FOS ACTIVATION IN HYPOTHALAMIC NEURONS DURING COLD OR WARM EXPOSURE: PROJECTIONS TO PERIAQUEDUCTAL GRAY MATTER

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Abstract—The hypothalamus, especially the preoptic area, plays a crucial role in thermoregulation, and our previous studies showed that the periaqueductal gray matter is important for transmitting efferent signals to thermoregulatory effectors in rats. Neurons responsible for skin vasodilation are located in the lateral portion of the rostral periaqueductal gray matter, and neurons that mediate non-shivering thermogenesis are located in the ventrolateral part of the caudal periaqueductal gray matter. We investigated the distribution of neurons in the rat hypothalamus that are activated by exposure to neutral (26 °C), warm (33 °C), or cold (10 °C) ambient temperature and project to the rostral periaqueductal gray matter or caudal periaqueductal gray matter, by using the immunohistochemical analysis of Fos and a retrograde tracer, cholera toxin-b. When cholera toxin-b was injected into the rostral periaqueductal gray matter, many doublelabeled cells were observed in the median preoptic nucleus in warm-exposed rats, but few were seen in cold-exposed rats. On the other hand, when cholera toxin-b was injected into the caudal periaqueductal gray matter, many double-labeled cells were seen in a cell group extending from the dorsomedial nucleus through the dorsal hypothalamic area in coldexposed rats but few were seen in warm-exposed rats. These results suggest that the rostral periaqueductal gray matter receives input from the median preoptic nucleus neurons activated by warm exposure, and the caudal periaqueductal

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Abbreviations: ABC, avidin-biotin complex; AHA, anterior hypothalamic area; BAT, brown adipose tissue; BST, bed nucleus of the stria terminalis; cPAG, caudal periaqueductal gray matter; CTb, cholera toxin-b; CTb-IR, cholera toxin-b immunoreactivity; DAB, 3,3'-diaminobenzidine; DHA, dorsal hypothalamic area; DMH, dorsomedial hypothalamic nucleus; Fos-IR, Fos immunoreactivity; LH, lateral hypothalamic area; LPO, lateral preoptic area; MnPO, median preoptic nucleus; MPA, medial preoptic area; MPO, medial preoptic nucleus; PAG, periaqueductal gray matter; PeF, perifornical area; PH, posterior hypothalamic area; PMD, dorsal premammillary nucleus; POA, preoptic area; PS, parastrial nucleus; PVH, paraventricular hypothalamic nucleus; rPAG, rostral periaqueductal gray matter; Ta, ambient temperature; VLH, ventral part of the lateral hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; Zi, zona incerta. gray matter receives input from neurons in the dorsomedial nucleus/dorsal hypothalamic area region activated by cold exposure. These efferent pathways provide a substrate for thermoregulatory skin vasomotor response and non-shivering thermogenesis, respectively. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: Fos, cholera toxin-b, thermoregulation, preoptic area, periaqueductal gray matter, median preoptic nucleus.

Regulation of body temperature in homeothermic animals engages behavioral, endocrine, and autonomic processes that are controlled by the CNS. The preoptic area (POA) occupies a crucial position in the neuronal circuit for thermoregulation, detecting local brain temperature as well as receiving thermal information from the body core and the skin (Boulant, 1980; Nakayama, 1985; Kanosue et al., 1998; Nagashima et al., 2000; Kanosue et al., 2001). Other areas in the hypothalamus, such as the paraventricular (PVH), dorsomedial (DMH), and ventromedial (VMH) nuclei, and the posterior (PH) and lateral (LH) hypothalamic areas also participate in thermoregulation (Thornhill and Halvorson, 1994; Kanosue et al., 1998, 2001; Lu et al., 2001; Cano et al., 2003). Although we know the POA sends efferent signals to various thermoregulatory effectors, hypothalamic efferent pathways that mediate thermoregulation are not well understood.

