Social defeat stress induces hyperthermia through activation of thermoregulatory sympathetic premotor neurons in the medullary raphe region

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Abstract

Psychological stress-induced hyperthermia is a fundamental autonomic response in mammals. However, the central circuitry underlying this stress response is poorly understood. Here, we sought to identify sympathetic premotor neurons that mediate a hyperthermic response to social defeat stress, a psychological stress model. Intruder rats that were defeated by a dominant resident conspecific exhibited a rapid increase in abdominal temperature by up to 2.0°C. In these defeated rats, we found that expression of Fos, a marker of neuronal activation, was increased in the rostral medullary raphe region centered in the rostral raphe pallidus and adjacent raphe magnus nuclei. In this region, Fos expression was observed in a large population of neurons expressing vesicular glutamate transporter 3 (VGLUT3), which are known as sympathetic premotor neurons controlling non-shivering thermogenesis in brown adipose tissue (BAT) and thermoregulatory constriction of skin blood vessels, and also in a small population of tryptophan hydroxylase-positive, serotonergic neurons. Intraperitoneal injection of diazepam, an anxiolytic agent, but not indomethacin, an antipyretic, significantly reduced both the stress-induced hyperthermia and Fos expression in these medullary raphe neuronal populations. Systemic blockade of β_3-adrenoreceptors, which are abundantly expressed in BAT, also attenuated the stress-induced hyperthermia. These results suggest that psychological stress signals activate VGLUT3-expressing medullary raphe sympathetic premotor neurons, which then drive hyperthermic effector responses including BAT thermogenesis through β_3-adrenoreceptors.

Introduction

Psychological stress elicits an elevation of body core temperature (T_c) in mammals. This is a fundamental physiological response called psychological stress-induced hyperthermia (PSH) and observed in many species including rats (Briese & De Quijada, 1970; Singer *et al.*, 1986; Vinkers *et al.*, 2009), mice (Zethof *et al.*, 1995; Oka *et al.*, 2003; Olivier *et al.*, 2003), rabbits (Yokoi, 1966; Snow & Horita, 1982) and humans (McNeil *et al.*, 1984; Timmerman *et al.*, 1992; Oka & Oka, 2007; Hiramoto *et al.*, 2009; Kaneda *et al.*, 2009). However, the brain circuitry mechanism underlying PSH is poorly understood.

The development of PSH involves sympathetic effector responses including non-shivering thermogenesis in brown adipose tissue (BAT) and constriction of skin blood vessels for heat conservation, as shown by previous studies using restraint stress (Shibata & Nagasaka, 1984; Ootsuka *et al.*, 2008). In central sympathetic efferent mechanisms, sympathetic premotor neurons play a pivotal role by transmitting command signals collected from various brain sites to a subset of sympathetic preganglionic spinal neurons to drive specific effector responses (Nakamura, 2004; Nakamura *et al.*, 2005a; Morrison *et al.*, 2008). We have identified a population of sympathetic premotor neurons that mediate BAT thermogenesis and cutaneous vasoconstriction for fever development and cold defense (Nakamura *et al.*, 2004a). These neurons express vesicular glutamate transporter 3 (VGLUT3) and are distributed in a rostral medullary raphe region including the rostral raphe pallidus nucleus (rRPa) and the adjacent raphe magnus nucleus (RMg) (Nakamura *et al.*, 2004a). However, it is unknown whether these premotor neurons mediate the sympathetic thermal responses for PSH.

Previous studies have suggested that psychological stress elicits hyperthermia via mechanisms distinct from fever (Oka *et al.*, 2001).

Mice lacking the receptors of prostaglandin (PG) E_2, a pyrogenic mediator, fail to develop fever, but exhibit PSH (Oka *et al*., 2003) and anxiolytic drugs, such as diazepam, attenuate PSH, but not fever (Zethof *et al*., 1995; Olivier *et al*., 2003; Vinkers *et al*., 2009). In addition, studies using different stress models have shown inconsistent results on stress-evoked activation of spinally projecting neurons in the rostral medullary raphe region (Senba *et al*., 1993; Carrive & Gorissen, 2008).

These earlier results prompted us to investigate the involvement of the sympathetic premotor neurons in the rostral medullary raphe region in hyperthermic responses to social defeat stress, a psychological stress model close to human social stress (Vinkers *et al*., 2009), in rats. We studied whether social defeat stress-induced hyperthermia is associated with expression of Fos, a marker of neuronal activation (Sagar *et al*., 1988), within the medullary raphe region by examining the effects of diazepam on the hyperthermia and on the stress-induced expression of Fos. We also determined the involvement of β_3-adrenoreceptor-mediated BAT thermogenesis in the stress-induced hyperthermia. Finally, we examined whether social defeat stress induces Fos expression in VGLUT3-immunoreactive medullary raphe neurons as well as in serotonergic neurons in the same region, which are known to modulate bulbospinal thermoregulatory signaling (Madden & Morrison, 2006; Hodges *et al*., 2008).

Materials and methods

Animals

Male Wistar rats weighing 190–290 g and male Long-Evans rats weighing 400–550 g (SLC, Shizuoka, Japan) were used as intruders and residents, respectively. Wistar rats were individually caged and Long-Evans rats were pair-caged with age-matched females. Both strains

were housed in separate rooms air-conditioned at 24 ± 2°C with a standard 12 h light/dark cycle (lights on 0700–1900 h) and allowed *ad libitum* access to food and water. All procedures conform to the guidelines of animal care by Kyushu University and by the Institute of Laboratory Animals, Faculty of Medicine, Kyoto University and were approved by the Ethics Committees of Kyushu University (A22-165-0) and by the Animal Research Committee, Graduate School of Medicine, Kyoto University (MedKyo10082).

Surgery and T_c monitoring

We measured T_c using a telemetry system (Data Sciences International, St. Paul, MN, USA). A battery-operated telemetric transmitter (TA10TA-F40) was implanted into the peritoneal cavity of each Wistar rat via a midline incision under anesthesia with sodium pentobarbitone (50 mg/kg, i.p.) as described previously (Hayashida *et al*., 2010). After closure of the cavity with suture, the animals were housed individually for 1 week to recover from the surgery under regular health checks. T_c signals were received by an antenna below the rat cage and relayed to a signal processor (Dataquest A.R.T.™ System, Data Sciences International) connected to a server computer. At least one day before the experiment, the telemetric transmitters were activated using a magnet to start recording T_c every 5 min. Only rats that showed stable diurnal changes in T_c were used for the following experiments.

Drugs

Diazepam (Wako, Osaka, Japan) was dissolved in saline with 40 mM hydrochloric acid (Shannon & Herling, 1983). In a pilot study, we determined the minimum effective dose of diazepam (i.p.) based on its inhibitory effect on social defeat stress-induced increase in T_c and found

that 4 mg/kg gave the most consistent effect. SR59230A (Sigma), a selective β_3-adrenoreceptor antagonist, was diluted in pyrogen-free saline containing 2% dimethyl sulfoxide (DMSO). Indomethacin (Wako, Osaka, Japan) was dissolved in 0.2 M Tris-HCl (pH 8.3). A fresh solution of lipopolysaccharide (LPS), phenol-extracted from *Escherichia coli* 0111:B4 (Sigma), was prepared by diluting in pyrogen-free saline before every use. The doses and preparation of SR59230A, indomethacin and LPS followed previous studies (Sugimoto *et al*., 1997; Steiner & Branco, 2000; Sprague *et al*., 2004; Bexis & Docherty, 2009).

Social defeat stress

On the experimental day, Wistar rats were exposed to social defeat stress following a modified resident-intruder confrontation procedure (Miczek *et al*., 1982; Tornatzky & Miczek, 1993). Briefly, from the home cage of paired Long-Evans rats, the female was removed, and in exchange, a Wistar rat (the intruder) was placed into the cage (40 cm long × 25 cm wide × 20 cm high) of the male Long-Evans rat (the resident) for 60 min. In most cases, the intruder was attacked and defeated by the resident within 5 min as was evident from freezing behavior or a submissive posture. As soon as the intruder was found defeated, the animals were separated by inserting a wire-mesh partition to avoid physical injury. Thereby, the intruder was protected from direct physical contact, but remained in olfactory, visual and auditory contact with the resident for the rest of the stress period. No wound was found on the intruders after this stress procedure. Following the stress period, the intruder was returned to its home cage. This stress procedure was performed in the morning, between 1000 and 1200 h, when the circadian changes in T_c were minimal.

In the experiments not involving drug injections (Figs. 1, 3 and 4), Wistar rats in the stress group were subjected to the social defeat stress procedure and control rats were kept in their home cages for 60 min instead of being placed into Long-Evans cages, but were gently lifted by their tails twice at the start and at the end of the period (sham handling). Following the stress exposure or sham handling, the animals in both groups were left undisturbed overnight. In the experiments involving drug injections (Figs. 2 and 5), Wistar rats received an i.p. injection of diazepam (4 mg/kg, 0.8–1.2 ml), SR59230A (5 mg/kg, 0.2–0.3 ml), indomethacin (5 mg/kg, 0.2–0.3 ml) or either of their respective vehicles. To minimize stress from injection procedure, the lower part of the body was gently bent backward by holding the base of the tail and the lower back to expose the lower abdominal skin to the experimenter, who then quickly injected there. This procedure was performed within the home cages of the rats and their head and forelegs had little movement during the procedure. Sixty minutes after the injection (or 30 min for SR59230A), the rats were subjected to the social defeat stress procedure (Stress group) or left undisturbed in their home cages (Control group). Following the stress or control period, the animals were left undisturbed overnight. In the experiment testing the antipyretic effect of indomethacin, Wistar rats received an i.p. injection of LPS (10 μg/kg, 0.1–0.2 ml) or a mixture of LPS and indomethacin (5 mg/kg, 0.3–0.5 ml) and were left undisturbed overnight. Animals for each group were randomly chosen.

Mean T_c for a 30-min period prior to stress/sham handling (Fig. 1) or injection (Fig. 2) was considered the baseline T_c. Peak T_c was taken during the 60-min stress or sham/control period (post-injection peak value was taken in the LPS injection experiment) and the change from the baseline T_c is presented as peak ΔT_c.

