Prostaglandin-mediated inhibition of serotonin signaling controls the affective component of inflammatory pain

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Pain is fundamentally unpleasant and induces a negative affective state. The affective component of pain is mediated by circuits that are distinct from those mediating the sensory-discriminative component. Here, we have investigated the role of prostaglandins in the affective dimension of pain using a rodent pain assay based on conditioned place aversion to formalin injection, an inflammatory noxious stimulus. We found that place aversion induced by inflammatory pain depends on prostaglandin E$_2$ that is synthesized by cyclooxygenase 2 in neural cells. Further, mice lacking the prostaglandin E$_2$ receptor EP$_3$ selectively on serotonergic cells or selectively in the area of the dorsal raphe nucleus failed to form an aversion to formalin-induced pain, as did mice lacking the serotonin transporter. Chemogenetic manipulations revealed that EP$_3$ receptor activation elicited conditioned place aversion to pain via inhibition of serotonergic neurons. In contrast to their role in inflammatory pain aversion, EP$_3$ receptors on serotonergic cells were dispensable for acute nociceptive behaviors and for aversion induced by thermal pain or $\kappa$ opioid receptor agonist. Collectively, our findings show that prostaglandin-mediated modulation of serotonergic transmission controls the affective component of inflammatory pain.

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Introduction

Pain is an adaptive warning signal under physiological conditions, but it also causes a lot of suffering during various pathological states. Pain is a complex phenomenon with many components. The sensory component provides information about location, intensity, and quality of the painful event (1). In addition, pain is inherently unpleasant and induces a negative affective state (2, 3). Converging evidence indicates that the brain regions mediating the sensory and affective components of pain are, at least partly, distinct (1, 4–6).

Prostaglandins are key regulators of nociceptive processing during inflammation, and prostaglandin synthesis inhibitors in the form of nonsteroidal antiinflammatory drugs are widely used for their analgesic properties. Prostaglandins modulate nociceptive signaling at many levels of the neuraxis (7), but it is unclear how they are involved in the affective dimension of pain.

Results and Discussion

To investigate the affective component of pain in mice, we used conditioned place avoidance induced by inflammatory pain. This test, in which mice learn to avoid a chamber where they experienced pain induced by formalin injection to the hind paw, has been used extensively to investigate the affective component of pain in rodents (3, 5, 6, 8). To explore the role of prostaglandins, we first interfered with the cyclooxygenases COX1 and COX2 (also known as PTGS1 and PTGS2), enzymes responsible for prostaglandin synthesis. Normal (WT) mice, mice lacking COX1, and mice pretreated with a COX1 selective inhibitor (sc560, 5 mg/kg, i.p.; Figure 1B) avoided the chamber paired with formalin injections (Figure 1, A and B; for figures showing the behavioral experiments with individual values indicated, see Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI90678DS1). In contrast, the aversion was completely blocked in genetically modified mice lacking COX2 activity (Figure 1C) and when COX2 was inhibited (parcoxib, 10 mg/kg) during the pain sessions (Figure 1D). To identify the cell type producing the critical prostaglandins, we next used the Cre/loxP system to delete Cox2 in specific cell types. Mice without COX2 in myeloid cells (Cox2<sup>−/−</sup> LysM-Cre; Figure 1E) or in brain endothelial cells (Cox2<sup>−/−</sup> Sloolci-Cre mice; Figure 1F) displayed aversion comparable to that of littermates without Cre (WT mice). In contrast, mice without COX2 in neural cells (Cox2<sup>−/−</sup> Nes-Cre mice), including peripheral and central neurons and glia, showed no avoidance behavior (Figure 1G).