Recent reports indicate that the periaqueductal gray matter (PAG) also plays important roles in thermoregulation. Chemical stimulation of the ventrolateral portion of the rostral periaqueductal gray matter (rPAG) produces tail skin vasodilation, which is the major nonevaporative heat loss mechanism in rats (Zhang et al., 1997a,b). This vasodilatory area extends rostrally to the caudal edge of the hypothalamus. On the other hand, stimulation of the ventrolateral part of the caudal periaqueductal gray matter (cPAG) elicits thermogenesis by interscapular brown adipose tissue (BAT), a major organ of heat production in the rat (Chen et al., 2002). These two regions, the rPAG and the cPAG, are separated by a zone where stimulation produces neither vasomotor nor BAT response. Although signals produced by local preoptic warming reach the PAG (Yoshida et al., 2002), the specific thermoregulatory pathways to the rPAG and cPAG are not known.

The purpose of the present study was to elucidate the afferent connection of the rPAG and cPAG from the hypothalamus that are engaged during thermoregulation. We first injected a retrograde tracer, cholera toxin-b (CTb), into the rPAG or cPAG and exposed rats to warm (33 °C), cold (10 °C), or neutral environments (26 °C). We then used

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dual immunohistochemical analysis to identify hypothalamic neurons that were CTb-labeled and expressed Fos protein, a marker for recent neuronal activation, to identify neurons that receive warm or cold signals and project to the PAG.

EXPERIMENTAL PROCEDURES

Animals and surgery

A total of 18 male crj-Wistar rats (300–360 g; Charles River Japan, Osaka, Japan) were used in this study. Rats were individually housed in a cage with bedding at an ambient temperature (Ta) of 26 °C in a 12-h light/dark photoperiod with free access to food and water. The experiment was done according to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences of the Physiological Society of Japan and approved by the Institutional Animal Care and Use Committee, School of Allied Health Sciences, Faculty of Medicine, Osaka University. Throughout the experiment we tried to minimize the number of animals used and their suffering.

Each rat was anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg). Then CTb (0.1% solution in saline; Sigma-Aldrich Co., St. Louis, MO, USA) was injected stereotaxically from a glass micropipette into the right rPAG (0.4 mm from midline, 5.2 mm posterior to bregma and 6.4 mm below the skull surface), or the right cPAG (0.8 mm from midline, 8.0 mm posterior to bregma and 6 mm below the skull surface) (Paxinos and Watson, 1998). CTb was injected iontophoretically by applying discontinuous positive pulses of DC current (10 μ A, 7-s on and 7-s off) from a precision current source (Dye injector, DIA Medical System Co., Tokyo, Japan) for 10 min. After the injection the glass micropipette was left in place for 10 min.

Experimental protocol

After CTb injection, rats were returned to the cages and housed in a climate chamber (Espec, Tokyo, Japan) for 7 days. The Ta in the climate chamber was 26 °C with a 12-h light/dark photoperiod with free access to food and water. On the experiment day, temperature of the climatic chamber was changed to 33 °C (warm) or to 10 °C (cold), or unchanged at 26 °C (neutral) for 2.5 h from 10:30–13:00 h. It took 10 and 20 min for the temperature to be stabilized at 33 °C and 10 °C, respectively.

Rats were divided into six groups; neutral temperature-caudal injection group (n=3), cold exposed-caudal injection group (n=3), warm exposed-caudal injection group (n=3), neutral temperature-rostral injection group (n=3), cold exposed-rostral injection group (n=3) and warm exposed-rostral injection group (n=3).

Histology and immunohistochemistry

After the exposure to warm, cold, or neutral Ta, rats were deeply anesthetized with sodium pentobarbital (125 mg/kg, i.p.) and immediately perfused transcardially with 30 ml of heparinized (10 U/ml, Novo Nordisk A/S, Denmark) PBS followed by 500 ml of 10% neutral buffered formalin (Sigma-Aldrich Co.). The brains were removed and submerged in 20% sucrose in 0.1 M PBS with 0.02% sodium azide (Sigma-Aldrich Co.) overnight. Brains were cut on a freezing microtome into 40 μ m sections that were stored at 4 °C in PBS with 0.01% sodium azide.

