

# Immune-Induced Fever Is Dependent on Local But Not Generalized Prostaglandin E<sub>2</sub> Synthesis in the Brain

Anna Eskilsson,<sup>1\*</sup> Takashi Matsuwaki,<sup>1\*</sup>  Kiseko Shionoya,<sup>1\*</sup>  Elahe Mirrasekhian,<sup>2</sup>  Joanna Zajdel,<sup>2</sup>  Markus Schwaninger,<sup>3</sup>  David Engblom,<sup>2</sup> and  Anders Blomqvist<sup>1</sup>

<sup>1</sup>Division of Neurobiology and <sup>2</sup>Center for Social and Affective Neuroscience, Department of Clinical and Experimental Medicine, Faculty of Medicine and Health Sciences, Linköping University, S-581 85 Linköping, Sweden, and <sup>3</sup>Institute of Experimental and Clinical Pharmacology and Toxicology, University of Lübeck, 23538 Lübeck, Germany

Fever occurs upon binding of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) to EP<sub>3</sub> receptors in the median preoptic nucleus of the hypothalamus, but the origin of the pyrogenic PGE<sub>2</sub> has not been clearly determined. Here, using mice of both sexes, we examined the role of local versus generalized PGE<sub>2</sub> production in the brain for the febrile response. In wild-type mice and in mice with genetic deletion of the prostaglandin synthesizing enzyme cyclooxygenase-2 in the brain endothelium, generated with an inducible CreER<sup>T2</sup> under the *Slco1c1* promoter, PGE<sub>2</sub> levels in the CSF were only weakly related to the magnitude of the febrile response, whereas the PGE<sub>2</sub> synthesizing capacity in the hypothalamus, as reflected in the levels of cyclooxygenase-2 mRNA, showed strong correlation with the immune-induced fever. Histological analysis showed that the deletion of cyclooxygenase-2 in brain endothelial cells occurred preferentially in small- and medium-sized vessels deep in the brain parenchyma, such as in the hypothalamus, whereas larger vessels, and particularly those close to the neocortical surface and in the meninges, were left unaffected, hence leaving PGE<sub>2</sub> synthesis largely intact in major parts of the brain while significantly reducing it in the region critical for the febrile response. Furthermore, injection of a virus vector expressing microsomal prostaglandin E synthase-1 (mPGES-1) into the median preoptic nucleus of fever-refractive mPGES-1 knock-out mice, resulted in a temperature elevation in response to LPS. We conclude that the febrile response is dependent on local release of PGE<sub>2</sub> onto its target neurons and not on the overall PGE<sub>2</sub> production in the brain.

**Key words:** cyclooxygenase-2; endothelial cells; fever; median preoptic nucleus; microsomal prostaglandin E synthase-1; prostaglandin E<sub>2</sub>

## Significance Statement

By using mice with selective deletion of prostaglandin synthesis in brain endothelial cells, we demonstrate that local prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production in deep brain areas, such as the hypothalamus, which is the site of thermoregulatory neurons, is critical for the febrile response to peripheral inflammation. In contrast, PGE<sub>2</sub> production in other brain areas and the overall PGE<sub>2</sub> level in the brain do not influence the febrile response. Furthermore, partly restoring the PGE<sub>2</sub> synthesizing capacity in the anterior hypothalamus of mice lacking such capacity with a lentiviral vector resulted in a temperature elevation in response to LPS. These data imply that the febrile response is dependent on the local release of PGE<sub>2</sub> onto its target neurons, possibly by a paracrine mechanism.

## Introduction

It is now well established that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is the final mediator of inflammation-induced fever (Li et al., 1999; Engblom et al., 2003). Fever has long been associated with elevated

brain levels of PGE<sub>2</sub> (Splawinski, 1977); in response to a peripheral immune stimulus, PGE<sub>2</sub> levels in the CSF increase concomitant with the febrile response (Inoue et al., 2002), and injection of PGE<sub>2</sub> into the CSF elicits fever in a dose-dependent way (Nilsberth et al., 2009b). PGE<sub>2</sub> is synthesized by brain endothelial cells through the concerted action of the inducible enzymes cycloo-

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\*A.E., T.M., and K.S. contributed equally to this work.

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Correspondence should be addressed to Dr. Anders Blomqvist, Division of Neurobiology, Department of Clinical and Experimental Medicine, Faculty of Medicine and Health Sciences, Linköping University, S-581 85 Linköping, Sweden. E-mail: anders.blomqvist@liu.se.

T. Matsuwaki's present address: Department of Veterinary Physiology, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8657 Japan.

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genase (Cox)-2 and microsomal prostaglandin E synthase-1 (mPGES-1; Ek et al., 2001; Yamagata et al., 2001). These correlational studies have later been complemented by functional genetic studies, which have shown that genetic deletion of Cox-2 or mPGES-1 in the brain endothelium strongly attenuates the febrile response (Wilhelms et al., 2014), hence demonstrating that the brain endothelium plays a critical role for the generation of the PGE<sub>2</sub> that is seen in the brain during fever.

Because a peripheral immune stimulus elicits the induction of prostaglandin-synthesizing enzymes throughout the brain vasculature (Ek et al., 2001; Yamagata et al., 2001), suggesting a generalized PGE<sub>2</sub> release, the specificity of the PGE<sub>2</sub>-elicited responses comes about through the distinct distribution of its receptor subtypes (Zhang and Rivest, 1999; Ek et al., 2000; Oka et al., 2000), as demonstrated by the attenuation of fever through the deletion of EP<sub>3</sub> receptors in the median preoptic nucleus but not by deletion of these receptors at other sites (Lazarus et al., 2007). However, it is less clear whether the responses evoked by PGE<sub>2</sub> binding to its receptors is the result of PGE<sub>2</sub> release locally, in a paracrine fashion, or whether PGE<sub>2</sub> released at other sites also can elicit fever under physiological conditions (Matsumura et al., 1997). Although studies using injections of PGE<sub>2</sub> or cyclooxygenase inhibitors support the importance of local synthesis (Scammell et al., 1996, 1998), the critical site of prostaglandin synthesis has not been investigated using modern functional genetic techniques. Here we addressed this question by examining in normal mice and in mice with the deletion of Cox-2 in the brain endothelium the relationship between the magnitude of the febrile response to a peripheral immune stimulus and the level of induced PGE<sub>2</sub> in the CSF, as seen in individual animals. We also examined the relationship between the magnitude of the febrile response and the level of Cox-2 mRNA in the hypothalamus, as well as the PGE<sub>2</sub> levels in plasma, and we determined by using immunohistochemistry how Cox-2 expression in different types of vessels was related to the febrile response. Finally, we examined whether local endogenous immune-induced production of PGE<sub>2</sub> in the anterior hypothalamus resulted in a temperature response. Our data show that local PGE<sub>2</sub> release onto brain PGE<sub>2</sub> receptors is the mechanism governing the febrile response to peripheral immune challenge.

## Materials and Methods

**Animals.** Mice with specific deletion of Cox-2 in brain endothelial cells were generated by crossing animals in which exons 4–5 of the Cox-2 gene (*Ptgs2*) are flanked by *loxP*-sites (Ishikawa and Herschman, 2006) with animals expressing a tamoxifen-inducible CreER<sup>T2</sup> under the *Slco1c1* promoter (expressed in the cerebrovascular endothelium; Ridder et al., 2011). The tamoxifen (Sigma-Aldrich; 1 mg diluted in a mixture of 10% ethanol and 90% sunflower seed oil) was injected (0.1 ml, i.p.) twice a day for 5 d, followed by a 5 week recovery period before any further experiments were performed. The inducible Cre line was also crossed with a Cre reporter line, which expresses a *Gt(ROSA)26Sor* locus with a *loxP*-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato; The Jackson Laboratory; RRID: IMSR\_JAX:007914), and the offspring were treated with tamoxifen, as described above. Mice with deletion of the *Ptgs2* gene (Trebino et al., 2003), encoding mPGES-1, were from our own breeding and on a C57BL/6 background. All animal experiments were approved by the local animal care and use committee and followed international guidelines.

