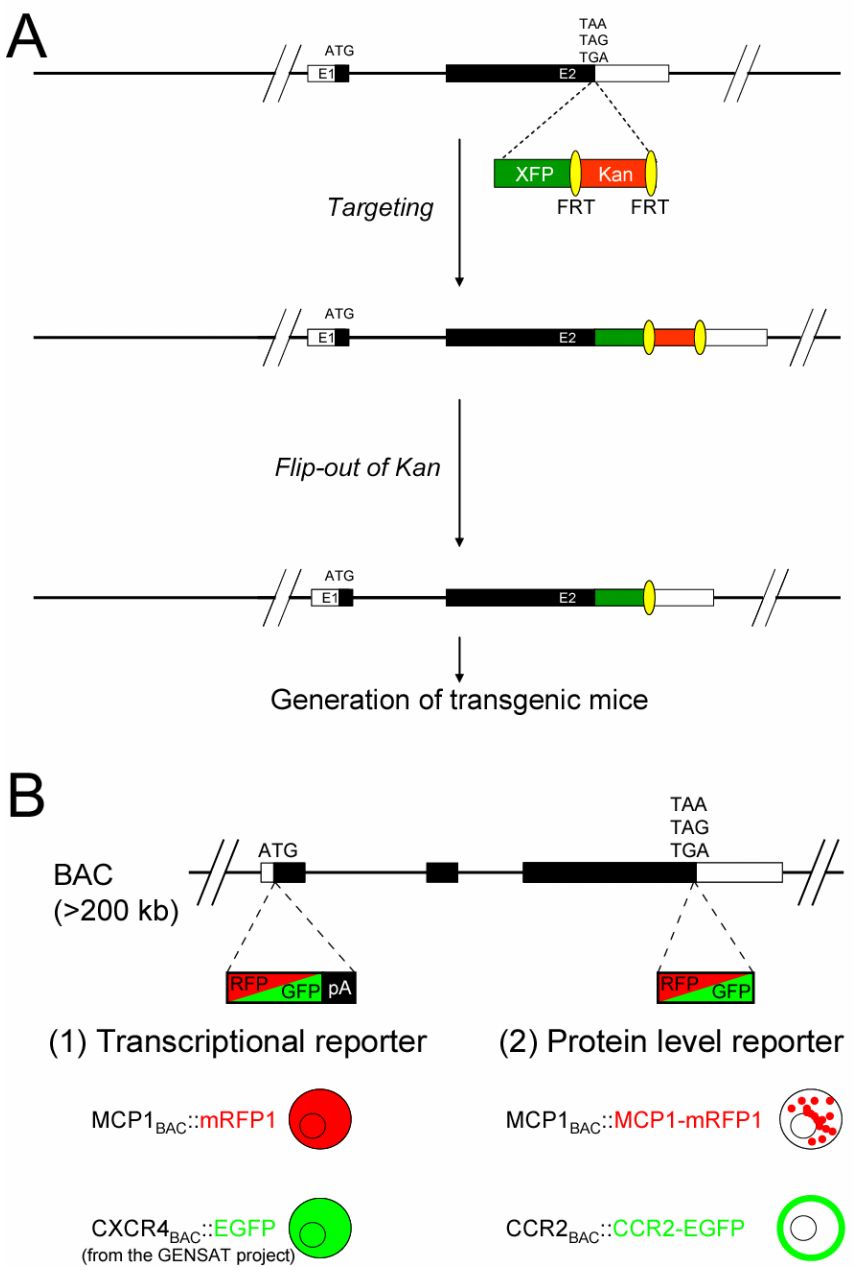

Figure 4.4. Generation of BAC transgenic reporter mice. (A) BAC clones were modified by *recombineering*. By homologous recombination in *E. coli*., EGFP-FRT-KAN-FRT was inserted into an appropriate location within the BAC, after which the KAN cassette was excised by site-specific recombination. (B) EGFP or mRFP1 was inserted in place of the start codon to generate a transcriptional reporter (1), or the stop codon to generate a protein level reporter (2). CXCR4::EGFP transcriptional reporter mice were obtained from the GENSAT project.

Expression of EGFP-tagged neuropeptides in neurons *in vitro* and *in vivo* has been widely used to study dynamics of neuropeptide release (Lou et al. 2005; Burke et al. 1997). However, most of these experiments utilized the exogenous promoter to ectopically express the tagged proteins. Recently, it was established that by using bacterial artificial chromosomes (BACs) reliable transcriptional reporter mice could be generated without detailed knowledge of the genes' promoter structure (Heintz 2004). It is assumed that a single BAC clone of a sufficiently large size (around 200 kb) contains most regulatory cis-elements responsible for expression of a single gene. Using an efficient homologous recombination system in bacteria, a reporter gene (e.g. EGFP) can be inserted into a precise location in a BAC clone, so that endogenous regulatory elements may control the expression of the inserted reporter gene (Figure 4.4A). By combining these two approaches, transgenic reporter mice that express MCP1-mRFP1 and CCR2-EGFP under the control of their respective endogenous regulatory elements were created. mRFP1 coding sequence was inserted in place of the MCP1 stop codon, and EGFP was inserted in place of the CCR2 stop codon (MCP1::MCP1-mRFP1 and CCR2::CCR2-EGFP, respectively) (Figure 4.4B). In parallel, a conventional BAC transcriptional reporter mouse for MCP1 (MCP1::mRFP1) was also generated.

Faithful expression of reporter genes in BAC transgenic mice

MCP1 is not expressed at a high level in the nervous system under normal conditions. Rather, its expression is induced under various pathological conditions accompanied by the activation of the innate immune response (Serbina et al. 2008). The cells which upregulate MCP1 depending on

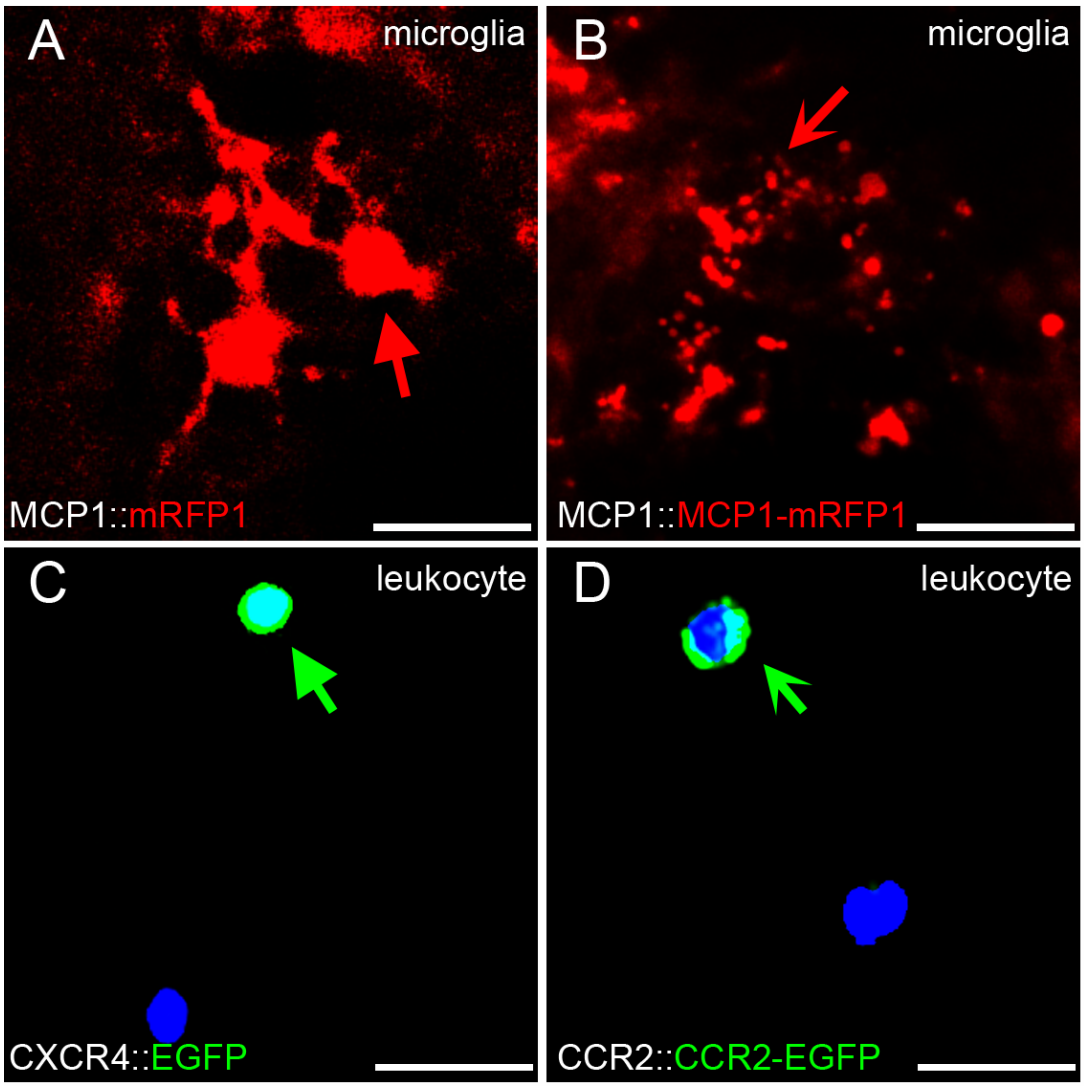

Figure 4.5. Faithful cellular and subcellular localization of the transgene expression. (A, B) MCP1 reporter genes were expressed by microglia during the inflammatory response. (C, D) The CXCR4 and CCR2 reporter genes were expressed by a subset of leukocytes under normal conditions. The reporter gene products (i.e. EGFP or mRFP1) of the transcriptional reporter mice (A, C) filled entire cells, while those of the protein level reporter mice (B, D) were limited to the appropriate subcellular localizations (i.e. MCP1-mRFP1 in vesicles, and CCR2-EGFP in membranes). Scale bars, 20 μm.

circumstances include microglia, astrocytes, and neurons (Majumder, Zhou, and Ransohoff 1996; Lund et al. 2006; White et al. 2005). In agreement with known expression patterns, there was little basal expression of RFP in transcriptional and protein level reporter mice (data not

shown). The RFP expression was, however, induced by various manipulations which are known to upregulate MCP1. For example, acute activation of the inflammatory response by injection of bacterial endotoxin lipopolysaccharide (LPS) induced the upregulation of the reporter gene in microglia across the central nervous system (Figure 4.5A, B). Several independent lines made of the same construct showed similar patterns of expression. Moreover, cellular localization (i.e. microglia in the case of LPS injection) of the reporter gene products was identical in the transcriptional (MCP1::mRFP1) and the protein reporter (MCP1::MCP1-mRFP1) mice, further confirming that expression of the reporter gene products is consistently regulated (Figure 4.5A, B). However, as expected, subcellular localization of the reporter was clearly different. Unlike the transcriptional reporter where mRFP1 filled entire cells, MCP1-mRFP1 was localized to vesicular structures in the protein reporter mice as previously shown in cultured cells (Figure 4.2 and 4.5A-B).

CCR2 is normally expressed by neurons, neural progenitors, and leukocytes, and its expression may be upregulated by neurons and microglia under pathological conditions (Abbadie et al. 2003; Banisadr, Queraud-Lesaux et al. 2002; Gosselin et al. 2005; Jung et al. 2008). In CCR2-EGFP protein reporter mice (CCR2::CCR2-EGFP), the reporter gene was expressed by leukocytes in bone marrow and circulating leukocytes under normal conditions and also expressed by neurons under pathological conditions (Figure 4.5D and Figure 2.1). CCR2-EGFP was localized mainly to the plasma membrane in unstimulated cells (Figure 4.5D). In contrast, a similarly-generated transcriptional reporter mouse for the chemokine receptor, CXCR4, showed diffusive localization of EGFP (CXCR4::EGFP, a kind gift of the GENSAT project) (Figure 4.5C). By crossing these mice, MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP bi-transgenic mice

were generated. These bi-transgenic mice faithfully reported cellular and subcellular localization of MCP1 and CCR2 in a single animal.

Visualization of chemokine-chemokine receptor interactions *in vivo*

In bone marrow: regulated release of MCP1 during inflammation

The bone marrow of naïve animals contained a number of MCP1-expressing cells surrounded by numerous CCR2-positive cells often making physical contacts (Figure 4.6A, C). However, the activation of CCR2 in these cells was minimal, because most of the CCR2-EGFP localized to the plasma membrane (Figure 4.6A, C). Moreover, there was no MCP1-mRFP1 signal in or around CCR2-EGFP-expressing cells (Figure 4.6C). This expression pattern changed dramatically during activation of the inflammatory response. Animals that were injected with 10 mg/kg lipopolysaccharide (LPS), a bacterial endotoxin that elicits strong immune responses by activating Toll-like receptors (TLRs), showed an significant increase in MCP1 release in the bone marrow (Figure 4.6D, G, H). Two observations supported this conclusion. First, the cell surface expression of CCR2-EGFP was decreased, while intracellular CCR2-EGFP was increased (Figure 4.6B, D, G, H). Second, CCR2-expressing cells now had numerous MCP1-mRFP1-containing vesicles both inside and around the plasma membrane (Figure 4.6D, H). These data indicated an increase in MCP1 release and consequent activation of CCR2.

The activation of CCR2 during inflammation was likely due to increased release rather than increased expression, because the number of MCP1-expressing cells did not increase when the

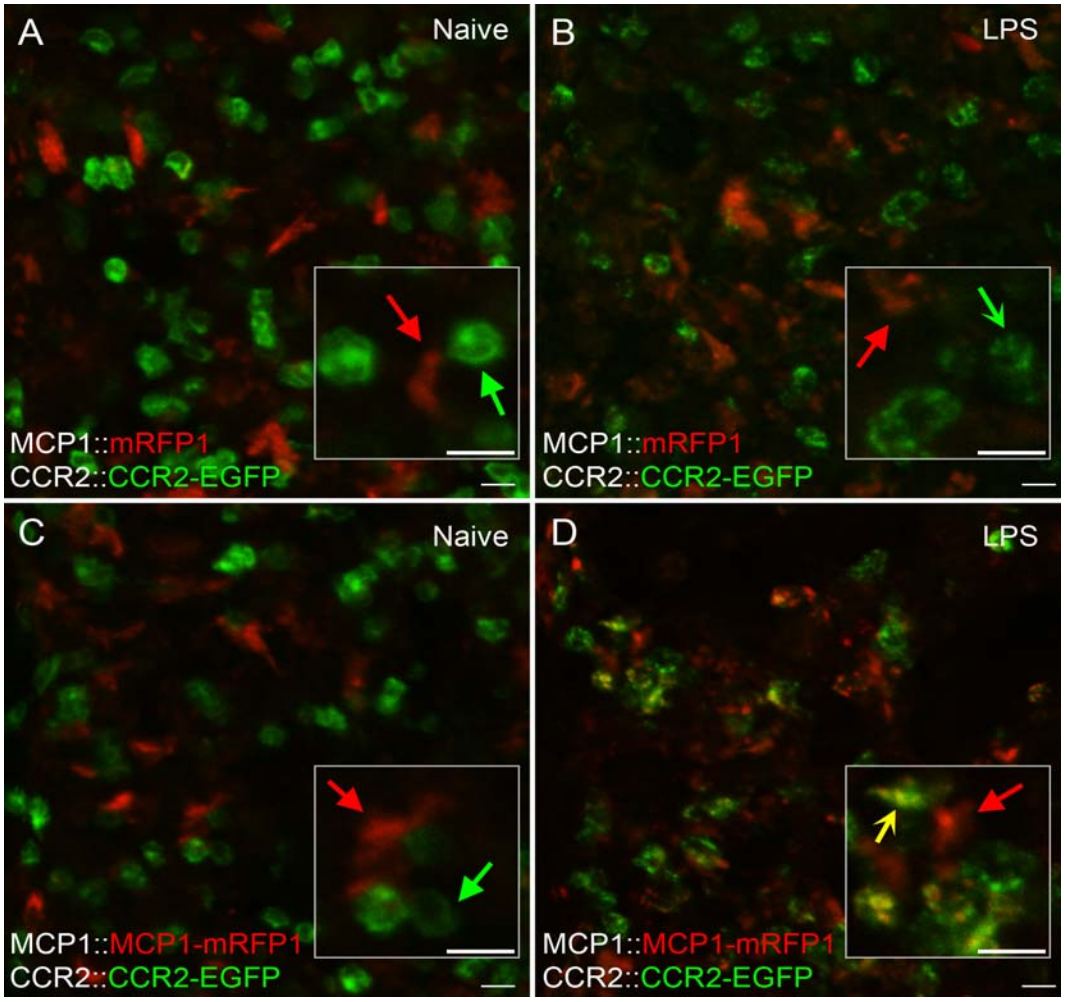

Figure 4.6. Release of MCP1 and the activation of CCR2 receptors can be visualized in the BAC transgenic mice. (A-D) MCP1::mRFP1; CCR2::CCR2-EGFP mice (A, B) and MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP (C, D) mice were injected with LPS (B, D). After 24 h, bone marrow sections were imaged using a confocal microscope. Compared to CCR2 receptor expression under naïve conditions (A, C: green arrows), the activation of CCR2 receptors by LPS was evident: internalization and loss of membrane localized CCR2-EGFP (B, D: concave green arrows). When MCP1 protein reporter mice were used, MCP1 uptake by CCR2-expressing cells could also be visualized (yellow concave arrow) (D). (E-F) The activation of CCR2 receptors in the bone marrow was reversed by the CCR2 receptor antagonist (CCR2-RA). MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP mice were injected with LPS alone (G, H) or LPS and CCR2-RA (I, J). The CCR2-RA increased membrane localization and decreased vesicular localization of CCR2-EGFP (J). Scale bars, 10 μm.

