Prostaglandin E<sub>2</sub> Attenuates Preoptic Expression of GABA<sub>A</sub> Receptors via EP3 Receptors

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Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) has been shown to produce fever by acting on EP3 receptors within the preoptic area of the brain. However, there is little information about the molecular events downstream of EP3 activation in preoptic neurons. As a first step toward this issue, we examined PGE<sub>2</sub>-induced gene expression changes at single-cell resolution in preoptic neurons expressing EP3. Brain sections of the preoptic area from PGE<sub>2</sub>- or saline-injected rats were stained with an anti-EP3 antibody, and the cell bodies of EP3-positive neurons were dissected and subjected to RNA amplification procedures. Microarray analysis of the amplified products demonstrated the possibility that gene expression of γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor subunits is decreased upon PGE<sub>2</sub> injection. Indeed, we found that most EP3-positive neurons in the mouse preoptic area are positive for the α2 or γ2 GABA<sub>A</sub> receptor subunit. Moreover, PGE<sub>2</sub> decreased the preoptic gene expression of these GABA<sub>A</sub> subunits via an EP3-dependent and pertussis toxin-sensitive pathway. PGE<sub>2</sub> also attenuated the preoptic protein expression of the α2 subunit in wild-type but not in EP3-deficient mice. These results indicate that PGE<sub>2</sub>-EP3 signaling elicits G<sub>i/o</sub> activation in preoptic thermocenter neurons, and we propose the possibility that a rapid decrease in preoptic GABA<sub>A</sub> expression may be involved in PGE<sub>2</sub>-induced fever.

Body temperature is controlled by central and peripheral mechanisms such as heart rate, muscle tone, metabolic rate, vasoconstrictor action, and shivering, thereby reaching a new thermoregulatory set point. Fever is characterized as an elevation in body temperature by 1–2 °C and is one of the representative systemic responses against inflammation (1, 2). Fever is a clinically important symptom associated with many serious diseases such as infections, toxonosis, and neoplasia. Fever responses are initiated by cytokines such as interleukin-1 and interleukin-6, which are released from immune cells activated by exogenous pyrogens such as lipopolysaccharide. These cytokines act on the brain vasculature and therein stimulate prostaglandin (PG) production (3).

PGE<sub>2</sub> has been shown to elicit fever, because aspirin-like drugs exert anti-pyretic actions through the suppression of PGE<sub>2</sub> biosynthesis by inhibiting cyclooxygenase, a rate-limiting enzyme of PGE<sub>2</sub> synthesis (4, 5). PGE<sub>2</sub> displays a broad range of actions including fever generation through its binding to specific receptors on target cells. There are four PGE receptor subtypes, EP1, EP2, EP3, and EP4, all of which are expressed in the central nervous system (6). We have shown that EP3 plays a pivotal role in inflammation-associated fever, because EP3-deficient mice fail to exhibit fever in response to central administration of PGE<sub>2</sub> and interleukin-1β or peripheral administration of interleukin-1β and lipopolysaccharide (7, 8). However, there is still little information about the molecular events downstream of PGE<sub>2</sub>-EP3 signaling in the central nervous system.

The preoptic area (POA), which is the rostral region of the basal forebrain, has been shown to contain a thermocenter because destruction of this region leads to the inability to generate fever and also because the POA is rich in thermosensory neurons and is sensitive to PGE<sub>2</sub>-induced fever (reviewed in Ref. 9). Moreover, the level of PGE<sub>2</sub> in the POA is well associated with lipopolysaccharide-induced fever, and indomethacin completely abolishes both lipopolysaccharide-induced fever and the increase in PGE<sub>2</sub> levels in the POA (2). We previously showed that a number of EP3-expressing neurons exist in the POA (10, 11), and it was recently demonstrated that selective genetic deletion of the EP3 receptors in the POA results in abrogation of the PGE<sub>2</sub>-induced fever response (12). These results indicated that PGE<sub>2</sub> transmits febrile input by acting on the EP3 receptors expressed in the POA. How then does acti—

