Role of inferior olive and thoracic IML neurons in nonshivering thermogenesis in rats

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Uno, Tadashi, and Masaaki Shibata. Role of inferior olive and thoracic IML neurons in nonshivering thermogenesis in rats. Am J Physiol Regulatory Integrative Comp Physiol 280: R536–R546, 2001.—Removal of the midbrain tonic inhibitory mechanism on nonshivering thermogenesis (NST) results in increased temperatures of the interscapular brown adipose tissue (IBAT) and rectum (TIBAT and Trec, respectively) via an enhanced central sympathetic output. Because it is unlikely that neurons (primary) of the midbrain inhibitory mechanism tonically inhibit the IBAT monosynaptically, there must be secondary or tertiary neurons posterior to the midbrain. Such neurons, therefore, may increase their activity during enhanced NST after removal of the midbrain tonic inhibition. The aim of the present experiments was to localize these secondary or tertiary neurons and establish descending neuronal pathway(s) that may project to the major NST effector IBAT. TIBAT and Trec increases induced by removal of the tonic inhibition by midbrain procaine microinjections were accompanied with appearance of c-Fos-positive neurons in the inferior olive (IO) and the intermediolateral (IML) cell column of the thoracic spinal cord. Electrical stimulation of and L-glutamate microinjections into the IO increased TIBAT and Trec. Midbrain procaine-induced TIBAT and Trec increases were blocked by electrolytic IO lesions. These results suggest that central thermal signals produced from the lower midbrain are transmitted to IBAT through the IO and IML and that the IO has a role in the central sympathetic functions.

TIBAT and Trec consistently increase. On the other hand, when L-glutamate (Glut), an excitatory amino acid, is microinjected into the midbrain site where procaine increases TIBAT and Trec, it decreases these temperatures, probably as a result of enhanced midbrain tonic inhibition (27). This result suggests the existence of nerve cell bodies in the midbrain that may tonically inhibit NST. Because skin temperature (Tskin) does not change during either the increase caused by procaine/tetrodotoxin or the decrease caused by Glut of TIBAT and Trec and because animals decerebrated at the posterior forebrain level are still able to increase TIBAT and Trec in response to midbrain procaine administration, putative neurons in the midbrain tonic inhibitory mechanism may directly control NST by tonically suppressing it (26, 27). This possibility is in contrast to the generally held belief that NST is reciprocally stimulated and inhibited by excitation of hypothalamic cold- and warm-sensitive neurons, respectively (4).

Recent studies (9, 26, 27) have confirmed the above results in rats by showing that procaine, a local anesthetic, transiently increases interscapular BAT (IBAT) and rectal temperatures (TIBAT and Trec, respectively) by 1–2°C, but only when it is microinjected into the lower midbrain area that was delineated by the two brain tissue transections reported in those earlier studies. That area appears to include the nuclei of the retrorubral field (RRF) and rubrospinal tract (rs). Tetrodotoxin, an Na⁺-channel blocker, microinjected into the RRF and rs areas also increases TIBAT and Trec temperatures (27). Thus, regardless of the method used, when the midbrain tonic inhibition is removed, TIBAT and Trec consistently increase. On the other hand, when L-glutamate (Glut), an excitatory amino acid, is microinjected into the midbrain site where procaine increases TIBAT and Trec, it decreases these temperatures, probably as a result of enhanced midbrain tonic inhibition (27). This result suggests the existence of nerve cell bodies in the midbrain that may tonically inhibit NST. Because skin temperature (Tskin) does not change during either the increase caused by procaine/tetrodotoxin or the decrease caused by Glut of TIBAT and Trec and because animals decerebrated at the posterior forebrain level are still able to increase TIBAT and Trec in response to midbrain procaine administration, putative neurons in the midbrain tonic inhibitory mechanism may directly control NST by tonically suppressing it (26, 27). This possibility is in contrast to the generally held belief that NST is reciprocally stimulated and inhibited by excitation of hypothalamic cold- and warm-sensitive neurons, respectively (4).

Because it is unlikely that these putative inhibitory neurons in the lower midbrain tonically inhibit BAT monosynaptically, it may be presumed that secondary

WHEREAS BRAIN TISSUE TRANSECTIONS at the postmamillary level produce no temperature changes of interscapular brown adipose tissue (BAT) and the rectum, those at the prepontine level increase these temperatures 3–4°C above the pretransection level within 4–5 h in anesthetized rats. The hyperthermia is accompanied by increased BAT blood flow, oxygen consumption, and levels of circulating epinephrine and norepinephrine. In addition, the hyperthermia is attenuated by an adrenergic β-blocker and completely blocked by pretreatment with 6-hydroxydopamine. Skeletal muscle paralysis by end-plate blockade has no effect on the hyperthermia (3, 21, 25). It was concluded, therefore, that removal of a tonic inhibitory mechanism on nonshivering thermogenesis (NST) increases body temperature through disinhibition-induced activation of the central sympathetic nervous system. It has been suggested that a tonic inhibitory mechanism of NST, similar to this rodent model, may also exist in the midbrain of adult cats, monkeys, and perhaps humans (7, 15, 24).

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or tertiary neurons located caudal to the lower midbrain are transsynaptically inhibited by the midbrain neurons and that they, therefore, may increase their activity during enhanced NST after the removal of the midbrain tonic inhibition. The aim of the present study was to localize these neurons that may be involved in transmitting thermal signals originating in the midbrain RRF and rs and projecting to the major NST effector in rodents, the IBAT pad. To achieve this aim, neurons were visualized immunohistochemically, using Fos expression, in male rats.