For immunohistochemical analyses, five groups of Wistar rats, whose T_c was monitored as described above, were treated with one of the following procedures: 1) social defeat stress (Stress), 2) sham handling (Sham), 3) diazepam injection followed by social defeat stress (Diazepam/Stress), 4) vehicle injection followed by social defeat stress (Vehicle/Stress) and 5) diazepam injection, then being left undisturbed (Diazepam/Control). These procedures were conducted in the same time course as described above. These animals were re-anesthetized at 15 min after the end of the 60-min period of social defeat stress or sham/control handling and immediately perfused transcardially with 100–150 ml of saline and then with 200–300 ml of 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed, postfixed in the fixative at 4°C for 2 h, and then cryoprotected with a 30% sucrose solution overnight. The tissues were cut into 30-μm-thick frontal sections on a freezing microtome.

Immunohistochemistry

Immunohistochemical procedures were based on our previous studies (Nakamura *et al*., 2000, 2002, 2004a). For single staining for Fos, the brain sections were incubated overnight with an anti-Fos rabbit serum (1:10,000–20,000; Ab-5; Oncogene, Cambridge, MA, USA) and then for 1 h with a biotinylated donkey antibody to rabbit IgG (10 μg/ml; Chemicon, Temecula, CA, USA). The sections were further incubated for 1 h with avidin-biotinylated peroxidase complex (ABC-Elite; 1:50; Vector, Burlingame, CA, USA). Bound peroxidase was visualized by incubating the sections with a solution containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma), 0.001% hydrogen peroxide and 50 mM Tris-HCl (pH 7.6) (DAB solution).

For double staining for Fos and VGLUT3, sections were incubated overnight with an anti-VGLUT3 guinea pig antibody (0.5 μg/ml; Hioki *et al*., 2004) and then for 1 h with a biotinylated donkey antibody to guinea pig IgG (10 μg/ml; Jackson ImmunoResearch, West Grove, PA, USA). The sections were further incubated for 1 h with ABC-Elite. Bound peroxidase was visualized by incubation of the sections with a solution containing 0.02% DAB, 0.0002% hydrogen peroxide, 0.5% ammonium nickel sulfate hexahydrate and 50 mM Tris-HCl (pH 7.6) (Ni-DAB solution) to develop VGLUT3 immunoreactivity as a blue-black reaction product of Ni-DAB. After the peroxidase reaction, 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. The sections were further processed for Fos immunohistochemistry as described above. In this manner, Fos immunoreactivity was developed as a brown reaction product of DAB.

For double staining for Fos and tryptophan hydroxylase (TPH), sections were processed for Fos immunohistochemistry as described above, except that a Ni-DAB solution instead of a DAB solution was used for the visualization. After blocking the bound peroxidase and unreacted avidin and biotin in the sections, the sections were incubated overnight with an anti-TPH mouse monoclonal antibody (1:1,000; clone WH-3, Sigma). The sections were further incubated for 1 h with a biotinylated donkey antibody to mouse IgG (10 μg/ml; Chemicon) and then for 1 h with ABC-Elite. Bound peroxidase was visualized by incubating the sections with a DAB solution. Through this procedure, Fos and TPH immunoreactivities were developed as blue-black and brown products, respectively. This anti-TPH antibody was raised against a recombinant rabbit TPH and reacts specifically with TPH in immunoblotting assays (55 kDa, manufacturer's technical information).

This antibody stains a pattern of neuronal morphology and distribution identical to that of serotonin-containing neurons in previous reports (Takeuchi *et al*., 1982) and has been widely used to label serotonergic neurons in rat brain (Burman *et al*., 2003; Mulkey *et al*., 2007).

By omitting one of the primary antibodies used for these double-staining procedures, we confirmed that there was no cross-reactivity between the reagents involved in the two different sequential immunoperoxidase-staining steps.

Anatomy and cell counting

Following the brain atlas of Paxinos and Watson (1998), the raphe pallidus nucleus was nomenclaturally divided into two parts: rostral (rRPa) and caudal (cRPa) to the rostral end of the inferior olivary complex (IO) (Nakamura *et al*., 2002, 2004a). The number of immunolabeled cell bodies was counted in every sixth 30-μm-thick frontal section of the medulla oblongata over the rostrocaudal levels at which VGLUT3-immunoreactive cells were distributed: between the rostral end of the facial nucleus and the caudal end of the fourth ventricle. In the cell counting, the ventromedial medullary region was anatomically divided into five subregions: 1) rRPa, 2) RMg and parapyramidal region (PPy), 3) a zone near the ventral surface of the pyramidal tract, 4) cRPa and 5) raphe obscurus nucleus (ROb) and ventral part of the gigantocellular reticular nucleus (Giv). The rRPa, RMg and PPy are located rostral to the rostral end of the IO and the cRPa, ROb and Giv are caudal to the rostral end of the IO. A zone near the ventral surface of the pyramidal tract was not divided rostrocaudally.

Statistics

All data are presented as means ± S.E.M.   Treatment effects on T_c over time were evaluated using a two-way repeated ANOVA (groups × time; SPSS for Windows, Version 17) and when a significant P value was attained in the ANOVA, pairwise comparisons of T_c at each time point were performed with an unpaired t-test.   Drug effects on peak ΔT_c were analyzed using an unpaired t-test (Prism 4, GraphPad, La Jolla, CA, USA).   To examine the effect of a drug injection itself on the T_c level, baseline T_c and post-injection T_c were compared using a paired t-test. Inter-group comparisons of baseline T_c were performed using an unpaired t-test or a one-way ANOVA.   Statistical analyses for cell-counting data were performed using an unpaired t-test or a one-way ANOVA followed by a Bonferroni *post hoc* test.   All tests were two-tailed and results with a P value of < 0.05 were considered significant.

Results

Effect of social defeat stress on T_c

We first examined the effect of social defeat stress on T_c in Wistar rats. Soon after being placed into the cages of resident Long-Evans rats, intruder Wistar rats were defeated by the residents and exhibited an increase in T_c by 2.0 ± 0.1°C (peak ΔT_c, n = 6) with a peak observed 25 min after the beginning of their contact.   This T_c peak was followed by a gradual decrease over the subsequent 2 h (Fig. 1).   In contrast, animals that received sham handling showed small changes in T_c (peak ΔT_c: 0.7 ± 0.1°C, n = 6; Fig. 1).   There was a significant difference in T_c across time between these two groups (groups, $F_{1,24}$ = 106.26, P < 0.001; time, $F_{1,24}$ = 12.56, P < 0.001; groups × time, $F_{1,24}$ = 7.07, P = 0.004; two-way repeated measures ANOVA) and pairwise comparisons at each time point with t-tests revealed significant differences between 15 and 105 min after the beginning of the stress or sham handling period (Fig. 1).   Baseline T_c

before the stress or sham handling did not differ between the groups (sham, 36.9 ± 0.1°C; stress, 37.0 ± 0.1°C; $t_{10} = 0.65$, $P = 0.53$, unpaired t-test).

Effects of diazepam, SR59230A and indomethacin on social defeat stress-induced hyperthermia

Although diazepam, a benzodiazepine anxiolytic drug, is reported to attenuate hyperthermia elicited by some kinds of stress that provoke anxiety (Zethof *et al.*, 1995), its effect on hyperthermic responses to social defeat stress is unknown. We injected intruder rats i.p. with diazepam 60 min before exposure to social defeat stress. Diazepam injection itself did not affect the post-injection T_c level (30-min average before diazepam, 37.0 ± 0.1°C; 30-min average before stress or control handling, 36.8 ± 0.1°C; $n = 10$ per group, $t_9 = 0.93$, $P = 0.38$, paired t-test), but significantly attenuated the social defeat stress-induced increase in T_c compared to the Vehicle/Stress group across time (Fig. 2A; groups, $F_{3,24} = 30.88$, $P < 0.001$; time, $F_{3,24} = 13.83$, $P < 0.001$; groups × time, $F_{3,72} = 7.86$, $P < 0.001$; two-way repeated measures ANOVA). Diazepam injection significantly reduced social defeat stress-evoked increases in T_c as measured by peak ΔT_c (Stress in Fig. 2B; $t_8 = 3.61$, $P = 0.007$, unpaired t-test), but did not affect the maintenance of T_c levels (Fig. 2A) or peak ΔT_c (Control in Fig. 2B; $t_8 = 1.00$, $P = 0.345$) in control animals, which remained undisturbed instead of being exposed to stress. Baseline T_c did not differ among the groups (Fig. 2A; $F_{3,16} = 0.17$, $P = 0.92$, one-way ANOVA).

To determine whether BAT thermogenesis contributes to the social defeat stress-induced hyperthermia, we next examined the effect of blockade of the β_3-adrenoreceptor, which is primarily expressed in BAT (Muzzin *et al.*, 1991) and is the predominant adrenoreceptor subtype that

mediates BAT thermogenesis (Zhao *et al*., 1994). SR59230A, a selective β_3-adrenoreceptor antagonist (Manara *et al*., 1996; Nisoli *et al*., 1996; Sprague *et al*., 2004; Bexis & Docherty, 2009), was injected i.p. 30 min prior to the exposure to social defeat stress. Injection of this drug did not affect the T_c level (30-min average before SR59230A, 37.2 ± 0.04°C; 30-min average before stress or control handling, 37.1 ± 0.04°C, $n = 10$ per group, $t_9 = 1.23$, $P = 0.25$, paired *t*-test), but significantly attenuated the social defeat stress-evoked increase in T_c compared to the Vehicle/Stress group both across time (Fig. 2C; groups, $F_{3,24} = 91.40$, $P < 0.001$; time, $F_{3,24} = 32.96$, $P < 0.001$; groups × time, $F_{3,72} = 14.41$, $P < 0.001$; two-way repeated measures ANOVA) and also as measured via peak ΔT_c (Stress in Fig. 2D; $t_8 = 6.18$, $P < 0.001$, unpaired *t*-test). SR59230A injection did not affect the maintenance of T_c levels in control animals (Fig. 2C) or their peak ΔT_c (Control in Fig. 2D; $t_8 = 0.34$, $P = 0.74$). Baseline T_c did not differ among the groups (Fig. 2C; $F_{3,16} = 2.11$, $P = 0.14$, one-way ANOVA).