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<sup>5</sup>The abbreviations used are: PG, prostaglandin; POA, preoptic area; GABA, γ-aminobutyric acid; MnPO, median preoptic nucleus; MPO, medial preoptic nucleus; i.c.v., intracerebroventricularly; PT, pertussis toxin; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
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The activation of EP3 receptors in the POA neurons lead to fever generation. It has been demonstrated that EP3-expressing neurons project to the rostral raphe pallidus nucleus, where thermogenic sympathetic premotor neurons are located (13), and make γ-aminobutyric acid (GABA)-mediated inhibitory synapses (14). Subsequently, these neurons transneuronally innervate brown adipose tissue, which is the major thermogenic organ in rodents (15). It has been suggested that PGE<sub>2</sub> affects the activity of thermosensitive neurons in the POA. For example, it was reported that PGE<sub>2</sub> reduces the firing activities of heat-sensitive neurons in the ventromedial preoptic area (16). Thus, as a mechanism of fever, PGE<sub>2</sub> has been suspected to alter temperature sensitivity in the POA neurons, in most cases via suppressing the firing activities in these neurons. If such effects of PGE<sub>2</sub> could be mediated by EP3 receptor, EP3 activation may desensitize temperature sensitivity in these neurons. However, it remains unknown as to what happens to the EP3-expressing neurons in the POA when they receive PGE<sub>2</sub> and how EP3 activation leads to fever generation.

As a first step to understanding the molecular basis of PGE<sub>2</sub>-induced fever generation and to characterizing the EP3-expressing neurons in the POA, we undertook to detect gene expression changes in the POA EP3-expressing neurons upon central administration of PGE<sub>2</sub>. To analyze the changes in gene expression, specifically in EP3-expressing neurons, we employed the technique of microarray analysis at single-cell resolution. Here we show that the POA EP3-expressing neurons co-express GABA type A (GABAA) receptor subunits and that PGE<sub>2</sub>-EP3 signaling attenuates GABAA expression levels in the POA in a fever-associated manner.

**EXPERIMENTAL PROCEDURES**

**Animals**—Adult male pathogen-free Sprague-Dawley rats (250–350 g; Taconic, Germantown, NY) were used for microarray analysis (Fig. 1 and Table 1). The experiments using rats were approved by the Harvard Medical School and Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committees. Adult male pathogen-free C57BL/6 mice (25–30 g; SLC Japan, Hamamatsu, Japan) and EP3<sup>(piger3)</sup>-deficient (EP3<sup>−/−</sup>) mice with a C57BL/6 genetic background (7, 17) were used for subsequent analyses (Figs. 2–5 and Table 2). The experiments using mice were approved by the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences at Kyoto University. The animals were housed in a light (12 h on/off) and temperature (22–23 °C)-controlled environment with food and water available ad libitum.

**Microarray Analysis of POA Neurons Expressing EP3 Receptors**—A flowchart of the experimental strategy used to analyze the gene expression changes that occur in EP3-expressing POA neurons upon PGE<sub>2</sub> injection is shown in Fig. 1A. A stainless steel cannula was implanted stereotaxically into the lateral cerebral ventricle of a rat and fixed to the skull with acrylic dental cement (8). PGE<sub>2</sub> (500 ng; Sigma) in 5 μl of pyrogen-free 0.9% saline or saline only was injected into the ventricle, and the rectal temperature was monitored. The rats were sacrificed 30 min after the injection and fixed by transcardial perfusion with 4% paraformaldehyde. The brains were postfixed, saturated with a sucrose solution, and then cut into coronal sections (10 μm), which were stained with an anti-rat EP3 rabbit antibody as described previously (11). Single-cell microdissection and RNA amplification were performed essentially as described previously (18). Briefly, sections were incubated with the oligo(dT)-T7 primer and subjected to first-strand cDNA synthesis with reverse transcriptase. With the use of a glass micropipette, the cell bodies of individual EP3-positive neurons were carefully microdissected from the surrounding neuropil and subsequently aspirated (19). Photomicrographs were taken before and after aspiration to assure that the cells had positive staining and that only a single cell was detached (Fig. 1B). Twelve single cells derived from three rats per group (PGE<sub>2</sub>-injected and saline-injected groups) were pooled and subjected to two-round RNA amplification. The labeled probe was synthesized by converting the amplified antisense RNA to either a Cy3- or Cy5-labeled cDNA probe. The resultant probes were hybridized to Incyte Lifearray™ Chips RAT GEM2. The data were normalized, and the genes were ranked based on the magnitude of differential expression calculated across dye swap experiments as described previously (18). Microarray analysis was repeated independently, and genes showing similar expression changes upon PGE<sub>2</sub> injection were selected and presented.

