Optogenetic activation of spinal microglia triggers chronic pain in mice

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Abstract
Spinal microglia are highly responsive to peripheral nerve injury and are known to be a key player in neuropathic pain. However, there has not been any direct evidence showing selective microglial activation in vivo is sufficient to induce chronic pain. Here we used optogenetic approaches in microglia to address this question employing CX3CR1creER+/ R26LSL-ReaChR+/ transgenic mice, in which red-activated channelrhodopsin (ReaChR) is inducibly and specifically expressed in microglia. We found that activation of ReaChR by red light in spinal microglia evoked reliable inward currents and membrane depolarization. In vivo optogenetic activation of microglial ReaChR in the spinal cord triggered chronic pain hypersensitivity lasting for 5-7 days. In addition, activation of microglial ReaChR upregulated neuronal c-fos expression and enhanced C-fiber responses. Mechanistically, ReaChR activation led to a reactive microglial phenotype with increased IL-1β production. IL-1 receptor antagonist was able to reverse the pain hypersensitivity and neuronal hyperactivity induced by microglial ReaChR activation. Therefore, our work demonstrates that optogenetic activation of spinal microglia is sufficient to trigger chronic pain phenotypes by increasing neuronal activity via IL-1 signaling.
Introduction

Microglia are CNS-resident immune cells that constantly survey the microenvironment through their ramified processes in the brain and spinal cord \(^1,2,3\). Following peripheral nerve injury, spinal microglia transition to reactive states in a time period correlated with pain behaviors \(^4,5\). In addition, many studies have shown genetic knockout of microglial signaling molecules or depletion of microglia partially reverse neuropathic pain \(^6,7\), suggesting microglia as a key player in chronic pain pathogenesis. However, it is still unclear whether direct microglial activation is sufficient to trigger pain behaviors. Given the complex interactions between neurons, glia, and immune cells in pain modulation, dissecting the specific role of microglia requires the continuous development of new tools.

Microglia utilize ionotropic signaling to interact with the brain microenvironment during injury and pathology \(^8\). Unlike neurons, which rely heavily on voltage-gated ion channels, microglia have very few voltage-gated sodium or calcium channels in vivo. Well-known ion channels mediating ionic flux in microglia include potassium channels \(^9\), proton channels \(^10\), TRP channels \(^11\), and purinergic ionotropic receptors \(^12\). Purinergic signaling can directly activate ionotropic P2X4 and P2X7 receptors that are highly calcium permeable. In spinal microglia, P2X4 is upregulated following nerve injury \(^13\). Its prolonged activation in an injury context can lead to p38-MAPK dependent BDNF release \(^14\), pain sensitization, and the maintenance of neuropathic pain \(^15,16\). P2X7 receptors also promote microglial depolarization and pain responses in vivo \(^17\). Their prolonged activation can lead to NLRP3 inflammasome assembly and IL-1\(\beta\) release \(^18,19\), as well as microglial activation and proliferation.

Optogenetics allows manipulation of specific cell populations in real-time through the activation of a light-responsive ion channel. In neurons, optogenetics has been used to drive the depolarization or hyperpolarization of selective neurons, allowing for complex circuit interrogation underlying behaviors \(^20\). In electrically-silent astrocytes, optogenetics has also been used to dissect astrocyte function in breathing, and learning and memory \(^21,22\). Interestingly, channelrhodopsin-2 (ChR2) can depolarize spinal astrocytes and induce hypersensitive pain behaviors through ATP release \(^23\). However, so far, optogenetic tools have not been applied to study microglia. It is not yet known how experimental changes in microglia ionic conductance via optogenetic manipulation affect their cellular function or subsequent behaviors in vivo.
Using CX3CR1<sup>creER<sup>/+</sup>: R26<sup>LSL-ReaChR/+</sup> transgenic mice, we expressed the red-activated channelrhodopsin (ReaChR) specifically in microglia. ReaChR has improved membrane trafficking and enhanced photocurrents compared to existing red-shifted channelrhodopsins<sup>24</sup>. Also, red light reduces tissue scattering and absorption by chromophores, such as blood, relative to blue/green wavelengths of light. Here we find that optogenetic stimulation of spinal cord microglia by ReaChR is sufficient to produce a reactive microglial phenotype, neuronal hyperactivity and long-lasting behavioral pain sensitization.

**Results**

**Inducible expression of ReaChR in microglia in CX3CR1<sup>creER<sup>/+</sup>: R26<sup>LSL-ReaChR/+</sup> transgenic mice**

CX3CR1 is highly expressed by microglia in the CNS but also other cells of mononuclear origin in the periphery<sup>25</sup>. We generated CX3CR1<sup>creER<sup>/+</sup>: R26<sup>LSL-ReaChR/+</sup> transgenic mice to enable tamoxifen (TM) inducible Cre-dependent expression of ReaChR in CX3CR1<sup>+</sup> cells (Fig. 1A). To confirm ReaChR expression, we stained for rhodopsin, as ReaChR is a subfamily of retinylidene proteins<sup>24</sup>. In the spinal cord sections harvested 3 weeks post-TM injection, rhodopsin expression was observed at both the thoracic and lumbar levels of the spinal cord dorsal horn (Fig. 1Ba-b). To characterize the cellular distribution of ReaChR expression in CX3CR1<sup>creER<sup>/+</sup>: R26<sup>LSL-ReaChR/+</sup> transgenic mice, we performed co-immunofluorescence staining of Iba1 (microglia marker), GFAP (astrocyte marker), and NeuN (neuronal marker) with rhodopsin (Fig. 1C-D). Rhodopsin expression was not observed in mice that did not receive TM treatment (Supplementary Fig. 1A). In mice receiving TM, the expression of rhodopsin was observed in Iba1<sup>+</sup> microglia but not GFAP<sup>+</sup> astrocytes or NeuN<sup>+</sup> neuronal cells (Fig. 1C-D). Similarly, in the cortex of CX3CR1<sup>creER<sup>/+</sup>: R26<sup>LSL-ReaChR/+</sup> transgenic mice, we observed the co-localization of rhodopsin and Iba1 in TM treated mice but not in mice without TM treatment (Supplementary Fig. 1B). Taken together, these results indicate that we are able to induce Cre-dependent expression of ReaChR in CX3CR1<sup>+</sup> microglia in mouse CNS. Herein, CX3CR1<sup>creER<sup>/+</sup>: R26<sup>LSL-ReaChR/+</sup> transgenic mice treated with TM referred to as “ReaChR mice” while CX3CR1<sup>creER<sup>/+</sup> mice undergoing similar treatment but without ReaChR expression are referred to as the “control group”.