**Telemetric temperature recordings.** The mice were briefly anesthetized with isoflurane (Abbot Scandinavia) and implanted intraperitoneally with a transponder that records core body temperature (Mini Mitter). Immediately after surgery, the mice were transferred to a room in which

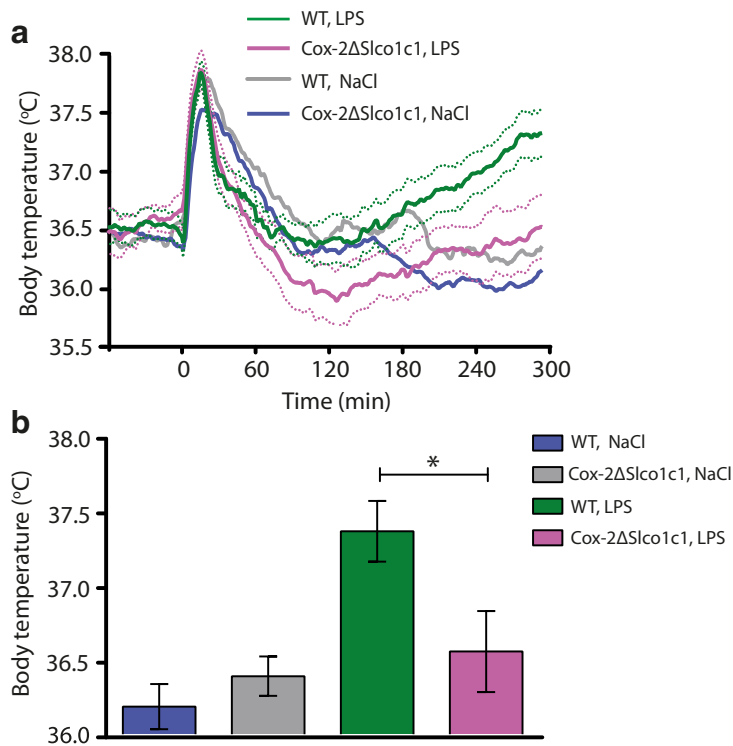
the ambient temperature was set to 29°C, providing near-thermoneutral conditions (Rudaya et al., 2005).

**Injection of lentiviral vectors.** Mice were anesthetized with isoflurane and mounted onto a stereotaxic frame. The scalp was exposed, and two small holes were drilled at the level of the bregma on each side of the midline. A Hamilton syringe was lowered to a position that was 0.0 mm anteroposterior to bregma, 0.3 mm mediolateral to bregma, and 5.5 mm dorsoventral to bregma. A lentiviral (lenti) vector was then injected at a rate of 180 nl/min over 3 min. The syringe remained in place for at least 3 min after the infusion and was then slowly removed, after which the skin was closed. Mice were injected either with a lentiviral vector, in Dulbecco's PBS containing MgCl<sub>2</sub> and CaCl<sub>2</sub> (catalog #D8662, Sigma-Aldrich), expressing GFP [lenti-5-KP-pgk-GFP ( $5 \times 10^8$  to  $1 \times 10^9$  transducing units/ml), produced as previously described (Zufferey et al., 1997; Georgievska et al., 2004; a gift from Johan Jakobsson, Lund University, Sweden)] or a lentiviral vector expressing human mPGES-1 [suCMV promoter-human *Ptgs2* (NM\_004878)-Rsv promoter-puromycin resistance,  $10^8$  infectious units/ml; AMS Biotechnology], to which was added 10% of the lenti-GFP virus to permit subsequent immunofluorescent identification of the injection site (we found no available antibody that could detect mPGES-1). During the same surgical session, a temperature transponder was implanted in the abdominal cavity, as described above.

**Immune stimulation.** Mice were injected intraperitoneally with bacterial wall lipopolysaccharide (LPS) from *Escherichia coli* (catalog #O111: B4, Sigma-Aldrich; 120 μg/kg body weight, diluted in 100 μl), 1 or 3 weeks (virus-injected mice) following implantation of the temperature transponder.

**Tissue collection.** Mice injected with LPS only were killed 5 h after injection (this time point was selected since it corresponds to the time of peak fever in this experimental paradigm; Hamzic et al., 2013). Blood was drawn from the right atrium, transferred to EDTA-coated tubes (Sarstedt) to which was added indomethacin (10 μM; Sigma-Aldrich), and centrifuged at  $7000 \times g$  for 7 min at 4°C. The plasma was immediately frozen on dry ice and kept at –70°C. The animals were then placed in a stereotaxic frame, the atlanto-occipital membrane was exposed, and CSF was withdrawn from the cisterna magna using a Hamilton syringe mounted on a micromanipulator and immediately frozen. Samples that contained traces of blood were discarded. The whole procedure from when the animals were killed until CSF was withdrawn took <10 min. A hypothalamic block was then dissected and placed in RNAlater stabilization reagent (Qiagen) and stored at –70°C until analysis. This block was first isolated by two coronal cuts, one placed 0.5 mm rostral to the apex of the optic chiasm and the other at the caudal margin of the mammillary bodies. The resulting slab was then trimmed by sagittal cuts on each side through the sulcus between the hypothalamus and the temporal lobe. Finally, a horizontal cut was placed slightly above the anterior commissure. Mice injected with viral vectors were killed the day after the immune challenge with LPS. After asphyxiation with CO<sub>2</sub>, one group of mice was fixed by transcardial perfusion with a phosphate-buffered (0.1 M) paraformaldehyde solution (4%). The brains were removed and post-fixed for 3 h in the same fixative and then cryoprotected with 25% sucrose in PBS. In another group of mice, the brain was immediately removed. The hypothalamus was dissected and stored in RNAlater (Qiagen) at –70°C.

**Immunohistochemistry.** Brains were cut in the frontal plane at 30 μm on a freezing microtome. The immunohistochemical procedures were performed according to standardized protocols (Engström et al., 2012). In brief, sections were incubated in a blocking solution [PBS containing 3% normal donkey serum (Jackson ImmunoResearch), 1% bovine serum albumin (Sigma-Aldrich), and 0.3% Triton X (Merck)] for 45 min, followed by incubation overnight at room temperature with rabbit anti-Cox-2 antibody (1:500; sc-1747 M-19, Santa Cruz Biotechnology; RRID: AB\_2084976) and goat anti-lipocalin-2 antibody (1:500; AF1857, R&D Systems; RRID: AB\_355022), rinsed in PBS and then incubated with Alexa Fluor 555 donkey anti-rabbit antibody and Alexa Fluor 488 donkey anti-goat antibody (both 1:500; Life Technologies). Sections from lentivector-injected brains were incubated with chicken anti-GFP antibody (1:10,000; ab13970, Abcam; RRID: AB\_300798), followed by Alexa Fluor 488 goat anti-chicken IgG (heavy chain and light chain) antibody (Life Technologies). The sections were finally mounted on SuperFrost



**Figure 1.** Attenuated fever in mice with gene deletion of Cox-2 in brain endothelial cells. **a**, Temperature recordings from WT and Cox-2<sup>ΔSlco1c1</sup> mice immune challenged by intraperitoneal injection of LPS (120 μg/kg). The initial temperature peak is due to the handling stress in conjunction with the injection procedure. It is prostaglandin independent (Saha et al., 2005) and does not differ between genotypes. **b**, Bar graph showing mean fever 5 h after LPS injection in WT type and Cox-2<sup>ΔSlco1c1</sup> mice. \**p* < 0.05. In **a** and **b**: *n* = 15 for WT LPS; *n* = 11 for Cox-2<sup>ΔSlco1c1</sup> LPS; *n* = 9 for WT NaCl; and *n* = 6 for Cox-2<sup>ΔSlco1c1</sup> NaCl. Error bars = SEM.