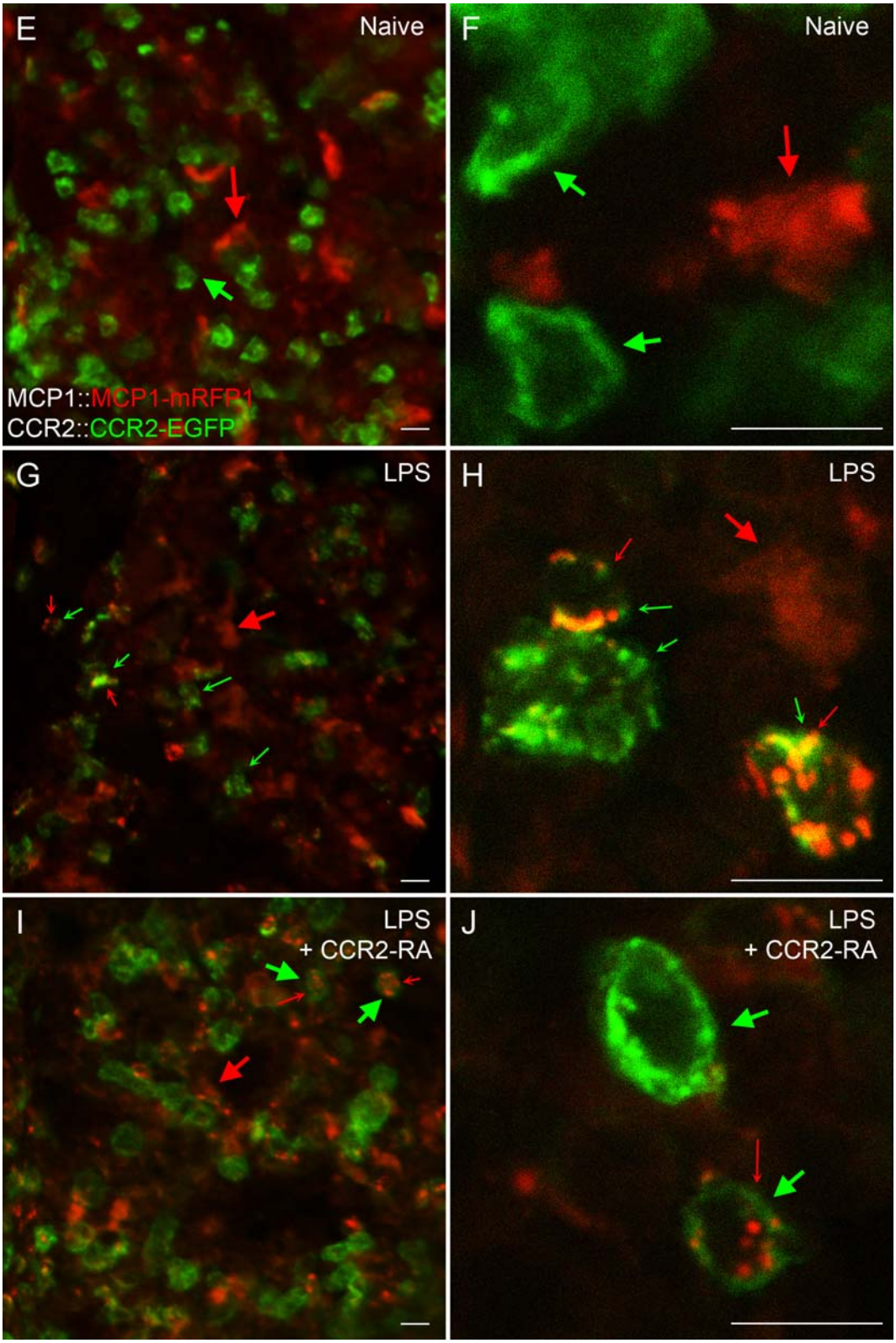

Figure 4.6 (Continued).

transcriptional reporter mice (MCP1::mRFP1) were used instead of the protein reporter mice (MCP1::MCP1-mRFP1) (Figure 4.6A, B). However, the activation of CCR2 receptors in monocytes during this process was evident, because the surface expression of CCR2-EGFP was decreased while the expression in endocytosed vesicles increased (Figure 4.6B). These data clearly suggest that the activation of CCR2 in monocytes in the bone marrow is due to the increased release of pre-synthesized MCP1 by bone marrow cells.

Finally, specificity of MCP1-mRFP1 and CCR2-EGFP interactions was confirmed by the systemic injection of the specific CCR2-RA. The CCR2-RA was injected three times at 4 hr intervals, 12 hr after the injection of LPS. Animals were sacrificed after a total of 24 hr. The injection of the CCR2-RA restored the cell surface expression of CCR2-EGFP (Figure 4.6I, J). Some MCP1-mRFP1 containing-vesicles remained inside the CCR2-EGFP expressing cells (Figure 4.6J). This may be due to different stability of MCP1-mRFP1 and CCR2-EGFP in endocytic vesicles. In this example, it was shown that various aspects of MCP1-CCR2 signaling such as MCP1 release and CCR2 activation can be examined *in vivo* using these bi-transgenic reporter mice.

In the brain: interactions between microglia and leukocytes

MCP1-CCR2 signaling in the brain was also examined using the LPS injection model. MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP bi-transgenic mice were injected with 10 mg/kg LPS ***i.p.*** and sacrificed 48 hr later. In this paradigm, MCP1 was widely upregulated by microglia (i.e. IBA-1-immunoreactive cells) across the brain (Figure 4.7). These microglia were often in

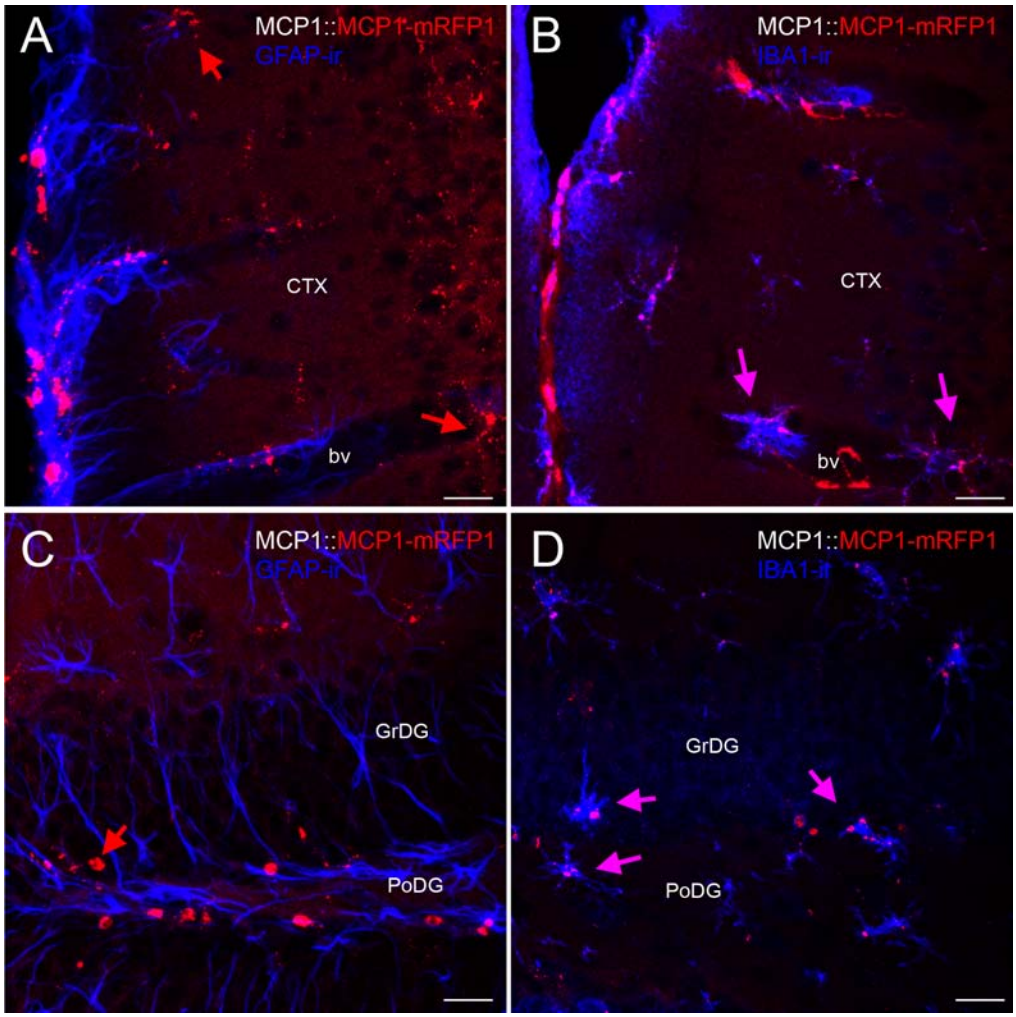

Figure 4.7. Inducible expression of MCP1 is recapitulated in the BAC transgenic mice. MCP1::MCP1-mRFP1 mice were injected with LPS. MCP1 is significantly upregulated across the brain in these animals in contrast to naïve animals which showed no MCP1 expression (data not shown). MCP1 was upregulated mostly by microglia, which were identified by IBA-1-ir (B, D), but not by astrocytes which were identified by GFAP-ir (A, C). The pictures were taken from the mediofrontal cortex (A, B) and the hippocampal dentate gyrus (C, D). Scale bars, 20 μm.

close contact with CCR2-expressing leukocytes attached to the walls of blood vessels (Figure 4.8). Importantly, CCR2-EGFP-expressing leukocytes possessed many vesicles containing both CCR2-EGFP and MCP1-mRFP1, suggesting that these vesicles are the product of ligand binding-induced endocytosis (Figure 4.8). These vesicles have not been observed in CCR2-

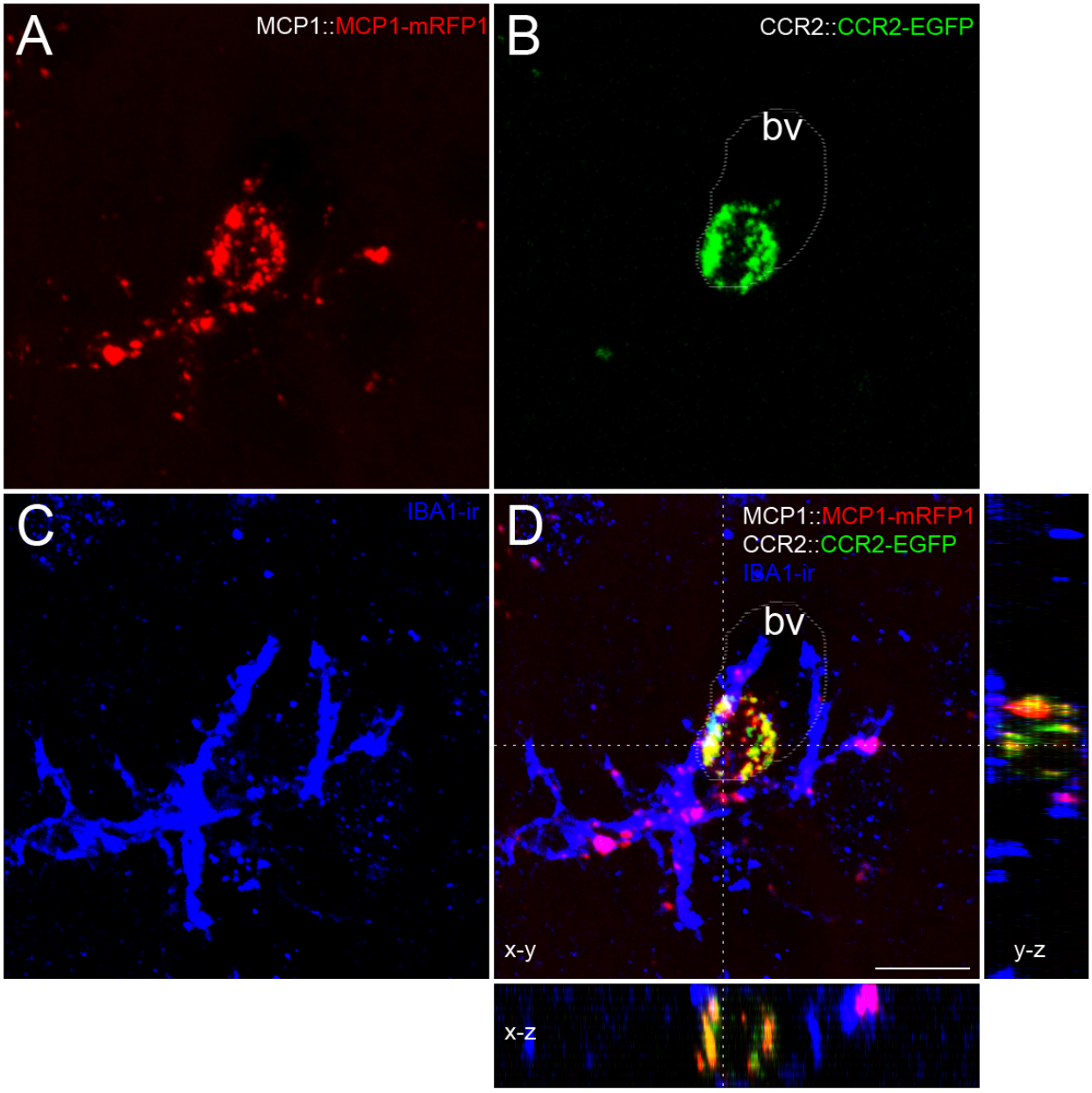

Figure 4.8. Interactions between MCP1 and CCR2 expressing cells can be visualized in the BAC transgenic reporter mice. MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP mice were injected with LPS. In this figure, two perivascular microglia (C: IBA-ir) are interacting with one circulating leukocyte (B: CCR2-EGFP+). Microglia not only upregulated MCP1 but also released MCP1 to the CCR2-EGFP expressing leukocyte (A). The internalized vesicles contained both MCP1-mRFP1 and CCR2-EGFP (D), which indicates that the MCP1-expressing microglia had activated CCR2 receptors expressed by the leukocyte (D). Scale bars, 10 μm.

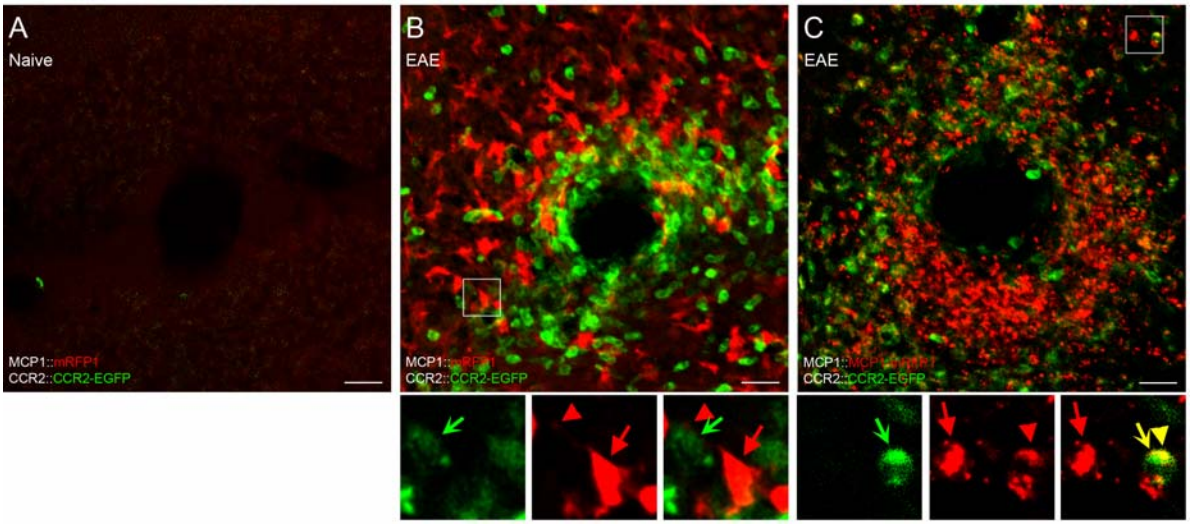

Figure 4.9. Breakage of the blood brain barrier is visualized by the BAC transgenic reporter mice. EAE was induced in MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP mice (A, B) and MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP mice (C). Unlike under naïve conditions (A and data not shown), there is heavy infiltration of leukocytes (B and C: CCR2-EGFP+) into the parenchyma of the cerebellum. Although there are close interactions between MCP1-expressing cells (red arrow) and CCR2-expressing cells (green arrow) (B and C), only MCP1-mRFP1 (C), but not mRFP1 alone (B), is transferred to CCR2-EGFP expressing leukocytes (yellow arow). Scale bars, 20 μm.