**Optogenetic stimulation of spinal microglia evokes inward currents and depolarization**
To verify the functional expression of microglial ReaChR, we performed whole-cell patch clamp recordings in microglia from acute spinal cord slices obtained from ReaChR mice (Fig. 2A). Since mCitrine is linked with ReaChR 26, we recorded mCitrine positive microglia and examined light-induced responses. Consistent with our previous studies 27, spinal microglia exhibited small currents with a linear current-voltage relationship in response to voltage steps from -140 mV to 40 mV. We found that stimulation with red light (625 nm) via LED dramatically increased the inward currents, with little deactivation, and drove the reversal potential to around -10 mV in microglia (Fig. 2B-C). We further characterized ReaChR currents by voltage ramp test with a gradual increase of light intensity (0-20% from 0.2 to 121.8 mW). The ReaChR current increased in amplitude with increasing light intensity (Fig. 2D-E). In addition, we performed current clamp recordings in spinal microglia to examine ReaChR-dependent changes in membrane potentials in response to different frequencies of light stimulation (persistent, 20 Hz, 10 Hz, 5 Hz, and 2 Hz). Light stimulation significantly depolarized microglia in a frequency-dependent manner with minimal desensitization, which was not observed in control group of CX3CR\textsuperscript{creER\^T} mice receiving TM (Fig. 2F). These results indicate that ReaChR is functionally expressed in spinal microglia and mediates light-induced, non-selective channel currents and membrane depolarization.

Optogenetic stimulation of spinal microglia induces pain hypersensitivity

To test the function of microglial ReaChR in vivo, we turned to pain behaviors in which microglia have a well-established role 6, 28, 29. To this end, we optogenetically stimulated spinal microglia in vivo and monitored pain behaviors in ReaChR mice or control mice. To target spinal microglia, a ferrule was stereotaxically implanted above the vertebra of the lumbar spinal cord, and an optogenetic fiber was inserted through the ferrule for light delivery (Fig. 3A). The optogenetic fiber was localized above the dural membrane of the dorsal horn to avoid damage to the spinal cord. Three weeks after TM injection, mice were exposed to red LED (625 nm, 45 ms light on, 5 ms light off, 20 Hz) through the optic fiber and then behavioral tests were performed (Fig. 3B). First, we investigated the potential impacts of vertebral ferrule implantation and spinal optogenetic stimulation on motor coordination. We evaluated the motor function of each group (Sham, Ferrule–implanted controls, and Ferrule-implanted ReaChR) with 30-min light stimulation using gait analysis (Fig. 3C). All groups demonstrated similar levels of locomotor ability. Using the rotarod test, we also found there were no significant change in motor coordination across all three groups (Fig. 3D). These results suggest that the procedures of ferrule implantation and optogenetic stimulation did not induce motor deficits.
Next, we investigated the effect of light stimulation (5, 15, and 30 min) on pain responses in ReaChR mice (Fig. 3E-F). We found that mechanical allodynia (Fig. 3E) and thermal hyperalgesia (Fig. 3F) were elevated one hour after 30-min light stimulation on the ipsilateral side, but not the contralateral side (Fig. 3E-F). Additionally, elevated pain behaviors were only elicited after 30 min of stimulation, and could not be achieved with 5 or 15 min stimulation of ReaChR mice. Thus, we used 30 min light stimulation to characterize optogenetic activation of microglia in ReaChR and control mice in the following experiments (Fig. 3G-H). We found that light-induced mechanical allodynia in the ipsilateral side persisted for 7 days and recovered after 9 days (Fig. 3G). In addition, light-induced allodynia could be re-introduced after the second optogenetic stimulation (Fig. 3G). Interestingly, light-induced thermal hypersensitivity was relatively transient and only lasted for 1 day after optogenetic stimulation (Fig. 3H). Similarly, a second round of light stimulation could still induce transient thermal hyperalgesia. We did not observe mechanical or thermal hyperalgesia on the contralateral side of ReaChR mice or in control mice (Fig. 3G-H). The light-induced pain responses were not due to damage in DRG neurons, as we did not observe the expression of ATF3, a well-established injury marker, after light stimulation in ReaChR or control mice (Supplementary Fig. 2A). The high expression of ATF3 was observed in ipsilateral L4 dorsal root ganglion (DRG) but not in contralateral side after spinal nerve transection (SNT) surgery (Supplementary Fig. 2B). Together, these results indicate that optogenetic stimulation of spinal microglia is sufficient to trigger multiple forms of pain hypersensitivity in ReaChR mice.

It has been reported that microglia play a sex-dependent role in neuropathic pain. Here we wanted to examine whether chronic pain induced by microglial ReaChR activation is dependent on sex. To this end, we compared the effect of light stimulation on pain behaviors in male and female ReaChR mice. Interestingly, we found that ReaChR activation induced mechanical allodynia lasting for at least 5 days in both male and female mice (Supplementary Fig. 3A). However, there were noticeable differences in light-induced thermal hypersensitivity, which was transient in male mice but lasted for 5 days in female mice (Supplementary Fig. 3B).

**Optogenetic stimulation induces microglial activation in the spinal cord dorsal horn**

Activation of microglia is critical for central sensitization in various pain-related conditions, including neuropathic pain after peripheral nerve injury. Therefore, we evaluated microglial activation in the spinal cord of ReaChR mice after optogenetic stimulation. After light stimulation,
the number of Iba1+ microglia was significantly increased in the ipsilateral dorsal horn compared to the contralateral side of the dorsal horn in ReaChR mice (Fig. 4A-B). Light stimulation did not change Iba1+ microglia number in control mice. Next, we analyzed the morphological changes in microglia after light stimulation, which is correlated with their activation state. Using Sholl analysis, we compared the complexity of spinal microglia after light stimulation in ReaChR mice and control mice. Indeed, light stimulation induced shorter processes and less complexity compared to controls (Fig. 4C-D). In addition, we examined Kv1.3, another marker of microglial activation after ReaChR stimulation. We found that there was an up-regulation of Kv1.3 in Iba1+ microglia in ipsilateral spinal dorsal horn compared with the contralateral side after light stimulation (Fig. 4E). These results indicate that the optogenetic stimulation of ReaChR induces spinal microglia activation.

Optogenetic stimulation of spinal microglia increases neuronal activity

Next, to elucidate how light-induced microglial activation translates into pain behaviors, we explored the potential effects of ReaChR stimulation on nociceptive transmission. To this end, we performed in vivo recordings of C-fiber-evoked field potentials in the spinal cord dorsal horn in anesthetized mice (Fig. 5A). After obtaining stable baseline recordings for up to 60 min, we introduced 30 min of light stimulation (625 nm, 45 ms light on, 5 ms light off, 20 Hz) and assessed changes in C-fiber evoked responses (Fig. 5B). We found that light stimulation did not affect the C-fiber-evoked field potential in control mice, but significantly increased the responses in ReaChR mice (Fig. 5C-D). The light-induced increase persisted for more than 90 min and returned to baseline over the course of 2 hours. After normalization to pre-light stimulation values (baseline; 0-60 min before light stimulation), C-fiber-evoked field potentials were maximally increased by 298.0% ± 24.5% after light stimulation in ReaChR mice (Fig. 5C-D). These results indicate that optogenetic stimulation of spinal microglia is able to facilitate nociceptive transmission in vivo.

