

CCR2 chemokine receptor signaling mediates pain in experimental osteoarthritis

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Osteoarthritis is one of the leading causes of chronic pain, but almost nothing is known about the mechanisms and molecules that mediate osteoarthritis-associated joint pain. Consequently, treatment options remain inadequate and joint replacement is often inevitable. Here, we use a surgical mouse model that captures the long-term progression of knee osteoarthritis to longitudinally assess pain-related behaviors and concomitant changes in the innervating dorsal root ganglia (DRG). We demonstrate that monocyte chemoattractant protein (MCP)-1 (CCL2) and its high-affinity receptor, chemokine (C-C motif) receptor 2 (CCR2), are central to the development of pain associated with knee osteoarthritis. After destabilization of the medial meniscus, mice developed early-onset secondary mechanical allodynia that was maintained for 16 wk. MCP-1 and CCR2 mRNA, protein, and signaling activity were temporarily up-regulated in the innervating DRG at 8 wk after surgery. This result correlated with the presentation of movement-provoked pain behaviors, which were maintained up to 16 wk. Mice that lack *Ccr2* also developed mechanical allodynia, but this started to resolve from 8 wk onwards. Despite severe allodynia and structural knee joint damage equal to wild-type mice, *Ccr2*-null mice did not develop movement-provoked pain behaviors at 8 wk. In wild-type mice, macrophages infiltrated the DRG by 8 wk and this was maintained through 16 wk after surgery. In contrast, macrophage infiltration was not observed in *Ccr2*-null mice. These observations suggest a key role for the MCP-1/CCR2 pathway in establishing osteoarthritis pain.

Osteoarthritis is the most common joint disorder and is one of the leading causes of chronic pain (1, 2). Osteoarthritis commonly affects knees, hips, and hands and is characterized by radiographic changes (primarily joint space narrowing, subchondral bone sclerosis, and osteophytes) accompanied by clinical symptoms, most prominently pain. Treatments that alter the progression of the structural damage in the joint are not yet available. Options for treating the pain include nonsteroidal anti-inflammatory drugs, steroids, and viscosupplementation, but analgesia is often inadequate, and uncontrolled pain is the number one reason why people with osteoarthritis undergo joint-replacement surgery (3). Despite the enormous health and economic burden of osteoarthritis and associated pain (4), very few studies have examined the molecular pathways that mediate osteoarthritis pain. As in all types of chronic pain, osteoarthritis pain is the dynamic result of a complex interaction between local tissue damage and inflammation, peripheral and central sensitization, and the brain (5–7). Joint pain associated with osteoarthritis, however, has unique clinical features that provide insight into the mechanisms that cause it. First, joint pain has a strong mechanical component: it is typically triggered by specific activities (for example, climbing stairs elicits knee pain) and is relieved by rest. As structural joint disease advances, pain may also occur in rest (8). Signs of central sensitization (6) have been described, including mechanical allodynia (9, 10) (pain caused by a stimulus that does not normally evoke pain), and reduced pain-pressure thresholds (11).

The development of pain behavior involves fundamental changes in the properties of neurons that signal pain, including alterations in gene transcription and protein expression in the sensory neurons of the dorsal root ganglia (DRG) (12, 13). This has been specifically

shown in nerve-injury models in rodents. In such models, chemokines and their receptors, particularly monocyte chemoattractant protein-1 (MCP-1 or CCL2) and its high-affinity receptor, chemokine (C-C motif) receptor 2 (CCR2), mediate excitatory effects at the level of both the DRG and the spinal cord (12, 14).

The importance of MCP-1/CCR2 signaling remains to be explored in animal models of complex disorders associated with chronic pain, such as osteoarthritis. Therefore, we decided to study the role of MCP-1/CCR2 in osteoarthritis pain using a surgical mouse model, induced by destabilization of the medial meniscus (DMM) (15). In this model, structural damage of the knee joint progresses slowly and in defined stages over 16 wk after surgery (15, 16). The protracted nature of the model uniquely enables longitudinal analysis of pain behaviors and concomitant molecular events in the sensory neurons that innervate the knee (16, 17).