EGFP-expressing leukocytes, when the MCP1 transcriptional reporter mice were used instead (MCP1::RFP1; CCR2::CCR2-EGFP) (data not shown. See Figure 4.9B.). This data supports the interpretation that, in the previous experiment, MCP1-mRFP1 released *in trans* entered CCR2-EGFP-expressing leukocytes (Figure 4.8). Using these mice, it was possible to appreciate the cellular landscape of CCR2 receptor activation under pathological conditions where MCP1-CCR2 signaling is known to play a role such as in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, and in neuropathic pain. In the EAE model, an animal develops an autoimmune response characterized by an immune attack against the white matter of the central nervous system (Karpus and Ransohoff 1998). Specifically, the cerebellar white matter is the most vulnerable structure. Accordingly, the transgenic animals that developed

EAE showed dramatic infiltration of peripheral leukocytes expressing CCR2-EGFP into the cerebellar parenchyma (Figure 4.9B). These cells were juxtaposed with MCP1-mRFP1-expressing cells (Figure 4.9). Many of the infiltrated cells had subcellular vesicles containing both MCP1-mRFP1 and CCR2-EGFP confirming that the activation of CCR2-mediated signaling plays a role in the pathogenesis of EAE. As in the LPS model, no dual-color vesicles were observed when the transcriptional reporter mice for MCP1 were used (MCP1::mRFP1; CCR2::CCR2-EGFP), suggesting that these vesicles represent the activation of CCR2 receptors (Figure 4.9B, C). The next chapter in this thesis investigates MCP1-CCR2 signaling in the pain pathway specifically in the context of neuropathic pain, using these transgenic mice.

DISCUSSION

To our knowledge, the use of bi-transgenic mice discussed here represents the first direct visualization of ligand-receptor interactions *in vivo*. Fluorescent protein tagging has been widely used to study receptor mobilization and neuropeptide release. However, most of these studies were done *in vitro* (Lou et al. 2005; de Jong et al. 2005) with a few exceptions (Shakiryanova, Tully, and Levitan 2006) and most utilized ectopic expression. With recent advances in BAC modification technology, it has become practical to generate gene-specific promoters without much knowledge of information about the promoter itself. The key to generating a specific promoter is being able to clone a sufficiently large portion of the DNA sequence flanking the gene of interest. By bacterial homologous recombination, it is possible to precisely insert an exogenous DNA fragment (e.g. EGFP coding sequences) into a BAC clone. BAC clones are, in fact, so large (e.g. more than 400 times larger than the protein coding region of MCP1, and more

than 100 times larger than the MCP1 gene) that it is generally believed that most regulatory cis-elements can be accommodated in one BAC clone. The BAC modification technology has been used to generate specific transcriptional reporter mice and tissue-specific cre recombinase drivers (Heintz 2004). By inserting EGFP or mRFP1 coding sequences, in frame, in place of the stop codons of MCP1 and CCR2, it was feasible to generate the reliable protein level reporter mice for MCP1 and CCR2.

In these mice, constitutive as well as inducible expression of MCP1 and CCR2 was faithfully recapitulated. Moreover, subcellular processes such as MCP1 release and CCR2 endocytosis could be clearly visualized. The interactions between MCP1 and CCR2 after the activation of CCR2 could also be observed. Therefore, it can be concluded that novel transgenic reporter mice have been developed in which the diverse aspects of MCP1 and CCR2 signaling can be examined *in vivo*. In this chapter, MCP1-CCR2 signaling in bone marrow and in the brain was thoroughly examined.

In the bone marrow, MCP1-CCR2 signaling has been implicated in the emigration of monocytes into the circulation. This known signaling interaction provided an ideal platform for perfecting visualization techniques, and it also allowed the researchers an opportunity to shed new light on the MCP1-CCR2 signaling process. In MCP1 and CCR2 knockout mice, monocyte egress during the inflammatory response is significantly impaired resulting in accumulation of monocytes in the bone marrow and impaired immune responses (Serbina and Pamer 2006). Consequently, these mice cannot suppress the growth of infecting bacteria. Although it was known that MCP1-CCR2 signaling plays an important role in monocyte egress, the molecular mechanisms

governing this process are not known. One important question concerned the source of the MCP1 which activates bone marrow monocytes (e.g. infected tissues or bone marrow). By direct visualization of MCP1 and CCR2, it was observed that MCP1 is pre-synthesized and stored in bone marrow cells under normal conditions (Figure 4.6). MCP1-expressing cells were often seen making physical contacts with CCR2-expressing monocytes suggesting that they may have low levels of basal communication. During the inflammatory response induced in the bi-transgenic mice, stored MCP1 was rapidly secreted in the bone marrow and activated nearby monocytes. It is still unclear how CCR2 activation induces monocyte egress in the bone marrow.

MCP1-CCR2 signaling in the brain serves a very similar function. In this case, microglia upregulate MCP1 during acute inflammatory responses. These microglia were often found in close contact with CCR2-expressing leukocytes. Leukocytes contained vesicles filled with both MCP1 and CCR2, suggesting that they were activated by microglia as parts of a brain inflammatory response. Although MCP1 is a well known pro-inflammatory cytokine expressed by microglia, its upregulation, processing, and release are still unclear. Using the bi-transgenic mice, the researchers sought to address this question. Live imaging was used in tissue slices to study the mechanisms of MCP1 release in microglia (e.g. by multiphoton microscopy) (Figure 6.2). Although not attempted in this study, these dual-color mice will allow simultaneous electrophysiological recordings from MCP1- and CCR2-expressing cells.

It is known that MCP1-CCR2 signaling plays a critical role in the pathogenesis of EAE. One study has shown, for example, that genetic deletion of the CCR2 gene confers resistance to EAE (Fife et al. 2000). Specifically, activation of CCR2-mediated signaling in monocytes and T cells

mediates breakage of the blood brain barrier (BBB) (Mahad et al. 2006), although this phenomenon is not well understood. For instance, the source of MCP1 expression in the brain during EAE is unclear. Using the BAC reporter mice developed for this thesis research, it was possible to study the cellular origin of MCP1 upregulation (Figure 4.9). It was found that microglia and astrocytes around blood vessels upregulated MCP1 in close association with the pathology of EAE (data not shown). Using these mice, breakage of the BBB could be directly visualized (Figure 4.9). In this paradigm, it is simple to appreciate the general topography of BBB breakage. Therefore, not only will these mice provide a good model to screen the efficacy of drugs to treat EAE in animals, but they will be a useful tool in live imaging, allowing researchers to dissect the cellular and subcellular events leading to BBB breakage.

Finally, this approach can be extended to the study of GPCRs in general. The construction of large scale GPCR and protein ligand reporter mouse libraries may be practical through rapid BAC modification. Thus, this technique will provide invaluable tools to the community of neuroscientist as well as pharmacologist and immunologists.

EXPERIMENTAL METHODS

Plasmid construction

To make chemokine-RFP fusion constructs, MCP1 and SDF1α protein coding sequences were amplified by PCR and cloned into pmRFP1-N1 and pmCherry-N1 (kind gifts of Dr. Roger Tsien, University of California, San Diego, CA, USA) using the following primers: for MCP1 upstream, 5'-TTGAATTCATGCAGGTCCCTGTCATGCTT-3'; for MCP1 downstream, 5'-AAGGA-

TCCAAGTTCACTGTCACACTGGTCA-3'; for SDF1 upstream, 5'- AAGAATTCATGG-ACGCCAAGGTCGTCG-3'; for SDF1 downstream, 5'-AACCGCGGCTTGTTTAAAG-CTTTCTCCAG-3'. The CCR2-expressing vector was made by cloning the CCR2 protein coding sequence into pIRES2-EGFP (Clontech) using the following primers and then deleting the IRES-EGFP sequence: for upstream 5'-CACAGATCTAAAGGAAATGGAAGACAA-3'; for downstream, 5'-CTTCTGCAGCAACCCAACCGAGACCTCTT-3'. The CXCR4-EYFP-expressing vector was a kind gift from Dr. Dongjun Ren (Northwestern University, Chicago, IL). Sequence identity was confirmed by dideoxy-sequencing methods. All chemicals were purchased from Sigma (Grand Island, NY, USA) unless stated otherwise.

Cell culture and transfection

Human embryonic kidney cells (HEK293; a tSA201 subclone) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 0.5% penicillin–streptomycin (P/S) at 37°C under 5% CO_2. One microgram of plasmid DNA was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfected cells were plated on poly-L-lysine-coated glass coverslips on the following day and antagonists were added. Materials for cell culture were purchased from Invitrogen (Carlsbad, CA, USA) unless stated otherwise.

Recombineering

The standard recombineering protocol described by (Lee et al. 2001) was used (Figure 4.4). The EGFP-FRT-KAN-FRT targeting cassette was generated by self-ligation of the blunt-ended BglI/SmaI fragment of pIGCN21 (kind gift from Dr. Neal Copeland, NCIF, Frederick, MD). The

mRFP1-FRT-KAN-FRT targeting cassette was generated by replacing the EGFP protein coding sequence with the mRFP1 protein coding sequence. The EGFP or mRFP1 targeting cassette was amplified by PCR using the chimeric primers, in which the 3' end was homologous to the targeting cassette and the 5' end was homologous to the gene of interest. The amplified cassette was inserted in place of MCP1's or CCR2's start or stop codon by λ-Red-mediated recombination to generate the transcriptional reporter (gene::reporter) and protein level reporter (gene::gene-reporter), respectively.

The following reporter transgenes were made: (1) CCR2::CCR2-EGFP (protein level reporter), (2) MCP1::mRFP1 (transcriptional reporter), and (3) MCP1::MCP1-mRFP1 (protein level reporter). In case of the MCP1::mRFP1 transcriptional reporter, the poly-A signal of SV40 (5'-AATAAAGCAATAGCATCACAAAT-TTCACAAATAAAGCATTTTTTTCACTGC-3') was added at the end of the mRFP1 protein coding sequence. The CCR2-containing BAC clone (MSM-529G05) was obtained from RIKEN DNA Bank (Ibaraki, Japan), and the MCP1-containing BAC clone (RP23-328G11) from Invitrogen. The chimeric primers used to generate the targeting cassettes are as follows: for CCR2::CCR2-EGFP upstream, 5'-TGAGCTCTACATTCACTCCTTCCACTGGGGAGCAAG-AGGTCTCGGTTGGGTTGGA-TGATAATATGGCCACAACC-3' ; for CCR2::CCR2-EGFP upstream: 5'-CTGTCTTTGAGGCTT-GTTGCTATGTACAAACTGCTCCCTCCTTCCCTGCTCTATT-CCAGAAGTAGTGAGGA-3'; for MCP1::mRFP1 upstream: 5'-CCAGCACCAG-CCAACTCTCACTGAAGCCAGCTCTC-TCTTCCTCCACCACCCCGGTCGCCACCATGG-CCTCC-3'; for MCP1::mRFP1 downstream: 5'- CTCCAGCCGGCAACTGTGAACAGCAGG-CCCAGAAGCATGACAGGGACCTGCTATTCCAGAAGTAGTGAGGA-3'; for

MCP1::MCP1-mRFP1 upstream: 5'-CCACAA-CCACCTCAAGCACTTCTGTAGGAGT-GACCAGTGTGACAGTGAACCCGGTCGCCACCATGGCCTCC-3'; for MCP1::MCP1-mRFP1 downstream: 5'-ATAAGTTAAATAAGTTTAATATTAATTAAGGCATCACA-GTCCGAGTCACACTATTCCAGAAGTAGTGAGGA-3'. The CXCR4::EGFP mouse was a kind gift from the GENSAT project (Rockefeller University, New York, NY).

Genotyping

The mice were genotyped for the presence of the transgene by PCR. The CCR2 genotyping primers were designed to flank the inserted cassette so that the wildtype allele and the transgene would generate two bands of different lengths. The downstream MCP1 genotyping primers were used with the upstream mRFP1 primer to detect the transgenes. Primer sequences are as follows: for CCR2::CCR2-EGFP, upstream: 5'-TGACAAGCACTTAGACCAGG-3', downstream: 5'-ATATGGTTCAGTCACGGC-3'; for MCP1::mRFP1, upstream (mRFP1): 5'-AGTTCCA-GTACGGCTCCAAG-3', downstream: 5'-GCTGAGCCAACACGTGGATG-3'; for MCP1::MCP1-mRFP1, upstream (mRFP1): 5'-AGTTCCAGTACGGCTCCAAG-3', downstream: 5'- GAAAGGGAATACCATAACATC-3'.

LPS injection

Mice were injected *i.p.* with 10 mg/kg lipopolysaccharide (LPS) diluted in saline and were sacrificed after 24 or 72 hours. For some experiments, animals were injected *i.p.* with the CCR2 receptor antagonist (CCR2-RA: kind gift from Eli Lilly and Company, IN) diluted in saline. In this case, animals were injected with 50 mg/kg CCR2-RA three times at 4 h intervals during the last 12 hours of LPS treatment.

Experimental Autoimmune Encephalomyelitis (EAE)

Detailed methods for the induction of EAE are described elsewhere (Bailey et al. 2007). Briefly, female mice at 12-15 weeks of age were immunized subcutaneously with 100 μl of emulsified incomplete Freund's adjuvant (Difco) supplemented with 200 μg *Mycobacterium tuberculosis* H37Ra (Difco) and 100 μg MOG(35–55: MEVGWYRSPFSRVVHLYRNGK), and they received *i.p.* injections of 200 ng pertussis toxin (Sigma) at the time of immunization and 48 h later.

Tissue sectioning, immunohistochemistry, and imaging

To sacrifice, animals were transcardially perfused with 4% paraformaldehyde. Tissues including the brain were post-fixed in the same solution overnight before being sectioned. Brains were sectioned at 40 μm using a vibratome (Leica). Free floating brain sections were immunostained for specific antigens using the standard protocol (see the chapter 3). Primary antibodies used are as follows: a rabbit polyclonal anti-IBA1 antibody (WAKO, 1:400) and a rabbit polyclonal anti-GFAP antibody (Sigma, 1:400). Corresponding secondary antibodies conjugated with Cy5 were used. Fluorescent images were taken by a laser scanning confocal microscope. The following settings were used to detect each fluorophore: for EGFP, excitation at 488 nm and emission at 500-520 nm; for mRFP1, excitation at 543 nm and emission at 610 nm and above; and for Cy5, excitation at 633 nm and emission at 670 nm and above. When mRFP1 and Cy5 were imaged in the same section, each fluorophore was sequentially excited. A 20x objective and a 60x oil-immersion objective were used, and the thickness of optical sections was 2 μm and 0.4 μm, respectively.

CHAPTER 5

Cellular Mechanisms Underlying MCP1-CCR2 Signaling-Mediated Hyperalgesia in States of Neuropathic Pain

ABSTRACT

It has previously been shown that upregulation of the chemokine, monocyte chemoattractant protein-1 (MCP1, also known as CCL2), and its receptor, CC chemokine receptor 2 (CCR2), signaling plays an important role in the genesis of neuropathic pain. However, the exact location of the MCP-CCR2 signaling-cells which contribute to pain hypersensitivity is not clear. This chapter shows that MCP1-CCR2 signaling is important not only in the development phase but also in the maintenance phase of neuropathic pain. This is supported by the fact that established pain hypersensitivity was reversed by a systemic injection of a CCR2 receptor antagonist (CCR2-RA). The rapid action of the CCR2-RA suggests that ongoing activation of CCR2-mediated signaling contributes to the pain hypersensitivity observed in states of neuropathic pain. Using MCP1-mRFP1; CCR2-EGFP protein level reporter mice, the cellular localization of active MCP1-CCR2 signaling in dorsal root ganglia (DRG) as well as their central and peripheral connections was examined using a focal demyelination model of neuropathic pain. These experiments show that MCP1-CCR2 signaling is activated in neurons and satellite glia of the DRG, as well as in the leukocytes infiltrating at the site of nerve injury. The activation of CCR2-mediated signaling in these cells was inhibited by the CCR2-RA that reversed behavioral hypersensitivity in pain model animals. In contrast to DRG and peripheral nerves, there was no detectible expression of CCR2 in the spinal cord under both naïve and pathological conditions. These data suggest that, in a focal demyelination model of neuropathic pain, major sites of MCP1-CCR2 activation are found in the peripheral nervous system, and explain the rapid anti-nociceptive effects of the *i.p.* administered CCR2-RA.

INTRODUCTION

Upregulation of MCP1-CCR2 signaling has been observed in many different models of neuropathic pain including sciatic nerve transsection (Subang and Richardson 2001; Taskinen and Roytta 2000), partial ligation of the sciatic nerve (Abbadie et al. 2003; Tanaka et al. 2004), chronic constriction injury of the sciatic nerve (Kleinschnitz et al. 2005; Zhang et al. 2007), chronic compression of the L_4L_5 DRG (CCD) (Sun et al. 2006; White et al. 2005), lysophosphatidylcholine-induced focal nerve demyelination (S. Bhangoo et al. 2007; Jung et al. 2008), bone cancer pain (Vit et al. 2006; Khasabova et al. 2007), and zymosan-induced inflammatory pain (Xie et al. 2006). Using the MCP1 and CCR2 transgenic reporter mice (MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP), this chapter aimed the cellular localization of active MCP1-CCR2 signaling-cells under conditions of neuropathic pain. A focal demyelination model of neuropathic pain (S. Bhangoo et al. 2007; Jung et al. 2008) was used. In this model, a detergent (lysophosphatidylcholine: LPC)-soaked sponge was implanted near the sciatic nerve at the level of the thigh. The sponge slows releases LPC which induces focal demyelination of the nerve. As a result, animals develop hyperalgesia in the hind paws because they are innervated by the sciatic nerve, and this lasts for at least three weeks. At the peak of hyperalgesia, animals were injected with the CCR2-RA. By comparing the cellular localization of activated CCR2 receptors under these conditions, this study investigated the sites of MCP1-CCR2 signaling activation that play a role in neuropathic pain.

