Dorsomedial hypothalamus mediates autonomic, neuroendocrine, and locomotor responses evoked from the medial preoptic area

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Hunt JL, Zaretsky DV, Sarkar S, DiMicco JA. Dorsomedial hypothalamus mediates autonomic, neuroendocrine, and locomotor responses evoked from the medial preoptic area. Am J Physiol Regul Integr Comp Physiol 298: R130–R140, 2010. First published November 18, 2009; doi:10.1152/ajpregu.00574.2009.—Previous studies suggest that sympathetic responses evoked from the preoptic area in anesthetized rats require activation of neurons in the dorsomedial hypothalamus. Disinhibition of neurons in the dorsomedial hypothalamus in conscious rats produces physiological and behavioral changes resembling those evoked by microinjection of muscimol, a GABA<sub>A</sub> receptor agonist and neuronal inhibitor, into the medial preoptic area. We tested the hypothesis that all of these effects evoked from the medial preoptic area are mediated through neurons in the dorsomedial hypothalamus by assessing the effect of bilateral microinjection of muscimol into the DMH on these changes. After injection of vehicle into the dorsomedial hypothalamus, injection of muscimol into the medial preoptic area elicited marked increases in heart rate, arterial pressure, body temperature, plasma ACTH, and locomotor activity and also increased c-Fos expression in the hypothalamic paraventricular nucleus, a region known to control the release of ACTH from the adenohypophysis. Prior bilateral microinjection of muscimol into the dorsomedial hypothalamus produced a modest depression of baseline heart rate and body temperature but completely abolished all changes evoked from the medial preoptic area. Microinjection of muscimol just anterior to the dorsomedial hypothalamus had no effect on autonomic and neuroendocrine changes evoked from the medial preoptic area. Thus, activity of neurons in the dorsomedial hypothalamus mediates a diverse array of physiological and behavioral responses elicited from the medial preoptic area, suggesting that the latter region represents an important source of inhibitory tone to key neurons in the dorsomedial hypothalamus.

The preoptic area plays a primary role in mammalian thermoregulation (7). In anesthetized and conscious rats, perfusion or microinjection of the medial preoptic area (mPOA) with the neuronal inhibitor muscimol, a GABA<sub>A</sub> receptor agonist, elicits changes resembling thermoregulatory responses to cold exposure, including increased body temperature, heart rate, and plasma ACTH (22, 54, 55, 57, 94). Conversely, disinhibition of the mPOA with bicuculline, a GABA<sub>A</sub> receptor antagonist, blocks cold-induced thermoregulatory responses in anesthetized rats (49, 54), and produces hyperthermia in conscious rats kept at 5°C (22). Extracellular GABA in the mPOA increases during cold exposure and decreases in a warm environment (22).

Thus, neurons in the mPOA that tonically suppress downstream neural circuitry mediating thermoregulatory responses to cold are, in turn, under tonic GABAergic inhibition. This neural circuitry, although not fully understood, includes 1) the medullary rostral raphe pallidus (rRP), the location of sympathetic premotor neurons controlling nonshivering thermogenesis by interscapular brown fat in rats (43), and 2) the hypothalamic paraventricular nucleus (PVN), the location of neurons controlling the hypothalamic-pituitary-adrenal axis (for review, see Ref. 20). Neurons in the mPOA send direct projections to both regions, and these projections have been proposed to mediate mPOA-evoked sympathetic responses and neuroendocrine effects (17, 20, 23, 27, 34, 47).

However, evidence now supports a role for the dorsomedial hypothalamus (DMH) in sympathetic thermoregulatory responses evoked from the mPOA. In anesthetized rats, microinjection of muscimol into the DMH attenuates hyperthermia, increases in sympathetic nerve activity to interscapular brown fat, and tachycardia evoked by microinjections of PGE<sub>2</sub> into the mPOA (32, 48, 93), an established model for fever. Disinhibition of neurons in the DMH by local microinjection of bicuculline evokes hyperthermia, tachycardia, and increased plasma ACTH and locomotor activity in conscious rats (6, 10, 69, 76, 77, 92). Neurons in the DMH project to the rRP and PVN (65, 85, 86, 88), and GABAergic neurons in the mPOA innervate rRP-projecting neurons in the DMH (48). Therefore, inhibition of the mPOA may elicit sympathetically mediated tachycardia and hyperthermia by disinhibiting neurons in the DMH. While the DMH appears to mediate stress-induced increases in plasma ACTH (for a review, see Ref. 13), no evidence exists implicating the DMH in increases in plasma ACTH or locomotor activity evoked from the mPOA.

Thus, we determined the effect of microinjection of muscimol into the DMH on increases in heart rate, blood pressure, body temperature, locomotor activity, plasma ACTH, and c-Fos expression in the PVN evoked by microinjection of muscimol into the mPOA in conscious rats. We chose to study the effects of muscimol because of the evidence linking GABAergic mechanisms acting through GABA<sub>A</sub> receptors in the mPOA with thermoregulation and because microinjection of this agent into the mPOA elicits autonomic, neuroendocrine, and behavioral changes.

