Role of an excitatory preoptic-raphé pathway in febrile vasoconstriction of the rat’s tail

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Tanaka M, McKinley MJ, McAllen RM. Role of an excitatory preoptic-raphé pathway in febrile vasoconstriction of the rat’s tail. Am J Physiol Regul Integr Comp Physiol 305: R1479–R1489, 2013. First published October 16, 2013; doi:10.1152/ajpregu.00401.2013.—Heat dissipation from the rat’s tail is reduced in response to cold and during fever. The sympathetic premotor neurons for this mechanism, located in the medullary raphé, are under tonic inhibitory control from the preoptic area. In parallel with the inhibitory pathway, an excitatory pathway from the rostromedial preoptic region (RMPO) to the medullary raphé mediates the vasoconstrictor response to cold skin. Whether this applies also to the tail vasoconstrictor response in fever is unknown. Single- or a few-unit tail sympathetic nerve activity (SNA) was recorded in urethane-anesthetized, artificially ventilated rats. Experimental fever was induced by PGE2 injected into the lateral cerebral ventricle (50 ng in 1.5 μl icv) or into the RMPO (0.2 ng in 60 nl); in both cases, there was a robust increase in tail SNA and a delayed rise in core temperature. Microinjection of glutamate receptor antagonist kynurenate (50 mM, 120 nl) into the medullary raphé completely reversed the tail SNA response to intracerebroventricular or RMPO PGE2 injection. Inhibiting RMPO neurons by microinjecting glycine (0.5 M, 60 nl) or the GABA A receptor agonist, muscimol (2 mM, 30–60 nl), reduced the tail SNA response to PGE2 injected into the same site by approximately half. Vehicle injections into the medullary raphé or RMPO were without effect. These results suggest that the tail vasoconstrictor response during experimental fever depends on a glutamatergic excitatory synaptic relay in the medullary raphé and that an excitatory output signal from the RMPO contributes to the tail vasoconstrictor response during fever.

prostaglandin E2; kynurenate; glycine

Fever is a coordinated thermoregulatory response to infection, and is organized in the central nervous system. In fever, body temperature is raised by increased heat production and decreased heat loss. Brown adipose tissue and skeletal muscle generate extra heat by nonshivering and shivering thermogenesis. The tail circulation is the major effector organ for heat exchange in rodents, and heat loss from there is regulated by sympathetic vasoconstrictor nerves. PGE2 is considered to be the final chemical mediator in the signaling pathways that trigger fever (37, 42). PGE2, produced centrally (39) and perhaps also peripherally (30), gains access to the brain where it binds to its receptors on neurons. The preoptic area is the key brain structure for body temperature regulation and for the febrile action of prostaglandins (41, 42, 53). Microinjection of PGE2 into this area induces thermogenesis and tail vasoconstriction, thereby raising body temperature (1, 22, 44).

In normal thermoregulation, neurons in the preoptic area hold tail vasomotor tone under inhibitory restraint (33, 46, 54). We found previously that in anesthetized rats, this inhibitory restraint originated from two distinct preoptic areas: a rostromedial preoptic region (RMPO) surrounding the organum vasculosum of the lamina terminalis (OVLT) and including the ventral part of the median preoptic nucleus, and a second preoptic region located ~1 mm caudolaterally (CLPO) (46). The RMPO corresponds to the most sensitive site for microinjected PGE1 to cause fever in rats (41), and we also found it to be highly sensitive to PGE2 to cause tail vasoconstrictor activity in rats (46). The CLPO was much less sensitive to microinjected PGE2 (46).

The sympathetic premotor neurons that control tail vasoconstrictor tone are located in the medullary raphé (28, 29, 31, 40, 47, 48). This is also the site where premotor neurons for shivering and nonshivering thermogenesis are located (16, 20, 22, 23, 49). Activity of these raphé premotor neurons is required to induce tail vasoconstriction and thermogenesis in response to cold and also in experimental fever induced by central injections of PGE2 or PGE1 (7, 15, 36). In the case of thermogenic responses, the signals from the preoptic area to the medullary raphé are mediated through an obligatory synapse in the dorsomedial hypothalamic nucleus (DMH) (11, 23). The pathways for these functions from the DMH to the medullary raphé are excitatory and glutamatergic (2, 10, 23). The tail vasoconstrictor pathway, by contrast, has no obligatory synaptic relay in the DMH during cold or experimental fever (36). It has, therefore, been suggested that preoptic neurons control the raphé premotor neurons for tail vasoconstriction by a direct connection, which is inferred to be inhibitory (14, 18, 36). In this model, preoptic neurons control tail vasoconstriction by modulating the degree of tonic inhibition on the raphé premotor neurons.

In contrast to that model, we recently identified an excitatory glutamatergic connection from the RMPO to the medullary raphé, which mediates the tail vasoconstrictor response to cold skin (45). Here, we tested the hypotheses that 1) the tail vasoconstrictor response to experimental fever also depends on a glutamatergic excitatory synaptic relay in the medullary raphé, and that 2) it is driven by an excitatory signal from the RMPO.

