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Development of opioid-induced hyperalgesia depends on reactive astrocytes controlled by Wnt5a signaling

Short title: Astrogliosis in opioid-induced hyperalgesia

One sentence summary: Neuron-to-astrocyte Wnt5a signaling controls the pathogenesis of opioid-induced hyperalgesia via astrogliosis

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Abstract.

Opioid analgesics are the frontline pain medicine for managing various types of pain. Paradoxically, repeated use of opioid analgesics may cause an exacerbated pain state known as opioid-induced hyperalgesia (OIH). OIH significantly contributes to dose escalation and consequently opioid overdose. In addition to neuronal malplasticity, emerging evidence suggests a critical role of reactive glia in OIH development. A potential astrocytic underpinning of OIH pathogenesis is indicated by their prominently activation in OIH animal models. However, this hypothesis has not been conclusively tested and the mechanism underlying the astrocyte activation remains unclear. Here, we show that reactive astrocytes (a.k.a. astrogliosis) are critical for OIH development in mice. Genetic ablation of astrogliosis inhibited the expression of OIH and morphine-induced neural circuit polarization (NCP) in the spinal dorsal horn (SDH). We also found that Wnt5a is a neuron-to-astrocyte signal that is required for morphine-induced astrogliosis. Conditional knock-out of Wnt5a in neurons or its co-receptor ROR2 in astrocytes blocked not only morphine-induced astrogliosis but also OIH and NCP. Furthermore, we showed that the Wnt5a-ROR2 signaling-dependent astrogliosis contributes to OIH via inflammasome-regulated IL-1β. Our results reveal an important role of morphine-induced astrogliosis in OIH pathogenesis and elucidate a neuron-to-astrocyte intercellular Wnt signaling pathway that controls the astrogliosis.

INTRODUCTION

Opioid analgesics such as morphine are the gold standard for treating severe pain. However, among other side effects, chronic use or abuse of opioids paradoxically increases pain sensitivity, known as opioid-induced hyperalgesia (OIH)[1-4]. OIH is a critical contributor to the common clinical practice of dose escalation for effective pain relief and opioid overdose[4-6]. Despite its clinical significance, the mechanisms of OIH pathogenesis are still poorly understood, hampering the development of effective interventions.

Several forms of malplasticity in pain neuronal circuits are implicated in OIH pathogenesis. These include opioid-induced sensitization of nociceptors in peripheral, sensitization of second order neurons in the spinal cord, and enhanced descending facilitation[4,7]. Long-term potential (LTP) of synapses between nociceptive C fibers and second order neurons in the spinal cord dorsal horn is considered as a critical synaptic mechanism facilitating OIH expression[7,8]. Indeed, LTP and OIH appear to use similar molecular signaling pathways that involve NMDA receptors, CaMKII and BDNF to support their expression[7]. LTP might provide a synaptic support to enhance communication between pronociceptive neurons. It is less clear whether other forms of synaptic plasticity in the pain neuronal circuits contribute to OIH expression.

In addition to neuronal malplasticity, opioids also induce reactions of glia,

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including microglia and astrocytes[7]. Microglia were reported to play a critical role in OIH [9] [but see also refs [1,10]]. Astrocytes are activated in the spinal cords following different paradigms of opioid administration[11,12]. However, the potential role of reactive astrocytes in OIH expression has not been conclusively established, and the mechanism by which opioids cause the activation of astrocytes has not elucidated.

Astrocytes intimately interact with neurons, especially with synapses to form tripartite synaptic structures[13]. By interacting with synapses, astrocytes are positioned to sense and respond to synaptic activity-regulated signals from neurons. Because reactive astroglia are widely observed in pain pathways of various models of pathological pain[14], it is tempting to conceive that pain-associated hyper-synaptic activity may modulate the activation of astrocytes. However, such neuron-to-astrocyte signals have not been elucidated, and their contribution to OIH expression has not been established.

On the other hand, astrocytes may also regulate neuronal functions. Under normal physiological conditions, astrocytes play essential roles in regulating central nervous system homeostasis[15], plasticity of neural circuits[16], and synaptic transmission [13,17,18]. However, how reactive astrocytes may regulate synaptic transmission and plasticity remains elusive. In particular, it is unclear whether reactive astrocytes contribute to malsynaptic plasticity of pain circuits associated with OIH development. Although reactive astrocytes are thought to dysregulate neuronal circuits by secreting bioactive molecules such as chemokines and cytokines, the involvement of such a mechanism in OIH pathogenesis has not been tested directly.

In this study, we use a mouse OIH model to show that reactive astrocytes are critical for morphine-induced OIH and malsynaptic plasticity in the SDH. We also demonstrate that the morphine-elicited astrocyte activation is controlled by a neuron-to-astrocyte intercellular Wnt5a-ROR2 signaling pathway. We further elucidate that the reactive astrocytes promote OIH and malsynaptic plasticity via inflammasome-regulated IL-1β.

RESULTS

Reactive astrocytes play essential role in OIH expression and maintenance.

To induce OIH, we repeatedly injected wild-type (WT; C57BL) mice with morphine (i.p.; 20 mg/kg/day for 4 days). We observed the expression of mechanical OIH following the morphine administration (Fig. 1C), as reported previously [2,19]. In these animal models, we found microglia and astrocyte activation in the spinal dorsal horn (SDH) (Fig. 1A-1B), consistent with previous observations[20,21]. A critical role of microglia in OIH was proposed in an early study[9], but inconsistent findings were reported later[1,10]. In this study, we aimed to examine the potential role of reactive astrocytes in OIH development. To this end, we employed a genetic approach to selectively ablate reactive astrocytes, using GFAP-thymidine kinase (TK) transgenic mice[22]. The transgenic mice were administered with ganciclovir (GCV), which would be metabolized to nucleotide analogues by TK in astrocytes. The GCV-derived nucleotide analogues are toxic to proliferating cells, and thus would specifically induce cell death of reactive astrocytes.

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Von Frey tests showed that the GFAP-TK transgenic mice expressed mechanical OIH similarly as WT mice (Fig. 1C). To determine the contribution of astrogliosis to the expression of OIH, we intrathecally (i.t.) injected GCV (5 mg/kg) during the first 2 days of morphine administration. We observed that GCV administration blocked the morphine-induced increase of GFAP but not Iba1 (Fig. 1A-1B), indicating selective ablation of reactive astrocytes without affecting microglia. Importantly, the GCV treatment impaired OIH expression in GFAP-TK but not in WT mice (Fig. 1C). Significant GCV-induced impairment of OIH in the transgenic mice started to develop one day after the first GCV injection, suggesting a critical role of reactive astrocytes in early expression of OIH.

We then sought to determine if astrogliosis contributes to OIH maintenance. To this end, we tested the effect of ablation of reactive astrocytes on established OIH. In this experiment, GCV was administered six days after the first injection of morphine, when OIH was fully expressed (Fig. 1D). We observed that the GCV administration significantly reversed the established OIH in GFAP-TK but not in WT mice (Fig. 1D), indicating an important role of reactive astrocytes in OIH maintenance. These results collectively showed that reactive astrocytes were essential for both expression and maintenance of OIH.

Reactive astrocytes mediate morphine-induced neural circuit polarization in the SDH.

To gain insight into the neural circuitry mechanism by which astrogliosis contributes to OIH, we tested the effect of astrogliosis ablation on morphine-induced malplasticity in pain neural circuits. When we performed whole-cell recording of SDH neurons in morphine-treated GFAP-TK mice, we found that morphine increased both the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) and the amplitude of evoked EPSCs (eEPSCs) of excitatory neurons (Fig. 2A-2C), identified by their characteristic non-tonic firing patterns [23,24]. By contrast, morphine decreased the sEPSC frequency and eEPSC amplitude of inhibitory neurons (Fig. 2D-2F), identified by their characteristic tonic firing pattern [23,24]. These findings indicate that morphine administration polarizes neural circuits in the SDH, by increasing excitatory inputs to excitatory neurons and meanwhile decreasing excitatory inputs to inhibitory neurons. The observed neural circuit polarization (NCP) might contribute to OIH expression, by facilitating activation of the SDH pain pathway.

Astrocytes normally play important role in maintaining homeostasis of neural circuits [15], but it is unclear how astrogliosis modulates neural circuits during pain pathogenesis. Hence, we set to test the contribution of astrogliosis to morphine-induced NCP. To this end, we determined the effect of astrogliosis ablation on EPSCs of excitatory and inhibitory neurons in the SDH of GFAP-TK transgenic mice. We found that GCV treatment abolished the morphine-induced increase in sEPSC frequency and eEPSC amplitude of excitatory neurons (Fig. 2A-2C).