MATERIALS AND METHODS

Animals. The experiments were performed in accordance with the guidelines of the Ethics Committee for Animal Experiments, Yamanashi Institute of Environmental Sciences. Male Wistar rats (n = 67) weighing between 290 and 330 g were reared at a room temperature of 24 ± 1°C with free access to water and standard rat chow with a 12:12-h light-dark regimen. Animals were anesthetized with a bolus intraperitoneal injection of urethan (1.0–1.2 g/kg) and mounted in a rat stereotaxic apparatus (model SR-6, Narishige Sci. Instr. Lab., Tokyo) according to the rat brain atlas of Paxinos and Watson (20). Three copper-constantan thermocouples were used to monitor changes of the rectum (Trec), right or left lobe of the IBAT (TIBAT), and Tskin. Trec was determined by inserting the probe 5 cm beyond the anus, TIBAT by inserting the probe between the IBAT pad and its connecting tissues, Tskin by attaching the probe along the ventral side of the tail at the point one-third from its base. Electrical temperature signals were calibrated by a standard 0°C (±0.02°C) calibrator (Zero-con, Komatsu Electronics, Tokyo), amplified with a high-gain direct-current amplifier (AD-611G, Nihonkoden, Tokyo), and recorded on a four-channel wide-scale pen recorder (Unicorder UR-4P, Unique Medical, Tokyo). All experiments were performed under room temperature between 25 and 26°C without using a heating device for animals. Instead, they were wrapped with layers of cotton cloth to maintain their initial rectal temperatures between 36°C and 37°C.

Experimental protocols. The present study consists of eight individual immunohistochemical and physiological experiments. They were grouped into three large experiments. Experiment I (n = 30): the brain and spinal cord were examined for c-Fos-positive neurons in control and experimental animals that, respectively, received bilateral microinjections of physiological saline and procarce solution into the lower midbrain. In this experiment, the brain of the first group was sectioned parasagittally, and the second group was sectioned coronally including the spinal cord. The first group globally surveyed the lower midbrain and medulla oblongata, and the second group specifically examined certain areas of the nervous system (e.g., individual nucleus of the inferior olive (IO) and the raphe pallidus (RPa)) for c-Fos-positive neurons. Experiment II (n = 27): in the first group, the medulla oblongata (e.g., the IO) was examined for c-Fos-positive neurons after passive whole body warming. In the second and third groups, thermal response of animals to unilateral electrical stimulation (ES) of two IO nuclei and the IO nucleus and the RPa nucleus were tested, respectively. In the fourth group, thermal response of animals to microinjections of Glut solution into the IO was examined. Experiment III (n = 10): thermal response of animals to bilateral microinjections of procarce solution into the lower midbrain was examined before and after electrolytic lesioning of the IO in the first group and severing the inferior cerebellar peduncle (ICP) in the second group.

Administration of substances. Solutions of saline (0.9%) in control animals (n = 13) and procarce hydrochloride (10% or 3.7 × 10−1 M, pH adjusted to 7.4) in experimental animals (n = 17) were bilaterally microinjected (1.0 μl/site) into the lower midbrain. To remove the midbrain tonic inhibition and increase TIBAT and Trec maximally, procarce solution was microinjected bilaterally for the period of 2 min into the lower midbrain, as reported earlier (26), with coordinates of 1.7–2.7 mm anterior to ear bar, 1.5 mm lateral to the midline, and 7.5–8.0 mm deep from the cerebral cortical surface. Two stainless steel guide cannulas (0.7 mm OD, 20.0 mm long, and 3.0 mm apart from each other) with injection needles (0.4 mm OD, 20.5 mm long) attached to a 10-μl microsyringe via polyethylene tubing were used. Unilateral microinjection of Glut solution (10−5 M) into the IO (n = 5) through an injection needle (0.4 mm OD) was performed in the same way as those of procarce solution except for the injection time (1.0 μl/1.5 min for 4.5 min) and brain coordinates (3.7 mm posterior to the interaural line, 1.0 mm lateral to the midline, and 8.0–9.0 mm from the cerebellar cortical surface). Microinjection protocols were so determined because the present preliminary experiments indicated that a bolus injection of 200 nl of Glut solution into the IO was without effect. However, if it is repeated five times within 1.5 min, TIBAT increased in the range of 0.3–0.5°C.

Fos immunohistochemistry. Ninety minutes after the TIBAT had peaked after procarce microinjections into the lower midbrain of the experimental animals (n = 17), their brains were perfused with 4% paraformaldehyde and processed according to established methods for immunohistochemical detection of c-Fos protein (12). The brain of the control animal (n = 13) was similarly perfused after bilateral saline microinjections. After the animal was removed from the stereotaxic apparatus, the chest of the animal was opened and 50 ml of heparinized PBS followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer were perfused through the left ventricle of the heart. The brain tissues posterior to the injection cannula that included the lower midbrain, medulla oblongata, and spinal cord (thoracic segments) were reixed for 24 h with the same fixative at 4°C. Blocks of tissues containing the lower midbrain and medulla oblongata were frozen and sectioned (40 μm thick) using a microtome along the parasagittal axes in one group and the coronal axes in another group. Blocks of the spinal cord were sectioned coronally. Sectioned brain tissues were treated with 1.0% hydrogen peroxide for 10 min to prevent generation of endogenous peroxidase. With the use of a standard avidin-biotin peroxidase complex (ABC) method, c-Fos was stained immunohistochemically as follows. Each brain section was incubated in PBS containing an affinity-purified polyclonal antibody raised against a synthetic peptide (Fos 4–17) from the NH2-terminal end of Fos (1:3,000 dilution, Santa Cruz Biotechnology, CA) for 48 h at 4°C. The section was further incubated in biotinylated anti-rabbit IgG (1:2,000 dilution, Vector Labs, CA) and in elite ABC reagents. Fos protein was visualized by treating the brain sections with diaminobenzidine. c-Fos-expressing neurons were identified as positive only when the cell nucleus exhibited a very clear outline with increasing immunoreactivity against the background level, as reported earlier (12).