**In Situ Hybridization and β-Galactosidase Staining**—The DNA probes for GABA<sub>A</sub> (ionotropic) receptor subunits α2 (944 bp), γ1 (1,302 bp), and γ2 (1,168 bp) were amplified from mouse whole brain cDNA and subcloned into pBluescript (Stratagene). Antisense riboprobes were transcribed by T7 RNA polymerase in the presence of [35S]CTP. Hybridization in situ was carried out as described previously (10). Serial sections were hybridized with EP3, α2, γ1, and γ2 probes. The specific hybridization of the probe for each GABA<sub>A</sub> receptor subunit was confirmed by the loss of significant hybridization in experiments in the presence of an excess of unlabeled probe (data not shown). For β-galactosidase staining, brain sections were fixed with glutaraldehyde and then stained with X-gal at 30 °C for 6 h.

**Immunofluorescence Microscopy**—Coronal sections (14 μm) of adult male EP3<sup>+/−</sup> mice were fixed and incubated in phosphate-buffered saline containing a blocking reagent. The slides were incubated in phosphate-buffered saline containing an anti-β-galactosidase antibody (1:500; Biogenesis) in combination with an anti-GABA<sub>A</sub> α2 antibody (1:100, Alomone Labs, Jerusalem, Israel) or an anti-GABA<sub>A</sub> γ2 antibody (1:30, Alomone Labs). To enhance the signals for GABA<sub>A</sub> subunits, we employed the Tyramide signal amplification system (Molecular Probes) and antibodies conjugated to Alexa Fluor 488 (Molecular Probes) or to Cy3 (Jackson ImmunoResearch). TOPRO-3 (Molecular Probes) was also used as a nuclear marker. Images were collected on a Zeiss Axiosvert 200M microscope using Axiovision software (Carl Zeiss). Backgrounds were defined as the fluorescence intensity in non-neuronal fields.

**Dissection of POA and Real-Time-Polymerase Chain Reaction (PCR)**—Wild type and EP3<sup>−/−</sup> mice anesthetized with α-chloralose-HBC (40 mg/kg intraperitoneally; Sigma) were administered intracerebroventricularly (i.c.v.) with PGE<sub>2</sub> (1 nm, 5 μl) or saline (20), and rectal temperatures were monitored. Thirty or 60 min after the injection, the mice were sacrificed and brain sections (14 μm) prepared. The POA regions were scraped and subjected to RNA extraction. The POA was defined as follows:
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the initiation of the third ventricle was determined as the rostral limit, the site where the bilateral anterior commissure joins together as the caudal limit, bottom of the anterior commissure as the dorsal limit, the upper side of the optic chiasm as the ventral limit, and an extension of both lateral ventricles as the side limit. The RNA was reverse-transcribed and subjected to quantitative PCR (LightCycler; Roche Applied Science). Primers used in this study are as follows: \( \text{GABA}_A \alpha_2 \), forward (5'-CAGACCTATCTGCCCTTG-3') and reverse (5'-ACTACACTCTCCCCG-3'); \( \text{GABA}_A \gamma_1 \), forward (5'-ACATGGGTCTTGGCAC-3') and reverse (5'-AGGCGATTAGCGTGGT-3'); \( \text{GABA}_A \gamma_2 \), forward (5'-ATTCCCTGCACTCA-3') and reverse (5'-GTGCCATACTCCCAAC-3'); \( \alpha_1 \)-tubulin, forward (5'-AGAGTGCCTGGTCGAAG-3') and reverse (5'-GTCTACGACCAATCACGGC-3'); neurofilament-M, forward (5'-TGAACTTCGGGGAACCA-3') and reverse (5'-GCTGTGCGTGTTGTA-3'); and 28 S ribosomal RNA, forward (5'-CAGTCAACGAGTCCG-3') and reverse (5'-GGCACAACACATCATCAG-3'). The relative mRNA levels of \( \text{GABA}_A \alpha_2 \), \( \gamma_1 \), \( \gamma_2 \), and \( \alpha_1 \)-tubulin for individual animals were normalized with the mRNA levels of 28 S ribosomal RNA (Fig. 4) or neurofilament-M (Fig. 6) and expressed as fold change values.

