Excitatory amino acid receptors in the dorsomedial hypothalamus mediate prostaglandin-evoked thermogenesis in brown adipose tissue

C. J. Madden
S. F. Morrison

Neurological Sciences Institute
Oregon Health & Science University
Beaverton, OR  97006

Corresponding author:
Christopher J. Madden, Ph.D.
Neurological Sciences Institute / OHSU
505 NW 185th Avenue
Beaverton, OR 97006
Phone: (503) 418-2671
FAX: (503) 418-2501
Email: maddench@ohsu.edu

Running Head: Role of dorsomedial hypothalamus in fever
Abstract

We determined whether the dorsomedial hypothalamus (DMH) plays a role in the thermogenic, metabolic and cardiovascular effects evoked by centrally-administered prostaglandin E2 (PGE2). Microinjection of PGE2 (170pmol/60nl) into the medial preoptic area of the hypothalamus in urethane-chloralose anesthetized, artificially ventilated rats increased brown adipose tissue (BAT) sympathetic nerve activity (SNA; +207 ± 18% of control), BAT temperature (+1.5 ± 0.2 ºC), expired CO2 (+0.9 ± 0.1%), heart rate (HR; +106 ± 12 beats/min, bpm) and mean arterial pressure (+22 ± 4 mmHg). Within five minutes of subsequent bilateral microinjections of the GABA_A receptor agonist, muscimol (120pmol/60nl/side), or the ionotropic excitatory amino acid antagonist, kynurenate (6nmol/60nl/side) into the DMH, the PGE2-evoked increases were respectively attenuated by 91 ± 3% and 108 ± 7% for BAT SNA, by 73 ± 12% and 102 ± 28% for BAT temperature, by 100 ± 4% and 125 ±21% for expired CO2, by 72 ± 11% and 70 ± 16 % for HR and by 84 ±19% and 113 ± 16% for mean arterial pressure. Microinjections outside the DMH within the dorsal hypothalamic area adjacent to the mamillothalamic tracts or within the ventromedial hypothalamus were less effective for attenuating the PGE2-evoked thermogenic, metabolic and cardiovascular responses. These results demonstrate that activation of excitatory amino acid receptors within the DMH is necessary for the thermogenic, metabolic and cardiovascular responses evoked by microinjection of PGE2 into the medial preoptic area.

Keywords: febrile; thermoregulation; sympathetic; glutamate; medial preoptic area
Introduction

Prostaglandin E₂ acting within the preoptic area of the hypothalamus has been implicated in the acute phase component of the febrile response. Fever can be elicited by injection of prostaglandin directly into the medial preoptic area (MPA) (11,27,28,31) and blockade of prostaglandin synthesis within the preoptic area of the hypothalamus attenuates fever (16,25). Furthermore, the prostaglandin EP3 receptor is found in the MPA (10,19,22) and EP3 receptor knockout mice have an impaired febrile response to systemic pyrogen administration (30). Recent observations suggest that ventromedial medullary neurons, including those in the rostral raphe pallidus (RPa), are an essential site of synaptic integration in the central pathways mediating the thermogenic and cardiovascular components of the febrile response in the rodent model (15,17,20). However, the neural pathway(s) responsible for conveying the thermogenic and cardiovascular drive from the MPA to neurons in the RPa are poorly understood. Several lines of evidence suggest that the dorsomedial hypothalamus (DMH) plays a role in the thermogenic and cardiovascular components of the PGE₂-evoked febrile response. Anatomically, MPA neurons project to the DMH (29) and, in turn, DMH neurons send direct projections to the RPa (12,21). Physiologically, disinhibition of neurons within the DMH produces activation of brown adipose tissue (BAT) thermogenesis and tachycardia (6,33) and inhibition of neurons within DMH attenuates the rise in core body temperature and heart rate (HR) evoked by microinjection of PGE₂ into MPA (32). In the present study, we sought to determine the role of sympathetically-mediated BAT thermogenesis in the attenuation of the PGE₂-evoked febrile response that results from inhibition of neurons in the DMH. In addition, we tested the hypothesis that activation of excitatory amino acid (EAA) receptors within the DMH is necessary for PGE₂-evoked BAT thermogenesis.
Experimental Procedures