The expression of c-fos in spinal dorsal horn neurons is increased after noxious stimulation and associated with the development of central sensitization, neuropathic pain, and inflammatory pain. To further study how microglial ReaChR activation affects neuronal activity, we examined c-fos expression 2 hr after light stimulation in the dorsal horn (Fig. 5E). We found that c-fos immunoreactivity was markedly upregulated after light stimulation in the ipsilateral dorsal horn of ReaChR mice, compared with the contralateral side or control mice (Fig. 5F). Since, our results showed that short term light stimulation (30 min) of microglial ReaChR resulted in long-
lasting mechanical allodynia (5-7 days), we posited that microglial ReaChR activation may trigger multiple, long-term effects in neuronal activity in addition to c-fos expression. The role of PKCα in the spinal dorsal horn plays a critical role in central sensitization, chronic pain, and maintenance of persistent pain. Here we found a significant increase in PKCα expression mostly in NeuN+ neurons at 3 days after light stimulation in ReaChR mice, compared with control mice. However, PKCα expression was not observed in Iba1+ microglia after light stimulation (Fig. 5G). Taken together, these results suggest that microglial optogenetic stimulation increases neuronal activity as suggested by increased neuronal c-fos and PKCα expression in spinal cord dorsal horn.

Optogenetic stimulation of spinal microglia induces pain through IL-1β signaling

During pathogenesis of neuropathic pain, IL-1β is a pivotal proinflammatory mediator released from microglia. Indeed, we found that a single intrathecal injection of recombinant IL-1β protein alone induces both mechanical and thermal pain responses lasting for multiple hours (Supplementary Fig. 4A-B). In addition, our exogenous application IL-1β protein was able to upregulate endogenous IL-1β in microglia (Supplementary Fig. 4C), suggesting an amplified response for microglial IL-1β signaling. Therefore, we first tested whether light stimulation is able to induce IL-1β expression. In our results, we observed that IL-1β was largely induced and selectively expressed in Iba1+ microglia in the ipsilateral dorsal horn at 1 day after light stimulation. However, there was very little IL-1β expression in control mice (Fig. 6A). Consistently, in vitro experiment using cultured primary microglia confirmed that optogenetic activation of microglial ReaChR increases IL-1β expression and release (Fig. 6B-C). We observed that light stimulation induced IL-1β release in the culture media from ReaChR expressing microglia at 1, 6, or 24 hr compared with the control group in vitro (Fig. 6D).

Next, to delineate whether IL-1β is the causal factor that mediated the increase of synaptic transmission and pain, we tested the effect of an IL-1 receptor antagonist (IL-1ra) to inhibit IL-1β signaling. To this end, we recorded C-fiber-evoked field potentials using in vivo recordings in the spinal cord of anesthetized mice. Three groups of mice were used: ReaChR+vehicle, ReaChR+IL-1ra, and Control+IL-1ra. Vehicle or IL-1ra was applied after 30 min of baseline recording. IL-1ra application alone did not change the baseline C-fiber-evoked responses in any group. We then gave light stimulation at 30 mins after the administration of IL-1ra (Fig. 7A). After normalization to pre-light stimulation values, we consistently found increased C-fiber-evoked field potentials in the ReaChR group by light stimulation (Fig. 7B). However, IL-1ra
administration prevented light-induced increase in C-fiber-evoked field potentials (Fig. 7B). Thus, these results demonstrate that increased nociceptive transmission after microglia ReaChR activation is mediated by IL-1β signaling.

We further investigated the effects of IL-1ra administration on light-induced pain hypersensitivity. Animals were intrathecally injected with IL-1ra (30 min) before light stimulation (Fig. 7C-D). We found that IL-1ra administration significantly alleviated light-induced mechanical allodynia (Fig. 7C) and thermal hyperalgesia (Fig. 7D) compared with the vehicle injected group (Fig. 7C-D). Consistently, IL-1ra administration also inhibited neuronal expression of PKCα in spinal neurons by light stimulation, although it cannot prevent light-induced microglial activation at 3 days after light stimulation (Supplementary Fig. 5). Taken together, these findings suggest that the IL-1β from optogenetically activated microglia mediates synaptic potentiation and pain hypersensitivity.

Discussion

In this study, we used a microglial optogenetic approach as a novel way to induce microglial activation and examined its function in pain behaviors. Taking advantage of CX3CRcreER/+; R26LSL-ReaChR/+ transgenic mice, we were able to controllably and specifically activate microglial ReaChR to induce the depolarization of spinal microglia. We found short-term activation of microglia via direct optogenetic stimulation leads to long-lasting changes in neuronal activity and chronic pain behaviors. Mechanistically, optogenetic stimulation of microglia leads to IL-1β production that increases C-fiber evoked responses, which could mediate the long-lasting pain hypersensitivity (Fig. 7E). Our results demonstrate that optogenetic activation of ReaChR in spinal microglia is sufficient to trigger chronic pain behaviors, proposing the interesting concept of “microgliogenic” pain phenomenon. Microglial optogenetic approaches represent a unique and controlled way for studying microglial function in awake mice.

Optogenetics has been mainly used to interrogate the neuronal circuits underlying various brain functions. Recent studies have also applied optogenetics to manipulate glial activity, particularly astrocytes, in the normal and diseased brain. Despite the increasing interest in microglial function in the CNS, optogenetic tools have not been used to study microglia due to the lack of effective viral tools for microglial research. To this end, we first generated CX3CRcreER/+; R26LSL-ReaChR/+ transgenic mice, which inducibly express ReaChR in CNS microglia, and validated its function. In addition, ReaChR-activated spinal microglia
exhibited morphological signs of activation alongside IL-1β expression. Most importantly, we found that short-term optogenetic stimulation of spinal microglia is sufficient to trigger long-lasting pain hypersensitivity. Our current study is exciting in several regards. First, this is the first genetic mouse model to manipulate microglia functions in vivo using optogenetic approaches. Second, our results suggest that transient optogenetic activation of spinal microglia is sufficient to trigger chronic pain behaviors. Third, we showed that microglial IL-1β via optogenetic activation is a critical mediator for synaptic potentiation and pain hypersensitivity. Therefore, this study provides proof-of-principle evidence that optogenetics is a viable tool for understanding microglia function in chronic pain. We are aware of several caveats in this study. (1) CX3CR1 is expressed in some peripheral immune cells and resident CNS macrophages in addition to microglia. Although our TM injection paradigm excludes mostly the peripheral immune cells, we cannot exclude the possibility of ReaChR expression in perivascular macrophages. (2) Recent reports suggest leaky expression with CX3CR1CreER mice, depending on the length of the STOP cassette in this loxP system. Nevertheless, our control and experimental expression data sets do not indicate that issue. Also, we confirmed the specific expression of ReaChR in Iba1+ microglia after TM injection in the CNS. To circumvent these issues of CX3CR1CreER mice, future studies should use the newly available microglia specific cre lines including TMEM119CreER and HexbCreER mice.

