Functional topography of the dorsomedial hypothalamus.

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Abstract

The dorsomedial hypothalamus (DMH) has been proposed to play key roles in both the defense reaction to acute stress and in the thermoregulatory response to cold. We reasoned that the autonomic / respiratory motor patterns of these responses would be mediated by at least partly distinct DMH neuron populations. To test this we made simultaneous recordings of phrenic nerve and plantar cutaneous vasoconstrictor (CVC) activity in 14 vagotomized, ventilated, urethane-anesthetized rats. Microinjections of D,L-homocysteic acid (DLH; 15 nl, 50 mM) were used to cause localized, short-lasting (< 1 min) activation of DMH neuron clusters. Cooling the rat’s trunk skin by perfusing cold water through a water jacket activated plantar CVC activity but depressed phrenic burst rate (cold-response pattern). The expected ‘stress/defense response’ pattern would be phrenic activation, with increased blood pressure, heart rate and possibly CVC activity. DLH microinjections into 76 sites within the DMH region never reduced phrenic activity. They frequently increased phrenic rate and/or plantar CVC activity, but the magnitudes of those two responses were not significantly correlated. Plantar CVC responses were evoked most strongly from the dorsal hypothalamic area and most dorsal part of the dorsomedial nucleus, while peak phrenic rate responses were evoked from more caudal sites; their relative magnitudes varied systematically with rostrocaudal position. Tachycardia correlated with plantar CVC responses but not phrenic rate. These findings
indicate that localized activation of DMH neurons does not evoke full ‘cold-response’ or ‘stress/defense response’ patterns, but demonstrate the existence of significant functional topography within the DMH region.

Key words: cutaneous vasoconstrictor, phrenic, cold, defense, stress
Introduction

The dorsomedial hypothalamus (DMH) has been implicated as mediating autonomic, neuroendocrine and behavioral responses to acute stress (6-8). Inhibition of DMH neurons has been shown to block both the increase in systemic adenocorticotropin hormone (ACTH) and the tachycardia following acute air jet stress in conscious rats (40, 41). Moreover, when neurons in this region are activated or disinhibited by cell body-selective stimuli, they evoke a cardiovascular response pattern identified as that of the ‘defense reaction’ (3, 39).

On the other hand, it has been proposed that besides mediating stress/defense responses, DMH neurons integrate thermoregulatory responses to cold and fever (9). Disinhibition of DMH neurons by microinjections of bicuculline causes strong activation of the sympathetic nerves to intrascapular brown adipose tissue (BAT SNA) (2), and drives non-shivering thermogenesis (47). More critically, inhibition of DMH neurons by injections of muscimol has been shown to block shivering in rats exposed to cold (44), and to reverse the activation of BAT SNA in response to cold exposure (27) or experimental fever after preoptic injections of prostaglandin E$_2$ (24, 28, 46). Although no direct evidence yet exists, it has been predicted that cutaneous vasoconstriction would be part of the thermoregulatory actions of DMH neurons (9).

Despite some overlap in the output pathways activated (e.g. tachycardia (25, 27, 31) and non-shivering thermogenesis (11, 27, 38), the autonomic and
respiratory response patterns during cold exposure and the ‘stress/defense reaction’ are not identical. One clear difference between the two patterns may be in their respiratory responses: tachypnoea is a consistent component of the defense reaction (12, 25), and this may be evoked by disinhibition of DMH neurons (4, 26). By contrast, respiratory responses to cold are inconsistent, and are most likely secondary to increased metabolic rate (1, 10).

The co-existence within the DMH region of critical synaptic relays for two distinct motor output patterns raises the question of how they might both be organized within the same small brain region. One possibility is that there are two distinct cell populations, one of which mediates the ‘stress/defense’ response pattern and the other of which mediates ‘thermoregulatory’ cold-response pattern. Alternatively, the two response patterns might be mediated in ‘pick and mix’ fashion, by co-activating selective combinations of DMH cells with more specific output functions (e.g. cutaneous vasoconstriction or tachycardia). The latter arrangement implies that the full ‘stress/defense’ or ‘thermoregulatory’ motor pattern has to be co-ordinated by common inputs from antecedent neurons, rather than being integrated within the DMH itself. In either case, it is possible that the different functions (patterns or unitary outputs) mediated by DMH neurons are organized topographically. We therefore sought to clarify the functional organization of the DMH by making functional maps of the region.