MATERIALS AND METHODS

Twenty-three adult male Sprague-Dawley rats (310–380 g) were used in this study. All experiments were carried out in accordance with guidelines of the National Health and Medical Research Council of Australia and were approved by the Animal Experimentation Ethics Committee of Florey Institute of Neuroscience and Mental Health.
Animals were anesthetized initially with pentobarbital sodium (60 mg/kg ip), and the hair over the trunk was shaved. The trachea was cannulated, and animals were then artificially ventilated with 2.0% isoflurane (Forthane; Abbott Australia, North Ryde, NSW, Australia) in pure oxygen. Respiratory pressure was monitored via a pressure transducer attached to a side tube, and expired CO2 concentration was monitored by a CO2 analyzer (ADC, Hoddesdon, Herts, UK). Ventilation was adjusted to keep peak expired CO2 between 3.5 and 4.5%. The right femoral artery and vein were cannulated for monitoring blood pressure and intravenous administration of drugs, respectively. A water-perfused Silastic jacket was positioned around the animal’s shaven trunk, and the temperature of the perfusion water was used to manipulate skin and core temperature. Skin temperature was measured as the average of three thermocouples placed across the trunk between the skin and water jacket. Core temperature was measured by a thermocouple inserted 5 cm into the rectum. Core temperature was maintained between 37°C and 38°C during surgery by perfusion of the jacket at 150–180 ml/min with water from a reservoir maintained at 39–42°C.

The animal was then mounted prone in a stereotaxic apparatus, and burr holes were drilled in the skull over the preoptic area and the medulla. When surgery was complete, isoflurane was gradually withdrawn and replaced by urethane (1.0–1.2 g/kg iv). The depth of anesthesia was assessed at intervals throughout the experiment by testing withdrawal and corneal reflexes, and small additional doses of urethane (25–50 mg iv) were administered, if necessary, to abolish those reflexes.

**Tail sympathetic nerve fiber recording.** Postganglionic sympathetic nerve activity (SNA) was recorded from the rat tail tail lateral collector nerve, which was exposed by removing the skin over the lateral vein (34). A pool filled with liquid paraffin was constructed around the tail. Connective tissue was removed from a branch of the nerve trunk, which was then desheathed. Nerve filaments were placed over a silver-wire hook electrode, and few-fiber spike activity was recorded differentially with respect to a nearby thread of connective tissue, amplified (10,000-fold), and filtered (30–600 Hz). The activity was monitored continuously using an oscilloscope, and recorded to a computer at a sampling rate of 5 kHz using a CED Power 1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK). Spikes that passed across a selected threshold voltage were detected with a time-window discriminator and counted as spikes per 10 s for online reference. Each single unit was discriminated off-line using a computer-based spike shape analysis system (Spike 2).

Tail SNA was identified functionally by the excitatory response to passing cold water through the water jacket for 30–60 s. This stimulus was repeated at intervals throughout the experiment. All subsequent series of brain microinjections were made from baseline conditions at which tail SNA was low and stable. This was achieved by maintaining resting core temperature at a sufficiently high level (see RESULTS). To identify brain injection sites, 2% yellow-green fluorescent microspheres (FluoSpheres, Molecular Probes, Eugene, OR) were added to the injectate. Pontamine sky blue (2% in normal saline, 1.5 μl) was given through the injection cannula at the end of the experiment to confirm the position of the cannula for intracerebroventricular injection.

At the end of experiment, hexamethonium chloride (Sigma; 50 mg/kg in saline) was given intravenously to confirm that the recorded activity was postganglionic sympathetic.

**PGE2 microinjections.** Microinjections of PGE2 were made into the lateral cerebral ventricle (n = 6) and into the preoptic area (n = 17). PGE2 (Sigma) was initially dissolved in pure ethanol and then diluted with artificial cerebrospinal fluid (aCSF) to give a final dose of 50 ng in 1.5 μl (intracerebroventricular injections; n = 6) and 0.2 ng in 60 nl (preoptic injections; n = 14), with less than 0.1% ethanol. In three rats, higher doses of PGE2 (0.5, 2, or 4 ng in 30–60 nl aCSF) were used for preoptic injections (experimental series 4). aCSF consisted of (in mM) 128 NaCl, 2.6 KCl, 1.3 CaCl2, 0.9 MgCl2, 20 NaHCO3, and 1.3 NaHPO4. For intracerebroventricular PGE2 injections, a stainless-steel guide cannula (0.7 mm OD) was implanted into the left lateral cerebral ventricle using the following stereotaxic coordinates: 1.0 mm posterior to the bregma, 1.8 mm lateral to midline, and 3.8 mm below from the skull surface. The position of the guide cannula was confirmed by backflow of cerebrospinal fluid. An injection cannula (0.4 mm OD) was then inserted into the guide cannula so that its tip protruded 0.5 mm beyond the tip of the guide cannula. A polyethylene tube connected the injection cannula to a 10-μl microsyringe (Hamilton). Intracerebroventricular injections of PGE2 were made over 60 s.

For injections of PGE2 into the preoptic area, a single-barreled glass micropipette (tip OD ~20 μm) was aimed at the rostromedial preoptic region (RMPO), which previous work has implicated in both febrile and thermoregulatory control of tail SNA (46). The RMPO was localized by the site at which microinjection of GABA (Sigma, 300 mM in distilled water, 15–30 nl) excited tail SNA (46). Microinjections were made using air pressure (NeuroPhore BH-2; Digitimer, Welwyn Garden City, UK), and were monitored by observing the meniscus through a dissecting microscope. Whenever the injectate was changed, the pipette was flushed with distilled water at least twice.