Results

MCP-1/CCR2 Signaling Is Up-Regulated in Innervating DRG Following Induction of Osteoarthritis. We began by examining the levels of MCP-1 and CCR2 expressed in the context of progressive osteoarthritis in the DMM model. Gene-expression levels of MCP-1 and CCR2 were analyzed by quantitative RT-PCR in the ipsilateral innervating DRG, L3–L5, at 4, 8, and 16 wk after surgery. Four weeks postsurgery, no changes in DMM DRG expression levels were observed compared with age-matched naïve DRG (Fig. 1A). Eight weeks postsurgery, both MCP-1 and CCR2 mRNA were greatly up-regulated in DMM DRG compared with age-matched naïve and with sham DRG. Finally, at 16 wk after DMM, MCP-1 and CCR2 expression had returned back to base levels, and they were even somewhat down-regulated compared with age-matched naïve DRG.

In situ hybridization at 8 wk postsurgery on L3–L5 DRG sections showed expression of MCP-1 and CCR2 in DRG neuronal cells of different sizes, and this was strongly up-regulated after DMM (Fig. 1B; higher magnification images in Fig. S1).

To assess protein levels of MCP-1, DRG were harvested at 4 and 8 wk postsurgery, and neurons were acutely isolated and cultured for 4 d. Four weeks postsurgery, supernatants from DMM cultures contained similar MCP-1 protein levels compared with sham and age-matched naïve cultures (Fig. 1C). By 8 wk postsurgery, DMM cultures produced increased amounts of MCP-1 compared with both naïve and sham mice (Fig. 1C). Together with the in situ hybridization results, these data suggest that neurons are a source of the increased levels of MCP-1 observed in the DRG in progressive DMM.

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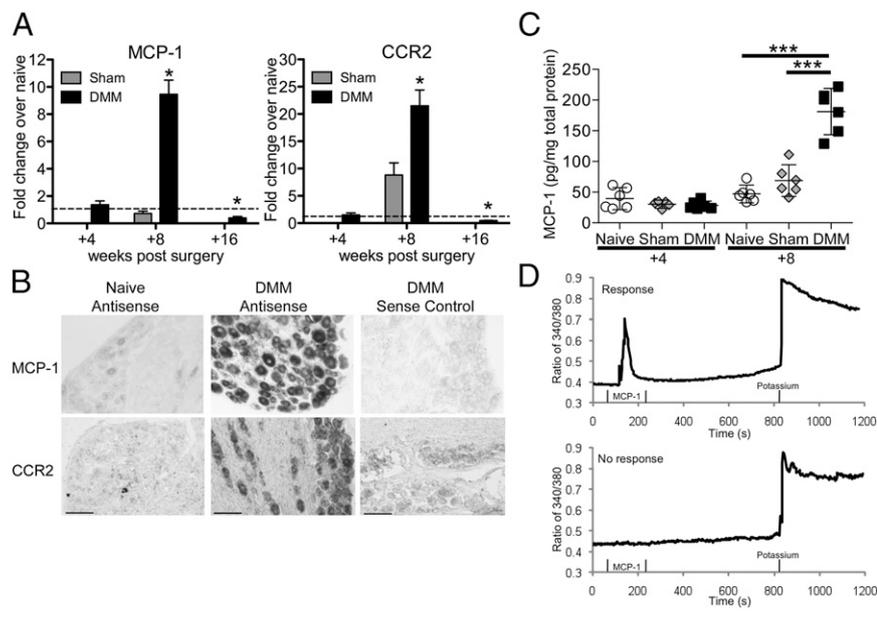