Although psychological stress induces hyperthermic responses similar to fever, previous studies have suggested differences in the mechanisms of PSH and fever (Oka *et al*., 2001). Fever is triggered by an action of PGE_2, a pyrogenic mediator produced centrally in response to immune signals during infection (Matsumura *et al*., 1998; Lazarus *et al*., 2007) and PGE receptor-deficient mice fail to develop LPS-induced fever, but exhibit PSH (Oka *et al*., 2003). Therefore, we examined whether social defeat stress-induced hyperthermia involves a PGE_2-mediated mechanism by testing the effect of indomethacin, an inhibitor of the rate-limiting enzyme for PGE_2 synthesis, cyclooxygenase, on the stress-induced increase in T_c. Social defeat stress significantly increased T_c compared to control handling (Fig. 2E; groups, $F_{3,24} = 38.75$, $P < 0.001$; time, $F_{3,24} = 32.74$, $P < 0.001$; groups × time, $F_{3,72} = 11.50$, $P <$

0.001; two-way repeated measures ANOVA). However, rats that received an i.p. injection of either indomethacin or vehicle exhibited a comparable rise in T_c in response to stress (Stress in Fig. 2F; $t_8 = 0.61$, $P = 0.56$; unpaired t-test). Indomethacin did not affect the maintenance of T_c levels in control animals, either (Control in Fig. 2F; $t_8 = 0.59$, $P = 0.57$). In contrast, the same dose of indomethacin exerted a prominent antipyretic effect on fever evoked by LPS: there was a significant difference in T_c across time between LPS-injected and LPS/indomethacin-injected groups (Fig. 2G; groups, $F_{1,14} = 97.96$, $P < 0.001$; time, $F_{1,14} = 24.76$, $P < 0.001$; groups × time, $F_{1,24} = 23.21$, $P < 0.001$; two-way repeated measures ANOVA). Post-injection peak ΔT_c in the group injected with LPS and indomethacin was significantly lower than that in the LPS-injected group (Fig. 2H; $t_8 = 11.60$, $P < 0.001$, unpaired t-test). This antipyretic effect of indomethacin is consistent with other studies (Wilkinson & Kasting, 1993; Zampronio et al. 1995; Steiner & Branco, 2000). These results indicate that social defeat stress-induced hyperthermia is sensitive to anxiolytic drugs and involves non-shivering thermogenesis in BAT, but does not require PGE_2.

Induction of Fos expression in the medullary raphe region in response to social defeat stress

We next investigated whether social defeat stress signals activate neurons in medullary raphe subregions by immunohistochemically detecting Fos expression and by comparing the numbers of Fos-expressing neurons between intruder rats that received social defeat stress and those that received sham handling ($n = 4$ per group). Baseline T_c of these groups was comparable (sham, 37.0 ± 0.1°C; stress, 37.0 ± 0.2°C; $t_6 = 0.06$, $P = 0.96$, unpaired t-test), but the stress group exhibited a significantly larger increase in T_c during the stress period than the sham group (peak ΔT_c:

sham, 0.4 ± 0.1°C; stress, 1.9 ± 0.3°C; t_6 = 5.45, P = 0.002, unpaired t-test).   As shown in Figure 3A, the stressed animals exhibited remarkable expression of Fos in the rRPa and the adjacent RMg subregion, whereas the same medullary area of the sham-handled animals contained few Fos-immunoreactive cells.   Counting Fos-immunoreactive cells in ventromedial medullary subregions of these two groups showed that social defeat stress remarkably increased Fos-immunoreactive cells in the rostral medullary raphe subregions: the rRPa and RMg/PPy (Fig. 3B; rRPa, t_6 = 15.87, P < 0.001; RMg/PPy, t_6 = 16.60, P < 0.001; unpaired t-test).   A significant increase in Fos-immunoreactive cells in response to social defeat stress was also observed in the ventral surface of the pyramidal tract throughout the rostrocaudal axis of the medulla oblongata (Fig. 3B; t_6 = 7.45, P < 0.001).   Although a stress-evoked increase in Fos-expressing cells was also significant in the cRPa (Fig. 3B; t_6 = 4.13, P = 0.006), the number of Fos-immunoreactive cells was small even in the stress group (19.8 ± 3.0 cells, counted in every sixth 30-μm-think section).   The number of Fos-immunoreactive cells in the ROb/Giv was not significantly different between the groups (Fig. 3B; t_6 = 2.44, P = 0.051).

Social defeat stress-induced Fos expression in VGLUT3- or TPH-positive neurons in the medullary raphe region

We further examined whether the medullary raphe cells that expressed Fos in response to social defeat stress were VGLUT3-expressing neurons by double-immunolabeling for Fos and VGLUT3.   Medullary raphe neurons expressing VGLUT3 were distributed at the rostrocaudal levels between the rostral end of the facial nucleus and the caudal end of the fourth ventricle (Nakamura *et al*., 2004a).   In the stressed animals, Fos immunoreactivity was detected in many VGLUT3-immunoreactive

neurons, which were distributed mostly in the caudal one third of the rRPa and the adjacent RMg (*i.e*. immediately rostral to the rostral pole of the IO; Fig. 4A and B).   Some double-labeled neurons were observed in the areas surrounding the pyramidal tract, which includes the PPy and the ventral surface of the tract (Fig. 4B).   A few double-labeled neurons were also found in the caudal medullary raphe subregions, cRPa and ROb/Giv, where VGLUT3 neurons were distributed sparsely (Fig. 4B). In contrast, such double-labeled cells were few in the sham-handled animals (Fig. 4A and B).   Quantitative and statistical analyses showed that social defeat stress, compared with sham handling, significantly increased the Fos-expressing population in VGLUT3-immunoreactive neurons in all the medullary raphe subregions that we observed (Fig. 4C; rRPa, t_6 = 14.89, P < 0.001; RMg/PPy, t_6 = 13.61, P < 0.001; ventral surface of pyramidal tract, t_6 = 9.70, P < 0.001; cRPa, t_6 = 4.98, P = 0.003; ROb/Giv, t_6 = 3.27, P = 0.017; unpaired t-test).

We also measured social defeat stress-induced Fos expression in serotonergic neurons in the medullary raphe subregions by double-immunolabeling for Fos and TPH, a marker for serotonergic neurons.   In the stressed animals, overall expression of Fos in TPH-immunoreactive neurons was much lower than that in VGLUT3-immunoreactive neurons (Fig. 4B and E), although some Fos-expressing, TPH-immunoreactive neurons were found in the rRPa and RMg (Fig. 4D and E).   Supporting these observations, social defeat stress significantly increased Fos expression in TPH-immunoreactive neurons in the rRPa and RMg/PPy, but not in the other medullary raphe subregions (Fig. 4F; rRPa, t_6 = 4.72, P = 0.003; RMg/PPy, t_6 = 3.70, P = 0.01; ventral surface of pyramidal tract, t_6 = 0.63, P = 0.55; cRPa, t_6 = 1.15, P = 0.29; ROb/Giv, t_6 = 2.38, P = 0.055; unpaired t-test).

Effect of diazepam on social defeat stress-induced Fos expression

To determine whether the medullary raphe Fos expression induced by social defeat stress can be reduced by diazepam, we examined Fos expression in the rRPa, the RMg/PPy and the ventral surface of the pyramidal tract, where remarkable Fos expression was induced by social defeat stress (Fig. 3B), in Vehicle/Stress ($n = 5$), Diazepam/Stress ($n = 7$) and Diazepam/Control ($n = 4$) groups. Baseline T_c of these groups was comparable (Vehicle/Stress, 37.1 ± 0.1°C; Diazepam/Stress, 37.0 ± 0.1°C; Diazepam/Control, 36.9 ± 0.1°C; ($F_{2,13} = 1.10$, $P = 0.36$, one-way ANOVA). Consistent with the diazepam-injection experiment described above (Fig. 2A and B), animals that received a prior injection of diazepam exhibited a significantly attenuated increase in T_c during the stress period compared to those that received a vehicle injection (peak ΔT_c: Vehicle/Stress, 2.6 ± 0.1°C; Diazepam/Stress, 0.9 ± 0.1°C; $t_{10} = 7.94$, $P < 0.001$, unpaired t-test). Changes in T_c of the Diazepam/Control group during the control period were small (peak ΔT_c: 0.1 ± 0.1°C). The Vehicle/Stress group exhibited expression of Fos in many neurons distributed in the medullary raphe subregions and the numbers of Fos-immunoreactive cells in these subregions were significantly reduced following diazepam injection as seen in the Diazepam/Stress group (Fig. 5A and B; one-way ANOVA: rRPa, $F_{2,13} = 22.55$, $P < 0.001$; RMg/PPy, $F_{2,13} = 33.96$, $P < 0.001$; ventral surface of pyramidal tract, $F_{2,13} = 13.57$, $P = 0.001$). The numbers of Fos-immunoreactive cells in the Diazepam/Stress group were all comparable to those in the Diazepam/Control group (Fig. 5A and B).

To examine whether the diazepam-sensitive, stress-induced expression of Fos occurs in VGLUT3-expressing neurons or in serotonergic neurons, we performed double immunohistochemical staining for Fos and VGLUT3 or TPH in the rRPa, the RMg/PPy and the

ventral surface of the pyramidal tract in the Vehicle/Stress, Diazepam/Stress and Diazepam/Control groups. In the Vehicle/Stress group, Fos immunoreactivity was detected in 49–63% of VGLUT3-immunoreactive neurons (Fig. 5C) and in 5–19% of TPH-immunoreactive neurons (Fig. 5D) in these medullary raphe subregions, consistent with the social defeat stress-induced expression of Fos in these neuronal populations (Fig. 4). Diazepam injection prior to the stress exposure (Diazepam/Stress) significantly reduced the stress-induced expression of Fos in VGLUT3-immunoreactive neurons to levels comparable to those observed in the Diazepam/Control group (Fig. 5C; one-way ANOVA: rRPa, $F_{2,13} = 122.8$, $P < 0.001$; RMg/PPy, $F_{2,13} = 103.3$, $P < 0.001$; ventral surface of pyramidal tract, $F_{2,13} = 236.0$, $P < 0.001$). Diazepam injection also reduced the stress-induced expression of Fos in TPH-immunoreactive neurons in the rRPa and the RMg/PPy to levels comparable to those observed in the Diazepam/Control group, but had no effect on Fos expression in TPH-immunoreactive neurons in the ventral surface of the pyramidal tract (Fig. 5D; one-way ANOVA: rRPa, $F_{2,13} = 36.22$, $P < 0.001$; RMg/PPy, $F_{2,13} = 17.82$, $P < 0.001$; ventral surface of pyramidal tract, $F_{2,13} = 3.66$, $P = 0.055$).

