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EP3 prostaglandin receptors in the median preoptic nucleus are critical for fever responses

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Fever is a result of the action of prostaglandin E2 (PGE2) on the brain and appears to require EP3 prostaglandin receptors (EP3Rs), but the specific neurons on which PGE2 acts to produce fever have not been definitively established. Here we report that selective genetic deletion of the EP3Rs in the median preoptic nucleus of mice resulted in abrogation of the fever response. These observations demonstrate that the EP3R-bearing neurons in the median preoptic nucleus are required for fever responses.

Fever is a ubiquitous response among animals that has an adaptive function, improving survival during bacterial infection. During inflammation, a variety of proinflammatory cytokines and lipid mediators are produced. However, E-type prostaglandins have been considered to be the principal mediators of fever. The genetic deletion of their synthetic enzymes, cyclooxygenase or glutathione-dependent, membrane-bound prostaglandin E synthase, prevent fever responses[1,2]. These genes are widely expressed by macrophages and endothelial cells that are associated with small penetrating venules along the surface of the brain[3]. The identity of the neurons on which PGE2 acts to produce fever has been a pressing question in the field. Injection of PGE2 into the preoptic area produces fever, and injection of a cyclooxygenase inhibitor in this area attenuates (but does not entirely prevent) fever responses to systemic injection of lipopolysaccharide (LPS)[4]. EP1, EP3 and EP4 receptors for PGE2 have been identified in the preoptic area[5]. Although initial experiments have suggested that only the EP3 receptors produce fever[6], more exhaustive studies have found that genetic knockouts of either the EP1 or the EP3 receptors from the entire animal reduce fever responses[7]. However, both EP1 and EP3 receptors are expressed by many different cell groups in the preoptic area, as well as elsewhere in the CNS and systemically, and it is not clear as to which site these receptors are required for causing fever responses.

We prepared animals with a genetic construct allowing for the conditional disruption of the mouse EP3 prostaglandin receptor (*Ptger3*) gene (**Fig. 1** and **Supplementary Methods** online). We inserted flanking *loxP* sites into the 5′-untranslated region (UTR) and the first intron of the *Ptger3* gene to create a mouse line with a *Ptger3* gene that is amenable to conditional deletion by Cre recombinase ($Ptger3^{flox}$). Although one *loxP* sequence is located in the 5′-UTR of the *Ptger3* mRNA, *in situ* hybridization for *Ptger3* mRNA by using a ^{35}S-labeled riboprobe against exon 1 of *Ptger3* showed that the EP3R gene was expressed in the brain in a pattern identical to that of wild-type mice (**Fig. 2a** and **Supplementary Fig. 1** online). Baseline and