The advantage of optogenetics is that it targets cells with high spatiotemporal precision. Although microglia cannot fire action potentials, they respond dramatically to ion flux due to the high membrane resistance. Indeed, we found that activation of microglial ReaChR reliably induces membrane depolarization in microglia. To further validate this new optogenetic tool in microglia in vivo, we chose to study pain behaviors, because a microglial role in pain has been well established. For instance, spinal microglia are known to participate in synaptic plasticity, central sensitization, and neuropathic pain. Furthermore, Nasu-Hakola patients having TREM2 mutations only in microglia show pain symptoms. Consistently, our results demonstrate that optogenetic activation of spinal microglia is sufficient to induce reversible pain hypersensitivity. These results suggest an intriguing possibility for “microgliogenic” pain that originates from microglial dysfunction in the CNS. We used ReaChR activated by red light that is more suitable for spinal activation without penetration of optic fiber into spinal parenchyma. Thus, ReaChR is advantageous than ChR2 (activated by blue light) in studying spinal mechanism of pain. We also found that ReaChR is widely expressed in different brain regions such as the hippocampus and cortex. Therefore, CX3CR1CreER/+; R26LSL-ReaChR/+ transgenic mice...
will be a useful tool to study supraspinal microglia in brain function, such as pain-related comorbidity \(^{53}\), memory \(^{54}\), and epilepsy \(^{55}\).

Our optogenetic approach to alter microglial function is based on the known literature of ion channel conductance and modeling their role in pain condition. A variety of ion channels including \(K^+\) channels, \(Cl^-\) channels, TRP channels, proton channels, and ligand-gated ion channels such as P2X4 and P2X7 play important functions in cell proliferation, production of cytokines and cytotoxic substances, morphological changes, and the migration of microglial cells \(^{56, 57, 58}\). Here we artificially express ReaChR in microglia and observe a light-induced inward current, consistent with ReaChR as a non-selective cation channel \(^{24}\). We found that microglial ReaChR activation in vivo can increase synaptic transmission, neuronal excitability (c-fos and PKC\(\alpha\) expression), and long-lasting pain behaviors. Thus, these results suggest that opening of non-selective cation channel like ReaChR in microglia is sufficient to enhance neuronal activity and pain. In line with this idea, there are several non-selective cation ion channels in microglia, including P2X4, P2X7, and TRPM2 that are reported to be critical for neuropathic pain induction \(^{6}\). Particularly, our results by optogenetic activation of ReaChR in spinal microglia are reminiscent of a previous study in which the transplantation of P2X4-activated microglia induces pain behaviors \(^{13}\). Therefore, ReaChR likely mimics other endogenous, non-selective cation channels in microglia and is a unique optogenetic tool to understand microglia function.

How ReaChR activation in the spinal microglia leads to neuronal hyperactivity and chronic pain? There are two potential consequences after ReaChR activation, including membrane depolarization and Ca\(^{2+}\) elevation. Currently, we know little about the function of membrane potential alterations in microglia function. It has been reported that K channel activation and hyperpolarization is critical for microglial motility \(^{27, 59}\). In addition, spinal microglia reactivity is correlated with K channel activation in neuropathic pain \(^{60, 61}\). Therefore, these results suggest membrane hyperpolarization (associated with K channels) instead of depolarization in microglia may activate microglia, likely due to the increase of Ca\(^{2+}\) driving force. Indeed, a recent report demonstrates that inhibition of K channel in microglia decrease P2X4-channel dependent Ca\(^{2+}\) elevation \(^{9}\). Therefore, we believe that ReaChR-mediated Ca\(^{2+}\) elevation other than membrane depolarization might be more important in light-induced microglial activation and subsequence pain behaviors.
Microglial Ca\(^{2+}\) activity was not well studied in vivo but was suggested to be critical for microglial functions such as motility and cytokine release \(^{62, 63}\). Although we did not directly detect intracellular Ca\(^{2+}\) increase in microglia after optogenetic ReaChR stimulation, the light-induced inward current likely contains Ca\(^{2+}\) influx through ReaChR. Considering Ca\(^{2+}\) increase is a common signaling pathway in potential release and expression of gliotransmitters, cytokines and chemokines \(^{64}\), we believe that ReaChR-induced Ca\(^{2+}\) might be critical in microglial regulation of neuronal activity. Consistently, we demonstrate that IL-1\(\beta\) is the downstream molecule after ReaChR activation for following reasons: (1) ReaChR activation induces IL-1\(\beta\) upregulation in microglia; (2) Inhibition of IL-1\(\beta\) by IL-1ra ameliorates microglial ReaChR-dependent synaptic potentiation in the spinal cord; (3) IL-1ra also inhibits light-induced neuronal activation and chronic pain. At least two possibilities may link ReaChR and with IL-1\(\beta\) in mediating microgliogenic pain: (1) ReaChR-dependent Ca\(^{2+}\) elevation could facilitate the release of IL-1\(\beta\) which potentiates synaptic transmission and initiate pain, and (2) ReaChR activation could increase IL-1\(\beta\) expression, which maintains chronic pain hypersensitivity. Interestingly, activation of P2X4 in spinal microglia is coupled with BDNF production critical for neuropathic pain \(^{14}\). In addition, optogenetic activation of spinal astrocytes induced pain hypersensitivity via ATP release \(^{23}\). Therefore, future studies are needed to test whether other factors such as BDNF and ATP mediate microglial ReaChR-dependent chronic pain. Since ReaChR is an exogenous cation channel, Ca\(^{2+}\) signaling induced by ReaChR activation may not represent the physiological microglia function. Therefore, development of new optogenetic tools other than ChR2, such as stim1 channel \(^{65}\) which is highly expressed in microglia, might be useful in dissecting microglia function in neuronal circuits and behaviors.

As resident immune response cells, microglia are well-known to employ their function in a variety of neurological diseases, such as bacterial meningitis, ischemic stroke, epilepsy, Alzheimer’s disease, Parkinson’s disease, multiple sclerosis \(^{55, 66, 67}\). By understanding microglia in the normal and disease brain, researchers are developing tools to manipulate microglia with high spatial and temporal resolution. The new optogenetic approach using ReaChR offers such an opportunity and is unique in studying microglial Ca\(^{2+}\) signaling in vivo. This novel tool will advance our ability to understand how microglia respond to the microenvironment as well as regulate neuronal activity and subsequent behaviors. Interestingly, the safety of using viral vectors and opsins in optogenetics was demonstrated in preclinical studies \(^{68, 69}\). Although the idea is far-fetched, optogenetic manipulation of microglia might be theoretically explored as therapeutic target in pain management and in other microglia-related neurological disorders.
Materials and methods

Animals. Mice (7-12 weeks old) were used following institutional guidelines as approved by the Institutional Animal Care and Use Committee (IACUC) at Mayo Clinic. C57BL/6J, R26LSL-ReaChR (026294) 26, and CX3CR1CreER/CreER mice (021160) 70 were purchased from Jackson Laboratory (Bar Harbor, ME). CX3CR1CreER/CreER mice were crossed with R26LSL-ReaChR mice to obtain CX3CR1CreER+/+: R26LSL-ReaChR/+ offspring used in experiments. Male mice were used throughout the study unless the use of female mice was specifically indicated. These mice were assigned to an experimental group randomly within a litter. Experimenters were blind to drug treatments.

Cre-dependent ReaChR expression. To induce ReaChR expression in microglia, 150 mg/kg tamoxifen (TM; T5648, Milipore-Sigma, Burlington, MA) in corn oil (20 mg/mL) was administered via intraperitoneal (i.p.) injection once daily for 3 days.