On inhibitory neurons, we observed that GCV treatment by itself decreased sEPSC frequency and eEPSC amplitude of inhibitory neurons in GFAP-TK mice without morphine administration (Fig. 2D-2F), indicating excitatory inputs on inhibitory

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neurons under physiological conditions are more sensitive to astrocyte ablation than excitatory inputs on excitatory neurons. Importantly, after GCV treatment morphine failed to induce further decease of sEPSC frequency and eEPSC amplitude on inhibitory neurons (Fig. 2D-2F). Together, these results indicated that astrogliosis was critical for morphine to induce NCP, including the increase of sEPSC frequency and eEPSC amplitude on excitatory neurons and the decrease of sEPSC frequency and eEPSC amplitude on inhibitory neurons.

Neuronal Wnt5a is critical for morphine to induce astrogliosis, NCP and OIH.

Having shown the essential role of astrogliosis in OIH and NCP, we next sought to elucidate the mechanism by which morphine induces astrocyte activation. To this end, we focused on the potential role of Wnt5a signaling, which is implicated in pain pathogenesis[25-27]. Wnt5a is secreted protein mainly expressed by neurons[28], and is upregulated by in the SDH by pain signals[29]. Wnt5a secretion is controlled by synaptic activity[30,31]. The involvement of Wnt5a in regulating astrogliosis is suggested by the observations that Wnt5a antagonist inhibits astrocyte activation in pain models[32,33] and JNK, a critical downstream target in the Wnt5a signaling pathway, is critical for astrogliosis in a neuropathic pain model[34]. Based on these prior findings, we hypothesized that Wnt5a secreted from neurons hyperactivated following morphine treatment activated astrocytes. To test this hypothesis, we generated neuronal Wnt5a conditional knock-out (CKO) mice (Wnt5a-CKO-S) by crossing floxed Wnt5a mice[35] with synapsin 1-Cre mice[36]. We observed that morphine administration significantly upregulated spinal Wnt5a protein in the WT but not in Wnt5a-CKO-S mice (Fig. 3A), indicating morphine stimulated Wnt5a upregulation predominantly in spinal neurons. Importantly, we found that, unlike WT mice, morphine completely failed to induce GFAP upregulation in the spinal cord of the Wnt5a-CKO-S mice (Fig. 3B), indicating deletion of Wnt5a in neurons abolished morphine-induced astrogliosis. To confirm these findings, we also generated another Wnt5a CKO mutant to delete Wnt5a in neural stem cells using nestin-Cre[37] (Wnt5a CKO-N). We found that this Wnt5a mutation also abolished morphine-induced Wnt5a upregulation and astrogliosis (Sup. Fig. 1). These findings collectively showed that neuronal Wnt5a was essential for morphine to induce astrogliosis.

As reactive astrocytes were crucial for OIH induced by morphine (Fig. 1C, 1D) and neuronal Wn5a was required for the astrocyte activation (Fig. 3B), we hypothesized that the OIH expression depended on neuronal Wnt5a. To test this hypothesis, we determined the effect of neuronal Wnt5a CKO on OIH. The result showed that OIH expression was abolished in Wnt5a CKO-S mice (Fig. 3C). We observed similar inhibitory effect on OIH in Wnt5a CKO-N mice (Fig. 3D). These data together demonstrated the requirement of neuronal Wnt5a for OIH development.

Because of the observed role of astrogliosis in NCP (Fig. 2) and neuronal Wnt5a in the astrogliosis (Fig. 3B), we further hypothesized that neuronal Wnt5a was important for NCP expression. To test this, we determined the expression of NCP in the SDH of Wnt5a CKO-S mice. We found that the morphine-induced increase of sEPSC frequency of excitatory neurons (Fig. 3E) and the decrease of sEPSC

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frequency of inhibitory neurons (Fig. 3F) were abolished in Wnt5a CKO-S mice. These results showed that neuronal Wnt5a mediated the expression of NCP in the SDH.

Astrocytic ROR2 receptor is crucial for astrogliosis, NCP and OIH induced by morphine.

The results described above demonstrate a key role of Wnt5a from neurons in mediating morphine-induced astrocyte activation. Next, we sought to further elucidate the underlying mechanism by which Wnt5a activates astrocytes. We hypothesized that Wnt5a co-receptor ROR2 on astrocytes [28] was critical for the astrogliosis. To test this hypothesis, we generated astrocytic CKO of ROR2 by crossing floxed ROR2[38] and GFAP-Cre[39] mouse lines. Similar to the effect of neuronal Wnt5a CKO (Fig. 3), we observed that the astrocytic ROR2 CKO also blocked morphine-induced GFAP upregulation, indicating inhibition of astrogliosis (Fig. 4A).

As astrogliosis and neuronal Wnt5a are critical for OIH development (Fig. 1; Fig. 3), the finding of the astrogliosis blockage by the ROR2 CKO led us to predict that the mutant mice would be impaired in OIH development. In support of this hypothesis, we indeed found that the expression of OIH was abolished in the ROR2 CKO mice (Fig. 4B), as in the Wnt5a CKO-S and Wnt5a CKO-N mice (Fig. 3).

Because of the observed contribution of astrogliosis and neuronal Wnt5a to NCP (Fig. 2; Fig. 3), we further hypothesized that the ROR2 CKO would impair the expression of NCP. We tested this hypothesis by determining effect of the ROR2 CKO on sEPSCs of neurons in the SDH. The results showed that both the morphine-induced increase in sEPSC frequency of excitatory neurons and decrease in sEPSC frequency of inhibitory neurons in the SDH were blocked in ROR2 CKO mice (Fig. 4C-4D). The findings suggested that, similar to neuronal Wnt5a, astrocytic ROR2 also played an important role in morphine-induced astrogliosis, OIH, and NCP.

Wnt5a signaling-regulated reactive astrocytes control OIH and NCP via IL-1β.

The above results from Wnt5a CKO and ROR2 CKO mice collectively suggest that Wnt5a secreted from neurons stimulates astrocytic ROR2 receptor to promote morphine-induced astrogliosis, and that this neuron-to-astrocyte Wnt5a-ROR2 signaling pathway contributes to OIH expression via astrogliosis. Next, we sought to further understand the mechanism by which Wnt5a signaling-mediated astrogliosis regulates OIH pathogenesis. Previous studies reveal that reactive astrocytes contribute to pain pathogenesis by releasing proinflammatory mediators[14]. Indeed, we observed morphine-induced upregulation of spinal activated (cleaved) IL-1β (Fig. 5A), a key proinflammatory cytokine that is implicated in OIH[40] and regulated by Wnt5a signaling[28]. Importantly, CKO of Wnt5a in either Wnt5a CKO-S or CKO-N mice abolished the IL-1β upregulation (Fig. 5A). CKO of ROR2 in astrocytes also blocked IL-1β upregulation (Fig. 5A). These results suggested that the neuron-to-astrocyte Wnt5a-ROR2 signaling controlled morphine-induced IL-1β upregulation.

Because the neuron-to-astrocyte Wnt5a-ROR2 signaling controls both astrocyte activation (Fig. 3; Fig.4) and IL-1β upregulation induced by morphine (Fig. 5A), we

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hypothesized that the reactive astrocytes produced IL-1β. To test this hypothesis, we determined the effect of ablating reactive astrocytes on the IL-1β upregulation. We found that astrogliosis ablation by GCV in GFAP-TK transgenic mice significantly impaired morphine-induced IL-1β upregulation in the spinal cord (Fig. 5B). On the other hand, ablation of microglia by the CSF1R inhibitor PLX5622[41-43] did not significantly affect morphine-induced spinal IL-1β upregulation (Fig. 5C). These results suggested that reactive astrocytes, rather than microglia, were the major cell source of morphine-induced IL-1β. Previous studies suggest astrocytic IL-1β in other pain models[11].

Because reactive astrocytes contributed to morphine-induced OIH (Fig. 1) and produced IL1β (Fig. 5B), we hypothesized that astrogliosis promoted OIH via IL-1β. Hence, we tested the pathogenic role of IL-1β in OIH expression. In these experiments, we used the endogenous IL-1 receptor antagonist IL-1Ra to block Il-1β signaling [40]. We observed that IL-1Ra administration (20 μg/kg/day for the first 3 days, i.t.) abolished the expression of OIH, without affecting baseline mechanical sensitivity (Fig. 6A). These results suggested that IL-1β produced by reactive astrocytes was critical for OIH pathogenesis.

IL-1Ra administration also blocked the morphine-induced increase in sEPSC frequency (Fig. 6B) and eEPSC amplitude (Fig. 6C-6D) in excitatory neurons in the SDH. Furthermore, using GAD67-GFP transgenic mice[44], we found that IL-1Ra blocked the morphine-induced decrease in sEPSC frequency (Fig. 6E) and sEPSC amplitude (Fig. 6F-6G) of GABAergic inhibitory neurons in the SDH. Together, these results indicated that the morphine-induced expression of NCP was controlled by IL-1β produced by Wnt5a-ROR2 signaling-activated astrocytes (Fig. 5A-5B).