Plus glasses (ThermoFisher Scientific) with Prolong Gold Anti-Fade Reagent (Life Technologies).

**Assays for PGE<sub>2</sub> levels in CSF and plasma.** The concentration of PGE<sub>2</sub> in CSF (diluted 1:100) was determined using a High Sensitivity Prostaglandin E2 Enzyme Immunoassay Kit (Assay Designs). The values were calculated using a standard curve ranging from 7.81 to 1000 pg/ml (*R*<sup>2</sup> = 1). The kit antiserum shows the following cross-reactivity, according to the manufacturer: PGE<sub>2</sub>, 100%; PGE<sub>1</sub>, 70%; PGE<sub>3</sub>, 16.3%; PGF<sub>1α</sub>, 1.4%; PGF<sub>2α</sub>, 0.7%; 6-keto-PGF<sub>1α</sub>, 0.6%; PGA<sub>2</sub>, 0.1%; PGB<sub>1</sub>, 0.1%; and <0.1% for 13,14-dihydro-15-keto-PGF<sub>2α</sub>, 6, 15-keto, 13, 4-dihydro-PGF<sub>1α</sub>, thromboxane B<sub>2</sub>, 2 arachidonoylglycerol, anandamide, PGD<sub>2</sub>, and arachidonic acid. The concentration of PGE<sub>2</sub> metabolites in plasma (diluted 1:20) was determined with a Prostaglandin E Metabolite EIA Kit (Cayman Chemical). The values were calculated using a standard curve ranging from 0.2 to 50 pg/ml (*R*<sup>2</sup> = 0.999). The kit antiserum recognizes derivatized 13,14-dihydro-15-ketoPGE<sub>1</sub>, 13,14-dihydro-15-ketoPGE<sub>2</sub>, and bicycloPGE<sub>1</sub>, but has <0.01% cross-reactivity with arachidonic acid, leukotriene B<sub>4</sub>, tetranor-PGEM, tetranor-PGFM, PGD<sub>2</sub>, PGE<sub>1</sub>, 6-keto PGE<sub>1</sub>, PGE<sub>2</sub>, PGF<sub>1α</sub>, 6-keto PGF<sub>1α</sub>, PGF<sub>2α</sub> and thromboxane B<sub>2</sub>.

**Real-time quantitative PCR analyses.** RNA was extracted with RNeasy Universal Plus kit or RNeasy Micro Kit (Qiagen), and reverse transcription was performed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time quantitative PCR (qPCR) was then performed using Gene Expression Master Mix (Applied Biosystems) on a 96-well plate (7900HT Fast RT-PCR System, Applied Biosystems). The following assays (all from Applied Biosystems) were used: *Ptgs2*: Mm00478374\_m1; human *Ptges* (Hs01115r610\_m1); and *Gapdh* (Mm99999915\_g1).

**Experimental design and statistical analysis.** For all comparisons between genotypes, littermates were used. Mice were of both sexes, and experimental groups were balanced with respect to sex and age. Sample size is reported in the figure legends. All statistical analyses were performed in GraphPad Prism (GraphPad Software). Analysis of differences in body temperature at 5 h between LPS-treated WT mice and mice with

endothelial-specific deletion of Cox-2 was performed with a one-way ANOVA followed by Sidak's multiple-comparisons test. The same analysis was used for differences between groups with respect to PGE<sub>2</sub> in CSF and qPCR data. For PGE<sub>2</sub> metabolites in plasma, nonparametric statistics were used (Kruskal–Wallis test followed by Dunn's multiple-comparisons test). Temperature responses after injections of viral vectors were analyzed with a two-way repeated-measures ANOVA followed by Tukey's *post hoc* test. Regression analysis was performed with *F* statistics. Qualitative data were analyzed with Fisher's exact test. Results were considered significant at *p* < 0.05.

## Results

### Deletion of Cox-2 in brain endothelial cells results in attenuated fever response to LPS

As reported previously (Wilhelms et al., 2014), mice with deletion of Cox-2 in brain endothelial cells (Cox-2<sup>ΔSlco1c1</sup>) show attenuated fever following intraperitoneal injection of LPS (Fig. 1*a*). At 5 h after injection, when the animals were killed and tissue was collected for analysis, LPS-treated Cox-2<sup>ΔSlco1c1</sup> mice displayed significantly lower body temperatures than their wild-type (WT) Cox-2<sup>fl/fl</sup> littermates (*F*<sub>(3,37)</sub> = 6.283, *p* = 0.0015; LPS Cox-2<sup>ΔSlco1c1</sup> vs LPS WT: *p* = 0.0219; Fig. 1*b*).

### No difference in PGE<sub>2</sub> levels in CSF between Cox-2<sup>ΔSlco1c1</sup> mice and WT mice

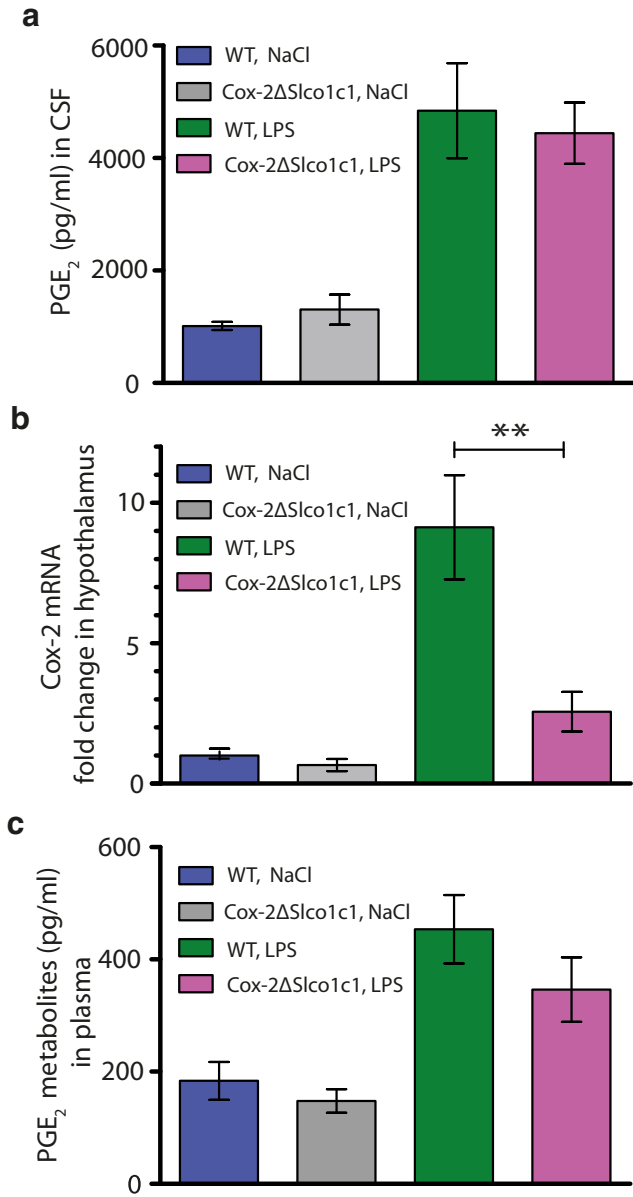
We next examined whether the difference in the febrile response to LPS between Cox-2<sup>ΔSlco1c1</sup> mice and WT mice was associated with a difference in CSF levels of PGE<sub>2</sub>. LPS treatment resulted in elevated levels of PGE<sub>2</sub> in CSF at 5 h after injection (*F*<sub>(3,37)</sub> = 8.003, *p* < 0.003; LPS WT vs NaCl WT: *p* = 0.0005; LPS Cox-2<sup>ΔSlco1c1</sup> vs NaCl Cox-2<sup>ΔSlco1c1</sup>: *p* = 0.0198), but there was no difference between genotypes (Fig. 2*a*).