Passive whole body warming. As described above, anesthetized rats (n = 6) were mounted in a stereotaxic apparatus. Two guide cannulas with inserted injection needles were implanted into the lower midbrain in four animals. No solution was microinjected into the brain. The remaining two
animals were not cannulated. After wrapping the animals with cotton cloth, changes in $T_{\text{rect}}$, $T_{\text{IBAT}}$, and $T_{\text{skin}}$ were continuously monitored while they were passively warmed by an electric heating bulb (60 W, set 15 cm above animals). They were warmed such that their body temperature increases would mimic those induced by the midbrain procaine administrations in the present first experiment. As soon as $T_{\text{rect}}$ increased by 1.2°C above the prewarming levels, the electric heating bulb was switched off. After 90 min, the brains were perfused with 4% paraformaldehyde and processed according to the method for immunohistochemical detection of c-Fos protein described above.

ES. Unilateral ES was applied to the IO and RPa nuclei in two groups of animals ($n = 16$) using a concentric electrode for its greater accuracy of the stimulated site. The concentric electrode consists of a stainless steel outer tube (0.4 mm in diameter) and an inner wire (0.2 mm in diameter). Both materials were electrically insulated by chemical coating. The inner wire was inserted into the outer tube until the latter protruded slightly from the former. They were fixed together. The whole length of the protruded part of the inner wire was then tapered off completely with a metal file. Insulating chemical coating was removed by 0.2 mm from the tip of the outer tube. Thus the inner wire acted as a negative and the outer tube as a positive electrode.

In the first group ($n = 5$), the IO principal nucleus and the IO medial nucleus were stimulated at coordinates of 1.0 and 0.2 mm lateral to the midline, respectively, with the same anterocaudal (3.7 mm posterior from the interaural line) and depth readings (9.0 mm from the cerebellar surface). ES to both nuclei was applied for a duration of 15 min (stimulator: SEN-3391; insulator: SS-403J, Nihonkoden, Tokyo). In the second group ($n = 11$), the IO principal nucleus and the RPa nucleus were stimulated at coordinates of 1.0 and 0.0 mm lateral to the midline, respectively, with the same anterocaudal (3.7 mm posterior from the interaural line) and three depth readings (8.0, 8.5, and 9.0 mm from the cerebellar surface). Duration of ES was 15 min for both nuclei but extended to 30 min if there was a thermal response. In both groups of animals, the order of stimulating brain sites were randomized. The present study employed a stimulating frequency of 10 Hz (square pulse of 0.8 mA with a 100-ms duration) because it has been reported (7a) that the tremor-producing agent harmaline, at the dose of 40 mg/kg ip, induces a fine synchronous firing discharge of maximally 8–12 Hz from the baseline of 3–4 Hz from the inferior olivary neurons. Electric current of 0.8 mA was chosen because the present preliminary experiments showed that 0.5 mA was without effect in inducing $T_{\text{IBAT}}$ and $T_{\text{rect}}$ increases by the IO stimulation.

Brain tissue lesioning. For making electrolytic lesions of the IO ($n = 5$), a direct current (2.0 mA) was passed between two stainless steel electrodes (0.5 mm OD, insulated except for the tip 0.2 mm) for 30 s with brain coordinates of 3.0 mm posterior to the interaural line, 0.8 mm lateral to the midline, and 9.0 mm from the cerebellar surface. The ICP was bilaterally transected ($n = 5$) by a retracting microwire knife (27), inserting it horizontally from the posterior aspect of the cerebellum through small openings at coordinates 3.0 mm dorsal to the interaural line and 1.0 mm lateral to the midline. The ICP was cut from 2.5 mm posterior to and 1.0 mm anterior from the interaural line, extending the cutting edge by 2.0 mm laterally. Cutting procedures were always repeated twice on both ICP to ensure discrete and reproducible lesions.

Histological verification. Positions of microinjection cannulas, stimulating electrode, and extents of lesions and transections were histologically verified according to the rat atlas of Paxinos and Watson (20). After completion of experiments, 0.5 μl of blue dye were deposited into the brain to verify intracranial microinjection sites of saline, procaine, or Glut solutions. These and other brains were perfused with 4% formaldehyde through the lateral carotid artery. They were removed, frozen, and coronally sectioned (50-μm thick) using a microtome (models MC-802A and TU-213, Komatsu Electronics, and Yamato Koki, Tokyo). Brain sections were lightly

![Fig. 1. Temperature changes of the interscapular brown adipose tissue (IBAT), rectum, and tail skin after bilateral microinjections of saline in the control (A) and procaine solutions in the experimental animal (B)]. Whereas procaine solution microinjected into the lower midbrain (small filled circles with vertical bars in both insets) increased the IBAT and rectal temperatures, saline solution had no effect. Ninety minutes after the IBAT temperature had peaked following procaine microinjections into the lower midbrain of the experimental animal, the brain was perfused (Perfusion) with formaldehyde and processed according to the methods for immunohistochemistry of c-Fos protein. The brain of the control animal was similarly perfused after bilateral saline microinjections. Insets, top and bottom, depict coronal and parasagittal sections of the rat brain, indicating sites of both saline and procaine microinjections. CG, central gray; Hyp, hypothalamus; MB, mamillary body; Pn, pons; RRF, reticulorubral field; rs, rubrospinal tract; Th, thalamus.
stained with 1% eosin yellowish solution for 10 min, and excess dye was washed out with ethyl alcohol. All brains that exhibited histological evidence of inaccurate operational procedures were excluded from final analysis of data.