**Kynurenate microinjection into the medullary raphé.** The NMDA receptor antagonist, kynurenic acid (kynurene; Sigma, St. Louis, MO) was dissolved in hypotonic aCSF with 10 M NaOH, and then the pH was adjusted to ~7.4 by adding 1 M HCl. Hypotonic aCSF consisted of (in mM) 103 NaCl, 2.6 KCl, 1.3 CaCl2, 0.9 MgCl2, 20 NaHCO3, and 1.3 NaHPO4, calculated to make the 50 mM kynurenate solution approximately isotonic. A glass micropipette was first filled with 50 mM sodium glutamate (Sigma; dissolved in aCSF), positioned stereotaxically into the rostral medulla (3.0 mm posterior to lambda, 0.0–0.5 mm lateral to midline, and 9.5 mm deep to the dorsal surface), according to the brain atlas of Paxinos and Watson (1998), and aimed at the ventral medullary raphé. Its appropriate location was confirmed by the tail SNA response to injecting 60 nl of 50 mM glutamate (44). It was then withdrawn, and the solution was replaced with 50 mM kynurenic acid or vehicle (aCSF) before it was reinserted to the original position.

PGE2 was first injected either into the lateral cerebral ventricle (6 rats, experimental series 1) or into the RMPO (6 rats, experimental series 2), as described above. Injections of 50 mM kynurene were then made into the medullary raphé in volumes of 120 nl over 60 s, starting 5 min after completion of PGE2 injection. Vehicle (aCSF, 120 nl) was also microinjected into the medullary raphé in all 12 rats.

**Glycine or muscimol microinjection into the preoptic area.** In 11 rats, we tested the effect of local neuronal inhibition of the RMPO on the tail SNA response to PGE2 injected into that site, by subsequent microinjection of glycine (n = 7) or muscimol (n = 4). In the case of glycine microinjection (experimental series 3), PGE2 (0.2 ng) was first injected into the RMPO with a single-barreled glass micropipette in volumes of 60 nl, as described above. Five minutes after the completion of the RMPO PGE2 injection, 0.5 M glycine (Ajax Finechem, dissolved in aCSF) was injected into the same site, in volume of 60 nl over 60 s. In 6 of 7 rats, vehicle (aCSF, 60 nl) was also microinjected into the same position.

In the case of muscimol microinjection (experimental series 4), different doses of PGE2 (0.2, 0.5, 2, or 4 ng in 30–60 nl) were injected into the RMPO of the four rats; 2 mM muscimol (Sigma, dissolved in aCSF) was subsequently injected into the same site in volume of 60 nl over 60 s. In 6 of 7 rats, vehicle (aCSF, 60 nl) was also microinjected into the same position.

Control experiments (using animals taken from experimental series 2, 3, and 4) examined the actions of microinjections of glycine or muscimol alone into the RMPO, without prior PGE2. For this purpose, nine rats received microinjections of 0.5 M glycine (15–60 nl), and two rats received 2 mM muscimol (30–60 nl) into the RMPO.
At the end of experiments, animals were deeply anesthetized with pentobarbital sodium (160 mg iv) and perfused transcardially with saline followed by 4% paraformaldehyde. The brain was removed and placed overnight in the same fixative. After cryoprotection with 20% sucrose in PBS, 40-μm frozen coronal sections were made of the preoptic area and the medullary raphé. The locations of injection sites were identified by detecting fluorescent microspheres and pontamine sky blue, using fluorescence microscopy (AxioPlan 2 imaging; Carl Zeiss, Oberkochen, Germany). Relevant sections were photographed under both light and fluorescence optics using a digital camera (AxioCam HRc; Carl Zeiss). Anatomical detail was revealed by contrast reversal of the light microscopic image in Adobe Photoshop. All microinjection sites were identified and mapped in relation to local structures on a set of drawings made from serial coronal sections of the preoptic area and the medulla.

Statistical analysis. The resting core temperature, skin temperature, blood pressure, heart rate, peak expired CO₂ and few-fiber tail SNA (spikes/10 s) were measured as the average values of these variables over 2 min just before each stimulation (baseline). The resting conditions were compared between raphé vehicle and kynurenate injection (experimental series 1 and 2), and between RMPO vehicle and glycine injection (experimental series 3), using Student’s t-test (unpaired).

To assess the effect of kynurenate injection into the medullary raphé (experimental series 1 and 2) or glycine injection into the RMPO (experimental series 3) on tail SNA response to PGE₂ injections, tail SNA was averaged over 1-min periods: 1) from 4 to 5 min after PGE₂ injections (febrile responses) and 2) from 1 to 2 min after the completion of the subsequent kynurenate or glycine injection (subsequent injections). One-way repeated-measures ANOVA, followed by Tukey’s test, was used to compare tail SNA responses at each time period (baseline, febrile responses, and subsequent injections). The same procedure was used for blood pressure, heart rate, and expired CO₂. The Friedman test was used for comparisons of nonnormally distributed data. Averaged core temperature between 5 and 6 min after subsequent injections was compared with the 1-min average just before injection, using a paired t-test (normally distributed data) or Wilcoxon signed rank test (nonnormally distributed data). To assess the effect of muscimol injection into the RMPO on the tail SNA response to PGE₂ (experimental series 4), a paired t-test was used to compare the 1-min averages of tail SNA taken just before, and from 1–2 min after the completion of the muscimol injection.