Wn5a-ROR2 signaling activates IL-1β via Inflammasomes.

Having identified a critical of IL-1β in OIH, we wanted to further understand the mechanism by which morphine regulates astrocytic IL-1β. We hypothesized inflammasome, a key protein complex that controls IL-1β processing[45] and is regulated by morphine to prolong neuropathic pain[46], played a key role in this process. We observed that morphine administration upregulated the active forms of both IL-1β and caspase 1 (Cas-1), the inflammasome proteinase that processes pro-IL-1β protein (Fig. 7A). In addition, pharmacological blockade of the inflammasome with AC-YVAD-CMK, a selective inhibitor of Cas-1, impaired the upregulation of Cas-1 and IL-1β (Fig. 7A). These results indicated that morphine stimulated spinal IL-β activation via the inflammasome. Furthermore, inhibition of the inflammasome by eAC-YVDA-CMK or Cas-1 siRNA also blocked the expression of OIH (Fig. 7B-7C). These data collectively demonstrated a critical role of inflammasome in the regulation of IL-1β during OIH expression.

Because morphine induced IL-1β via Wnt5-ROR2 signaling-regulated astrogliosis (Fig. 3; Fig. 4; Fig. 5B), we hypothesized the neuron-to-astrocyte Wnt5a-ROR2 signaling regulated morphine-induced inflammasome activation. To test this idea, we determined the effect of neuronal Wnt5a CKO and astrocytic ROR2 CKO on inflammasome activation, by measuring active Cas-1 levels. We found that either the

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Wnt5a CKO-S or ROR2 CKO abolished the morphine-induced increase of active Cas-1 (Fig. 7D). These findings suggested that Wnt5a-ROR2 signaling regulated the activation of inflammasome to process IL-1β in astrocytes.

DISCUSSION

We show that astrogliosis is crucial for morphine-induced OIH and expression of NCP in the SDH. The morphine-induced astrogliosis, NCP, and OIH requires Wnt5a in neurons and the ROR2 receptor in astrocytes. Furthermore, the effects of morphine on neural circuits and pain behavior are mediated by IL-β controlled by Wnt5a-ROR2 pathway-regulated inflammasome activation. These findings suggest bidirectional interactions between neurons and astrocytes during the pathogenesis of OIH. We propose that morphine induces Wnt5a release from neurons to stimulate astrocytic ROR2 receptor to activate astrocytes and inflammasome to produce IL-1β, and active IL-1β released from reactive astrocytes in return stimulates pain processing neurons in the SDH to promote NCP and OIH (Fig. 8).

Neuronal circuitry mechanism in the development of OIH.

Malplasticity of multiple components in the pain transmission pathway is implicated in OIH. Sensitization of peripheral nociceptors and second-order neurons in the spinal cord, long-term potentiation (LTP), and descending facilitation are among the best characterized forms of neuronal malplasticity proposed for OIH expression [3,5,7,8,47,48]. These and other lines of evidence suggest the importance of malplasticity of excitatory systems in OIH expression[7]. However, because functional homeostasis of normal neural circuits is maintained by the coordinated activity of excitatory and inhibitory neurons, it is also important to understand if and how this coordination is altered during OIH development. Yet, we currently know little in this aspect. We find that morphine indeed induces hyper-activity of excitatory inputs on excitatory neurons and hypo-activity of excitatory inputs on inhibitory neurons (Fig. 2). The increased synaptic drive on excitatory neurons and decreased synaptic drive on inhibitory neurons reveal a form of morphine-induced polarization in the SDH neural circuit. This form of neural circuit polarization (NCP) would facilitate hyper-activation of the pain neural circuit in the SDH and thus promote the expression of OIH. This neural circuitry mechanism may also play a critical role in HIV-1 gp120-induced pain pathogenesis[49].

The increase of excitatory synaptic drive on excitatory neurons is consistent with the involvement of sensitization of pain processing neurons in the SDH during OIH expression, as suggested by previous studies[7]. It is unexpected for us to observe the decrease of excitatory synaptic drive on inhibitory neurons. Inhibitory loss, manifested as loss of inhibitory neurons or synapses, has been suggested previously as a potential mechanism underlying pain pathogenesis[50-52]. Our findings reveal novel form of loss of inhibition implicated in OIH development.

Our finding of NCP reveals a novel circuit mechanism that supports the expression of OIH. The increase excitatory inputs to excitatory neurons and the decrease of excitatory inputs to inhibitory neurons in NCP may promote OIH by facilitating hyper-activation of pain transmission circuits.

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Astrocytic mechanism in regulation of OIH expression.

Previous studies on OIH mechanism mainly concentrate on the contribution of neuronal plasticity. The involvement of glial cells is recognized later, as indicated by the activation of microglia and astrocytes in rodent OIH models[7]. Microglia were suggested to modulate neuronal activity via BDNF during OIH expression[9]. However, other studies raise questions about the role of microglia[1,10]. Astrogliosis is associated with various chronic pain conditions, including OIH models, but its pathogenic contribution has not been tested conclusively [7,14]. Previous work suggests a contribution of activated astrocytes to pain development in animal models[53,54]. However, a recent study by Sasaki et al. show that inhibition of astrocyte activation by gap junction blockade did not ameliorate OIH[55]. It is important to note that the GFAP upregulation induced by morphine was not blocked in this study, raising uncertainty about the degree of astrogliosis inhibition.

Our current speculations of astrocytic function in pain pathogenesis are still largely based on circumventive evidence such as the correlation between astrogliosis and pain and the effect of glial inhibitors[53-55]. We take a genetic approach to specific ablate reactive astrocytes and thus directly evaluate the contribution of reactive astrocytes to OIH. We show that astrogliosis ablation during the early phase of OIH induction impaired the expression of mechanical OIH (Fig. 1C), whereas astrogliosis ablation after the establishment of OIH significantly reversed the OIH (Fig. 1D). These results strongly suggest an important role of reactive astrocytes in both expression and maintenance of OIH.

Our conclusion described above is also supported by the effect of astrogliosis inhibition induced by the CKO of Wnt5a or ROR2. We show that when morphine-induced astrogliosis is blocked by either neuronal Wnt5a CKO or astrocytic ROR2 CKO OIH is abolished (Fig. 3; Fig. 4). These cell-type-specific approaches of astrogliosis inhibition provide strong complementary evidence for a crucial contribution of reactive astrocytes to OIH.

How would reactive astrocytes promote OIH? Little is known about the answer to this important question. Astrocytic processes intimately interact with synapses to form a tripartite synaptic structure[13]. Under physiological conditions, normal astrocytes play key roles in neural circuit homeostasis, by maintaining cation (e.g. K+) equilibrium, uptaking released neurotransmitters and releasing gliotransmitters[56]. However, it is unclear how reactive astrocytes would disturb the circuit homeostasis, especially in during OIH development. We find that morphine induces NCP and ablation of astrogliosis suppresses the NCP expression (Fig. 2). These findings suggest that morphine-induced reactive astrocytes disturb circuit homeostasis and mediate the expression of NCP. This notion is supported by the findings that inhibition of astrogliosis in either the neuronal Wnt5a CKO or the astrocytic ROR2 CKO mouse also blocks NCP expression (Fig. 3; Fig. 4). Based on these results, we propose that reactive astrocytes facilitates OIH by disrupting homeostasis of neural circuits in the SDH and promoting NCP expression.

Then, how would reactive astrocytes promote NCP? Based on the characterized

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biological activity of reactive astrocytes, it has been speculated that they could facilitate OIH expression by releasing excitatory substances to stimulate synapses. The suggested excitatory factors include proinflammatory cytokines and chemokines, ATP and gliotransmitters[7]. However, this hypothesis has not been directly and systematically tested in the context of OIH. Our results suggest that the reactive astrocytes in the OIH models generate active IL-1β (Fig. 5). We further show that blockade of IL-1 signaling by IL-1Ra impairs both OIH and NCP (Fig. 6). Based on these findings, we suggest that reactive astrocytes contribute to NCP via IL-1β.

Wnt signaling in regulation of astrogliosis during OIH pathogenesis.

Mechanism of astrocyte activation during OIH pathogenesis remains poorly understood. We find that neuronal Wnt5a and astrocytic ROR2 receptors are critical for morphine to activate astrocytes in the spinal cord (Fig. 3; Fig. 4). These findings suggest that Wnt5a secreted from neurons stimulates its receptor ROR2 on astrocytes to activate astrocytes. Our results also show that this neuron-to-astrocyte Wnt5a-ROR2 signaling pathway is required for OIH and NCP (Fig. 3; Fig. 4), both of which are dependent on reactive astrocytes (Fig.1; Fig. 2).