### Lower levels of induced Cox-2 mRNA in the hypothalamus of Cox-2<sup>ΔSlco1c1</sup> mice

qPCR analysis of the levels of Cox-2 mRNA in the hypothalamus of Cox-2<sup>ΔSlco1c1</sup> mice and WT mice showed significantly lower induction following LPS administration in the gene-deleted mice than in the WT mice (*F*<sub>(3,39)</sub> = 21.78, *p* < 0.0001; LPS WT vs LPS Cox-2<sup>ΔSlco1c1</sup>: *p* = 0.0021; Fig. 2*b*). As expected, there was also a small (but statistically not significant) reduction of Cox-2 mRNA in the NaCl-treated Cox-2<sup>ΔSlco1c1</sup> mice compared with the WT mice (Fig. 2*b*).

### No difference in PGE<sub>2</sub> metabolite levels in plasma between Cox-2<sup>ΔSlco1c1</sup> mice and WT mice

To examine whether the gene deletion in the Cox-2<sup>ΔSlco1c1</sup> mice, which should occur only in brain endothelial cells (Ridder et al., 2011), had influenced prostaglandin synthesis peripherally, the levels of PGE<sub>2</sub> metabolites in plasma were analyzed. This assay was chosen instead of the direct measurement of PGE<sub>2</sub> because PGE<sub>2</sub> in plasma is difficult to measure reliably (Samuelsson et al., 1975) since it is rapidly converted *in vivo* to its 13,14-dihydro-15-

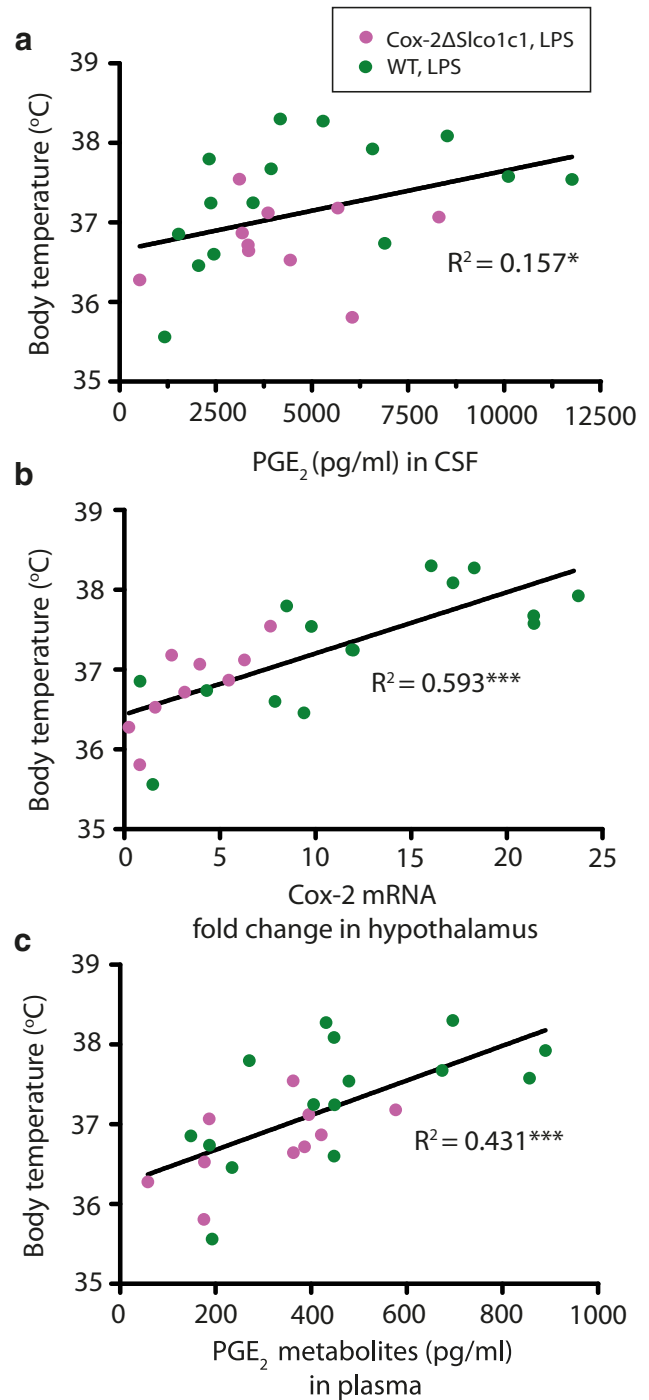


**Figure 2.** PGE<sub>2</sub> levels in brain and plasma and Cox-2 mRNA expression in the hypothalamus in WT mice and in mice with gene deletion of Cox-2 in brain endothelial cells. **a**, Immune stimulation with LPS (120 μg/kg, i.p.) increases the PGE<sub>2</sub> concentration in the CSF to similar levels in both WT and Cox-2<sup>ΔSlco1c1</sup> mice. **b**, The immune-induced Cox-2 mRNA induction in the hypothalamus is significantly attenuated in Cox-2<sup>ΔSlco1c1</sup> mice. \*\**p* < 0.01. **c**, The immune-induced levels of PGE<sub>2</sub> metabolites in plasma do not differ between WT and Cox-2<sup>ΔSlco1c1</sup> mice. In all graphs: *n* = 15 for WT LPS; *n* = 10–11 for Cox-2<sup>ΔSlco1c1</sup> LPS; *n* = 9–10 for WT NaCl; and *n* = 5–8 for Cox-2<sup>ΔSlco1c1</sup> NaCl. Error bars = SEM.

keto metabolite, with >90% of circulating PGE<sub>2</sub> being cleared by a single passage through the lungs (Hamberg and Samuelsson, 1971). The levels of PGE<sub>2</sub> metabolites in plasma were elevated following immune challenge with LPS (Kruskal-Wallis statistic = 19.84, *p* = 0.0002; LPS WT vs NaCl WT: *p* = 0.0022; LPS Cox-2<sup>ΔSlco1c1</sup> vs NaCl Cox-2<sup>ΔSlco1c1</sup>: *p* = 0.0327), but there was no difference between the genotypes (Fig. 2c).