Optogenetic ferrule implantation and light stimulation. Mice were anesthetized by 2% isoflurane anesthesia on a stereotaxic apparatus. An incision was made along the skin on the back of the animal, and then connective tissue and muscles were removed, exposing the bones of the lumbar spinal cord region. The spinal cord was immobilized in place using a metal bar along the vertebrae. A small craniotomy was made through the bone using a high-speed dental drill. A ceramic ferrule (Thorlabs, CF230-10, ø2.5 mm, 10.5 mm) securing a length of optic fiber (Thorlabs, FT200UMT, ø200 µm) was inserted so that the optic fiber tip reached 0.3 mm below the bone surface, in the epidural space. The ferrule was secured in place using dental glue (Tetric EvoFlow). In this procedure, we ensured the dura membrane was left intact and the spinal cord tissue was unscathed by the procedure. Mice were provided 2 weeks to recover from the surgery.

To activate ReaChR in microglia, light stimulation was delivered through the optic fiber implanted with the ferrule. We used 625 nm, red LED light (20 Hz: 45 ms light on, 5 ms light off, Thorlabs, M625F2, 13.2 mW) to activate the ReaChR. As a control, CX3CR1CreER/+ mice with TM injection (i.p.) were also given identical stimulation patterns and protocols.

Behavioral measurement. Mechanical allodynia was assessed by measuring the paw withdraw threshold using a set of von Frey filaments (0.04-2 g; North Coast medical). Mice were placed on an elevated metal grid. The filament was applied to the plantar surface from the
bottom at a vertical angle for up to 3 s. Fifty percent of withdrawal threshold values were determined using the up-down method.

Thermal hyperalgesia was assessed by measuring the paw withdrawal latency in response to radiant heat stimuli. Mice were placed in elevated chambers with a plexiglass floor and allowed to habituate for 20 min. The radiant heat source (IITC Inc life science) was applied to the center of the plantar surface of the hind paw four times with 3-min intervals. The average withdrawal latency of the four trials was recorded as the response latency.

Rotarod tests were performed using a four-lane Rotarod apparatus (Med Associates Inc). The rotarod speed started at 4 RPM and uniformly accelerated over a 5 min period to 40 RPM. Each mouse was tested 3 times in a day with a 15-min interval. The average daily latency per mouse from the three trials was used for analysis.

Gait analysis was performed using the footprint test. The forepaws and hindpaws of the mice were coated with red and blue nontoxic paints, respectively. The mice were trained to walk along a covered runway (50 cm length, 10 cm width, with 10 cm walls). All the mice were given three runs per test using a fresh sheet of white paper. Hind limb stride length was calculated using the average length between four sequential steps. This value was averaged among the three daily trials per mouse.

**Fluorescent immunostaining.** Mice were deeply anesthetized with isoflurane (5% in O2) and perfused transcardially with 20 ml PBS followed by 20 ml of cold 4% paraformaldehyde (PFA) in PBS. The spinal cord was removed and post-fixed by submersion in the same 4% PFA solution for 4-6 h at 4 °C. The samples were then transferred to a 30% sucrose in PBS solution overnight for cryoprotection. Sample sections (15 μm in thickness for IHC or 30 μm thickness for Sholl analysis) were prepared on gelatin-coated glass slides using a cryostat (Leica). The sections were blocked with 5% goat serum and 0.3% triton X-100 (Sigma) in PBS buffer for 60 min, and then incubated overnight at 4 °C with primary antibody: rabbit-anti-rhodopsin (1:200, Abcam Cat# ab5417, RRID:AB_304874), rabbit-anti-Iba1 (1:500, Abcam Cat# ab178847, RRID:AB_2832244), rabbit-anti-NeuN (1:500, Abcam Cat# ab104225, RRID:AB_10711153), rabbit-anti-GFAP (1:500, CST Cat#12389s, RRID:AB_2631098), mouse-anti-PKC alpha (1:200, Abcam Cat# ab31, RRID:AB_303507), mouse-anti-IL-1β (1:400, Cell Signaling Technology Cat# 12242, RRID:AB_2715503), mouse-anti-Kv1.3 (NeuroMab Cat# 73-009,
RRID:AB_10673575), rabbit-anti-c-fos (Cell Signaling Technology Cat# 2250, RRID:AB_2247211) and rabbit-anti-ATF3 (Santa Cruz Biotechnology Cat# sc-188, RRID:AB_2258513). The sections were then washed and incubated for 60 min at room temperature with an appropriate secondary antibody: anti-rat (1:500, Thermo Fisher Scientific Cat# A-11006, RRID:AB_2534074), anti-rabbit (1:500, Thermo Fisher Scientific Cat# A-11035, RRID:AB_2534093) or anti-mouse (1:500, Thermo Fisher Scientific Cat# A-11029, RRID:AB_2534088). The sections were mounted with Fluoromount-G (SouthernBiothech) and fluorescent images were obtained with the EVOS FL Imaging System.

**Sholl analysis.** Fixed tissue (30 μm) was used to acquire Z stack images of microglia (3-μm step size) using a 40× objective and a confocal microscope (LSM510, Zeiss). Consecutive Z-stack Images were converted to a maximum intensity projection image using Fiji software. Over the maximum intensity projection image, concentric circles were drawn (concentric circles plugin, fiji), centered on the soma, beginning at a 0.5 μm radius and increasing in radius by 0.1 μm steps. Sholl analysis was manually performed for each cell by counting the number of intersections between microglia branches along each concentric circle to create a Sholl plot. Additional measures to characterize each cell included the process branch number, process length, and cell soma area.

**In vivo extracellular recordings in the dorsal horn.** Mice were anesthetized with urethane (Sigma, catalogue #U2500 1.5 g/kg, i.p.). A T12-L1 laminectomy was performed to expose the lumbar enlargement of the spinal cord. Under a surgical microscope, the dura was carefully removed, and the left sciatic nerve was gently dissected free for electrical stimulation using a bipolar platinum hook electrode. Stabilizing clamps were affixed to the spinal column, head, and tail (model STS-A; Narishige) to minimize movement-associated artifacts during recording. A small recording well was formed on the dorsal surface of the spinal cord for drug application (IL-1ra, 50 ng). Single, test stimuli (0.5 ms duration, 1 min intervals, at C-fiber intensity) were delivered to the sciatic nerve. C-fiber-evoked fEPSP were recorded from the dorsal horn of the mouse spinal cord using a glass microelectrode filled with 0.5 M sodium acetate (impedance 0.5–1 MΩ). The optimal recording position for C-fiber-evoked fEPSP was at a depth of around 200–350 μm from the surface of the L4 lumbar enlargement. Data was acquired at a 10 kHz sampling rate. An A/D converter card (National Instruments M-Series PCI-6221) was used to digitize the data. C-fiber-evoked fEPSPs were analyzed by the WinLTP Standard Version program (WinLTP, RRID:SCR_008590). In each experiment, the amplitude of five consecutive
fEPSPs was averaged for overall analysis. To investigate the effects of ReaChR activation, the mean fEPSP amplitude was compared between a pre-optogenetic stimulation phase (baseline) and following light stimulation. Light stimulation (20 Hz: 45 ms light on, 5 ms light off) was applied for 30 min after a stable C-fiber-evoked fEPSP was established.