Discussion

The present study revealed that social defeat stress elicits activation of VGLUT3-expressing sympathetic premotor neurons in the rostral medullary raphe region. Sympathetic premotor neurons play a pivotal role in the bulbospinal transmission for controlling various effector responses related to homeostatic regulation. We previously identified VGLUT3-expressing rostral medullary raphe neurons as sympathetic premotor neurons that mediate febrile and cold-defensive thermal responses (Nakamura *et al.*, 2004a). Transsynaptic retrograde tracing

from peripheral sympathetic effectors using pseudorabies virus showed that many VGLUT3-expressing medullary raphe neurons innervate sympathetic preganglionic neurons that control BAT or skin blood vessels, the two major thermoregulatory effectors (Nakamura *et al*., 2004a). A dominant population of these VGLUT3-expressing neurons are activated in response to central administration of PGE$_2$ or to cold exposure of animals (Nakamura *et al*., 2004a) and inhibition of neurons in this raphe region blocks sympathetic thermogenesis in BAT and constriction of skin blood vessels that are evoked by central injection of PGEs or by body cooling (Nakamura *et al*., 2002; Morrison, 2003; Korsak & Gilbey, 2004; Ootsuka *et al*., 2004; Nakamura & Morrison, 2007; Rathner *et al*., 2008). Furthermore, stimulation of neurons in this raphe region elicits BAT thermogenesis and cutaneous vasoconstriction (Blessing *et al*., 1999; Morrison, 1999; Rathner & McAllen, 1999), leading to an increase in T_c. These findings indicate the essential role of the sympathetic premotor neurons in the rostral medullary raphe region in driving the sympathetic thermal responses for fever development and cold defense. In the present study, the anxiolytic agent, diazepam attenuated both the social defeat stress-induced hyperthermia and the stress-induced Fos expression in rostral medullary raphe neurons. Based on the present results, we propose that social defeat stress induces hyperthermia by driving BAT thermogenesis and cutaneous vasoconstriction through activation of the medullary raphe sympathetic premotor neurons.

In support of the involvement of BAT thermogenesis in social defeat stress-induced hyperthermia, SR59230A reduced the stress-evoked increase in T_c. The β_3-adrenoreceptor is expressed primarily in BAT (Muzzin *et al*., 1991) and mediates a dominant part of noradrenergic activation of BAT thermogenesis (Zhao *et al*., 1994). Therefore, the

attenuation of the stress-induced hyperthermia by SR59230A is likely caused by inhibition of BAT thermogenesis through the blockade of β_3-adrenoreceptors therein.   However, the β_3-adrenoreceptor is also very weakly expressed in skeletal muscles (Evans *et al.*, 1996) and a β_3-adrenoreceptor agonist increased oxygen consumption in isolated rat hindlimb muscles without changing blood flow rate (Ye *et al.*, 1995). Therefore, inhibition of non-shivering thermogenesis in skeletal muscles by SR59230A might also contribute to the attenuation of social defeat stress-induced hyperthermia.   The incomplete attenuation by SR59230A might be due to the involvement of other effector responses in the stress-induced hyperthermia, such as cutaneous vasoconstriction, which is mediated by α-adrenoreceptors (Roberts *et al.*, 2002).

The present findings highlight the role of the VGLUT3-expressing medullary raphe neurons in the bulbospinal signaling for PSH.   Because VGLUT3 accumulates glutamate into vesicles (Takamori, 2006), VGLUT3-expressing sympathetic premotor neurons in the medullary raphe region potentially release glutamate in the intermediolateral cell column (IML) of the thoracic spinal cord, where sympathetic preganglionic neurons are located.   Consistent with this notion, VGLUT3-immunoreactive axon terminals in the IML, observed with immunoelectronmicroscopy, were found to form asymmetric synapses (Nakamura *et al.*, 2004b), which are characteristic of excitatory synapses, although VGLUT3 was also found in GABAergic raphe-spinal neurons providing symmetric terminals in the IML (Stornetta *et al.*, 2005). Furthermore, the BAT thermogenic response evoked by stimulation of rRPa neurons is blocked by application of glutamate receptor antagonists into the thoracic IML and nanoinjections of glutamate into the IML elicit BAT thermogenesis (Nakamura *et al.*, 2004a).   Together with the present results, these lines of evidence suggest that glutamate is an

essential neurotransmitter in the raphe-spinal sympathetic premotor signaling, which is probably mediated by the VGLUT3-expressing neurons, for driving thermal responses including PSH.

In contrast to the large number of VGLUT3-expressing medullary raphe neurons that were activated by social defeat stress, the number of activated serotonergic neurons in this region was limited. This result is consistent with our previous observations that central application of PGE_2 induced Fos expression in 70–80% of VGLUT3-expressing rostral medullary raphe neurons (Nakamura *et al*., 2004a), but did not induce significant Fos expression in serotonergic neurons (Nakamura *et al*., 2002). Since 10–15% of VGLUT3-immunoreactive neurons in the rostral medullary raphe region contain serotonin (Nakamura *et al*., 2004a), it is likely that such VGLUT3-expressing serotonergic neurons in the rRPa and RMg/PPy were activated in response to social defeat stress in the present study. Both serotonergic and VGLUT3-expressing populations of medullary raphe neurons innervate sympathetic preganglionic neurons in the IML (Loewy, 1981; Nakamura *et al*., 2004a) and 6–22% (depending on the thoracic levels) of VGLUT3-containing axon terminals in the IML also contain serotonin (Nakamura *et al*., 2004b). Taken together, a population of VGLUT3-expressing neurons in the rostral medullary raphe region, although not predominant, likely release both glutamate and serotonin in the IML. Since nanoinjection of serotonin into the thoracic IML does not evoke rapid BAT thermogenesis (Madden & Morrison, 2006), serotonin by itself seems unlikely to have a potency to depolarize sympathetic preganglionic neurons controlling BAT. However, serotonin can play a modulatory role in the IML by potentiating the effect of excitatory glutamatergic input to sympathetic preganglionic neurons controlling BAT (Madden & Morrison, 2006) and

subcutaneous administration of a serotonin$_{2A}$ receptor antagonist suppresses social defeat stress-induced hyperthermia (Beig *et al*., 2009).

Sympathetic thermogenesis in BAT and cutaneous vasoconstriction for fever and cold defense are driven through efferent neural pathways from the preoptic area (POA), a febrile and thermoregulatory center (For reviews, see Nakamura, 2004; Nakamura *et al*., 2005a; Morrison *et al*., 2008). Many studies support a model of the efferent mechanism, in which tonic GABAergic inhibition from the POA regulates the activity of neurons in the dorsomedial hypothalamus (DMH) and those in the rostral medullary raphe region. This tonic inhibition can be attenuated by an action of PGE_2 in the POA (for fever) or by a cold-sensory afferent input from the skin to the POA (for cold defense) and thereby, disinhibits sympathoexcitatory neurons in the DMH. The activated DMH neurons then provide an excitatory input to activate sympathetic premotor neurons in the rostral medullary raphe region, which then drive BAT thermogenesis and cutaneous vasoconstriction (Nakamura *et al*., 2002, 2004a, 2005b; Zaretskaia *et al*., 2003; Madden & Morrison, 2003, 2004; Ootsuka *et al*., 2004; Nakamura & Morrison, 2007, 2008a, 2008b, 2010; Rathner *et al*., 2008). Consistent with this model of the efferent mechanism for fever, LPS injection induces Fos expression in the DMH and this Fos expression is reduced by indomethacin (Lacroix & Rivest, 1997).

However, it has been uncertain whether PSH is mediated by a similar efferent mechanism because there are differences in sensitivities to drugs between fever and some kinds of PSH. Anxiolytic drugs such as diazepam can attenuate hyperthermic responses to many types of psychological stress, yet have no effect on fever (Zethof *et al*., 1995; Olivier *et al*., 2003; Vinkers *et al*., 2009). In contrast, cyclooxygenase inhibitors can attenuate fever, but have no effect on many kinds of PSH

(Wilkinson & Kasting, 1993; Zampronio *et al.*, 1995; Steiner & Branco, 2000; Vinkers *et al.*, 2009). Our study demonstrates that the pharmacological properties of social defeat stress-induced hyperthermia are shared with many kinds of PSH, but not with fever. Nevertheless, the social defeat stress-induced activation of a large population of VGLUT3-expressing rostral medullary raphe neurons strongly supports the idea that sympathetic thermal effector responses to psychological stress, pyrogens and cooling all share the same outflow pathways, at least from the premotor level.

In the present study, systemic injection of diazepam attenuated both social defeat stress-induced hyperthermia and activation of VGLUT3-expressing neurons and serotonergic neurons in the rostral medullary raphe region, further supporting the role of the medullary raphe sympathetic premotor neurons in social defeat stress-induced hyperthermia. We chose the minimum dose of diazepam that exerted significant inhibitory effect on the stress-induced hyperthermia. Although diazepam can cause hypothermia by increasing skin blood flow (Echizenya *et al.*, 2003), the present dose did not affect the basal T_c level. It seems unlikely that the attenuation of the stress-induced hyperthermia by diazepam was caused by a direct inhibitory action of the drug on medullary raphe sympathetic premotor neurons. If diazepam directly inhibited these neurons, it would have caused hypothermia, as was observed following microinjection of muscimol, a neuronal inhibitor, into the rRPa of conscious rats (Zaretsky *et al.*, 2003). Therefore, the diazepam-induced reduction of stress-induced Fos expression in the rostral medullary raphe region was likely caused by an action of this drug at a site antecedent to the sympathetic premotor neurons. Because diazepam does not block the mechanism of fever (Vinkers *et al.*, 2009), the major targeting site(s) of this drug is most likely in the stress-

processing brain structures, such as the limbic system, rather than on the febrile efferent pathways from the POA to thermal effectors.