Materials and Methods

Animals. Experimentally naïve male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300 ± 20 g were used in all experiments. Animals were housed singly with free access to rat chow and water under a 12:12-h light-dark cycle. All procedures and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine and followed the guidelines set forth by the National Institutes of Health. Experiments were performed between 1000 and 1400 to control for circadian variability.

Surgical procedures. A Dataquest telemetry system (Transoma Medical, St. Paul, MN) was used to monitor heart rate, mean arterial...
pressure, core body temperature, and locomotor activity. Rats were anesthetized (80 mg/kg ip ketamine and 11.5 mg/kg ip xylazine, and supplemented as needed) for implantation of telemetric probes (TL11M2-C50-PXT; Transoma Medical, St. Paul, MN). The body of the probe was placed in the peritoneal cavity and fixed to the abdominal wall. The flexible catheter of the probe was inserted into the abdominal aorta through the right femoral artery. The animals were then allowed to recover from anesthesia on a heated plate in their home cages.

After at least three full days of recovery, rats were reanesthetized (80 mg/kg ip ketamine and 11.5 mg/kg ip xylazine, and supplemented as needed) before being placed in a stereotaxic device for implantation of guide cannulas targeting the mPOA (left) and DMH (bilaterally). The technique used to implant guide cannulas for microinjection of drug solutions was slightly modified from those previously described (6, 9, 10, 71, 94). With the incisor bar of the stereotaxic device initially set at 3.3 mm below the interaural line, two stainless-steel guide cannulas (26 gauge; Plastics One, Roanoke, VA) were implanted bilaterally at a 10° angle from the sagittal plane into the DMH using bregma as the reference point (3.2 mm posterior, 1.9 mm lateral, 7.2 mm ventral). After guide cannulas targeting the DMH were fixed to the skull with veterinary adhesive and dental acrylic, the incisor bar was raised to 5.0 mm above the interaural line. A single cannula was then targeted to the left mPOA in a similar manner (1.9 mm anterior, 1.9 mm lateral, 6.9 mm ventral). Guide cannulas were then securely fixed to the skull with two stainless-steel screws, veterinary adhesive, and dental acrylic. The dental acrylic was allowed to set, and sterile dummy wires were inserted into the cannulas, and the rats were returned to their home cages. A similar technique was used in animals with bilateral guide cannulas targeting the PVN instead of the DMH (1.8 mm posterior, 1.8 mm lateral, 6.9 mm ventral). Negative controls outside the DMH and PVN were obtained serendipitously in rats with misplaced guide cannulas.

After 5 days of recovery from surgery, rats were once again anesthetized (80 mg/kg ip ketamine and 11.5 mg/kg ip xylazine, and supplemented as needed) for the implantation of chronic intra-arterial catheters for blood sampling. Catheters were prepared by inserting a 6-cm piece of Teflon tubing (0.015 in ID; Small Parts, Miami, FL) ~1 cm into a 20-cm piece of Tygon tubing (0.02 in ID; Small Parts). A small incision was made in the left inguinal skin, and the femoral artery was carefully dissected. The Teflon leader of the catheter was threaded ~5 cm into the artery. The Tygon portion of the catheter was routed subcutaneously to the nape of the neck and secured with suture and a jacket (Kent Scientific; Torrington, CT). Animals were then returned to their home cages and allowed to recover from surgery for at least three full days before experimentation. Catheters were flushed daily with heparinized sterile saline to maintain patency until experimentation.

All animals received postoperative care following survival surgeries as mandated by the protocols of IACUC. Posturgical analgesia (buprenorphine, 0.02 mg/kg sc) was administered following each survival surgery. Subsequent surgeries or experiments were not performed unless the animal displayed daily weight gain, a normal level of activity and mobility, and normal grooming patterns. No animals were removed from the current experiments because of signs of poor health.

**Effect of pretreatment with muscimol in the DMH on mPOA-evoked responses.** Each animal was subjected to two experimental sessions separated by at least two days of rest. On the morning of the experiment, rats were brought to the testing facilities in their home cages and placed on telemetric receiver plates. Dummy wires were removed from the guide cannulas, PE-50 tubing extensions were attached to arterial catheters, and the rats were left undisturbed for at least 2 h to establish resting baselines. A 33-gauge microinjector (Plastics One) was connected to a 10-μl syringe (Hamilton, Reno, NV) with Teflon tubing (ID 0.12 mm, OD 0.65 mm; Bioanalytical Systems, West Lafayette, IN) and loaded with either solutions of muscimol (0.8 mM) or vehicle (aCSF: 122 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl2, 1.2 mM MgSO4, 20 mM NaH2PO4, and 11 mM glucose in deionized water). Just prior to the injection, the microinjector was inserted into the guide cannula of the conscious animal and fixed in place with a modified plastic dust cap (Plastics One). Using an infusion pump (KD Scientific) we infused either 80 pmol of muscimol or vehicle (100 nl total volume) into the right DMH over a 30-s period. The microinjector was left in place for an additional 60 s to prevent the backflow of solution up the cannula track, followed 1 min later by identical microinjection into the left DMH. Identical microinjections of muscimol into the DMH have been previously shown to inhibit stress-induced cardiovascular and neuroendocrine changes, and this effect was highly anatomically specific to the region of the DMH (79). After the microinjector was removed from the guide cannula, successful injection was verified by visualizing the flow of solution within seconds of reactivating the infusion pump.