**Body temperature following immune stimulation correlates with Cox-2 mRNA levels in the hypothalamus but not with PGE<sub>2</sub> levels in CSF**

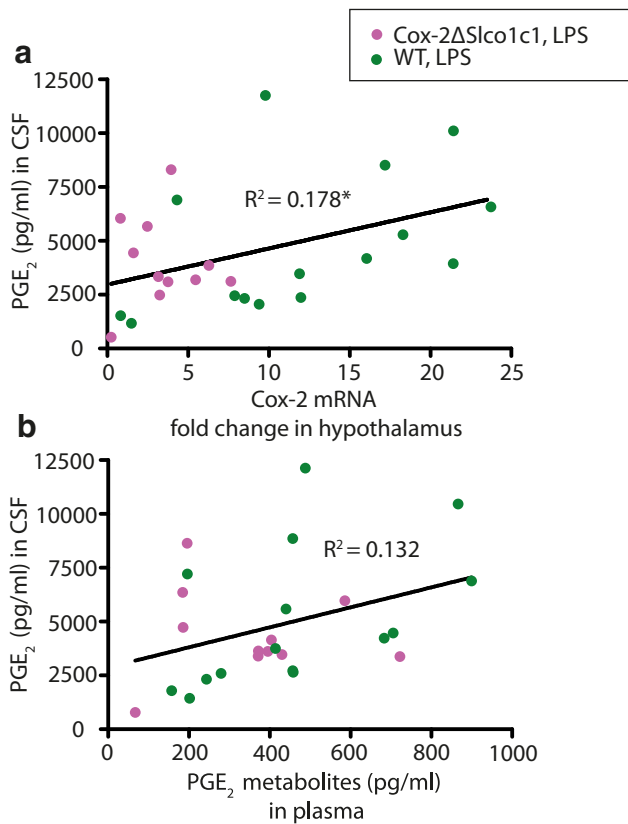
To further examine the relationship between body temperature and central prostaglandin synthesis, we next performed a regres-



**Figure 3.** Relationship among body temperature and PGE<sub>2</sub> in the CSF, Cox-2 mRNA in the hypothalamus, and PGE<sub>2</sub> metabolites in plasma, respectively, following immune stimulation with LPS (120 μg/kg, i.p.). **a**, Weak relationship between body temperature and PGE<sub>2</sub> in the CSF. **b**, Strong relationship between body temperature and Cox-2 mRNA in the hypothalamus. **c**, Moderately strong relationship between body temperature and PGE<sub>2</sub> metabolites in plasma. \**p* < 0.05, \*\*\**p* < 0.001.

sion analysis of the individual temperatures and the levels of PGE<sub>2</sub> in CSF in Cox-2<sup>ΔSlco1c1</sup> mice and WT mice 5 h after LPS injection. As shown (Fig. 3a), there was only weak relationship between these two parameters (*R*<sup>2</sup> = 0.157, *F*<sub>(1,23)</sub> = 4.292, *p* = 0.0497; Fig. 3a). In contrast, there was a strong correlation between temperature and Cox-2 mRNA in the hypothalamus (*R*<sup>2</sup> = 0.593, *F*<sub>(1,22)</sub> = 32.01, *p* < 0.0001; Fig. 3b). There was also a





**Figure 4.** Relationship between PGE<sub>2</sub> levels in the CSF and Cox-2 mRNA and PGE<sub>2</sub> metabolites in plasma, respectively. *a*, Weak relationship between PGE<sub>2</sub> in the CSF and Cox-2 mRNA in the hypothalamus. *b*, No significant relationship between PGE<sub>2</sub> in the CSF and PGE<sub>2</sub> metabolites in plasma. \* $p < 0.05$ .

moderately strong relationship between body temperature and PGE<sub>2</sub> metabolites in plasma ( $R^2 = 0.4310$ ,  $F_{(1,23)} = 17.42$ ,  $p = 0.0004$ ; Fig. 3*c*).

#### The levels of PGE<sub>2</sub> in CSF correlate only weakly with Cox-2 mRNA expression in the hypothalamus and not with levels of PGE<sub>2</sub> metabolites in plasma

To examine what affects the PGE<sub>2</sub> levels in CSF, regression analysis was performed for the relationship between PGE<sub>2</sub> in CSF and Cox-2 mRNA expression in the hypothalamus of LPS-treated mice (Fig. 4*a*) and PGE<sub>2</sub> metabolites in plasma in these mice (Fig. 4*b*), respectively. PGE<sub>2</sub> levels in CSF showed only a weak relationship with the Cox-2 mRNA expression in the hypothalamus ( $R^2 = 0.1783$ ,  $F_{(1,25)} = 5.424$ ,  $p = 0.0282$ ; Fig. 4*a*). In the same vein, there was no significant relationship between PGE<sub>2</sub> levels in CSF and the levels of PGE<sub>2</sub> metabolites in plasma ( $R^2 = 0.1321$ ,  $F_{(1,24)} = 3.654$ ,  $p = 0.0679$ ; Fig. 4*b*), indicating that they have different sources of origin.

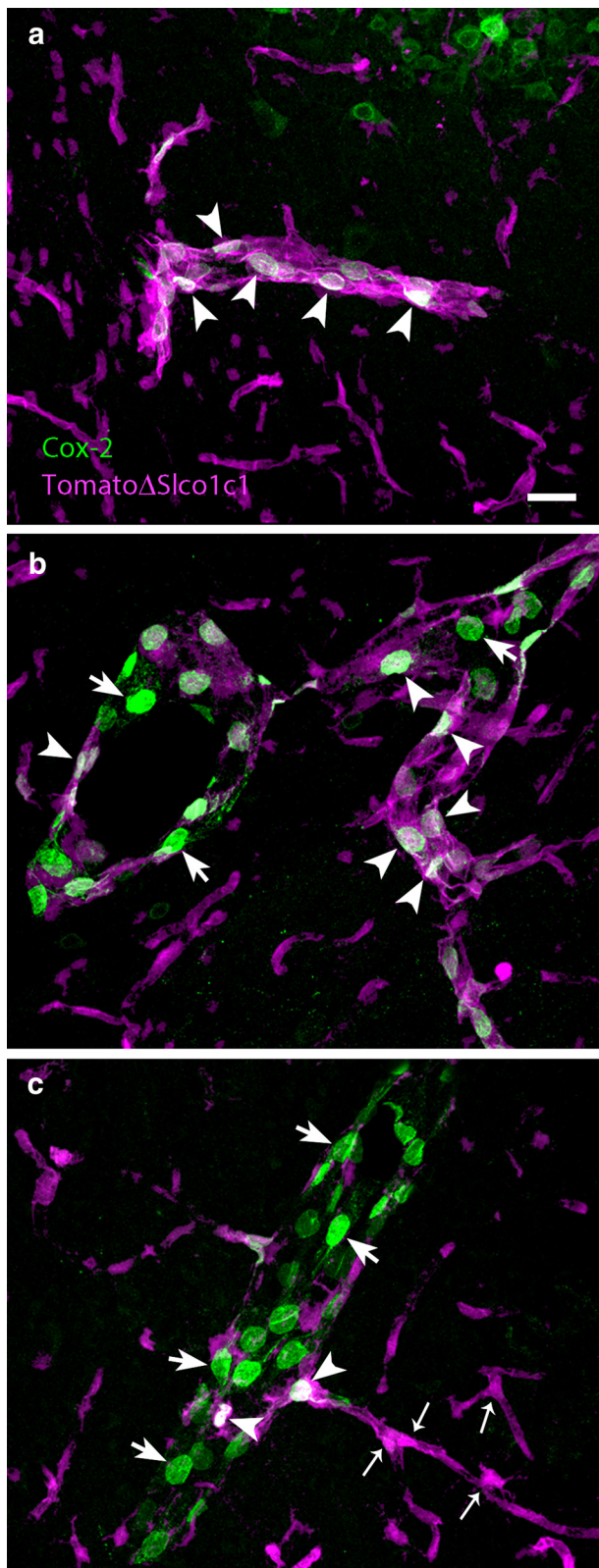
#### The gene deletion in Cox-2<sup>ΔSlo1c1</sup> mice mainly occurs in smaller vessels in the brain parenchyma but not in large vessels