Wnt5a expression and secretion are evoked by synaptic activity[30] and pain[29]. In addition, morphine is known to upregulate Wn5a in the spinal cord[32]. Hence, we propose that OIH induced by morphine elicits Wnt5a release from neurons to activate astrocytes. The requirement of neuronal Wnt5a indicates that morphine does not cause astrocyte activation by directly stimulating astrocytes in vivo. Instead, it activate astrocytes indirectly via neuronal Wnt5a. Consistent with this notion, μ-opioid receptor (MOR), which is critical for OIH development and critical for astrocyte activation induced by ultra-low-dose morphine [12], is not expressed in spinal astrocytes [57].

Our data further show that Wnt5a receptor ROR2 on astrocytes is critical for morphine to activate astrocytes (Fig. 4). Because JNK signaling is an important intracellular mediator of the Wnt5a-ROR2 pathway[58] and the JNK signaling are critical for astrocyte activation induced in OIH and neuropathic pain models [12,34], it is likely that morphine induced Wnt5a-ROR2 signaling activate astrocytes via JNKs.

The neuron-to-astrocyte Wnt5a-ROR2 intercellular signaling pathway is also critical for spinal astrocyte activation induced by gp120 in an HIV pain model[49], indicating that this pathway is a general mechanism that controls astrocyte activation induced by different neurological conditions.

Collectively, our results suggest that astrogliosis is a key cell target for OIH intervention. Furthermore, the results also suggest that the Wnt5a-ROR2 signaling pathway is a potential molecular target to block the astrogliosis during OIH pathogenesis.

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FIGURES

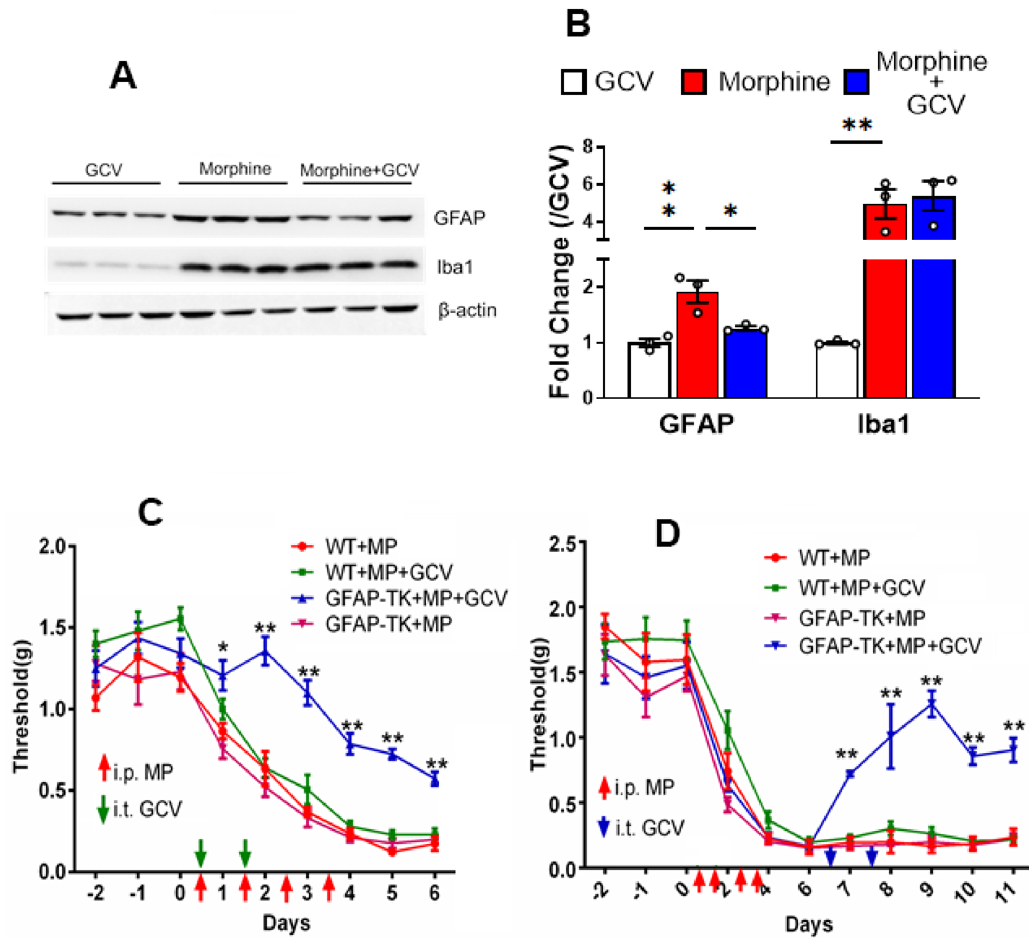

Figure 1. Reactive astrocytes are critical for morphine-induced hyperalgesia. (**A**) Immunoblotting analysis of GFAP and Iba1 in the spinal cord of the GFAP-TK transgenic mice treated with GCV, morphine or GCV+morphine using the drug administration paradigm shown in C. Mice were euthanized for spinal cord collection at day 7. (**B**) Quantitative summary of band intensity of immunoblots. GCV abolished morphine-induced up-regulation of GFAP but not Iba1, indicating GCV ablated specifically reactive astrocytes but not reactive microglia in GFAP-TK mice. GFAP and Iba1 protein levels were determined by immunoblotting analysis. *p<0.05; **p<0.01; ns, p>0.05. (**C**) Effect of astrogliosis ablation on OIH. GFAP-TK mice expressed mechanical OIH similarly as WT mice. Injections of GCV (green arrows; 5 mg/kg; i.t.) during the early phase of morphine (MP) administration (red arrows; 20 mg/kg; i.p.) inhibited the morphine-induced increase of mechanical sensitivity, measured by von Frey tests, specifically in GFAP-TK transgenic but not in WT mice (n=6/group). (**D**)

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Effect of astrogliosis ablation on OIH maintenance. Injections of GCV (blue arrows; i.t.) after OIH was established by morphine administration (red arrows; i.p.) reversed the morphine-induced increase of mechanical sensitivity in GFAP-TK transgenic but not in WT mice. (n=6/group).