**Whole cell recording of microglia in spinal cord slices.** Mice were anesthetized with isoflurane and coronal slices of spinal cord (350 µm) were prepared in ice-cold, sucrose-substituted artificial cerebral spinal fluid (ACSF, in mM): Sucrose 185, KCl 2.5, CaCl₂ 0.5, MgCl₂ 10, NaHCO₃ 26, NaH₂PO₄ 1.2, glucose 25. Slices were then incubated in normal ACSF containing (in mM): NaCl 120, KCl 2.5, CaCl₂ 2, MgCl₂ 2, NaHCO₃ 26, NaH₂PO₄ 1, glucose 11 for 1h recovery. Whole-cell, patch-clamp recordings from microglia were obtained from spinal cord slices perfused with room temperature ACSF. Recordings were made using a 5-7MΩ glass pipette filled with intracellular solution, containing (in mM): K-Gluconate 135, HEPES 10, Mg-ATP 4, Na2-GTP, Phosphocreatine disodium salt hydrate 10 with pH at 7.5 and 290–300 mOs. Slices were protected from light throughout the whole process of slice recovery and recordings. Microglia were held at -60 mV. In assessing a cell’s current-voltage relationship in response to ReaChR activation, two protocols were used under voltage clamp mode: (1) a ramping protocol was used to shift the membrane voltage from the holding voltage of -60 mV to a voltage between -140 mV and 40 mV (500 ms duration); (2) Constant membrane voltage changes from -140 mV and 40 mV (500 ms duration) with a 20 mV step size. These tests were performed in both the presence and absence of red light stimulation (625 nm), which was specifically applied during the 500 ms duration of the voltage shift. In addition, current clamp mode was used to examine membrane potential shifts in response to red light stimulation. Recordings were amplified and filtered at 2 kHz by a patch-clamp amplifier (Axon 700B), digitized (DIGIDATA 1550), and analyzed by pCLAMP software (Molecular Devices, Union City, CA). Data were discarded when the input resistance changed >20% during recording.

**Primary microglia cultures and IL-1β ELISA.** Mixed glia cultures were prepared from P0-P2 CX3CR1creER⁻/⁻ : R26LSL-creERT₂⁺/+ mice and CX3CR1creERT₂⁺/+ mice as a control group following an established procedure. Briefly, extracted forebrains were dissociated in 0.25% trypsin-EDTA (Thermo Fisher Scientific Gibco, cat#25200-056) and DNase I (15 unit/mL, Sigma-Aldrich, cat#DN25). Cells were seeded in DMEM/F12 (Thermo Fisher Scientific Gibco, cat#11330-032) containing 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific Gibco, cat#26140-079) and 1× penicillin/streptomycin (Thermo Fisher Scientific Gibco, cat#15140-122) on 75-
cm² tissue culture flasks coated with poly-D-lysine (100 µg/mL, Sigma Aldrich, cat#P6407) and incubated at 37 °C in 5% CO₂ atmosphere. The culture medium was changed after 3 days and then 4 days until confluency (11–12 days in vitro). To obtain pure microglia cell, flasks of mixed glial cultures were shaken at 200 rpm for 1 hr at 37 °C. Floating microglia were collected by centrifugation (1000 rpm for 10 min) and seeded at ∼1×10⁵ cells/ml on poly-D-lysine-coated dishes containing 50% old/50% new media. On the following day, culture medium was supplemented with 5 µM 4-OHT and incubated for 24 h for optimal induction of ReaChR expression in microglia. ReaChR-expressing microglia were activated for 30 min with 625 nm, red LED light (20 Hz: 45 ms light on, 5 ms light off). The conditioned media was used to evaluate levels of IL-1β using the mouse IL-1β ELISA kit (Thermo Fisher Scientific Cat#88-7013-22, RRID: AB_2574942) following the manufacturer’s protocol.

**Statistical analysis.** Quantification of fluorescent immunostaining results was done with Fiji software (Fiji, RRID:SCR_002285). Pain behaviors were analyzed using two-way ANOVA with multi comparisons to test for main effects between groups followed by post-hoc testing for significant differences. Two-group analysis utilized the Student’s t-test. Three-group analysis utilized a one-way ANOVA design. Data are presented as mean ± SEM. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Prism 8, RRID:SCR_002798). Level of significance is indicated with *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

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**Author Contributions:** M.H.Y and L.J.W. designed the studies. M.H.Y. performed the experiments and collected data. Y.U. L. helped the spinal cord slice recording. T.C., D.B.B. and H.D. assisted with some experimental design. Y.Y. and A.D. assisted with analysis. M.H.Y., A.D.U. and L.J.W. wrote and revised the manuscript.

**Conflict of Interest:** The authors declare no competing financial interests.
Figure Legends:

**Figure 1.** CX3CR1<sup>creER</sup>/+; R26<sup>LSL-reaChR</sup>/+ transgenic mice enable selective expression of ReaChR in spinal microglia.

(A) Generation of CX3CR1<sup>creER</sup>/+; R26<sup>LSL-reaChR</sup>/+ transgenic mice by crossing CX3CR1<sup>creER/creER</sup> mice with R26<sup>LSL-reaChR</sup> mice. (B) Immunofluorescence images indicate rhodopsin expression in the thoracic and lumbar sections of the spinal cord. Scale bar, 40 μm. (C) Representative images of rhodopsin immunostaining (red) with either Iba1 (green), GFAP (green), or NeuN (green) in the lumbar region of the spinal dorsal horn in CX3CR1<sup>creER</sup>/+;R26<sup>LSL-reaChR</sup>/+ mice after TM injection. Scale bar, 40 μm. n=4 mice/group. (D) Summarized data show the co-localization of rhodopsin with Iba1<sup>+</sup> cells but not with GFAP<sup>+</sup> or NeuN<sup>+</sup> cells. n=4 mice/group, ****P<0.0001, unpaired Student’s t-test.

**Figure 2.** Optogenetic activation of spinal microglia evokes inward currents and membrane depolarization.

(A) Representative image of mCitrine<sup>+</sup> microglia being recorded through whole-cell patch-clamp electrophysiology in spinal cord slices of ReaChR (CX3CR1<sup>creER</sup>/+: R26<sup>LSL-reaChR</sup>/+) mice. (B) Microglia membrane currents from baseline (before red light stimulation, black traces) or ReaChR activation (after red light stimulation [intensity 8% from 54.5 mW], red traces) in ReaChR mice. Microglia were held at -60 mV, then underwent 500 ms voltage steps from -140 to +40 mV (20 mV steps) (C) Summarized plot of current (I, [pA]) versus holding potential (Vm, [mV]) in spinal microglia from ReaChR mice before light (black traces) and after light stimulation (red traces). (D) Voltage ramp tests from -140 to +40 mV in response to the gradual increase of light intensity (0-20% from 0.2 to 121.8 mW). (E) A summarized graph depicts that the increase of optogenetic current in microglia correlates with the increase in light intensity. (F) The current clamp recording of microglia shows the changes in membrane potential in response to 5 s light stimulation (intensity 8% from 54.5 mW, pulse duration 45 ms) at indicated frequencies in control (CX3CR1<sup>creER</sup>/+) mice (upper) and ReaChR mice (lower).