As noted by others, the functionally relevant parts of this brain region
extend from the anatomically defined dorsomedial hypothamic nucleus (DMN) to include part of the dorsal hypothalamic area (DA) and the posterior hypothalamic nucleus (PH) (9). Previous studies in which this region has been functionally mapped with cell body-selective stimuli have used comparatively large injections of agents or those whose actions lasted for tens of minutes (2-5, 15, 26, 37, 47). Such long-lasting actions provide ample time for the agent to diffuse to neighboring brain regions, limiting the spatial resolution that may be achieved. We sought to use smaller injections of a short-acting (< 1 min) agent, combined with accurate marking of the centers of injection sites, to make a higher resolution functional map of the region. With the aim of discriminating between ‘stress/defense’ and ‘thermoregulatory’ cold-response patterns, we made simultaneous neural recordings of phrenic and cutaneous vasoconstrictor sympathetic nerve activity. To help in this process, we also sought to clarify the nature of phrenic responses to skin and core cooling.

Materials and Methods

Fourteen adult male Sprague-Dawley rats (330-470 g) were used in this study. All experiments were carried out in accordance with guidelines of the National Health and Medical Research Council of Australia and were approved by the Animal Experimentation Ethics Committee of the Howard Florey Institute.

Animals were anesthetized initially with pentobarbital sodium (30 mg/kg,
i.p.) and the hair over the trunk was shaved. The trachea was cannulated and animals were then artificially ventilated with 2.0% isoflurane (Forthane; Abbott Australia Pty Ltd., NSW, Australia) in pure oxygen (60-65 cycles/min, tidal volume 3-5 ml). Respiratory pressure was monitored via a pressure transducer attached to a side tube and expired CO₂ concentration was monitored by CO₂ analyzer (ADC, Hoddesdon, Herts, UK). Ventilation was adjusted to keep expired CO₂ between 3.5 and 5% during surgery. Both cervical vagi were cut to desynchronize phrenic activity from the artificial ventilation, and 6/14 rats were given a unilateral pneumothorax to reduce respiratory movements. The right femoral artery and vein were cannulated for monitoring blood pressure and intravenous administration of drugs, respectively. A water-perfused silastic jacket was positioned around the animal’s shaved trunk, and the temperature of the perfusion water was used to manipulate skin and body temperature. Skin temperature was measured as the average of three thermocouples placed across the trunk between the skin and water jacket. Core temperature was measured by a thermocouple inserted 5 cm into the rectum. Core temperature was maintained between approximately 37 and 38°C by perfusion of the jacket at 150-180 ml/minute with water from a reservoir maintained at 43-45°C.

Each animal was then mounted prone in a stereotaxic apparatus according to the coordinate system of Paxinos and Watson (33), and a burr hole was made in the skull over the caudal hypothalamus. The left phrenic nerve and
left tibial nerves were dissected as described below. When surgery was complete, isofluorane was gradually withdrawn and replaced by urethane (1.0-1.2 g/kg, i.v.). The depth of anesthesia was frequently assessed throughout the experiment by testing withdrawal and corneal reflexes, and small additional doses of urethane (25-50 mg, i.v.) were administered if necessary to abolish those reflexes. Animals were not paralyzed.

Recording of nerve activity

Simultaneous recordings were made from the whole left phrenic nerve and cutaneous vasoconstrictor fibers dissected from the left tibial nerve. The phrenic nerve was exposed at the brachial plexus by a dorsolateral approach, after lateral retraction of the scapula. The phrenic nerve was cut distally, desheathed and placed over paired silver wire hook electrodes under a pool of liquid paraffin. Activity was recorded differentially, amplified (10,000 fold) and filtered (100-3,000 Hz). The depth and rate of artificial ventilation were adjusted to maintain spontaneous respiratory activity in the phrenic nerve signal.

Cutaneous vasoconstrictor (CVC) activity was recorded from thin filaments dissected from the central cut end of either the lateral or medial plantar branch of the tibial nerve (17). The Achilles tendon was cut, the gastrocnemius and soleus muscles were reflected proximally to access these plantar branches and a paraffin pool was constructed over the area. Paired silver wire electrodes
were used to make differential recordings between the central end of the nerve filaments and a thread of connective tissue. The activity was amplified (10,000 fold) and filtered (70-800 Hz). Efferent filaments were split from the nerve until unitary spike activity could be discriminated. Brief trunk skin cooling was performed to confirm the recorded activity was thermosensitive (17).