**Immunoblot Analysis**—Wild-type and EP3\(^{-/-} \) mice were injected with saline or \( \text{PGE}_2 \) and sacrificed 30 min after the injection. The POA was dissected, homogenized in lysis buffer (66 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin, apro- tin, and pepstatin, respectively). Protein (10 μg) was loaded into separate lanes in a 7.5% SDS-polyacrylamide gel, and blotting was performed as described previously (21). After blocking in nonfat milk, the membranes were incubated with primary antibodies diluted with nonfat milk (\( \text{GABA}_A \alpha_2 \), 1:500), washed, and incubated with horseradish peroxidase-conjugated secondary antibodies. After washing, the membranes were stained with an ECL kit (GE Healthcare) and visualized with the LAS system (Fujifilm, Japan). The densities of the bands for \( \text{GABA}_A \) were quantified using Image Gauge software (Fujifilm). As a normalizing control, an anti-actin antibody (Chemicon, 1:2000 dilution) was used. The specificity of the anti-\( \text{GABA}_A \alpha_2 \) antibody was confirmed by the loss of the corresponding band when the antigen peptide-preabsorbed antibody was used (data not shown).

**POA Slice Culture**—We performed slice culture based on a method described previously (22). Briefly, mouse brains were removed and cut into coronal slices containing POAs of 500 μm in thickness under sterile conditions. The slices were transferred onto a 30-mm Millicell-CM insert membrane (Millipore) in 6-well plates. The slices were maintained at 37°C in 3 ml/well culture medium consisting of 50% minimum essential medium/HEPES, 25% Hanks’ balanced salt solution, and 25% heat-inactivated horse serum supplemented with 6.5 mg/ml D-glucose and 2 mM L-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate. The slices were pretreated with or without pertussis toxin (PT) (200 ng/ml; Seikagaku Japan) for 6 h followed by the addition of \( \text{PGE}_2 \) (1 μM). After incubation with \( \text{PGE}_2 \) for 30 min, the POA was dissected from the slices and subjected to RNA extraction and reverse transcription-PCR.

**Data Analysis**—Data are shown as means ± S.E. A comparison between two groups was performed using Student’s t test; \( p \) values <0.05 were considered significantly different.

**RESULTS**

**Microarray Analysis of Rat POA EP3-positive Neurons upon \( \text{PGE}_2 \) Injection**—As a first step to elucidate the molecular mechanism underlying EP3-mediated fever generation, we utilized cDNA microarray analysis at single-cell resolution to detect genes in POA EP3-positive neurons with altered expression levels upon \( \text{PGE}_2 \) injection (Fig. 1A). \( \text{PGE}_2 \)- and saline-injected rats were sacrificed 30 min after injection, and POA sections from these rats were fixed, stained with an anti-rat EP3 antibody, and subjected to reverse transcription in situ. The positively stained cell bodies were picked up with glass capillaries (Fig. 1B), and pooled. The cDNAs derived from 12 positive cell bodies/group were extracted and amplified as antisense and subjected to quantitative PCR (LightCycler; Roche Applied Science). Primers used in this study are as follows: \( \text{GABA}_A \alpha_2 \), forward (5'-CAGACCTATCTGCCCTTG-3') and reverse (5'-ACTACACTCTCCCCG-3'); \( \text{GABA}_A \gamma_1 \), forward (5'-ACATGGGTCTTGGCAC-3') and reverse (5'-AGGCGATTAGCGTGGT-3'); \( \text{GABA}_A \gamma_2 \), forward (5'-ATTCCCTGCACTCA-3') and reverse (5'-GTGCCATACTCCCAAC-3'); \( \alpha_1 \)-tubulin, forward (5'-AGAGTGCCTGGTCGAAG-3') and reverse (5'-GTCTACGACCAATCACGGC-3'); neurofilament-M, forward (5'-TGAACTTCGGGGAACCA-3') and reverse (5'-GCTGTGCGTGTTGTA-3'); and 28 S ribosomal RNA, forward (5'-CAGTCAACGAGTCCG-3') and reverse (5'-GGCACAACACATCATCAG-3'). The relative mRNA levels of \( \text{GABA}_A \alpha_2 \), \( \gamma_1 \), \( \gamma_2 \), and \( \alpha_1 \)-tubulin for individual animals were normalized with the mRNA levels of 28 S ribosomal RNA (Fig. 4) or neurofilament-M (Fig. 6) and expressed as fold change values.