Five minutes after the completion of the second microinjection into the DMH, either 80 pmol of muscimol or vehicle (100 nl total volume) was injected into the mPOA in an identical manner. Microinjection of this dose of muscimol into the mPOA produces robust increases in heart rate, blood pressure, body temperature, locomotor activity, and plasma ACTH (94). Exactly 15 min after the microinjection into the mPOA, a blood sample (0.35 ml) was collected from the arterial catheter using a syringe containing a solution of EDTA and aprotinin. The sample was immediately centrifuged to obtain plasma that was stored at ~80°C until analysis for ACTH. Normal saline was used for volume replacement after blood sampling. The animal was then left undisturbed as telemetric monitoring continued for at least 60 min. For the second experimental session, the treatment in the mPOA (i.e., muscimol or saline vehicle) was held constant for a given animal, while the injection of drug or vehicle into the DMH was switched. Order effect was controlled by randomly assigning an equal number of animals to each sequence.

A subset of these rats whose final treatment was either microinjected with vehicle in both regions (n = 4), muscimol in the mPOA and vehicle in the DMH (n = 3), or muscimol in both regions (n = 4) was selected at random for analysis of c-Fos in the PVN. Because 2 or 3 days had elapsed since the first experimental session, any effect of the initial intervention on c-Fos expression was likely to have completely dissipated owing to the relatively transient nature of the response (16, 37, 39, 40, 64). Ninety minutes after the final microinjection of the second experimental session, the rats were deeply anesthetized with pentobarbital sodium (65 mg/kg ia). Within 2 min of establishing anesthesia, animals were perfused transcardially with 40 ml of 0.9% normal saline containing 15,000 IU heparin sulfate followed by 100 ml of an ice-cold solution containing 3% paraformaldehyde and 1% acrolein in 0.1 M phosphate buffer (pH 7.4). Brains were postfixed in 3% paraformaldehyde in 0.1 M phosphate buffer at room temperature for 60 min, followed by storage in 20% sucrose in 0.1 M PBS overnight at 4°C. Brains were then blocked and frozen on dry ice before serial coronal sections of 25-μm thickness were cut with a cryostat for verification of sites of injection and immunohistochemical processing.

**Verification of sites of injection.** All final preparations of drug and vehicle solutions contained fluorescent-embedded, polystyrene microspheres (5% vol/vol, Molecular Probes; Carlsbad, CA). The fluorescent microspheres were reconstituted and injected simultaneously with the injectate at the time of the experiment and presumably concentrated at the sites of injection until microscopic evaluation. The location of intense fluorescence was identified as the site of injection based on the atlas of Paxinos and Watson (60).

**Immunohistochemistry.** The antibody against c-Fos was obtained from Calbiochem (rabbit anti-c-Fos 4–17 polyclonal antibody; San Diego, CA) and has been used extensively in functional neuroanatomical studies (67, 75, 96, 97). A dilution of 1:20,000 was determined to produce an optimal signal-to-noise ratio in preliminary
experiments. In brief, sections were immersed in 1% sodium borohy-
dride for 25 min. Sections were then treated with 0.5% H₂O₂ for 12
min, incubated in 0.5% Triton-X-100 for 30 min, and placed in 10% normal horse serum for 20 min. The sections were incubated in antiserum (1:20,000) for 2–3 days at 4°C.

The sections were incubated in biotinylated goat anti-rabbit IgG (1:400; Jackson ImmunoResearch, West Grove, PA) for 3 h prior to visualization of immunoreactivity with a Vectastain ABC Elite Kit (Vector Laboratories Elite Kit, Vector Laboratories, Burlingame, CA) for 60 min. Nickel-diaminobenzidine tetrahydrochloride was used to inten-
sify the signal in c-Fos-immunoreactive nuclei. Thus, staining was developed in freshly prepared Tris-buffer solution containing 0.025% diaminobenzidine tetrahydrochloride, 0.06% nickel-ammonium sul-
fate, and 0.0027% H₂O₂ for ~2 min to yield a dark purple color in reacting nuclei, while minimizing nonspecific background staining. The color reaction was stopped by dilution of the section in Tris buffer. The sections were mounted on charged slides before being dehydrated and coverslipped.