Data analysis. With the use of a light microscope, c-Fos-positive neurons in any given nucleus or area of the lower midbrain, medulla oblongata, and spinal cord (thoracic T1–T4 segments) were visually counted from four tissue sections per nucleus or area per animal. Each segment of the spinal cord was identified using the T2 vertebra, which has a long spinous process as a landmark (28). If any nucleus and/or area of the nervous system were involved in body temperature increase produced by midbrain procaine administrations, that nucleus or area should express a higher number of c-Fos-positive neurons than other nuclei or areas. On the basis of this assumption, we first selected a tissue slice with the highest number of c-Fos-positive neurons in a given nucleus or area. Second, we selected three neighboring tissue slices containing the same nucleus or area in the same animal. All c-Fos-positive neurons in that nucleus or area were counted from four tissue slices, and the mean number was calculated. After repeating the same analytical procedures on the same nucleus or area in all other animals, the final mean number of c-Fos-positive neurons from that nucleus or area was obtained for each group. In control animals, four tissue slices similar to those of the experimental animals for any given nucleus or area were selected, and the number of c-Fos-positive neurons was similarly counted and analyzed. The results from the experimental animals were compared with those from the control animals. Maximum changes of TIBAT and Trec after procaine or Glut injections or ES were calculated by subtracting baseline from maximum levels. If TIBAT and Trec increased maximally 0.2°C, such data were discarded because noxious stimuli, such as tail pinch, alone produce a similar thermal response. Duration of changes of any parameters was measured from the moment of the change to that of the return to its baseline level. All data were expressed as means ± SE and treated with two-tailed Student’s t-test. A 95% level of confidence (P < 0.05) was accepted as statistically significant in all the analyses.

RESULTS

Immunohistochemical analysis of c-Fos-positive neurons. Figure 1B illustrates typical TIBAT and Trec increases in an experimental animal after bilateral microinjections of a procaine solution into the area between the RRF and RS. It shows that TIBAT and Trec increased by 2.6 and 1.8°C above the baseline with onsets of 1.3 and 2.9 min, respectively, after midbrain procaine administrations. Tskin temperature was not affected by the stimulation. Overall, TIBAT and Trec increased 1.5 ± 0.2 (mean ± SE) and 1.2 ± 0.2°C with the onset latency of 1.0 ± 0.1 and 3.8 ± 1.0 min for the TIBAT and Trec increases, respectively (n = 8). Time from onset of temperature rise to maximum was, respectively, 23.2 ± 2.5 and 28.1 ± 4.3 min for TIBAT and Trec. Animals that received physiological saline microinjections (n = 5) showed no temperature change (Fig. 1A). As shown in Fig. 2, immunohistochemical analysis of these brains revealed that, both in control and experimental animals, c-Fos-positive neurons were evident in various areas of the lower midbrain and medulla oblongata. A total of 10 histologically distinct nuclei and/or areas was analyzed in parasagittal brain sections from the lateral to the midline regions (histogram). Of these, the IO was the only nucleus in which the experimental animals showed a significantly larger number of c-Fos-positive neurons (59 ± 14 neurons, 27.8 ± 2.05) than in control animals (14 ± 9 neurons, 1.6 ± 0.2) (Fig. 2A). The IO was located at the level of the peduncular tegmental nucleus (tz) in the lower midbrain. Those nuclei with high c-Fos-positive neurons or with a significant increase of c-Fos-positive neurons in the experimental animals were the IO, peduncular tegmental nucleus (tz), paragigantocellular nucleus; GiaA, gigantocell reticular nucleus; MdV, medullary reticular nucleus; Pmn, paramedian reticular nucleus; py, pyramidal tract; Rp, raphe pallidus nucleus; SPTg, subpeduncular tegmental nucleus; tz, trapezoid body.

Fig. 2. c-Fos-positive neurons in the lower midbrain and medulla oblongata after bilateral microinjections of saline (control, n = 5) and procaine solutions (experimental, n = 8) into the lower midbrain. Ten histologically distinct nuclei and/or areas were analyzed for c-Fos-positive neurons (histogram) using parasagittal brain sections. Because only the inferior olive (IO) showed significant difference, representative photomicrographs from the control (A) and experimental animal (B) of the IO were shown in parasagittal sections. *P < 0.05 compared with the corresponding control value. AP, area postrema; DC, dorsal cochlear nucleus; dIO, area dorsal to the inferior olive; DMTg, dorsomedial tegmental area; DPGi, dorsal paragigantocellular nucleus; GIa, gigantocell reticular nucleus; MdV, medullary reticular nucleus; Pmn, paramedian reticular nucleus; py, pyramidal tract; Rp, raphe pallidus nucleus; SPTg, subpeduncular tegmental nucleus; tz, trapezoid body.
than did the control animals (7 ± 1 neurons). Figure 2 also depicts two microphotographs of parasagittal sections of the medulla oblongata showing the IO from a control (A) and an experimental (B) animal. In general, c-Fos-positive neurons [dark dots in the microphotograph (B)] were localized in the rostral half of the IO of the experimental animals.