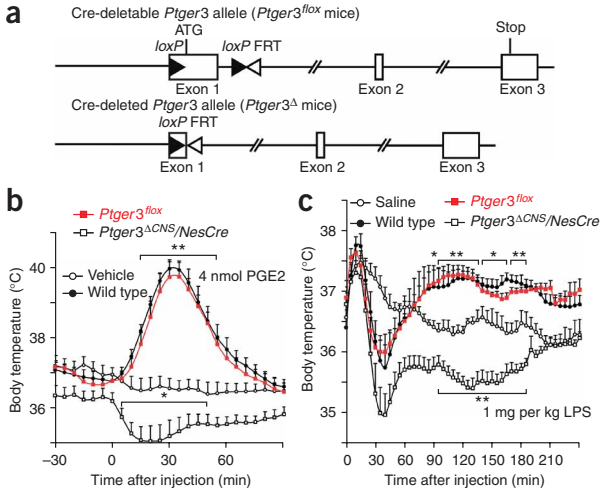

Figure 1 Generation of conditional EP3R mice. (**a**) *loxP* sites were inserted in the 5′-UTR of the first exon and in the first intron of the *Ptger3* gene. This allele was bred to homozygosity to generate $Ptger3^{flox}$ mice. The Cre-mediated removal of exon 1 eliminates the translational start site, thus preventing expression of a functional EP3R. Care of all mice followed Institutional Animal Care and Use Committee (IACUC) guidelines, and all procedures were approved by the Standing Committee on Animals at Harvard University. (**b**) Effects of ICV injection of PGE2 on body temperature in wild-type, $Ptger3^{flox}$ and $Ptger3^{flox}$/*NesCre* mice. The mice were injected with PGE2 at 4 nmol or vehicle (0.9% saline with 10% DMSO) at time zero. The $Ptger3^{flox}$ mice had a normal fever response, but the $Ptger3^{flox}$/*NesCre* mice had a hypothermic response to PGE2. (**c**) Effects of intraperitoneal injection of LPS on body temperature in wild-type, $Ptger3^{flox}$ and $Ptger3^{flox}$/*NesCre* mice. The mice were injected with LPS (1 mg per kg) or 0.9% saline at time zero. Between 90–180 min, the $Ptger3^{flox}$ mice had a fever response of 0.7 °C, which was identical to that of wild-type mice. The $Ptger3^{flox}$/*NesCre* mice instead showed hypothermia of about 1.0 °C. Each point in **b** and **c** represents the mean ± s.e.m. of six animals. Symbols represent level of significance for the $Ptger3^{flox}$ and $Ptger3^{flox}$/*NesCre* mice when compared with vehicle-injected control at each time point. $^{*}P < 0.05$ and $^{**}P < 0.01$.

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circadian rhythms of body temperature were intact in mice with transgenically modified *Ptger3* alleles (**Supplementary Fig. 1**).

We implanted the animals with chronic cannulas for intracerebroventricular (ICV) injection of PGE2 and intraperitoneal telemetric thermal sensors, and tested the PGE2-dependent fever of the *Ptger3*^{flox} mice at 22 °C with an ICV injection of 4 nmol of PGE2 versus vehicle (**Fig. 1b**). We recorded a rapid and robust hyperthermic response with a peak at 39.7 ± 0.4 °C that was indistinguishable from that of their wild-type littermates (39.9 ± 0.2 °C, $P = 0.56$). We also injected the same animals intraperitoneally with saline or with LPS (1 mg per kg of body weight) and studied the fever response of the *Ptger3*^{flox} mice (**Fig. 1c**). All animals showed an initial stress fever as a result of handling during the injection procedure, irrespective of whether LPS or saline was given. An elevation of body temperature between 90 and 180 min after injection was observed in LPS-injected, but not in saline-injected, mice. During this period, the mean temperature difference of 0.7 ± 0.0 °C ($P < 0.001$) between LPS and saline injection in *Ptger3*^{flox} mice was identical to that of the wild-type littermates (0.7 ± 0.0 °C, $P < 0.001$).

We next crossed *Ptger3*^{flox} mice with mice expressing Cre recombinase under the rat nestin (*Nes*) promoter, which is expressed selectively in neuronal and glia-cell precursors[8]. In these mice, the first exon of the EP3 receptor mRNA is deleted in the central and peripheral nervous system only. This exon contains the start site for translation, and we found no *Ptger3* exon 1 mRNA in the brain (**Supplementary Fig. 1**). The *Ptger3*^{flox} mice containing the Nes-Cre construct (*Ptger3*$^{\Delta CNS}$/*NesCre*) failed to show a fever response when injected ICV with PGE2, but instead developed a mild hypothermia (**Fig. 1b**). When *Ptger3*$^{\Delta CNS}$/*NesCre* mice were injected intraperitoneally with LPS (1 mg per kg), they showed a profound biphasic hypothermic response with a fall in body temperature of about 2.2 °C from 25 to 60 min after injection, and a second phase from 90 to 180 min, with the maximal decrease (1.2 ± 0.3 °C) at 140 min after injection (**Fig. 1c**).

