Supporting Information

Nakamura and Morrison 10.1073/pnas.0913358107

SI Materials and Methods

Cholera Toxin B-Subunit–Fos Study. Rats deeply anesthetized with chloral hydrate (280 mg/kg, i.p.) received a pressure-injection of 1.0 mg/mL Alexa594-conjugated cholera toxin b-subunit (CTb) (70–120 nL; Molecular Probes) through a glass micropipette to the median preoptic nucleus (MnPO), medial preoptic area (POA), or lateral POA. At the end of the surgery, 5 mg/mL penicillin G solution (200 μL) was injected into femoral muscles to avoid infection. The animals were caged individually and allowed to access food and water ad libitum. Three or four days after the surgery, the cages were placed in a climate chamber air-conditioned to 24°C, and the animals were acclimatized to the chamber for 3 days (1). On the fourth day in the chamber, they were exposed to 36°C, 24°C, or 4°C for 4 h (from 10:00 AM to 2:00 PM). During the exposure to 4°C, the animals huddled, shivered, and consumed food, but none of them displayed any signs of discomfort, anxiety, or pain. Immediately after the exposure, the animals were reanesthetized and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline, and during the exposure to 4°C, the animals showed a spread posture and consumed food, but none of them displayed any signs of discomfort, anxiety, or pain. Immediately after the exposure, the animals were reanesthetized and transcardially perfused with 4% formaldehyde, and the brains were subjected to CTb and Fos immunohistochemistry.

Immunohistochemistry. After perfusion of the animals, the brain was postfixed, cryoprotected, and cut into frontal sections 30 μm thick. The sections were incubated overnight with an anti-Fos rabbit serum (1:20,000; Ab-5; Oncogene). After rinsing, the sections were incubated for 1 h with a biotinylated donkey antibody to rabbit IgG (10 μg/mL; Chemicon) and then with ABC-Elite (1:50; Vector) for 1 h. After thorough washing of the sections, the bound peroxidase was reacted with 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB), 0.0002% hydrogen peroxide, and 0.5% ammonium nickel sulfate hexahydrate in 50 mM Tris-HCl (pH 7.6) to visualize Fos immunoreactivity as a blue-black reaction product. The bound peroxidase and unreacted avidin and biotin in the sections were blocked with 3% hydrogen peroxide and an avidin-biotin–blocking kit (Vector), respectively. After thorough washing, the sections were incubated with an anti-CTb goat antiserum (1:20,000; List Biological Laboratories). After rinsing, the sections were incubated with a biotinylated donkey antibody to goat IgG (10 μg/mL; Chemicon) for 1 h and then with ABC-Elite for 1 h. The sections were incubated with 0.02% DAB and 0.001% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6) to visualize CTb immunoreactivity as a brown reaction product. By omitting one of the primary antibodies, we confirmed that there was no cross-reactivity between the reagents involved in the two different immunoperoxidase-staining steps.

Anatomy. The nomenclature of lateral parabrachial (LPB) subnuclei basically followed that in a previous study (2), except for inclusion of the ventral subnucleus into the central part of the lateral parabrachial nucleus.

In Vivo Physiology. The basic procedures for the animal preparation and skin-temperature manipulation followed our previous protocol (3). Rats were anesthetized with i.v. urethane (0.8 g/kg) and α-chloralose (70 mg/kg), paralyzed with D-tubocurarine, and artificially ventilated with 100% O2. The trunk was shaved, a thermocouple to monitor skin temperature was taped onto the abdominal skin, and the trunk was wrapped with a plastic water jacket to control the temperature of the trunk skin. The animals were placed in a stereotaxic apparatus allowing access to targeted brain sites with glass nanoinjection pipettes, microstimulating electrodes, and single-cell recording electrodes.

