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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 a κ opioid receptor agonist. Collectively, our findings show that prostaglandin-mediated modulation of serotonergic transmission controls the affective component of inflammatory pain.

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 the 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 (parecoxib, 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*$^{fl/fl}$ *LysM-Cre*; Figure 1E) or in brain endothelial cells (*Cox2*$^{fl/fl}$ *Slco1c1-Cre* mice; Figure 1F) displayed aversion comparable to that of littermates without Cre (WT mice). In contrast, mice without COX2 in neural cells (*Cox-2*$^{fl/fl}$ *Nes-Cre* mice), including peripheral and central neurons and glia, showed no avoidance behavior (Figure 1G).

Prostaglandin E_2 is the principal proinflammatory prostanoid 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 E_2 binds to 4 G protein–coupled receptors called EP_1 to EP_4 (encoded by *Ptger1-4*). We tested mice lacking EP_1 or EP_3 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_1Rs (*Ptger1*$^{-/-}$ mice, referred to as EP1R KO mice) did not show any difference in

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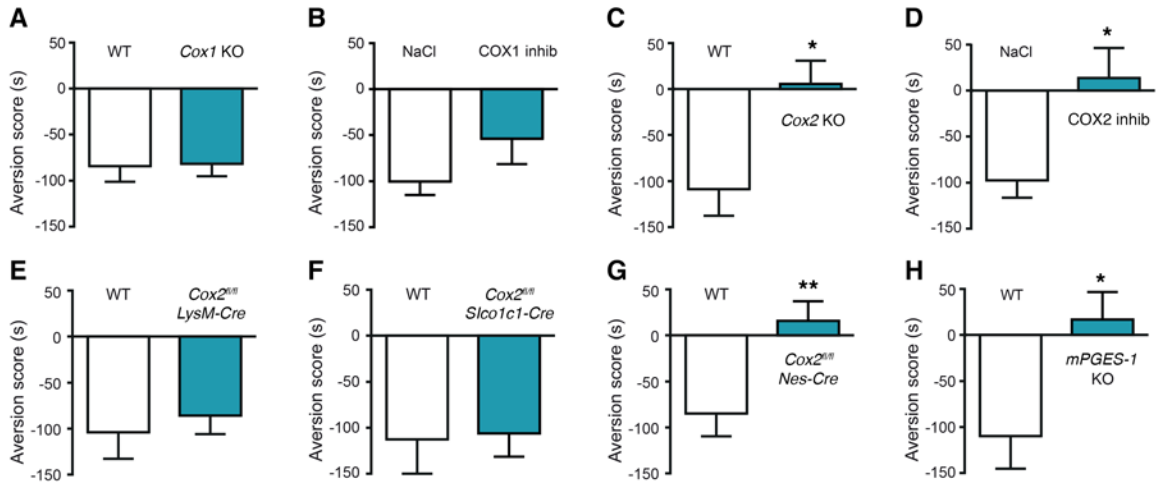

Figure 1. The affective component of inflammatory pain is dependent on prostaglandin E2 generated by COX2 in neural cells. (**A**–**D**) Conditioned place aversion induced by injection of diluted formalin in the dorsal part of the hind paws in mice lacking COX1 (*Cox1* KO) (**A**), mice treated with a COX1 inhibitor (COX1 inhib) (sc560, 5 mg/kg, i.p.; **B**), mice lacking COX2 (*Cox2* KO) (**C**), mice treated with a COX2 inhibitor (parecoxib, 10 mg/kg, i.p.; **D**), and corresponding control mice. (**E**) Aversion scores in mice lacking COX2 in myeloid cells (*Cox2$^{fl/fl}$ LysM-Cre*). See Supplemental Methods for calculation of aversion scores. (**F** and **G**) Aversion scores in mice lacking COX2 in brain endothelial cells (*Cox2$^{fl/fl}$ Slco1c1-Cre*) or mice lacking COX2 in neural cells (*Cox2$^{fl/fl}$ Nes-Cre*). (**H**) Aversion is also blocked in mice lacking the inducible microsomal prostaglandin E synthase (*mPGES-1* KO). *P < 0.05; **P < 0.01, Student's t test.

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

EP_3 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 EP_3 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 EP_3 receptors on serotonergic neurons, we next used *Ptger3$^{fl/fl}$ 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*-α 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 abrogation 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 (AAV5) 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 E_2 can modulate such transmission by a direct effect on EP_3 receptors on serotonergic neurons.