EP3 receptors are expressed in several nuclei in the preoptic area in mice, in a pattern nearly identical to that reported previously in rats (**Fig. 2a** and **Supplementary Fig. 1**)[5]. As all of these EP3R-positive cell groups are in the 'febrogenic region' of the preoptic area, we stereotaxically injected adeno-associated viral (AAV) vectors that contained the gene for Cre recombinase under the CMV promoter into the median preoptic nucleus (MnPO) in *Ptger3*^{flox} mice to determine the contribution of the MnPO to fever response (**Fig. 2a–d** and **Supplementary Fig. 2** online). Because our injections were targeted along the midline, none of them included the ventrolateral preoptic EP3R-positive neurons. At 2, 3 and 4 weeks after injection, we tested the animals with ICV injection of PGE2, vehicle and PGE2, respectively (**Fig. 2e–g**). The deletion of EP3Rs from the MnPO attenuated the hyperthermic responses of *Ptger3*^{flox} mice as tested by ICV injection of PGE2. In control animals with AAV-GFP injections into the MnPO, or with AAV-Cre injections that did not involve the MnPO, fever responses were

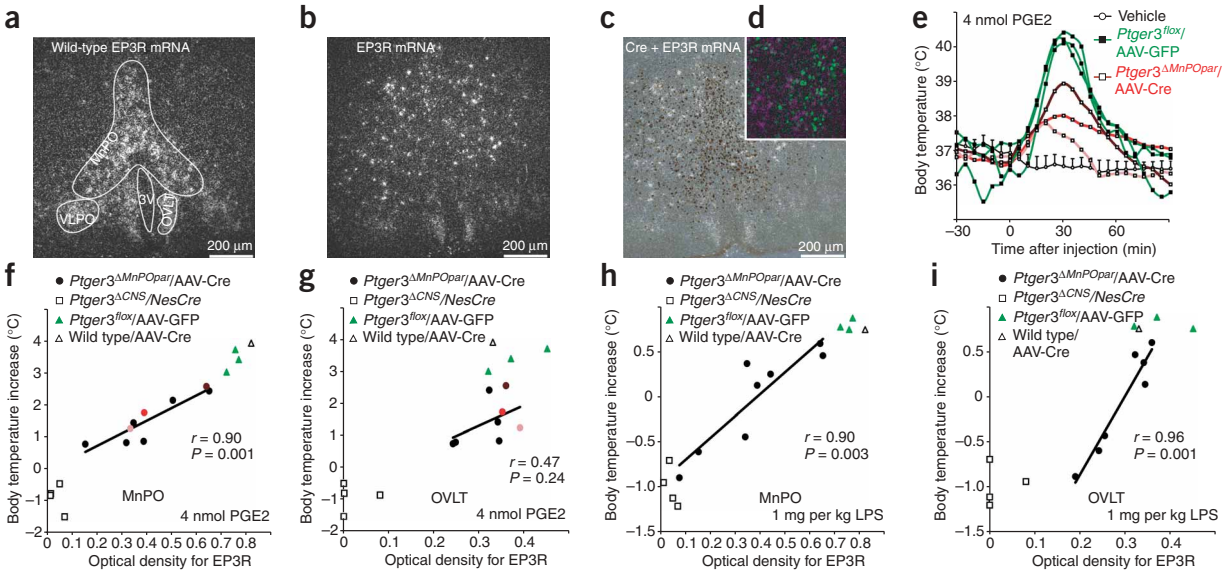

Figure 2 Site-specific deletion of EP3 receptors in the MnPO. (**a**) Robust expression of EP3Rs is seen in the PGE2-sensitive preoptic region of mice. Dark field photomicrographs of emulsion-coated sections from a wild-type mouse show clusters of silver grains marking EP3R mRNA hybridization signals in the febrogenic region of the preoptic area surrounding the third ventricle (3V), including the MnPO and the OVLT and the ventrolateral preoptic nucleus (VLPO), as indicated by white lines. This is essentially identical in *Ptger3*^{flox} mice (**Supplementary Fig. 1**). (**b**) A typical section from a *Ptger3*^{flox} mouse injected with AAV-Cre into the MnPO shows depleted expression of EP3R mRNA in the MnPO (compare with **a** and **Supplementary Fig. 1**). (**c**) An adjacent section was stained with rabbit polyclonal antibody to Cre (Novagen). The overlay of the photomicrograph of this section photographed in brightfield with the darkfield photomicrograph in **b** is shown, demonstrating that patterns of EP3R mRNA and Cre expression are complementary. (**d**) At a single-cell level, dual *in situ* hybridization (magenta) and immunofluorescence (green) for Cre shows that individual neurons that express Cre do not express EP3R, indicating deletion of EP3Rs by Cre in the MnPO. (**e**) Effects of ICV injection of PGE2 in week 4 on body temperature in three typical *Ptger3*^{flox} mice after injection with AAV-Cre (different red curves) or *Ptger3*^{flox} mice after injection with AAV-GFP (green) as control animals. The fever response in *Ptger3*^{flox} mice after AAV-GFP was identical to that of wild-type littermates (**Fig. 1b**), whereas the response in mice that received AAV-Cre was significantly attenuated. (**f**,**g**) The loss of fever response to PGE2 in *Ptger3*^{flox} mice that received AAV-Cre injections correlated closely with the loss of silver grain density for EP3R mRNA in the MnPO (**f**), but not for EP3R mRNA in the OVLT (**g**). The red and green data points correspond to the curves in **e**. (**h**,**i**) The loss of fever response to LPS in *Ptger3*^{flox} mice that received AAV-Cre injection in the MnPO correlated with the loss of the silver grain density for EP3R mRNA in the MnPO (**h**) and the OVLT (**i**), although in multivariate analysis the OVLT correlation was mainly due to the loss of OVLT cells in animals with the largest MnPO injection, and did not make a significant independent contribution. The body temperature increase is the difference of the mean body temperature between the saline and LPS response of each animal (mean body temperature between 90 and 180 min after the injection). Each point in **f**–**i** represents a single animal.

indistinguishable from *Ptger3*^{flox} animals without AAV injections. We measured the optical density of silver grains for *Ptger3* mRNA in the MnPO on *in situ* hybridization autoradiograms of sections containing the rostral, central and caudal MnPO, and found a Pearson correlation of $r = 0.90$ ($P = 0.001$) with fever response after ICV PGE2 in week 4. Thus, Cre-mediated disruption of *Ptger3* alleles in MnPO neurons was directly proportional to the loss of PGE2 fever response.

We also examined the correlation of the fever responses with loss of EP3Rs in the organum vasculosum of the lamina terminalis (OVLT). Although this is only a small component of the EP3R-positive region, previous work has implicated the OVLT as a site that is possibly involved in the pathogenesis of fever. We found that the Pearson correlation for the OVLT was only $r = 0.47$ ($P = 0.24$). Because the largest MnPO deletions might also be the ones most likely to include the OVLT, we separated the contributions of these two cell groups with a multiple regression analysis, which showed that the best-fit multiple regression model was affected significantly only by changes in optical density for EP3Rs in the MnPO ($P < 0.001$) and not by those in the OVLT ($P = 0.06$), but that the body temperature in response to ICV PGE2 could be best predicted by a linear combination of optical density for EP3R-positive neurons in the MnPO and OVLT ($r = 0.98$, $P < 0.001$). Thus, the small number of EP3R-positive cells in the OVLT can be considered to be a continuation of the MnPO febrogenic population.