To record postganglionic sural CVC neuronal activity, we used thin nerve filaments that were split from the central cut end of the sural nerve until unitary spike activity could be discriminated, placed on bipolar hook electrodes, and tested for the presence of an individual or a few active axons whose activity was stimulated by skin cooling and inhibited by skin warming (Fig. S5), as has been done for the rat plantar cutaneous vasconstrictor (CVC) discharge (4). At the end of each experiment, hexamethonium chloride (30 mg/kg) was injected i.v. to test whether the nerve activity recorded was postganglionic sympathetic (Fig. S5). Even in cases of recording from a few active axons, we confirmed that activities of all individual units observed were inhibited by skin warming and were completely eliminated by the hexamethonium administration. In some animals, postganglionic brown adipose tissue (BAT) sympathetic nerve activity (SNA) was recorded from the right interscapular BAT pad, as reported (3). Nerve activity was filtered (30–1,000 Hz for sural CVC, 1–300 Hz for BAT SNA) and amplified (±50,000 for sural CVC; ±5,000–50,000 for BAT SNA) with a CyberAmp 380 (Axon Instruments). Physiological variables were digitized and recorded to a computer hard disk using Spike 2 software (CED).

Discharge rate (spikes/10 s) analysis of single or a few sural CVC nerve fiber recordings was performed using Spike 2 software. Spike 2 software also was used to obtain a continuous measure (4-s bins) of BAT SNA amplitude by calculating the root mean square amplitude of the BAT SNA (square root of the total power in the 0–20 Hz band) from the autospectra of sequential 4-s segments of BAT SNA.

Stereotaxic pressure injection of drugs into the brain in nanoliter volumes (nanoinjections) was performed using glass micropipettes (tip inner diameter, 20–30 μm) as described (3). The concentrations of injected drugs were 5 mM each mixture of DL-2-amino-5-phenylpentanopentanoic acid (AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 0.2 mM NMDA, and their injection volumes (60 nL/site of AP5/CNQX into the LPB, 36 nL/site of NMDA into the LPB and 100–200 nL into the MnPO) followed our previous in vivo physiological studies (3, 5) in which we obtained consistent effects from these drug doses. To mark the injection sites, fluorescent microspheres (Molecular Probes) were injected at the same stereotaxic coordinates through the same micropipettes, as described (3). After the physiological recordings, the animals were perfused with 10% formaldehyde, and the brain tissues were sectioned. The locations of the nanoinjections were identified by detecting the fluorescent microspheres in the sections under an epifluorescence microscope.

As with most homeostatic autonomic functions, thermoregulation is likely impaired by the effect of urethane-chloralose anesthesia to depress neuronal responses. Nonetheless, brisk thermoregulatory effector responses paralleling those in awake animals to skin warming and cooling were well maintained, providing strong support for our physiological findings identifying the fundamental neural pathways for thermoregulation derived from experiments in anesthetized animals.

Physiological Data Analysis. In the analysis of data from skin-warming experiments (Figs. 3B and 5G), the baseline values of all of the physiological variables were the averages during the 30-s period immediately before each skin-warming episode. Skin warming-evoked response values for tail skin temperature and heart rate (HR) were taken at the end of the skin-warming episode, and those for sural CVC and mean arterial pressure were the
averages during the 30-s period immediately before the end of skin warming. Skin warming-evoked changes in these variables from their baseline values were compared between warming episodes after AP5/CNQX injections into the dorsal part of the lateral parabrachial nucleus (LPBd) and after saline injections into the LPBd or before injections (control) (Fig. 3B), and between warming episodes after AP5/CNQX injection into the MnPO and after saline injection into the MnPO (Fig. 5G).

In the analysis of data from experiments involving nanoinjection of NMDA into the LPBd during sural CVC recording (Figs. 4B and 5B), the baseline values of all physiological variables were the averages during the 30-s period immediately before each injection into the LPBd. Because of the differential onset delay of NMDA-evoked changes in these physiological variables, injection-evoked response values for HR and rectal and brain temperatures were taken at 120 s (HR) or 180 s (rectal and brain temperatures) after the injection into the LPBd, those for sural CVC and mean arterial pressure were the averages during the 30-s period 90 s after the injection, and that for tail skin temperature was the peak value within 180 s after the injection. Injection-evoked changes in these variables from their baseline values were compared between saline and NMDA injections into the LPBd (Fig. 4B) and between NMDA injections into the LPBd after AP5/CNQX injection into the MnPO and after saline injection into the MnPO (Fig. 5B).