Fig. 1. MCP-1/CCR2 gene and protein expression in DRG is elevated 8 wk after DMM surgery. (A) Real-time RT-PCR of *Mcp-1* and *Ccr2* in sham and DMM wild-type mice normalized to age-matched naive levels: at 4 wk postsurgery, $n = 5$, two-tailed t test, $P > 0.05$; at 8 wk postsurgery, $n = 4$ for naive, $n = 5$ for sham and DMM, one-way ANOVA with Bonferroni posttest, MCP-1: $*P < 0.001$ vs. sham and naive, CCR2: $*P < 0.001$ vs. naive, and $P < 0.01$ vs. sham; at 16 wk postsurgery, $n = 4$ for naive, $n = 7$ for DMM, two-tailed t test $*P = 0.01$ for MCP-1 vs. naive, $*P = 0.003$ for CCR2 vs. naive. Results show mean \pm SEM. (B) Representative images of in situ hybridization using antisense probes for MCP-1 and CCR2 in DRG sections (L3–L5) taken from DMM wild-type mice at 8 wk postsurgery and age-matched naive mice. Sense probe control is shown for the DMM condition. Magnification 10 \times . (Scale bars, 100 μ m.) (C) Protein levels of MCP-1 in the supernatants of cells cultured from age-matched naive, sham, and DMM mice at 4 and 8 wk postsurgery, $n = 6$ wells, representative of two independent experiments, one-way ANOVA with Bonferroni posttest, $***P < 0.001$. Results show mean \pm 95% confidence interval. (D) Representative traces of individual cells during calcium mobilization assay indicating a response to MCP-1. Response to 50 mM potassium solution, used as a positive control, is also shown.

One way that chemokines can produce pain is by directly exciting DRG nociceptive neurons, which is associated with increases in intraneuronal calcium concentrations, $[Ca^{2+}]_i$ (18). We therefore assessed the sensitivity of DRG neurons to stimulation by MCP-1 by measuring the effects of MCP-1 on neuronal $[Ca^{2+}]_i$. Mobilization can be measured on an individual cell basis by using a fluorescent calcium indicator combined with digital video microfluorometry (18) (Fig. 1D). At 4 wk postsurgery, a similar percentage of neurons in cultures from DMM, sham, and age-matched naive mice responded to MCP-1 (Table 1). At 8 wk postsurgery, an increased percentage of DMM neurons displayed enhanced $[Ca^{2+}]_i$ in response to MCP-1 stimulation compared with neurons from age-matched naive and sham mice (Table 1), implying that an increased number of DMM neurons expressed functional CCR2 receptors. The increased effects of MCP-1 were specific, as responses to capsaicin or potassium did not change over the same time period.

Macrophage Infiltration of Innervating DRG. In addition to the cell bodies of sensory neurons, the DRG contain glial cells and may be infiltrated by immune cells, particularly macrophages. All these cell types may contribute to pain signaling (5, 19, 20). MCP-1 can act on all of these cell types (12) and has been shown to promote macrophage infiltration into the spinal cord and DRG following nerve injury (21–23). Therefore, we examined changes in the DRG macrophage population following DMM surgery to assess whether these cells were infiltrating the innervating DRG as a result of the up-regulation of MCP-1. Four weeks postsurgery, few macrophages appeared in DMM and age-matched naive DRG (Fig. S2). By 8 wk postsurgery, macrophages infiltrated the DRG in wild-type DMM only (Fig. 2 and Fig. S2). This increase in macrophages in DMM DRG was maintained through 16 wk postsurgery (Fig. 2). Macrophage infiltration was not noted in *Ccr2*-null DRG at 8 wk after DMM nor in age-matched naive *Ccr2*-null mice (Fig. 2).

Table 1. Number of cells with $[Ca^{2+}]_i$ response to MCP-1

Weeks postsurgery	Naive	Sham	DMM	χ^2 test
+4	190/631 (30.1%)	99/258 (38.4%)	95/306 (31.0%)	$P = 0.051$
+8	165/416 (39.7%)	125/339 (36.9%)	273/498 (54.8%)	$P < 0.0001$