Of interest is which brain sites relay psychological stress signals to activate these sympathetic premotor neurons. One of the candidates is the DMH. There is a direct projection from the DMH to the rostral medullary raphe region (Hermann *et al*., 1997; Samuels *et al*., 2004; Nakamura *et al*., 2005b; Yoshida *et al*., 2009) and DMH neurons that project to the rostral medullary raphe region are activated (express Fos) in response to air-jet or restraint stress (Sarkar *et al*., 2007). Inhibition of DMH neurons with muscimol injections suppresses tachycardic and pressor responses to air-jet stress (Stotz-Potter *et al*., 1996), although the effect of such inhibition on stress-induced hyperthermia remains to be tested. Stimulation of DMH neurons induces Fos expression in the rostral medullary raphe region (Zaretskaia *et al*., 2008) and elicits BAT thermogenesis that can be blocked by inhibition of rostral medullary raphe neurons (Cao *et al*., 2004). Stimulation of DMH neurons also elicits cutaneous vasoconstriction (Rathner *et al*., 2008). These findings support the view that the direct pathway from the DMH to the rostral medullary raphe region delivers stress-driven hyperthermic signals to activate sympathetic premotor neurons controlling thermal effectors. Another brain site that could provide an input to the medullary raphe sympathetic premotor neurons is the POA. A direct GABAergic projection from the POA to the rostral medullary raphe region has been proposed to mediate febrile responses through the inhibitory regulation of the activity of the sympathetic premotor neurons (Nakamura *et al*., 2002), especially the ones driving cutaneous vasoconstriction (Rathner *et al*., 2008; Nakamura *et al*., 2009). However, the involvement of the POA in psychological stress-induced autonomic responses is unknown.

There is one primary limitation of the current study. We cannot necessarily generalize our findings obtained by social defeat stress to the mechanisms of all kinds of PSH. For example, conditioned fear stress is reported to increase T_c without an involvement of interscapular BAT (Marks *et al*., 2009). In accordance with this, conditioned fear stress does not induce Fos expression in the rostral medullary raphe region, but does in spinally projecting neurons in the perifornical area and paraventricular nucleus of the hypothalamus and in the A5 noradrenergic area of the pons (Carrive & Gorissen, 2008). These findings suggest that conditioned fear stress elicits hyperthermia through a central mechanism different from that for social defeat stress-induced hyperthermia. The reason we employed social defeat stress as a psychological stress model is that this stress is caused by social interaction and as such may be closer to stress in human society compared to more artificial stressors, such as needle injection or placement into a new cage (Vinkers *et al*., 2009). Therefore, we consider the social defeat stress model more useful to understand the development of chronic hyperthermia caused by psychosocial stress in humans – called "psychogenic fever" by clinicians (McNeil *et al*., 1984; Timmerman *et al*., 1992; Oka & Oka, 2007; Hiramoto *et al*., 2009; Kaneda *et al*., 2009). The autonomic efferent mechanism proposed in the present study might be relevant to the mechanisms underlying such clinically observed stress symptoms.

In summary, the present study provides an important piece of evidence that sympathetic hyperthermic effector responses to social defeat stress are driven through activation of sympathetic premotor neurons in the rostral medullary raphe region. Taken together with previous findings on sympathetic premotor pathways for fever and cold defense, the present study suggests that thermoregulatory responses to

some types of psychological stress, infection (pyrogens) and cooling all share the common medullospinal sympathetic output mechanisms. However, further studies are awaited to determine how psychological stress signals, potentially from the limbic system, enter the thermoregulatory efferent pathways between the POA and the rostral medullary raphe region.

Acknowledgements

Abbreviations

BAT, brown adipose tissue; cRPa, caudal raphe pallidus nucleus; DAB, 3,3'-diaminobenzidine tetrahydrochloride; DMH, dorsomedial hypothalamus; Giv, ventral part of the gigantocellular reticular nucleus; IML, intermediolateral cell column; IO, inferior olivary complex; LPS, lipopolysaccharide; PG, prostaglandin; POA, preoptic area; PPy, parapyramidal region; PSH, psychological stress-induced hyperthermia; RMg, raphe magnus nucleus; ROb, raphe obscurus nucleus; rRPa, rostral raphe pallidus nucleus; T_c, body core temperature; TPH, tryptophan hydroxylase; VGLUT3, vesicular glutamate transporter 3

References

Beig, M.I., Baumert, M., Walker, F.R., Day, T.A. & Nalivaiko, E. (2009) Blockade of 5-HT2A receptors suppresses hyperthermic but not cardiovascular responses to psychosocial stress in rats. *Neuroscience*, **159**, 1185–1191.

Bexis, S. & Docherty, J.R. (2009) Role of α_1- and β_3-adrenoceptors in the modulation by SR59230A of the effects of MDMA on body temperature in the mouse. *Br. J. Pharmacol.*, **158**, 259–266.

Blessing, W.W., Yu, Y.H. & Nalivaiko, E. (1999) Raphe pallidus and parapyramidal neurons regulate ear pinna vascular conductance in the rabbit. *Neurosci. Lett.*, **270**, 33–36.

Briese, E. & De Quijada, M.G. (1970) Colonic temperature of rats during handling. *Acta Physiol. Lat. Am.*, **20**, 97–102.

Burman, K.J., Ige, A.O., White, J.H., Marshall, F.H., Pangalos, M.N., Emson, P.C., Minson, J.B. & Llewellyn-Smith, I.J. (2003) GABA$_B$ receptor subunits, R1 and R2, in brainstem catecholamine and serotonin neurons. *Brain Res.*, **970**, 35–46.

Cao, W.H., Fan, W. & Morrison, S.F. (2004) Medullary pathways mediating specific sympathetic responses to activation of dorsomedial hypothalamus. *Neuroscience*, **126**, 229–240.

Carrive, P. & Gorissen, M. (2008) Premotor sympathetic neurons of conditioned fear in the rat. *Eur. J. Neurosci.*, **28**, 428–446.

Echizenya, M., Mishima, K., Satoh, K., Kusanagi, H., Sekine, A., Ohkubo, T., Shimizu, T. & Hishikawa, Y. (2003) Heat loss, sleepiness, and impaired performance after diazepam administration in humans. *Neuropsychopharmacology*, **28**, 1198–1206.

Evans, B.A., Papaioannou, M., Bonazzi, V.R. & Summers, R.J. (1996) Expression of β_3-adrenoceptor mRNA in rat tissues. *Br. J. Pharmacol.*, **117**, 210–216.

Hayashida, S., Oka, T., Mera, T. & Tsuji, S. (2010) Repeated social defeat stress induces chronic hyperthermia in rats. *Physiol. Behav.*, **101**, 124–131.

Hermann, D.M., Luppi, P.H., Peyron, C., Hinckel, P. & Jouvet, M. (1997) Afferent projections to the rat nuclei raphe magnus, raphe pallidus and reticularis gigantocellularis pars α demonstrated by iontophoretic application of choleratoxin (subunit b). *J. Chem. Neuroanat.*, **13**, 1–21.

Hioki, H., Fujiyama, F., Nakamura, K., Wu, S.X., Matsuda, W. & Kaneko, T. (2004) Chemically specific circuit composed of vesicular glutamate transporter 3- and preprotachykinin B-producing interneurons in the rat neocortex. *Cereb. Cortex*, **14**, 1266–1275.

Hiramoto, T., Oka, T., Yoshihara, K. & Kubo, C. (2009) Pyrogenic cytokines did not mediate a stress interview-induced hyperthermic response in a patient with psychogenic fever: a case report. *Psychosom. Med.*, **71**, 932–936.

Hodges, M.R., Tattersall, G.J., Harris, M.B., McEvoy, S.D., Richerson, D.N., Deneris, E.S., Johnson, R.L., Chen, Z.F. & Richerson, G.B. (2008) Defects in breathing and thermoregulation in mice with near-complete absence of central serotonin neurons. *J. Neurosci.*, **28**, 2495–2505.

Kaneda, Y., Tsuji, S. & Oka, T. (2009) Age distribution and gender differences in psychogenic fever patients. *Biopsychosoc. Med.*, **3**, 6.

Korsak, A. & Gilbey, M.P. (2004) Rostral ventromedial medulla and the control of cutaneous vasoconstrictor activity following i.c.v. prostaglandin E_1. *Neuroscience*, **124**, 709–717.

Lacroix, S. & Rivest, S. (1997) Functional circuitry in the brain of immune-challenged rats: partial involvement of prostaglandins. *J. Comp. Neurol.* **387**, 307–324.

Lazarus, M., Yoshida, K., Coppari, R., Bass, C.E., Mochizuki, T., Lowell, B.B. & Saper, C.B. (2007) EP3 prostaglandin receptors in the median preoptic nucleus are critical for fever responses. *Nat. Neurosci.*, **10**, 1131–1133.

Loewy, A.D. (1981) Raphe pallidus and raphe obscurus projections to the intermediolateral cell column in the rat. *Brain Res.*, **222**, 129–133.

Madden, C.J. & Morrison, S.F. (2003) Excitatory amino acid receptor activation in the raphe pallidus area mediates prostaglandin-evoked thermogenesis. *Neuroscience*, **122**, 5–15.

Madden, C.J. & Morrison, S.F. (2004) Excitatory amino acid receptors in the dorsomedial hypothalamus mediate prostaglandin-evoked thermogenesis in brown adipose tissue. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **286**, R320–R325.

Madden, C.J. & Morrison, S.F. (2006) Serotonin potentiates sympathetic responses evoked by spinal NMDA. *J. Physiol.*, **577**, 525–537.

Manara, L., Badone, D., Baroni, M., Boccardi, G., Cecchi, R., Croci, T., Giudice, A., Guzzi, U., Landi, M. & Le Fur, G. (1996) Functional identification of rat atypical β-adrenoceptors by the first β_3-selective antagonists, aryloxypropanolaminotetralins. *Br. J. Pharmacol.*, **117**, 435–442.

Marks, A., Vianna, D.M.L. & Carrive, P. (2009) Nonshivering thermogenesis without interscapular brown adipose tissue involvement during conditioned fear in the rat. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **296**, R1239–R1247.

Matsumura, K., Cao, C., Ozaki, M., Morii, H., Nakadate, K. & Watanabe, Y. (1998) Brain endothelial cells express cyclooxygenase-2 during lipopolysaccharide-induced fever: light and electron microscopic immunocytochemical studies. *J. Neurosci.*, **18**, 6279–6289.

McNeil, G.N., Leighton, L.H. & Elkins, A.M. (1984) Possible psychogenic fever of 103.5 degrees F in a patient with borderline personality disorder. *Am. J. Psychiatry.*, **141**, 896–897.

