HMGB1 released from nociceptors mediates inflammation

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Inflammation, the body’s primary defensive response system to injury and infection, is triggered by molecular signatures of microbes and tissue injury. These molecules also stimulate specialized sensory neurons, termed nociceptors. Activation of nociceptors mediates inflammation through antidromic release of neuropeptides into infected or injured tissue, producing neurogenic inflammation. Because HMGB1 is an important inflammatory mediator that is synthesized by neurons, we reasoned nociceptor release of HMGB1 might be a component of the neuroinflammatory response. In support of this possibility, we show here that transgenic nociceptors expressing channelrhodopsin-2 (ChR2) directly release HMGB1 in response to light stimulation. Additionally, HMGB1 expression in neurons was silenced by crossing synapsin-Cre (Syn-Cre) mice with floxed HMGB1 mice (HMGB1f/f). When these mice undergo sciatic nerve injury, nociceptors respond with HMGB1 release as part of the neurogenic inflammatory response. Here, by combining optogenetics, neuronal-specific ablation, and molecular mechanistic analysis, we show nociceptor HMGB1 release mediates pain and inflammation during sciatic nerve injury and collagen antibody–induced arthritis. HMG1 | cytokine | arthritis | DAMP | sciatic nerve injury

The dual threat of infection and injury exerts a significant influence on the evolution of the mammalian immune and nervous systems. The nervous system detects changes in the environment, generates reflexive responses to those changes, and integrates these responses across space and time to establish adaptive memories of events (1, 2). Sensory neurons, termed nociceptors, innervate tissues subjected to injury and mediate the body’s primary defensive response system to injury and infection. Nociceptor-related pain and inflammation can be prevented by targeting HMGB1.
Since evolution selects for reflexively controlled homeostatic systems acting antagonistically, here we reasoned it likely also selected for neural reflex circuits which stimulate inflammation and enhance immunity.

High mobility group box 1 protein (HMGB1), a ubiquitous nuclear protein conserved across evolution from archaea to mammals, is a necessary and sufficient mediator of sterile injury and infection-elicted inflammation and immunity (27, 28). It is passively released by cells undergoing necrosis and secreted by innate immune cells activated by inflammation (27, 29). HMGB1 stimulates inflammation by activating the receptor for advanced glycation end products (RAGE) and Toll-like receptor 2, 4, and 9 (TLR 2, 4, and 9) expressed by many different cell types (30–32). Signal transduction via these receptors culminates in increased expression of cytokines, and in recruiting and stimulating monocytes, lymphocytes, and neutrophils. HMGB1 also stimulates dendritic cell maturation and enhances antibody responses to antigen, an important early stage in memory formation (33, 34).

Administration of HMGB1 antagonists significantly attenuates the severity of inflammation in preclinical models of sepsis, inflammatory bowel disease, arthritis, and neuroinflammation (31, 32, 35, 36).

HMGB1 is expressed in the nervous system in cortical neurons and in nociceptors (37–40). In preclinical models of nerve injury, HMGB1 levels are increased in the cell bodies of nociceptors residing in the dorsal root ganglia (DRG) (39, 40). In brain neurons, HMGB1 translocates into the cytoplasm and is released during ischemia (38, 41). Exposure of cortical neurons to ethanol activates HMGB1 release via a mechanism dependent upon NOX2/NLRP1 inflammasome (42). Neuronal HMGB1 expression is also significantly enhanced during nerve injury (39, 43, 44). Because HMGB1 stimulates inflammation, and is expressed by nociceptors, and since nociceptors provide a ubiquitous network of nervous system connectivity to peripheral tissues, we reasoned HMGB1 released by nociceptors is a mechanism of neurogenic inflammation. Here, utilizing neuronal-specific ablation of HMGB1 (Syn-Cre/HMGB1fl/fl mice), we discovered a specific role for neuron-derived HMGB1 in inflammation. Genetic silencing of HMGB1 in neurons attenuates inflammation after sciatic nerve injury and in collagen antibody–induced polyarthritis revealing a previously unexpected, but essential, mechanistic role for neuronal HMGB1 in inflammation.

