Brain regions expressing Fos during thermoregulatory behavior in rats

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Maruyama, Megumi, Maiko Nishi, Masahiro Konishi, Yuko Takashige, Kei Nagashima, Toshikazu Kiyohara, and Kazuyuki Kanosue. Brain regions expressing Fos during thermoregulatory behavior in rats. Am J Physiol Regul Integr Comp Physiol 285: R1116–R1123, 2003. First published July 31, 2003; 10.1152/ajpregu.00166.2002.—We surveyed the neural substrata for behavioral thermoregulation with immunohistochemical analysis of the expression of Fos protein in the rat brain. We used an operant system in which a rat exposed to heat (40°C) could get cold air (0°C) for 30 s when it moved into the reward area. Rats moved in and out of the reward area of the system periodically and thus maintained their body temperature at a normal level. In the rats performing heat escape behavior (active group), strong Fos immunoreactivity (Fos-IR) was found in the median preoptic nucleus (MnPO), parastrial nucleus (PS), and dorsomedial hypothalamus (DMH) compared with the controls. Another group of rats (passive group) were given the same temperature changes, regardless of the rat’s movement, as those obtained by rats of the active group. Fos-IR in the MnPO was also seen in this group. The present results suggest that the PS and DMH play an important role in the genesis of thermoregulatory behavior, whereas the MnPO may be important for detecting changes in ambient and/or body temperatures.

HOMEOThERMIC ANIMALS regulate their body temperature by using behavioral as well as autonomic processes. The autonomic responses consist of shivering, nonshivering thermogenesis, skin vasomotion, and evaporative heat loss (sweating in humans) (15). Behavioral thermoregulation is the behavior of finding or establishing an appropriate thermal environment and in humans includes wearing clothes and building houses. Autonomic responses cannot be sustained indefinitely, especially in a severely hot or cold environment, and in the long term temperature regulation depends mainly on behavior (1). Both autonomic and behavioral processes are controlled primarily by the central nervous system. The brain mechanism for the autonomic processes has been well documented (15, 21). Temperature signals from receptors in the skin and body core project to the central nervous system, which in turn sends efferent signals to activate the appropriate thermoeffector responses (12). The preoptic area of the hypothalamus plays a key role in detecting local temperature as well as integrating temperature signals from all over the body. The anatomic loci in the brain that are involved in behavioral thermoregulation, on the other hand, are not so well understood. Interestingly, destruction of the preoptic area does not impair behavioral thermoregulation (28), although the preoptic area does function as a thermosensitive site since warming or cooling the preoptic area appropriately alters behavioral thermoregulation (4, 24).

Behavioral thermoregulation has been studied in the laboratory with two kinds of devices: temperature gradients and an operant system. When an animal is placed in a temperature gradient, the temperature of the place where the animal spends the most time is considered the “preferred temperature” (9, 18). In an operant system, an animal in a cold or hot environment gets warm or cool reinforcements when it performs a required act (2, 3, 11, 27, 35). To elucidate the neuronal mechanism underlying behavioral thermoregulation, it is necessary to analyze neuronal activities in animals when they regulate their body temperature behaviorally. Although animals readily learn to regulate their body temperature in temperature gradients, such devices are less well suited for analyzing neuronal responses, because the animal may stay at its preferred temperature for a long period of time without activating the neuronal circuitry involved in behavioral thermoregulation. With most operant systems, on the other hand, animals have to be extensively trained, because responses such as lever pressing are somewhat unnatural. Chen et al. (5) recently developed a new operant system for studying behavioral thermoregulation. In this system, a rat in a box at a high temperature can get cold reinforcement by moving into a specific area in the box. Because this system depends on the rat’s natural behavior in seeking a comfortable environment, the necessary response is readily acquired.

The purpose of the present study was to elucidate the neural substrates for behavioral thermoregulation. To this end, we assessed neuronal activation by immuno-
histochemical analysis of the expression of Fos protein (20, 26, 30) in the brains of rats that had performed heat escape behavior in the new operant system. Several studies have investigated the pattern of Fos immunoreactivity during thermal stimulation (16, 19). However, no study has been made with regard to behavioral thermoregulation.