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GABA<sub>A</sub> receptor genes such as tu via EP3 receptor. On the other hand, the expression of tor. In the following studies, we therefore surveyed the GABA<sub>A</sub> (located genes such as syntaxin 1B2 (stx1B2) were down-regulated by PGE2 injection (Table 1). Surprisingly, than a 1.5-fold increase and 16 genes showing more than a 1.5-fold decrease upon PGE2 injection (Table 1). Surprisingly, expressed genes. After the exclusion of functionally unknown expressed sequence tags, we detected two genes showing more genes down-regulated by PGE2 include vesicle transport-related genes such as syntaxin 1B2 (stx1B2) and synaptophysin (syp), suggesting that PGE2 may alter the vesicle transport status via EP3 receptor. On the other hand, the expression of GABA<sub>A</sub> receptor genes such as gabrg2 and gabra6 were decreased upon PGE2 stimulation (Fig. 1C and Table 1). Although α6 is the GABA<sub>A</sub> subunit specifically expressed in the cerebellum, we considered that this result might reflect changes in gene expression of some structurally close GABA<sub>A</sub> receptor subunits such as α1 or α2, because the cDNA microarray sometimes gives signals for cross-hybridization to structurally close probes. Although GABA is known to be a primary inhibitory transmitter in the central nervous system, it has not been reported that EP3-positive neurons co-express GABA<sub>A</sub> receptor. In the following studies, we therefore surveyed the GABA<sub>A</sub> receptor subunits showing expression overlaps with EP3 receptor in the POA.

GABA<sub>A</sub> Subunits α2 and γ2 Are Expressed in EP3-positive Neurons in Mouse POA—Because Wisden et al. (24) previously reported that mRNAs for the α2 and γ1 subunits of GABA<sub>A</sub> receptor are abundantly expressed in POA, we examined RNA expression of α2, α6, γ1, and γ2 subunits in the mouse POA section. As reported previously (10), the signals for EP3-RNA were detected in the neurons of the median pre-optic nucleus (MnPO), medial preoptic nucleus (MPO), and rostral wall of the third ventricle. Similarly, the signals for GABA<sub>A</sub> γ2-RNA were highly concentrated to the regions surrounding the third ventricle in the POA (Fig. 2). The signals for GABA<sub>A</sub> α2-RNA were distributed more widely than those of EP3 and γ2, but strong signals were observed in the regions surrounding the third ventricle in the POA. The signals for GABA<sub>A</sub> γ1-RNA were observed only in a few brain areas but were present in the neurons adjacent to the third ventricle in the POA. However, no significant signals for GABA<sub>A</sub> α6 were found in the POA. The signals for GABA<sub>A</sub> α2-, γ1-, and γ2-RNA were confined to neurons (data not shown). Thus, distribution of EP3 appears to overlap with those of three GABA<sub>A</sub> receptor genes, and we selected α2 and γ2 as candidate subunits colocalized with EP3 receptors in the POA. Although an anti-EP3 antibody was used to detect EP3-expressing neurons in the POA, this antibody could not detect mouse EP3 receptor protein effectively (25). Moreover, because all anti-mouse EP3 antibodies that we tested were not suitable for immunohistochemistry, we used EP3<sup>+/−</sup> mice in which the β-galactosidase (β-gal) gene was “knocked in” at the EP3 gene locus (7). In these animals, EP3-positive cells can be detected by the anti-β-galactosidase antibody (Fig. 4A) (26). Simultaneous staining of the POA sections of EP3<sup>+/−</sup> mice for β-galactosidase and GABA<sub>A</sub> γ2 revealed colocalization of the positive signals in the MnPO and MPO neurons (Fig. 3 and Table 2). In MnPO and MPO, 71.1 and 90.5%, respectively, of EP3-positive neurons were also positive for γ2. Moreover, when we stained the POA sections with anti-β-galactosidase and anti-GABA<sub>A</sub> α2 antibodies, most of the EP3-positive neurons were also positive for the α2 sub-
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**TABLE 2**
EP3-positive neurons co-express GABAA receptor in the POA