Results and Discussion

HMGB1 Is Actively Released by Stimulated Sensory Neurons. HMGB1 expression is up-regulated during nerve and tissue injury, but the cellular source and function of HMGB1 was previously unknown. We used an optogenetic approach to investigate whether stimulated neurons release HMGB1 upon activation. Channelrhodopsin-2 (ChR2) is a light-gated ion channel that is activated by exposure to blue light (45). To selectively activate sensory neurons, we generated transgenic Vglut2-Cre/ChR2-eYFP mice that express ChR2 coupled to an enhanced yellow fluorescent protein (ChR2-eYFP) directed by vesicular glutamate transporter type 2 (VGLUT2) promoter. VGLUT2 is expressed in peripheral glutamatergic sensory neurons (46). Sensory neurons harvested from the DRG of Vglut2-Cre/ChR2-eYFP mice were cultured and stimulated with 470 nm light in vitro. Consistent with previous reports (40), quiescent sensory neurons constitutively express HMGB1 and maintain an intracellular pool of HMGB1 predominantly in the nucleus (Fig. 1 A, optogenetic control). Sixty minutes after acute optogenetic activation, significant levels of HMGB1 are observed in the cytoplasm of sensory neurons (Fig. 1A, optogenetic stimulated), confirming that stimulated nociceptors translocate nuclear HMGB1 to the cytoplasm before releasing it into the extracellular milieu. We observe a time-dependent increase in the extracellular HMGB1 levels after optogenetic stimulation of nociceptors (Fig. 1B). In contrast, photostimulation of DRG sensory neurons from Vglut2-Cre/ChR2-eYFP mice using yellow light (595 nm), which does not activate ChR2, fails to induce HMGB1 release (Fig. 1B, control). Lactate dehydrogenase (LDH), a soluble cytoplasmic enzyme released upon membrane disruption or cell death (47), is not released during light exposure (Fig. 1C), further confirming that HMGB1 is actively secreted by stimulated nociceptors.

Generation and Characterization of Syn-Cre/HMGB1fl/fl Mice. To investigate the functional role of nociceptors and HMGB1 in inflammation, we selectively ablated HMGB1 expression in neurons by crossing synapsin-Cre (Syn-Cre) mice with floxed Hmgb1 mice (HMGB1fl/fl) (SI Appendix, Fig. S1A). Although global deletion of Hmgb1 leads to early postnatal death (48), mice lacking HMGB1 expression in neurons (Syn-Cre/HMGB1fl/fl) develop normally. No significant differences are observed in body weights of 8- to 10-wk-old Syn-Cre/HMGB1fl/fl mice as compared to age- and sex-matched wild-type (WT) and HMGB1fl/fl control mice (SI Appendix, Fig. S1B). Specific genetic ablation of HMGB1 expression in neurons significantly depletes HMGB1 protein levels in DRG sensory neurons in Syn-Cre/HMGB1fl/fl mice as compared to WT and HMGB1fl/fl mice (SI Appendix, Fig. S1 C and D). Immunos- taining of the cultured DRG neurons confirms absence of HMGB1 in the nucleus of Syn-Cre/HMGB1fl/fl sensory neurons as compared to WT (SI Appendix, Fig. S1E). In addition, HMGB1 expression is nondetectable in the brain, but abundant in splenic immune cells in Syn-Cre/HMGB1fl/fl mice (SI Appendix, Fig. S1 F and G).

Neuronal HMGB1 Ablation Leads to Reduced Local Inflammation and Hyperalgesia after Sciatic Nerve Injury. To test whether neuronal HMGB1 is required for mediating tissue inflammation following nerve injury, we utilized ligature constriction of the sciatic nerve, a standard preclinical model, which produces inflammation, swelling, and hyperalgesia in the paw (49, 50). Syn-Cre/HMGB1fl/fl and control WT and HMGB1fl/fl mice were subjected to sciatic nerve injury (49). HMGB1 is significantly increased in paw tissue from WT and HMGB1fl/fl control mice (Fig. 2 A and B), while Syn-Cre/HMGB1fl/fl mice do not exhibit increased paw HMGB1 expression (Fig. 2 A and B). Following sciatic nerve injury, proinflammatory cytokine levels in paw tissues are significantly elevated in WT and HMGB1fl/fl control mice, whereas a significant reduction is observed in Syn-Cre/HMGB1fl/fl mice as compared to HMGB1fl/fl mice (HMGB1fl/fl mice; CXCL1: 206 ± 91 pg/mg; IL-18: 340 ± 91 pg/mg; IL-1β: 22 pg/mg) (Fig. 2 C–E). No significant differences in CXCL1, TNF, and IL-18 levels are observed between WT and HMGB1fl/fl mice following sciatic nerve injury (Fig. 2 C–E). Next, we examined mechanical hypersensitivity (alldynia) in Syn-Cre/HMGB1fl/fl mice to analyze the contribution of neuronal HMGB1 in developing mechanical hyperalgesia, a cardinal feature of inflammation. Mechanical hyperalgesia after sciatic nerve injury is significantly reduced in Syn-Cre/HMGB1fl/fl mice as compared to WT and HMGB1fl/fl control mice (threshold responses in sciatic nerve injury groups: WT = 0.48 ± 0.03, HMGB1fl/fl = 0.53 ± 0.1, Syn-Cre/HMGB1fl/fl = 1.11 ± 0.06 g. P < 0.0001 vs. HMGB1fl/fl) (Fig. 2F).