Radioimmunoassay for ACTH. A double-antibody radioimmuno-
assay was used to quantify ACTH, as described previously (29, 92). Briefly, duplicate samples were incubated in a rabbit ACTH antiserum (1:30,000; IgG, Nashville, TN) at 4°C for 24 h. Reconstituted 125I-
ACTH (Diasorin; Stillwater, MN) was added and incubated overnight at 4°C. Goat anti-rabbit y-globulin (1:50; Calbiochem; San Diego, CA) was added and incubated at 4°C for an additional 24 h. After centrifugation and aspiration of the supernatant, radioactivity present in the precipitate was determined with a gamma counter (Cobra II, Packard) and compared with known standards (ACTH 1–39; Bachem; Torrance, CA) to calculate unknown sample concentrations of ACTH.

Data analysis. All data were reported as means ± SE. The level of significance was set at P < 0.05 for all statistical tests. Between-group differences were analyzed by one-way ANOVA with Bonferroni post hoc tests.

RESULTS

Effect of prior microinjection of muscimol into the DMH on mPOA-evoked sympathetic and locomotor responses. The sites of microinjection were confirmed by postmortem histology (Fig. 1). Sites of injection in the DMH were comparable in rats that received intrapreoptic injections of muscimol and intrapreoptic injections of vehicle. Only data from rats with left and right sites of injection in the DMH that were roughly symmetrical and either both within or both outside the borders of the dorsomedial hypothalamic nucleus as defined by the atlas of Paxinos and Watson (60) were used for analysis. There were no significant differences in basal heart rate, arterial pressure, body temperature, or locomotor activity prior to microinjections for any treatment group (one-way ANOVA, Table 1).

Fig. 1. Schematic coronal sections adapted from the atlas of Paxinos and Watson (60), illustrating approximate location of microinjections in all animals receiving dual microinjections. A: sites of injection in the medial preoptic area (mPOA)-muscimol (black circles and all triangles and squares) or vehicle (gray circles) followed by treatment in either the dorsomedial hypothalamus (DMH) (circles) or at sites in the paraventricular nucleus (PVN) (black triangles) or other adjacent regions (black squares). B: sites of injection in the DMH for either muscimol (black circles) or vehicle (gray circles). C: sites of injection of vehicle and muscimol either in the PVN (black triangles) or at sites outside either the DMH or the PVN (black squares). Numbers indicate the distance in millimeters from bregma. ac, anterior commissure; oc, optic chiasm; v, third ventricle; DMN, dorsomedial hypothalamic nucleus; f, fornix; mt, mammillothalamic tract; me, median emi-
ence; ot, optic tract.
Table 1. Baseline HR, BP, BT, and LA in conscious rats just prior to microinjections into the mPOA and into the DMH

<table>
<thead>
<tr>
<th>Treatment (mPOA/DMH)</th>
<th>HR, beats/min</th>
<th>BP, mmHg</th>
<th>BT, °C</th>
<th>LA, counts/min</th>
</tr>
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<tbody>
<tr>
<td>Microinjection into DMH</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vehicle/Vehicle (n = 9)</td>
<td>340±7</td>
<td>128±3</td>
<td>37.7±0.2</td>
<td>1.3±0.5</td>
</tr>
<tr>
<td>Muscimol/Vehicle (n = 12)</td>
<td>356±7</td>
<td>117±7.4</td>
<td>37.4±0.2</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>Muscimol/Muscimol (n = 12)</td>
<td>351±8</td>
<td>117±3</td>
<td>37.6±0.2</td>
<td>1.7±0.5</td>
</tr>
<tr>
<td>Vehicle/Muscimol (n = 9)</td>
<td>340±8</td>
<td>125±5</td>
<td>37.5±0.2</td>
<td>0.6±0.4</td>
</tr>
<tr>
<td>Microinjection outside DMH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscimol/Vehicle (n = 14)</td>
<td>359±9</td>
<td>132±6</td>
<td>37.9±0.3</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Muscimol/Muscimol (n = 14)</td>
<td>350±7</td>
<td>131±6</td>
<td>37.8±0.2</td>
<td>0.4±0.2</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. HR, heart rate; BP, arterial pressure; BT, body temperature; LA, locomotor activity; mPOA, medial preoptic area; DMH, dorsomedial hypothalamus. There were no significant differences among any of the groups (one-way ANOVA; P > 0.05).