RESULTS

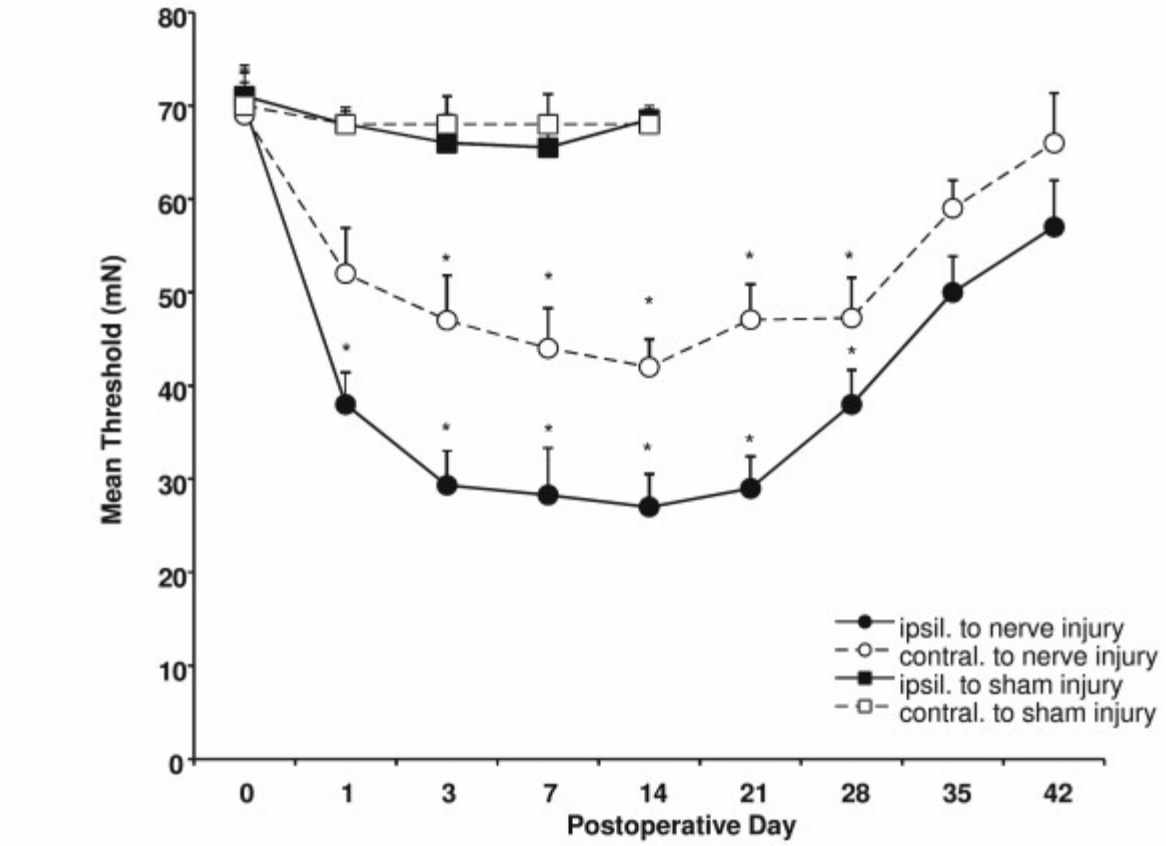

Figure 5.1. Development of focal demyelination-induced neuropathic pain. Graph shows the mean threshold force required for paw withdrawal in response to Von Frey stimulation at 1, 3, 7, 14, 21, 28, 35 and 42 days following LPC-induced focal nerve demyelination. Each data point represents the mean threshold (± SE) force on the hindpaw ipsilateral (black circle) or contralateral (white circle) to the focal nerve injury site eliciting a withdrawal response (n = 10). Reduced behavioral thresholds for the hindpaw ipsilateral to the nerve lesion were significantly different from the pre-operative baseline on postoperative days 1–28. The threshold force for the hindpaw contralateral to the nerve lesion did not reach significance until postoperative day 3, and significant differences were observed until postoperative day 28. The time course of sham injury (n = 6) is also represented but did not differ from the uninjured animals. Analysis was performed using two-way ANOVA followed by the Bonferroni post-hoc pair-wise comparisons (*$p < 0.01$). (From SK Bhangoo et al., 2007)

MCP1-CCR2 signaling plays an important role in the development and maintenance phases of neuropathic pain.

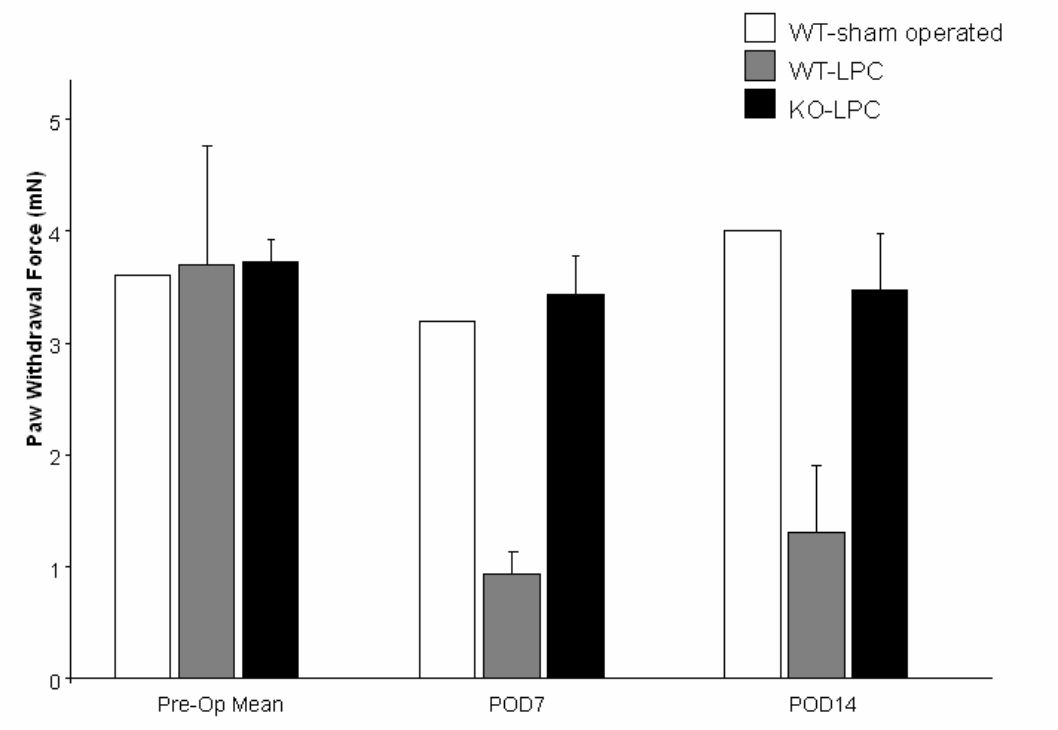

Figure 5.2. CCR2 signaling is important in the development phase of neuropathic pain. Wildtype and CCR2 knockout (KO) mice were administered the LPC model of neuropathic pain. Foot withdrawal thresholds were measured in *von Frey* tests. Mechanical hyperalgesia developed early and was evident on POD7 and on POD14 (gray bars) which is compared with naive controls (white bars). However, CCR2 KO mice did not develop neuropathic pain (black bars). (Modified form unpublished data of SK Bhangoo *et al*.)

First, the importance of CCR2-mediated signaling in the LPC model of neuropathic pain was confirmed by behavioral tests. Pain hypersensitivity was examined by a *von Frey* test in which the mechanical threshold to elicit a paw withdrawal response was recorded. Mechanical allodynia (i.e. decrease in the paw withdrawal threshold) developed shortly after surgery and peaked around postoperative day (POD) 14 (Figure 5.1). Development of mechanical allodynia was significantly impaired in CCR2 knockout mice confirming the notion that CCR2-mediated signaling plays a pivotal role in the genesis of neuropathic pain (Figure 5.2). To examine

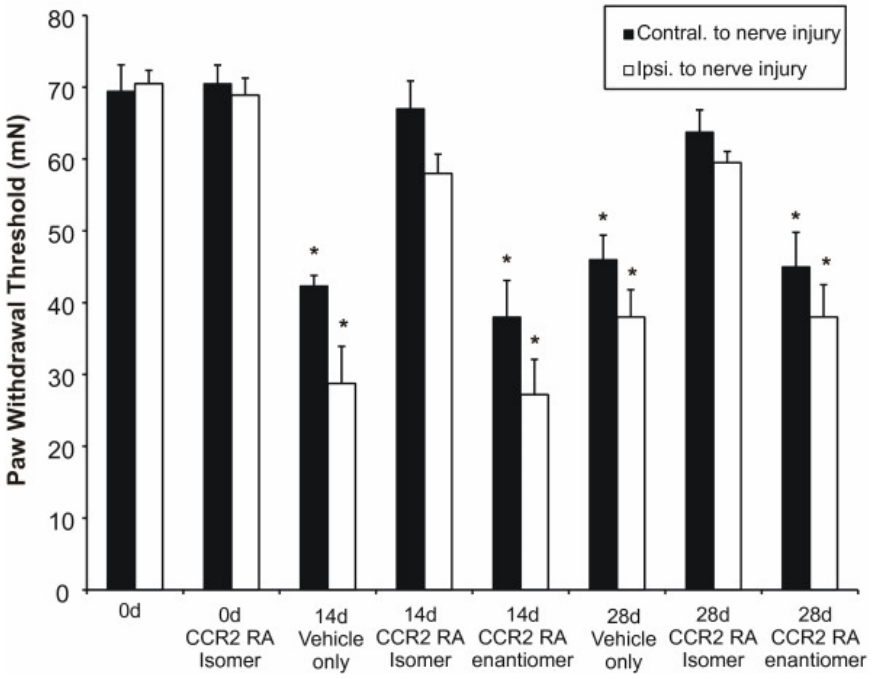

Figure 5.3. CCR2 signaling is important in the maintenance phase of neuropathic pain. CCR2 receptor antagonist (CCR2 RA-[R]) administration reversed existing nociceptive behavior. Animals were subjected to a nerve demyelination injury on day 0 and nociceptive behavior was assessed for 28 days. On days 14 and 28 post-surgery, animals received 5 mg/kg CCR2 RA-[R], 5 mg/kg of its inactive enantiomer (CCR2 RA-[S]), or saline by intraperitoneal injection, and behavioral responses were tested 1 h later. Administration of the CCR2 RA-[R] to rats with a focal nerve demyelination injury resulted in a significant bilateral increase in the force required to elicit a paw withdrawal compared with vehicle-treated controls and animals injected with CCR2 RA-[S]. Nociceptive behavior in vehicle-treated controls and animals given CCR2 RA-[S] differed significantly from day 0 pre-injury baseline responses (*$p < 0.01$). Data represent means ± SE. (From SK Bhangoo *et al*., 2007)

whether MCP1-CCR2 mediated signaling also plays a role in the maintenance phase of neuropathic pain, the CCR2-RA was injected (10 mg/kg, *i.p.*) on POD14 by which time pain hypersensitivity in the animals had reached a peak. Injection of the CCR2-RA rapidly reversed established nociceptive behavior (1 hour post injection), however, the effect was relatively short-lived (around 4 hours) (Figure 5.3). These data indicate that MCP1-CCR2 signaling is activated and ongoing activation contributes to pain hypersensitivity in these animals. The rapid anti-

nociceptive effect of the peripherally injected CCR2-RA suggests that the major sites of this drug's action include the peripheral nervous system.

The sites of active MCP1-CCR2 signaling

Nerve

A significant number of CCR2-expressing leukocytes (Figure 5.4: green arrow) infiltrated to the site of nerve demyelination. This was in contrast to the uninjured nerve where few leukocytes were present (Figure 5.4A, B). Infiltrated leukocytes remained at the site during the course of the experiments. In addition, the number of MCP1-expressing cells was significantly increased in the demyelinated nerves (Figure 5.4B: red arrow). Many of the CCR2-expressing leukocytes contained vesicles with MCP1-mRFP1 suggesting that they had undergone CCR2 receptor activation (Figure 5.4C). Injection of the CCR2-RA induced a decrease in the number of cells with activated CCR2, confirming ongoing CCR2 activation in these cells (Figure 5.4D). The main source of MCP1 was endoneurial fibroblasts, which do not express the Schwann cell marker, S100, and were identified in the sciatic nerve as cells not directly associated with axons (Joseph et al. 2004).

DRG

In agreement with previous results (S. Bhangoo et al. 2007; Jung et al. 2008), the expression of MCP1 and CCR2 increased in association with the development of neuropathic pain (Figure

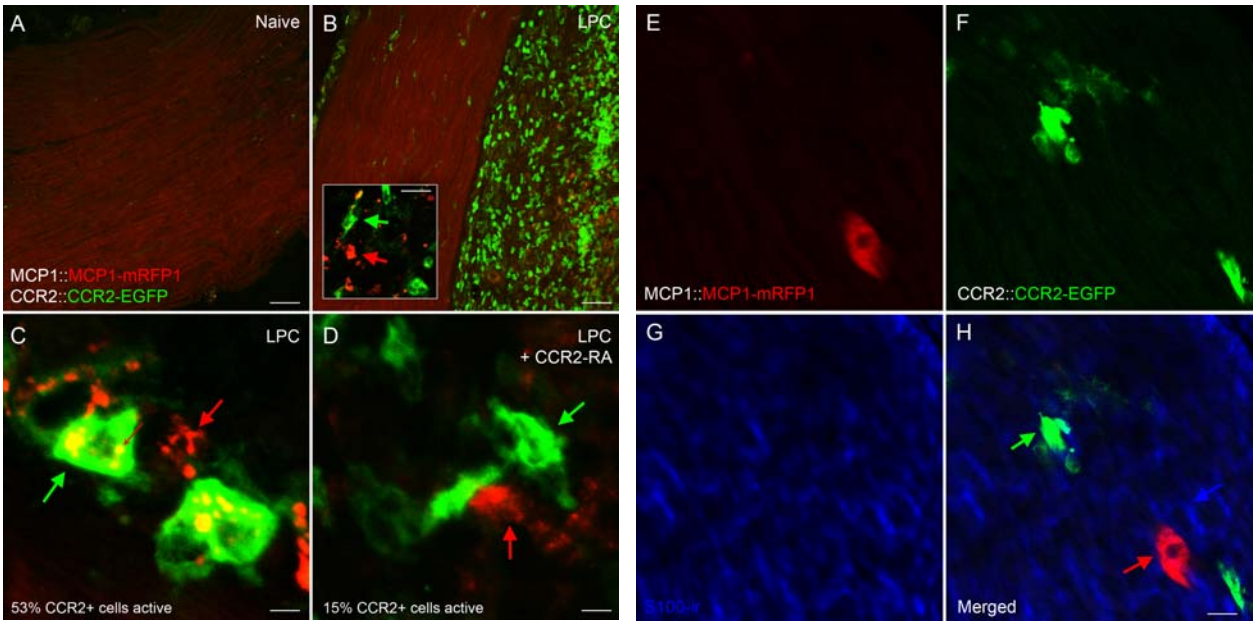

Figure 5.4. CCR2 signaling is activated in the injured nerve. MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP mice were given the LPC model of neuropathic pain. At the injury site, MCP1-expressing cells increased significantly in the nerve (red arrow), and there was infiltration of leukocytes both in and around the nerve (green arrow) (B). Many of the CCR2-expressing leukoctyes were observed undergoing active CCR2 receptor-mediated signaling as evidenced by the internalized red vesicles (C). Injection of the CCR2-RA reversed the CCR2 receptor activation (D). (E-H). Endoneurial fibroblasts upregulated MCP1. Endoneurial fibroblasts (red arrow) were identified as cells which were not associated with axons and did not express S100 (G), a marker for Schwann cells (blue arrow). Expression of S100 was examined by immunohistochemistry. The green arrow indicates a leukocyte. Scale bars, A, B, 60 μm; B inset, 20 μm; C, D, 4 μm.