METHODS

Twenty-one male crj-Wistar rats (300–400 g, Charles River Japan, Osaka, Japan) were used in this study. The rats were housed at 22°C on a 12:12-h light-dark schedule (on 0700–1900) with free access to food and water. The experimental procedures were approved by the Institutional Animal Care and Use Committee, School of Allied Health Sciences, Faculty of Medicine, Osaka University. Under pentobarbital sodium anesthesia (50 mg/kg, ip), a biotelemetry device (Physiotel; DataScience, St. Paul, MN) was implanted in the peritoneal cavity of each rat for the measurement of body temperature. Experiments were conducted at least 1 wk after the surgery.

The system for testing behavioral thermoregulation consists of a chamber (60 × 50 × 50 cm high) with an inlet and an outlet that are connected to two air supply units (CAU-210; TABAI ESPEC, Osaka, Japan) (5). One unit supplies cold air (0–30°C, 3 m³/min) and the other hot air (20–45°C, 3 m³/min). Circulation in the chamber is switched between the two air supply units by computer-controlled valves (Macintosh 7200/80 with an interface Mac ADIOS II; GW Instruments, Somerville, MA; Fig. 1). The temperature of the air from the supply unit that was used to produce thermal stress was termed the “load temperature.” Air from the other supply unit reduced the thermal stress, and its temperature was termed the “reward temperature.”

To begin an experiment, the rat was placed in a plastic box (50 × 10 × 30 cm high) within the chamber. The top of the box was covered with metallic mesh, and the side of the box was perforated to facilitate the airflow. The location of the rat in the box was monitored by light-emitting diodes placed 2 cm above the bottom of the box at 10-cm intervals along the longer side of the box and directed toward photoelectric cells on the opposite side. The rat’s position could thus be located within one of five 10 × 10-cm² areas. Air at load temperature was blown into the chamber. When the animal entered a predetermined reward zone, the air was replaced by a reward temperature for 30 s. To receive a subsequent reinforcement, the animal had to leave the reward zone and reenter it. Two telemetry receivers (CTR86; DataScience) for detecting body temperature, body temperature, and the position of the animal were recorded at 1-s intervals. The behavior of the rat was monitored with a video camera throughout the experiment.

Six pairs of randomly selected rats (n = 12) formed the experimental group. One of each pair performed in an “active” experiment and the other in a “passive” experiment. In the active experiment, the load temperature was set at 40°C and reward temperature at 0°C. In the passive experiment, movement of the rat had no consequence, and switching of warm and cold air was made with the same sequence as that of the active experiment on the counterpart of the pair. In the active experiment, the rats’ behavior determined when they were rewarded with cold air. On the other hand, in the passive experiment, the rats’ behavior did not alter the environmental temperature. As utilized in this experiment, the term “passive” makes no reference to the actual movement or behavior of the rat. Thus the rats of each pair were exposed to identical temperature changes, even though one obtained it “actively” and the other “passively.” A similar active experiment was performed on three additional rats; however, these animals were allowed to drink freely from a bottle in the chamber. In the control group (n = 6), both load and reward temperatures were set at 25°C. Thus, in this case, although the air flow was changed when the rats entered the reward zone, the temperature in the box did not change and stayed at 25°C. All rats were given four training sessions to habituate them to the experimental box, and all were utilized in the experimental session. In the training session, both load and reward temperatures were set at 25°C. Each session lasted 3 h and was done during the light phase between 0800 and 1700. The training runs occurred on alternate days, and the experimental run was carried out 2 days after the last training session.

Immediately after the experimental session, all of the rats were deeply anesthetized with pentobarbital sodium (100 mg/kg ip) and perfused transcardially; they were first flushed with 30 ml of heparinized (1,000 U/ml, Novo Nordisk, Bagsvaerd, Denmark) phosphate-buffered saline (PBS), followed by 400 ml of phosphate-buffered 4% paraformaldehyde. The brains were removed, stored in the same fixative for 48 h at 4°C, and submerged in 12.5% sucrose in PBS for 24 h and then in 25% sucrose in PBS for 48 h. Whole brain sections were made at 40-µm thickness.