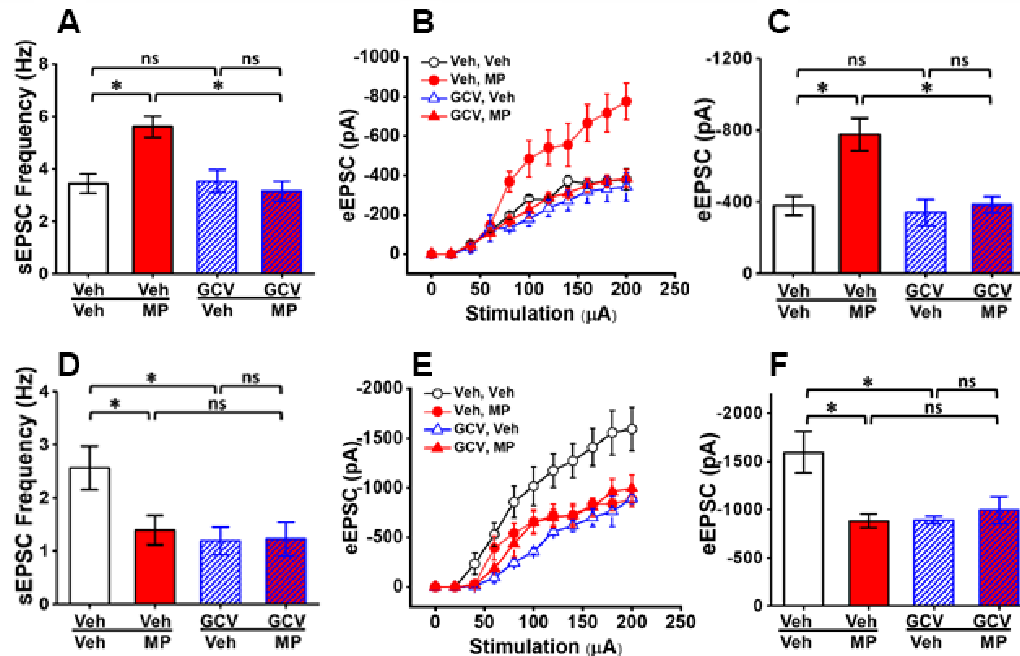

Figure 2. Reactive astrocytes are critical for morphine-induced NCP in the SDH. (**A**) Effect of astrocytic ablation on sEPSCs of non-tonic firing neurons in the SDH of GFAP-TK transgenic mice. Whole-cell patch recording was performed from spinal slices prepared from GFAP-TK transgenic mice (day 7) treated with morphine and/or GCV according to the paradigm shown in Fig. 1C. Morphine increased sEPSC frequency, whereas GCV abolished this increase. GCV did not affect basal sEPSC frequency. Veh/Veh: 21/3 (cells/mice); veh/MP: 60/5; GCV/Veh: 29/4; GCV/MP: 50/5. (**B**) Effect of astrogliosis ablation on eEPSCs of non-tonic firing neurons in the SDH of GFAP-TK transgenic mice. eEPSC amplitudes of patched SDH neurons were recorded from spinal slices prepared as in A. Morphine increased eEPSC amplitude, whereas GCV abolished this increase. GCV did not affect basal eEPSC amplitude. Veh/Veh: 24/3 (cells/mice); veh/MP: 48/4; GCV/Veh: 35/3; GCV/MP: 48/4 (**C**) Statistical analysis of eEPSC amplitude evoked by 200 μA stimulation shown in B. (**D**) Effect of astrogliosis ablation on sEPSCs of tonic firing neurons in the SDH of GFAP-TK transgenic mice. Morphine decreased sEPSC frequency. GCV alone also decreased sEPSC frequency. However, morphine did not further decrease sEPSC frequency after GCV treatment. Veh/Veh: 13/3 (cells/mice); veh/MP: 12/4; GCV/Veh: 20/5; GCV/MP: 26/5. (**E**) Effect of astrogliosis ablation on eEPSCs of tonic firing neurons in the SDH of GFAP-TK transgenic mice. Morphine decreased eEPSC amplitude. GCV alone also decreased eEPSC amplitude. However, morphine did not

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further decrease eEPSC amplitude after GCV treatment. Veh/Veh: 14/3 (cells/mice); veh/MP: 12/4; GCV/Veh: 20/3; GCV/MP: 26/4. (**F**) Statistical analysis of eEPSC amplitude evoked by 200 μA stimulation shown in E**.** *p<0.05; **p<0.01; ns, p>0.05.

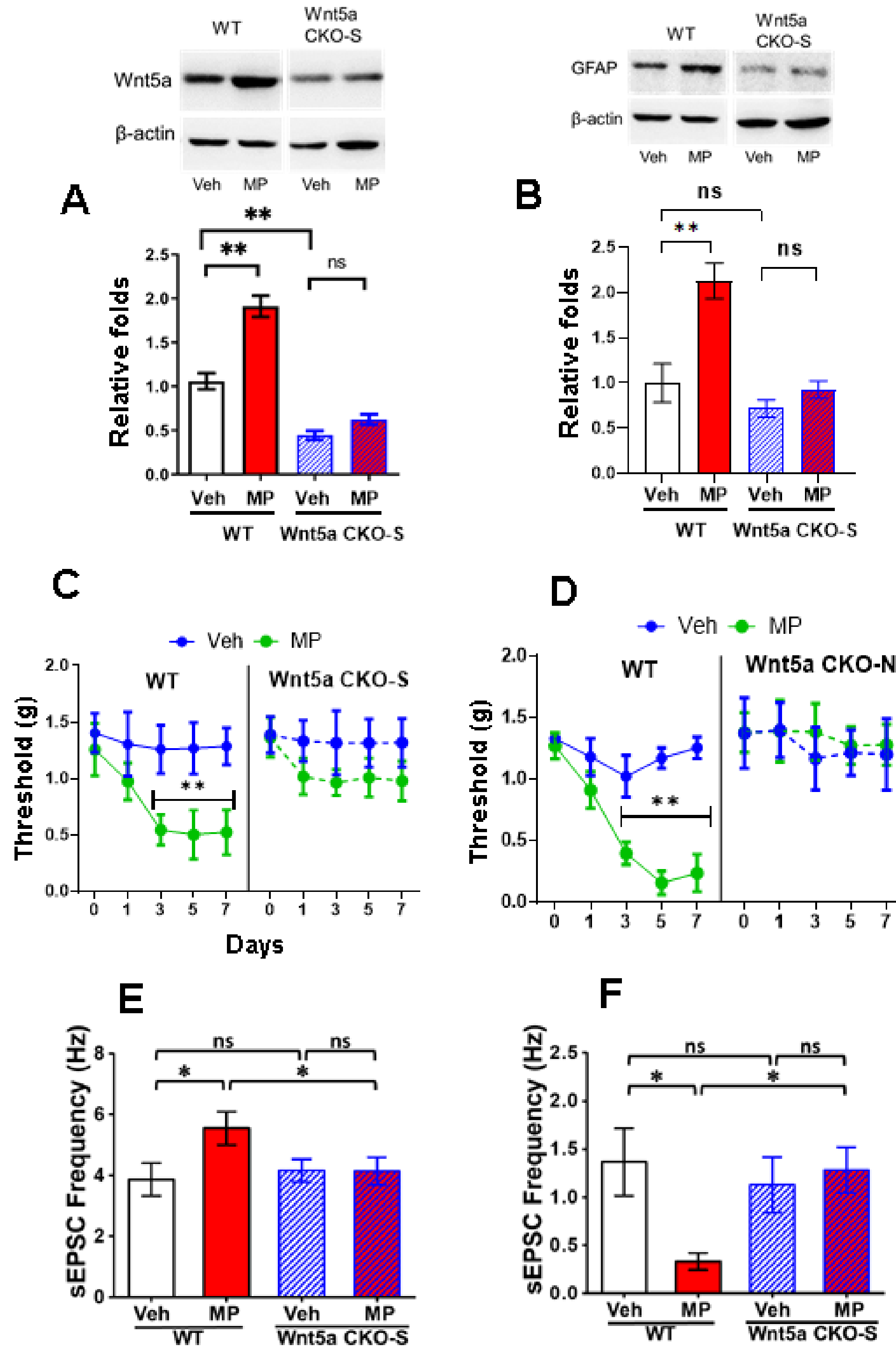

Figure 3. Neuronal Wnt5a is essential for morphine-induced astrogliosis, hyperalgesia, and NCP in the SDH. Two Wnt5a CKO mouse lines were generated,

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one by crossing floxed Wnt5a with nestin-Cre mice (Wnt5a CKO-N) and the other by crossing floxed Wnt5a with synapsin 1-Cre mice (Wnt5a CKO-S). (**A**) Wnt5a CKO-S (and Wnt5a CKO-N, not shown) blocked morphine (MP)-induced upregulation of Wnt5a in the spinal cord. (**B**) Wnt5a CKO-S (and Wnt5a CKO-N, Sup. Fig. 2) blocked morphine-induced spinal astrogliosis, as shown by GFAP upregulation. Tissues used in **A** and **B** were collected from mice (n=4/group) on day 7 after morphine administration according to the paradigm shown in Fig. 1A. (**C**) Wnt5a CKO-S abolished OIH expression (n=6/group). (**D**) Wnt5a CKO-N abolished OIH expression (n=6/group). (**E**) Wnt5a CKO-S blocked the morphine-induced increase in sEPSC frequency of non-tonic firing neurons in the SDH. Veh/WT, 19/3 (cells/mice); MP/WT: 47/5; Veh/Wnt5a CKO-S, 57/5; MP/Want5a CKO-S, 33/4. (**F**) Wnt5a CKO-S blocked the morphine-induced decrease in sEPSC frequency of tonic firing neurons in the SDH. Veh/WT, 12/3 (cells/mice); MP/WT: 18/3; Veh/Wnt5a CKO-S, 28/5; MP/Want5a CKO-S, 13/4.