To assess the effect of glycine or muscimol injection into the RMPO under resting warm conditions (control experiments), the peak response in each variable was averaged over 30-s periods within 5 min of injection and was compared with the 1-min average just before injection, using a paired t-test. All values were shown as means ± SE, and P < 0.05 was considered significant.

RESULTS

Effect of kynurenate microinjections in the medullary raphé on the response to intracerebroventricular PGE₂. As previously described for PGE₁ (7), injections of PGE₂ (50 ng icv in 1.5 μl) into the lateral cerebral ventricle of six anesthetized rats (experimental series 1) produced a robust increase in tail SNA, which appeared within 3 min of the injection, followed by a delayed rise in core temperature (Fig. 1 and Table 1). To test whether this tail SNA response depends upon an excitatory synaptic relay in the medullary raphé, the NMDA receptor antagonist kynurenate (50 mM, 120 nl) or vehicle (aCSF, 120 nl) was microinjected into the medullary raphé 5 min after the intracerebroventricular injection of PGE₂.

In Fig. 1, A and B, representative chart records show the effect of microinjecting kynurenate or vehicle into the medullary raphé during the febrile response to intracerebroventricular PGE₂. The resting conditions before intracerebroventricular PGE₂ injections were not different between the raphé vehicle and kynurenate injections (Fig. 1 and Table 1). The microinjection of vehicle into the medullary raphé had no effect on the firing rate of tail SNA after intracerebroventricular PGE₂, and core temperature continued to rise (n = 6; Fig. 1 and Table 1). By contrast, microinjection of kynurenate into the medullary raphé immediately reversed the increase in tail SNA and suppressed the rise in core temperature evoked by intracerebroventricular PGE₂ (n = 5; Fig. 1 and Table 1). Intracerebroventricular PGE₂ also significantly increased blood pressure, and raphé kynurenate (but not vehicle) reversed this response (Table 1). Following intracerebroventricular PGE₂ injections, increases in heart rate and expired CO₂ were inconsistent, and grouped data showed no significant changes (Table 1).

Effect of kynurenate microinjections in the medullary raphé on the response to microinjections of PGE₂ into RMPO. In six rats (experimental series 2), a febrile response was induced by a much smaller dose of PGE₂ (0.2 ng in 60 nl) microinjected into the rostromedial preoptic region (RMPO), which our previous work had found was highly sensitive to PGE₂ affecting tail SNA (46). To locate the RMPO, the position of the injection pipette was first confirmed by an excitatory response of tail SNA to microinjection of GABA (300 mM, 15–30 nl, Fig. 2C), as described previously (46).

Figure 2, A and B, are representative chart records showing the effect of microinjection of kynurenate or vehicle into the medullary raphé during experimental fever induced by PGE₂ injected into the RMPO. The resting conditions before RMPO PGE₂ injections were not different between raphé vehicle and kynurenate injections (Fig. 2 and Table 2). Microinjection of PGE₂ into the RMPO induced a rapid increase in tail SNA, which was followed by a rise in core temperature (Fig. 2 and Table 2). Subsequent microinjection of vehicle (aCSF, 120 nl) into the medullary raphé (n = 6) had no effect on tail SNA, and core temperature continued to rise, but microinjection of kynurenate (50 mM, 120 nl) into the medullary raphé (n = 5) rapidly reversed the tail SNA and core temperature responses to RMPO PGE₂ (Fig. 2 and Table 2). A small but significant increase in blood pressure was observed in response to RMPO PGE₂, but raphé kynurenate injections did not significantly reduce that rise (Table 2). Grouped data showed no significant increase in heart rate or expired CO₂ in response to RMPO PGE₂ (Table 2).

Neuronal inhibition of the RMPO. The ability of raphé kynurenate injections to block the tail SNA response to PGE₂ (intracerebroventricular or in the RMPO) suggests that the febrile response depends on excitatory glutamatergic transmission in the medullary raphé. Our previous work demonstrated that two cell groups, with opposing actions on tail SNA, exist side by side in the RMPO (45). RMPO cells with an inhibitory effect on tail SNA are tonically active in warm conditions, as may be shown by the excitation of tail SNA in response to local GABA microinjections. A second set of RMPO neurons is inhibited under warm baseline conditions but can be activated by cool skin and by microinjection of the GABA_A receptor antagonist, bicuculline. The latter neurons are excitatory to tail SNA, most likely by a direct glutamatergic connection to the medullary raphé (45). We, therefore, hypothesized that the increase in tail SNA in response to RMPO PGE₂ also depends on an excitatory signal from the RMPO. To examine this
hypothesis, we tested whether inhibition of neurons in the RMPO by microinjection of glycine (experimental series 3) or muscimol (experimental series 4) could reduce the tail SNA response to PGE2 given into the same region.