| GABA\(_A\) subunit | % Double positive per β-galactosidase (EP3)-positive cells* |
|---------------------|----------------------------------------------------------|
|                     | MnPO | MPO |
| α2                  | 86.1 ± 9.3 | 95.2 ± 4.8 |
| γ2                  | 71.1 ± 4.2 | 90.5 ± 5.8 |

* Data represent mean ± S.E.

PGE\(_2\) Attenuates Gene Expression of GABAA Receptors in the POA—Because the EP3-positive cell bodies and neuropils are distributed throughout the POA and because most EP3-positive neurons appear to express GABA\(_A\) subunits, we next tested whether total GABA\(_A\) transcript levels in the POA could be attenuated by PGE\(_2\) stimulation. We therefore examined the effect of central administration of PGE\(_2\) on POA gabr gene expression and body temperature in wild-type and EP3\(^{-/-}\) mice. When wild-type mice were injected i.c.v. with PGE\(_2\), their rectal temperatures were increased by 2 °C at 30 min after the injection, which gradually decreased at 60 min but was still higher than before the injection (Fig. 4A). No such response was observed upon saline injection. Saline injection failed to alter POA gabr expression until 60 min after the injection (Fig. 4B). In contrast, PGE\(_2\) significantly attenuated gabr\(_1\) and gabr\(_2\) expression levels at 30 min, and such reduced levels were restored to the original level at 60 min. Furthermore, PGE\(_2\) down-regulated gabr\(_2\) expression levels at 30 min, and although this was not statistically significant, the PGE\(_2\)-elicited down-regulation in gabr\(_2\) expression was still found at 60 min; the effect of PGE\(_2\) at this time was significant. In contrast, PGE\(_2\) failed to alter the expression of α-tubulin (tuba1a) and other housekeeping genes in the POA (data not shown), indicating that the effect of PGE\(_2\) is GABA\(_A\) gene-specific. Furthermore, the PGE\(_2\)-elicited reduction in gabr expression was not observed in whole brain (data not shown), indicating that such effect of PGE\(_2\) is POA-specific. We then examined whether PGE\(_2\)-elicited attenuation in GABA\(_A\) gene expression could be observed in the EP3\(^{-/-}\) mice, which do not show fever generation in response to PGE\(_2\) injection (Fig. 4A). Interestingly, PGE\(_2\) failed to affect the expression of any gabr genes in the POA of EP3\(^{-/-}\) mice (Fig. 4B). These results indicate that PGE\(_2\)-elicited attenuation in POA GABA\(_A\) gene expression is mediated by EP3 receptor. Thus, PGE\(_2\)-EP3 signaling appears to down-regulate GABA\(_A\) gene expression in the POA in a fever-associated manner.

**FIGURE 3.** GABA\(_A\) receptors are co-expressed in POA neurons expressing EP3 receptors. A, the X-gal staining pattern coincides with the distribution pattern of EP3-mRNA signals. B and C, brain tissues isolated from EP3\(^{+/+}\) mice expressing the β-galactosidase (red) gene at the EP3 gene locus were stained with anti-β-galactosidase (red). The sections were simultaneously stained with an anti-GABA\(_A\) α2 antibody (B) or an anti-GABA\(_A\) γ2 antibody (C) (green). Representative photomicrographs of the MnPO and MPO are shown. The regions corresponding to the MnPO and MPO are illustrated in Fig. 2. Arrows in the merged images indicate the double positive cells. Scale bars, 20 μm.
PGE₂ failed to affect the GABA<sub>A</sub> protein expression levels in the POA. These results indicate that PGE₂ rapidly down-regulates at least GABA<sub>A</sub> protein levels within the POA via EP₃ receptor.