5.5C, F). Most CCR2-expressing neurons contained MCP1 in their cell bodies suggesting the activation of CCR2 receptors in these cells (Figure 5.5F: large yellow arrow). Most CCR2-expressing satellite glia also contained vesicles with MCP1 (Figure 5.5F: small yellow arrow). Some neurons contained only MCP1 suggesting that they were the local source of MCP1 (Figure 5.5D-F: red arrow). When animals were injected with the CCR2-RA, the pattern of MCP1 and CCR2 localization changed. First, the intensity of the CCR2-EGFP signal in neurons increased (Figure 5.5E, H: compare intensity of large and small green arrows in each panel). Moreover,

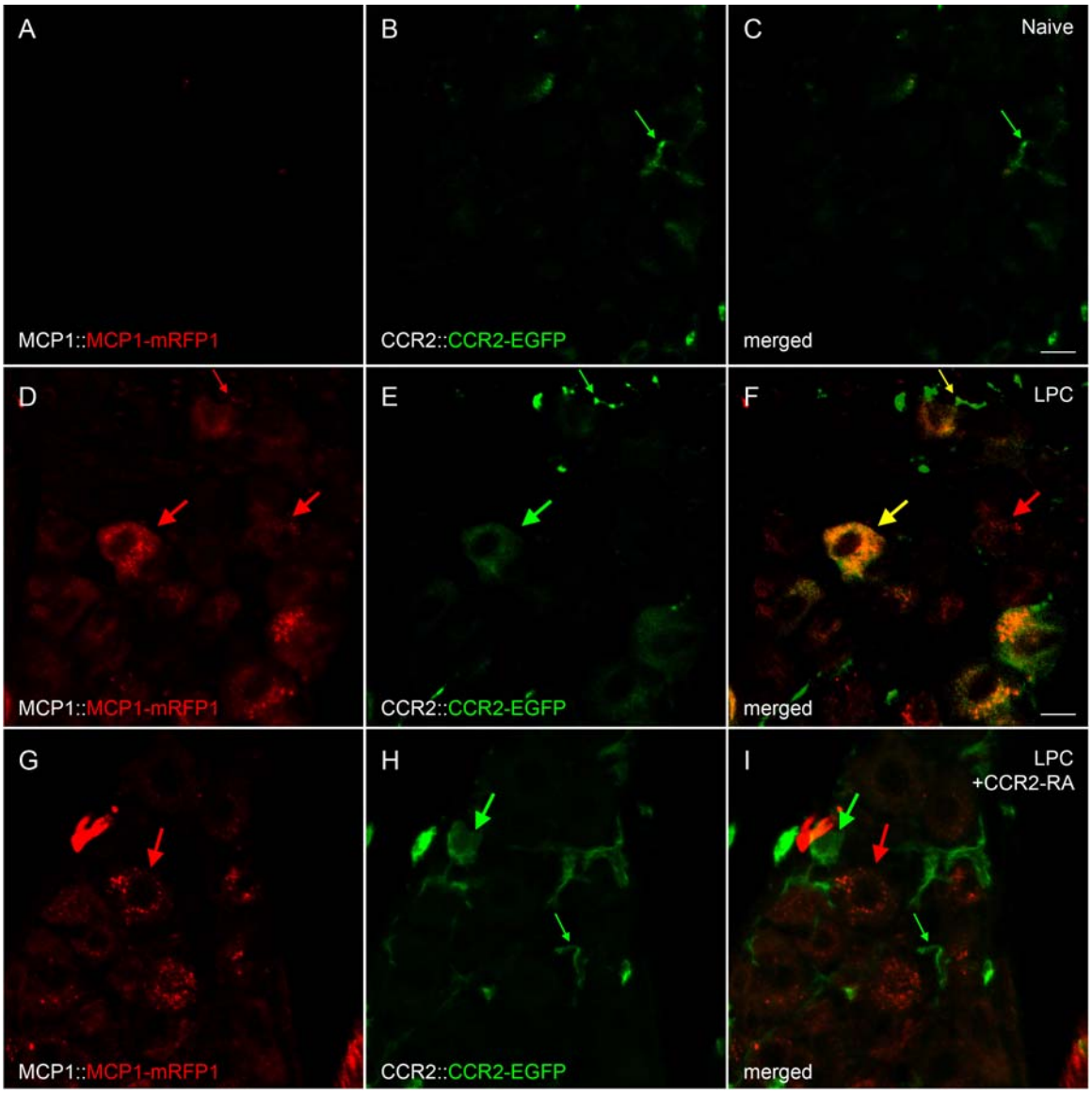

Figure 5.5. CCR2 signaling is activated in the affected DRG. MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP mice were subjected to the LPC model of neuropathic pain. In the affected DRG, expression of both MCP1 and CCR2 increased (D-F), while there was little expression of MCP1 or CCR2 under naïve conditions (A-C). MCP1-mRFP1 mainly localized to neurons (large red arrows) and, to some extent, to satellite glia (small red arrows) (D). CCR2-EGFP localized to neurons (large green arrows) and satellite glia (small green arrows) (E). Most CCR2-EGFP-expressing neurons also contained MCP1-mRFP1 (F). Injection of the CCR2-RA eliminated MCP1-mRFP1 in satellite glia (G-I). Also, it separated MCP1-mRFP1 and CCR2-EGFP expressing cells (I). Scale bars, 15 μm.

there was now clear separation of MCP1- and CCR2-expressing neurons (Figure 5.5I: large green and red arrows). Finally, satellite glia expressing CCR2 contained no MCP1 (Figure 5.5I: small green arrow). These data suggest that MCP1 upregulated in a subset of neurons activates CCR2 receptors expressed by neighboring neurons and satellite glia in this model of neuropathic pain.

Spinal cord

There is evidence in the literature that MCP1 is transported to central terminals where it is released and affects neuronal and microglial activities in the spinal cord (Thacker et al. 2008; Gosselin et al. 2005; Zhang et al. 2007). However, no cells expressing CCR2-EGFP were observed in the parenchyma of the spinal cord under either normal or pathological conditions. However, a few leukocytes were closely associated with blood vessels or meninges (Figure 5.6). Moreover, no significant accumulations of MCP1-mRFP1 were observed at the dorsal horn of the spinal cord, suggesting that most MCP1 upregulated by a DRG neuron is secreted locally within its ganglion. Finally, injection of the CCR2-RA did not change the expression patterns of MCP1 and CCR2.

DISCUSSION

This research has shown that expression of MCP1 and CCR2 increases in the DRG of animals with a demyelination injury. CCR2 knockout mice have impaired development of neuropathic pain suggesting that CCR2-mediated signaling plays a role in the generation of neuropathic pain.

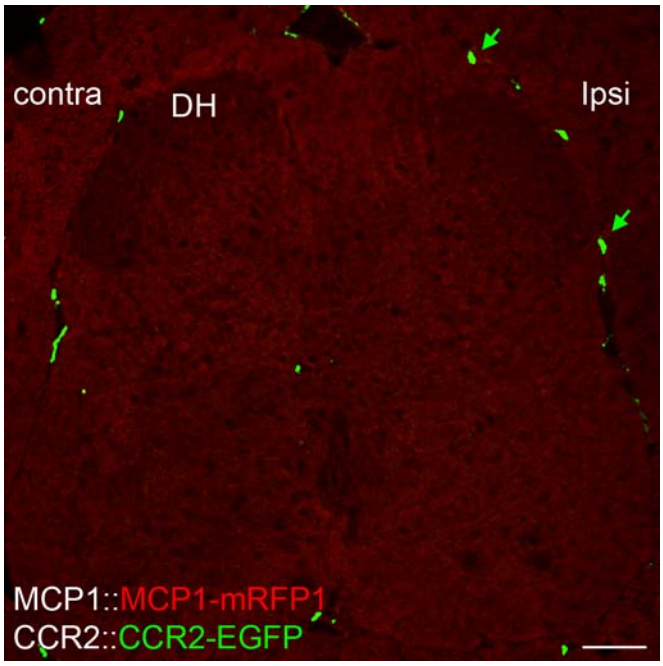

Figure 5.6. CCR2 signaling is not activated in the spinal cord. MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP mice were treated with LPC to model neuropathic pain. There was no expression of MCP1 or CCR2 at a detectible level. Leukocytes outside the spinal cord are clearly visible (green arrow). Scale bar, 60 μm.

Significantly, research points to a role for CCR2-mediated signaling in the maintenance phase of neuropathic pain, because administration of a CCR2-RA alleviates established pain hypersensitivity. This suggests that on going activation of CCR2 receptors at the periphery contributes to pain hypersensitivity. Using the transgenic reporter mice, the cellular localization of activated CCR2 receptors was examined at the level of the peripheral nerve, DRG, and the spinal cord.

Many models have been proposed to explain the potential role of CCR2 in neuropathic pain. However, the details concerning the time course and cellular anatomy of CCR2 upregulation vary among injury models. Occasionally, studies of the same animal model yielded conflicting results. The combined results of several studies have generated the following cascade of events

leading to increased MCP1-CCR2 signaling. First, Schwann cells and/or endoneurial fibroblasts in injured axons upregulate MCP1 which attracts macrophages into the nerve. Infiltrating macrophages secrete inflammatory molecules that sensitize the nerve. Secondly, neurons in DRG to which injured axons project upregulate both MCP1 and CCR2. The activation of CCR2 signaling in the DRG neurons is excitatory and therefore pro-nociceptive. Thirdly, DRG neurons transport MCP1 to central endings in the spinal cord where it is released. Once there, MCP1 activates resident microglia which express CCR2. The activation of microglia potentiates synaptic transmission between primary and secondary nociceptors in the dorsal horn of the spinal cord. Finally, centrally transported MCP1 is released to neurons in the spinal cord. A population of GABAergic neurons there also express CCR2, and the activation of CCR2 in these neurons inhibits their response to GABAergic input.

The results shown in this chapter support the idea that the sites of MCP1-CCR2 action under conditions of chronic pain are located in the periphery. And the injured nerve and DRG, it has been observed that CCR2-expressing cells (e.g. DRG neurons, satellite glia, and leukocytes in the nerve) undergo active CCR2-mediated signaling in correlation with hyperalgesic behavior. The activation of CCR2 signaling in these cells could also be inhibited by the injection of the CCR2-RA. These data suggest that activation of CCR2 receptors in leukocytes in the nerve, and in neurons and satellite glia in the DRG likely contribute to pain hypersensitivity.

Chapter two offered one possible mechanism by which CCR2 signaling may contribute to pain hypersensitivity. This research shows that activation of CCR2 receptors in DRG neurons sensitizes them by transactivating TRP channels. Further, satellite glia in the DRG are a source

of many proinflammatory molecules such as NGF (Scholz and Woolf 2007). Therefore, the activation of CCR2 receptors in these satellite glia may also sensitize DRG neurons. Similar mechanisms may also apply to leukocytes in the nerve, as they secrete molecules such as prostaglandins and TNFα that sensitize the injured nerve (Scholz and Woolf 2007).

The effectiveness of peripherally injected CCR2-RA does provide an argument that the major sites of drug action, at least in this model, do not reside in the spinal cord but rather in the peripheral nervous system. As expected no significant axonal transport of MCP1 to the dorsal horn of the spinal cord was observed, nor were there a significant number of CCR2-expressing cells (e.g. neurons or microglia) in the spinal cord. However, several additional possibilities cannot be discounted, including: (1) the methods for MCP1-CCR2 visualization were not sensitive enough to detect low yet functional levels of MCP1 and CCR2 in the spinal cord and (2) upregulation of MCP1-CCR2 signaling in the spinal cord is strictly dependent upon the animal models being studied. Wherever the precise site of action is, it is clear that CCR2 activation in the peripheral nervous system plays a critical role in pain hypersensitivity in states of neuropathic pain. Therefore, the antagonism of MCP1-CCR2 signaling may be a novel and approachable point of therapeutic intervention to treat chronic pain syndromes.

EXPERIMENTAL METHODS

Sciatic nerve demyelination

Animals were anesthetized with 4% isoflurane and maintained on 2% isoflurane (Halocarbon, River Edge, NJ, USA) in O_2. For all demyelination experiments, lysophosphatidylcholine (LPC),

(type V, 99% pure; Sigma-Aldrich, St Louis, MO, USA) was dissolved in buffered sterile saline (pH 7.2) to yield a final concentration of 10 mg/mL. The right sciatic nerve of the mouse was exposed at mid-thigh level under sterile conditions. A sterile polyvinyl acetal sponge (Ivalon, San Diego, CA, USA), 2 mm × 2 mm, was soaked in 7 μL of LPC and then placed adjacent to the sciatic nerve. The dermal incision site was closed with 4.0 suture thread. Sham control animals were prepared as described above, but buffered sterile saline was used in place of LPC. Animals were allowed to survive for 14 days. For some experiments, animals were injected with a CCR2 receptor antagonist (CCR2-RA: kind gift from Eli Lilly and Company, IN). Three injections of CCR2-RA (50 mg/kg) were given *i.p.* at 4 hour intervals during the last 12 hours of the experiments. All experiments complied with protocols approved by the Northwestern University and Loyola University Chicago Institutional Animal Care and Use Committees.

Tissue processing and sectioning

DRG (L3, L4, and L5), spinal cords, and sciatic nerves were isolated and fixed in 4% paraformaldehyde at 4°C overnight. Tissues were then saturated with 30% sucrose in 1X PBS before sectioning using a cryostat. Tissue samples were sectioned at 20 μm and stored at -70 °C until used.

Von Frey test for mechanical allodynia

The von Frey test was performed on the area of the hind paw as previously described (S. Bhangoo et al. 2007).

CHAPTER 6

Conclusions

Questions

The chemokine family of signaling molecules first attracted researchers' attention when their role as inflammatory mediators, particularly their role in trafficking of leukocytes, was discovered. Since that time, chemokines have been found to have many other functions. An early connection to the nervous system was made with the observation that several chemokines traffic neural progenitors during development, a role analogous to that in the immune system. During the course of these studies, it was further found that some neurons continued to express a number of chemokine receptors even after they had finished migrating to their targets. This expression in the adult nervous system indicated that chemokines must have roles beyond those as mediators of cell migration.

This discovery led many researchers to seek out other locations where chemokines and receptors were expressed and to explore the conditions which governed their expression. One study showed that cultured primary sensory neurons expressed multiple functional chemokine receptors. Researchers went on to show that activation of these receptors had an excitatory effect on the sensory neurons. This led to the hypothesis that chemokine expression by peripheral neurons might be pronociceptive. Animal studies *in vivo* have shown that while DRG neurons do not express chemokines and their receptors under normal conditions, they are instead upregulated in states of neuropathic pain as seen in many animal models of chronic pain.

Among the various chemokines and chemokine receptors, MCP1 and its cognate receptor, CCR2, have been most frequently implicated in chronic pain. CCR2 is strongly upregulated by dorsal

root ganglia (DRG) in injured animals, and acutely isolated DRG neurons are depolarized after application of MCP1. Furthermore, studies have shown that MCP1 itself is also upregulated in the same DRG (Sun et al. 2006; White et al. 2005; Jung et al. 2008; S.K. Bhangoo et al. 2007).

The contribution of MCP1-CCR2 signaling to establishment and maintenance of pain hypersensitivity appears critical. Specifically, experiments showed that that injured animals were resistant to development of neuropathic pain and showed short-term reversal of pain hypersensitivity, when this signaling was disrupted by CCR2 knockout and administration of a CCR2 receptor antagonist, respectively. However, many questions still remained about the mechanisms which underlie the expression of MCP1 and CCR2 in a variety of cells. This thesis aimed to answer some of these questions.