Neuronal inhibition of the RMPO under resting warm conditions. As described above, the RMPO was located by the excitatory response of tail SNA to GABA microinjection. Also, neuronal inhibition of the RMPO with glycine or muscimol (see Table 1). Fig. 1. Effect of kynurenate microinjection into the medullary raphé on responses to intracerebroventricular prostaglandin E2 (PGE2) injection. A and B: chart records from a representative experiment showing single-unit tail sympathetic nerve activity (SNA; 10-s counts), core (rectal) temperature, peak expired CO2, heart rate, and blood pressure when PGE2 (50 ng in 1.5 μl) was injected into the lateral cerebral ventricle at the times indicated by open arrowheads. Vehicle [artificial cerebrospinal fluid (aCSF); 120 nl] or kynurenate (kyn; 50 mM, 120 nl) were then injected into the medullary raphé at the times indicated by solid arrowheads. C and D: averaged time courses of tail SNA (C) and core temperature (D) following intracerebroventricular PGE2 microinjection and subsequent microinjection of vehicle (○; n = 6) or kynurenate (●; n = 5) into the medullary raphé. Error bars show means ± SE.

Table 1. Experiment 1: Responses to intracerebroventricular PGE2 and raphé kynurenate

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>aCSF (n = 6)</th>
<th>kyn (n = 5)</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>aCSF icv</td>
<td>aCSF Raphé</td>
</tr>
<tr>
<td>Tail SNA, spikes/10 s</td>
<td>4 ± 1</td>
<td>27 ± 4*</td>
</tr>
<tr>
<td>Core temperature, °C</td>
<td>37.7 ± 0.3</td>
<td>38.2 ± 0.3</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>105 ± 4</td>
<td>112 ± 4*</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>409 ± 16</td>
<td>429 ± 20</td>
</tr>
<tr>
<td>Expired CO2, %CO2</td>
<td>4.0 ± 0.1</td>
<td>4.1 ± 0.1</td>
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</table>

Values are expressed as means ± SE. Responses to PGE2 and subsequent microinjections in experiment 1 of tail SNA, core temperature, blood pressure, heart rate and peak expired CO2. Columns show averaged values just before PGE2 injection, 4–5 min after PGE2 injection, and 1–2 min (5–6 min in the case of core temperature) after the subsequent vehicle (aCSF) or kynurenate (kyn) microinjection. The baseline conditions were not significantly different between aCSF and kynurenate injections. *P < 0.05, compared to baseline. †P < 0.05, compared to PGE2 injection.
below) activates tail SNA under these conditions (Figs. 3 and 4). Six rats from experimental series 2 and three rats from experimental series 3 were tested for the tail SNA response to microinjection of glycine into the RMPO under warm resting conditions (baseline skin and core temperatures 40.2 ± 0.1°C and 39.0 ± 0.2°C, respectively). As illustrated in Fig. 3C, microinjection of 0.5 M glycine (15–60 nl) into the RMPO significantly increased tail SNA (7 ± 1 6 spikes/10 s; \( P < 0.01, n = 9 \)). Repeat

Table 2. Experiment 2: Responses to RMPO PGE\(_2\) and raphe kynurenate

| Experiment 2 |   | aCSF (n = 6) | aCSF Raphe
<table>
<thead>
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<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td>PGE(_2) RMPO</td>
<td>PGE(_2) RMPO</td>
</tr>
<tr>
<td>Tail SNA spikes/10 s</td>
<td>8 ± 2</td>
<td>39 ± 7*</td>
<td>36 ± 6</td>
</tr>
<tr>
<td>Core temperature, °C</td>
<td>38.9 ± 0.3</td>
<td>39.1 ± 0.3</td>
<td>39.3 ± 0.2†</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>108 ± 9</td>
<td>113 ± 9*</td>
<td>113 ± 9</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>456 ± 19</td>
<td>480 ± 21</td>
<td>480 ± 19</td>
</tr>
<tr>
<td>Expired CO(_2), %CO(_2)</td>
<td>4.0 ± 0.05</td>
<td>4.3 ± 0.2</td>
<td>4.3 ± 0.2</td>
</tr>
</tbody>
</table>

|   | kyn (n = 5) | kyn Raphe
<table>
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</thead>
<tbody>
<tr>
<td>Baseline</td>
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<td>PGE(_2) RMPO</td>
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<tr>
<td>Tail SNA spikes/10 s</td>
<td>6 ± 2</td>
<td>35 ± 8*</td>
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<tr>
<td>Core temperature, °C</td>
<td>38.7 ± 0.2</td>
<td>38.9 ± 0.2</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>114 ± 4</td>
<td>119 ± 5*</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>457 ± 15</td>
<td>472 ± 22</td>
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<tr>
<td>Expired CO(_2), %CO(_2)</td>
<td>3.9 ± 0.1</td>
<td>4.2 ± 0.1</td>
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Values are expressed as means ± SE. Responses to RMPO PGE\(_2\) and kynurenate microinjections in experiment 2 of tail SNA, core temperature, blood pressure, heart rate and peak expired CO\(_2\). Columns show averaged values just before PGE\(_2\) injection, 4–5 min after PGE\(_2\) injection, and 1–2 min (5–6 min in the case of core temperature) after the subsequent vehicle (aCSF) or kyn microinjection. The baseline conditions were not significantly different between aCSF and kynurenate injections. *\( P < 0.05 \), compared to baseline.
Glycine injections in six rats produced tail SNA responses that did not differ significantly from those to the first injection ($P > 0.05$; $n = 6$, Fig. 3C). There was a small but significant increase in blood pressure (116 ± 4 to 121 ± 4 mmHg, $P < 0.05$; $n = 9$), but no significant change was detected in heart rate or expired CO2.