These data indicate neuronal HMGB1 is required to mediate nerve injury–induced tissue inflammation and neuropathic pain. Following sciatic nerve injury, a partial denervation of the skin occurs (51), followed by variable regeneration and sprouting from adjacent nociceptors and recruitment of inflammatory cells, which may be another cellular source of HMGB1.

Anti-HMGB1 mAb Administration Ameliorates Sciatic Nerve Injury–Induced Hyperalgesia. Since neuronal HMGB1 is strongly correlated with inflammation and hyperalgesia following nerve injury, we tested whether neutralizing HMGB1 ameliorates sciatic nerve injury–induced hyperalgesia. Administration of monoclonal
anti-HMGB1 antibody (anti-HMGB1 mAb) (52) to rats (300 μg/rat, intraperitoneally [i.p.]) attenuates sciatic nerve injury–induced mechanical hypersensitivity (Fig. 3A). Significant improvement occurs in a dose-dependent manner in animals receiving anti-HMGB1 mAb (anti-HMGB1 mAb [300 μg/rat] = 2.3 ± 0.2 vs. IgG [300 μg/rat] = 0.9 ± 0.1 g, P < 0.0001) (Fig. 3A). Sciatic nerve injury also produces thermal hypersensitivity, a sign of inflammation-induced hyperalgesia (53). Significant increases in the paw withdrawal latency are observed in the treated animals in an antibody dose-dependent manner as compared to controls (IgG vs. anti-HMGB1 mAb [300 μg/rat]: 4.1 ± 0.2 vs. 7.2 ± 0.3 s, P < 0.0001) (Fig. 3B). To determine whether increased amounts of mAb could improve over the 300 μg/rat dose initially tested, additional MAb doses were administered on successive days to rats (Fig. 3 C and D) and mice (SI Appendix, Figs. S2 A and B). The results show that additional doses provide no further improvement over the initial single dose. Additionally, a significant reduction is observed in thermal hypersensitivity in rats receiving anti-HMGB1 mAb (Fig. 3E). These results indicate antibody neutralization of extracellular HMGB1 following nerve injury attenuates inflammatory hyperalgesia.

**Ablation of Neuronal HMGB1 Reduces Joint Inflammation and Cartilage Destruction and Improves Hyperalgesia in Murine Collagen Antibody–Induced Arthritis.** HMGB1 levels are significantly increased in synovial tissues and synovial fluid in experimental models of arthritis and in patients with rheumatoid arthritis and tendonitis (54–56). HMGB1-specific antagonists attenuate joint inflammation in several preclinical models of arthritis (54, 57). To investigate the role of neuronal HMGB1 in the pathogenesis of arthritis, we utilized a clinically relevant collagen antibody–induced arthritis (CAIA) mouse model (58). In this model, both pain and the development of arthritis depend upon nociceptor-induced neuroinflammation (59, 60). Following anti-collagen antibody administration, both male and female WT and HMGB1fl/fl control mice develop polyarthritis, as indicated by increased swelling of ankle joints and paws (Fig. 4A), elevated arthritis scores (Fig. 4B, SI Appendix, Fig. S3A), and mechanical hypersensitivity (Fig. 4C, SI Appendix, Fig. S3B). No significant difference in the severity of arthritis score is observed between WT and HMGB1fl/fl mice (Fig. 4B). However, a significantly delayed onset and reduced severity of polyarthritis occurs in Syn-Cre/HMGB1fl/fl control mice develop polyarthritis, as indicated by increased swelling of ankle joints and paws (Fig. 4A), elevated arthritis scores (Fig. 4B, SI Appendix, Fig. S3A), and mechanical hypersensitivity (Fig. 4C, SI Appendix, Fig. S3B). This is accompanied by a significant reduction in mechanical hyperalgesia (Fig. 4C, SI Appendix, Fig. S3B).