CCR2 Signaling-Mediated Pain Hypersensitivity

A significant question is whether such data, mostly obtained in cell culture studies, has relevance to the situation in chronic pain states *in vivo*. In this study, the DRG as well as its peripheral (i.e. the sciatic nerve) and central (i.e. the dorsal horn of the spinal cord) connections were examined, because these locations are major sites where sensitization of the pain pathway occurs. As previously mentioned, upregulation of chemokines and chemokine receptors has been observed in DRG neurons in many different rodent models of neuropathic pain. Thus, in association with chronic pain nearby DRG neurons may upregulate both a chemokine and its receptor, suggesting that a form of paracrine regulation of DRG excitability by these molecules may occur. For

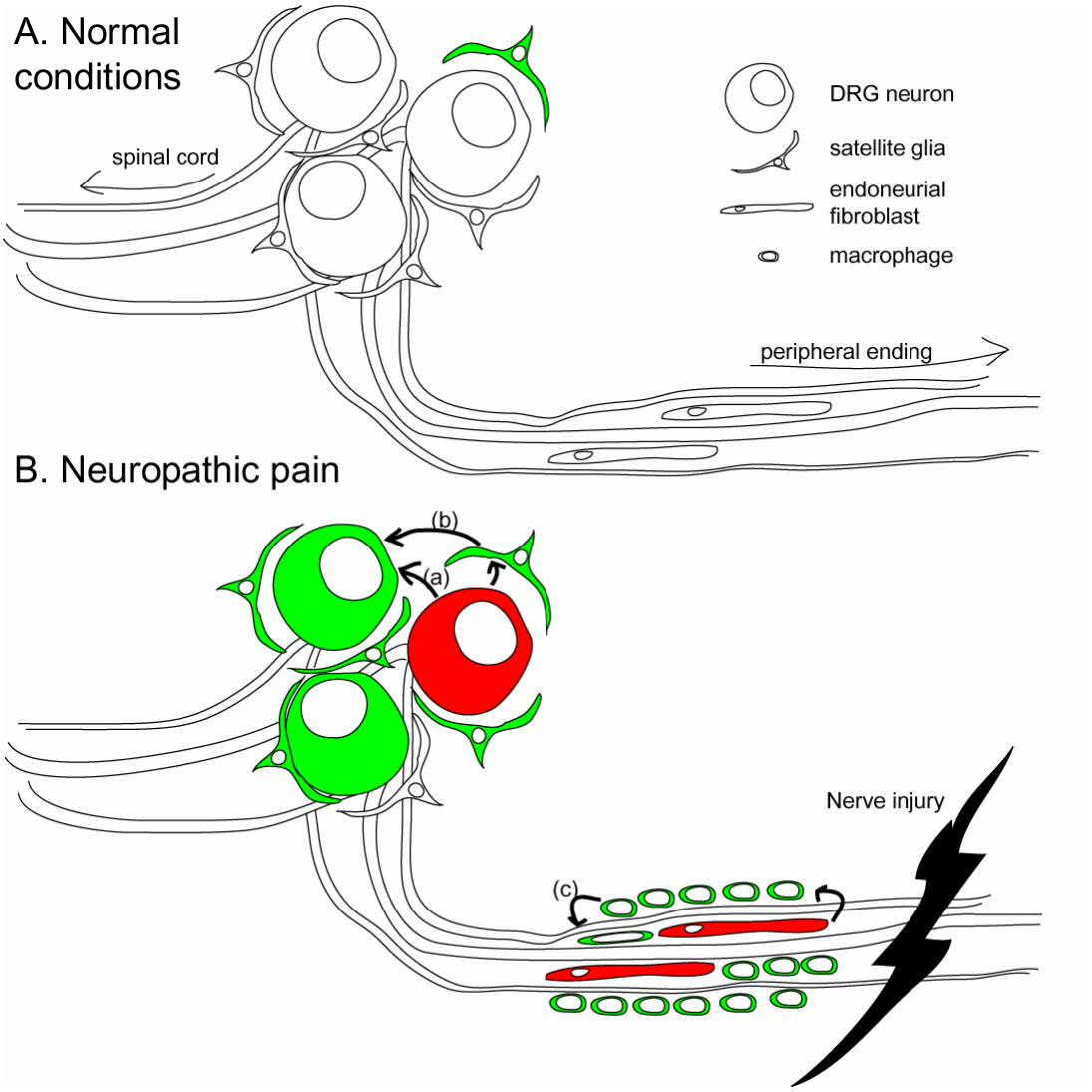

Figure 6.1. A proposed model of peripheral sensitization by MCP1-CCR2 signaling. (A) Under normal conditions, DRG neurons express neither MCP1 nor CCR2. A few satellite glia express CCR2. (B) Nerve injury induces upregulation of MCP1 (red) by DRG neurons via activation of the NFκB pathway. CCR2 (green) expression is upregulated by DRG neurons via activation of the NFAT pathway. CCR2 is also upregulated by satellite glia. MCP1-expressing DRG neurons release MCP1 in an activity-dependent manner which activates CCR2 receptors expressed by neighboring neurons and glia. CCR2 activation in DRG neurons directly sensitizes them (a) by mechanisms including transactivation of TRP channels. CCR2 activation in satellite glia induces release of pro-nociceptive molecules which sensitize neurons in the vicinity (b). Endoneurial fibroblasts at the injury site also upregulate MCP1, which recruit CCR2-expressing macrophages. Activation of CCR2 receptors in macrophages induces release of various chemical mediators of pain which sensitize nearby nerves (c).

example, it can be speculated that under these circumstances DRG neurons release chemokines which then activate receptors expressed by neurons in the vicinity. Chemokines have an excitatory effect on DRG neurons, therefore, this process may contribute to the neuronal hyperexcitability that accompanies chronic pain conditions (Figure 6.1B-a). Chemokines released by neurons may also sensitize neighboring neurons by other methods. For example, chemokines may sensitize DRG neurons indirectly by binding to receptors on satellite glia. Satellite glia then release pro-inflammatory molecules into the DRG (Figure 6.1B-b). Chemokines are also of vital importance to the recruitment of leukocytes (Figure 6.1B-c), thus, they are a likely candidate for the simultaneous coordination of inflammation and neuronal excitability. Indeed, MCP1 is upregulated at the site of nerve injury, and it signals to CCR2 expressed by the infiltrated leukocytes. MCP1-CCR2 signaling may stimulate the release of pro-nociceptive molecules from these leukocytes, resulting in sensitization of the injured nerves.

Finally, CCR2 signaling may have roles in the spinal cord in chronic pain. Although the methods used here did not detect a significant number of CCR2-expressing cells in the spinal cord, there are several reports which described the effects of MCP1-CCR2 signaling in the spinal cord. Several groups have reported that intrathecal injection of MCP1 induces the activation of microglia, which contributes to sensitization at the level of the spinal cord. However, whether resident microglia in the spinal cord express functional CCR2 is under debate. A study by Abbadie et al. detected in microglia by immunohistochemistry, however, most groups were unable to visualize CCR2 immunoreactivity in microglia. A couple of possible explanations for this discrepancy exist. One explanation may be that a local increase of MCP1 in the spinal cord recruits circulating monocytes which express CCR2, and infiltrated monocytes secrete molecules

that activate microglia. Alternatively, infiltrated monocytes may differentiate into microglia in the spinal cord and then downregulate their CCR2 expression. Indeed, it has been reported that circulating monocytes can enter the spinal cord and differentiate into microglia under some circumstances (Zhang et al. 2007). Further, Gosselin et al. have reported that some spinal cord neurons express functional CCR2, the activation of which inhibits GABAergic responses in these neurons. Although this thesis did not examine this phenomenon, it provides an interesting possible mode of pro-nociceptive action of MCP1-CCR2 signaling at the level of the spinal cord.

Transcriptional Regulation of MCP1 and CCR2

Inducible expression is a unique property of MCP1-CCR2 signaling in the nervous system. In naïve animals, the cells of the nervous system express neither MCP1 nor CCR2 at high levels. Instead, their expression is closely related to various pathological conditions associated with activation of the inflammatory response. In the case of neuropathic pain, neuronal upregulation of MCP1 and CCR2 is a well accepted, although the underlying mechanisms are not understood.

This thesis sought to shed light on this question. This research has shown that MCP1 expression in DRG neurons is under the control of the NFκB pathway. This substantiated findings in cultured fibroblasts (Ping, Jones, and Boss 1996). In DRG neurons, the NFκB pathway can be activated by TNFα through the activation of TNFα receptor type I (TNFRI) (Li et al. 2004). It is also well known that the serum concentration of TNFα increases in many pathological states with associated inflammation including neuropathic pain (Empl et al. 2001). In DRG, the source of the TNFα may be satellite glia as they have been shown to upregulate TNFα in response to

inflammatory stimuli (Li et al. 2004). The local increase in TNFα in the DRG may activate the TNFRI receptors expressed by neurons, and this will ultimately activate downstream transcription of MCP1.

This work revealed that the transcription of CCR2, however, was not under the control of the same pathway. Experiments have shown that CCR2 is a target gene of the NFAT pathway. The CCR2 promoter contains a conserved binding element for NFAT, the activation of which induces *de novo* synthesis of CCR2 mRNA. In DRG neurons, the NFAT pathway was activated by depolarization suggesting that activity-dependent transcription may mediate the upregulation of CCR2 in DRG neurons by activation of the NFAT pathway. In agreement with this concept, CCR2 mRNA was found to be abundant in cells where the endogenous activity of the NFAT pathway is high (e.g. WEHI265.1 monocytic cells and PC12 pheochromocytoma cells). Further, pharmacological inhibition of the pathway resulted in a decrease in the baseline level of CCR2 expression (Figure 3.7). Ectopic discharge of action potentials in DRG neurons is a typical feature of neuropathic pain (Omana-Zapata et al. 1997). This leads to a sustained increase in intracellular Ca in these neurons, which results in the activation of the NFAT pathway. Therefore, it is likely that the NFAT pathway mediates the upregulation of CCR2 in DRG neurons under conditions of neuropathic pain.

Therapeutic Implications

Chemokines and chemokine receptors are widely expressed by cells of the immune and nervous systems. Injury- or disease-induced changes in the expression of diverse chemokines and their

receptors have been demonstrated in the neural and non-neural elements of the pain pathway. Under these circumstances, chemokines have been shown to modulate the electrical activity of neurons by multiple regulatory pathways including increases in neurotransmitter release through Ca-dependent mechanisms and transactivation of transient receptor potential (TRP) channels. These mechanisms alone, or in combination, may contribute to the sustained excitability of primary afferent and secondary neurons within spinal pain pathways. Chemokines may also influence sustained neuronal excitability by their ability to function as excitatory neurotransmitters within the peripheral and central nervous systems. As is the case for traditional neurotransmitters, chemokines upregulated during injury are found within synaptic vesicles. Chemokines released after depolarization of the cell membrane can then act on other chemokine receptor-expressing neurons, glia, or immune cells. Upregulation of chemokines and their receptors may be one mechanism that directly or indirectly contributes to the development and maintenance of chronic pain, therefore, these molecules may represent novel targets for therapeutic intervention in chronic pain states.

As shown in the chapter five, specific receptor antagonists may be an effective therapy to treat neuropathic pain. In fact, several pharmaceutical companies are now targeting new drugs at CCR2 in order to treat neuropathic pain. Additionally, receptor antagonists for TRP channels may be effective drugs to treat neuropathic pain. Under conditions of neuropathic pain, serum concentrations of many inflammatory mediators increase, and many of these molecules can sensitize TRP channels. TRP channels are not only expressed by DRG neurons but also by many cells including neurons in the central nervous system, thus, it will be necessary to develop a peripherally-targeted drug to minimize complications from side effects.

In most cases, MCP1 and CCR2 expression is upregulated in close association with the development and maintenance of the pathology. Therefore, in addition to inhibition of MCP1 action, inhibition of MCP1 synthesis may serve as an alternative approach to treatment of neuropathic pain. This thesis has shown that MCP1 upregulation is controlled by the NFκB pathway. The NFκB pathway is known to regulate a number of other 'pro-inflammatory genes', therefore, use of specific inhibitors of the NFκB pathway may be an effective way to reverse chronic pain syndromes. Further, activators of signaling pathways that are known to antagonize the NFκB pathway (e.g. peroxisome proliferator-activated receptor agonists) may have anti-nociceptive effects. Although pharmacological inhibitors of the NFAT pathway (e.g. cyclosporin A and FK506) are currently in clinical use (e.g. for post-transplant graft rejection, psoriasis, and atopic dermatitis), their potential as a treatment for chronic pain has never been critically assessed. Therefore, it would be compelling to examine the effects of these drugs in patients with neuropathic pain.

In vivo Monitoring of G-Protein Coupled Receptor Signaling

In the course of this study, several novel transgenic reporter mice were developed in order to create a system in which MCP1 and CCR2 can be simultaneously visualized. This is, according to the literature reviewed, the first direct visualization of GPCR activation *in vivo*. One of the many advantages of transgenic animals that express a ligand or receptor tagged with a fluorescent protein is the opportunity to perform real-time imaging either *in vivo* or in slice preparation. The mechanisms underlying neuropeptide release and receptor endocytosis in

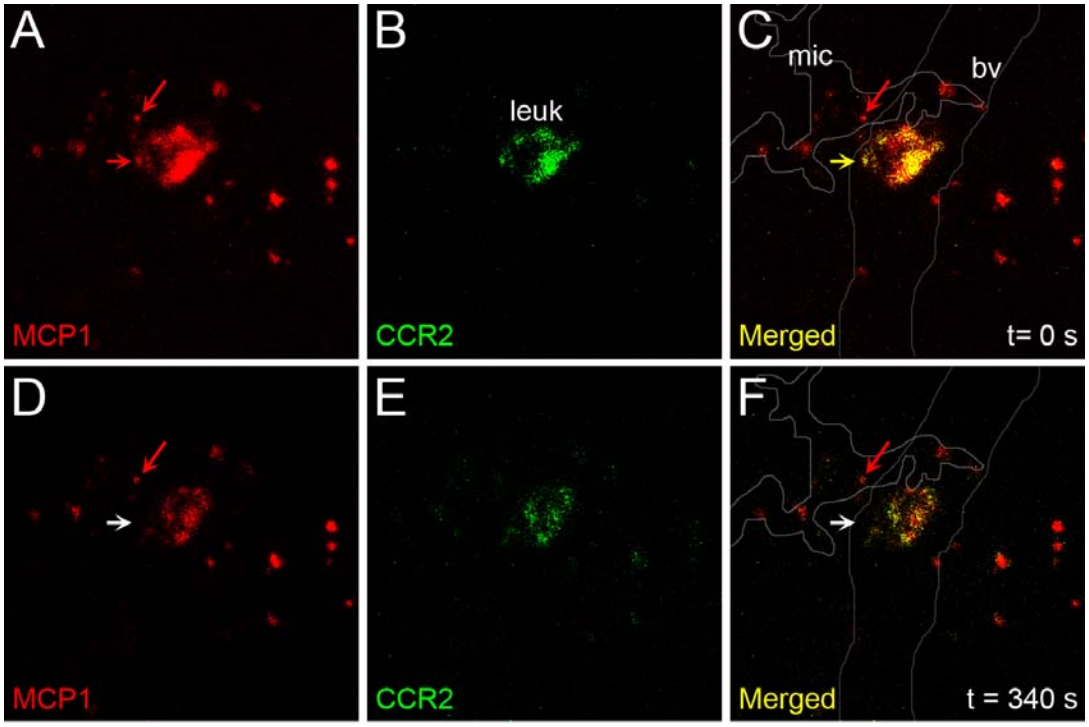

Figure 6.2. Live imaging of MCP1-contanining vesicle dynamics by 2PM microscopy. Brain slices were taken from MCP1::MCP1-mRFP1; CCR2::CCR2-EGFP mice injected with LPS. Microglia expressing MCP1-mRFP1 (red) in close contact with a CCR2-EGFP expressing leukocyte (green) were imaged by a two-photon (2PM) confocal microscope. The vesicles inside the leukocyte (green arrow) were dynamic in contrast to the vesicles outside the leukocyte which appeared to be localized in the microglia (red arrow).

particular have been studied using such approaches. Moreover, the bi-transgenic mice described in this thesis were shown to express the tagged proteins in the appropriate cellular locations. Thus, the localization of the fluorescent proteins is expected to be almost identical to the endogenous proteins. These mice provide a unique opportunity to study the dynamic signaling of MCP1-CCR2 in many systems. For example, the nature of the chemokine-containing vesicles as well as their mechanisms of release in microglia is poorly understood. Using a two-photon laser microscope, it is possible to examine the mechanisms of MCP1 release in microglia in live tissues (Figure 6.2). Additionally, dynamic changes in MCP1-CCR2 signaling in the bone

marrow during inflammation can also be visualized in real time. This new technology provides a novel cell biological insight into the mechanisms by which such a dynamic process occurs.

Overall Conclusions

Recent research has made it clear that inflammatory processes are critical for the development of chronic pain states and for the changes in behavior of pain neurons that accompany chronic pain syndromes. The development of such behavior may involve reciprocal signaling interactions between different cellular elements in the central and peripheral nervous systems. As discussed here, chemokines are one family of molecules that play a key role in coordinating injury-associated nociceptive events, because they play a role in regulating inflammatory responses and simultaneously act on elements of the nervous system. Importantly, chemokines in DRG neurons seem to act as upregulatable neurotransmitters which can produce excitatory effects in the DRG through a variety of mechanisms. The ability of small molecule antagonists to CCR2 receptors to ameliorate ongoing pain hypersensitivity in animal models clearly indicates the importance of chemokine signaling in this behavior. Therefore, it can be concluded that targeting peripheral MCP1-CCR2 signaling may provide a novel form of therapeutic intervention into states of chronic pain.

REFERENCES

Abbadie, C., J. A. Lindia, A. M. Cumiskey, L. B. Peterson, J. S. Mudgett, E. K. Bayne, J. A. DeMartino, D. E. MacIntyre, and M. J. Forrest. 2003. Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. *Proc Natl Acad Sci U S A* 100, no. 13 (Jun 24): 7947-52.

Adler, M. W., and T. J. Rogers. 2005. Are chemokines the third major system in the brain? *J Leukoc Biol* 78, no. 6 (Dec): 1204-9.

Bagri, A., T. Gurney, X. He, Y. R. Zou, D. R. Littman, M. Tessier-Lavigne, and S. J. Pleasure. 2002. The chemokine SDF1 regulates migration of dentate granule cells. *Development* 129, no. 18 (Sep): 4249-60.

Bailey, S. L., B. Schreiner, E. J. McMahon, and S. D. Miller. 2007. CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4+ T(H)-17 cells in relapsing EAE. *Nat Immunol* 8, no. 2 (Feb): 172-80.

Banisadr, G., P. Fontanges, F. Haour, P. Kitabgi, W. Rostene, and S. Melik Parsadaniantz. 2002. Neuroanatomical distribution of CXCR4 in adult rat brain and its localization in cholinergic and dopaminergic neurons. *Eur J Neurosci* 16, no. 9 (Nov): 1661-71.

Banisadr, G., F. Queraud-Lesaux, M. C. Boutterin, D. Pelaprat, B. Zalc, W. Rostene, F. Haour, and S. Melik Parsadaniantz. 2002. Distribution, cellular localization and functional role of CCR2 chemokine receptors in adult rat brain. *Journal of Neurochemistry* 81, no. 2: 257-69.

Banisadr, G., D. Skrzydelski, P. Kitabgi, W. Rostene, and S. M. Parsadaniantz. 2003. Highly regionalized distribution of stromal cell-derived factor-1/CXCL12 in adult rat brain: constitutive expression in cholinergic, dopaminergic and vasopressinergic neurons. *Eur J Neurosci* 18, no. 6 (Sep): 1593-606.