**Neuronal inhibition of the RMPO after microinjections of PGE2.** Fig. 3, A and B, show representative chart records of the response to RMPO glycine or vehicle injection following PGE2. The resting conditions before RMPO PGE2 injections were not different between glycine and vehicle injections (Fig. 3C).

### Table 3. Experiment 3: Responses to RMPO PGE2 followed by RMPO glycine

<table>
<thead>
<tr>
<th></th>
<th>aCSF (n = 6)</th>
<th>aCSF RMPO</th>
<th>gly (n = 7)</th>
<th>gly RMPO</th>
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<tbody>
<tr>
<td><strong>Tail SNA, spikes/10 s</strong></td>
<td>5 ± 1</td>
<td>17 ± 4*</td>
<td>17 ± 5</td>
<td>6 ± 1</td>
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<tr>
<td>Core temperature, °C</td>
<td>39.1 ± 0.2</td>
<td>39.4 ± 0.2</td>
<td>39.6 ± 0.3†</td>
<td>39.1 ± 0.2</td>
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<tr>
<td>Blood pressure, mmHg</td>
<td>113 ± 3</td>
<td>120 ± 3*</td>
<td>120 ± 3</td>
<td>114 ± 3</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>415 ± 8</td>
<td>445 ± 15</td>
<td>443 ± 11</td>
<td>424 ± 10</td>
</tr>
<tr>
<td>Expired CO2, %CO2</td>
<td>3.7 ± 0.2</td>
<td>4.1 ± 0.3</td>
<td>4.1 ± 0.3</td>
<td>3.9 ± 0.1</td>
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</tbody>
</table>

Values are expressed as means ± SE. Responses to PGE2 and subsequent microinjections in experiment 3 of tail SNA, core temperature, blood pressure, heart rate and peak expired CO2. Columns show averaged values just before PGE2 injection, 4–5 min after PGE2 injection into the RMPO, and 1–2 min (5–6 min in the case of core temperature) after the subsequent vehicle (aCSF) or gly microinjection into the same site. The baseline conditions were not significantly different between aCSF and glycine injections. *P < 0.05, compared to baseline. †P < 0.05, compared to PGE2 injection.
As described above, microinjection of 0.2 ng PGE2 (60 nl) into the RMPO produced a prompt increase in tail SNA, which was followed by a rise in core temperature (Fig. 3 and Table 3). Subsequent injection of vehicle (aCSF, 60 nl) into the same site had no effect on tail SNA, and core temperature continued to rise (n = 6; Fig. 3 and Table 3). In contrast, subsequent microinjection of glycine (0.5 M, 60 nl) into the same site significantly attenuated the tail SNA response and suppressed the rise in core temperature (n = 7; Fig. 3 and Table 3). Blood pressure also increased in response to RMPO PGE2, but glycine had no significant effect on this (Table 3). As noted above, heart rate and expired CO2 were not significantly affected by RMPO PGE2 injections (Table 3).

**Muscimol microinjections.** Because glycine may have actions other than direct neuronal inhibition [e.g., modulating NMDA receptors (3, 35)], we confirmed the effect of neuronal inhibition in the RMPO after PGE2 using a second inhibitory agent, muscimol (experimental series 4). As described above, microinjection of PGE2 into the RMPO (0.2–4 ng, 30–60 nl in this case) produced a rapid increase in tail SNA (n = 4; Fig. 4 A and C). Subsequent microinjection of muscimol (2 mM, 30–60 nl) into the same site significantly attenuated the tail SNA response to PGE2 (22 ± 2 to 15 ± 2 spikes/10 s, P < 0.05; n = 4, Fig. 4, A and C). Microinjecting muscimol into the RMPO under resting warm conditions (without PGE2) produced a rapid, long-lasting increase in tail SNA (11 to 21, 8 to 19 spikes/10 s; n = 2; Fig. 4B).

**Location of RMPO and raphé microinjection sites.** The locations of microinjection into the preoptic area (PGE2, glycine and muscimol) and the medullary raphé (kynurenate) were reconstructed and are shown in Fig. 5. The centers of injection sites aiming at the medullary raphé were located within the raphé magnus and the raphé pallidus at the level of the caudal half of the facial nucleus, while those in the preoptic area were all located in the rostromedial preoptic area, as defined previously (46).

**DISCUSSION**

It has been shown that neuronal activity in the medullary raphé is required to support the increased sympathetic vasoconstrictor drive to the rat’s tail during experimental fever (7, 36). The present study confirms that the tail vasoconstrictor response to the pyrogenic mediator PGE2 depends upon activation of neurons in the medullary raphé and provides evidence that this is through a glutamatergic excitatory synapse. In addition, we obtained evidence that an excitatory neural signal emerging from the RMPO—the most PGE2-sensitive preoptic region—mediates much of the tail vasoconstrictor response to this stimulus. This latter result would not have been predicted from the current view (14, 17, 18) that PGE2 acts by inhibiting preoptic neurons that tonically suppress the thermogenic and vasoconstrictor neurons of the DMH and/or medullary raphé. In combination with our previous work (45), we now have evidence that excitatory glutamatergic connections from the RMPO to the medullary raphé mediate tail SNA responses to both cold skin and experimental fever.