Miczek, K.A., Thompson, M.L. & Shuster, L. (1982) Opioid-like analgesia in defeated mice. *Science*, **215**, 1520–1522.

Morrison, S.F. (1999) RVLM and raphe differentially regulate sympathetic outflows to splanchnic and brown adipose tissue. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **276**, R962–R973.

Morrison, S.F. (2003) Raphe pallidus neurons mediate prostaglandin E$_2$-evoked increases in brown adipose tissue thermogenesis. *Neuroscience*, **121**, 17–24.

Morrison, S.F., Nakamura, K. & Madden, C.J. (2008) Central control of thermogenesis in mammals. *Exp. Physiol.*, **93**, 773–797.

Mulkey, D.K., Rosin, D.L., West, G., Takakura, A.C., Moreira, T.S., Bayliss, D.A. & Guyenet, P.G. (2007) Serotonergic neurons activate chemosensitive retrotrapezoid nucleus neurons by a pH-independent mechanism. *J. Neurosci.*, **27**, 14128–14138.

Muzzin, P., Revelli, J.P., Kuhne, F., Gocayne, J.D., McCombie, W.R., Venter, J.C., Giacobino, J.P. & Fraser, C.M. (1991) An adipose tissue-specific β-adrenergic receptor. Molecular cloning and down-regulation in obesity. *J. Biol. Chem.*, **266**, 24053–24058.

Nakamura, K. (2004) Fever-inducing sympathetic neural pathways. *J. Therm. Biol.*, **29**, 339–344.

Nakamura, K., Kaneko, T., Yamashita, Y., Hasegawa, H., Katoh, H. & Negishi, M. (2000) Immunohistochemical localization of prostaglandin EP3 receptor in the rat nervous system. *J. Comp. Neurol.*, **421**, 543–569.

Nakamura, K., Matsumura, K., Hübschle, T., Nakamura, Y., Hioki, H., Fujiyama, F., Boldogköi, Z., König, M., Thiel, H.J., Gerstberger, R.,

Kobayashi, S. & Kaneko, T. (2004a) Identification of sympathetic premotor neurons in medullary raphe regions mediating fever and other thermoregulatory functions. *J. Neurosci.*, **24**, 5370–5380.

Nakamura, K., Matsumura, K., Kaneko, T., Kobayashi, S., Katoh, H. & Negishi, M. (2002) The rostral raphe pallidus nucleus mediates pyrogenic transmission from the preoptic area. *J. Neurosci.*, **22**, 4600–4610.

Nakamura, K., Matsumura, K., Kobayashi, S. & Kaneko, T. (2005a) Sympathetic premotor neurons mediating thermoregulatory functions. *Neurosci. Res.*, **51**, 1–8.

Nakamura, K. & Morrison, S.F. (2007) Central efferent pathways mediating skin cooling-evoked sympathetic thermogenesis in brown adipose tissue. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **292**, R127–R136.

Nakamura, K. & Morrison, S.F. (2008a) A thermosensory pathway that controls body temperature. *Nat. Neurosci.*, **11**, 62–71.

Nakamura, K. & Morrison, S.F. (2008b) Preoptic mechanism for cold-defensive responses to skin cooling. *J. Physiol.*, **586**, 2611–2620.

Nakamura, K. & Morrison, S.F. (2010) A thermosensory pathway mediating heat-defense responses. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 8848–8853.

Nakamura, K., Wu, S.X., Fujiyama, F., Okamoto, K., Hioki, H. & Kaneko, T. (2004b) Independent inputs by VGLUT2- and VGLUT3-positive glutamatergic terminals onto rat sympathetic preganglionic neurons. *NeuroReport*, **15**, 431–436.

Nakamura, Y., Nakamura, K., Matsumura, K., Kobayashi, S., Kaneko, T. & Morrison, S.F. (2005b) Direct pyrogenic input from prostaglandin EP3 receptor-expressing preoptic neurons to the dorsomedial hypothalamus. *Eur. J. Neurosci.*, **22**, 3137–3146.

Nakamura, Y., Nakamura, K. & Morrison, S.F. (2009) Different populations of prostaglandin EP3 receptor-expressing preoptic neurons project to two fever-mediating sympathoexcitatory brain regions. *Neuroscience*, **161**, 614–620.

Nisoli, E., Tonello, C., Landi, M. & Carruba, M.O. (1996) Functional studies of the first selective β_3-adrenergic receptor antagonist SR 59230A in rat brown adipocytes. *Mol. Pharmacol.*, **49**, 7–14.

Oka, T. & Oka, K. (2007) Age and gender differences of psychogenic fever: a review of the Japanese literature. *Biopsychosoc. Med.*, **1**, 1–5.

Oka, T., Oka, K. & Hori, T. (2001) Mechanisms and mediators of psychological stress-induced rise in core temperature. *Psychosom. Med.*, **63**, 476–486.

Oka, T., Oka, K., Kobayashi, T., Sugimoto, Y., Ichikawa, A., Ushikubi, F., Narumiya, S. & Saper, C.B. (2003) Characteristics of thermoregulatory and febrile responses in mice deficient in prostaglandin EP1 and EP3 receptors. *J. Physiol.*, **551**, 945–954.

Olivier, B., Zethof, T., Pattij, T., van Boogaert, M., van Oorschot, R., Leahy, C., Oosting, R., Bouwknecht, A., Veening, J., van der Gugten, J. & Groenink, L. (2003) Stress-induced hyperthermia and anxiety: pharmacological validation. *Eur. J. Pharmacol.*, **463**, 117–132.

Ootsuka, Y., Blessing, W.W. & McAllen, R.M. (2004) Inhibition of rostral medullary raphé neurons prevents cold-induced activity in sympathetic nerves to rat tail and rabbit ear arteries. *Neurosci. Lett.*, **357**, 58–62.

Ootsuka, Y., Blessing, W.W. & Nalivaiko, E. (2008) Selective blockade of 5-HT2A receptors attenuates the increased temperature response in brown adipose tissue to restraint stress in rats. *Stress*, **11**, 125–133.

Paxinos, G. & Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates, 4th edn*. Academic Press, San Diego.

Rathner, J.A., Madden, C.J. & Morrison, S.F. (2008) Central pathway for spontaneous and prostaglandin E$_2$-evoked cutaneous vasoconstriction. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **295**, R343–R354.

Rathner, J.A. & McAllen, R.M. (1999) Differential control of sympathetic drive to the rat tail artery and kidney by medullary premotor cell groups. *Brain Res.* **834**, 196–199.

Roberts, M., Rivers, T., Oliveria, S., Texeira, P. & Raman, E. (2002) Adrenoceptor and local modulator control of cutaneous blood flow in thermal stress. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.*, **131**, 485–496.

Sagar, S.M., Sharp, F.R. & Curran, T. (1988) Expression of *c-fos* protein in brain: metabolic mapping at the cellular level. *Science*, **240**, 1328–1331.

Samuels, B.C., Zaretsky, D.V. & DiMicco, J.A. (2004) Dorsomedial hypothalamic sites where disinhibition evokes tachycardia correlate with location of raphe-projecting neurons. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **287**, R472–R478.

Sarkar, S., Zaretskaia, M.V., Zaretsky, D.V., Moreno, M. & DiMicco, J.A. (2007) Stress- and lipopolysaccharide-induced c-fos expression and nNOS in hypothalamic neurons projecting to medullary raphe in rats: a triple immunofluorescent labeling study. *Eur. J. Neurosci.*, **26**, 2228–2238.

Senba, E., Matsunaga, K., Tohyama, M. & Noguchi, K. (1993) Stress-induced *c-fos* expression in the rat brain: activation mechanism of sympathetic pathway. *Brain Res. Bull.*, **31**, 329–344.

Shannon, H.E. & Herling, S. (1983) Discriminative stimulus effects of diazepam in rats: evidence for a maximal effect. *J. Pharmacol. Exp. Ther.*, **227**, 160–166.

Shibata, H. & Nagasaka, T. (1984) Role of sympathetic nervous system in immobilization- and cold-induced brown adipose tissue thermogenesis in rats. *Jpn. J. Physiol.*, **34**, 103–111.

Singer, R., Harker, C.T., Vander, A.J. & Kluger, M.J. (1986) Hyperthermia induced by open-field stress is blocked by salicylate. *Physiol. Behav.*, **36**, 1179–1182.

Snow, A.E. & Horita, A. (1982) Interaction of apomorphine and stressors in the production of hyperthermia in the rabbit. *J. Pharmacol. Exp. Ther.*, **220**, 335–339.

Sprague, J.E., Brutcher, R.E., Mills, E.M., Caden, D. & Rusyniak, D.E. (2004) Attenuation of 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy)-induced rhabdomyolysis with α_1- plus β_3-adrenoreceptor antagonists. *Br. J. Pharmacol.*, **142**, 667–670.

Steiner, A.A. & Branco, L.G.S. (2000) Central CO-heme oxygenase pathway raises body temperature by a prostaglandin-independent way. *J. Appl. Physiol.*, **88**, 1607–1613.

Stornetta, R.L., Rosin, D.L., Simmons, J.R., McQuiston, T.J., Vujovic, N., Weston, M.C. & Guyenet, P.G. (2005) Coexpression of vesicular glutamate transporter-3 and γ-aminobutyric acidergic markers in rat rostral medullary raphe and intermediolateral cell column. *J. Comp. Neurol.*, **492**, 477–494.

Stotz-Potter, E.H., Willis, L.R. & DiMicco, J.A. (1996) Muscimol acts in dorsomedial but not paraventricular hypothalamic nucleus to suppress cardiovascular effects of stress. *J. Neurosci.*, **16**, 1173–1179.

Sugimoto, N., Shido, O., Sakurada, S. & Nagasaka, T. (1997) Lipopolysaccharide-induced fever in rats prolonged by a needle prick. *Jpn. J. Physiol.*, **47**, 199–204.

Takamori, S. (2006) VGLUTs: 'exciting' times for glutamatergic research? *Neurosci. Res.*, **55**, 343–351.

Takeuchi, Y., Kimura, H. & Sano Y. (1982) Immunohistochemical demonstration of the distribution of serotonin neurons in the brainstem of the rat and cat. *Cell Tissue Res*., **224**, 247–267.