Proinflammatory cytokine and chemokine levels are significantly increased in the inflamed paw tissues in both male and female WT and HMGB1fl/fl mice (Fig. 4 D–I, SI Appendix, Fig. S3 D–I). HMGB1 levels are also significantly increased in the inflamed paw tissues in both male and female WT and HMGB1fl/fl mice, but not in Syn-Cre/HMGB1fl/fl mice (male mice: WT = 1.9 ± 0.2, HMGB1fl/fl = 2.1 ± 0.3 vs. Syn-Cre/HMGB1fl/fl = 0.8 ± 0.1 (P < 0.0001) (Fig. 4D, SI Appendix, Fig. S3 C and D)). Chemokine and cytokine levels in the arthritic paw tissues are significantly elevated in WT and HMGB1fl/fl control mice, whereas these levels
are significantly reduced in Syn-Cre/HMGB1\textsuperscript{fl/fl} mice as compared to HMGB1\textsuperscript{fl/fl} mice (HMGB1\textsuperscript{fl/fl} mice: CXCL1 = 317 ± 58, IL-6 = 146 ± 15, TNF = 162 ± 21, vs. Syn-Cre/HMGB1\textsuperscript{fl/fl} mice; CXCL1 = 100 ± 16 [P < 0.0001], TNF = 38 ± 17 [P < 0.001] pg/mg protein) (Fig. 4 F–H, SI Appendix, Fig. S3 F and G). No significant differences are observed in IL-18 levels (IL-18 levels in HMGB1\textsuperscript{fl/fl} = 66 ± 3 vs. Syn-Cre/HMGB1\textsuperscript{fl/fl} = 66 ± 11 pg/mg protein) Fig. 4I, SI Appendix, Fig. S3F).

Significant reductions in immune cell infiltration, synovitis, pannus formation, and cartilage destruction are observed in Syn-Cre/HMGB1\textsuperscript{fl/fl} mice as compared to WT and HMGB1\textsuperscript{fl/fl} mice (histological score = 3.8 ± 0.1 in WT, 2.8 ± 0.4 in HMGB1\textsuperscript{fl/fl} and 0.6 ± 0.2 in Syn-Cre/HMGB1\textsuperscript{fl/fl}, P = 0.002 vs. HMGB1\textsuperscript{fl/fl} group) (Fig. 4 J–K). Because gender differences regarding neuropathic pain in rodents have been reported (61, 62), we also examined responses to CAIA in female WT, HMGB1\textsuperscript{fl/fl}, and Syn-Cre/HMGB1\textsuperscript{fl/fl} mice (SI Appendix, Fig. S3 A–H). Selective ablation of neuronal HMGB1 in female mice also significantly delays the onset of arthritis and attenuates the severity of inflammation, hyperalgesia, and expression of inflammatory cytokines and chemokines (SI Appendix, Fig. S3 A–H). Together these results reveal a sex-independent role for neuronal HMGB1 in mediating inflammation, tissue damage, and hyperalgesia in experimental arthritis.

The results of these experiments establish that nociceptor-derived HMGB1 is a required mediator of inflammation and arthritis. Although numerous studies have previously implicated HMGB1 in inflammatory responses and in initiating neuropathic hyperalgesia following nerve injury, the cellular source of HMGB1 that contributes to a persistent inflammatory phenotype and to chronic hypersensitivity was previously unknown. Because global HMGB1 gene deletion is lethal to mice, and administration of HMGB1 antagonists cannot reveal the cellular source of HMGB1 release, here we developed neuronal-specific ablation to study the
role of neuronal HMGB1 in arthritis and neuropathy. The selective ablation of neuronal HMGB1 in Syn-Cre/HMGB1fl/fl mice significantly attenuates inflammation and hyperalgesia and provides direct evidence for a critical role of neurons stimulating inflammation through release of HMGB1.