The tissue sections were rinsed with PBS and then incubated in 1) 0.3% hydrogen peroxide in PBS for 20 min, 2) 0.3% Triton X-100 in PBS for 20 min at room temperature, 3) rabbit primary polyclonal IgG (1:4,000 in PBS; Santa Cruz Biotechnology, catalog no. sc-52) for 48 h at 4°C, and 4) biotinylated goat anti-rabbit IgG (1:400 in PBS; Vector Laboratories) for 2 h at room temperature. After a rinsing, the sections were incubated in 0.02% dianinobenzidine tetrahydrochloride (Sigma Chemical), 0.02% nickel (II) sulfate (Wako), and 0.017% hydrogen peroxide dissolved in 0.1 M
Tris·HCl buffer. The tissues were mounted onto gelatin-coated slides and air dried. Each slide was covered with a glass microcoverslip.

Photomicrographs were produced by capturing images with a digital camera (HC-2500; Fuji) mounted directly on a microscope (Eclipse E600; Nikon). Data were fed into an Apple Macintosh computer, and only the sharpness, contrast, and brightness were adjusted. All figures were printed (Pictography 3000; Fuji).

The amount of Fos immunoreactivity (Fos-IR) was counted in all rats of each group for a given brain region. The same experimenter made all determinations, utilizing constant criteria as much as possible. Only clearly stained cells were counted. The numbers of cells in the median preoptic nucleus (MnPO) and the parastrial nucleus (PS) were counted for the section at the level of the anterior commissure. In the dorsomedial hypothalamus (DMH) and its surrounding area, cell counting was performed in the area surrounded by the mammilotegmental tract, fornix, and the third ventricle. For each region, we counted both sides of the three continual sections and calculated the average of the six areas. Statistical analyses were performed by use of one-way ANOVA followed by Bonferroni’s test. Results where $P < 0.05$ were taken as the level of significance.

RESULTS

Figure 2A shows the behavior of a rat in an active experiment. During the first 1 h, the rat moved around restlessly in the box, and body temperature rose. Then, the rat periodically moved in and out of the reward area and maintained a stable body temperature. Figure 2B shows the record of behavior and temperature in the passive experiment involving the rat paired with the active animal from Fig. 2A. Note that the change in ambient temperature was identical to that in the active experiment in Fig. 2A. Although the rat moved around similarly to its active counterpart for the 1st h, it moved only slightly afterward. The body temperature of the rat was stable throughout the experiment. In the control experiment, the rats’ behavior was very similar to that seen in the passive experiment; their movements were minimal after first 30 min (Fig. 2C).

Figure 3 shows the sum of activity and the average body temperature for every 30 min of a 3-h experimental session. In the first 30 min, all groups were quite active, and there were no significant differences among the groups. This reflects the typical orienting behavior of rats when they encounter a new environment. Because of higher levels of activity (and likely arousal), body temperature is higher in this period but without any differences among groups. In the passive and control groups, the amount of activity decreased to low levels in the second 30-min period and stayed at that level until the end of the experiment. In contrast, in the active group, although activity levels decreased slightly in the second 30-min period, they stayed high throughout the experiment. After the first 30 min, body temperature was maintained in the range of 37.0–37.5°C in all three groups with no significant differences among groups.

Positive staining for Fos-IR was recognized as a dark reaction product that was localized in the neuronal nuclei (Figs. 4, 5, and 6). No change in the size or shape of the nuclei was evident among the groups. Particularly strong Fos-IR was found in the MnPO in both the active and passive group (Fig. 4, C and D) compared with that of the control group (Fig. 4B). In the active group, definite Fos expression was also seen in the PS (Fig. 5C) and in the DMH and its surrounding area (Fig. 6C). Strong Fos-IR in the MnPO, PS, and DMH was also seen in the “free-drinking active” group (not shown). No Fos-IR was seen in the supraoptic nucleus (SON) or in the amygdala in any groups of rats. Likewise, no Fos-IR was observed in any other brain areas, which was stronger in the active or passive group than in the control group.

Figure 7 shows the numbers of Fos-IR cells in three hypothalamic regions in three groups of rats. In the MnPO, the numbers were significantly greater in the active and passive groups than in the control group, and there was no difference between the active and passive groups. In the PS, the number of Fos-IR cells in the active group was significantly greater than in the control and passive groups. Likewise, in the DMH and its surrounding area, the number of Fos-IR cells in the active group was significantly greater than in the control and passive groups.