Basbaum, A. I., and C. J. Woolf. 1999. Pain. *Curr Biol* 9, no. 12 (Jun 17): R429-31.

Bautista, D. M., S. E. Jordt, T. Nikai, P. R. Tsuruda, A. J. Read, J. Poblete, E. N. Yamoah, A. I. Basbaum, and D. Julius. 2006. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* 124, no. 6 (Mar 24): 1269-82.

Belmadani, A., P. B. Tran, D. Ren, S. Assimacopoulos, E. A. Grove, and R. J. Miller. 2005. The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors. *J Neurosci* 25, no. 16 (Apr 20): 3995-4003.

Belmadani, A., P. B. Tran, D. Ren, and R. J. Miller. 2006. Chemokines regulate the migration of neural progenitors to sites of neuroinflammation. *J Neurosci* 26, no. 12 (Mar 22): 3182-91.

Berger, O., G. Li, S. M. Han, M. Paredes, and S. J. Pleasure. 2007. Expression of SDF-1 and CXCR4 during reorganization of the postnatal dentate gyrus. *Dev Neurosci* 29, no. 1-2: 48-58.

Bhangoo, S. K., D. Ren, R. J. Miller, D. M. Chan, M. S. Ripsch, C. Weiss, C. McGinnis, and F. A. White. 2007. CXCR4 chemokine receptor signaling mediates pain hypersensitivity in association with antiretroviral toxic neuropathy. *Brain Behav Immun* 21, no. 5 (Feb 8): 581-91.

Bhangoo, S., D. Ren, R. J. Miller, K. J. Henry, J. Lineswala, C. Hamdouchi, B. Li, P. E. Monahan, D. M. Chan, M. S. Ripsch, and F. A. White. 2007. Delayed functional expression of neuronal chemokine receptors following focal nerve demyelination in the rat: a mechanism for the development of chronic sensitization of peripheral nociceptors. *Mol Pain* 3, no. 1 (Dec 12): 38.

Bhattacharyya, B. J., G. Banisadr, H. Jung, D. Ren, D. G. Cronshaw, Y. Zou, and R. J. Miller. 2008. The chemokine stromal cell-derived factor-1 regulates GABAergic inputs to neural progenitors in the postnatal dentate gyrus. *J Neurosci* 28, no. 26 (Jun 25): 6720-30.

Bhave, G., H. J. Hu, K. S. Glauner, W. Zhu, H. Wang, D. J. Brasier, G. S. Oxford, and R. W. th Gereau. 2003. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc Natl Acad Sci U S A* 100, no. 21 (Oct 14): 12480-5.

Bouhassira, D., M. Lanteri-Minet, N. Attal, B. Laurent, and C. Touboul. 2008. Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain* 136, no. 3 (Jun): 380-7.

Boukhris, S., L. Magy, M. Khalil, P. Sindou, and J. M. Vallat. 2007. Pain as the presenting symptom of chronic inflammatory demyelinating polyradiculoneuropathy (CIDP). *J Neurol Sci* 254, no. 1-2 (Mar 15): 33-8.

Brenneman, D. E., G. L. Westbrook, S. P. Fitzgerald, D. L. Ennist, K. L. Elkins, M. R. Ruff, and C. B. Pert. 1988. Neuronal cell killing by the envelope protein of HIV and its prevention by vasoactive intestinal peptide. *Nature* 335, no. 6191 (Oct 13): 639-42.

Bruck, W. 2005. The pathology of multiple sclerosis is the result of focal inflammatory demyelination with axonal damage. *J Neurol* 252 Suppl 5 (Nov): v3-9.

Burke, N. V., W. Han, D. Li, K. Takimoto, S. C. Watkins, and E. S. Levitan. 1997. Neuronal peptide release is limited by secretory granule mobility. *Neuron* 19, no. 5 (Nov): 1095-102.

Campbell, J. N., S. N. Raja, R. A. Meyer, and S. E. Mackinnon. 1988. Myelinated afferents signal the hyperalgesia associated with nerve injury. *Pain* 32, no. 1 (Jan): 89-94.

Carter, G. T., M. P. Jensen, B. S. Galer, G. H. Kraft, L. D. Crabtree, R. M. Beardsley, R. T. Abresch, and T. D. Bird. 1998. Neuropathic pain in Charcot-Marie-Tooth disease. *Arch Phys Med Rehabil* 79, no. 12 (Dec): 1560-4.

Charo, I. F., and R. M. Ransohoff. 2006. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* 354, no. 6 (Feb 9): 610-21.

Chuang, H. H., E. D. Prescott, H. Kong, S. Shields, S. E. Jordt, A. I. Basbaum, M. V. Chao, and D. Julius. 2001. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature* 411, no. 6840 (Jun 21): 957-62.

Chuang, Huai-hu, Elizabeth D Prescott, Haeyoung Kong, Shannon Shields, Sven-Eric Jordt, Allan I Basbaum, Moses V Chao, and David Julius. 2001. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature* 411, no. 6840 (2001/06/21/print): 957-62.

Clipstone, N. A., D. F. Fiorentino, and G. R. Crabtree. 1994. Molecular analysis of the interaction of calcineurin with drug-immunophilin complexes. *J Biol Chem* 269, no. 42 (Oct 21): 26431-7.

Coughlan, C. M., C. M. McManus, M. Sharron, Z. -Y. Gao, D. Murphy, S. Jaffer, W. Choe, W. Chen, J. Hesselgesser, and H. Gaylord. 2000. Expression of multiple functional chemokine

receptors and monocyte chemoattractant protein-1 in human neurons. *Neuroscience* 97, no. 3 (2000/5): 591-600.

Creagh, E. M., and L. A. O'Neill. 2006. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol* 27, no. 8 (Aug): 352-7.

de Jong, E. K., I. M. Dijkstra, M. Hensens, N. Brouwer, M. van Amerongen, R. S. Liem, H. W. Boddeke, and K. Biber. 2005. Vesicle-mediated transport and release of CCL21 in endangered neurons: a possible explanation for microglia activation remote from a primary lesion. *J Neurosci* 25, no. 33 (Aug 17): 7548-57.

Empl, M., S. Renaud, B. Erne, P. Fuhr, A. Straube, N. Schaeren-Wiemers, and A. J. Steck. 2001. TNF-alpha expression in painful and nonpainful neuropathies. *Neurology* 56, no. 10 (May 22): 1371-7.

Fan, S. F., K. F. Shen, M. A. Scheideler, and S. M. Crain. 1992. F11 neuroblastoma x DRG neuron hybrid cells express inhibitory mu- and delta-opioid receptors which increase voltage-dependent K+ currents upon activation. *Brain Res* 590, no. 1-2 (Sep 11): 329-33.

Fife, B. T., G. B. Huffnagle, W. A. Kuziel, and W. J. Karpus. 2000. CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. *J Exp Med* 192, no. 6 (Sep 18): 899-905.

Francel, P. C., K. Harris, M. Smith, M. C. Fishman, G. Dawson, and R. J. Miller. 1987. Neurochemical characteristics of a novel dorsal root ganglion X neuroblastoma hybrid cell line, F-11. *J Neurochem* 48, no. 5 (May): 1624-31.

Fryer, A. D., L. H. Stein, Z. Nie, D. E. Curtis, C. M. Evans, S. T. Hodgson, P. J. Jose, K. E. Belmonte, E. Fitch, and D. B. Jacoby. 2006. Neuronal eotaxin and the effects of CCR3 antagonist on airway hyperreactivity and M2 receptor dysfunction. *J Clin Invest* 116, no. 1 (Jan): 228-36.

Ge, S., E. L. Goh, K. A. Sailor, Y. Kitabatake, G. L. Ming, and H. Song. 2006. GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature* 439, no. 7076 (Feb 2): 589-93.

Gillard, S. E., M. Lu, R. M. Mastracci, and R. J. Miller. 2002. Expression of functional chemokine receptors by rat cerebellar neurons. *J Neuroimmunol* 124, no. 1-2 (Mar): 16-28.

Giovannelli, A., C. Limatola, D. Ragozzino, A. M. Mileo, A. Ruggieri, M. T. Ciotti, D. Mercanti, A. Santoni, and F. Eusebi. 1998. CXC chemokines interleukin-8 (IL-8) and growth-related gene product alpha (GROalpha) modulate Purkinje neuron activity in mouse cerebellum. *J Neuroimmunol* 92, no. 1-2 (Dec 1): 122-32.

Gong, S., C. Zheng, M. L. Doughty, K. Losos, N. Didkovsky, U. B. Schambra, N. J. Nowak, A. Joyner, G. Leblanc, M. E. Hatten, and N. Heintz. 2003. A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* 425, no. 6961 (Oct 30): 917-25.

Gosselin, R. D., C. Varela, G. Banisadr, P. Mechighel, W. Rostene, P. Kitabgi, and S. Melik-Parsadaniantz. 2005. Constitutive expression of CCR2 chemokine receptor and inhibition by MCP-1/CCL2 of GABA-induced currents in spinal cord neurones. *J Neurochem* 95, no. 4 (Nov): 1023-34.

Graef, I. A., P. G. Mermelstein, K. Stankunas, J. R. Neilson, K. Deisseroth, R. W. Tsien, and G. R. Crabtree. 1999. L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. *Nature* 401, no. 6754 (Oct 14): 703-8.

Groth, R. D., L. G. Coicou, P. G. Mermelstein, and V. S. Seybold. 2007. Neurotrophin activation of NFAT-dependent transcription contributes to the regulation of pro-nociceptive genes. *J Neurochem* (May 4).

He, T. C., S. Zhou, L. T. da Costa, J. Yu, K. W. Kinzler, and B. Vogelstein. 1998. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* 95, no. 5 (Mar 3): 2509-14.

Heintz, N. 2004. Gene expression nervous system atlas (GENSAT). *Nat Neurosci* 7, no. 5 (May): 483.

Hempenstall, K., T. J. Nurmikko, R. W. Johnson, R. P. A'Hern, and A. S. Rice. 2005. Analgesic therapy in postherpetic neuralgia: a quantitative systematic review. *PLoS Med* 2, no. 7 (Jul): e164.

Hernandez-Ochoa, E. O., M. Contreras, Z. Cseresnyes, and M. F. Schneider. 2007. Ca(2+) signal summation and NFATc1 nuclear translocation in sympathetic ganglion neurons during repetitive action potentials. *Cell Calcium* 41, no. 6 (Jun): 559-71.

Hesselgesser, J., M. Halks-Miller, V. DelVecchio, S. C. Peiper, J. Hoxie, D. L. Kolson, D. Taub, and R. Horuk. 1997. CD4-independent association between HIV-1 gp120 and CXCR4: functional chemokine receptors are expressed in human neurons. *Curr Biol* 7, no. 2 (Feb 1): 112-21.

Horuk, R, AW Martin, Z Wang, L Schweitzer, A Gerassimides, H Guo, Z Lu, J Hesselgesser, HD Perez, J Kim, J Parker, TJ Hadley, and SC Peiper. 1997. Expression of chemokine receptors by subsets of neurons in the central nervous system. *J Immunol* 158, no. 6 (March 15, 1997): 2882-90.

Huang, J., X. Zhang, and P. A. McNaughton. 2006. Modulation of temperature-sensitive TRP channels. *Semin Cell Dev Biol* 17, no. 6 (Dec): 638-45.

Huang, L. Y., and E. Neher. 1996. Ca(2+)-dependent exocytosis in the somata of dorsal root ganglion neurons. *Neuron* 17, no. 1 (Jul): 135-45.

Huising, M. O., R. J. Stet, C. P. Kruiswijk, H. F. Savelkoul, and B. M. Lidy Verburg-van Kemenade. 2003. Molecular evolution of CXC chemokines: extant CXC chemokines originate from the CNS. *Trends Immunol* 24, no. 6 (Jun): 307-13.

Jackson, J. G., Y. M. Usachev, and S. A. Thayer. 2007. Bradykinin-Induced NFAT-Dependent Transcription in Rat Dorsal Root Ganglion Neurons. *Mol Pharmacol* (May 7).

Jahnel, R., O. Bender, L. M. Munter, M. Dreger, C. Gillen, and F. Hucho. 2003. Dual expression of mouse and rat VRL-1 in the dorsal root ganglion derived cell line F-11 and biochemical analysis of VRL-1 after heterologous expression. *Eur J Biochem* 270, no. 21 (Nov): 4264-71.

Ji, R. R., and G. Strichartz. 2004. Cell signaling and the genesis of neuropathic pain. *Sci STKE* 2004, no. 252 (Sep 28): reE14.

Joseph, N. M., Y. S. Mukouyama, J. T. Mosher, M. Jaegle, S. A. Crone, E. L. Dormand, K. F. Lee, D. Meijer, D. J. Anderson, and S. J. Morrison. 2004. Neural crest stem cells undergo

multilineage differentiation in developing peripheral nerves to generate endoneurial fibroblasts in addition to Schwann cells. *Development* 131, no. 22 (Nov): 5599-612.

Jung, H., and R. J. Miller. 2008. Activation of the nuclear factor of activated T-cells (NFAT) mediates upregulation of CCR2 chemokine receptors in dorsal root ganglion (DRG) neurons: a possible mechanism for activity-dependent transcription in DRG neurons in association with neuropathic pain. *Mol Cell Neurosci* 37, no. 1 (Jan): 170-7.

Jung, H., P. T. Toth, F. A. White, and R. J. Miller. 2008. Monocyte chemoattractant protein-1 functions as a neuromodulator in dorsal root ganglia neurons. *J Neurochem* 104, no. 1 (Jan): 254-63.

Jung, J., S.K. Bhangoo, M.P. Fitzgerald, R. J. Miller, and F. A. White. 2007. Expression of functional chemokine receptors in bladder-associated sensory neurons following focal demyelination of sciatic nerve. *2007 Neuroscience Meeting Planner.* . San Diego, CA

Kallal, L., and J. L. Benovic. 2000. Using green fluorescent proteins to study G-protein-coupled receptor localization and trafficking. *Trends Pharmacol Sci* 21, no. 5 (May): 175-80.

Karpus, W. J., and R. M. Ransohoff. 1998. Chemokine regulation of experimental autoimmune encephalomyelitis: temporal and spatial expression patterns govern disease pathogenesis. *J Immunol* 161, no. 6 (Sep 15): 2667-71.

Khasabova, I. A., C. L. Stucky, C. Harding-Rose, L. Eikmeier, A. J. Beitz, L. G. Coicou, A. E. Hanson, D. A. Simone, and V. S. Seybold. 2007. Chemical interactions between fibrosarcoma cancer cells and sensory neurons contribute to cancer pain. *J Neurosci* 27, no. 38 (Sep 19): 10289-98.

Kim, D., M. A. Kim, I. H. Cho, M. S. Kim, S. Lee, E. K. Jo, S. Y. Choi, K. Park, J. S. Kim, S. Akira, H. S. Na, S. B. Oh, and S. J. Lee. 2007. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. *J Biol Chem* 282, no. 20 (May 18): 14975-83.

Kleinschnitz, C., J. Brinkhoff, C. Sommer, and G. Stoll. 2005. Contralateral cytokine gene induction after peripheral nerve lesions: Dependence on the mode of injury and NMDA receptor signaling. *Brain Res Mol Brain Res* 136, no. 1-2 (May 20): 23-8.

Knaut, H., P. Blader, U. Strahle, and A. F. Schier. 2005. Assembly of trigeminal sensory ganglia by chemokine signaling. *Neuron* 47, no. 5 (Sep 1): 653-66.

Knopp, KL, and GS Oxford. 2006. Is enhanced intraganglion excitability a paracrine effect of CGRP release from DRG somata? *Neuroscience Meeting Planner. Atlanta, GA: Society for Neuroscience, 2006. Online.* 441.16.

Kolodziej, A., S. Schulz, A. Guyon, D. F. Wu, M. Pfeiffer, V. Odemis, V. Hollt, and R. Stumm. 2008. Tonic activation of CXC chemokine receptor 4 in immature granule cells supports neurogenesis in the adult dentate gyrus. *J Neurosci* 28, no. 17 (Apr 23): 4488-500.

Kuang, Y., Y. Wu, H. Jiang, and D. Wu. 1996. Selective G protein coupling by C-C chemokine receptors. *J Biol Chem* 271, no. 8 (Feb 23): 3975-8.

Kurihara, T., and R. Bravo. 1996. Cloning and functional expression of mCCR2, a murine receptor for the C-C chemokines JE and FIC. *J Biol Chem* 271, no. 20 (May 17): 11603-7.

Kwan, K. Y., A. J. Allchorne, M. A. Vollrath, A. P. Christensen, D. S. Zhang, C. J. Woolf, and D. P. Corey. 2006. TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron* 50, no. 2 (Apr 20): 277-89.

Lee, E. C., D. Yu, J. Martinez de Velasco, L. Tessarollo, D. A. Swing, D. L. Court, N. A. Jenkins, and N. G. Copeland. 2001. A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* 73, no. 1 (Apr 1): 56-65.

Levitan, E. S. 2004. Using GFP to image peptide hormone and neuropeptide release in vitro and in vivo. *Methods* 33, no. 4 (Aug): 281-6.