**Limitations.** Necessarily, these invasive experiments were done entirely on animals under general anesthesia. This undoubtedly reduced the magnitude of thermoregulatory and febrile responses. Nevertheless, PGE2 injections still caused appropriate febrile responses and allowed us to investigate their neural pathways. We deliberately set core and skin temperatures high to minimize baseline tail SNA. Against this low background, the actions of PGE2 could be most clearly...
demonstrated. It may be that these conditions, in combination with general anesthesia, accentuate inhibitory tone in the pathway we studied. The data should be interpreted with this in mind.

A further potential bias is that kynurenate injections into the medullary raphé would also have blocked glutamatergic inputs from sources other than RMPO, which might then lower the excitability of the raphé premotor neurons. Our previous study found that while raphé kynurenate injections completely blocked the tail SNA response to cold skin (attributed to a direct excitatory connection from RMPO), they merely reduced the response to core cooling (attributed to other pathways) (45). From this result, we infer that if kynurenate treatment does reduce the excitability of raphé premotor neurons, that reduction is insufficient to block their output entirely. The full suppression of tail SNA responses to PGE2 by raphé kynurenate injections in the present study was, therefore, most likely to due to direct blockade of a glutamatergic pathway carrying the fever signal.

Febrogenic actions of PGE2. It has been demonstrated that PGE2 microinjected either into the lateral cerebral ventricle or into the preoptic area increases body temperature, blood pressure, heart rate, and oxygen consumption in a dose-dependent manner, even in anesthetized animals (1, 6, 12, 13, 32). Our experimental conditions were optimized to detect changes in tail SNA from a low but suprathreshold baseline. Under these conditions, however, the grouped data showed no significant change in heart rate or expired CO2 (indicative of oxygen consumption), although some individual cases appeared to respond. It seems most likely that the low doses of PGE2 used here were near the threshold for those responses in warm resting animals. There was, however, a consistent, strong increase in tail SNA and a small but significant increase in blood pressure.

How does PGE2 activate tail SNA? The EP3 receptor is thought to be the critical receptor responding to PGE2 to mediate the febrile response (9, 50). It is strongly expressed in the ventral parts of the median preoptic nucleus and adjacent areas close to the OVLT (4, 19), corresponding to the RMPO (46). This region is also where Stitt (41) found the most sensitive site for microinjections of PGE1 to cause fever and where we found the most sensitive site for PGE2 microinjections to activate tail SNA (46). Furthermore, Lazarus and colleagues found that EP3 receptors within this region were responsible for at least most of the febrile action of intracerebroventricular PGE2 in mice (9). Therefore, it is reasonable to presume that PGE2, whether delivered intracerebroventricularly or by direct injection into RMPO, acts primarily on EP3 receptors in RMPO to activate tail SNA. But how does this happen?

The EP3 receptor is most commonly coupled to inhibitory G proteins and is, thus, considered to inhibit its target neurons by reducing intracellular cAMP levels (24, 26). Until recently, the control of cutaneous vasoconstriction from the preoptic region...
was also considered to be predominantly, if not exclusively, inhibitory (33, 46, 54). The simplest hypothesis combining these two observations was, therefore, that PGE₂, via EP₃ receptors, inhibited those preoptic neurons—possibly warm-sensitive cells—that held the cutaneous vasoconstrictor pathway under tonic restraint. PGE₂’s action would, thus, release tail SNA from that inhibitory restraint, causing it to increase (14, 17, 18).

But that explanation cannot account for our new findings. The fact that kynurenate microinjected into the medullary raphé reverses the tail SNA response to PGE₂ indicates that the descending febrile signal depends on an excitatory connection to the raphé premotor neurons. It does not tell us the origin of that excitatory drive. Our second new finding, that microinjection of glycine or muscimol into the RMPO significantly reduces the tail SNA response to PGE₂, suggests that the increase in tail SNA after PGE₂ is not only due to the withdrawal of tonic inhibitory drive; it must involve preoptic neurons with an excitatory action. Only the existence of RMPO output neurons with an excitatory action on tail SNA can explain such a result.

These apparently conflicting accounts may be reconciled once it is accepted that the RMPO contains at least two sets of neurons with opposing actions on tail SNA. As outlined in our recent articles (45, 46), RMPO cells providing inhibitory drive to tail SNA are tonically active, as shown by the release of tail SNA in response to local microinjection of the inhibitory agents, GABA, glycine, or muscimol [(46); this study, Figs. 2–4]. A second set of RMPO neurons is tonically inhibited under resting warm conditions but activated by cold skin or by local microinjection of the GABAₐ receptor antagonist bicuculline (45). The latter neurons provide excitatory drive to tail SNA, most likely by a direct glutamatergic connection to the medullary raphé, and this RMPO-raphé excitatory drive accounts fully for the tail SNA response to cold skin (45). Support for the idea that neurons in the RMPO are activated as part of the febrile response is provided by studies that have shown strong c-Fos expression by neurons in the ventromedial part of the preoptic region, corresponding to the RMPO, during experimental fever produced by systemic LPS (5, 38), systemic PGE₂ (52), or intracerebroventricular PGE₂ (8).