In the experiments monitoring skin cooling-evoked thermogenesis (Fig. S6B), NMDA or saline was injected into the LPBd during the BAT thermogenic response to skin cooling, and skin cooling was continued for at least 100 s after the intra-LPBd injection. Baseline values of all physiological variables were the averages during the 30-s period immediately before skin cooling episodes. Skin cooling-evoked response values for BAT temperature and HR were those at the time of the injections into the LPBd, and those for BAT SNA, expired CO$_2$, and mean arterial pressure were the averages during the 30-s period immediately before the injection. Injection-induced effect values for BAT temperature and HR were taken 60 s after the injection, and those for BAT SNA, expired CO$_2$, and mean arterial pressure were the averages during the 30-s period 30 s after the injection.

**Unit Recording.** Rats received urethane and α-chloralose anesthesia. Unit recordings were made with glass microelectrodes filled with 0.5 M sodium acetate (DC resistance: 20–43 MΩ) containing 5% biotinamide to allow juxtacellular labeling of the recorded neurons. A monopolar tungsten stimulating microelectrode was stereotaxically positioned in the MnPO. The LPB was explored for neurons showing a constant onset latency response to electrical stimulation in the POA (0.8–5 mA, 1 ms, 0.25–0.33 Hz). Standard criteria (6) were used to establish the antidromic nature of the responses of LPB neurons to POA stimulation. After time-controlled collision tests (Fig. 2C), the thermosensitivity of LPB neurons was tested by warming the skin for 100–300 s, and this warming challenge was repeated at least twice. For juxtacellular labeling (7) of the recorded neurons with biotinamide, positive current pulses (0.5–4.0 nA, 400-ms duration, 50% duty cycle) were delivered for 1–5 min to entrain the neuron. After the recording, the animals were perfused with 4% formaldehyde, and the labeled neurons were visualized by incubating the brain sections with 1 μg/mL Alexa594-conjugated streptavidin.

For firing rate analysis of each recorded neuron, the rate of spontaneous firing was measured as spikes/10 s, and all response values represent the average of the two or three skin-warming episodes. During the skin-warming stimulus, there was a clearly identifiable skin temperature range, occupying only a portion of the skin temperature ramp, during which the LPBd unit firing rate exhibited a highly significant, positive correlation with the skin temperature. We have termed this portion of the unit response to the skin temperature ramp the “dynamic phase” to distinguish it from the portions of the skin temperature ramp in which the unit firing did not change significantly with skin temperature (i.e., the early portion of the skin temperature ramp during which the skin temperature was presumably below the response threshold of the cutaneous thermal receptors whose activation contributed to the activity of the LPBd neuron and the later portion of the skin temperature ramp during which the LPBd neuron discharge rate remained at an elevated, “plateau” level, perhaps because skin temperature was greater than that eliciting a maximal discharge from the relevant skin warm receptors or because of some factor limiting the activity of the dorsal horn warm neurons in contributing to the activity of the LPBd neuron). The mean temperatures between which warm-responsive LPB neurons exhibited their dynamic increase in firing rate during skin warming were the means of the beginning (i.e., low end) and the ending (i.e., high end) temperatures of the dynamic phases of each LPBd neuronal response to skin warming. In cases where the skin temperature ramps were repeated for a given unit, the means of the beginning and ending temperatures for these trials were used in the calculation of the overall population means (i.e., a single onset and offset temperature from each neuron contributed to the population means).

Fig. S1. POA-projecting neurons in the LPBel are activated in a cold environment (see also Fig. 1). Fos expression in LPB neurons that were retrogradely labeled with CTb injected into the POA was examined in rats exposed to cold (4 °C; A–D and G), control (24 °C; E), or warm (36 °C; F) temperature. Photomicrographs in A–D and G were taken from the same animal, and those in E and F were from the animals shown in Fig. 1. (A) CTb injection site (red) in the cold-exposed rat. (B–G) CTb (brown) and Fos (blue-black) immunoreactivities in the LPB. Many CTb-labeled neurons in the LPBel (arrows, G), but not those in the LPBd (arrowheads, D), expressed Fos following cold exposure, whereas most CTb-labeled neurons in the LPBel following control- (E) or warm-exposure (F) were Fos-negative (arrowheads). scp, superior cerebellar peduncle. [Scale bars, 0.5 mm (A–C); 30 μm (D–G).] Some images from the 24 °C- and 4 °C-exposed animals were adapted from our previous study (1).