Wild-Type Mice Develop Mechanical Allodynia and Decreases in Locomotion at Different Stages After DMM. Our findings suggest a temporary role for MCP-1/CCR2 signaling in the DRG after DMM surgery, with 8 wk postsurgery apparently being a crucial time point. To understand the biological role of MCP-1/CCR2 signaling in mediating pain-related behaviors, we compared mechanical allodynia and locomotive behaviors for 16 wk after DMM in wild-type vs. *Ccr2*-null mice. In wild-type mice, mechanical allodynia occurred in the operated hindpaw after DMM but not sham surgery, as we previously reported (17). This allodynia progressed up to 4 wk and was maintained at a constant level up to 16 wk after surgery; age-matched naive and sham-operated mice did not develop mechanical allodynia (Fig. 3A). We previously reported that morphine and, to a lesser extent, acetaminophen reversed this mechanical allodynia (17). During the second stage, beginning at 8 wk after DMM surgery, behaviors indicative of movement-provoked pain were observed. When monitored overnight on a LABORAS (Laboratory Animal Behavior Observation Registration Analysis System) platform (24), DMM mice 4-wk postsurgery and age-matched naive mice traveled similar distances (Fig. 3B). The mice also climbed (hanging upside down from the cage lid) and reared (standing on hind paws) the same number of times per hour throughout the night (Fig. 3B). By 8 wk, DMM mice covered less distance, climbed less often, as has been reported previously (16), and reared less often than age-matched naive and sham mice. These decreases continued up to 16 wk postsurgery (Fig. 3B). Decreases in locomotive behaviors could be substantially reversed by indomethacin, suggesting that they are indicative of movement-provoked pain (Fig. S3).

***Ccr2*-Null Mice Present Different Pain-Related Behaviors After DMM Surgery.** *Ccr2*-null mice displayed similar joint-damage scores at 8 wk postsurgery compared with wild-type mice (total joint score, mean \pm SEM: wild-type sham = 1.4 ± 1.0 ; wild-type DMM = 11.5 ± 2.1 ; *Ccr2*-null DMM = 8.2 ± 1.5) (Figs. S4 and S5), but they presented a very different sequence of pain-related behaviors. These mice developed marked progressive mechanical allodynia in the operated hind limb over the first 8 wk following DMM, but unlike in wild-type mice, allodynia abruptly began to resolve after 8 wk and was completely resolved by 16 wk (Fig. 4A). *Ccr2*-null mice were protected from locomotion changes at 8 wk postsurgery: despite the allodynia present at this time point, and despite comparable knee joint damage, *Ccr2*-null DMM mice traveled, climbed, and reared as much as age-matched naive *Ccr2*-