PGE₂ Attenuates GABA<sub>A</sub> Gene Expression via a PT-sensitive Pathway—Although EP₃ receptor has been shown to be critical in PGE₂-induced fever generation, it remains uncertain as to which G protein pathway is activated by PGE₂-EP₃ signaling in the POA neurons. To address this issue, we established a slice culture model of the mouse brain POA. In this culture system, PGE₂ significantly attenuated gabrg1 and gabrg2 gene expression levels within the POA at 30 min after PGE₂ addition, and a similar tendency was observed for gabra2 expression (Fig. 6). PT treatment of slices abolished the PGE₂-elicited decrease in gabr expression. These results indicate that PGE₂ attenuates GABA<sub>A</sub> gene expression via a PT-sensitive pathway.

DISCUSSION

Although EP₃ receptor has been shown to most preferentially couple to G<sub>i1</sub> and G<sub>i2</sub> (27), it is not certain which G protein pathway is involved in the EP₃-mediated physiological actions of PGE₂ including fever generation. The current study indicates that EP₃ receptor couples to Gi/o proteins in the POA neurons, leading to attenuation of GABA<sub>A</sub> gene expression. If such a molecular event reflects the functional changes of the GABA<sub>A</sub> channel in thermal regulation as discussed below, fever generation could be triggered by the activation of G<sub>i/o</sub> in EP₃-expressing POA neurons.

One of the most important findings in this study is that most EP₃-positive neurons co-express at least the α₂ and γ₂ subunits of GABA<sub>A</sub> receptor in the POA. Nakamura et al. (14) previously revealed that most EP₃-positive neurons in the POA (86%) are GABA<sub>A</sub>-agonist muscimol injection into the raphe pallidus nucleus blocks PGE₂-induced fever. Based on these results, they proposed the following mechanism of PGE₂-induced fever: the EP₃-expressing neurons are GABAergic and exert tonic inhibition on raphe pallidus neurons, which couples to stimulation of sympathetic innervation.
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nerves in the steady state (in the absence of PGE2), and when cytokine signals stimulate PG production within the POA, PGE2 somehow inhibits the GABAergic activity of EP3-expressing neurons allowing the excitation of raphe neurons, leading to thermogenesis (14). The present results demonstrate that the EP3-expressing neurons themselves express GABAA receptors; thus the EP3-expressing neurons are also susceptible to negative regulation by GABA. Indeed, local application of muscimol into the POA has been shown to induce hyperthermia in an nonsteroidal anti-inflammatory drug (NSAID)-insensitive manner (28, 29). This effect of muscimol may reflect the existence of a more universal thermal regulation mechanism by GABA within the POA. If this is true, PGE2-EP3 signaling may stimulate the susceptibility to GABA inhibition by affecting the GABAA channel properties such as sensitivity to GABA or GABA-elicited responses of the POA neurons.

GABAA receptors are pentameric and are assembled from 18 subunits (α1–6, β1–3, γ1–3, δ, ε, θ, ρ1–3), and GABAA receptors comprising different subunits show distinct properties in channel pharmacology, cellular localization, and dynamic transport regulation (30, 31). In this study, we found that PGE2 rapidly down-regulates protein expression of the GABAA α2 subunit in the POA. Thus, PGE2-EP3 signaling dynamically modulates the subunit components of GABAA channels within the POA, which may be an interesting hypothesis to be explored as a mechanism of fever. Alternatively, however, considering the lag time for transcript-protein conversion (decreases in GABAA protein levels), the GABAA receptor number could be decreased during the recovery from the fever response (30–60 min). Hence the phenomenon of PGE2-elicited down-regulation of POA gabr gene expression alternatively contributes to recovery from fever (a decrease in body temperature); because of the reduced number of GABAA receptors, the EP3-expressing neurons could become insensitive to negative regulation by GABA. Further analysis should be conducted to address these points.

In conclusion, we have demonstrated that PGE2-EP3 signaling attenuates GABAA gene expression within the POA via a PT-sensitive pathway in close association with fever generation. These results will contribute to our understanding of the mechanisms underlying PGE2-induced fever generation and GABA-elicited thermal regulation in the central nervous system.

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