Timmerman, R.J., Thompson, J., Noordzij, H.M. & van der Meer, J.W. (1992) Psychogenic periodic fever. *Neth. J. Med*., **41**, 158–160.

Tornatzky, W. & Miczek, K.A. (1993) Long-term impairment of autonomic circadian rhythms after brief intermittent social stress. *Physiol. Behav*., **53**, 983–993.

Vinkers, C.H., Groenink, L., van Bogaert, M.J.V., Westphal, K.G.C., Kalkman, C.J., van Oorschot, R., Oosting, R.S., Olivier, B. & Korte, S.M. (2009) Stress-induced hyperthermia and infection-induced fever: two of a kind? *Physiol. Behav*., **98**, 37–43.

Wilkinson, M.F. & Kasting, N.W. (1993) Vasopressin release within the ventral septal area of the rat brain during drug-induced antipyresis. *Am. J. Physiol. Regul. Integr. Comp. Physiol*., **264**, R1133–R1138.

Ye, J.M., Clark, M.G. & Colquhoun, E.Q. (1995) Constant-pressure perfusion of rat hindlimb shows α- and β-adrenergic stimulation of oxygen consumption. *Am. J. Physiol. Endocrinol. Metab*., **269**, E960–E968.

Yokoi, Y. (1966) Effect of ambient temperature upon emotional hyperthermia and hypothermia in rabbits. *J. Appl. Physiol*., **21**, 1795–1798.

Yoshida, K., Li, X., Cano, G., Lazarus, M. & Saper, C.B. (2009) Parallel preoptic pathways for thermoregulation. *J. Neurosci*., **29**, 11954–11964.

Zampronio, A.R., Silva, C.A.A., Cunha, F.Q., Ferreira, S.H., Pelá, I.R. & Souza, G.E.P. (1995) Indomethacin blocks the febrile response induced by interleukin-8 in rabbits. *Am. J. Physiol. Regul. Integr. Comp. Physiol*., **269**, R1469–R1474.

Zaretskaia, M.V., Zaretsky, D.V. & DiMicco, J.A. (2003) Role of the dorsomedial hypothalamus in thermogenesis and tachycardia caused by microinjection of prostaglandin E_2 into the preoptic area in anesthetized rats. *Neurosci. Lett.*, **340**, 1–4.

Zaretskaia, M.V., Zaretsky, D.V., Sarkar, S., Shekhar, A. & DiMicco, J.A. (2008) Induction of Fos-immunoreactivity in the rat brain following disinhibition of the dorsomedial hypothalamus. *Brain Res.*, **1200**, 39–50.

Zaretsky, D.V., Zaretskaia, M.V. & DiMicco, J.A. (2003) Stimulation and blockade of $GABA_A$ receptors in the raphe pallidus: effects on body temperature, heart rate, and blood pressure in conscious rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **285**, R110–R116.

Zethof, T.J.J., Van der Heyden, J.A.M., Tolboom, J.T.B.M. & Olivier, B. (1995) Stress-induced hyperthermia as a putative anxiety model. *Eur. J. Pharmacol.*, **294**, 125–135.

Zhao, J., Unelius, L., Bengtsson, T., Cannon, B. & Nedergaard, J. (1994) Coexisting β-adrenoceptor subtypes: significance for thermogenic process in brown fat cells. *Am. J. Physiol. Cell Physiol.*, **267**, C969–C979.

Figure legends

Figure 1.   Changes in T_c in response to social defeat stress

Abdominal temperature was measured in rats that received social defeat stress (filled circle, $n = 6$) or sham handling (open circle, $n = 6$) during the period indicated by the horizontal bar.   *$P < 0.05$, **$P < 0.01$, compared with the sham group (unpaired t-test following a two-way repeated measures ANOVA).

Figure 2.   Effects of diazepam, SR59230A and indomethacin on social defeat stress-induced increase in T_c and effect of indomethacin on LPS-induced fever

A–F, Rats ($n = 5$ per group) received an i.p. injection of diazepam (A and B), SR59230A (C and D), indomethacin (E and F) or their respective vehicles at the time point indicated by arrows (A, C and E) and were subsequently exposed to social defeat stress (Stress) or left undisturbed (Control) during the period indicated by the horizontal bars (A, C and E). B, D and F show peak changes in T_c from their pre-injection baseline values (peak ΔT_c) that were taken during the 60-min stress or control period.   The effects of vehicle and the drugs on the peak ΔT_c within the control or stress groups were compared using an unpaired t-test (**$P < 0.01$, ***$P < 0.001$).   G and H, LPS or a mixture of LPS and indomethacin was injected into rats (i.p., $n = 5$ per group) at the time point indicated by an arrow (G).   H shows post-injection peak ΔT_c. The effect of indomethacin on the LPS-induced rise in T_c was compared using an unpaired t-test (***$P < 0.001$).

Figure 3.   Social defeat stress-induced expression of Fos in the medullary raphe region

A, Fos immunohistochemistry in rostral medullary raphe subregions of rats that received sham handling (Sham) or social defeat stress (Stress). Many cells that exhibited Fos immunoreactivity in their nuclei were distributed in the rRPa and adjacent RMg of stressed animals, whereas such cells were very few in sham-handled animals.　py, pyramidal tract. Scale bar: 200 μm.　B, numbers of Fos-immunoreactive cells in medullary raphe subregions of sham-handled and stressed animals ($n = 4$ per group).　**$P < 0.01$, ***$P < 0.001$, compared with the sham group (unpaired t-test).

Figure 4.　Fos expression in VGLUT3-immunoreactive or TPH-immunoreactive medullary raphe neurons in response to social defeat stress

Social defeat stress-induced Fos expression in VGLUT3-immunoreactive (A–C) or TPH-immunoreactive (D–F) neurons was examined in medullary raphe subregions.　A and D, double immunolabeling for Fos (brown in A and blue-black in D) and VGLUT3 (blue-black in A) or TPH (brown in D) in the rRPa of rats that received sham handling or social defeat stress.　Arrows indicate cell bodies double-labeled for Fos and VGLUT3 (A) or TPH (D) and arrowheads indicate VGLUT3-immunoreactive (A) or TPH-immunoreactive (D) cell bodies that were negative for Fos.　Scale bars: 30 μm.　B and E, distribution of Fos-immunoreactive cells and VGLUT3-immunoreactive cells (B) or TPH-immunoreactive cells (E) in medullary raphe subregions of sham-handled and stressed animals.　One-in-six series of 30-μm-thick frontal sections are aligned in a rostrocaudal order.　DAO, dorsal accessory olivary nucleus; MAO, medial accessory olivary nucleus; PIO, principal inferior olivary nucleus.　Scale bars: 500 μm.　C and F, percentages of Fos-immunoreactive neurons in VGLUT3-positive (C) or TPH-positive (F)

populations in medullary raphe subregions of sham-handled and stressed animals ($n = 4$ per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with the sham group (unpaired t-test).

Figure 5.   Effect of diazepam on social defeat stress-induced expression of Fos in the rostral medullary raphe region