Male and female Sprague-Dawley rats (Charles River, Indianapolis, IN, n=20) weighing 250-450g were given ad libitum access to standard rat chow and water in a colony room maintained at 22-23 °C and kept on a 12:12-hour light-dark cycle. Rats were anesthetized with isoflurane (2-3% in oxygen) and implanted with femoral arterial and venous catheters and transitioned to urethan and chloralose anesthesia (500mg/kg and 80mg/kg intravenous, respectively) over a 10-minute period. In order to record arterial pressure and HR, the arterial catheter was attached to a pressure transducer. The trachea was cannulated, and the animals were paralyzed with d-tubocurarine (0.5 mg iv, supplemented with 0.1 mg every hour) and ventilated (tidal volume: 1ml/100g body weight, 60 cycles per minute) with 100% oxygen. Mixed-expired CO₂ was monitored using a capnometer. Colonic temperature was monitored using a copper-constantan thermocouple inserted 6 cm into the rectum and core body temperature was maintained at 37.5 ± 0.5° C with a heating plate and a heat lamp. Animals were placed in a stereotaxic instrument with the incisor bar positioned 4 mm below the interaural line. For microinjections into the MPA, small portions of the parietal and frontal bones were removed. Based on the coordinates at which microinjection of prostaglandin elicited hyperthermia (31), our coordinates for injections into the MPA were 0.0 to 0.3 mm caudal and 0.8 mm lateral to bregma and 8.0 mm ventral to dura. For microinjections into the DMH, small portions of the parietal bones were removed. The coordinates for injections into the DMH were 3.0-3.5 mm caudal to bregma, 0.5-0.7 mm lateral to the midline and 8.2-9.0 mm ventral to dura. These coordinates correspond to the area at which a microinjection of bicuculline evokes an increase in BAT SNA (6) and an increase in HR (6,26). Microinjections located within 100µm of the boundaries of the DMH designated in the atlas of Paxinos & Watson (24) were considered
effective DMH injection sites. All other microinjections were considered to be control injections and were analyzed as a separate group. Glass micropipettes (outer tip diameter, 20-30 μm) were used for all microinjections which were given over a 10-20 second period using a pressure injection system. All drugs were obtained from Sigma (St. Louis, MO) except isoflurane, which was obtained from Abbott Laboratories (North Chicago, IL).

Postganglionic BAT sympathetic nerve activity (SNA) was recorded from the central cut end of a nerve bundle isolated from the ventral surface of the right interscapular fat pad after dividing it along the midline and reflecting it laterally. The nerve bundle was placed on bipolar hook electrodes, and covered in mineral oil. Nerve activity was filtered (1-300 Hz) and amplified (20,000-50,000) with a Cyberamp 380 (Axon Instruments, Union City, CA). The BAT temperature was monitored with a copper-constantan thermocouple (Physitemp, Clifton, NJ) inserted beneath the intact, left interscapular fat pad. Physiological variables were digitized (Digidata 1320A, Axon Instruments) and recorded onto a PC hard drive using AxoScope acquisition software (Axon Instruments).

Rats received a microinjection of PGE2 (170pmol/60nl) into the medial preoptic area (MPA) followed 15-20 minutes later by bilateral microinjections into the dorsomedial hypothalamus (DMH) of either the GABA<sub>A</sub> agonist muscimol (120pmol/60nl/side; n=6), the non-selective ionotropic EAA receptor antagonist, kynurenic acid (6nmol/60nl/side; n=10), or saline (60nl/side; n=4). The dose of kynurenic acid used in the present study was effective in blocking PGE2-evoked BAT thermogenesis when microinjected into the RPa (15).

At the conclusion of each experiment, the DMH and MPA microinjection sites were marked by retracting the microinjection pipettes vertically, filling them with 2% fast green dye, repositioning them to the appropriate dorso-ventral coordinates of the microinjection sites and
electrophoretically (20 μA anodal direct current for 10 minutes) depositing the dye. Rats were perfused transcardially with a 10% paraformaldehyde solution. The brains were removed, post-fixed for 12-24 hours and sectioned at 60 μm. Brain sections containing fast green dye deposits were mounted on slides and photographed using a digital microscope camera (Kodak DC 220). Photomicrographs were downloaded to a PC computer and assembled using Adobe Photoshop software; only brightness, sharpness, and contrast were adjusted. The locations of microinjection sites were plotted on atlas drawings (Paxinos and Watson, 1986).

BAT SNA amplitude was derived from autospectral analysis using Datapac 2000 software (Run Technologies Co). For each experimental condition, the average autospectrum of a 101.6-second BAT SNA data segment was computed by dividing the data into 20 equal segments, computing the autospectrum for each of these 5.08 second segments and then averaging these individual autospectra (i.e., the average power at each frequency value is the mean of the powers at that frequency value in the 20 individual autospectra). The root mean square amplitude of the BAT SNA for each experimental condition was taken as the square root of the total power in the 0.19 to 20 Hz band of the averaged autospectrum. Resting (baseline) values were obtained from the data during the 2-minute period immediately prior to microinjection of PGE₂ into MPA. Response values of BAT SNA were obtained from the data during the 2-minute period of peak change in BAT SNA following microinjection of PGE₂ into MPA.