Fig. S2. Distribution of CTb-labeled and Fos-expressing neurons in the LPB following exposure to environment of 24 °C, 36 °C, or 4 °C. (A–C) Sites of CTb injections in the POA of animals exposed to 24 °C (A), 36 °C (B), and 4 °C (C). CTb injection sites from all of the animals are delineated with green (A), red (B), and blue (C) lines and are overlaid. (D) Percentages of Fos-immunoreactive neurons in CTb-labeled populations in the LPB subnuclei in 24 °C-, 36 °C-, and 4 °C-exposed animals (n = 4 per group). Neurons were counted in every sixth 30-μm-thick frontal section. ***, P < 0.001, compared with the 24 °C-exposed group; †††, P < 0.001 comparing the 36 °C-exposed and 4 °C-exposed groups (two-way ANOVA followed by a Bonferroni post hoc test). (E–G) Distribution of Fos-immunoreactive cells (filled circles), CTb-labeled cells (open circles), and Fos-immunoreactive, CTb-labeled cells (red circles) in 24 °C- (E), 36 °C- (F), and 4 °C-exposed (G) animals. The one-in-six series of 30-μm-thick frontal sections is aligned in rostrocaudal order. Me5, mesencephalic trigeminal nucleus. (Scale bar, 0.5 mm.) The results from 24 °C- and 4 °C-exposed animals were adapted from our previous study (1).

Fig. S3. Composite mapping of CTb injection sites in the POA and percentages of Fos-immunoreactive neurons in CTb-labeled populations in the LPBd of animals exposed to 36 °C (see also Fig. 1 and Fig. S2). Percentages colored in red are from the injection cases shown in Fig. S2B. The number of neurons that were positive for both CTb and Fos and the total number of CTb-labeled neurons for each injection case are shown in parentheses. Neurons were counted within the LPBd in every sixth 30-μm-thick frontal section. LPO, lateral preoptic area.

Fig. S4. Supplementary data from in vivo unit recording (see also Fig. 2). (A1–A3 and B) Positive correlation between skin temperature and firing rate of warm-responsive LPBd neurons during dynamic responses to skin warming. Linear regression analysis indicates a significant (Pearson's correlation test) correlation between skin temperature and the discharge frequency of the warm-responsive LPB neuron shown in Fig. 2A (A1–A3) and the warm-responsive LPB neuron with the regression coefficient (r) closest to 1 (B). A1–A3 show linear regression data from the three skin-warming challenges in the order shown in Fig. 2A. (C) In vivo extracellular unit recording from a warm-responsive LPBd neuron (Unit) that exhibited a plateau phase following a dynamic increase in the firing rate during skin warming. (D and E) Effect of tail pinch on the discharge of a warm-responsive neuron [two examples from repeated tail pinch stimuli (D)] and a non-thermoreponsive neuron (E) during in vivo unit recording in the LPB. Double horizontal lines indicate the period of tail pinch. No increase was observed in the firing rate of the warm-responsive neuron in response to tail pinch (D), whereas the non-thermoreponsive neuron showed a high-frequency burst discharge in response to tail pinch, which was followed by a postexcitatory depression period (E). The tail pinch evoked a pressor response in each tail pinch trial.
Fig. S5. Identification of sympathetic CVC properties of single-fiber sural nerve recording. (A) Discrimination (based on amplitude and spike shape shown in Inset) of the action potentials from a single sural CVC axon (red spikes). When core body temperature was below 38.5–39.0 °C, the unit was spontaneously active, and tail skin temperature ($T_{tail}$) was closer to room temperature than to body core temperature, indicating that tail cutaneous blood vessels were constricted. Brief warming of the trunk skin ($T_{skin}$) with a water jacket, which did not substantially affect body core (rectal, $T_{rec}$) or brain ($T_{brain}$) temperature, reduced the spontaneous unit activity and evoked a subsequent increase in tail skin temperature, indicating that tail cutaneous vasodilation occurred. Vertical scale bar for the sural CVC trace represents 40 μV. (B) Expanded view of the sural CVC unit trace shown in A during a period of spontaneous discharge before skin-warming challenges. The spontaneous sural CVC activity was synchronized with the respiratory cycle, which is identified with the expired CO$_2$ trace (Exp. CO$_2$). Vertical scale bar for the sural CVC trace represents 40 μV. (C) Complete inhibition of sural CVC activity following i.v.-administered hexamethonium confirms that the activity was sympathetic postganglionic nerve activity. (Vertical scale bar for the sural CVC trace represents 20 μV.)
Fig. S6. (A) Effect of unilateral NMDA nanoinjection (dashed line) into the LPBd on skin cooling-evoked changes in BAT SNA, BAT temperature ($T_{BAT}$), expired CO$_2$ (Exp. CO$_2$), HR, arterial pressure (AP), $T_{rec}$, and $T_{brain}$. (Vertical scale bar for the BAT SNA trace represents 100 μV.) (B) Average changes in BAT SNA, $T_{BAT}$, Exp. CO$_2$, HR, and mean arterial pressure (MAP) along the trunk skin cooling and the midcooling nanoinjection of saline ($n = 4$) or NMDA ($n = 6$) into the LPBd (injection sites shown in C). *, $P < 0.05$; **, $P < 0.01$, compared with the saline value (two-way ANOVA followed by a Bonferroni post hoc test). (C) Sites of injections in the LPBd.