**Figure 3.** Optogenetic stimulation of microglia induces mechanical allodynia and thermal hyperalgesia. (A) Image displaying LED stimulation (red light: 625 nm, 45 ms light on, 5 ms light off, 20 Hz) in a mouse (left) and a schematic representation of the optogenetic ferrule placement region for light stimulation of mouse spinal cord (right). (B) Timeline of experimental procedures. (C) Gait analysis using the footprint test displays the stride length of mice after
sham surgery, control mice after implantation of the optic ferrule (ferrule-implanted control), and ReaChR mice after implantation of the optic ferrule with ReaChR activation with 30-min duration (ferrule-implanted ReaChR). n=5 mice/group. (D) Rotarod test of motor coordination in the 3 groups described in C. n=5-7 mice/group. (E, F) Mechanical (E) and thermal (F) pain hypersensitivity of ReaChR mice at 1 hr after light stimulation with different durations (5-, 15- and 30- min). Both contralateral (Contra.) and ipsilateral (Ipsi.) side of light stimulation were measured. n=5 mice/group. *****P<0.0001 Two-way ANOVA with multi comparisons. (G, H) Mechanical (G) and thermal (H) pain hypersensitivity of ReaChR mice after the first and second light stimulation for 30 min. Red bar indicates the time points of light stimulation at day 0 and day 9. n=7-8 mice/group. ***P<0.001, ****P<0.0001 Control Ipsi. vs ReaChR Ipsi., Two-way ANOVA with multi comparisons.

Figure 4. Optogenetic stimulation induces microglia activation. (A) Representative images of Iba1+ microglia after light stimulation in control mice (left) and ReaChR mice (right). Scale bar, 40 μm. (B) Summarized data showing that the number of Iba1+ microglia was increased in the ipsilateral dorsal horn at 1-5 days after light stimulation in ReaChR mice as compared to the contralateral side and control group. n=3 mice/group. ns. P>0.9999, *P=0.0476, **P=0.0049, ****P<0.0001 for Contra. vs. Ipsi., Two-way ANOVA with multiple comparisons. (C) Representative single microglia images in the spinal cord using Iba1 immunostaining and after being skeletonized following optogenetic stimulation. Scale bar, 40 μm. (D) Sholl analysis indicates that optogenetic stimulation reduced the complexity of ReaChR microglia compared with the control group. n=5 mice/group, ****P < 0.0001. Multiple paired Student’s t-test. (E) Representative immunostaining images showing the increased expression of Kv1.3 in Iba1+ microglia after light stimulation in the ipsilateral dorsal horn compared with the contralateral side without light stimulation in ReaChR mice. Scale bar, 40 μm.

Figure 5. Optogenetic stimulation of microglia increases neuronal activity. (A) A schematic figure shows optogenetic stimulation and C-fiber-evoked field potential in vivo recording in mice. (B) Timeline of experimental procedures for optogenetic stimulation and in vivo electrophysiology. (C) Pooled results showing the time course of C-fiber-evoked field potentials following optogenetic stimulation of spinal microglia in ReaChR or control mice. Light stimulation enhances C-fiber responses for more than 60 mins in ReaChR mice but not in control mice. C-fiber-evoked field potential was normalized to the baseline. n=4 mice/group. ****P<0.0001. Two-way ANOVA with multi comparisons. (D) Representative traces of C-fiber-
evoked field potentials from ReaChR mice recorded at 45 min of baseline (i) and after light stimulation, 60 min (ii). The amplitude of C-fiber-evoked response (red vertical line) was determined with parameter extraction software WinLTP. n=5 mice/group. Scale bars, 100 ms (X) and 0.2 mV (Y). (E) Timeline of experimental procedures for immunostaining after optogenetic stimulation. (F) Representative immunostaining images showing the c-fos expression in ipsilateral side (Ipsi.) of spinal cord in ReaChR mice at 2 hr after light stimulation but not in contralateral side (Contra.) or in control mice. The insets show the magnified images of boxed areas. Scale bar, 40 μm. (G) Immunofluorescence images of PKCα (red) with either Iba1 (green) or NeuN (green) in the spinal cord in ReaChR or control mice after light stimulation. PKCα was not detected in the control group. At 3 days after light stimulation in ReaChR mice, PKCα was co-localized with NeuN+ neurons but not with Iba1+ microglia in the spinal dorsal horn. Scale bar, 40 μm.

**Figure 6. Optogenetic stimulation of microglia increases IL-1β production and release.** (A) Representative immunostaining images showing IL-1β upregulation in Iba1+ cells in the spinal cord of ReaChR mice at 1 day after light stimulation but not in the control group. Scale bar, 40 μm. (B) Timeline of experimental procedures for ReaChR induction, optogenetic stimulation, and IL-1β ELISA from cultured microglia. (C) Image showing light stimulation of primary ReaChR microglia in culture. (D) Pooled ELISA results indicating the released IL-1β in culture media of primary microglia following optogenetic stimulation. Light stimulation increases IL-1β release at 1, 6, or 24 hr from ReaChR expressing microglia but not from control microglia culture. ***P<0.001, ****P<0.0001. Two-way ANOVA with multi comparisons.

**Figure 7. Inhibition of IL-1β alleviated microglial ReaChR-induced pain hypersensitivity.** (A) Timeline of experimental procedures for IL-1ra administration and light stimulation during in vivo recordings. (B) The time course of C-fiber-evoked field potentials before and after optogenetic stimulation of spinal microglia in each group (ReaChR+vehicle, ReaChR+IL-1ra, and control+IL-1ra). Vehicle and IL-1ra (10μL, 50 ng/mL) was topically administered after 30 min baseline recording and subsequently light stimulation was applied at 60 min. Black arrows and red bar indicate the time points of IL-1ra treatment and light stimulation, respectively (A, B). n=4-5 mice/group. ****P<0.0001 vehicle vs IL-1ra. Two-way ANOVA with multi comparisons. (C, D) Light-induced mechanical (C) and thermal (D) pain hypersensitivity in ipsilateral or contralateral side of ReaChR mice after treatment of IL-1ra or vehicle. Data represented as mean ± SEM, n=5-8 mice/group. ****P<0.0001 vehicle vs IL-1ra. Two-way ANOVA with multi
comparisons. (E) A schematic diagram illustrates that optogenetic stimulation of spinal microglia triggers chronic pain in ReaChR mice and highlights the possible mechanism involved. Red light stimulation (1) on the spinal dorsal horn induces microglial activation (2), which releases IL-1β (3) and increases neuronal hyperactivity (4), leading to chronic pain hypersensitivity (5).
Supplementary Figure Legends:

Supplementary Figure 1. Expression of microglial ReaChR in CX3CR<sup>creER<sup>+/</sup>: R26<sup>LSL-ReaChR</sup>/+ transgenic mice. (A) Representative immunostaining images of rhodopsin (red) with either Iba1, GFAP, or NeuN (green) in the spinal dorsal horn of CX3CR<sup>creER<sup>+/</sup>: R26<sup>LSL-ReaChR</sup>/+ mice without TM injection. Rhodopsin expression was not detected under these conditions. Scale bar, 40 μm. (B) Representative immunostaining images of rhodopsin with Iba1 in the cortex of CX3CR<sup>creER<sup>+/</sup>: R26<sup>LSL-ReaChR</sup>/+ mice with or without TM injection. Rhodopsin expression was co-localized with Iba1<sup>+</sup> cells in the cortex of CX3CR<sup>creER<sup>+/</sup>: R26<sup>LSL-ReaChR</sup>/+ mice when TM was administered, but was absent without TM injection. Scale bar, 40 μm.