All statistics were performed using Systat software (version 10, SPSS Inc.). Values are expressed as mean ± SE. Statistical significance was assessed using a two-way repeated measures ANOVA with drug treatment as the grouping variable and time as the repeated measure. Significant group by time interactions were followed by ANOVA tests at each time
point with Fisher’s LSD post-hoc testing where appropriate. Significance level was set at P<0.05.

Results

Inhibition of neurons within DMH following PGE2 in MPA

Under resting conditions in urethane-chloralose-anesthetized rats, BAT SNA was typically low with only a few, small-amplitude bursts being recorded every minute. Resting values for BAT temperature, expired CO2, HR and mean arterial pressure prior to microinjection of PGE2 into the MPA are shown in Table 1. [location of Table 1] Unilateral microinjection of PGE2 into the MPA produced a dramatic increase in BAT SNA and significant (p<0.01) increases in BAT thermogenesis, expired CO2, HR and mean arterial pressure (Figure 1A). [location of Figure 1] These responses were rapid in onset, with initial increases beginning within minutes and typical peak responses occurring within 20 minutes of the microinjection of PGE2. As illustrated in Figure 1, the PGE2-evoked responses were also long lasting: in rats receiving subsequent microinjections of saline vehicle into the DMH, the augmented levels of BAT SNA, BAT temperature and expired CO2 remained within 20% of peak values for at least forty minutes. In the control response to microinjection of PGE2 illustrated in Figure 1A, PGE2 produced peak increases in BAT SNA of + 209%, in BAT temperature of + 1.0 °C, in expired CO2 of + 0.8%, in HR of + 136 beats/min (bpm) and in mean arterial pressure of + 22 mmHg. Table 1 contains the mean values of the responses to microinjection of PGE2 into the MPA. Bilateral microinjection of saline vehicle into the DMH had no effect on the levels of the PGE2-evoked responses in any of the thermogenic or cardiovascular variables (Figure 1A, Table 1).
Bilateral microinjections of the GABA<sub>A</sub> receptor agonist, muscimol into the DMH significantly reduced the levels of BAT SNA, BAT temperature, expired CO<sub>2</sub>, HR and mean arterial pressure from those produced by PGE<sub>2</sub> administration into the MPA to levels that were not different from those under control conditions prior to PGE<sub>2</sub> administration (Figure 1B). BAT SNA began to decrease immediately following the microinjections of muscimol into DMH, with maximum reductions in BAT SNA occurring within 11 ± 4 minutes. In the example in Figure 1B, BAT SNA was reduced from a peak response level of + 235% of control to a nadir of +1% of control. Similarly, the increases in BAT temperature, expired CO<sub>2</sub>, HR and mean arterial pressure evoked by PGE<sub>2</sub> administration were reversed by microinjections of muscimol into the DMH (Figure 1B), returning to pre-PGE<sub>2</sub> levels within 9 ± 2, 6 ± 1, 14 ± 4 and 8 ± 2 minutes, respectively, of the inhibition of DMH neurons. As shown in Figure 1B, microinjection of muscimol into the DMH reduced BAT temperature from a peak of + 1.5ºC above the resting control level back to the resting control level, expired CO<sub>2</sub> from a peak of + 0.9% to - 0.3% below the resting control level, HR from a maximum increase of + 148 bpm to + 18 bpm, and mean arterial pressure from a peak of + 51 mmHg above the resting control to - 8 mmHg below the resting control level. Table 1 provides the mean values of these variables after microinjections of muscimol into the DMH.

Paralleling the effects produced by inhibiting neurons in DMH with microinjections of muscimol, blockade of EAA receptors in DMH with kynurenic acid subsequent to microinjection of PGE<sub>2</sub> into MPA also reversed the increases in BAT SNA, BAT temperature, expired CO<sub>2</sub>, HR, and mean arterial pressure produced by PGE<sub>2</sub> administration. BAT SNA began to decrease immediately following microinjections of kynurenic acid into DMH, with maximum reductions in BAT SNA occurring within 8 ± 2, minutes. In the example in Figure 1C, BAT SNA was
reduced from a peak response level of +336% of control to a nadir of +8% of control. Similarly, the increases in BAT temperature, expired CO₂, HR, and mean arterial pressure evoked by PGE₂ administration were reversed by microinjections of kynurenic acid into the DMH (Figure 1C), returning to pre-PGE₂ levels within 9 ± 2, 7 ± 2, 11 ± 2 and 7 ± 2 minutes, respectively, of the blockade of EAA receptors in DMH. As shown in Figure 1C, microinjection of kynurenic acid into the DMH reduced BAT temperature from a peak of + 0.9°C above the control level to - 0.5°C below the resting level, expired CO₂ from a peak of + 0.9% to - 0.2% below resting level, HR from a maximum increase of + 70 bpm to + 19 bpm, and mean arterial pressure from a peak of +6 mmHg above the resting control to - 4 mmHg below the resting control level. Table 1 provides the mean values of these variables after microinjections of kynurenic acid into the DMH.