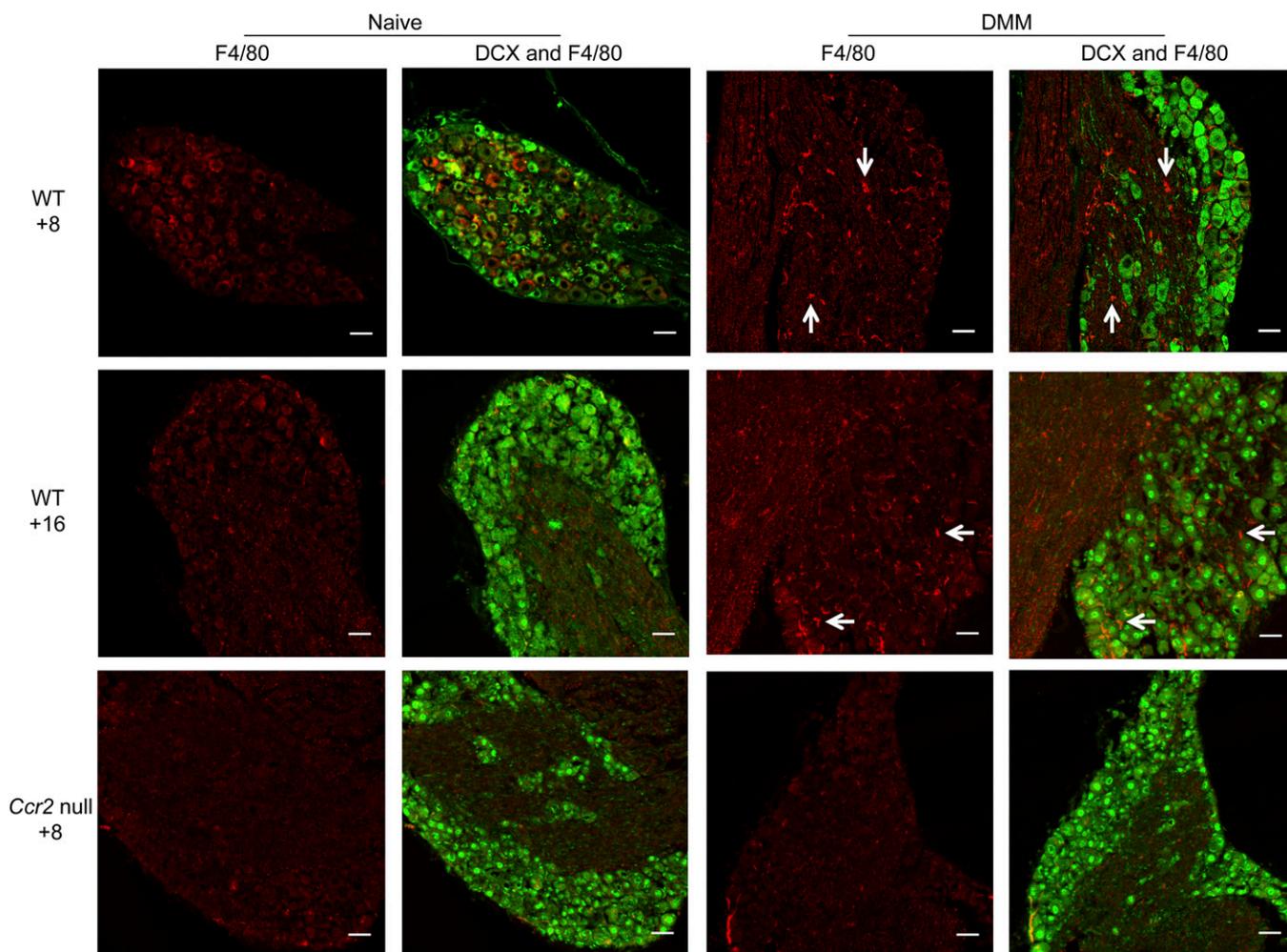


Fig. 2. Increases in macrophage DRG populations are seen at 8 and 16 wk after DMM surgery in wild-type mice. Doublecortin (DCX, neuron, green) and F4/80 (macrophage, red) staining of ipsilateral DRG in age-matched naïve and DMM wild-type (WT) mice at 8 wk postsurgery (*Top*), age-matched naïve and DMM wild-type mice at 16 wk postsurgery (*Middle*), and age-matched naïve and DMM *Ccr2*-null mice at 8 wk postsurgery (*Bottom*). White arrows indicate example macrophage staining. Magnification 20 \times . (Scale bars, 50 μ m.)

null mice (Fig. 4*B*). Sixteen weeks after DMM, these mice were still protected from movement-provoked pain behaviors (Fig. 4*B*). These findings suggest that *Ccr2*-null mice, despite the initially severe mechanical allodynia, did not progress to develop movement-provoked pain. Interestingly, supernatants from *Ccr2*-null DMM DRG neurons cultured at 8 wk postsurgery contained levels of MCP-1 below that of wild-type naïve mice (Fig. S6), suggesting the presence of a positive auto-feedback loop regulating MCP-1 and CCR2 expression in wild-type mice.

To confirm the key role of CCR2 signaling in development of the observed movement-provoked pain behavior after DMM surgery, we administered a CCR2 receptor antagonist to wild-type DMM mice at 9 wk postsurgery and found that this reversed the decrease in distance traveled (Fig. 4*C*).

Discussion

In a murine destabilization model of knee osteoarthritis, we found that early-onset mechanical allodynia is followed by an up-regulation of MCP-1/CCR2 signaling in the DRG 8 wk after surgery. This finding correlates with the onset of movement-provoked pain behaviors (decreased overnight distance traveled, climbing, and rearing) at 8 wk after DMM surgery. These decreased locomotion behaviors, indicative of pain that is triggered by movement, may be of translational significance in the context of osteoarthritis, where pain is typically triggered by specific activities (8). The significance

of mechanical allodynia in the context of clinical osteoarthritis pain is not yet understood. Current evidence confirms that subjects with osteoarthritis have lower pain-pressure thresholds, both at affected and unaffected sites, suggesting that central sensitization contributes to pain in osteoarthritis (reviewed in ref. 11), but it is unclear how these changed thresholds relate to the actual sensation of pain.