Raw signals for both phrenic nerve and plantar CVC fibers were monitored continuously on an oscilloscope, and were recorded using a computer-based analysis system (CED POWER1401 and Spike 2 software CED Cambridge, UK), at sampling rate of 5,000 Hz. Plantar CVC spikes that passed a selected threshold voltage were detected with a time-window discriminator, and each single unit was discriminated off-line using a computer-based spike shape analysis system (Spike 2).

At the end of experiment, hexamethonium chloride (Sigma; 50 mg/kg in saline) was given intravenously to confirm that the CVC activity recorded from the plantar branch was postganglionic sympathetic.

**Experimental procedures**

**Responses to skin and core cooling**

In 10 rats the skin was cooled by switching the perfusion of the water jacket from warm (43-45°C) to cold (iced) water for 30s every 3 mins, repeated 2-5 times. Phrenic and plantar CVC unit responses to this were measured,
along with blood pressure, expired CO₂, skin and core temperatures.

**DLH microinjections into the DMH region**

D,L-homocysteic acid (DLH, Sigma) (22) was diluted with artificial cerebrospinal fluid (aCSF). A double-barreled glass micropipette (tip o.d. ~20 μm) was used, and one barrel of the pipette was filled with 50mM DLH and the other was filled with 2% pontamine sky blue in 0.5 M sodium acetate for identifying injection sites (13). The pipette was positioned stereotaxically over the left caudal hypothalamus (0.2-1.0 mm from the midline, 2.7-3.8mm posterior to the bregma), and was first advanced to a position 6.5-7.0 mm below the skull surface. DLH was injected in volumes of 15 nl, using a pressure injection system (Neuro Phore BH-2 system, Medical Systems co., NY, USA). The volume of each injection was monitored by the movement of the meniscus. In some cases, repeat DLH injections were made at the same site. The pipette was then advanced 0.5 mm downwards and the injections repeated. At least 5 min was allowed between injections, and each injection was made at times when plantar CVC activity was low but not silent, and phrenic nerve activity and cardiovascular variables were stable. In each rat, 1-3 penetrations were made into the DMH region, and DLH injections were made into a total of 2-12 sites per rat. Pontamine sky blue was iontophoresed (10 μA cathodal current for 10 min) to mark the location of the pipette tip at two injection sites in every penetration. Data taken from one animal were discarded because the pontamine sky blue
marks were indistinct.

At the end of the experiment animals were deeply anesthetized with pentobarbital sodium (325 mg, i.v.) and perfused transcardially with saline followed by 4% paraformaldehyde. The brain was removed and placed in the same fixative at least overnight. After cryoprotection with 20% sucrose in phosphate buffered saline, 40 μm frozen coronal sections were made of the caudal hypothalamus. The locations of the two pontamine sky blue marks in each micropipette track were identified by microscopy (Axioplan 2 imaging, Carl Zeiss, Germany). Relevant sections were photographed under light and dark optics using a digital camera (AxioCam HRc, Carl Zeiss, Germany). Anatomical detail was revealed by adjusting contrast and brightness of the microscopic images using Adobe Photoshop®, and by counterstaining with cresyl violet. The precise position of each DLH microinjection was calculated in relation to the two pontamine blue marks in its micropipette track. All injection sites were then mapped in relation to local structures on a set of drawings made from serial transverse sections of the area, which interpolated between the standard atlas sections of Paxinos & Watson (33). Their exact rostrocaudal and dorsoventral positions were then calculated and plotted on standard parasagittal sections taken from the atlas of Paxinos & Watson (33).
**Statistical analysis**

Mean arterial pressure and heart rate were derived from arterial pressure. Phrenic nerve activity was rectified and integrated (time constant 50 ms). When multi-unit plantar CVC activity was recorded, one single unit with thermosensitivity was chosen for analysis. The baseline core temperature, skin temperature, blood pressure, heart rate, expired CO₂, plantar CVC activity (spikes/5sec), phrenic burst rate (5s averaged; bursts/min) and amplitude were measured as the average values of these variables over 30 sec just before each stimulation. The pre-stimulus phrenic burst amplitude was normalized as 100%. To quantify responses to skin cooling, the response of each variable was averaged over the 30s of maximum change, and Student's paired t test was used to compare these with pre-stimulus control values.