Prostaglandin E₂ is the principal proinflammatory prostaglandin and an important regulator of nociceptive and systemic inflammatory responses (7, 9–11). We tested mice lacking mPGES-1, the inducible form of prostaglandin E synthase, in our pain model (mPges-1 KO mice). These mice displayed significantly lower aversion scores compared with their WT littermates (Figure 1H). Prostaglandin E₂ binds to 4 G protein–coupled receptors called EP, to EP<sub>1</sub> (encoded by Ptger1-4). We tested mice lacking EP<sub>1</sub> or EP<sub>3</sub> receptors, since these are strongly expressed in brain structures related to motivation (12, 13) and have been shown to be implicated in nociceptive processing (14–16). Mice lacking EP<sub>3</sub> receptors (Ptger1<sup>−/−</sup> mice, referred to as EPIR KO mice) did not show any difference in

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aversion scores with WT littermates (Figure 2A). In contrast, Ptger3 knockout (EP3R KO) mice displayed no pain-induced aversion (Figure 2B).

EP3 receptors are expressed in many structures of the brain and the spinal cord. Given the important role of serotonin in the regulation of affective functions, the EP3 receptor expression in serotonergic neurons of the dorsal raphe nucleus and other serotonergic structures (12) is particularly interesting in the context of aversion. To explore the role of EP3 receptors on serotonergic neurons, we next used Ptger3fl/fl Sert-Cre mice (referred to as EP3R-SERTCre mice). In these mice, Ptger3 is deleted by Cre expressed under control of the serotonin transporter (Sert) promoter. As expected, Cre expression was specific to serotonergic neurons, identified by labeling for tryptophan hydroxylase (Tph) (Figure 2, C and D). Further, EP3R-SERTCre mice displayed a clear reduction of Ptger3-a mRNA in tissue punches from the dorsal raphe region, whereas no reduction was seen in the cortex or the hypothalamus/thalamus (Supplemental Figure 2A). Subsequently, we tested the EP3R-SERTCre mice in the conditioned pain-avoidance test. We observed a complete abolition of the aversion in EP3R-SERTCre mice (Figure 2E). Further, aversion was completely blocked in mice lacking the serotonin transporter SERT (Figure 2F). The serotonergic system can be divided into 2 main parts (17). One is ascending, involved in affective processing, and is constituted by the dorsal and median raphe nuclei. The other is descending and modulates nociceptive processing in the spinal cord (17, 18). To determine whether the ascending serotonergic pathways mediated the aversion, we deleted Ptger3 receptors in the region of the dorsal raphe nucleus. This was done by stereotaxic injection of Cre-expressing viral vectors (AAVs) into mice with floxed Ptger3. Mice with injections that affected the dorsal raphe nucleus (Figure 2G) without extending to the descending raphe nuclei showed blocked aversion (Figure 2H). Collectively, these findings strongly suggest that ascending serotonergic transmission is critical for pain-induced aversion and that prostaglandin E2 can modulate such transmission by a direct effect on EP3 receptors on serotonergic neurons.

Since most EP3R splice variants are coupled to inhibitory G proteins, we investigated whether EP3R activation elicits aversion by inhibition of serotonergic neurons. To test this, we used a designer receptor exclusively activated by designer drug–based (DREADD-based) chemogenetic approach (19) to maintain firing in serotonergic cells during the painful experience and monitored the aversive response of the mice. We injected viral vectors with Cre-dependent expression of Gq-coupled DREADDs (hM3Dq) or mCherry in EP3R-SERTCre mice (AAV-hM3Dq Sert-Cre and AAV-mCherry Sert-Cre mice, respectively; Figure 2I) and activated the cells by administration of clozapine N-oxide (CNO) before the formalin injections. Strikingly, chemogenetic activation of serotonergic cells blocked the aversion (Figure 2J). Further, mice without EP3Rs on serotonergic cells, which normally display no pain-induced aversion, showed a normal aversion when their serotonergic cells were inhibited during the formalin-pain session (Figure 2, K and L). This was achieved by stereotaxic injections of AAVs encoding Gq-coupled DREADDs (hM3Dq) or mCherry in EP3R-SERTCre mice (AAV-hM3Dq EP3R-SERTCre and AAV-mCherry EP3R-SERTCre mice, respectively; Figure 2K).