These data support the possibility that increased expression of both MCP-1 and its receptor CCR2 may mediate increased pain signaling in osteoarthritis through the direct excitation of DRG neurons, as well as through attracting macrophages to the DRG. It is known that macrophages can express numerous algogenic molecules that may contribute to the development of pain (25). We sought to confirm a role for MCP-1/CCR2 in pain development by assessing pain-related behaviors in *Ccr2*-null mutant mice. We found that these mice develop structural joint damage and mechanical allodynia during the first 8 wk following DMM surgery, but despite this they are completely protected from the onset of movement-provoked behaviors. Therefore, initiation of decreased locomotion indicative of movement-provoked pain appears to be dependent on MCP-1/CCR2 signaling.

Previous studies using rodent models of neuropathic pain have shown increases in MCP-1 and CCR2 expression in the DRG within 2 wk following peripheral nerve injury. These models included chronic constriction injury (26), unilateral gp120 sciatic

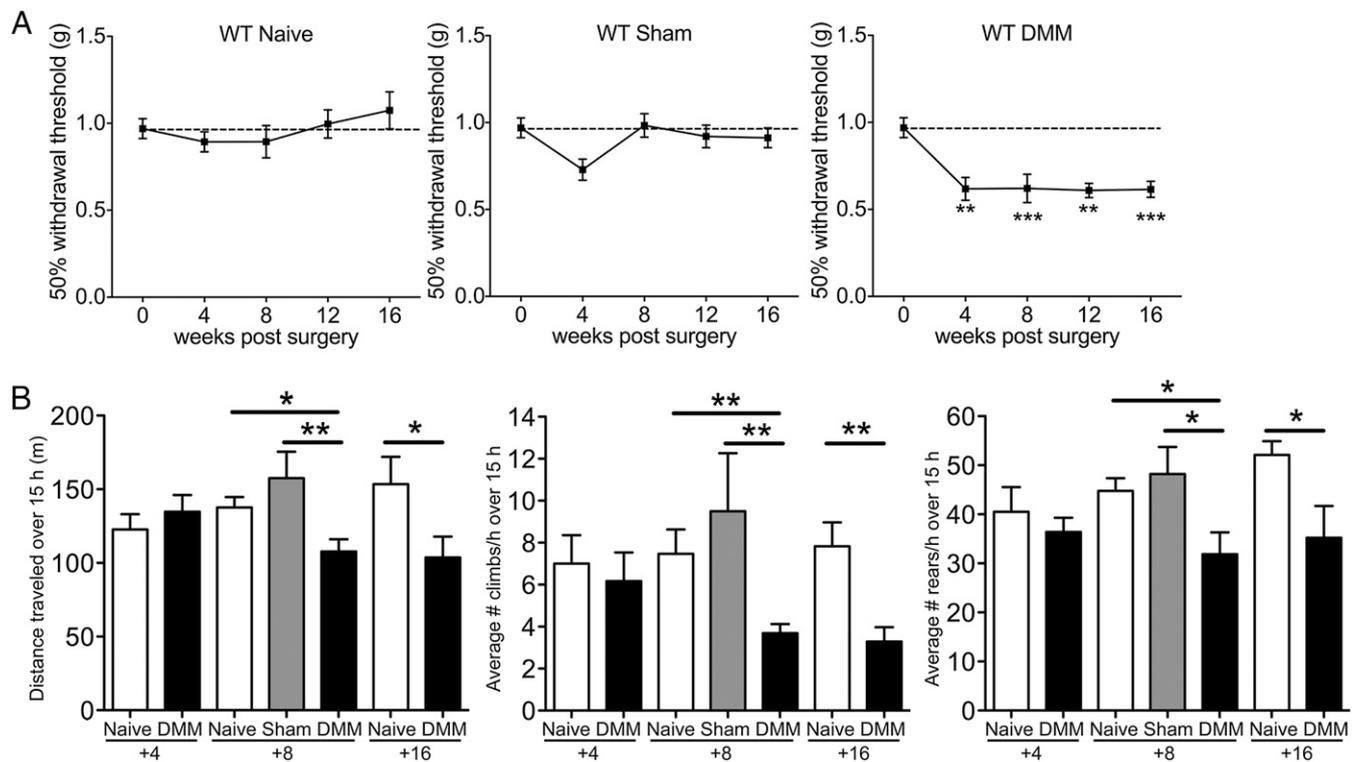


Fig. 3. Pain-related behaviors develop progressively in wild-type mice after DMM surgery. (A) Mechanical allodynia in the ipsilateral hindpaw of naïve ($n = 7-10$), sham ($n = 9$), and DMM mice ($n = 9-13$). Naïve time 0: $n = 30$, one-way ANOVA with Bonferroni's multiple comparison test, $**P < 0.01$, $***P < 0.001$ vs. Time 0. Results show mean \pm SEM. (B) Distance traveled, average number of climbs per hour, and average number of rears per hour during a 15-h period on a LABORAS platform. At 4 wk, $n = 8$ naïve, $n = 10$ DMM, $P > 0.05$ by two-tailed t test. At 8 wk, $n = 18$ naïve, $n = 10$ sham, $n = 15$ DMM, $*P < 0.05$ and $**P < 0.01$ by one-way ANOVA with Bonferroni's multiple comparison test. At 16 wk, $n = 9$ naïve and $n = 8$ DMM, $*P = 0.03$ for distance, $**P = 0.003$ for climbing, and $*P = 0.0487$ for rearing by two-tailed t test. For LABORAS results, data were log-transformed if necessary to ensure normality as determined by the D'Agostino-Pearson normality test before analysis. Results show mean \pm SEM.

nerve administration (27), partial ligation of the sciatic nerve (28), and focal nerve demyelination (29). Mechanical allodynia after nerve injury has been reported to be inhibited by treatment with a CCR2 receptor antagonist (27, 29), and *Ccr2*-null mice were protected from mechanical allodynia after nerve injury but not during acute inflammatory pain tests (21). We find here that *Ccr2*-null mice initially develop mechanical allodynia (first phase), but it resolves in the absence of MCP-1/CCR2 signaling. Resolution begins at 8 wk in the *Ccr2*-null mice, which directly correlates with the observed timing of MCP-1/CCR2 expression in wild-type DRG. Therefore, we conclude that the development of allodynia is not dependent on MCP-1/CCR2 signaling but, rather, that the persistence of allodynia is dependent on this signaling.