To test objectively whether plantar CVC, phrenic burst rate, phrenic amplitude, blood pressure or heart rate changed in response to each DLH microinjection, the significance of each response was tested by the moving F statistic method (35). For this purpose, baseline measures were taken from the 5s mean values over the 30s before the injection; the moving F statistic was calculated every 5s after the stimulus from a moving sample of three 5s mean values. In the case of phrenic amplitude, the mean amplitude of three consecutive bursts was used in place of three 5s mean values. Significant
changes in the time series of each variable were detected when the moving F value exceeded 5.786, indicating p<0.05 (35).

The maximum change in each variable (5s average) in response to DLH microinjection was measured after each injection. In the cases where DLH injections were repeated at the same site, response magnitudes after the second injection were compared with response magnitudes after the first injection by the Wilcoxon signed rank test. For all injections where DLH microinjection significantly increased at least one measured parameter ('effective injections'), we assessed the correlation between response magnitudes. Pairwise correlations were performed between changes in plantar CVC activity, phrenic rate, phrenic amplitude, heart rate and blood pressure. Their significance was tested by Spearman rank order correlation followed by step-wise Holm-Bonferroni correction for multiple comparisons (23).

When DLH microinjection significantly changed plantar CVC activity and/or phrenic burst rate, the response ratio ($\Delta$ plantar CVC / $\Delta$ phrenic rate) was calculated. Linear regression was used to test for a correlation between the logarithm of the response ratio and the rostrocaudal level of the DLH injection.

For all statistical tests, p<0.05 was considered significant. Values are reported in the text as mean ± S.E.

Contour maps
Color-coded contour maps of response magnitudes of plantar CVC, phrenic rate, phrenic amplitude, blood pressure and heart rate evoked by DLH microinjections into different sites over the DMH region were constructed in the sagittal plane, using SigmaPlot (SPSS Inc.). Color maps were normalized with respect to the maximum response of each variable, a sampling proportion of 0.2 was used and data were spatially smoothed to a resolution of 0.1 mm using a Cauchy weight function. Contour lines were generated by interpolation between the response magnitudes at all injection sites excluding those > 0.6 mm lateral to the midline.

Results

Responses to cooling

Figure 1 shows a representative example of the response of the phrenic nerve and a single CVC unit recorded from the plantar nerve (plantar CVC, displayed as 5 second spike counts) to repeated skin cooling. In grouped data from 9/10 rats (data from 1 animal were discarded because they were confounded by a change in ventilation), plantar CVC activity was low or silent (1±0.5 spikes/5s, n=9) under resting warm conditions (skin temperature 40.0±0.3°C, core temperature 38.4±0.2°C), while phrenic activity was stable (39±2 bursts/min). When animals were then subjected to a sequence of brief (30s) skin cooling
episodes by perfusing cold water through the water jacket, each cooling episode dropped skin temperature by 1.1-3.5°C, and the sequence of 2-5 cooling episodes caused core temperature to fall to 37.8±0.3°C (n=9). Peak expired CO₂ and heart rate also decreased after the cooling sequence (CO₂: 5.4±0.2 to 5.2±0.2%, heart rate: 464±10 to 438±8 bpm, p<0.01, n=9). Plantar CVC fibers were activated first by the fall in trunk skin temperature, and then by the subsequent fall in core temperature (Fig. 1), as established previously for tail and back skin CVC activity (29, 32, 43). This activity was abolished by hexamethonium (50 mg/kg) given at the end of experiment, demonstrating that it was of postganglionic sympathetic origin (not shown).