In the second c-Fos experiment, the IO and its neighboring nucleus (e.g., the RPa) were specifically examined because the IO of experimental animals showed a significantly larger number of c-Fos-positive neurons than that of controls. Detailed analysis of the IO in coronal sections revealed that those c-Fos expressing neurons in the brain of experimental animals (Fig. 3B) were concentrated in the medial nucleus and the ventrolateral aspect of the principal nucleus. Very few labeled neurons were observed in these regions of control animals (Fig. 3A, n = 4). The results of a quantitative analysis of c-Fos-positive neurons are shown in a histogram in Fig. 3. Significantly larger numbers of c-Fos-positive neurons (P < 0.05) were found in the IO medial (21 ± 2 neurons) and principal nuclei (8 ± 1 neurons, n = 5) of experimental animals than in those of control animals. A significantly small number of c-Fos-positive neurons were counted in the RPa of control and experimental animals. No c-Fos-positive neurons were found in any part of the cerebellum.

In the spinal cord of the experimental animals (Fig. 4), two major areas of c-Fos-positive neurons were noted (histogram): the intermediolateral cell column (IML, 3 ± 1 neurons, n = 4) and the combined areas of the layer 10 and the intermediomedial cell column (IMM, 3 ± 1 neurons) of the thoracic segments (T1-T4). A typical example of c-Fos-positive neurons in thoracic segments of experimental animals is shown in the microphotographs of Fig. 4, B and C. In the control animals, the number of c-Fos-positive neurons in the IML and the combined areas of the layer 10 and IMM was 1 ± 1 and 3 ± 1 neurons (n = 4), respectively, (histogram and microphotograph of Fig. 4A). A small number of c-Fos-positive neurons was also found in the dorsal horn (layers 1–6) of the control and experimental groups (2 ± 1 and 2 ± 1 neurons, respectively). A statistically significant difference in the number of c-Fos-positive neurons between experimental and control animals was observed only in the IML (P < 0.05).

Passive whole body warming. As shown in Fig. 5, passive warming of an anesthetized rat for 18.1 min increased TIBAT, Trec, and Tskin by 1.1, 1.4, and 2.9°C above their prewarming levels, respectively. The time necessary for TIBAT and Trec to reach their maximum was 17.1 and 19.2 min, respectively. Statistical analyses of all the data (n = 6) revealed that TIBAT, Trec, and Tskin by 1.1 ± 0.1, 1.1 ± 0.1, and 2.8 ± 0.2°C, respectively, above their prewarming levels after warming for 21.4 ± 1.4 min. The duration from onset of temperature rise to maximum was 24.4 ± 1.5 and 26.8 ± 1.6 min for TIBAT and Trec respectively. Thus passive warming of animals mimicked the way body temperature was increased by midbrain procaine administrations in the present first experiment. Immunohistochemical analysis of the brains, however, indicated that no animal, with or without cannulas in the lower midbrain, showed c-Fos-positive neurons in any nucleus of the IO as shown in a photomicrograph of Fig. 5.

Electrical and chemical stimulation of the IO and RPa. In the first group, the IO medial nucleus and the IO principal nucleus were stimulated electrically because these nuclei exhibited large numbers of c-Fos-positive neurons after TIBAT and Trec increase by the

![Figure 3](image-url)
midbrain procaine administration. As shown in Fig. 6, whereas ES of the IO medial nucleus did not change $T_{\text{IBAT}}$ and $T_{\text{rec}}$, that of the ventrolateral aspect of the IO principal nucleus for 15 min increased these temperatures by 0.7 and 0.3°C above the baseline level, respectively. Such thermal response to ES of the IO principal nucleus was reproducible. During the IO-principal nucleus stimulation, $T_{\text{IBAT}}$ and $T_{\text{rec}}$ increased on average 0.7 $\pm$ 0.1 and 0.4 $\pm$ 0.1°C ($n = 5$) above the baseline with significantly shorter time onsets for $T_{\text{IBAT}}$ (1.0 $\pm$ 0.1 min) than for $T_{\text{rec}}$ (3.5 $\pm$ 0.5 min), respectively. $T_{\text{skin}}$ did not significantly change during

Fig. 4. c-Fos-positive neurons in the spinal cord after bilateral microinjections of saline (control, $n = 4$) and procaine solutions (experimental, $n = 4$) into the lower midbrain. Results of quantitative analysis of c-Fos-positive neurons in the thoracic segments (T1–T4) were indicated in histogram. Photomicrographs A and B represent the right half of the thoracic segment of the control and experimental animal, respectively, and C is an enlarged intermediolateral (IML) nucleus from the same photomicrograph B. Arrows in C indicate c-Fos-expressing neurons in the IML. 10/IMM, spinal cord layer 10 and IML nucleus; CC, central canal.

Fig. 5. Temperature changes of the IBAT, rectum, and tail skin after passive whole body warming (between 2 arrows), and a photomicrograph presenting the right half of the IO. The brain was perfused (Perfusion) 90 min after body temperature had peaked for immunohistochemical staining of c-Fos protein. Note that no c-Fos-positive neurons were detected in any part of the IO.
the stimulation. Summary of ES sites of the IO nuclei is shown in Fig. 7. It shows that ES of the ventrolateral area of the IO principal nucleus and, to a lesser extent, the IO dorsal nucleus produced an increase in all five animals, whereas stimulation of the IO medial nucleus (in 4 animals) and the RPa (in 1 animal) did not alter these temperatures.