Animals receiving microinjections of vehicle into both the DMH and mPOA (n = 9) displayed modest and transient increases in heart rate, blood pressure, body temperature, and locomotor activity (Fig. 2). In rats pretreated with vehicle in the DMH, subsequent microinjection of muscimol into the mPOA (n = 12) was followed by immediate and dramatic increases in heart rate, arterial pressure, body temperature, and locomotor activity (i.e., increased movement in the cage with no apparent increase in sniffing, rearing, or grooming activity), replicating previously published findings (94). Maximal increases in heart rate, arterial pressure, and locomotor activity were attained ∼10 min after injection of muscimol into the mPOA (Fig. 2). Increases in body temperature developed more slowly, reaching a maximum ∼20 min after injection of muscimol into the mPOA (Fig. 4). The mean maximal increases in heart rate (+142 ± 8 beats/min), arterial pressure (+15 ± 2 mmHg), body temperature (+1.8 ± 0.1°C), and locomotor activity (+12 ± 2 counts/min) were significantly greater than those seen in animals receiving microinjections of vehicle only (+21 ± 12 beats/min, 0 ± 2 mmHg, +0.4 ± 0.1°C, 0 ± 1 counts/min, respectively).

Rats pretreated with muscimol in the DMH and subsequent microinjection of vehicle into the mPOA (n = 9) exhibited modest, but significant, reductions from baseline heart rate, arterial pressure, and body temperature (mean maximal changes: −19 ± 4 beats/min, −7 ± 2 mmHg, −0.4 ± 0.1°C; Fig. 2). Locomotor activity, which was minimal under baseline conditions, was unchanged in these animals (Fig. 2). After microinjection of muscimol into the DMH, microinjection of muscimol into the mPOA failed to elicit increases in heart rate, arterial pressure, body temperature, or locomotor activity. In fact, maximal changes from baseline in these rats (−20 ± 9 beats/min, −8 ± 3 mmHg, −0.6 ± 0.1°C, −1 ± 1 counts/min) were nearly identical to those seen after treatment with muscimol in the DMH and microinjection of vehicle into the mPOA.

Effect of prior microinjection of muscimol into the DMH on mPOA-evoked increase in plasma ACTH and c-Fos expression in the PVN. Plasma levels of ACTH were similar 15 min after microinjection of vehicle into the mPOA regardless of pretreatment of the DMH with vehicle (72 ± 15 pg/ml) or muscimol (77 ± 24; n = 9). Microinjection of muscimol into the mPOA resulted in markedly greater increases in plasma ACTH after pretreatment of the DMH with vehicle (390 ± 65 pg/ml) but not after pretreatment with muscimol (97 ± 30 pg/ml, n = 12; Fig. 3).

After the last treatment, c-Fos expression was assessed in a subset of rats as an index of neuronal activity in the PVN whose activation signals the release of ACTH from the anterior pituitary. Neurons immunoreactive for Fos were observed in all animals regardless of treatment (Fig. 4). In rats pretreated with vehicle in the DMH followed by microinjections of vehicle into the mPOA, moderate numbers of c-Fos-positive neurons were evident in the parvocellular PVN (133 ± 18 neurons) and magnocellular PVN (52 ± 9 neurons; Fig. 5). Significantly greater numbers were evident in both subregions of the PVN in rats receiving microinjection of vehicle in the DMH followed by microinjection of muscimol into the mPOA (parvocellular PVN: 630 ± 7 neurons; magnocellular PVN: 296 ± 12 neurons). In contrast, animals pretreated with muscimol in the DMH followed by microinjection of muscimol into the mPOA exhibited numbers of c-Fos-positive neurons in both regions that were not different from vehicle/vehicle-treated control animals (parvocellular PVN: 130 ± 16 neurons; magnocellular PVN: 52 ± 10 neurons).

Effect of prior microinjection of muscimol into regions adjacent to the DMH on mPOA-evoked sympathetic and locomotor responses, increase in plasma ACTH, and c-Fos expression in the PVN. The anatomical specificity of the effect of muscimol in the DMH was addressed by assessing the effect of similar microinjection of muscimol into sites anterior to the DMH (n = 14), including sites in the PVN (n = 4) and into sites anterior to the DMH but outside of the PVN (other sites: n = 7; Fig. 1C). Because corresponding data discussed below for heart rate, blood pressure, body temperature, and activity were equivalent for microinjections of muscimol into the PVN and for microinjections into other sites outside the DMH, the results from all injections were pooled for presentation (Fig. 5). After microinjection of vehicle into these sites, microinjection of muscimol into the mPOA evoked mean increases in heart rate, arterial pressure, body temperature, and locomotor activity (+132 ± 14 beats/min, +10 ± 4 mmHg, +1.5 ± 0.2°C, +11 ± 2 counts/min; Fig. 5) that were similar to those seen after microinjection of vehicle into the DMH (see above). After microinjection of muscimol into these sites, microinjection of muscimol into the mPOA evoked mean increases in heart rate, arterial pressure, body temperature, and locomotor activity (+133 ± 9 beats/min, +12 ± 3 mmHg, +1.8 ± 0.2°C, +6 ± 1 counts/min, n = 14; Fig. 5). As with the physiological parameters assessed, the increase in locomotor activity evoked from the mPOA was not abolished by microinjection of muscimol at sites outside the DMH, although unlike the other changes, this activity was significantly reduced (Fig. 5; compare with Fig. 2).