As shown by the example in Figure 1, phrenic nerve breath rate was significantly reduced by each fall in trunk skin temperature, and this was usually accompanied by a small decrease in phrenic burst amplitude. This inhibitory phrenic rate response to skin cooling occurred before any measurable change in core temperature or expired CO₂ (see Fig. 1). For the first 1-3 cooling episodes, phrenic rate generally returned to its control rate when the skin was rewarmed. Later in the cooling sequence, expired CO₂ and core temperature fell, confounding the interpretation of respiratory effects. Phrenic nerve activity ceased in 7/9 rats, and its rate was greatly decreased in 2 others (9±0.5 bursts/min). On rewarmed, plantar CVC activity and phrenic activity returned to their baseline states.
Microinjection of DLH into the DMH region

Microinjections of DLH were made into 76 sites throughout the caudal hypothalamus, including the dorsal hypothalamic area (DA), dorsomedial hypothalamic nucleus (DMN), and posterior hypothalamus (PH). The baseline plantar CVC unit activity before injections was 3±0.2 spikes/5s (mean of 76 measurements from 14 single units). Baseline skin and core temperatures (38.9±0.1°C and 37.8±0.1°C; n=76) were kept close to their thermal thresholds so as to maintain a low tonic level of plantar CVC unit activity. The baseline phrenic burst rate was 40±1 bursts/min, while blood pressure and heart rate were 82±1 mmHg and 459±3 bpm, respectively (n=76).

DLH microinjections in 25 sites significantly increased plantar CVC activity (median response +9.8 spikes/5s, range 3-20.5), phrenic rate in 43 sites (median + 6.9 bursts/min, range 1.5-25.4), phrenic amplitude in 30 sites (median + 11.4%, range 1.6-49.2), blood pressure in 43 sites (median + 6.1 mmHg, range 2.3-20.1) and heart rate in 25 sites (median +6.8 bpm, range 0.4-18.7). Repeat DLH injections were made into 56/76 sites. For all parameters measured, response magnitude after the second injection did not differ significantly from the response magnitude after the first injection (p> 0.05).

In 22 out of 76 sites tested, DLH microinjection simultaneously increased both plantar CVC activity and phrenic rate (Figs. 2 and 3A). Phrenic amplitude
increased in 13 of those 22 cases, blood pressure in 21 cases and heart rate in 19 (Fig. 3A). The sites which increased both plantar CVC and phrenic rate were located in the DA and the rostral part of the DMN (Fig. 4).

At 21 other sites, DLH microinjections increased phrenic burst rate without any plantar CVC response (Fig. 3B). These sites were distributed in the caudal part of the DMN and rostral part of the PH (Fig. 4). Phrenic amplitude increased in 11 of these cases. Accompanying the phrenic rate responses were increases in both blood pressure and heart rate in 4 cases, and just blood pressure in 10 other cases (Fig. 3B).

DLH microinjections increased plantar CVC activity without increasing phrenic phrenic rate and amplitude in 3/76 sites (Figs 3C and 4). In 2 of these responses there was also an increase in blood pressure.

In the remaining 30 sites, including 9 more lateral sites (0.6-1.0 mm lateral to midline), DLH microinjections had no significant affect on plantar CVC or phrenic burst rate (as in Fig. 2c). Phrenic burst amplitude increased in 6/30 of those cases. Blood pressure also increased in 6/30 cases (3 in common with phrenic amplitude), accompanied by a tachycardia in 2 cases. Injections into 21 sites evoked no significant change in any measured variable. Ineffective injection sites were distributed dorsal, ventral, caudal and lateral to the responsive region (Fig. 4).

Pairwise correlations were performed on all effective injections to
investigate the relations between plantar CVC, phrenic rate, phrenic amplitude, blood pressure and heart rate responses (n=55). Positive correlations were found between phrenic rate and phrenic amplitude (P<0.001); plantar CVC activity and heart rate (P=0.013); phrenic amplitude and heart rate (P=0.014). Blood pressure was correlated with all other responses except phrenic amplitude (P<0.001 for plantar CVC, phrenic rate and heart rate). Other correlations, including that between plantar CVC activity and phrenic rate, were not significant.

Figure 5 shows contour maps of the response magnitudes in each variable evoked by DLH microinjection into different locations in the DMH region. The sensitive region to evoke plantar CVC activity overlapped the DA and dorsalmost part of the DMN. Increases in phrenic burst rate were evoked most strongly from the rostral tip of the PH and adjacent parts of the caudal DA / dorsal DMN, distinctly caudal to plantar CVC region (Fig. 5). Phrenic amplitude increases were evoked from an overlapping, broader region. In confirmation of the different foci of plantar CVC and phrenic rate responses, their log response ratio (Δ plantar CVC / Δ phrenic rate) was strongly related to rostrocaudal position (p< 0.001, Fig. 4).