Following nerve injury, HMGB1 translocates from the nucleus into axons (63). The Syn-Cre/HMGB1fl/fl mice utilized here have pan-neuronal loss of HMGB1, which therefore includes higher-order neurons of the sensory system. The tactile allodynia which occurs following nociceptor injury is critically dependent on central neurons and their effects upon the microglial population within the spinal cord (64). In addition to a reduction in local inflammation, the beneficial effect of HMGB1 neutralization on pain behavior may extend to these higher-order neurons. In support of this concept, previous study of rats undergoing partial sciatic nerve ligation with subsequent mechanical allodynia has shown that neurons within the dorsal horn of the spinal cord (the second order neurons of the nociceptor system) increase synthesis of HMGB1. This augmented expression was associated with translocation of HMGB1 from the nucleus to the cytoplasm by neurons, but not in the surrounding astrocytes and microglia (65). Further, administration of HMGB1 antibody in this study significantly decreased the expression of the immediate early gene c-Fos within the dorsal horn, as well as of ionized calcium adapter protein 1, a specific marker of activated microglia, the cell type which mediates the development and maintenance of allodynia (64). Further study of how specific cell types involved in the neuroinflammation response in the acute and chronic stages of peripheral nerve injury is warranted.

It is important to note that neuronal release of HMGB1 may well confer advantageous or protective responses during the host response to infection or injury, when activation of inflammatory responses enhances host defense and promotes tissue repair. In the developing nervous system and after axonal damage, HMGB1 on the neuronal cell surface promotes axonal sprouting, neurtue outgrowth, and neuronal migration. The findings of the present study now warrant assessing the functional role of sensory neuron-derived HMGB1 in the pathogenesis of other HMGB1-dependent infectious and sterile inflammatory disease models, including sepsis (35), pneumonia (66), epilepsy (67), neuropathic pain (68), cancer (69), and arthritis (70). It is also interesting to consider whether clinical observations of disease attenuation in rheumatoid arthritis and psoriatic arthritis patients following central or peripheral neurologic damage can be attributed to reduced release of neuronal HMGB1 (71–73).

In summary, our optogenetic and neuronal-specific ablation strategies applied to nerve-injury and arthritis models reveal a critical role for nociceptor HMGB1 in neuroinflammation and pain. Because neuron-derived HMGB1 is key to inflammatory responses and injury, this work suggests a paradigm for nervous system integration of environmental signals to stimulate inflammation as a fundamental mechanism of host defense during infection and injury. The results also suggest that targeting HMGB1 may constitute a new therapeutic approach for the treatment of a wide variety of diseases characterized by pain and inflammation.