While microinjections of muscimol into the PVN and into other sites outside the DMH were similar in producing no...
effect on mPOA-evoked increases in heart rate, blood pressure, or body temperature, and equivalent modest suppression of increases in activity, clear differences were evident with respect to effects on increases in plasma ACTH. Consequently, these data were analyzed separately for each group. In rats pretreated with microinjection of vehicle into the PVN (n = 7) or into other sites outside the DMH (n = 6; samples from one animal lost), plasma ACTH was markedly elevated above that seen in vehicle/vehicle-treated animals 15 min after microinjection of muscimol in the mPOA (Fig. 6). However, after microinjection of muscimol into the PVN, plasma levels of ACTH 15 min after injection of muscimol in the mPOA were significantly reduced (168 ± 43 pg/ml) compared with levels after pretreatment with vehicle. In fact, the relative degree to which mPOA-evoked increases in plasma ACTH was suppressed was similar after microinjection of muscimol into the DMH (−75%) or the PVN (−77%). In contrast, after microinjections that targeted other adjacent hypothalamic sites, including those between the two regions, plasma levels of ACTH 15 min after the injection of muscimol into the mPOA were equivalent after pretreatment with muscimol (364 ± 97 pg/ml) and vehicle (322 ± 98 pg/ml, n = 6).
DISCUSSION

Our results clearly indicate that the autonomic, neuroendocrine, and behavioral effects of inhibiting neurons in the mPOA are a consequence of activation of neurons in the region of the DMH. Microinjection of muscimol into the mPOA of conscious rats elicited marked increases in heart rate, arterial pressure, body temperature, and locomotor activity, replicating previous findings (22, 54–57, 94). The cardiovascular changes evoked by muscimol in the mPOA are independent of the accompanying modest increase in locomotor activity since they are evident in anesthetized rats (55). The DMH, a region that includes the dorsomedial hypothalamic nucleus and the adjacent dorsal hypothalamic area, is recognized as a thermoregulatory center that may also be involved in autonomic, neuroendocrine, and behavioral responses to a variety of stressors (see Refs. 12–14, 49, 83). Inhibition or suppression of excitation of neurons in the DMH has been reported to attenuate the sympathetically mediated tachycardia, hypertension, and hyperthermia evoked by microinjection of PGE2 into the mPOA in anesthetized rats (32, 48, 93). In the present study, bilateral microinjection of muscimol, a neuronal inhibitor, into the DMH completely suppressed the increases in heart rate, arterial pressure, and body temperature evoked from the mPOA in the same conscious animals, confirming and extending these previous findings. However, we also found that the increases in plasma ACTH and locomotor activity that accompany these sympathetic effects were abolished. Identical injection of muscimol into areas adjacent to but outside the DMH failed to suppress autonomic responses or increases in plasma ACTH evoked from the mPOA, thus establishing the anatomical specificity of these effects. Microinjections of muscimol into the DMH also prevented mPOA-evoked induction of c-Fos protein, a marker for neuronal excitation, in the PVN, which is known to be densely innervated by neurons in the DMH (85, 86, 88) and is considered the final common pathway for activation of the hypothalamic-pituitary-adrenal axis. Thus, neurons in the region of the DMH appear to represent a key relay mediating diverse aspects of an integrated autonomic, neuroendocrine, and behavioral response evoked by inhibiting neurons in the mPOA.

Inhibition of neurons in the DMH by the mPOA: role in sympathetic thermoregulatory responses. The effects of injection of muscimol, a neuronal inhibitor, into the mPOA are consistent with the notion that neurons in the mPOA tonically inhibit the downstream effector circuits relevant to mechanisms for conserving or generating body heat in response to exposure to a cold environment in the conscious rat. In support of this idea, microinjection of PGE2 into the mPOA, a well-
known model for fever (5, 33, 38), produces autonomic responses nearly identical to those evoked by muscimol (94), including increased circulating levels of ACTH, reflecting activation of the hypothalamic-pituitary-adrenal axis (32, 44, 93, 94), and direct application of PGE2 has been shown to suppress the tonic activity of warm-sensitive neurons in the mPOA (61, 62). The POA is both one of the major sources of afferents to the DMH (87) and a primary source of GABAergic projection neurons in the brain (53). As discussed above, microinjections of muscimol into the DMH attenuated the hyperthermia, activation of sympathetic nerves innervating IBAT, and/or tachycardia evoked by microinjections of PGE2 into the mPOA in anesthetized rats (32, 48, 93) but not the accompanying cutaneous vasoconstrictor response in the tail (63). Nakamura and colleagues (48) found that many of the neurons in the mPOA that project to the DMH express EP3 receptors and that GABAergic terminals of projections from the mPOA are closely associated with neurons in the DMH that project to the rRP. Therefore, they suggested that GABAergic neurons in the mPOA tonically inhibit neurons in the DMH that mediate, at least in part, the increases in body temperature seen in this experimental model of fever (48). Disinhibition of neurons in the region of the DMH with the GABAA receptor antagonist bicuculline methiodide (BMI) has long been known to produce tachycardia, hypertension, hyperthermia, and increases in locomotor activity and plasma ACTH (6, 10, 11, 69, 76, 77, 92). However, the source of the inhibitory tone to the DMH has not until now been identified. The present findings thus suggest that the mPOA is a likely source of tonic inhibition of neurons in the DMH, whose activation is responsible not only for thermoregulatory effects as suggested by Nakamura and colleagues but for neuroendocrine and behavioral effects as well.