The strongest heart rate increases were evoked from the rostral tip of the DMN and adjacent DA. Blood pressure increases, however, were obtained from a broader area of the DA and dorsal DMN, which overlapped much of the territory evoking other responses (Fig. 5). The distinction between territories for
plantar CVC, phrenic rate and heart rate responses are shown clearly by the overlay plot in Figure 5f.

**Discussion**

In the present study we examined the functional topography of the DMH region, using small injections of a short-acting excitatory compound (DLH), to obtain a better spatial resolution than studies hitherto. We sought evidence that localized activation of cell groups within the DMH region could evoke ‘thermoregulatory’ (cold-response) or ‘stress/defense’ patterns of autonomic and respiratory activity. Cooling experiments showed that lowering skin temperature inhibited phrenic nerve activity, along with the expected increase in cutaneous vasoconstrictor drive to the hindpaw plantar skin (cf. tail and back skin (29, 32, 43)). This particular combination of responses was not seen in response to DLH injections, however; but our results did show for the first time that neuronal activation in the DA/DMN region increased plantar CVC activity. We confirmed that neuronal activation in the DMH region increased phrenic nerve activity (predominantly burst rate), but found that such responses were not significantly correlated with plantar CVC responses. Finally, we demonstrated significant functional topography within the DMH region, and related these findings to the local anatomy.
Phrenic response to cold

As far as we are aware, the inhibitory response of phrenic rate to skin cooling is a novel finding. However, this result is consistent with a previous study in which conscious dogs were exposed to cold (1). Core temperature did not fall in these animals, so the response was attributable to neural drive from the cold periphery. Metabolic rate increased, principally due to shivering, but ventilation did not, allowing arterial pCO₂ levels to rise. Similarly, Ingram and Legge found that ventilation rose proportionally much less than metabolic rate in cold-exposed pigs (18). Two mechanisms thus appear to be operating on respiratory drive during cold exposure: 1) increased metabolic rate generates CO₂ and drives ventilation chemically; 2) afferent signals from the cold periphery cause a downward shift in the ventilatory response to CO₂ (1). This relative underventilation has been interpreted as a heat-conserving mechanism. In pigs at 5°C, Ingram and Legge calculated that overall heat loss was reduced by 10-15% compared with what would have occurred had ventilation been allowed to increase in proportion to metabolic rate (18). Thus while an absolute increase in ventilation has been reported to occur in rats exposed to cold (10), this is attributable to the raised metabolic production of CO₂.

In the present experiments on anesthetized, vagotomized rats with controlled ventilation, we showed directly that phrenic burst rate decreased in
response to skin cooling before expired CO₂ levels changed. The neural pathways which mediate this reflex remain to be defined. From the present experiments we cannot tell whether core temperature also had a direct influence on phrenic activity because indirect mechanisms mediated by falling expired CO₂ levels always confounded our observations when core temperature fell.

**Involvement of DMH neurons in cutaneous vasoconstrictor activity**

The DMH neurons have been shown to be critical for the expression of shivering (44) and non-shivering (2, 47) thermogenesis. DiMicco and Zaretsky suggested that a common heat-conserving mechanism existed within the DMH region (9), and cutaneous vasoconstriction would form part of this. We indeed found that activating DMH neurons drove the cutaneous vasoconstrictor neurons to the plantar skin of the hindfoot. Unlike the case with thermogenic responses, however, it has not yet been demonstrated whether cutaneous vasoconstrictor responses to cooling can be blocked by inhibition of DMH neurons.

**Relation of responses to anatomical structures**

The present study used small injections (15 nl) of a short-acting neuroexcitant (actions lasting less than 1 min). Previous microinjection studies of this area have used either much larger volumes (≥ 100 nl) (4, 15, 37), and/or used agents (e.g. bicuculline) with much longer-lasting actions (> 20 min) (2-5, 26,
Such long-lasting actions allow the agent plenty of time to diffuse and affect neighboring regions. The brief actions of the stimuli that we used ensured that any distant effects due to diffusion must have been small. We therefore consider that the functional maps constructed from these data have better spatial resolution than those from previous work (see also Fig. 2). With this method, we found that the strongest responses were all evoked from a restricted region encompassing the DA, the dorsal DMN and the adjacent rostral parts of the PH, less than 0.6 mm from the midline.