Glycine or muscimol microinjections into the RMPO did not abolish the tail SNA response to PGE₂ but reduced it by approximately half. The reasons for this are not certain, but two main possibilities may be considered. First, glycine or muscimol silences the excitatory output neurons that drive tail SNA in response to PGE₂, but this action is offset by simultaneously suppressing the inhibitory output neurons (those whose tonic action is revealed by GABA in the resting warm state). If that is the case, it implies that the inhibitory output neurons are relatively unaffected by PGE₂. A second possibility is that the RMPO inhibitory output neurons are suppressed by PGE₂ at the same time as the excitatory output neurons are activated (or disinhibited). In that case, the remaining half of tail SNA is attributed to the release from inhibition (by RMPO inhibitory neurons) of excitatory neurons outside the reach of microinjected glycine or muscimol. Those excitatory neurons could be located within the broader preoptic area and/or be a more caudally located source of excitatory drive to the medullary raphé (but not the dorsomedial hypothalamus) (36).

How does PGE₂ activate RMPO neurons? The finding that some RMPO neurons are activated in response to PGE₂ and then provide an excitatory drive to tail SNA raises another question; how does PGE₂ activate those RMPO neurons? It is possible that PGE₂ directly activates those RMPO neurons through EP₃ receptors. In rodents, three different isoforms of EP₃ receptors have been identified (EP₃ₐ, EP₃ᵦ, EP₃ᵧ), and EP₃ₐ and EP₃ᵦ receptors are coupled to inhibitory G proteins, while EP₃ᵧ receptors are coupled to both inhibitory and stim-

![Diagram of proposed neuronal pathways regulating tail vasoconstriction](http://ajpregu.physiology.org/)
ulatory G proteins (25, 43). In the mouse preoptic area, expression of the EP3<sub>a</sub> receptor is predominant, but a moderate expression of EP3<sub>γ</sub> (15% of total EP3 receptor expression) is also observed (51). PGE<sub>2</sub>, thus, could activate RMPO neurons through EP3<sub>γ</sub> receptors, although some involvement of other EP receptors (EP<sub>1</sub>, EP<sub>2</sub>, and EP<sub>4</sub>) cannot be ruled out (27).

The other possibility is that the excitatory neurons that project out from the RMPO are activated indirectly, by disinhibition. Most (~85%) EP<sub>3</sub> receptor-expressing preoptic neurons are GABAergic (21) and probably include local inhibitory interneurons. If PGE<sub>2</sub> inhibits those GABAergic interneurons, the excitatory projection neurons of the RMPO would be released from inhibition, resulting in increased tail SNA (Fig. 6). In support of this proposed mechanism, we have already shown that RMPO excitatory projection neurons, when released from tonic inhibition by microinjections of bicuculline, drive tail SNA via a glutamatergic excitatory synapse in the medullary raphe (45).

Related findings in the literature. A similar disinhibitory mechanism may be involved in the preoptic control of febrile thermogenesis. Osaka (32) showed that pretreatment with muscimol microinjected in the peri-OVLT region of anesthetized rats greatly attenuated the metabolic and tachycardic responses to subsequent microinjections of PGE<sub>2</sub> into the same site. This suggests that the signal for febrile thermogenesis also involves an excitatory neural output from the area. A secondary measurement taken in those experiments was tail temperature, an indirect index of vasoconstrictor drive. Under Osaka’s experimental conditions, muscimol alone caused no fall in tail skin temperature (indicating vasoconstriction), perhaps because the tail vessels were already largely constricted, but Osaka did find that muscimol pretreatment prevented the small (~<0.5°C) fall in tail skin temperature (indicating vasoconstriction) that otherwise followed PGE<sub>2</sub> microinjections. The differences from the present study may be due to small differences in experimental conditions or the exact anatomical location of microinjections within the preoptic area (the present experiments always defined the RMPO functionally by the tail SNA response to microinjected GABA). But both studies support the idea that an excitatory signal emerges from the RMPO during PGE<sub>2</sub>-induced febrile responses.

In conclusion, we have found that in PGE<sub>2</sub>-induced fever, an excitatory drive from the RMPO contributes to the tail vasoconstrictor response and that the tail vasoconstrictor response to PGE<sub>2</sub> is entirely dependent on excitatory (glutamatergic) transmission in the medullary raphe. We recently found that the tail vasoconstrictor response to cold skin also depends on an excitatory signal from the RMPO and a glutamatergic excitatory input to the medullary raphe, mediated most likely by direct connections (45). It is not yet certain whether the same excitatory RMPO-raphe pathway drives tail SNA in response to cold skin and experimental fever but that would be the simplest explanation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.T., M.J.M., and R.M.M. conception and design of research; M.T. performed experiments; M.T. analyzed data; M.T., M.J.M., and R.M.M. interpreted results of experiments; M.T. prepared figures; M.T. and R.M.M. drafted manuscript; M.T., M.J.M., and R.M.M. approved final version of manuscript.

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