Supplementary Figure 2. Optogenetic stimulation of spinal microglia did not induce ATF3 expression in dorsal root ganglion (DRG). (A) Representative immunostaining images of ATF3 in L4-5 DRGs of ReaChR mice following light stimulation. Very few ATF3<sup>+</sup> cells were observed following stimulation. Scale bar, 40 μm. (B) SNT surgery induced significant ATF3 expression within ipsilateral L4 DRG (Ipsi.) but not contralateral side (Contra.). Scale bar, 40 μm.

Supplementary Figure 3. Sex differences were not observed for mechanical allodynia following optogenetic stimulation of microglia. (A, B) Measurement of mechanical (A) and thermal (B) pain hypersensitivity in male and female ReaChR mice. Results show that while no difference was observed between male and female mice in regards to mechanical allodynia, thermal hypersensitivity was observed to last longer in female mice when compared to male mice. n=5 mice/group ****P<0.0001, male versus female. Two-way ANOVA with multiple comparisons.

Supplementary Figure 4. Exogenous IL-1β induced pain hypersensitivity and increased microglial expression of IL-1β. (A, B) Measurement of mechanical (A) and thermal (B) pain hypersensitivity of control mice after intrathecal injection of recombinant IL-1β (10 μL, 20 ng/mL). Results show enhanced mechanical and thermal hypersensitivity following administration of IL-1β. n=5 mice/group *P<0.05, **P<0.01, ****P<0.0001, Vehicle versus IL-1β. Two-way ANOVA with multi comparisons. (C) Representative immunostaining images demonstrating the up-regulation of IL-1β expression in Iba1<sup>+</sup> cells within the spinal dorsal horn1 day after IL-1β intrathecal injection. Scale bar, 40 μm.
Supplementary Figure 5. IL-1ra prevents PKCα upregulation in the spinal cord following optogenetic stimulation. Representative images of PKCα immunostaining (red) with either Iba1 or NeuN (green) in the spinal dorsal horn of ReaChR mice following light stimulation and intrathecal injection of IL-1ra (10µL, 50 ng/mL). Light-induced PKCα expression was not detected in NeuN+ cells receiving IL-1ra 3 days after light stimulation. However, light-induced Iba1+ microglia activation was preserved in the presence of IL-1ra. Scale bar, 40 µm.
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Figure 1

A

CX3CR1<sup>creER</sup> X R26<sup>LSL-ReaChR</sup>

CX3CR1<sup>creER/+</sup>: R26<sup>LSL-ReaChR/+</sup>

B

DH

VH

Rhodopsin

Thoracic

Lumbar

C

Iba1

Rhodopsin

Iba1/Rhodopsin

Merge

GFAP

Rhodopsin

GFAP/Rhodopsin

Merge

NeuN

Rhodopsin

NeuN/Rhodopsin

Merge

CX3CR1<sup>creER/+</sup>: R26<sup>LSL-ReaChR/+</sup>

D

Iba<sup>+</sup> cells (%)

GFAP<sup>+</sup> cells (%)

NeuN<sup>+</sup> cells (%)

Colocalized with Rhodopsin

Not Colocalized with Rhodopsin
Figure 3

Optogenetic fiber implantation

Behavioral test

1 week

Recovery for 2 weeks

TM injection (i.p.)

1st light

Day 1

Day 5

2nd light

1 hr

Day 3

Day 7

A

B

C

D

E

F

G

H

Mechanical pain test

Thermal pain test

Light (625 nm, 20 Hz, 30 min)

Light
Figure 4

A. Immunofluorescence images showing the distribution of Iba1+ cells in control and ReaChR+light conditions.

B. Bar graph representing the number of Iba1+ cells per mm² for control and ReaChR conditions across different time points.

C. Magnified view of neuronal structures under control and ReaChR+light conditions.

D. Graph depicting the number of transections across different radial distances from the soma.

E. Confocal images showing the expression of Iba1 and Kv1.3 under control and ReaChR+light conditions.

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Figure 5

A. Diagram showing the experimental setup with LED light, glass pipette, and spinal cord.

B. Graph showing time (min) on the x-axis and C-fiber response (%) on the y-axis. The graph includes two conditions: Control+light and ReaChR+light.

C. Graph with two conditions: Control+light and ReaChR+light. The graph shows a comparison of the C-fiber response over time (min).

D. Graph showing before light (i) and after light (ii) conditions with a scale of 0.2 mV and 100 ms.

E. Immunostaining showing c-Fos and PKCα with light conditions specified.

F. Images showing c-Fos expression in control+light and ReaChR+light conditions in both ipsi. and contra. regions.

G. Images for NeuN, PKCα, NeuN/PKCα, and Merge showing control+light and ReaChR+light conditions.
Figure 6

A

Control+light

ReaChR+light

B

4-OHT (5 µM) treatment

Conditioned media (IL-1β ELISA)

Primary microglia

Seeding

C

D

IL-1β (pg/ml)

Control+light

ReaChR+light

Before 1 hr 6 hr 24 hr

ns **** ****
Figure 7

A. Thermal pain test

B. Mechanical pain test

C. Thermal pain test

D. Mechanical pain test

E. Schematic diagram of pain mechanisms:

1. Red LED 625 nm
2. Microglial activation
3. IL-1β
4. Neuronal activity increase
5. Chronic pain
A

CX3CR1<sup>creER/+</sup>: R26<sup>LSL-ReaChR/</sup> 

B

CX3CR1<sup>creER/+</sup>: R26<sup>L</sup>
A

| ReaChR+light | ReaChR+light |
|--------------|--------------|
| ATF3         | ATF3         |
| L4           | L5           |
| - Tamoxifen  | + Tamoxifen  |

B

| SNT |
|-----|
| ATF3 | ATF3 |
| L4   | L4   |
| Ipsi. | Contra. |

Suppl. 2

**A**

- Tamoxifen

**B**

+ Tamoxifen
Suppl. 3

ReaChR+light Contra. ♂
ReaChR+light Ipsi. ♂
ReaChR+light Contra. ♀
ReaChR+light Ipsi. ♀

A  Mechanical pain test

B  Thermal pain test

50% withdrawal threshold (g)
Latency (s)

Baseline  1 hr  1D  3D  5D  7D

**** ****

Baseline  1 hr  1D  3D  4D  5D  7D

0.0  0.5  1.0  1.5  2.0

0.0  5.0  10.0  15.0  20.0

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1 day after IL-1β recombinant protein intrathecal injection

**Mechanical pain test**

A

**Thermal pain test**

B

C

1 day after IL-1β recombinant protein intrathecal injection

**50% withdrawal threshold (g)**

**Latency (s)**

Vehicle

IL-1β

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IL-1ra intrathecal injection

| 3 days |  |
|--------|---|
| NeuN   | PKCα | NeuN/PKCα |
| Iba1   | PKCα | Iba1/PKCα |

ReaChR+light