**Stress/defense reponse pattern vs Thermoregulatory cold-response pattern**

The DMH region has also been implicated as a mediator of stress responses and an integrator of the ‘defense reaction’ (6-8). Increased respiratory rate has been identified as an integral component of the stress/defense response (12, 25) and is elicited by disinhibition of neurons in the DMH/PH region (4, 26). Cold exposure, by contrast, does not directly increase respiratory drive but depresses it (see above). Phrenic activation may thus be used to discriminate between ‘thermoregulatory’ (cold-response) and ‘stress/defense’ motor patterns. CVC activation is an obligatory component of the response to cold, but may also occur during arousal or stress responses (45). It could thus be part of both motor patterns.

We found that DLH injections into the DA and rostral DMN region
commonly activated both plantar CVC fibers and phrenic rate, generally with increases in blood pressure and heart rate (putative ‘stress/defense’ response pattern). If these combined responses were indeed evoked as part of a co-ordinated ‘stress/defense’ response, however, one would expect to see activation of the various output pathways in fixed proportion. In the event, we found that the magnitudes of the plantar CVC and phrenic responses were not correlated; nor were phrenic rate increases correlated with tachycardia (another obligatory component of the stress/defense response) (25).

With regard to putative ‘thermoregulatory’ (cold) pattern responses to DLH injections, we found that some sites selectively activated plantar CVC fibers, but this was never accompanied by any fall in phrenic burst rate. Thus, if a subset of DMH neurons integrates thermoregulatory responses to cold, that integration does not include the respiratory component. Besides being a component of the ‘stress/defense’ response, tachycardia often accompanies responses to cold (27, 31). In the present study we found that responses of heart rate to DLH injections were significantly correlated with plantar CVC responses. Those combined responses could be due to activating the dense group of DA/dorsal DMN neurons with axonal projections to the medullary raphé (14, 16), which mediates DMH actions on heart rate (36) and presumably contains the premotor neurons for the plantar CVC supply (cf. tail and back skin (30, 34, 42, 43)).
Our results thus do not convincingly support the idea that individual (or small clusters of) DMH neurons provide the coordinated drive to generate the full ‘stress/defense’ or ‘thermoregulatory’ cold-response patterns. What we did find, however, was a clear degree of functional topography: heart rate, plantar CVC and phrenic activity each followed their individual gradients of responsiveness across the DMH region. The variation in response patterns obtained from over the DMH region is most economically explained by a distribution of cell groups, each of which drives one or two motor outflows rather than a full motor pattern (the ‘pick and mix’ hypothesis outlined in the introduction). A limitation to this argument, however, is the fact that the clearest distinction made by our data is between respiratory and autonomic measures. It could still be argued that particular groups of DMH neurons drive the full autonomic patterns of the ‘stress/defense’ responses and the ‘thermoregulatory’ response to cold, but other neurons – in the DMH region or elsewhere – drive their respiratory components. Further experiments will be necessary to ascertain whether this is true. If so, however, one must still invoke antecedent ‘command’ neurons to co-ordinate the respiratory with the autonomic responses.

**Perspectives**

The present findings may be seen in the context of the task to define the
neuroanatomical basis of patterned autonomic activity. Which levels of the neuraxis may be said to 'integrate' a particular autonomic response pattern (e.g. exercise, defense, cold) and where it is co-ordinated with behavioural, endocrine or other activities are still unsettled questions. While neuroanatomical approaches (e.g. transynaptic retrograde transport of two viral tracers (19-21)) have successfully identified putative 'command neurons' with polysynaptic connections to two autonomic targets, physiological studies into the same questions have run into technical limitations. Electrical stimulation is no longer considered a suitable tool when one wishes to locate neuronal cell bodies that drive a response pattern. Microinjections of cell body-selective excitants usually activate large numbers of neurons, resulting in limited spatial resolution. Refinements of this approach to improve spatial resolution (e.g. as in the present study) may yield considerable further information on the functional anatomy of autonomic control nuclei, particularly when multiple outflows are measured. In combination with other approaches and new experimental tools, this will help to define the hierarchy of autonomic control by the brain.
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Figure legends