Since the above data indicated that the gene deletion in Cox-2<sup>ΔSlo1c1</sup> mice preferentially affected induced prostaglandin synthesis in the hypothalamus, but not generally in the brain, we examined histologically where the gene deletion took place. We crossed the mice with the tamoxifen-inducible CreER<sup>T2</sup> under the *Slo1c1* promoter with a Cre reporter line expressing a *Gt(ROSA)26Sor* locus with a *loxP*-flanked STOP cassette, which prevents tran-

scription of a CAG promoter-driven red fluorescent protein variant (tdTomato). Following tamoxifen treatment of the offspring, we subjected it to immune challenge with LPS. We found that Cre-induced recombination occurred preferentially in small- and medium-sized blood vessels; in the latter, tdTomato staining was found to be colocalized with induced Cox-2 immunoreactivity in endothelial cells (Fig. 5*a,b*). In contrast, Cox-2-positive cells in larger blood vessels rarely expressed tdTomato staining (Fig. 5*b,c*). We also examined brains from Cox-2<sup>ΔSlo1c1</sup> mice and WT mice for the coexpression of Cox-2 and lipocalin-2 immunoreactivity to evaluate whether the endothelial cells had responded to LPS since lipocalin-2 is expressed in endothelial cells in response to LPS together with, but independently of, Cox-2 (Hamzic et al., 2013; Vasilache et al., 2015). We found that lipocalin-2-expressing cells in larger vessels, and in particular in vessels close to the surface of the neocortex, coexpressed Cox-2 extensively in both genotypes (Fig. 6*a,b*). Strong Cox-2 labeling was also seen among lipocalin-2-expressing cells in meningeal vessels in both genotypes (Fig. 6*c,d*). However, smaller vessels in Cox-2<sup>ΔSlo1c1</sup> mice, preferentially in the more medial parts of the brain such as in the hypothalamic region, more rarely expressed Cox-2 while displaying extensive labeling for lipocalin-2 (Fig. 6*f*). This was in stark contrast to what was seen in WT mice, in which there was extensive coexpression of both proteins also in small vessels (Fig. 6*e*). Although there was some variation across animals, when a blinded investigator qualitatively determined the genotype of the animals by examining the degree of colocalization of Cox-2 and lipocalin-2 in differently sized vessels, the correct genotype was determined in 83% of the animals ( $p = 0.0073$ ;  $n = 13$  for Cox-2<sup>ΔSlo1c1</sup> mice, and  $n = 9$  for WT mice).

#### Restoration of PGE<sub>2</sub> synthesis in the preoptic hypothalamus of mPGES-1 knock-out mice results in a temperature response to LPS

To determine whether local PGE<sub>2</sub> production in the hypothalamus results in a temperature response to LPS, we used intracerebral injection of a viral vector to restore the PGE<sub>2</sub> synthesizing capacity in the median preoptic regions of mice with global deletion of mPGES-1, the inducible terminal PGE<sub>2</sub>-synthesizing enzyme (Jakobsson et al., 1999). We chose to use mPGES-1 knock-out (KO) mice, which previously have been shown to be unable to mount a temperature rise upon peripheral immune challenge (Engblom et al., 2003; Nilsberth et al., 2009*a*) instead of Cox-2 KO mice, because the latter are difficult to breed and suffer from various health problems, including chronic inflammation (Langenbach et al., 1999). mPGES-1 KO mice with injections of viral vectors expressing GFP aimed at the region of the median preoptic nucleus, the structure critical for the fever response to PGE<sub>2</sub> (Scammell et al., 1996; Lazarus et al., 2007), displayed the same hypothermic response to immune challenge with LPS as shown previously for these mice (Engblom et al., 2003; Nilsberth et al., 2009*a*; Engström et al., 2012). In contrast, mPGES-1 KO mice injected with a vector expressing mPGES-1 showed no sustained hypothermia after the initial temperature drop following the handling stress-induced temperature peak but displayed a body temperature elevation compared with mice injected with a vector expressing GFP (Fig. 7*a*). Mean body temperature during the period of 60–480 min after LPS injection (i.e., after the handling stress-induced temperature peak) was significantly different between treatments ( $F_{(2,29)} = 11.17$ ,  $p = 0.0003$ ; lenti-mPGES-1 vs lenti-GFP,  $p = 0.0042$ ). While injection sites were determined by immunofluorescence to GFP (GFP lentiviral vector was added at 10% to the mPGES-1 vector; Fig. 7*b,c*), transcription of mPGES-1



**Figure 5.** Gene deletion with *Slco1c1*-Cre targets mainly endothelial cells in small- and medium-sized vessels in the brain. *a*, Dual labeling (arrowheads) of the Cre reporter protein tdTomato and Cox-2 in a small vessel in the brain. *b*, Large vessel with smaller-sized branch. Most of the Cox-2-immunoreactive cells (green) in the large vessel do not express tdTomato (arrows), whereas those in the smaller sized branch do (arrowheads). *c*, Numerous Cox-2-expressing cells (arrows) in a large vessel are shown, but few of those cells also express tdTomato (arrowheads). All micrographs are from immune-challenged mice. Note that endothelial cells in capillaries express tdTomato (small arrows) but not Cox-2. Scale bar, 20  $\mu$ m.

was assured by qPCR analysis. In a total of eight mice injected with the viral vector expressing mPGES-1, all displayed mPGES-1 mRNA, whereas none of four mice injected with the GFP vector did so, as expected.

## Discussion

### The febrile response is dependent on PGE<sub>2</sub> synthesis by small- to medium-sized vessels in the hypothalamus but independent of global PGE<sub>2</sub> synthesis in brain