A, Fos immunohistochemistry in rostral medullary raphe subregions of rats that received social defeat stress (Stress) or were left undisturbed (Control) following an i.p. injection of diazepam or vehicle.   Scale bar: 200 μm.   B, numbers of Fos-immunoreactive cells in the rRPa, the RMg/PPy and the ventral surface of the pyramidal tract of Vehicle/Stress ($n = 5$), Diazepam/Stress ($n = 7$) and Diazepam/Control ($n = 4$) groups. **$P < 0.01$, ***$P < 0.001$, compared with the Vehicle/Stress group (Bonferroni $post\ hoc$ test following a one-way ANOVA).   C and D, percentages of Fos-immunoreactive populations in VGLUT3-positive (C) or TPH-positive (D) neurons in the rostral medullary raphe subregions of the Vehicle/Stress, Diazepam/Stress and Diazepam/Control groups.   **$P < 0.01$, ***$P < 0.001$, compared with the Vehicle/Stress group (Bonferroni $post\ hoc$ test following a one-way ANOVA).
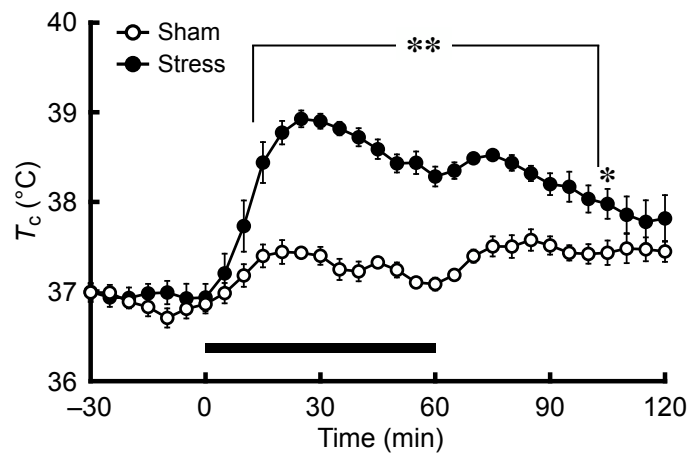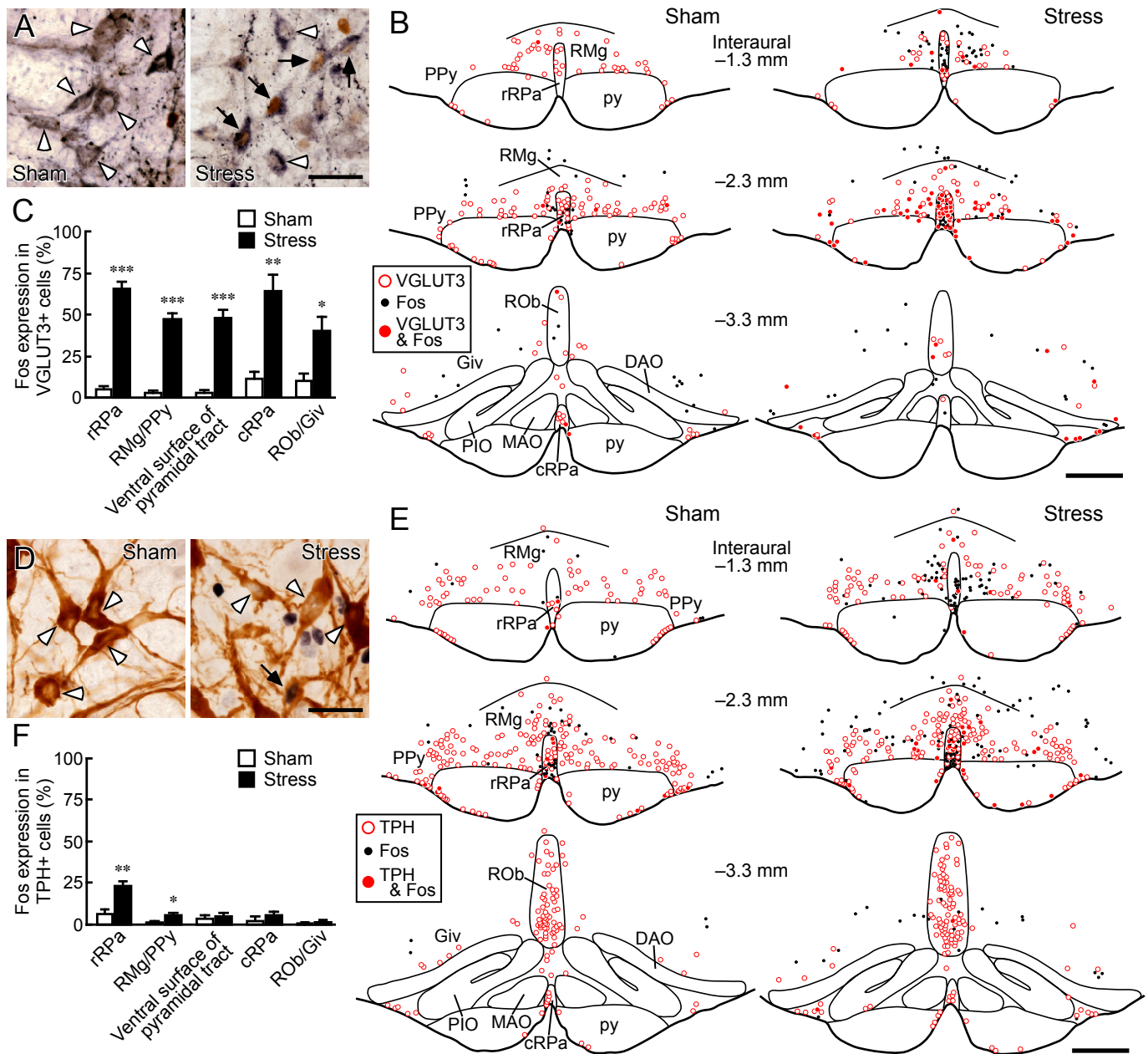
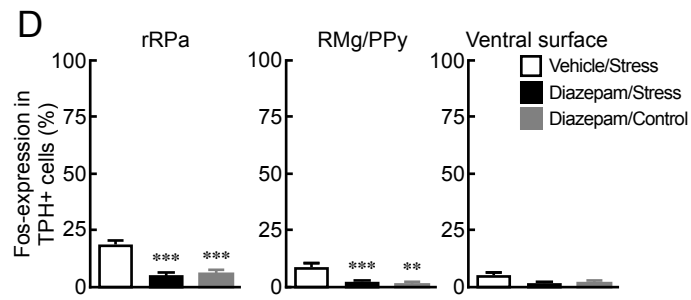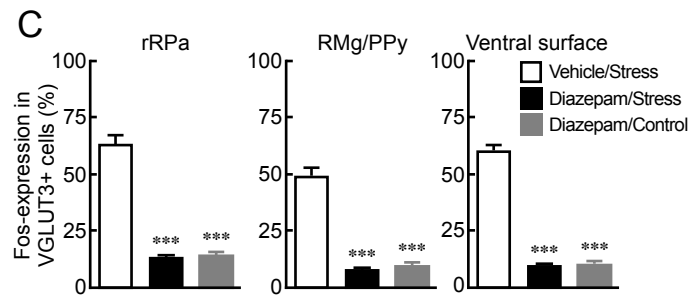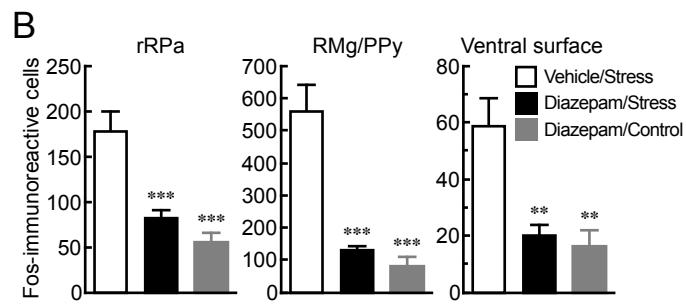Lkhagvasuren *et al.*, *Eur. J. Neurosci.*
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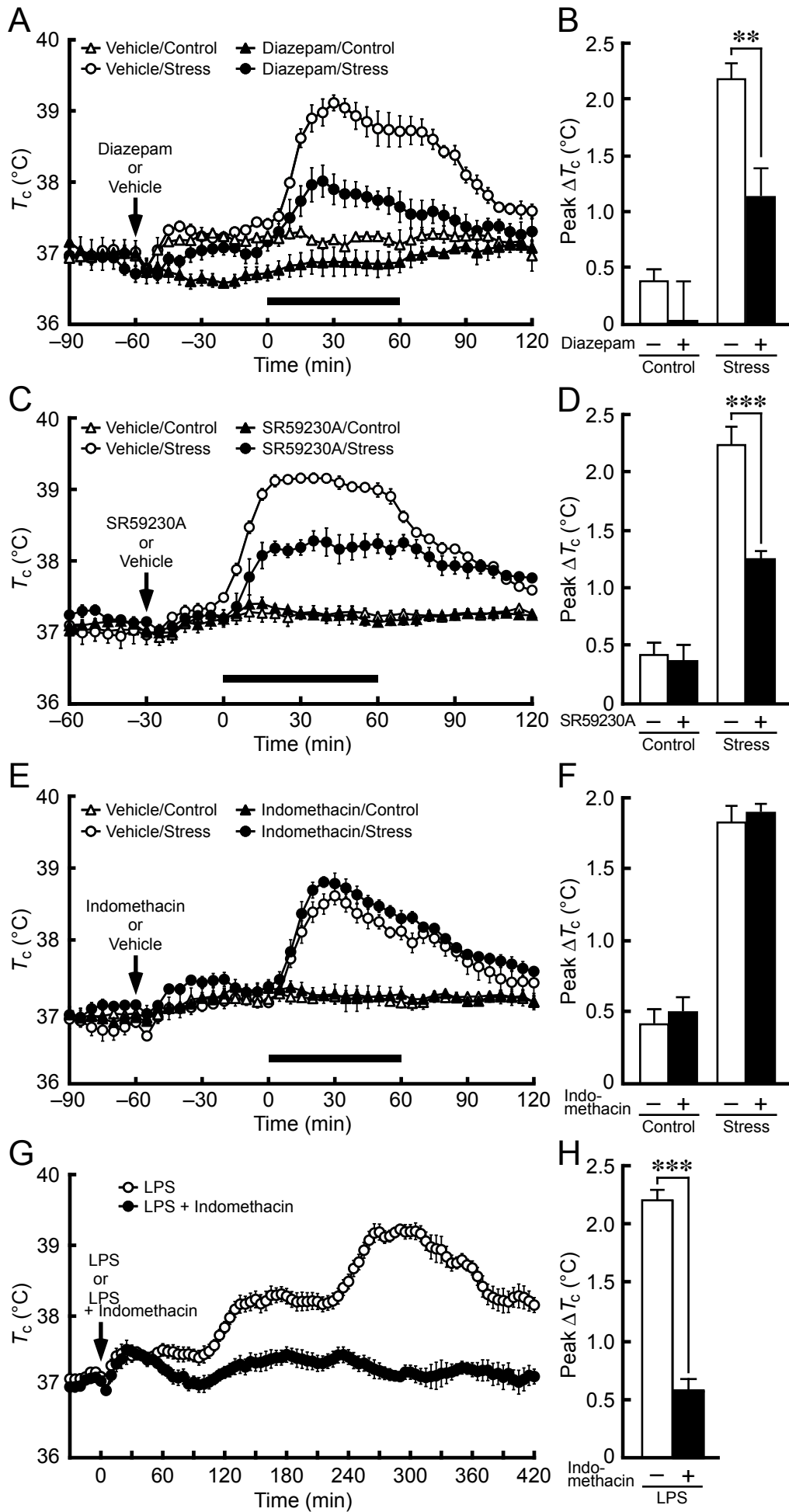
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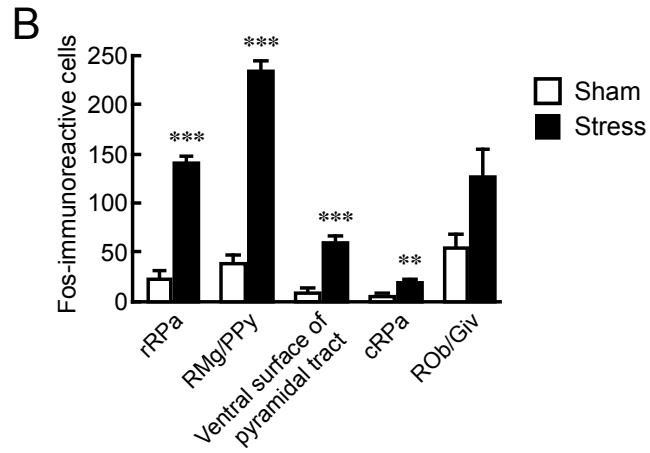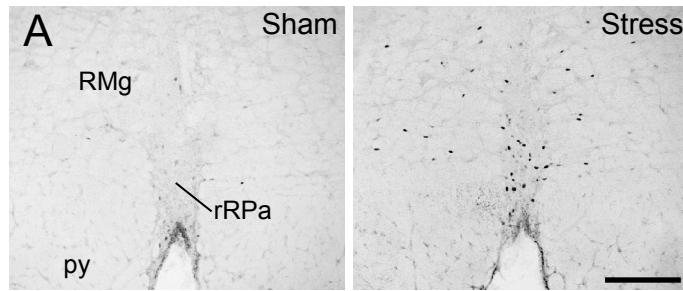
A

Sham   Stress

RMg

rRPa

py

B

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Figure 4. Two column width

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Figure 5. One column width