Next, we investigated whether the prostaglandin-dependent pathway identified was specific to the affective component of pain or whether it was necessary for all aspects of pain. We monitored acute formalin-induced nociceptive behaviors (lifting, shaking, and licking the injected paw) in mice subjected to pharmacological inhibition of COX2 in mPGES-1 KO mice and in EP3R-SERTCre mice. Pharmacological inhibition of COX2, as well as lack of mPGES-1, markedly reduced the nociceptive behaviors in the second phase of the formalin test (Figure 3, A and
displayed normal sucrose preference, normal withdrawal latencies in the hot-plate test, and normal real-time avoidance of a hot floor (Supplemental Figure 2, B–D). Thus, prostaglandin-mediated modulation of serotonergic signaling selectively controls aversion to inflammatory pain.

The finding that EP3 receptors on serotonergic neurons regulate the affective component of pain is in line with the central role of serotonin in affective functions (17) and may have relevance for the high comorbidity of pain and depression. Populations of serotonergic cells are modulated by aversive stimuli (22), including pain (23), as well as by rewarding stimuli (24).
as prostaglandins and serotonin modulate nociceptive processing at many levels, this mechanism selectively regulates the affective dimension of inflammatory pain.

**Methods**

Additional methods are provided in the Supplemental Methods.

**Statistics.** All the data are expressed as mean ± SEM and were analyzed (in GraphPad Prism) using 2-tailed Student’s *t* tests, except for body temperature measurements, which were analyzed with 2-way ANOVA. *P* < 0.05 was considered significant. The number of mice are specified in Supplemental Figure 1 or the figure legends.

**Study approval.** All experiments were approved by the Linköping Animal Care and Use Committee and followed national and international guidelines.

**Author contributions**

AKS, JZ, and DE were responsible for overall study design. AKS performed the behavioral/physiological experiments except for those involving EP1R-KOs (AMK and MF), fever (EM), or thermal pain and sucrose preference (NA, IF, and JZ). MF designed, implemented, and introduced others to the formalin conditioning protocol. Viral injections and histological validations were done by JZ. Quantitative PCR (qPCR) analysis was done by JZ, AKS, MJ, and IF. The manuscript was written by DE, AKS, and JZ. All authors were involved in the design of the parts of the study they executed, discussed the design and results, and commented on the manuscript.

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Further, serotonergic neurotransmission mediates adaptive danger avoidance (25), can induce fear and anxiety (26), and regulates affective responses to stress (27, 28). The affective responses to stress are mediated by a transient increase in serotonin reuptake in the ventral striatum, leading to a local hypo-serotonergic state (29). Prostaglandin-mediated inhibition of serotonergic cells could be another way of reducing serotonin levels in the ventral striatum. It is likely that the importance of ascending serotonergic neurons is not restricted to inflammatory pain. Thus, other types of pain may access the serotonergic cells by pathways not involving prostaglandins and EP3 receptors. We recently found that the aversion induced by systemic inflammation is mediated by prostaglandins (30). The characteristics of the aversive stimulus used in that study (LPS injected i.p.) are quite different from those of the stimulus used in this study. Thus, the systemic inflammation was presumably nonpainful and the signaling mechanisms involved the brain endothelium, COX1, and EP3 receptors on dopamine D1 receptor-expressing neurons in the striatum (30). In contrast, the aversion seen in this study was dependent on nociceptive input and could consequently be blocked by deletion of *Trpa1* (Supplemental Figure 2E), the receptor activated by formalin on the primary afferents (31). Thus, prostaglandins are involved in the aversive signaling induced by both systemic inflammation and localized inflammatory pain, but different peripheral signaling routes, cyclooxygenases, EP receptor subtypes, and monoaminergic neuronal populations are involved.

In summary, we show that persistent inflammatory pain induces aversion through a mechanism involving neural prostaglandin synthesis and EP3 receptors on serotonergic cells. Where
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