Fig. 3. Anti-HMGB1 mAb administration ameliorates sciatic nerve injury–induced hyperalgesia. (A and B) Female Wistar rats were subjected to sciatic nerve ligation surgery or sham surgery 2 wk prior to i.p. administration of anti-HMGB1 mAb (30 or 300 μg per rat) or control IgG (300 μg per rat). (A) Mechanical (von Frey) and (B) thermal (Hargreaves) hypersensitivity were assessed at 6 h after antibody was administered. Dose-dependent improvement in mechanical and thermal hypersensitivity is observed after anti-HMGB1 mAb administration. Data are represented as individual mouse data points with mean ± SEM. One-way ANOVA followed by Tukey’s multiple comparisons test between groups: ***P < 0.001, ****P < 0.0001. n = 10 per group (normal or sham) and n = 6 (sciatic nerve injury). (C–E) Repetitive administration of anti-HMGB1 mAb ameliorates sciatic nerve injury–induced hyperalgesia. (C) Two weeks postsciatic nerve ligation or sham surgery, female Wistar rats received anti-HMGB1 mAb or IgG (300 μg/rat) i.p. once a day for 3 consecutive days. (D) Mechanical and (E) thermal hypersensitivity were assessed daily for 3 d after anti-HMGB1 mAb administration. Data are represented as individual mouse data points with mean ± SEM. Two-way ANOVA followed by Sidak’s multiple comparisons test between groups: ***P < 0.001, ****P < 0.0001, n = 6 per group.
Fig. 4. Ablation of neuronal HMGB1 reduces joint inflammation and cartilage destruction and improves hyperalgesia in murine collagen antibody–induced arthritis. (A and B) Syn-Cre/HMGB1fl/fl mice are protected from CAIA. (A) Representative images of hind paws of WT, HMGB1fl/fl, and Syn-Cre/HMGB1fl/fl mice on day 10 after initiation of arthritis. (Scale bar, 0.2 cm.) (B) Arthritis scores are significantly reduced in Syn-Cre/HMGB1fl/fl mice as compared to WT and HMGB1fl/fl mice. Data are represented as mean ± SEM. Two-way ANOVA followed by Sidak’s multiple comparisons test between groups: Syn-Cre/HMGB1fl/fl vs. HMGB1fl/fl group: *P < 0.05, ****P < 0.0001. (C) Mechanical hypersensitivity (von Frey) is significantly improved in Syn-Cre/HMGB1fl/fl mice subjected to CAIA as compared to WT and HMGB1fl/fl mice. Data are represented as mean ± SEM. Two-way ANOVA followed by Sidak’s multiple comparisons test between groups: Syn-Cre/HMGB1fl/fl vs. HMGB1fl/fl group: ***P < 0.001, ****P < 0.0001. (D and E) HMGB1 levels are increased in the paw tissue in WT and HMGB1fl/fl mice but not in Syn-Cre/HMGB1fl/fl after CAIA. HMGB1 levels were assessed 10 d after initiation of arthritis in the paw tissues. (D) Representative images of Western blot for HMGB1 and GAPDH in the paw tissues post CAIA. (E) Ratio of HMGB1/GAPDH expression. Data are represented as individual mouse data points with mean ± SEM. Two-way ANOVA followed by Tukey’s multiple comparisons test between groups: Syn-Cre/HMGB1fl/fl vs. HMGB1fl/fl group: ****P < 0.0001. (F–I) Syn-Cre/HMGB1fl/fl mice mount reduced local chemokine and cytokines response. Levels of (F) CXCL1, (G) IL-6, (H) TNF, and (I) IL-18 in the paw tissue from WT (n = 8), HMGB1fl/fl (n = 5 to 6) or Syn-Cre/HMGB1fl/fl mice (n = 6 to 7). Data are represented as individual mouse data points with mean ± SEM. Two-way ANOVA followed by Sidak’s multiple comparisons test between groups: ****P < 0.0001, ****P < 0.0001. (J and K) Histological assessment of joints on day 10 after initiation of arthritis. (J) Reduced infiltration of inflammatory cells, pannus formation, and cartilage damage (arrows) is observed in Syn-Cre/HMGB1fl/fl mice as compared to WT and HMGB1fl/fl subjected to CAIA. (Scale bars, 50 μm.) (K) The degree of inflammatory cells infiltration and cartilage disruption was graded as described in Materials and Methods. A significant reduction in histological score is observed in Syn-Cre/HMGB1fl/fl mice. Data are represented as individual mouse data points with mean ± SEM. One-way ANOVA followed by Tukey’s multiple comparisons test between groups: ****P < 0.001, ****P < 0.0001.
Materials and Methods

Animals. All procedures with experimental animals were approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the Feinstein Institutes for Medical Research in accordance with NIH guidelines and the ethical guidelines of the International Association for the Study of Pain. Animals were maintained at 25 °C on a 12-h light/dark cycle with free access to food and water. Sprague-Dawley or Wistar rats, C57BL/6 mice, VGlut2-Ires-Cre (Slc17a8(IRES-Cre)Zfp450), ChR2-YFP-Flox mice (B6.Cg-Gt(ROSA)26Sor<Zsg-4Gt(CAG-CTAAAG-ACTTTTGGA)1Hsd>), and synapsin-Cre (Syn-Cre, B6.Cg-Tg 671xMrt) were obtained from The Jackson Laboratory or Charles River Laboratories. HMGB1fl/fl mice were a gift from Timothy Billiar, University of Pittsburgh Medical Center, Pittsburgh, PA. Animals were acclimated for 7 d before any experimental use and housed under standard temperature, light-dark cycles.

Aldrich), and allowed to adhere for 12 to 15 h at 37 °C (with 5% CO2). Neuronal Cultures. DRG from adult Vglut2-Cre/ChR2-YFP mice and Syn-Cre/HMGB1fl/fl mice (8 to 12 wk old) and rats (2 to 3 mo old) were used in these studies. VGlut2-Ires-Cre mice were bred with ChR2-YFP-flox mice to generate Vglut2-Ires-Cre/ChR2-YFP mice that express ChR2 alleles under control of the Vglut2 locus, which encodes vesicular glutamate transporter type 2 that is predominantly expressed in peripheral sensory neurons. Syn-Cre female mice were bred with HMGB1fl/fl male mice to generate Syn-Cre/HMGB1fl/fl mice. The genotypes of the transgenic strains were confirmed using PCR (Transnetyx).