The locations of the microinjection sites targeting the DMH and the MPA are shown in Figure 2. [location of Figure 2] The approximate centers of the injection sites targeting the DMH were located in an area extending dorsally from the dorsal aspect of the ventromedial hypothalamus (VMH) to just medial to the mamillothalamic tracts. The majority of injection sites that were capable of completely reversing the PGE₂-evoked response were located within or just dorsal to the borders of the DMH (according to the atlas of Paxinos and Watson, (24)). Injection sites located more dorsally, near the level of the mamillothalamic tract, or those located ventral to DMH, within the VMH, were less effective at reversing the PGE₂-evoked responses than those located within the DMH (Figure 2, Table 1). The approximate centers of the microinjections of PGE₂ into the MPA were located primarily dorsal and medial to the anteroventral preoptic nucleus within the boundaries of the MPA (24). These microinjection sites are in the same locations as those previously reported (31) to be thermogenically-responsive to prostaglandin.
Discussion

The present study is the first to demonstrate that ionotropic EAA receptor-mediated activation of neurons in the DMH is necessary for the increases in BAT SNA and thermogenesis as well as those in expired CO₂, HR and mean arterial pressure evoked by PGE₂ administration into the MPA of anesthetized rats. The present data are consistent with, and extend, an earlier study in which inhibition of DMH neurons indicated a critical role of DMH neurons in mediating the increase in core body temperature and the tachycardia evoked by central administration of PGE₂ (32).

It has been suggested that the RPa contains the sympathetic premotor neurons responsible for driving the discharge in the sympathetic nerves innervating BAT (15,17,18). This suggestion is based on the observations that neurons within the RPa are labeled by injections of pseudorabies virus into BAT (3,4,23), that neurons within the RPa project directly to the intermediolateral cell column (2,14), that BAT SNA can be driven by disinhibition of neurons within the RPa (18), and that inhibition of RPa neurons (17,20) or blockade of EAA receptors within the RPa (15) prevents the sympathetically-mediated increase in BAT thermogenesis evoked by central administration of PGE₂. Taken together with the results of the current study, these observations support a model for the central activation of BAT thermogenesis during the acute phase of fever in which PGE₂ acts within the MPA to produce an EAA-mediated increase in the discharge of neurons in DMH which, in turn, drive sympathetic outflow to BAT via activation of neurons within the RPa.

Our data do not allow discrimination between a direct activation of DMH neurons by glutamatergic neurons in MPA and an ‘indirect’ activation that might occur through disinhibition
of DMH neurons, allowing the influence of an active EAA input to be revealed. However, it is noteworthy that increases in BAT temperature can be produced either by disinhibition of neurons within the DMH using microinjections of the GABA<sub>A</sub> receptor antagonist, bicuculline, or by transection of the neuraxis between the MPA and the DMH (7,33). Both of these observations are consistent with the existence of a tonically-active inhibitory input from the MPA to the DMH, though this has yet to be directly tested. Although the present study reveals an EAA input to neurons of the DMH, the source of this excitatory drive remains to be determined.

Whether the thermogenic output of the DMH influences sympathetic premotor neurons of the RPa directly, as suggested previously (32), or whether this influence occurs via a multisynaptic pathway, remains unknown. Regarding the latter, a marked reduction in the cardiovascular responses to disinhibition of DMH neurons results from inhibition of neurons in the dorsolateral periaqueductal gray (9) and increases in BAT temperature can be elicited from the lateral region of the caudal periaqueductal gray (8). In the present study, many of the DMH sites at which microinjections of muscimol or kynurenic acid reversed the PGE<sub>2</sub>-evoked responses were within the dorsal region of the DMH, shown anatomically to contain retrogradely-labeled cells following tracer injections into RPa (12). However, in a significant number of animals, equally-effective DMH microinjections were made into sites outside of the dorsal-most aspect of the DMH and the area just dorsal to the DMH (the area that contains neurons that project directly to the RPa). In the latter cases, the possibility that the injectate diffused to the RPa-projecting cells of the dorsal region of the DMH cannot be rule out. It is also interesting to note that in the present study injection of Kyn into the VMH did not reverse the PGE<sub>2</sub>-evoked responses. Several studies have suggested that the VMH plays a role in BAT thermogenesis elicited from the preoptic area of the hypothalamus (1,13), however, Zaretskaia et
al (33) have convincingly argued that these studies which employed high doses and large
injection volumes into the VMH were likely to have had their inhibitory effects by diffusion of
the injectate to the DMH.