In the second group of animals, the RPa nucleus, the IO principal nucleus, and areas dorsal to these nuclei were electrically stimulated, and changes of the TIBAT, Trec, and Tskin were monitored. Figure 8 shows that ES of two sites dorsal to the RPa as well as the RPa itself did not change, whereas ES of two sites dorsal to the IO principal nucleus did not change, and ES applied to the IO principal nucleus increased TIBAT and Trec by 1.3 and 1.0°C, respectively, during a 30-min stimulation. Tskin tended to increase as body temperature was raised. Ten of eleven animals tested exhibited the same results as those shown in Fig. 8. With regard to ES of the IO principal nucleus, it increased TIBAT and Trec above the baseline by 1.1 ± 0.1 and 0.8 ± 0.1°C, with onset of 1.7 ± 0.2 and 2.5 ± 0.5 min (n = 11), respectively. When levels of these elevated TIBAT and Trec were read after 15 min (0.5 ± 0.1 and 0.4 ± 0.1°C, respectively), they showed similar values with those of the first group in which the IO principal nucleus was stimulated only for 15 min. Summary of stimulated sites in this group is shown in Fig. 9. It shows that whereas TIBAT and Trec increases were recorded from all 11 ventrolateral IO areas, no temperature increase was obtained from the RPa nucleus and medial areas of the IO. No TIBAT and Trec rise was observed from any area dorsal to these stimulated sites.

An excitatory amino acid, Glut, was microinjected unilaterally into the ventrolateral area of the IO principal nucleus to stimulate only nerve cell bodies in the vicinity of the ventrolateral IO principal nucleus. An example of the results is shown in Fig. 10; Glut admin-

![Fig. 6. Temperature changes of the IBAT, rectum, and tail skin during electrical stimulation (ES) of the IO nuclei. The IO was unilaterally stimulated at the medial nucleus (a or ES-a) and the ventrolateral aspect of the principal nucleus (b or ES-b) using a concentric electrode. Insets, left and right, depict coronal and parasagittal sections of the rat brain, respectively, indicating sites of IO ES. 4V, 4th ventricle; Hyp, hypothalamus; MB, mamillary body.](image)

![Fig. 7. Sites of ES of the IO nuclei. Filled circles in the left hemisphere indicate sites with increased IBAT and rectal temperatures, and open circles indicate sites without temperature change (n = 5). Each drawing depicting coronal section of the rat brain was taken from the atlas of Paxinos and Watson (20). Numbers under each section indicate the distance of that section from the interaural zero line (IAL). Amb, ambigus nucleus; IO dr, IO dorsal nucleus; IO md, IO medial nucleus; LPGi, lateral paragigantocellular nucleus; PMn, paramedian reticular nucleus; ROb, raphe obscurus nucleus; RVL, rostroventrolateral reticular nucleus; Sol, nucleus of the solitary tract.](image)

![Fig. 8. Temperature changes of the IBAT, rectum, and tail skin during ES of the IO pr and the RPa. Time of stimulation (between pairs of arrows) and site of the stimulation (small open circles) were indicated above temperature traces and in insets, respectively. Note that IBAT and rectal temperatures increased only when ventrolateral aspect of the IO pr (ES: b3), but not the RPa nucleus (ES: a3), was stimulated.](image)
istration increased $T_{\text{IBAT}}$ and $T_{\text{rec}}$ by 1.1 and 1.0°C, respectively. Glut significantly increased $T_{\text{IBAT}}$ and $T_{\text{rec}}$ by 0.7 ± 0.1 and 0.5 ± 0.1°C above the baseline ($n = 5$) with onset latencies of 3.7 ± 0.4 and 5.7 ± 0.6 min, respectively. $T_{\text{skin}}$ did not change after Glut administration. Averaged time from the onset of $T_{\text{IBAT}}$ and $T_{\text{rec}}$ increase to its maximum was 67.7 ± 17.9 and 64.8 ± 17.7 min, respectively. In this group, return of the increased body temperature to pre glutamate injection levels was not always observed because of exceptionally prolonged thermal response. All five animals showed injection sites in and around the ventrolateral area of the IO principal nucleus, similar to those shown in Fig. 7. We estimated, in preliminary experiments, that microinjected glutamate solution under the present experimental conditions may spread up to 0.5 mm in all the directions from the injection needle at the time of onset (mean 3.7 min) of $T_{\text{IBAT}}$ rise. It was, therefore, presumed that injecting a total volume of 3.0 μl within a 4.5-min period, the solution may have further spread and affected the RPa medially and the lateral paragigantocellular nucleus (LPGi) laterally. In the present study, these nuclei, however, did not exhibit significantly different numbers of c-Fos-positive neurons between control and experimental animals (data for LPGi are not shown).

Lesioning of the IO and severing the ICP. Figure 11 shows that electrolytically produced IO lesions completely blocked the $T_{\text{IBAT}}$ and $T_{\text{rec}}$ increases (1.1 and 0.7°C, respectively) caused by bilateral midbrain procaine microinjections. $T_{\text{skin}}$ abruptly increased by 4.4°C above the prelesioning level immediately after placing lesions in the IO. In five animals tested, all showed a complete blockade of midbrain procaine-induced $T_{\text{IBAT}}$ and $T_{\text{rec}}$ rise. In this group, $T_{\text{IBAT}}$ and $T_{\text{rec}}$ increases by the midbrain procaine administration before IO lesioning were 1.3 ± 0.3 and 1.0 ± 0.2°C ($n = 5$), respectively. In some animals ($n = 2$), a steep drop of body temperature was observed after IO lesioning due to increased heat dissipation through skin ($T_{\text{skin}}$ increase varied between 2.0 and 4.4°C). Histological examination of these brains revealed that the lesions extended to the area of the rostral ventrolateral medulla (RVLM). The remaining three brains showed lesions restricted around both IOs. Because ES of the RVLM produces a sustained decrease in $T_{\text{skin}}$ (11), lesioning this nucleus may have released a tonically controlled vasomotor tone of the skin resulting in an increased tail $T_{\text{skin}}$.