Fig. S7. Schematic thermal somatosensory pathways from the skin. Environmental warming (Left) is sensed by warm receptors in the cutaneous endings of primary somatosensory fibers, which transmit the thermal signals to dorsal horn (DH) neurons that project axon collaterals to the LPBd and the thalamus. The LPBd neurons are activated by glutamatergic inputs (Glu) from the DH neurons and then transmit the signals to the MnPO via a glutamatergic input. This feedforward warm afferent input activates, either directly or through excitatory interneurons (postulated) in the MnPO, GABAergic projection POA neurons that could be distributed both in the MPO and MnPO. The activated GABAergic neurons inhibit excitatory pathways that drive sympathetic thermoregulatory effectors, leading to cutaneous vasodilation and reduced thermogenesis. Environmental cooling (Right) is sensed by cutaneous cool receptors and transmitted to DH neurons that provide glutamatergic inputs to the LPBel and the thalamus. Cooling-activated LPBel neurons provide a glutamatergic excitation to GABAergic interneurons in the MnPO, which reduce the activity of the inhibitory projection POA neurons in the MPO. The resulting disinhibition of the descending efferent pathways that excite thermoregulatory effectors leads to induction of cold-defensive responses. Collaterals of the thermal responsive DH neurons that ascend to the thalamus innervate thalamocortical neurons mediating perception and discrimination of cutaneous temperature (dashed lines).
Table S1. Skin warming-induced changes in sural CVC activity, tail skin temperature ($T_{\text{tail}}$), and HR following bilateral AP5/CNQX nanoinjections into the LPBd or into the LPBel.

<table>
<thead>
<tr>
<th>Site</th>
<th>$n$</th>
<th>ΔSural CVC (% of prewarming activity)</th>
<th>$\Delta T_{\text{tail}}$ (°C)</th>
<th>ΔHR (beats per minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPBd</td>
<td>5</td>
<td>$-10.9 \pm 4.0$</td>
<td>$+0.0 \pm 0.2$</td>
<td>$+1 \pm 3$</td>
</tr>
<tr>
<td>LPBel</td>
<td>3</td>
<td>$-90.9 \pm 0.4^\dagger$</td>
<td>$+1.2 \pm 0.3^\dagger$</td>
<td>$+16 \pm 4^\dagger$</td>
</tr>
</tbody>
</table>

For injection sites, see Fig. 3D. Values represent mean ± SEM.

$^\dagger P < 0.05; ^\ddagger P < 0.001$, compared with the LPBd group (two-tailed unpaired t test).