As we have discussed previously (15), we interpret the PGE$_2$-evoked increases in BAT
temperature to be secondary to the increase in BAT SNA. Consistent with this interpretation is
our observation that the time course of the increase in BAT temperature always followed and
paralleled that of the BAT SNA. Similarly, we consider the increase in expired CO$_2$ following
PGE$_2$ administration to be secondary to the increased metabolism in BAT and cardiac muscle
associated with the PGE$_2$-evoked thermogenesis and tachycardia, respectively. Given that the
increase in HR evoked by disinhibition of neurons within the RPa is mediated by an increase in
cardiac sympathetic nerve activity (5) and that PGE$_2$-evoked tachycardia is mediated by neurons
within the RPa (15,17), we suggest that PGE$_2$-evoked tachycardia is mediated by an increase in
cardiac sympathetic outflow. It is likely that the PGE$_2$-evoked increase in HR is associated with
an increase in cardiac output which may contribute to the increase in mean arterial pressure,
though we cannot rule out a role of increased vascular resistance in this response.

In summary, the present study demonstrates that activation of EAA receptors within the
DMH is required for the thermogenic, metabolic, and cardiovascular effects resulting from
microinjection of PGE$_2$ into the MPA. Further studies will be required to determine the
source(s) of the EAA inputs responsible for the excitation of the neurons within the DMH that
are involved in thermogenic and tachycardic responses.
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References


Figure Legends

Figure 1. Reversal of the PGE₂-evoked increases in BAT thermogenesis and heart rate by microinjection of the GABA₅ receptor agonist, muscimol, or the excitatory amino acid (EAA) receptor antagonist, kynurenate (Kyn), into the dorsomedial hypothalamus (DMH). Microinjection of PGE₂ into the medial preoptic area (MPA) (filled arrows) increased brown adipose tissue (BAT) sympathetic nerve activity (SNA), BAT temperature, expired CO₂, heart rate, and mean arterial pressure. (A) Microinjection of saline vehicle into the DMH (open arrow) had no effect on any of the measured variables. In different rats, bilateral microinjections of muscimol (B) or kynurenate (C) into the DMH (open arrows) completely reversed the PGE₂-evoked responses. Vertical scale bar represents peak-to-peak BAT SNA of 80 μV in A, 80 μV in B, and 250μV in C.

Figure 2. Location of microinjection sites targeting the DMH and MPA. Upper panels: representative photomicrographs illustrating the fast green dye deposits (arrows) at the sites of microinjections within the area of the DMH (left) and MPA (right). Lower panels: Locations of the microinjection sites for rats receiving injections of saline (diamonds), muscimol (circles), or kynurenate (squares) plotted on atlas drawings of the rat hypothalamus (bregma -3.5 and -0.26 mm, respectively) (24). Open symbols represent sites at which injections attenuated the PGE₂-evoked brown adipose tissue sympathetic nerve response by less than 60%, whereas filled symbols represent sites at which injections attenuated this response by greater than 85%.
Table 1. Effect of saline, muscimol (Musc), or kynurenate (Kyn) into dorsomedial hypothalamus (DMH) on the cardiovascular, metabolic and thermogenic responses following PGE2 into MPA.

Table 1. Values for physiological variables are provided under resting (baseline) conditions, at the peak response within 20 minutes of the microinjection of PGE2 into the MPA and at the minimum level within five minutes after subsequent bilateral microinjections of saline, muscimol (Musc), or kynurenic acid (Kyn) into the DMH or surrounding areas. Values are mean ± SE (Saline, n=4; Musc, n=5; Kyn, n=5; Kyn outside the DMH, n=5). Note, one animal received an injection of Musc outside the DMH, the response in this animal was similar to those in rats receiving an injection of Kyn outside the DMH. However, since this was the only animal receiving this treatment it was excluded from analysis.

* indicates p<0.05, increase compared to the baseline value.
† indicates p<0.05, compared to the saline control response.
Figure 1
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