Fig. 5. A: Mean ± SE changes from baseline HR, BP, BT, and LA over time after microinjection of either muscimol (solid squares) or vehicle (open squares) into brain regions outside the DMH (at vertical dashed lines) in rats microinjected with muscimol into the mPOA (at vertical solid line). B: mean changes from baseline ± SE averaged over a time interval that encompassed the maximal response elicited by microinjection of the substances (10 to 29 min for HR, BP, and LA, and 25 to 44 min for BT). *Significantly different from corresponding values after microinjection of vehicle into the DMH (paired t-test; P < 0.05).
According to current theories regarding preoptic mechanisms related to thermoregulation, microinjection of muscimol, a GABA_A receptor agonist, into the mPOA may serve as a particularly appropriate model for the thermoregulatory response to cold exposure in the rat. Neurons in the nearby median POA (mnPOA) that are themselves activated by ascending input from peripheral cutaneous cold receptors appear to exert GABA_A receptor-mediated inhibition of neurons in the mPOA (50, 51). Thus, exposure to cold would result in increased activity of these inhibitory GABAergic neurons in the mnPOA, which would, in turn, suppress the activity of neurons in the neighboring mPOA to release appropriate downstream effector circuits from tonic GABA-mediated inhibition (51). Our findings thus highlight the potential importance of neuronal activation in the DMH in a range of thermoregulatory responses seen in cold defense (12).

Compared to vehicle pretreatment, microinjection of muscimol into the DMH modestly lowered basal heart rate, arterial pressure, and body temperature. A similar decrease in basal heart rate evoked by bilateral injection of muscimol into the DMH has been previously described (30). This finding suggests that tonic neuronal activity in the DMH contributes modestly to the maintenance of basal heart rate, arterial pressure, and body temperature in conscious rats held at room temperature. Alternatively, microinjection of muscimol into the DMH is known to block sympathetically mediated responses to stress (79), and it is possible that the muscimol-induced reduction in baseline heart rate, arterial pressure, and body temperature may reflect suppression of the low level of neuronal activity in the DMH caused by the minimal amount of stress related to the experimental setting. Regardless, the relatively modest effect of microinjection of muscimol into the DMH on baseline parameters cannot account for the complete abolishment of the increases evoked from the mPOA.

**Inhibition of neurons in the DMH by the mPOA: role in suppression of neuronal activity in the PVN and the hypothalamic-pituitary-adrenal axis.** Our findings demonstrate for the first time that neurons in the DMH play a key role in the activation of the hypothalamic-pituitary-adrenal axis evoked from the mPOA. After microinjection of vehicle into both regions, plasma levels of ACTH were similar to those reported previously for unstressed Sprague-Dawley rats (3, 18). Pretreatment of the DMH with muscimol failed to suppress these baseline levels, suggesting that activity of neurons in the DMH does not contribute to basal activity of the hypothalamic-pituitary-adrenal axis in conscious rats. Microinjection of muscimol into the mPOA markedly increased plasma levels of ACTH and c-Fos expression in the parvocellular PVN and magnocellular PVN, and all of these effects were abolished by prior microinjection of muscimol into the DMH. Interestingly, mPOA-evoked plasma levels of ACTH were even higher after microinjection of vehicle into the PVN than after vehicle treatment in other areas, an increment that may reflect the direct stimulation of hypophysiotropic neurons in the region. Not surprisingly, these elevated levels were suppressed by prior microinjection of muscimol into the PVN. However, microinjections of muscimol into adjacent sites outside the DMH, most of which were closer to the PVN, had no effect on the increases in plasma ACTH evoked from the mPOA. Thus, the effect of muscimol microinjected into the DMH is unlikely to have been a consequence of spread or diffusion to the nearby PVN. Similar increases in plasma ACTH have been evoked by intrapreoptic injections of PGE_2 (26, 41, 89, 94), an effect thought to represent the activation of the hypothalamic-pituitary-adrenal axis that serves to limit the intensity of the inflammatory response seen in systemic inflammation (for reviews, see Refs. 84, 90, 95). Interestingly, Scammell and colleagues (68) reported that intrapreoptic injections of PGE_2 evoked increased expression of c-Fos in the PVN that was largely restricted to the parvocellular PVN, while in the present study, microinjection of muscimol into the mPOA elicited marked increases in c-Fos expression in both subdivisions of the PVN. The difference may reflect the fact that muscimol inhibits the vast majority of adult mammalian neurons, while PGE_2 most likely acts on the subpopulation of neurons in the POA possessing EP3 receptors (45, 46). Thus, somewhat different populations of neurons in the mPOA may be responsive to microinjection of muscimol and of PGE_2, and this difference may account for the differential effects on c-Fos expression in the magnocellular PVN, as well as on locomotor activity noted here and previously (94). Previously, we reported that injections of muscimol into the DMH identical to those employed in the present study suppress stress-induced increases in plasma ACTH (80) and c-Fos expression in the parvocellular PVN and magnocellular PVN (42). Taken together with these previous results, the present findings suggest that recruitment of the hypothalamic-pituitary-adrenal axis in response to diverse stimuli is a consequence of activation of neurons in the region of the DMH.