**Figure 1. Effect of skin cooling on phrenic and plantar CVC activity.**

Above: representative chart record showing responses to three 30s episodes of skin cooling and the following fall in core temperature. Traces from above show heart rate, blood pressure, peak expired CO₂, core (rectal) temperature, skin temperature, phrenic nerve activity (rectified and integrated), phrenic burst rate (5s average, bursts/min) and single unit activity (5s spike counts) recorded from a plantar CVC fiber. Note that phrenic rate decreased reversibly with the first skin cooling episode and fell before any measurable decrease in core temperature or expired CO₂ (shaded period). Excerpts below show three expanded records of phrenic (rectified and integrated) and plantar CVC activities, taken from times denoted on the lower trace. Black dots in the second trace indicate discriminated spikes of a single plantar CVC unit, shown superimposed on an expanded timescale on the right.

**Figure 2. Responses to DLH microinjection at different sites.**

Above: representative photomicrograph showing pontamine blue dye marks (left) and reconstructed injection sites plotted on a line diagram (right). In the right panel, black dots and open squares indicate dye marks and calculated injection sites, respectively. Structures and rostrocaudal level are indicated with reference to Paxinos & Watson (33). Scale bar 500 μm. Below: chart records (a,b,c)
showing phrenic (rectified and integrated) and plantar CVC unit activity following injections of DLH into three sites denoted a, b and c in the line diagram above. Note the clear, but unequal, phrenic and CVC responses in a and b. No significant phrenic or CVC response was evoked from DLH injections into the other six sites shown above (i.e. c). Abbreviations: arc, arcuate nucleus; DA, dorsal hypothalamic area; DMN, dorsomedial hypothalamic nucleus; f, fornix; mt, mammillothalamic tract; VMN, ventromedial hypothalamic nucleus.

**Figure 3. Nerve and cardiovascular responses to DLH microinjection.**

Three different response patterns to DLH microinjections: From left to right, simultaneous increase in plantar CVC and phrenic rate (A), increase in phrenic rate only (B), and increase in plantar CVC only (C). Traces from above show heart rate, blood pressure, phrenic activity (rectified and integrated), phrenic rate (5s average) and single-unit activity (5s spike counts) recorded from plantar CVC fibers. Each injection site is shown below. Abbreviations as in Figure 2.

**Figure 4. DLH injection sites.**

Top, Grouped injection sites plotted onto standard parasagittal sections 0.4 and 0.9 mm lateral, drawn from the atlas of Paxinos & Watson (33). Dashed lines, from left, indicate three rostrocaudal levels: 3.0, 3.3 and 3.6 mm posterior to bregma. Black circles indicate sites where DLH activated plantar CVC without a
change in phrenic rate. Open circles indicate sites where phrenic rate was increased without plantar CVC activation. Pie symbols indicate sites where both plantar CVC and phrenic rate were increased, showing their proportionate increases by slice angle ($\Delta$ plantar CVC in black, and $\Delta$ phrenic rate in white). Crosses show sites where DLH had no significant effect on either plantar CVC or phrenic rate. Abbreviations are indicated in Figure 2. Bottom: plot of the logarithm of the response ratio ($\Delta$ plantar CVC / $\Delta$ phrenic rate) against the rostrocaudal level the injection site. Injections which failed to activate either nerve significantly were excluded from this plot, as was the single significant response to a lateral injection.

**Figure 5. Anatomical contour maps of response magnitudes.**

Panels a-e show contour maps representing the spatial distribution of response amplitudes for plantar CVC (a), phrenic rate (b), phrenic amplitude (c), heart rate (d) and blood pressure (e) following DLH microinjections into the DMH region. Data are taken from injections at all 66 medial (< 0.6 mm) sites shown in Figure 4, including non-significant responses. Sixteen color gradations represent the normalized data range in each case, as indicated. Note that the minima are not zero, but responses from the blue regions were mostly non-significant. Panel f shows an overlay of the upper 4 contours for plantar CVC (yellow), phrenic rate (green) and heart rate (blue) in relation to anatomical structures in the parasagittal
plane. Note the 2:1 difference between dorsoventral and rostrocaudal scales.

Abbreviations as in Figure 2.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5