Since most EP_3R splice variants are coupled to inhibitory G proteins, we investigated whether EP_3R 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 SERT-Cre 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 EP_3Rs 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 Gi-coupled DREADDs (hM4Di) or mCherry in EP3R-SERTCre mice (AAV-hM4Di 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

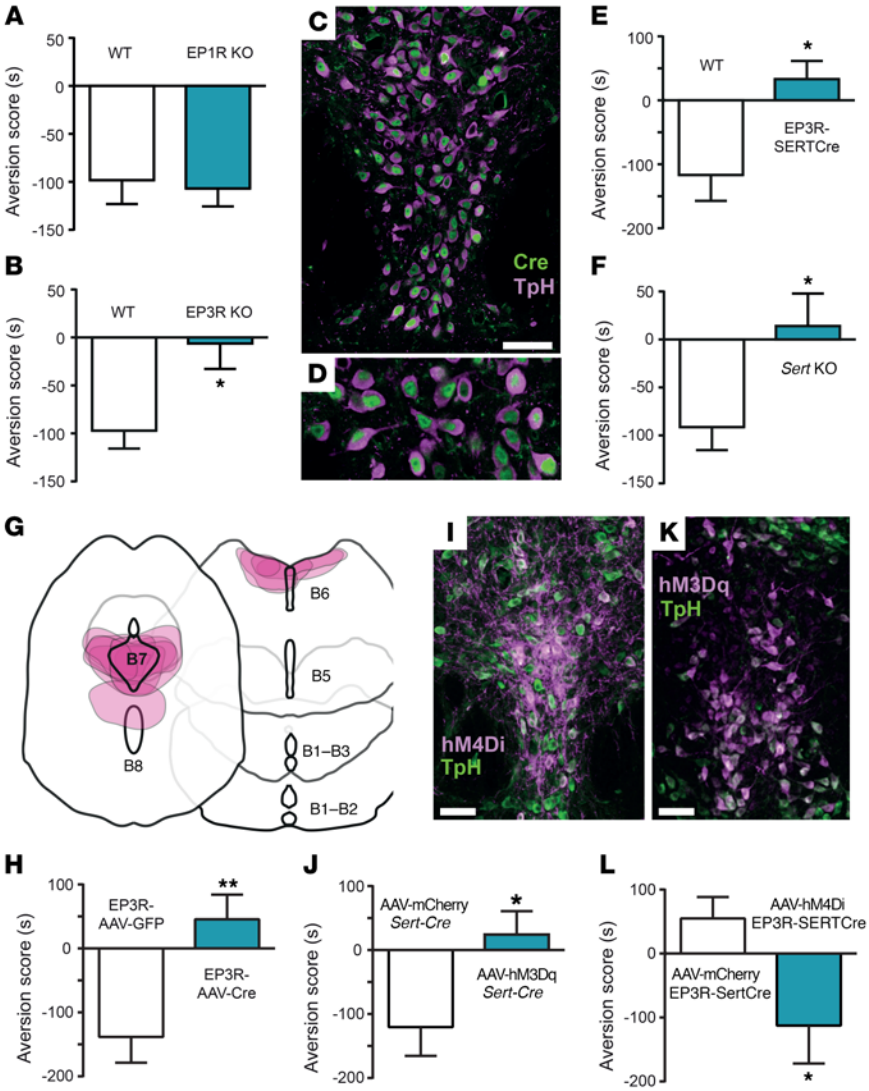

Figure 2. EP_3 receptors on serotonergic cells are critical for the affective component of pain. (**A** and **B**) Aversion scores in mice lacking EP_1 receptors (EP1R KO) (**A**) or EP_3 receptors (EP3R KO) (**B**). (**C** and **D**) Confocal micrographs from the dorsal raphe nucleus of a *Sert-Cre* mouse. Labeling for TpH, a marker for serotonergic neurons, is shown in purple, and Cre labeling is shown in green. **D** is a higher magnification of parts of **C**. (**E**) Graph showing the aversive reaction to inflammatory pain in mice lacking EP_3 receptors in serotonergic cells (EP3R-SERTCre) due to deletion driven by the *Sert* promoter. (**F**) Aversion scores in control mice and mice lacking the serotonin transporter (*Sert* KO). (**G**) Plot of the areas transfected with the viral vector encoding Cre in *Ptger3*$^{fl/fl}$ animals used in **H**. (**H**) Aversion scores in response to formalin-induced pain in mice with AAV-Cre–induced *Ptger3* deletion in the area of the dorsal raphe nucleus (B6–B7). (**I**) Expression of hM3Dq-mCherry in serotonergic cells of the dorsal raphe. (**J**) Aversion scores from mice in which serotonergic cells were chemogenetically activated (AAV-hM3Dq *Sert-Cre* + CNO) during the pain session and controls (AAV-mCherry/*Sert-Cre* + CNO). (**K**) Expression of hM4Di-mCherry in serotonergic cells of the dorsal raphe. (**L**) Aversion scores from EP3R-SERTCre mice in which serotonergic cells were chemogenetically inactivated (AAV-hM4Di EP3R-SERTCre + CNO) during the pain sessions and controls (AAV-mCherry EP3R-SERTCre + CNO). Scale bars: 50 μm (**C**, **I**, and **K**); 30 μm (**D**). **P* < 0.05; ***P* < 0.01, Student's *t* test.