The pronociceptive effects of chemokines and their receptors include the induction of hyperexcitability of neurons through transactivation of transient receptor potential cation channel subfamily V member 1 (TRPV1) and other ion channels (12). This process has been demonstrated on cultured (18) and previously injured adult DRG sensory neurons (27, 29–32). In addition, chemokines are of central importance in the recruitment of leukocytes. Thus, the unique ability of MCP-1/CCR2 signaling to simultaneously coordinate inflammation and neuronal excitability may well contribute to the proalgesic actions of the chemokine. This result was indeed evident in the current studies, where we found evidence for both mechanisms. First, MCP-1 directly excited DRG neurons derived from DMM mice 8 wk after surgery (Table 1). Interestingly, cultured neurons from DRG harvested 8 wk after DMM produced high levels of MCP-1 protein compared with naïve in wild-type but not in *Ccr2*-null mice, suggesting that in wild-type mice, an autologous feedback loop exists that may further contribute to neuronal hyperexcitability. Second, we found a CCR2-dependent influx of macrophages into the DRG. In a rat

model of inflammatory arthritis, it was recently demonstrated that DRG infiltration by macrophages correlated with pain-related behavior (33). It is interesting to note that the source of MCP-1 in these experiments appears to be the DRG neurons themselves. This up-regulated expression of both MCP-1 and CCR2 by DRG neurons is consistent with our previous findings (30, 34) in other models of chronic pain, and suggests that CCR2 signaling within the DRG may play an important role in the genesis of nociceptor hyperexcitability.

Interestingly, levels of MCP-1 and CCR2 return to baseline or lower by 16 wk when movement-provoked pain behaviors are observed. This finding may suggest that the MCP-1/CCR2 pathway is only involved in the initiation of changes in the DRG, but once macrophages are present, the process is no longer dependent on increased MCP-1/CCR2. In addition, although our findings are consistent with a role for MCP-1/CCR2 signaling within the DRG, it is also possible that CCR2 signaling is important in the spinal cord. MCP-1/CCR2 may be acting in the dorsal horn, as has been suggested (26, 35, 36), further contributing to the observed phenotype in the *Ccr2*-null mice.