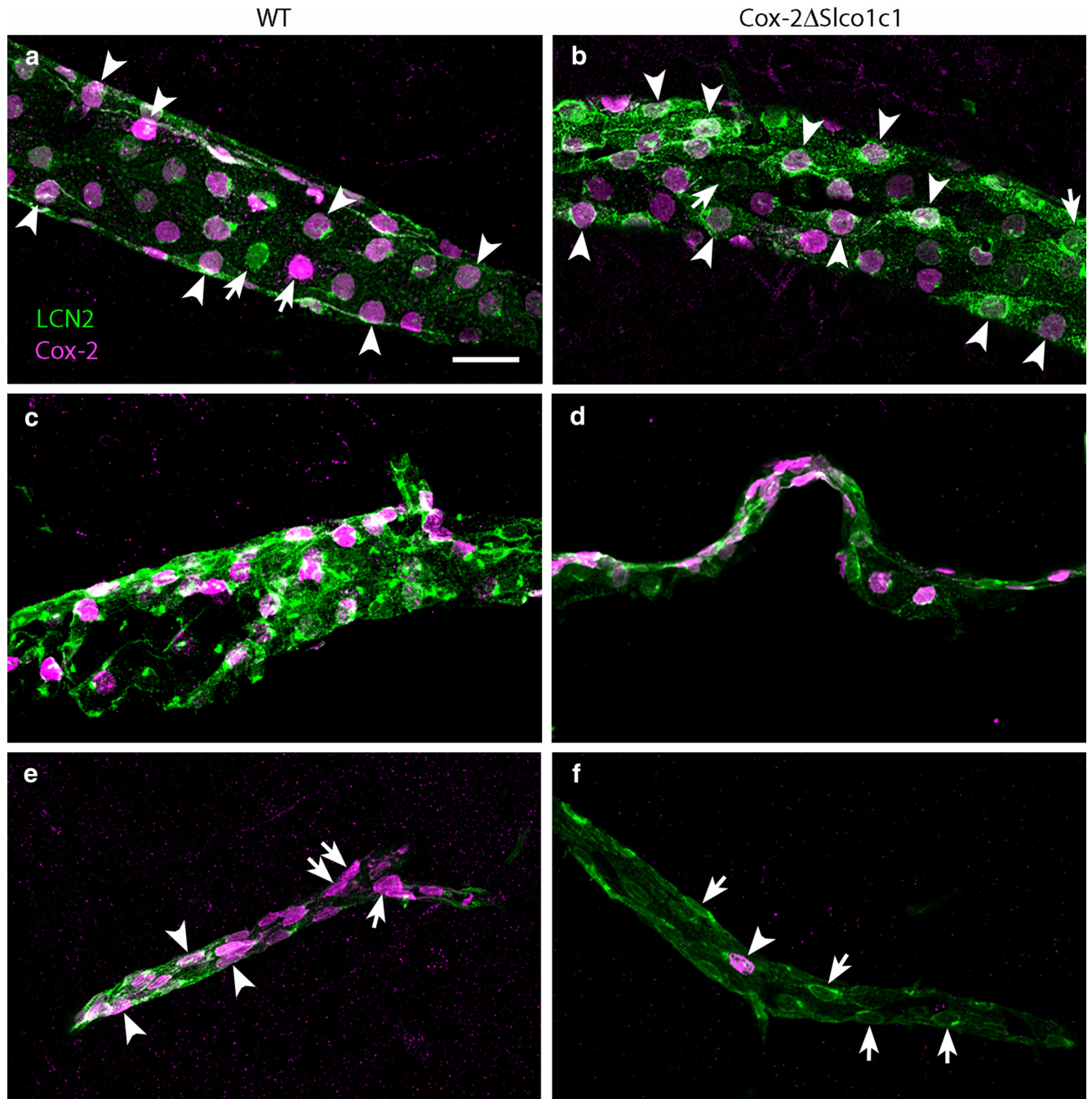
This study shows that the magnitude of the febrile response was strongly correlated with the PGE<sub>2</sub>-synthesizing capacity in the hypothalamus, as reflected in the levels of Cox-2 mRNA, but was only weakly related to the PGE<sub>2</sub> levels in CSF. These findings were corroborated by the histological demonstration that genetic deletion of Cox-2 in brain endothelial cells using a tamoxifen-inducible CreER<sup>T2</sup> under the *Slco1c1* promoter (Ridder et al., 2011) occurred preferentially in small- to medium-sized vessels deep in the brain parenchyma, such as in the hypothalamus, whereas larger vessels, particularly those close to the neocortical surface, and vessels in the meninges were left unaffected. Accordingly, whereas the gene deletion attenuated the PGE<sub>2</sub>-synthesizing capacity in the hypothalamus, in which the EP<sub>3</sub> receptor-expressing neurons that are critical for the febrile response are located (Lazarus et al., 2007), it left the PGE<sub>2</sub> synthesis intact in large parts of the brain. Together, these data imply that the febrile response is dependent on the local, possibly paracrine, release of PGE<sub>2</sub> onto the preoptic EP<sub>3</sub> receptors, whereas the overall PGE<sub>2</sub> level in the brain, as reflected in the levels measured in the CSF, is not involved. This conclusion was further supported by the finding that local restoration of induced PGE<sub>2</sub> synthesis in febrile-resistant mPGES-1 KO mice at loci involving the median preoptic nucleus, the critical site for immune-induced fever, resulted in a temperature elevation in response to intraperitoneal LPS.

Previous findings that the injection of PGE<sub>2</sub> into the cerebral ventricles causes fever in a dose-dependent manner (Engblom et al., 2003; Lazarus et al., 2007; Nilsberth et al., 2009b) would seem to indicate that PGE<sub>2</sub> synthesized at some sites in the brain other than in the immediate vicinity of the preoptic EP<sub>3</sub> receptor-expressing neurons that are critical for the febrile response also could influence the firing properties of these neurons and, hence, elicit fever. However, as noted previously (Nilsberth et al., 2009b), the concentration in the CSF of exogenously administered PGE<sub>2</sub> that is required for eliciting fever is on the order of 1000-fold higher than that seen in CSF during immune-induced fever. This observation suggests that the concentration of PGE<sub>2</sub> at its target neurons that is needed for eliciting fever in response to a peripheral immune stimulus is much higher than that measured in the CSF. It seems likely that such high concentrations of PGE<sub>2</sub> during physiological conditions could be achieved only by paracrine release. This idea is supported by the demonstration that intracerebral injection of a threshold dose of PGE<sub>2</sub> causes fever when localized to or in the immediate vicinity of the median preoptic nucleus but not when localized to more distant sites (Scammell et al., 1996). Similarly, microinjections of a cyclooxygenase inhibitor into the same area attenuated LPS-induced fever, whereas microinjections at other sites did not (Scammell et al., 1998).

### Relationship between fever and hypothermia

While injection of a viral vector encoding mPGES-1 resulted in a significantly higher body temperature after peripheral immune challenge than that displayed by mice subjected to control virus injections, the body temperature did not reach levels that could be classified as fever. The temperature response to immune chal-

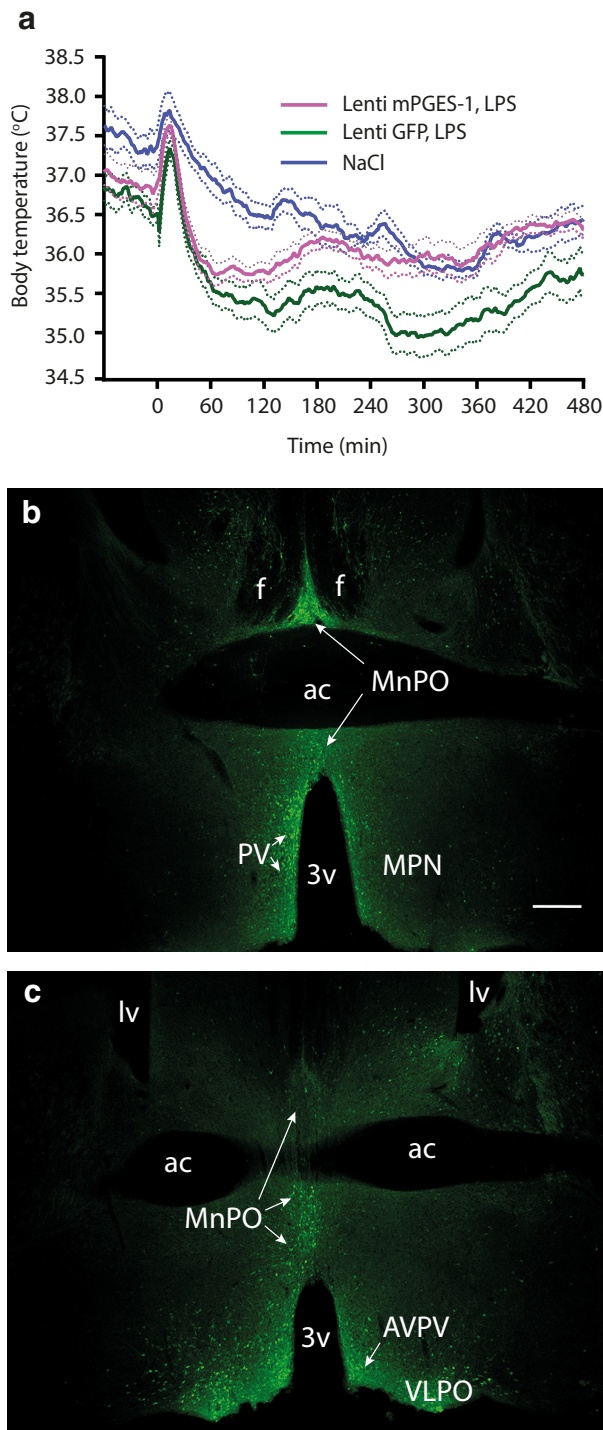




**Figure 6.** Cox-2 and lipocalin-2 expression following immune challenge. **a, b**, Abundant expression of Cox-2 among lipocalin-2 (LCN2)-positive cells in a large vessel in both WT (**a**) and Cox-2<sup>ΔSlco1c1</sup> mice (**b**). **c, d**, No difference in Cox-2 expression in the leptomeninges between WT (**c**) and Cox-2<sup>ΔSlco1c1</sup> mice (**d**). **e, f**, Reduced Cox-2 expression in small lipocalin-2-stained vessel in a Cox-2<sup>ΔSlco1c1</sup> mouse (**f**) and abundant expression in a WT mouse (**e**). Arrowheads point at dual-labeled cells, and arrows point at single-labeled cells. Scale bar, 20 μm.