B), whereas they were unaffected in EP3R-SERTCre mice (Figure 3, C and D). This indicates that prostaglandin E_2 modulates many components of pain, but that the effect on serotonergic transmission selectively controls the affective dimension. We also found a normal febrile response in EP3R-SERTCre mice (Figure 3E), indicating that responses driven by EP_3Rs in nonserotonergic cells were intact (20, 21). Further, aversion induced by the κ opioid receptor agonist U-50488 (2.5 mg/kg, i.p.) was intact in EP3R-SERTCre mice (Figure 3F), and both *Cox2*$^{fl/fl}$ *Nes-Cre* and EP3R-SERTCre mice displayed a robust aversion to thermal pain (Figure 3, G and H). EP3R-SERTCre mice also 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 EP_3 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).

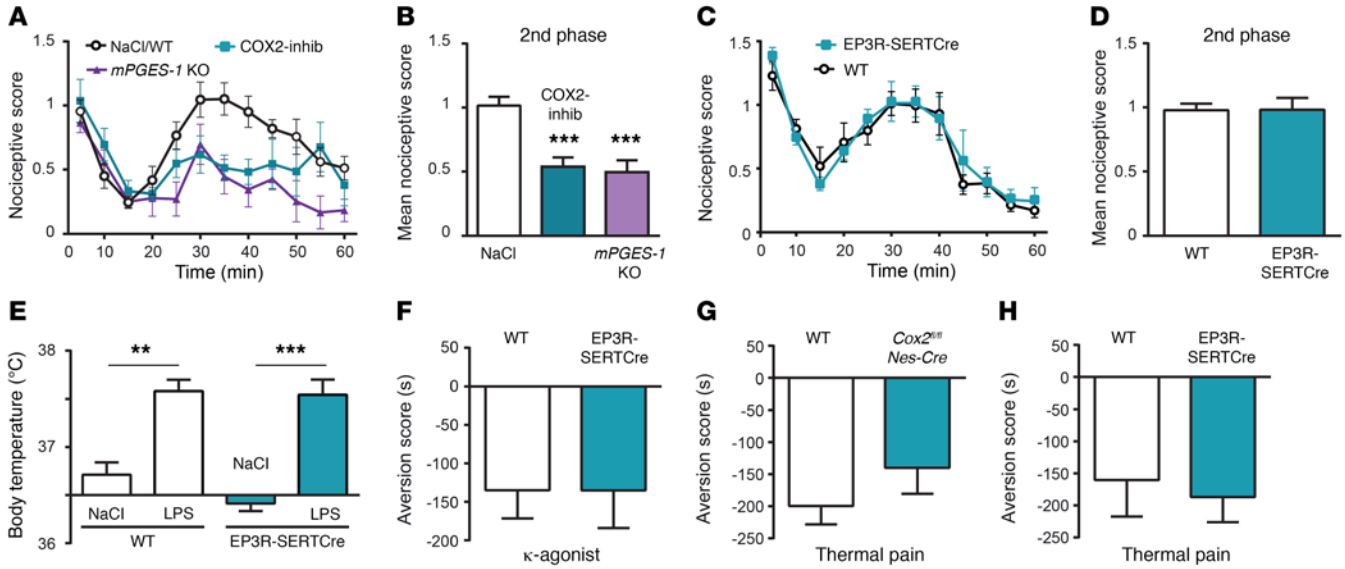

Figure 3. The involvement of EP_3 receptors on serotonergic cells is specific for the affective component of pain. (**A** and **B**) Nociceptive scores in mice given a COX2 inhibitor or *mPGES-1* KO mice (parecoxib, 10 mg/kg, i.p.; $n = 9 + 7 + 6$). **B** shows means of the scores during the peak of the second phase of the formalin-induced pain (defined as 30–40 minutes after injection). See Supplemental Methods for calculation of nociceptive scores. (**C** and **D**) Corresponding data for mice lacking EP_3 receptors in serotonergic cells (EP3R-SERTCre; $n = 5 + 5$). Note that the blunted response seen after COX2 inhibition cannot be seen in EP3R-SERTCre mice. (**E**) Febrile responses in WT and EP3R-SERTCre mice. Mean body temperatures 5 to 9 hours after an injection of lipopolysaccharide (100 μg/kg) are plotted. (**F**) Aversion scores in response to the κ receptor agonist U50488 (2.5 mg/kg, i.p.). Note that EP3R-SERTCre mice display normal acute nociceptive responses and a normal U-50488–induced aversion. (**G** and **H**) Conditioned place aversion induced by thermal pain in *Cox2*$^{fl/fl}$ *Nes-Cre* mice (**G**) and EP3R-SERTCre mice (**H**) and their WT littermates. $^{**}P < 0.01$; $^{***}P < 0.001$, Student's *t* test (**B**, **D**, and **F**–**H**); ANOVA (**E**).

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 hyposerotonergic 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 EP_3 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 EP_1 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 EP_3 receptors on serotonergic cells. Whereas 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 EP_1R-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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