Optogenetic Stimulation of DRGs. A transparent box with a metal mesh floor and allowed to acclimate for 30 min before testing. The cage was elevated so that stimulation was applied to each hind paw from beneath the rodent. Calibrated von Frey filament (capable of exerting forces of 0.4 to 7.3 g, Ugo Basile) were applied in ascending order on the plantar surface of the hind paw to define the threshold stimulus intensity required to elicit a paw withdrawal response. The duration of each stimulus was 5 to 7 s. The repetitive testing was performed with an interval of at least 5 min for the same paw. The behavioral responses were then used to calculate absolute threshold (50% probability of response), as described previously (76).

The paw withdrawal test was used to test the thermal hypersensitivity of rodents using a plantar analgesia instrument. Animals were placed in a transparent box and allowed to acclimate for 30 min before testing. A radiant heat source (Ugo Basile plantar test) was focused on the plantar surface of the hind paw; the time from switching on the radiant heat to paw withdrawal was measured automatically (53). The infrared heat intensity was adjusted as 50, and the cutoff latency at 15 s was used to prevent tissue damage. Each hind paw was tested three to four times with alternating paws, and with an interval of at least 5 min between tests. For some experiments, upon completion of behavioral testing, animals were killed, and tissue was collected for further analysis.

Histologic Analysis of CAIA Joints. Hind leg ankle joints from normal or CAIA mice (Day 10 after CAIA initiation) were dissected and fixed in 4% paraformaldehyde. The joints were then decalcified (Newcomer Supply), embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. The degree of inflammatory cell infiltration and cartilage disruption was graded on a 0 to 4 scale as described previously (77) with some modifications: 0 = normal; 1 = mild inflammatory cell infiltration/cartilage degradation; 2 = mild to moderate inflammatory cell infiltration/cartilage degradation; 3 = severe inflammatory cell infiltration/cartilage degradation; and 4 = maximal inflammatory cell infiltration/cartilage degradation. All tissues were scored in a blinded manner.

Immunohistochemistry. Mice were anesthetized and intracardially perfused with cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Lumbar and thoracic DRGs, brain, and spleen were harvested, fixed in 4% paraformaldehyde overnight at 4 °C, and then cryoprotected in 30% sucrose for 48 h. Tissues were embedded in optimal cutting temperature compound (VWR International), and 14-μm sections were collected on slides and stored at −20 °C until further analyses. For HMGB1 staining, frozen sections were air dried for 30 min and fixed with 4% paraformaldehyde for 15 min at room temperature. After a PBS wash, the sections were blocked with antibody host serum (10% rabbit or mouse serum, 0.3% TX-100 in PBS) for 1 h at room temperature, followed by staining with primary antibodies at 4 °C overnight, rinsed three times with PBS, and mounted using Vectashield anti-fade mounting medium with DAPI (Vector Laboratories). The antibodies used were Alexa Fluor 555–conjugated anti-HMGB1 rabbit antibody at 1:200 dilution (Abcam), Alexa Fluor 647–conjugated anti-CD3 rat monoclonal antibody at 1:100 dilution (BioLegend), and Alexa Fluor 594– or 647–conjugated anti-NeuN antibody at 1:100 dilution (Abcam). Fluorescence images were acquired on a confocal microscope (Zeiss LSM880) and analyzed using Fiji software.

Measurements of HMGB1, Chemokines, and Cytokines. Frozen tissues (paws and joints obtained from the normal or injured side) were homogenized in PBS with protease inhibitor (Sigma-Aldrich). Protein concentration was measured using the Bradford assay (BioRad, Inc.). Levels of TNF, CXCL1, IL-18, and IL-6 were measured using commercial ELISA kits per manufacturer’s instructions (R&D System). HMGB1 was measured by Western blotting (40 μg protein per lane) with anti-HMGB1 monoclonal antibodies (2G7 at 1 μg/mL final concentration). Membranes were also stained using anti-GAPDH antibody (Abclonal) in a loading control. In some experiments, HMGB1 levels were measured using ELISA (IBL International).

Preparations of Anti-HMGB1 Monoclonal Antibodies. Anti-HMGB1 monoclonal antibodies (mAb 2G7) were generated as described previously (52). As
controls, mouse IgG was used. The LPS content in protein solutions was less than 10 pg/mg protein as measured by the Limulus assay.

Statistical Analysis. Data were analyzed using GraphPad Prism software. Differences between groups were analyzed using one-way ANOVA followed by Statistical Analysis.

Differences between groups were analyzed using one-way ANOVA followed by Statistical Analysis.
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