Li, Yanzhang, Ailing Ji, Eberhard Weihe, and Martin K.-H. Schafer. 2004. Cell-Specific Expression and Lipopolysaccharide-Induced Regulation of Tumor Necrosis Factor {alpha} (TNF{alpha}) and TNF Receptors in Rat Dorsal Root Ganglion. *J. Neurosci.* 24, no. 43 (October 27, 2004): 9623-31.

Lindia, J. A., E. McGowan, N. Jochnowitz, and C. Abbadie. 2005. Induction of CX3CL1 Expression in Astrocytes and CX3CR1 in Microglia in the Spinal Cord of a Rat Model of Neuropathic Pain. *J Pain* 6, no. 7 (Jul): 434-8.

Lou, H., S. K. Kim, E. Zaitsev, C. R. Snell, B. Lu, and Y. P. Loh. 2005. Sorting and activity-dependent secretion of BDNF require interaction of a specific motif with the sorting receptor carboxypeptidase e. *Neuron* 45, no. 2 (Jan 20): 245-55.

Lu, M., E. A. Grove, and R. J. Miller. 2002. Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. *Proc Natl Acad Sci U S A* 99, no. 10 (May 14): 7090-5.

Lund, S., K. V. Christensen, M. Hedtjarn, A. L. Mortensen, H. Hagberg, J. Falsig, H. Hasseldam, A. Schrattenholz, P. Porzgen, and M. Leist. 2006. The dynamics of the LPS triggered inflammatory response of murine microglia under different culture and in vivo conditions. *J Neuroimmunol* 180, no. 1-2 (Nov): 71-87.

Ma, Q., D. Jones, P. R. Borghesani, R. A. Segal, T. Nagasawa, T. Kishimoto, R. T. Bronson, and T. A. Springer. 1998. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci U S A* 95, no. 16 (Aug 4): 9448-53.

Mahad, D J, S J L Howell, and M N Woodroofe. 2002. Expression of chemokines in the CSF and correlation with clinical disease activity in patients with multiple sclerosis. *J Neurol Neurosurg Psychiatry* 72, no. 4 (April 1, 2002): 498-502.

Mahad, D., M. K. Callahan, K. A. Williams, E. E. Ubogu, P. Kivisakk, B. Tucky, G. Kidd, G. A. Kingsbury, A. Chang, R. J. Fox, M. Mack, M. B. Sniderman, R. Ravid, S. M. Staugaitis, M. F. Stins, and R. M. Ransohoff. 2006. Modulating CCR2 and CCL2 at the blood-brain barrier: relevance for multiple sclerosis pathogenesis. *Brain* 129, no. Pt 1 (Jan): 212-23.

Majumder, S., L. Z. Zhou, and R. M. Ransohoff. 1996. Transcriptional regulation of chemokine gene expression in astrocytes. *J Neurosci Res* 45, no. 6 (Sep 15): 758-69.

Meucci, O., A. Fatatis, A. A. Simen, T. J. Bushell, P. W. Gray, and R. J. Miller. 1998. Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. *Proc Natl Acad Sci U S A* 95, no. 24 (Nov 24): 14500-5.

Miao, Z., K. E. Luker, B. C. Summers, R. Berahovich, M. S. Bhojani, A. Rehemtulla, C. G. Kleer, J. J. Essner, A. Nasevicius, G. D. Luker, M. C. Howard, and T. J. Schall. 2007. CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. *Proc Natl Acad Sci U S A* 104, no. 40 (Oct 2): 15735-40.

Milligan, E. D., V. Zapata, M. Chacur, D. Schoeniger, J. Biedenkapp, K. A. O'Connor, G. M. Verge, G. Chapman, P. Green, A. C. Foster, G. S. Naeve, S. F. Maier, and L. R. Watkins. 2004. Evidence that exogenous and endogenous fractalkine can induce spinal nociceptive facilitation in rats. *Eur J Neurosci* 20, no. 9 (Nov): 2294-302.

Mogil, J. S., L. Yu, and A. I. Basbaum. 2000. Pain genes?: natural variation and transgenic mutants. *Annu Rev Neurosci* 23: 777-811.

Moulin, D. E. 1998. Pain in central and peripheral demyelinating disorders. *Neurol Clin* 16, no. 4 (Nov): 889-98.

Nagasawa, T., S. Hirota, K. Tachibana, N. Takakura, S. Nishikawa, Y. Kitamura, N. Yoshida, H. Kikutani, and T. Kishimoto. 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 382, no. 6592 (Aug 15): 635-8.

Oh, S. B., P. B. Tran, S. E. Gillard, R. W. Hurley, D. L. Hammond, and R. J. Miller. 2001. Chemokines and glycoprotein120 produce pain hypersensitivity by directly exciting primary nociceptive neurons. *J Neurosci* 21, no. 14 (Jul 15): 5027-35.

Ohtani, Y., M. Minami, N. Kawaguchi, A. Nishiyori, J. Yamamoto, S. Takami, and M. Satoh. 1998. Expression of stromal cell-derived factor-1 and CXCR4 chemokine receptor mRNAs in cultured rat glial and neuronal cells. *Neurosci Lett* 249, no. 2-3 (Jun 19): 163-6.

Omana-Zapata, I., M. A. Khabbaz, J. C. Hunter, and K. R. Bley. 1997. QX-314 inhibits ectopic nerve activity associated with neuropathic pain. *Brain Res* 771, no. 2 (Oct 17): 228-37.

Ouyang, K., H. Zheng, X. Qin, C. Zhang, D. Yang, X. Wang, C. Wu, Z. Zhou, and H. Cheng. 2005. Ca2+ sparks and secretion in dorsal root ganglion neurons. *Proc Natl Acad Sci U S A* 102, no. 34 (Aug 23): 12259-64.

Oynebraten, I., O. Bakke, P. Brandtzaeg, F. E. Johansen, and G. Haraldsen. 2004. Rapid chemokine secretion from endothelial cells originates from 2 distinct compartments. *Blood* 104, no. 2 (Jul 15): 314-20.

Oynebraten, I., N. Barois, K. Hagelsteen, F. E. Johansen, O. Bakke, and G. Haraldsen. 2005. Characterization of a novel chemokine-containing storage granule in endothelial cells: evidence for preferential exocytosis mediated by protein kinase A and diacylglycerol. *J Immunol* 175, no. 8 (Oct 15): 5358-69.

Ping, D., P. L. Jones, and J. M. Boss. 1996. TNF regulates the in vivo occupancy of both distal and proximal regulatory regions of the MCP-1/JE gene. *Immunity* 4, no. 5 (May): 455-69.

Qin, X., Y. Wan, and X. Wang. 2005. CCL2 and CXCL1 trigger calcitonin gene-related peptide release by exciting primary nociceptive neurons. *Journal of Neuroscience Research* dx.doi.org/10.1002/jnr.20612: NA.

Scholz, J., and C. J. Woolf. 2007. The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci* 10, no. 11 (Nov): 1361-8.

Schonemeier, B., A. Kolodziej, S. Schulz, S. Jacobs, V. Hoellt, and R. Stumm. 2008. Regional and cellular localization of the CXCl12/SDF-1 chemokine receptor CXCR7 in the developing and adult rat brain. *J Comp Neurol* 510, no. 2 (Sep 10): 207-20.

Schreiber, R. C., K. Krivacic, B. Kirby, S. A. Vaccariello, T. Wei, R. M. Ransohoff, and R. E. Zigmond. 2001. Monocyte chemoattractant protein (MCP)-1 is rapidly expressed by sympathetic ganglion neurons following axonal injury. *Neuroreport* 12, no. 3 (Mar 5): 601-6.

Serbina, N. V., T. Jia, T. M. Hohl, and E. G. Pamer. 2008. Monocyte-mediated defense against microbial pathogens. *Annu Rev Immunol* 26: 421-52.

Serbina, N. V., and E. G. Pamer. 2006. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol* 7, no. 3 (Mar): 311-7.

Shakiryanova, D., A. Tully, and E. S. Levitan. 2006. Activity-dependent synaptic capture of transiting peptidergic vesicles. *Nat Neurosci* 9, no. 7 (Jul): 896-900.

Sierro, F., C. Biben, L. Martinez-Munoz, M. Mellado, R. M. Ransohoff, M. Li, B. Woehl, H. Leung, J. Groom, M. Batten, R. P. Harvey, A. C. Martinez, C. R. Mackay, and F. Mackay. 2007. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc Natl Acad Sci U S A* 104, no. 37 (Sep 11): 14759-64.

Stumm, R. K., J. Rummel, V. Junker, C. Culmsee, M. Pfeiffer, J. Krieglstein, V. Hollt, and S. Schulz. 2002. A dual role for the SDF-1/CXCR4 chemokine receptor system in adult brain: isoform-selective regulation of SDF-1 expression modulates CXCR4-dependent neuronal plasticity and cerebral leukocyte recruitment after focal ischemia. *J Neurosci* 22, no. 14 (Jul 15): 5865-78.

Subang, M. C., and P. M. Richardson. 2001. Influence of injury and cytokines on synthesis of monocyte chemoattractant protein-1 mRNA in peripheral nervous tissue. *Eur J Neurosci* 13, no. 3 (Feb): 521-8.

Sun, J. H., B. Yang, D. F. Donnelly, C. Ma, and R. H. LaMotte. 2006. MCP-1 enhances excitability of nociceptive neurons in chronically compressed dorsal root ganglia. *J Neurophysiol* 96, no. 5 (Nov): 2189-99.

Tachibana, K., S. Hirota, H. Iizasa, H. Yoshida, K. Kawabata, Y. Kataoka, Y. Kitamura, K. Matsushima, N. Yoshida, S. Nishikawa, T. Kishimoto, and T. Nagasawa. 1998. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* 393, no. 6685 (Jun 11): 591-4.

Tanaka, T., M. Minami, T. Nakagawa, and M. Satoh. 2004. Enhanced production of monocyte chemoattractant protein-1 in the dorsal root ganglia in a rat model of neuropathic pain: possible involvement in the development of neuropathic pain. *Neurosci Res* 48, no. 4 (Apr): 463-9.

Tanga, F. Y., N. Nutile-McMenemy, and J. A. DeLeo. 2005. The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A* 102, no. 16 (Apr 19): 5856-61.

Taskinen, H. S., and M. Roytta. 2000. Increased expression of chemokines (MCP-1, MIP-1alpha, RANTES) after peripheral nerve transection. *J Peripher Nerv Syst* 5, no. 2 (Jun): 75-81.

Tawfik, V. L., N. Nutile-McMenemy, M. L. Lacroix-Fralish, and J. A. Deleo. 2007. Efficacy of propentofylline, a glial modulating agent, on existing mechanical allodynia following peripheral nerve injury. *Brain Behav Immun* 21, no. 2 (Feb): 238-46.

Thacker, M. A., A. K. Clark, T. Bishop, J. Grist, P. K. Yip, L. D. Moon, S. W. Thompson, F. Marchand, and S. B. McMahon. 2008. CCL2 is a key mediator of microglia activation in neuropathic pain states. *Eur J Pain* (Jun 11).

Tominaga, M., and M. J. Caterina. 2004. Thermosensation and pain. *J Neurobiol* 61, no. 1 (Oct): 3-12.

Torebjork, H. E., L. E. Lundberg, and R. H. LaMotte. 1992. Central changes in processing of mechanoreceptive input in capsaicin- induced secondary hyperalgesia in humans. *J Physiol* 448: 765-80.

Tozuka, Y., S. Fukuda, T. Namba, T. Seki, and T. Hisatsune. 2005. GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. *Neuron* 47, no. 6 (Sep 15): 803-15.

Tran, P. B., G. Banisadr, D. Ren, A. Chenn, and R. J. Miller. 2007. Chemokine receptor expression by neural progenitor cells in neurogenic regions of mouse brain. *J Comp Neurol* 500, no. 6 (Feb 20): 1007-33.

Tran, P. B., and R. J. Miller. 2003. Chemokine receptors: signposts to brain development and disease. *Nat Rev Neurosci* 4, no. 6 (Jun): 444-55.

Valentin, G., P. Haas, and D. Gilmour. 2007. The chemokine SDF1a coordinates tissue migration through the spatially restricted activation of Cxcr7 and Cxcr4b. *Curr Biol* 17, no. 12 (Jun 19): 1026-31.

van der Meer, Peter, Anthony M. Ulrich, Francisco Gonzalez-Scarano, and Ehud Lavi. 2000. Immunohistochemical Analysis of CCR2, CCR3, CCR5, and CXCR4 in the Human Brain: Potential Mechanisms for HIV Dementia. *Experimental and Molecular Pathology* 69, no. 3 (2000/12): 192-201.

Verge, G. M., E. D. Milligan, S. F. Maier, L. R. Watkins, G. S. Naeve, and A. C. Foster. 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, no. 5 (Sep): 1150-60.

Vit, J. P., P. T. Ohara, D. A. Tien, J. R. Fike, L. Eikmeier, A. Beitz, G. L. Wilcox, and L. Jasmin. 2006. The analgesic effect of low dose focal irradiation in a mouse model of bone cancer is associated with spinal changes in neuro-mediators of nociception. *Pain* 120, no. 1-2 (Jan): 188-201.

Wallace, V. C., D. F. Cottrell, P. J. Brophy, and S. M. Fleetwood-Walker. 2003. Focal lysolecithin-induced demyelination of peripheral afferents results in neuropathic pain behavior that is attenuated by cannabinoids. *J Neurosci* 23, no. 8 (Apr 15): 3221-33.

Watkins, L. R., M. R. Hutchinson, A. Ledeboer, J. Wieseler-Frank, E. D. Milligan, and S. F. Maier. 2007. Glia as the "bad guys": Implications for improving clinical pain control and the clinical utility of opioids. *Brain Behav Immun* 21, no. 2 (Feb): 131-46.

Watson, C. P. 2000. The treatment of neuropathic pain: antidepressants and opioids. *Clin J Pain* 16, no. 2 Suppl (Jun): S49-55.

White, F. A., S. K. Bhangoo, and R. J. Miller. 2005. Chemokines: integrators of pain and inflammation. *Nat Rev Drug Discov* 4, no. 10 (Oct): 834-44.

White, F. A., H. Jung, and R. J. Miller. 2007. Chemokines and the pathophysiology of neuropathic pain. *Proc Natl Acad Sci U S A* 104, no. 51 (Dec 18): 20151-8.

White, F. A., J. Sun, S. M. Waters, C. Ma, D. Ren, M. Ripsch, J. Steflik, D. N. Cortright, R. H. Lamotte, and R. J. Miller. 2005. Excitatory monocyte chemoattractant protein-1 signaling is up-regulated in sensory neurons after chronic compression of the dorsal root ganglion. *Proc Natl Acad Sci U S A* 102, no. 39 (Sep 27): 14092-7.

Woolf, C. J. 1983. Evidence for a central component of post-injury pain hypersensitivity. *Nature* 306, no. 5944: 686-8.

Xia, M., S. Qin, M. McNamara, C. Mackay, and B. T. Hyman. 1997. Interleukin-8 receptor B immunoreactivity in brain and neuritic plaques of Alzheimer's disease. *Am J Pathol* 150, no. 4 (Apr): 1267-74.

Xie, W. R., H. Deng, H. Li, T. L. Bowen, J. A. Strong, and J. M. Zhang. 2006. Robust increase of cutaneous sensitivity, cytokine production and sympathetic sprouting in rats with localized inflammatory irritation of the spinal ganglia. *Neuroscience* 142(3):809-822, no. 3: 809-22.

Yamamoto, K., H. Takeshima, K. Hamada, M. Nakao, T. Kino, T. Nishi, M. Kochi, J. Kuratsu, T. Yoshimura, and Y. Ushio. 1999. Cloning and functional characterization of the 5'-flanking region of the human monocyte chemoattractant protein-1 receptor (CCR2) gene. Essential role of 5'-untranslated region in tissue-specific expression. *J Biol Chem* 274, no. 8 (Feb 19): 4646-54.

Zanzouri, M., I. Lauritzen, F. Duprat, M. Mazzuca, F. Lesage, M. Lazdunski, and A. Patel. 2006. Membrane potential-regulated transcription of the resting K+ conductance TASK-3 via the calcineurin pathway. *J Biol Chem* 281, no. 39 (Sep 29): 28910-8.

Zhang, J., and Y. De Koninck. 2006. Spatial and temporal relationship between monocyte chemoattractant protein-1 expression and spinal glial activation following peripheral nerve injury. *J Neurochem* 97, no. 3 (May): 772-83.

Zhang, J., X. Q. Shi, S. Echeverry, J. S. Mogil, Y. De Koninck, and S. Rivest. 2007. Expression of CCR2 in both resident and bone marrow-derived microglia plays a critical role in neuropathic pain. *J Neurosci* 27, no. 45 (Nov 7): 12396-406.

Zhang, X., J. Huang, and P. A. McNaughton. 2005. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *Embo J* 24, no. 24 (Dec 21): 4211-23.

Zimmermann, M. 2001. Pathobiology of neuropathic pain. *Eur J Pharmacol* 429, no. 1-3 (Oct 19): 23-37.

Zou, Y. R., A. H. Kottmann, M. Kuroda, I. Taniuchi, and D. R. Littman. 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 393, no. 6685 (Jun 11): 595-9.