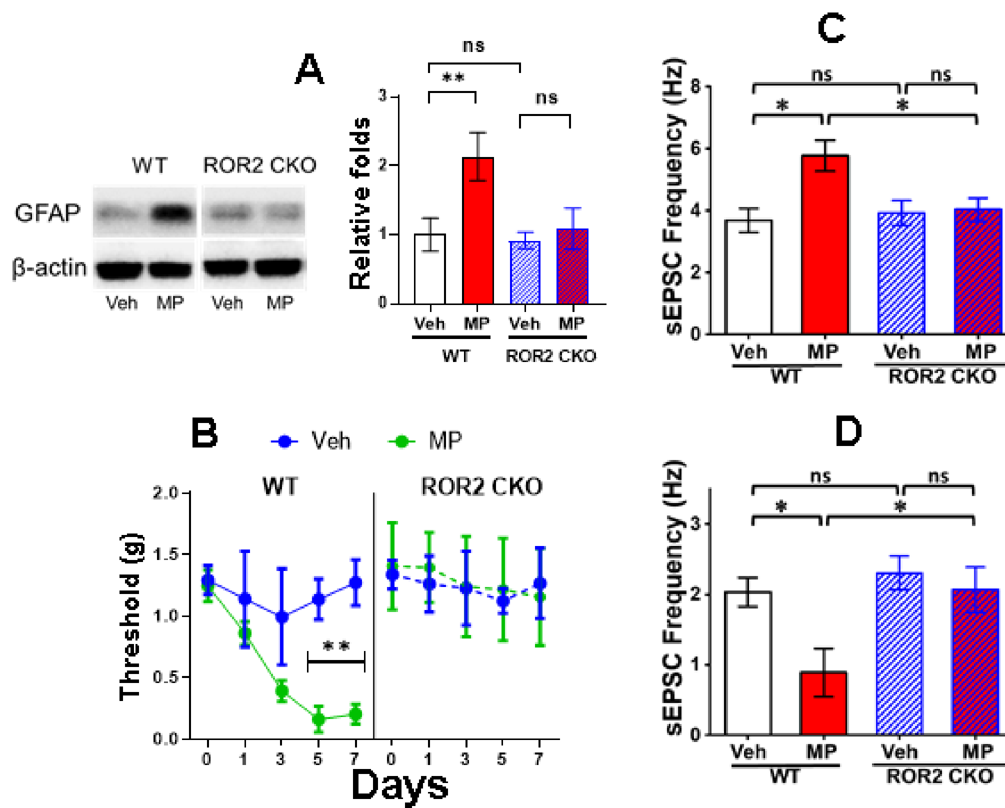

Figure 4. Astrocytic ROR2 is required for morphine-induced astrogliosis, hyperalgesia, and NCP in the SDH. (**A**) Astrocytic ROR2 CKO blocked morphine-induced astrogliosis. Spinal cords were collected from mice (n=4/group) on day 7 after morphine administration according to the paradigm shown in Fig. 1A. (**B**) Astrocytic ROR2 CKO blocked OIH expression (n=6/group). (**C**) Astrocytic ROR2 CKO blocked the morphine-induced increase in sEPSC frequency of non-tonic firing neurons in the SDH. Veh/WT, 32/3 (cells/mice); MP/WT: 53/5; Veh/ROR2 CKO, 45/3; MP/ROR2 CKO, 47/4. (**D**) Astrocytic ROR2 CKO blocked the morphine-induced decrease in

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sEPSC frequency of tonic firing neurons in the SDH. Veh/WT, 19/3 (cells/mice); MP/WT: 18/3; Veh/ROR2 CKO, 22/3; MP/ROR2 CKO, 12/3.

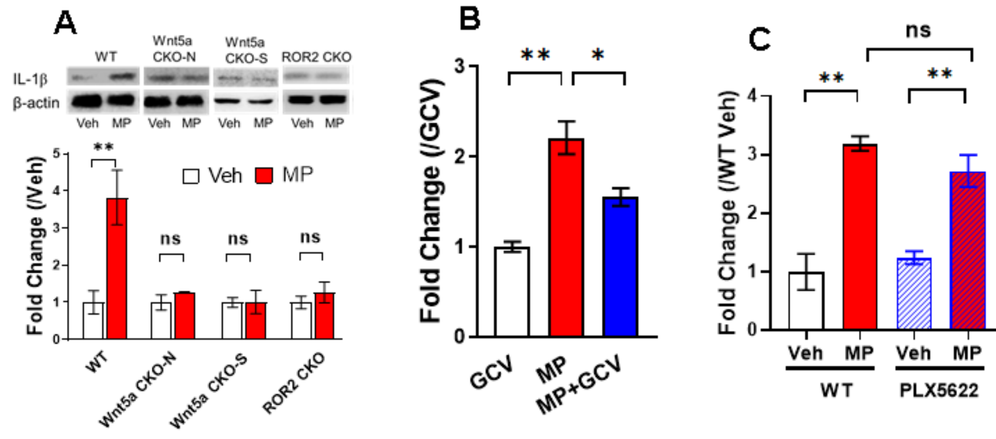

Figure 5. Morphine induces IL-1β activation (cleavage) via Wnt5a-ROR2 signaling-dependent astrogliosis. (**A**) Neuronal Wnt5a CKO and astrocytic ROR2 CKO blocked morphine-induced IL-1β upregulation. Spinal cords were collected for immunoblotting from mice (n=4/group) on day 7 after morphine administration according to the paradigm shown in Fig. 1A. (**B**) Astrogliosis ablation in GFAP-TK mice inhibited morphine-induced spinal IL-1β upregulation as revealed by immunoblotting (n=4/group). (**C**) Microglial ablation by PLX5622 did not affect morphine-induced spinal IL-1β upregulation as revealed by immunoblotting (n=4/group).

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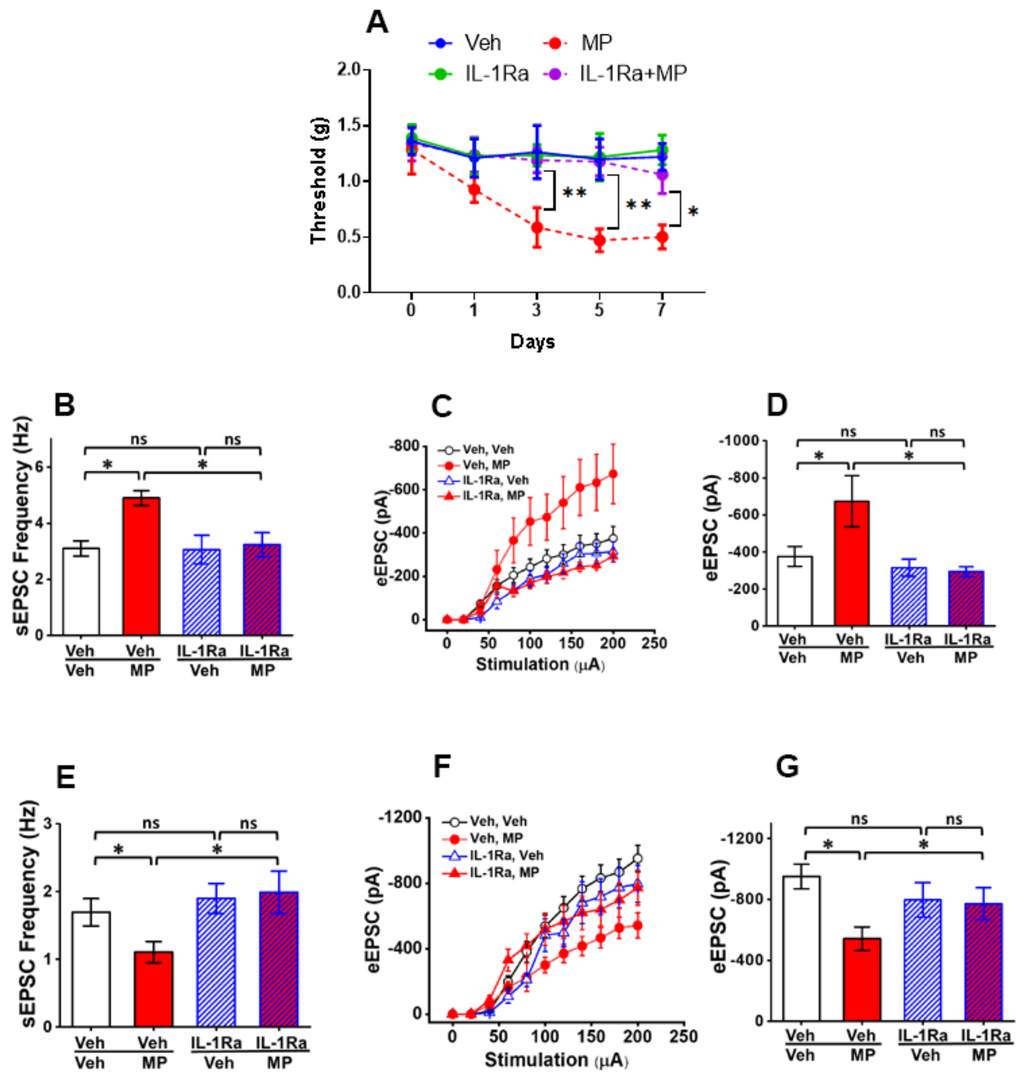

Figure 6. IL-1Ra blocks OIH and NCP. (**A**) The IL-1β receptor antagonist IL-1Ra blocked OIH expression. Morphine was administered according to the paradigm in Fig. 1C, and IL-1Ra was administered daily on the first 3 days (20 µg/kg, i.t.). (**B**) IL-1Ra impaired the morphine-induced increase in sEPSC frequency of non-tonic firing SDH neurons (Veh/Veh: 24/3 (cells/mice); veh/MP: 33/5; IL-1Ra/Veh: 22/5; IL-1Ra/MP: 44/4) in spinal slices prepared on day 7 from mice that received drug administration as infIG. 2A. (**C, D**) IL-1Ra abolished the morphine-induced increase in eEPSC amplitude of non-tonic firing neurons in the SDH; D shows statistical analysis of eEPSC amplitudes evoked by 200 µA stimulation shown in C (Veh/Veh: 24/3 (cells/mice); veh/MP: 28/5; IL-1Ra/Veh: 19/3; IL-1Ra/MP: 40/5). (**E**) IL-1Ra blocked the morphine-induced decrease in sEPSC frequency of SDH inhibitory neurons from GAD67-GFP transgenic mice (Veh/Veh: 67/3 (cells/mice); veh/MP: 51/5; IL-1Ra/Veh: 35/5; IL-1Ra/MP: 44/4). (**F, G**) IL-1Ra abolished the morphine-induced decrease in eEPSC amplitude of SDH inhibitory neurons; G shows statistical analysis of eEPSC amplitudes evoked by 200 µA stimulation shown in I (Veh/Veh: 42/4 (cells/mice);

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veh/MP: 43/5; IL-1Ra/Veh: 31/3; IL-1Ra/MP: 39/4). *p<0.05; **p<0.01; ns, p>0.05.