lenge with LPS is likely the central compilation of pyrogenic and hypothermic signaling, with the neuronal substrate being reciprocally interconnected cell groups that generate temperature-elevating and temperature-lowering signals, respectively (Zhao et al., 2017). In the absence of induced PGE<sub>2</sub> synthesis, animals immune challenged with LPS display hypothermia (Fig. 7; see also Engström et al., 2012), and such hypothermia also occurs in mice lacking EP<sub>3</sub> receptors (Oishi et al., 2015). Hypothermia, which is elicited by an as yet unidentified cryogen (Almeida et al., 2006), is thus the response to LPS when no pyrogenic PGE<sub>2</sub>–EP<sub>3</sub> signaling is present. While mPGES-1 KO mice, similar to WT mice, are fully responsive to intracerebroventricularly injected

PGE<sub>2</sub>, hence demonstrating intact EP<sub>3</sub> signaling (Engblom et al., 2003), it should be noted that the response of mice to the intracerebroventricularly injected PGE<sub>2</sub> is graded. Low doses give rise to only a slight temperature elevation, whereas high doses elicit a body temperature on the order of 40°C (Nilsberth et al., 2009b). The inability to fully restore the febrile response by virus vector injection in the present study is therefore likely explained by only partly restored PGE<sub>2</sub> synthesis, which in turn may be due to incomplete mPGES-1 expression and/or deficient mPGES-1 protein coupling to Cox-2. The latter is induced in brain endothelial cells (Engström et al., 2012); however, structures that had incorporated the virus (and hence were shown to express GFP) were



**Figure 7.** Mice injected into the preoptic hypothalamus with lentiviral vector encoding the terminal prostaglandin E<sub>2</sub>-synthesizing enzyme mPGES-1 display higher body temperature in response to LPS injection than mice injected with control vector. **a**, Temperature recordings of immune-challenged mPGES-1 knock-out mice injected with a viral vector encoding mPGES-1 (magenta trace) or given control injection of lentiviral vector encoding GFP (green trace).  $n = 12$  for lenti-mPGES-1 LPS;  $n = 10$  for lenti-GFP LPS; and  $n = 10$  for NaCl (mixed group of lenti-mPGES-1 and lenti-GFP-injected mice). Error bars = SEM. **b**, **c**, Micrographs showing immunofluorescent staining for GFP in the preoptic hypothalamus after injection with viral vector. **b** and **c** are from different animals; the plane of the chosen sections corresponds approximately to bregma  $+0.14/+0.145$  mm (**b**), and bregma  $+0.26/+0.245$  mm (**c**) in the atlas of Paxinos and Franklin (2001) and the Allen Reference Atlas (Dong, 2008), respectively. 3v, Third ventricle; ac, anterior commissure; AVPV, anteroventral periventricular nucleus; f, fornix; lv, lateral ventricle; MnPO, median preoptic nucleus; MPN, medial preoptic nucleus; PV, periventricular nucleus; VLPO, ventrolateral preoptic nucleus. Scale bar, 500  $\mu$ m.

preferentially of neuron/glia cell type and were only rarely suggestive of endothelial cells (data not shown). While speculative, mPGES-1 expressed by other cells than endothelial cells could perhaps couple with Cox-1 that is more ubiquitously expressed, hence resulting in PGE<sub>2</sub> synthesis, although at levels that did not permit a full-fledged restoration of the febrile response. Nevertheless, the finding of a temperature elevation in response to LPS injection, although modest, in animals in which mPGES-1 was re-expressed in the preoptic region demonstrates the critical role for localized PGE<sub>2</sub> synthesis for heat production in response to peripheral immune challenge.

#### Role of peripherally produced PGE<sub>2</sub> for the febrile response

The present data confirm that prostaglandin production in brain endothelial cells is important for the febrile response (Engström et al., 2012; Eskilsson et al., 2014a; Wilhelmsson et al., 2014). However, it has been suggested that peripherally produced circulating PGE<sub>2</sub> also is involved (Steiner et al., 2006), but data from mice chimeric for mPGES-1 imply that the role of PGE<sub>2</sub> produced by hematopoietically derived cells is, at most, very small (Engström et al., 2012). Here we found no significant relationship between the levels of PGE<sub>2</sub> metabolites in plasma and PGE<sub>2</sub> levels in the CSF, suggesting that peripherally and centrally produced PGE<sub>2</sub> are of distinct sources of origin, at least at the time point examined. Accordingly, although there was a moderately strong relationship in the present study between the levels of PGE<sub>2</sub> metabolites in plasma and the magnitude of fever, there is most likely no causality between these events; in the case of a strong peripheral immune response, there is also a strong central immune response, and, conversely, a weak peripheral immune response is associated with a weak central immune response. This conclusion is supported by observations in the mice chimeric for mPGES-1. Thus, whereas WT mice carrying mPGES-1 KO hematopoietic cells displayed normal LPS-induced fever, mPGES-1 KO mice carrying WT hematopoietic cells did not mount a febrile response, despite showing strong induction of PGE<sub>2</sub> metabolites in plasma (Engström et al., 2012). Furthermore, in those mice, central PGE<sub>2</sub> levels were not significantly increased, providing additional support for the distinct origin of centrally and peripherally produced PGE<sub>2</sub>.

#### Heterogeneous distribution of transporter proteins among brain endothelial cells

We observed in the genetically modified mice that Cre recombinase expressed under the control of the *Slco1c1* promoter produced recombination (and hence gene deletion) in brain endothelial cells in small- and medium-sized vessels but not in larger vessels. The *Slco1c1* gene encodes the organic anion transporter 14 (Oatp14), which has been shown to be expressed selectively in endothelial cells of the brain (Ridder et al., 2011). The organic anion transporter family transports hormones and other organic molecules to and from the brain (Westholm et al., 2008), and it has been demonstrated that other members of the Oatp family also show heterogeneous distributions among the vessels in the brain similar to Oatp14, with the main expression being in smaller vessels (Daneman et al., 2010). Furthermore, it has been shown that transporter proteins overall have a more prominent expression in capillaries than in venules (Macdonald et al., 2010), which is consistent with the idea that the exchange of molecules and nutrients between blood and tissue occurs preferentially in the capillaries. However, induced prostaglandin synthesis, as reflected by induced expression of Cox-2, seems to occur in large-, medium-, and small-sized vessels but not in capillaries (Fig. 5;



Eskilsson et al., 2014b), and it is not yet known which transporter is responsible for the transfer of PGE<sub>2</sub> into the brain. As shown here, PGE<sub>2</sub> synthesis in small- and medium-sized vessels is critical for the febrile response. The functional role of the PGE<sub>2</sub> that is synthesized in the larger vessels, including vessels in meninges, which seems to account for most of the PGE<sub>2</sub> that is seen in the CSF, remains to be clarified. It has been reported recently that the excitation of pyramidal cells in the cerebral cortex results in increased local cerebral blood flow via neuronal release of PGE<sub>2</sub> and its binding to vasodilatory EP<sub>2</sub> and EP<sub>4</sub> receptors on vascular smooth muscle cells or pericytes (Lacroix et al., 2015). Whether PGE<sub>2</sub> produced by endothelial cells in response to peripheral immune challenge also takes part in the vasodilation of cerebral vessels is not known, and neither is what functional role such vasodilation, if present, would subservise.

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