Brain sections were first stained immunohistochemically for Fos protein. Briefly, tissue was incubated in rabbit anti-Fos antiserum (Ab-5, 1:150,000, Oncogene Research Products, Cambridge, MA, USA) overnight at room temperature. A biotinylated donkey anti-rabbit secondary antiserum (Jackson Immuno-Research, West Grove, PA, USA) was used at a dilution of 1:1000. Tissue was then reacted with avidin–biotin complex (ABC; Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA, USA) for 1 h, and 1% 3,3'-diaminobenzidine (DAB; Sigma-Aldrich Co.), 0.01% H_2O_2 , 0.01% nickel ammonium sulfate and 0.01% CoCl₂ (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

After Fos staining, sections were rinsed in PBS and exposed to CTb primary antiserum raised in goat (1:100,000; List Biological Laboratories, Campbell, CA, USA) overnight at room temperature. Then, they were incubated in biotinylated donkey anti-goat antisera (1:1000; Jackson ImmunoReseach) for 1 h, followed by ABC and DAB, as above, except with the exclusion of the nickel ammonium sulfate and CoCl₂ in the DAB step to yield a brown cytoplasmic product.

The tissue sections were mounted onto gelatin-coated slides, dehydrated in alcohol, cleared (Lemosol, Wako Pure Chemical Industries, Ltd., Osaka, Japan), coverslipped (Permount, Fisher, Fair Lawn, NJ, USA), and analyzed with a microscope (Eclipse E600, Nikon, Tokyo, Japan). Photomicrographs were produced by capturing images with a digital camera (HC-2500, Fuji, Tokyo, Japan) mounted on the microscope. Image editing software (Adobe Photoshop, San Jose, CA, USA) was used to combine photomicrographs into plates.

Cell counts and statistical analysis

Cholera toxin-b immunoreactivity (CTb-IR) was observed exclusively in the cytoplasmic compartment whereas Fos immunoreactivity (Fos-IR) was found in the nucleus. Only cells that had a clearly distinguishable black nucleus surrounded by brown granular cytoplasmic staining were considered double-labeled. Retrogradely labeled cells with an unstained nucleus in the plane of section were recorded as singly labeled neurons.

The double-labeled neurons for Fos and CTb were analyzed qualitatively (Fig. 3) and quantitatively (Fig. 4). Within two brain areas the degree of double labeling was pronounced, namely (1) within the median preoptic nucleus (MnPO), and (2) from the dorsal part of the DMH and into the adjacent dorsal hypothalamic area (DMH/DHA region) (for details see Fig. 3). Therefore, these two brain structures were chosen for quantitative and statistical analysis. Counts were made on the computer from digital images of respective brain structures and also using the microscope by a blinded examiner. In detail, digital images of the brain sections were taken with the 20-fold objective for DMH/DHA region and 40-fold objective for MnPO. Three adjacent images surrounding the third ventricle were taken from one section to cover the main part of the MnPO (Fig. 3A). Cell counting in the DMH/DHA region was done on the side ipsilateral to the CTb injection. For the statistical analysis shown in Fig. 4, cell counts from three consecutive sections were averaged both for the MnPO and for the DMH/DHA region for each animal.

For statistical analysis, a two-way ANOVA followed by the post hoc Tukey test was used to compare cell counts between groups. Statistical significance was accepted for P<0.05.

RESULTS

Distribution of CTb-labeled cells

CTb injections were made into the rPAG in nine rats (Figs. 1A and 2A). CTb-IR in these animals was seen in the MnPO, medial preoptic nucleus (MPO), ventral part of the lateral hypothalamic nucleus (VLH), anterior hypothalamic area (AHA), DMH/DHA, perifornical area (PeF), zona incerta (Zi), LH, VMH, and the PH.

In another nine rats, CTb injections were made into the cPAG (Figs. 1B and 2B). In these cases CTb-IR was observed in the MnPO, lateral preoptic area (LPO), VLH,

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