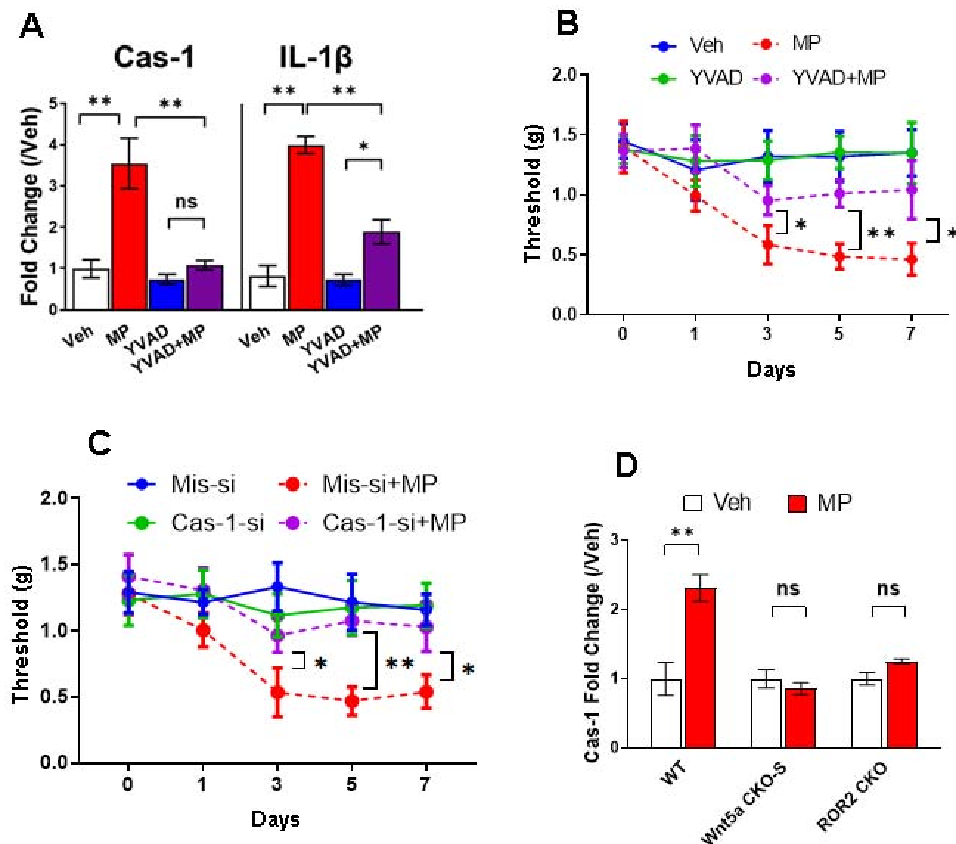

Figure 7. The Wnt5a-ROR2 signaling pathway controls inflammasome activation during OIH development. (**A**) Morphine activated the inflammasome in the spinal cord as measured by the upregulation of Cas-1 and IL-1β. Morphine-induced inflammasome activation was impaired by AC-YVAD-CMK (YVAD), a selective inhibitor of Cas-1. (**B**) YVAD impaired the expression of mechanical OIH. Morphine was administered according the paradigm shown in Fig. 1A, and YVAD was administered daily on the first 4 days (4 nmol/kg, i.t.). (**C**) Cas-1 siRNA (Cas-si) but not mismatch (silencer negative control) siRNA (Mis-si) blocked mechanical OIH expression. siRNA was administered daily on the first 4 days (3 nmol/kg, i.t.). (**D**) Morphine-induced inflammasome activation, measured by Cas-1 immunoblotting, was blocked by neuronal Wnt5a-CKO-S or astrocytic ROR2 CKO. *p<0.05; **p<0.01; ns, p>0.05.

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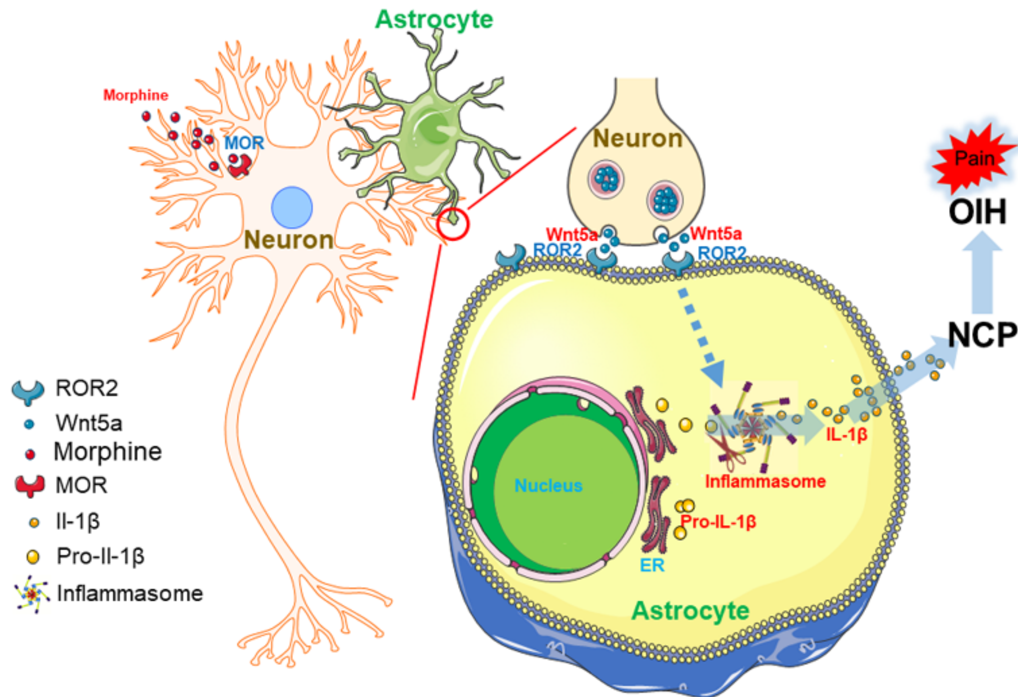

Figure 8. Model of the neuron-to-astrocyte Wnt5a-ROR2 signaling pathway in OIH development. Wnt5a-ROR2 signaling controls morphine-induced astrogliosis and astrocytic IL-1β activation. IL-1β is activated by the inflammasome, and feedback to neurons induces OIH.

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Materials & Methods

Animals

Adult male C57BL/6 mice (9–10 weeks of age) and GFAP-TK (B6.Cg-Tg(Gfap-TK)7.1Mvs/J) mice[59] were obtained from Jackson Laboratory (Bar Harbor, ME). Floxed Wnt5a mice were generated as previously described[35]. To delete Wnt5a in neurons, floxed Wnt5a mice were crossed with B6.Cg-Tg (Syn1-cre) 671Jxm/J mice[36] (Jackson Laboratory) to generate Syn1-cre/Wnt5a$^{flox/flox}$ mice. To delete Wnt5a in neural stem cells, floxed Wnt5a mice were crossed with B6.Cg-Tg(Nes-cre)1Kln/J mice[37] (Jackson Laboratory) to generate nestin-cre/Wnt5a$^{flox/flox}$ mice. To delete ROR2 in astrocytes, floxed ROR2 mice[38] (Jackson Laboratory) were crossed with B6.Cg-Tg(GFAP-cre)77.6Mvs/2J mice[39] (Jackson Laboratory) to generate GFAP-cre/ROR2$^{flox/flox}$ mice. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch.