DISCUSSION

In the active experiments, when the rats were exposed to 40°C, they periodically moved in and out of the reward area and obtained the cooler reward air. In contrast, in the control experiment, where the ambient temperature stayed at 25°C, the rats scarcely moved after the first 30 min in the box (Fig. 3). In the passive experiment, in which the ambient temperature was manipulated by the investigators to mimic changes in the active experiments, the rats also scarcely moved except for the first 30 min. From these results, we infer that the motivation behind the periodic movement in and out of the reward area in the active experiment was to get cold air, that is, thermoregulatory behavior.

In the present study, strong Fos-IR was observed in the MnPO in both the active and passive groups and in the PS and DMH only in the active group. In earlier experiments, when animals were given procedures involving stresses such as immobilization or pain, strong Fos-IR was seen in the SON, the amygdala, and the lateral septum (30, 31). In the present study, no Fos-IR was seen in those regions. Therefore, the protocol of both active and passive experiments was probably not stressful for the rats. The resultant Fos-IR is thus likely to be related specifically to thermal inputs or to thermoregulation.

In the present study, because Fos analysis occurred only at the 3-h point, we can provide no information on the time course of Fos staining. In experiments involving thermal stimulation or fever, Fos seems to be expressed as early as 1–2 h after stimulation (22, 29, 38). But Patronas et al. (23) and Miyata et al. (19) clearly showed that Fos analysis could also be applied to long-term events in some cases. The animals were killed 3 h after the start of experiment. As Fig. 2
illustrates, the rat learned the procedures in <1 h, and the movement in the last 2 h was very stable. However, the possibility cannot be excluded that the Fos expression in the present study may be related not only to thermoregulation but also to some aspect of the learning process.

The brain regions showing Fos-IR are very likely to be involved in the genesis of thermoregulatory behavior in some way. First, there was strong Fos-IR in the MnPO in both the active and passive groups. The only differences between the active and passive experiments were whether the changes in ambient tempera-
ture were the result of the rat's movements or not. The profile of ambient temperature change was identical in both experiments, and there was no significant difference in deep body temperature (Fig. 3B). Thus, if the MnPO is involved in behavioral thermoregulation, it may be important for detecting changes in the ambient and/or body temperature. Indeed, it was recently reported that Fos is expressed in the MnPO in response to heat exposure (23). Patronas et al. (23) have clearly shown that, in response to 2 days of heat exposure, Fos expression was especially strong in the dorsal MnPO compared with the anterior or ventral MnPO. In the present work, because there was no difference in Fos expression between subregions, counting was made in the MnPO as a whole.

The MnPO has long been known also to be a crucial site for drinking and body fluid regulation (8). In addition, strong Fos-IR is seen in the MnPO when rats are dehydrated. Thus the Fos-IR in the MnPO obtained in

![Fig. 3](image-url) Changes in activity (A) and body temperature (B) every 30 min in the experimental session. Amount of activity is the total for each 30-min period, and body temperature is the mean of the 30-min period. Open, gray, and filled bars indicate control, passive, and active experiments, respectively. Values are means ± SE; n = 6. * and §P < 0.01 vs. control and passive groups, respectively.

![Fig. 4](image-url) Photomicrographs demonstrating the distribution of Fos immuno-reactivity (Fos-IR) in the median preoptic nucleus (MnPO) after control (B), active (C), and passive experiments (D). Ac, anterior commissure; 3V, 3rd ventricle; ox, optic chiasm. Scale bar, 500 μm.

![Fig. 5](image-url) Photomicrographs demonstrating the distribution of Fos-IR in the parastrial nucleus (PS) after control (B), active (C), and passive experiments (D). Scale bar, 500 μm.