Fig. 9. Sites of ES in and around the RPa and the IO ($n = 11$). Small filled circles in the left hemisphere indicate stimulated sites where IBAT and rectal temperatures increased, and open circles indicate sites without temperature change. Coronal sections of the rat brain were taken from the brain atlas by Paxinos and Watson (20). Numbers under each section indicate the distance of that section from the IAL.

Fig. 10. Changes of the IBAT, rectum, and tail skin temperatures after microinjections of L-glutamate (Glut) into the IO. Insets, left and right, depict coronal and parasagittal sections of the rat brain, respectively, indicating the site of unilateral Glut microinjections (small filled circle with vertical bar) into the vicinity of the IO.

Fig. 11. Temperature changes of the IBAT, rectum, and tail skin induced by procaine microinjections into the lower midbrain before and after electrolytic lesioning of the IO. Note that IO lesions (dotted areas in both insets) blocked IBAT and rectal temperature increases by midbrain procaine administration. Note also that placing lesions in the IO abruptly increased tail skin temperature 4.4°C above the baseline.
In the last experiment \((n = 5)\), the ICP, one of three well-established major IO projections to and from the cerebellum that are traversed by nerve fibers of the olivocerebellar projection, was transected bilaterally with a retracting microwire knife. Figure 12 indicates that \(T_{\text{IBAT}}\) and \(T_{\text{rec}}\) increases by midbrain procaine administration before and after the pedunculotomy were, respectively, 0.8 and 0.3°C and 1.2 and 0.6°C. There was no significant difference in the \(T_{\text{IBAT}}\) and \(T_{\text{rec}}\) increases caused by midbrain procaine anesthesia before \((1.3 \pm 0.1\) and 0.9 \(\pm 0.2°C\)) and after \((1.3 \pm 0.1\) and 0.9 \(\pm 0.1°C\), respectively) the pedunculotomy. \(T_{\text{skin}}\) did not show significant change during the period of experiments. Gross anatomical examinations of pedunculotomized brains showed complete severing of the ICP in all the animals. However, heavy intracranial hemorrhage was often present in these brains.

**DISCUSSION**

Various attempts have been made in the past to elucidate the central neuronal mechanisms of thermoregulatory effector activation; progress, however, has been limited \((6, 10)\). The present findings suggest, for the first time, that thermal signals produced by the lower midbrain neurons are transmitted through the IO and the IML cell column of the spinal thoracic segments to one of the major NST effectors BAT.

The results suggested that these IO neurons may have expressed c-Fos as a result of postsynaptic activation due to the removal of midbrain tonic inhibition, as postulated in the introduction of this paper. On the other hand, it was also possible that these IO neurons expressed c-Fos as a result of the direct effect of increased brain temperature. If so, passively warming animals should lower IO neuronal activity resulting in a reduced number of c-Fos-positive neurons in this structure. The present results, however, indicated that the increased brain temperature was not the cause for the augmented expression of c-Fos reactivity.

The present results indicated that ES of the ventrolateral aspect of the IO principal nucleus increased \(T_{\text{IBAT}}\) and \(T_{\text{rec}}\) with shorter time onsets for \(T_{\text{IBAT}}\) than for \(T_{\text{rec}}\). ES of the IO medial nucleus, on the other hand, produced no body temperature change. Thus the results of the IO ES suggested that, although the medial nucleus showed a far larger number of c-Fos-positive neurons than the principal nucleus, the latter appears to be primarily responsible for the increased body temperature after midbrain procaine anesthesia. Taking the other present results into consideration that Glut, when microinjected into the ventrolateral vicinity of the IO, also increased \(T_{\text{IBAT}}\) and \(T_{\text{rec}}\), these IO stimulation results together support the possibility that the excitation of the nerve cell bodies was the cause of the increased body temperature. Because \(T_{\text{skin}}\) did not change, the results from both electrical and chemical stimulation thus suggest that 1) the expression of c-Fos by IO neurons is the cause and not the result of the increased brain temperature; 2) BAT is indeed the major heat-producing effector thus activated, because the \(T_{\text{IBAT}}\) increased more and sooner than the \(T_{\text{rec}}\). 3) these results lend support to our proposed notion that midbrain neurons in and around the RRF and RS transsynaptically suppress the activity of the IO (presumed above to be the secondary neuron) to tonically inhibit IBAT NST. In addition, considering the result that IO lesions completely blocked body temperature increase by midbrain procaine administration, it could be summarized that this result further supports our view that the IO forms an essential part of the central efferent pathway to thermogenic effectors, IBAT in particular.