**Inhibition of neurons in the DMH by the mPOA: effects on locomotor activity and potential implications for anxiety and panic.** In addition to blocking autonomic and neuroendocrine responses, microinjection of muscimol into the DMH prevented increases in locomotor activity evoked from the mPOA, suggesting that this behavioral response is also mediated through activation of neurons in this region. Microinjection of muscimol into sites immediately adjacent to but outside the DMH reduced mPOA-induced locomotor activity by nearly 50% but failed to affect increases in heart rate or body temperature. This finding further supports the independence of the autonomic and behavioral changes and also suggests that the neurons responsible for the increase in locomotor activity seen here, as well as after microinjection of BMI into the DMH.
(69), may actually be more widely distributed in the hypothalamus.

Although our data suggest that the behavioral response evoked from the mPOA is mediated through neuronal activity in the DMH, little is known about the relevant neural circuitry that may be involved beyond the latter region. However, the increase in activity, resulting from disinhibition of the DMH has recently been shown to be significantly reduced by injection of muscimol into the periaqueductal gray (9). Since the periaqueductal gray receives projections from neurons in the DMH (85, 88), the former region seems likely to play a role in the locomotor stimulation evoked from the mPOA as well.

The finding that neurons in the region of the DMH are responsible for the increase in activity elicited by inhibiting the mPOA suggests at least two distinct possibilities for its contextual meaning. Increased locomotor activity may represent another component of the thermogenic response evoked by cold stress in rats (19), a response in which the DMH has been implicated (12, 49, 51). However, the locomotor response evoked from the mPOA and suppressed by inhibition of the DMH may also be related to an intriguing animal model for human panic disorder developed by Shekhar and colleagues (24, 72, 73, 74). The injection of BMI into the DMH produces an “anxiety-like” response characterized by an increase in locomotor activity (69, 70), suggesting that neurons related to anxiety are located in this region and that these neurons are under tonic GABAergic inhibition. Chronic microinfusion of an agent that inhibits the synthesis of GABA into the DMH results in a “panic-prone” state in rats, in which systemic infusion of sodium lactate triggers a “panic-like” response (72, 73). This response consists of an anxiety-like state accompanied by increased heart rate and respiration, effects that mimic those seen upon disinhibition of the DMH in naïve rats and in response to infusion of sodium lactate in patients with panic disorder (8, 81). Most importantly, the effect of systemically infused sodium lactate in panic-prone rats appears to be mediated through an action in the region of the organum vasculosum laminae terminalis (73), a site immediately adjacent to both the mPOA and the mPOA. Our findings now suggest that the same projections from the mPOA to the DMH are involved and thus that the increases in locomotor activity evoked by microinjection of muscimol in the mPOA may represent an anxiety-like state. However, further behavioral experiments will be required to assess this latter possibility.

Perspective and Significance

A diverse array of physiological and behavioral changes that are evoked by inhibiting neurons in the mPOA appears to be mediated through neuronal activity in the DMH. These changes include increases in heart rate, arterial pressure, body temperature, locomotor activity, plasma ACTH, and c-Fos expression in the PVN, all of which were completely abolished by inhibiting neurons in the DMH bilaterally. Neuronal activity in the DMH has been suggested to play a key role in the generation of the similar autonomic, neuroendocrine, and behavioral changes seen in experimental stress, cold defense, and perhaps in anxiety and panic responses. Since all of these changes can be readily evoked by disinhibiting the DMH by local microinjection of GABA<sub>A</sub> receptor antagonists, the population of neurons appear to be tonically inhibited by GABAergic tone. However, the source of this tone has been unclear. The present findings along with the results of previous reports clearly indicate that 1) neurons in the DMH are likely to be relevant to the generation of cardiovascular, neuroendocrine, and behavioral responses seen in a variety of settings in which neurons in both the DMH and the mPOA have been implicated, including inflammation, arousal, and cold-stress, and 2) neurons in the mPOA represent a critical source of tonic inhibition of these hypothalamic neurons.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