![Fig. 6](image-url) Photomicrographs demonstrating the distribution of Fos-IR in the dorsomedial hypothalamus (DMH) and its surrounding region after control (B), active (C), and passive experiments (D). Mt, Mamillothalamic tract; DA, dorsal hypothalamic area; f, fornix. Scale bar, 500 μm.
the active and passive experiments that included exposure to heat might have been due to dehydration. But this is not likely to be the case because, although dehydration also induced Fos-IR in the SON (23), no Fos-IR was seen in the SON in this study. Additionally, those rats with free access to water, which should be in a hydrated state, expressed Fos in the MnPO to the same extent as those without water. Therefore, the Fos-IR seen in the MnPO is unlikely to be related to dehydration but rather is likely to be due to the heat exposure itself.

Fos-IR in the PS and DMH was observed only in the active group. This result strongly suggests that these areas are important for the mediation of thermoregulatory behavior. Attention has recently been paid to the PS in relation to fever, because it expresses Fos during lipopolysaccharide fever (7). Likewise, the DMH is related to thermoregulatory behavior. Indeed, the DMH receives telencephalic inputs from the ventral subiculum and the prefrontal cortex (34). A histological study showed that there is an afferent connection of the DMH from the PS and the MnPO (34). So it may be that the DMH receives signals from these two sites to produce thermoregulatory behavior; this should be elucidated in future studies.

Rats in a hot environment increase their evaporative heat loss by spreading saliva on their fur (10). Heat loss by this route requires not only the autonomic process of secreting a large amount of saliva (thermally induced salivary secretion) but also the behavioral process of spreading the secreted saliva (grooming) (36). Interestingly, the brain sites at which local warming elicits these responses are not the same. The only brain sites at which warming induces salivary secretion are in the preoptic area and the anterior hypothalamus (13). In contrast, the sites where local heating elicits grooming are located more caudally and include the DMH (32). In the present study, grooming was not observed in the active or passive experiments. But there is a possibility that the DMH neurons expressing Fos in the present study are involved not only in thermoregulatory operant behavior but also in the grooming behavior that accompanies the increased salivation seen in the heat-stressed rats.

It is known that the amygdala is important for the control of emotional behavior that is related to the maintenance of body homeostasis for such systems as food intake, drinking, and aversive behavior (25). Therefore, we expected that the amygdala would have to see Fos-IR expression during thermoregulatory behavior. Although no Fos-IR was actually observed in the amygdala, this does not necessarily mean that it is not important for behavioral thermoregulation. One explanation might be that the task required of rats in the present study was too easy, and thus the amygdala was not activated strongly enough to express Fos.

In conclusion, Fos-IR was seen in the MnPO, PS, and DMH of rats having performed the heat escape behavior. Fos-IR in the MnPO was also seen in rats passively given the same regime of ambient temperature, whereas the PS and DMH did not show Fos-IR in these latter rats. The MnPO may be important for detecting changes in ambient and/or body temperature, and the PS and DMH may play some role in the genesis of thermoregulatory behavior.

PERSPECTIVES

If the brain sites expressing Fos in the present study are important for behavioral thermoregulation, it would be interesting to test the effect of destruction of these sites on that behavior. It would also be interesting to see Fos expression after “cold escape” behavior. But compared with heat escape behavior, cold escape behavior with the same operant system as the present one is difficult to evoke. For example, in the situation where the load and reward temperatures are the converse of those in the present study, well-nourished rats prefer to simply curl up rather than perform an operant task for heat reward. Although curling up is also a thermoregulatory behavior, it involves a more stereo-
typed postural adjustment and it may not strongly activate Fos-IR specifically related to cold escape behavior. Recently, our laboratory found that rats perform cold escape behavior as vigorously as heat escape behavior when they are deprived of food (37). Thus another series of experiments is now planned in our laboratory to examine Fos expression after cold escape behavior in food-deprived rats.

Thermal comfort and discomfort are important in that they motivate an organism to make the appropriate behavioral corrective responses. That is, if one feels discomfort with heat or cold, he/she will look for a better condition that provides thermal comfort. If a better condition is found, the ensuing comfortable feeling confirms that the new condition is more suitable for keeping the body temperature normal. In some situations, the brain mechanism of thermal comfort/discomfort can be more easily studied in humans than in animals, since we can ask subjects how they feel and simultaneously apply positron emission tomography or functional magnetic resonance imaging scans to survey brain activation. A project is also currently going on in our laboratory to observe those brain areas activated in relation to thermal comfort/discomfort in humans (14).

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DISCLOSURES

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