According to recent reports by Morrison and colleagues \((18, 19)\), increased activity of intercostal sympathetic nerves (ISN) innervating the IBAT pad is observed after microinjections of bicuculline, a GABA\(_A\)-receptor antagonist, into the rostral area of the RPa of anesthetized rats. They concluded that disinhibition of neurons in the rostral RPa (by bicuculline) increases the ISN activity and that GABAergic neurons in the rostral RPa play a major role in the control of IBAT thermogenesis. It is, however, unlikely that the \(T_{\text{IBAT}}\) and \(T_{\text{rec}}\) increases induced by IO ES observed in the present experiments were caused by excited RPa neurons due to current spread for the following reasons. 1) Because, in the present experiments, ES was directly applied to the caudal area of the rostral RPa, the \(T_{\text{IBAT}}\) should have increased or decreased depending on circuits the rostral RPa neurons might form caudally. The present experiments, however, showed no \(T_{\text{IBAT}}\) change. Such no-temperature-change results were not due to the effect of anesthesia, because we have shown that, even under urethan anesthesia, rats are able to maintain metabolic heat production, albeit at slightly lowered levels, and, therefore, retain the ability to decrease \(T_{\text{IBAT}}\) below its baseline level when the mid-

![Fig. 12. Temperature changes of the IBAT, rectum, and tail skin induced by bilateral procaine microinjections into the lower midbrain before and after bilateral transections (Transec) of the inferior cerebellar peduncle (icp). Sites of the icp transections were indicated by darkened horizontal areas in both insets of coronal and parasagittal brain sections.](http://ajpregu.physiology.org/)

10.220.32.246 on October 22, 2017 http://ajpregu.physiology.org/ Downloaded from
brain tonic inhibitory mechanism is excited by Glut administration or ES (9, 27). 2) The above mentioned no-temperature-change results are supported by the other present finding that there was no significant difference in the number of c-Fos-positive RPa neurons between experimental and control animals, suggesting that the RPa is not involved in the central efferent pathway of thermal signals. Because Morrison and colleagues (18, 19) did not monitor levels of \( T_{\text{IBAT}} \) in their reports, it is not clear whether the reported ISN activity increase after RPa bicuculline administration is indeed involved in IBAT thermogenesis. But, from the present findings and animal model, it may be concluded that the RPa is not involved in IBAT thermogenesis. It is possible that ISN activity changes do not always induce altered levels of IBAT thermogenesis. For example, it was recently reported that pregnant rats at near term do not develop fever in response to intracerebroventricular prostaglandin \( E_2 \) administrations even though ISN activity is significantly increased (5).

The presence of c-Fos-positive neurons in the throracic IML of the experimental animals was expected, because this nucleus has been shown to contain sympathetic preganglionic neurons (28) and because postganglionic neurons at the cervical and thoracic levels exhibit retrogradely transported horseradish peroxidase injected directly into the IBAT pad (8). In addition, a recent report demonstrated that a viral trans-synaptic retrograde tract tracer, the pseudorabies virus, was injected into epidymidal or inguinal white adipose tissue of Siberian hamsters and rats was found in various regions of the nervous system including the IML of the spinal cord (1). The authors concluded that sympathetic nerve control of white adipose tissue originates from a higher structure of the central nervous system (CNS) and may play a significant role in lipid mobilization. The significance of the present findings on the IML would be that this nucleus was identified anterogradely in association with enhanced IBAT heat production.

As mentioned earlier, the cerebellum exhibited no c-Fos-positive neurons. This result, however, does not automatically exclude the cerebellum from participation in midbrain procaine-induced increases of body temperature for the following three reasons: 1) c-Fos may not be expressed in all the excited neurons of the CNS (17), 2) c-Fos may not be expressed at all in the cerebellum if the suppression of cerebellar neurons is necessary to enhance metabolic heat production by midbrain procaine administrations, and 3) the existence of powerful topographical projections from the IO to the various vermis of the cerebellar cortex (climbing fibers to Purkinje cells) and cerebellar nuclei have been well established (2, 13, 22). To address the question, therefore, whether the cerebellum plays a role in this regulation, the ICP, one of three well-established major IO projections to and from the cerebellum (22, 23, 29, 30) that are traversed by nerve fibers of the olive-cerebellar projection, was transected bilaterally with a retracting microwire knife. The results indicated that there was no significant difference in the \( T_{\text{IBAT}} \) and \( T_{\text{sec}} \) increases caused by midbrain procaine anesthesia before and after the peduncolotomy, suggesting that the cerebellum is not functionally involved in the body temperature increase induced by midbrain procaine microinjections.

The duration of the body temperature increase caused by a bolus Glut injection into the vicinity of the IO principal nucleus lasts longer than would normally be expected. The reason for this is unknown; it might be induced by activation of reverberating circuits between the IO and the cerebellum and/or red nucleus (22).

In conclusion, the present results indicate that neurons in and around the midbrain RRF and rs may transsynaptically activate neurons in the IO and the thoracic IML cell column and thereby tonically inhibit metabolic heat production by the IBAT. These findings thus demonstrate how thermal signals produced in the CNS may modulate peripheral thermoregulatory effectors such as IBAT. The present findings also suggest that the IO nucleus may have a role in the sympathetic nervous system control of NST and that this nucleus may also be involved in functional interactions between the motor and thermoregulatory systems (e.g., the body temperature increase associated with convulsions or shivering). This is an intriguing possibility because studies on the IO have mostly been limited to the field of predictive motor control and motor coordination (7b). Because recent studies have demonstrated the existence of mitochondrial uncoupling proteins not only in BAT but also in skeletal muscle (14, 31), some c-Fos-positive neurons found in the IO may also have a role in NST involving effectors other than BAT, e.g., skeletal muscle. Finally, the present findings together with other reports (6, 24–27) directly support the hypothesis that the CNS controls NST by tonically suppressing it rather than by reciprocally stimulating and inhibiting it. Currently, the latter view is prevalent.

REFERENCES