Materials

Morphine sulfate was purchased from West-Ward (Eatontown, NJ). PLX5622-containing rodent diet (1 kg containing 1200 mg PLX5622) was purchased from Research Diets (New Brunswick, NJ). Ganciclovir sodium was purchased from Advanced ChemBlocks Inc. (Burlingame, CA). Interleukin 1 receptor antagonist (IL-1Ra) protein was purchased from R&D Systems Inc. (Minneapolis, MN). AC-YVAD-CMK, a selective inhibitor of caspase-1, was purchased from Sigma-Aldrich (St. Louis, MO). Caspase-1 siRNA was purchased from Thermo Fisher Scientific (Waltham, MA). Antibodies used for immunoblotting were anti-IBa1 (1:1000; Wako, 016-20001); anti-GFAP (1:1000; Millipore, MAB360), anti-β-actin (1:1000; Santa Cruz Biotechnology, sc-1616-R), anti-IL-1β (1:500; Novus Biologicals, NB600-633), anti-Wnt5a (1:1000; R&D, MAB645), anti-caspase-1 (1:1000; Adiogen Corporation, AG-20B-0042), and anti-COX-2 (1:1000; Abcam, ab179800). Antibodies used for immunohistochemistry were anti-GFAP (1:200; Millipore, AB5541) and anti-IL-1β (1:200; Novus Biologicals, NB600-633).

Drug administration

Mice were intraperitoneally injected with morphine sulfate (20 mg/kg) daily for 4 consecutive days to establish morphine-induced hyperalgesia. For microglial ablation, C57BL/6 mice were fed a rodent diet containing PLX5622 starting 5 days prior to morphine administration and continuing until the end of experimentation. To ablate reactive astrocytes, GFAP-TK or control WT mice were administered ganciclovir (5 mg/kg) by intrathecal (i.t.) injection for 2 consecutive days during the inductive or maintenance phase of opioid-induced hyperalgesia. AC-YVAD-CMK (4 nmol/kg) or siRNA (3 nmol/kg) were injected i.t. daily for the first 4 days. IL-1Ra (20 μg/kg) was injected i.t. daily for the first 3 days.

Measurement of mechanical nociception by von Frey test

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Mechanical nociceptive hypersensitivity in mice was measured as previously described[60]. The plantar surface of the hind paw of mice in a resting state was stimulated with calibrated von Frey filaments (Stoelting, Wood Dale, IL), and paw withdrawal threshold was determined using the Dixon up and down paradigm. All tests were conducted 2 h prior to drug administration by an experimenter blind to the treatments received by individual animals.

Western blotting analysis

Mice were anesthetized with 3% isoflurane and sacrificed to collect L4-L6 lumbar spinal cord segments. Tissue was homogenized in RIPA lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, and 1 mM EDTA, pH 8.0) with a protease inhibitor cocktail (Sigma). BCA Protein Assay kits (Thermo Fisher) were used to determine protein concentrations. Equal amounts of protein (2 µg) were loaded and separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature and then incubated with primary antibodies in TBST buffer overnight at 4°C. After washing with TBST buffer (three times for 10 min at room temperature), membranes were incubated with HRP-conjugated secondary antibody. Enhanced chemiluminescence kits (Pierce) were used to visualize protein bands, and NIH ImageJ software was used for quantification. β-actin was used as a loading control.

Immunohistochemistry

Mice were anesthetized with 3% isoflurane and transcardially perfused with 20 ml of 0.01 M phosphate-buffered saline (PBS; 0.14 M NaCl, 0.0027 M KCl, 0.010 M PO_4^{3}) followed by 30 ml of 4% paraformaldehyde (PFA) in 0.01 M PBS. L4 and L5 lumbar spinal cord segments were fixed in 4% PFA solution for 12 h at 4°C, dehydrated with 30% sucrose solution in PBS for 24 h at 4°C, and embedded in optimal cutting temperature medium (Tissue-Tek). Tissue was sectioned (15 µm) on a cryostat (Leica CM 1900) and mounted onto Superfrost Plus microscope slides. Sections were incubated in blocking solution containing 5% bovine serum albumin (BSA) and 0.3% Triton X-100 in 0.01 M PBS for 1 h at room temperature and then in chicken anti-GFAP (1:200; Millipore, AB5541) or rabbit IL-1β (1:200; Novus Biologicals, NB600-633) in blocking solution overnight. Sections were washed with 0.01 M PBS (three times for 10 min at room temperature) and incubated with FITC- or Texas Red-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories) in dilution buffer (1% BSA and 0.3% Triton X-100 in 0.01 M PBS) before mounting. Finally, sections were stained with DAPI (Sigma) to visualize nuclei. Animal species-matched IgG was used as a negative control for primary antibodies. Images were collected on a confocal microscope (model A1, Nikon s).

Patch-clamp recording of dorsal horn neurons in ex vivo spinal cord slices

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Spinal cord slices were prepared as previously described[61]. Briefly, the spinal cord was sliced transversely at a thickness of 350 µm using a vibratome (Leica VT1200S, Buffalo Grove, IL) in cold (4°C) N-methyl-D-glucamine (NMDG) solution (93 mM NMDG, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 30 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 10 mM $MgSO_4$, and 0.5 mM $CaCl_2$, pH 7.4) saturated with 95% O_2 and 5% CO_2. Whole-cell recordings were performed from randomly selected neurons in lamina II in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 24 mM $NaHCO_3$, 5 mM HEPES, 12.5 mM glucose, 2 mM $MgSO_4$, and 2 mM $CaCl_2$, pH 7.4) using a Multiclamp 700B amplifier, DigiDATA, and pClamp software (version 10.6. Molecular Device, Sunnyvale, CA) with a 10-kHz sampling rate and 2-kHz filtering rate. Patch pipettes (4–8 MΩ) were filled with internal solution (120 mM K-gluconate, 10 mM KCl, 2 mM Mg-ATP, 0.5 mM Na-GTP, 0.5 mM EGTA, 20 mM HEPES, and 10 mM phosphocreatine, pH 7.3). After achieving a whole-cell recording configuration, spontaneous excitatory postsynaptic currents (sEPSCs) were recorded for 60 s at -65 mV in ACSF. Evoked EPSCs (eEPSCs) were elicited by focal electrical stimulation in the vicinity of recorded neurons with a metal bipolar electrode (MicroProbes, Gaithersburg, MD). Test pulses were delivered for 0.5 ms at 5-s intervals, with stimulation intensities ranging from 20–200 µA (20-µA steps). Recordings were performed only when eEPSCs were monosynaptic, based on characteristic waveforms with short latency, a single peak, and stable responses to repeated stimuli. Recordings showing polysynaptic responses were disregarded. Neurons were characterized by their action potential firing pattern upon depolarizing current injections in current clamp mode[62].

Statistical analysis

Statistical analysis was conducted with Prism 5 (GraphPad) software. Data are shown as mean ± standard error of the mean (SEM). One-way ANOVA was used for immunoblotting data, and two-way ANOVA with Bonferroni post hoc tests were used for pain behavior data. Electrophysiological data are expressed as mean ± SEM with n indicating the number of cells and N indicating the number of animals. sEPSC frequency and eEPSC amplitude were analyzed offline using Clampfit software (version 11, Molecular Devices, CA). sEPSC events were detected using the template event detection method. Electrophysiological data were analyzed using two-way ANOVA followed by Holm-Sidak multiple comparison tests. For all tests, $p < 0.05$ was considered statistically significant. Origin (version PRO 2020, OriginLab, Northampton, MA) was used to analyze data.

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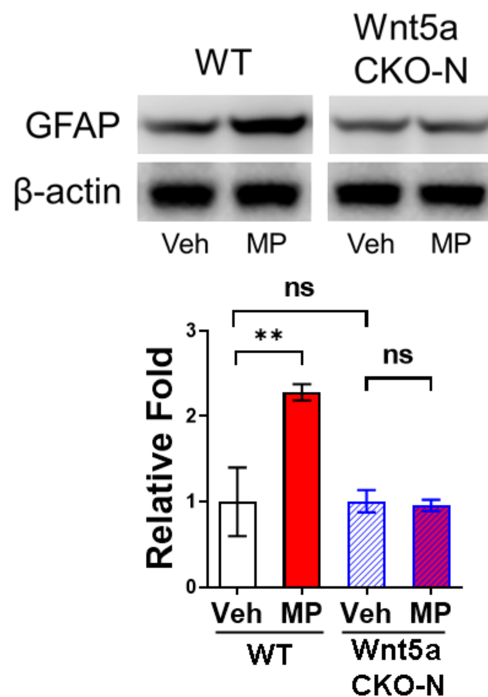
Supplemental Figures



Supplemental Figure 1. Wnt5a CKO-N blocked morphine-induced astrogliosis in the spinal cord.