This study represents an initial attempt to try and unravel molecular pathways of pain generation in a long-term model of osteoarthritis in a longitudinal fashion. To our knowledge, we are unique in showing that MCP-1/CCR2 signaling is linked to development of functional persistent pain behaviors in the context of a model for a complex disease associated with chronic pain.

There exists a dire need for new targets for the development of analgesics for osteoarthritis pain. Our data strongly implicate MCP-1/CCR2 signaling in the development of joint pain associated with osteoarthritis. In this respect, it is a promising finding that a CCR2 receptor antagonist reversed movement-provoked pain behavior, corroborating the finding that CCR2 signaling is

and laminin- (20 $\mu\text{g}/\text{mL}$) coated glass coverslips (18), and cultured at 37 °C with 5% CO_2 for 4 d in adult neurogenic medium: F12 with L-glutamine, 0.5% FBS, 1 \times N2 (Life Technologies), penicillin and streptomycin (100 $\mu\text{g}/\text{mL}$ and 100 U/mL) (29).

Protein Analysis of Supernatant. Cell culture supernatants were concentrated via 3-kDa molecular weight cutoff Millipore centrifugal filters. Total protein levels were determined by BCA assay (Thermo Fisher Scientific), and levels of MCP-1 protein were determined via ELISA (R&D Systems), following the manufacturers' recommendations.

Calcium Imaging. The response of cultured DRG neurons to the chemokine MCP-1 (200 ng/mL; R&D Systems) was recorded although intracellular Ca^{2+} -imaging, following standard protocols (18). In brief, MCP-1 was applied for 3 min by adding 1 mL of solution directly to the bath chamber. Cells were washed 3 min before applying positive control solution (potassium, 50 mM) (27). Three independent experiments each using DRG pooled from three to four mice were performed. Total number of neurons counted for each condition: age-matched naïve (4 wk, $n = 631$ neurons; 8 wk, $n = 416$), sham (4 wk, $n = 258$; 8 wk, $n = 339$), and DMM mice (4 wk, $n = 306$; 8 wk, $n = 498$).

Immunofluorescence. Mice were anesthetized by ketamine and xylazine and perfused transcardially with PBS followed by 4% (wt/vol) paraformaldehyde in PBS (40). The spinal column was dissected and postfixed in 4% paraformaldehyde overnight followed by cryopreservation in 30% (wt/vol) sucrose in PBS. Individual ipsilateral L3–L5 DRG were embedded with OCT (Tissue-Tek), frozen with dry ice, and cut into 12- μm sections. DRG sections were stained with the primary antibodies anti-F4/80 (Abcam ab6640) and anti-doublecortin (Abcam ab77450). Isotype-specific secondary AlexaFluor-488 or AlexaFluor-633

antibodies (Invitrogen) were used. All captured images were exported to Adobe Photoshop CS 5.1 (Adobe) and adjustments were made to the brightness and contrast to reflect true colors (40).

In Situ Hybridization. L3–L5 ipsilateral DRG were embedded and sectioned as above. Mouse-specific CCR2 probes were generated as described previously (40). For the generation of CCL2 probes, a 500-bp CCL2 cDNA fragment (nucleotides 51–551 of GenBank no. NM_011333.3) was cloned by PCR by using mouse brain cDNA. The resulting PCR product was subcloned into a pCR II-TOPO vector and verified by restriction analysis and automated DNA sequencing (Perkin-Elmer). The CCL2 template was linearized with XbaI to generate an antisense probe by using SP6 polymerase. The sense probe was linearized with HindIII by using T7 polymerase. In situ hybridization histochemistry for MCP-1 and CCR2 was performed by using digoxigenin-labeled riboprobes (Roche Applied Science), as previously described (40).

Statistical Analysis. We analyzed calcium imaging data by χ^2 test. We analyzed all other data using two-tailed t tests or one-way analysis of variance tests with Bonferroni posttests, as appropriate. Data were tested for normality by the D'Agostino–Pearson normality test before analysis and transformed if necessary.

Additional Methods. Detailed methodology is described in the *SI Materials and Methods*.

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