The deletion of EP3Rs from the MnPO also reduced the fever response to intraperitoneal LPS (**Fig. 2h,i**). A similar analysis of the effect of intraperitoneal injection of LPS (1 mg per kg) on body temperature during the second phase of fever, from 90 to 180 min, showed an $r = 0.90$ ($P = 0.003$) for the MnPO and $r = 0.96$ ($P = 0.001$) for the OVLT for the correlation of Cre-mediated disruption of the EP3R expression and loss of the fever response in *Ptger3*^{flox} mice. On multiple regression analysis, we found $r = 0.97$ ($P < 0.001$) for the combination of the MnPO and OVLT, but in the best-fit model EP3R in the MnPO had $P < 0.001$, whereas OVLT had $P = 0.053$. In addition, the deletion of the EP3Rs from the MnPO/OVLT unmasked a hypothermic process, so that mice with the most extensive (nearly total) loss of EP3Rs showed hypothermic responses that were comparable to those of the *Ptger3*$^{\Delta CNS}$*/NesCre* animals.

Our results definitively establish that the expression of EP3Rs by neurons in the MnPO is necessary for febrile responses in mice, as well as suggesting that the population of EP3R-positive neurons in the OVLT is probably an extension of the MnPO group and has a minor role in fever response. As EP3Rs in the hypothalamus are likely to be inhibitory, this model is consistent with the MnPO tonically inhibiting thermogenic responses[9,10]. The MnPO provides GABAergic inputs to a system of thermogenic neurons, including the paraventricular nucleus of the hypothalamus and the dorsomedial nucleus/dorsal area of the hypothalamus, as well as to the raphe pallidus (RPa) nucleus in the medulla (**Supplementary Fig. 3** online). All of these sites have descending projections that influence sympathetic preganglionic neurons in the spinal cord that are critical for thermogenesis. The RPa, in particular, is a key site for integrating sympathetic responses that are important for producing increased body temperature, including both increased thermogenesis through activation of brown adipose tissue and reduced passive heat loss through the skin by tail artery vasoconstriction[9,10].

EP3Rs are highly expressed by neurons in the MnPO with descending projections to the RPa[10]. In addition, the fever response is attenuated by lesions of the paraventricular nucleus of the hypothalamus, by inhibition of the dorsomedial nucleus of the hypothalamus or by injecting inhibitors of excitatory amino-acid neurotransmission into the RPa, indicating that the RPa receives both an excitatory amino-acid input and a reduced GABA input during fever responses[11,12]. Animals with large lesions of the preoptic area, which also include the MnPO, have high basal body temperature, which prevents further increases in body temperature caused by a pyrogen[13]. As our data show, the selective loss of EP3Rs in the MnPO has no effect on baseline body temperature, but prevents fever responses to PGE2 or LPS in our mouse models. Our data indicate that the EP3Rs in the MnPO are necessary for fever responses. Because fever is produced by focal injection of PGE2 in the preoptic area, but not at other sites in the brain that express EP3Rs (such as the RPa)[14], it is likely that the MnPO EP3Rs are also sufficient to produce a fever response. However, selective reinsertion of EP3Rs in the MnPO of mice lacking those receptors would be necessary to confirm this.

In the absence of EP3Rs in the MnPO, both PGE2 and LPS produce hypothermic responses. This response to LPS is identical for animals that have complete EP3R deletion in the entire body[7], or in our experiments, in the central nervous system. We previously showed that EP4 receptors may cause hypothermia, and because these are also expressed in the region of the MnPO, including in the ventromedial preoptic area which demonstrates Fos expression during LPS fever[5], they may have a role in the ultimate temperature response to inflammatory stimuli such as LPS[15]. In addition, EP1 receptors in the preoptic area may also promote hyperthermia, but in the absence of EP3Rs in the MnPO, they are clearly incapable of overcoming the hypothermic EP4 response. The focal conditional knockout approach used in this study has wide applicability to this and other problems involving the dissection of the effects of different receptors with complex brain distributions.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

M.L. was responsible for all phases of the experimental work, performing the analyses and writing of the manuscript. K.Y. participated in the physiological recording and histology. R.C. and B.B.L. assisted with design and execution of preparing the genetically altered mice. C.E.B. assisted with the design and production of the AAV-Cre vectors. T.M. assisted with physiological recordings. C.B.S. was responsible for overall design of the project and analysis of results, and edited the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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