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The dorsomedial hypothalamus and the response to stress Part renaissance, part revolution

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Abstract

Emotional stress provokes a stereotyped pattern of autonomic and endocrine changes that is highly conserved across diverse mammalian species. Nearly 50 years ago, a specific region of the hypothalamus, the hypothalamic defense area, was defined by the discovery that electrical stimulation in this area evoked changes that replicated this pattern. Attention later shifted to the hypothalamic paraventricular nucleus (PVN) owing to (1) elucidation of its role as the final common pathway mediating activation of the hypothalamic–pituitary–adrenal (HPA) axis, a defining feature of the stress response and (2) the finding that the PVN was the principal location of hypothalamic neurons that project directly to spinal autonomic regions. Consequently, a primary role for the PVN as the hypothalamic center integrating the autonomic and endocrine response to stress was inferred. However, our findings indicate that neurons in the nearby dorsomedial hypothalamus (DMH)—a region originally included in the hypothalamic defense area—and not in the PVN play a key role in the cardiovascular changes associated with emotional or exteroceptive stress. Indeed, excitation of neurons in the parvocellular PVN and consequent recruitment of the HPA axis that occurs in exteroceptive stress is also signaled from the DMH. Thus, the DMH may represent a higher order hypothalamic center responsible for integrating autonomic, endocrine and even behavioral responses to emotional stress. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

The physiological response to emotional stress consists of an integrated pattern of endocrine and autonomic changes that is highly conserved across mammalian species. Although these changes may be viewed in the context of enhancing the probability of survival in the face of a threatening circumstance, they have also been linked to various disease states in humans, including hypertension (Folkow, 1987; Henry et al., 1986), cardiac arrhythmias, sudden cardiac death and myocardial infarction (Meerson, 1994), gastrointestinal motility disorders and gastric or duodenal ulcer formation (Fossey and Lydiard, 1990), and increased susceptibility to infection (Kiecolt-Glaser and Glaser, 1995). In spite of these clinically important consequences, the central pathways and mechanisms responsible for the physiological changes associated with emotional stress remain unclear. However, for two decades, attention in this regard has been focused on the hypothalamic paraventricular nucleus (PVN).

2. The PVN: anatomic crossroad for endocrine and autonomic function in stress?

Most current thinking regarding the hypothalamus and autonomic cardiovascular control—especially relating to the response to stress—assumes a primary integrative role for the PVN (see Culman and Unger, 1992; Dampney, 1994; Loewy, 1991; Pacak et al., 1995; Swanson, 1991). This assumption rests on two lines of evidence. First, both anatomic and functional evidence support a role for neurons in the PVN as the final common pathway in the CNS mediating the activation of the adrenal cortex that is considered a hallmark of the stress response (Sawchenko et al., 2000; Whitnall, 1993). Stress-induced secretion of

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adrenal glucocorticoids results from the increased release of adrenal corticotrophic hormone (ACTH) from the adenohypophysis, which in turn is triggered principally by secretion of corticotrophin releasing hormone (CRH) into the hypothalamic portal system at the median eminence (ME; Antoni, 1986). Most of the CRH-containing neurons whose terminals impinge on this portal system are localized in the PVN; lesions of the PVN reduce tissue levels of CRH in the ME by more than 85% (Koegler-Muly et al., 1993; Palkovits et al., 1991), and reduce or abolish plasma ACTH or corticosterone responses to stress (Richardson-Morton et al., 1989). More recently, a host of diverse stressors have been shown to increase the expression of Fos (Ceccatelli et al., 1989; Chen and Herbert, 1995; but see Senba et al., 1993) and mRNA for CRH (Kalin et al., 1994; Makino et al., 1995) in neurons in the parvocellular PVN, suggesting that these neurons are, indeed, activated in stress.

In addition to the link with stress-induced activation of the hypothalamic–pituitary–adrenal (HPA) axis as described above, a second line of evidence has been taken to implicate the PVN in autonomic responses to emotional stress. From its inception, the majority of this evidence has been neuroanatomic. Direct projections from the PVN to regions of the spinal cord where sympathetic preganglionic neurons are located were first demonstrated 20 years ago (Kuypers and Maiky, 1975; Saper et al., 1976), and reports of projections to other important autonomic centers followed (for review, see Swanson and Sawchenko, 1980, 1983). From these purely *anatomic* findings, a major *functional* role for the PVN in the autonomic responses to stress was quickly inferred. This logic, wherein a functional role for the PVN as the location of hypothalamic "command neurons" for the cardiovascular defense reaction is derived wholly from anatomic findings, has continued to recent times (see Jansen et al., 1995).

The most significant *functional* data in support of such a role for the PVN derives from the results of studies that have employed the technique of microinjection in an attempt to chemically stimulate this region. Thus, microinjection of either the $GABA_A$ receptor antagonist bicuculline methiodide (BMI) or the excitatory amino acids kainate (KA) or *N*-methyl-D-aspartate (NMDA) into the PVN was reported to elicit increases in heart rate, increases in blood pressure, and/or changes in local blood flow (Jin and Rockhold, 1989; Martin and Haywood, 1993; Martin et al., 1991; Porter, 1993; Rockhold et al., 1987; Haywood et al., 2001; Schlenker et al., 2001), and these changes resemble those seen in acute stress. However, interpretation of these findings—or those of any studies where pharmacologic interventions have targeted the PVN using local microinjections—has generally failed to take into consideration two key issues: (1) the doses employed, particularly in the context of spread or diffusion to adjoining areas and (2) the relative proximity of the PVN to the dorsomedial hypothalamus (DMH).

3. The DMH: an effector center for the cardiovascular response to emotional stress

The DMH was considered part of the classical "hypothalamic defense area" based upon the finding that electrical stimulation of this region provoked the defense reaction, a pattern of adjustments characterized by behavioral and autonomic changes typically seen when the organism was confronted with a threatening stimulus (for review, see Hilton, 1979). Our search for central sites where $GABA_A$ receptor antagonists might act to increase sympathetic nervous activity first led us to roughly this same region of the hypothalamus in the rat. Microinjection of BMI or picrotoxin into this region elicited marked increases in heart rate and more modest pressor responses in anesthetized and conscious rats (DiMicco and Abshire, 1987; DiMicco et al., 1986; Fig. 1). These changes were accompanied by increases in sympathetic nerve activity and plasma catecholamines (Wible et al., 1988, 1989) and were in fact shown to be sympathetically mediated (DiMicco et al., 1986). Moreover, although changes in arterial pressure were relatively modest, examination of regional hemodynamics revealed opposite effects on visceral blood flow (i.e., splanchnic and renal arteries) which was greatly reduced, and skeletal muscle (hindquarter) blood flow which increased by as much as eight-fold (DiMicco et al., 1992). Similar marked increases in heart rate accompanied by relatively modest pressor effects were evoked by microinjection of excitatory amino acids KA, NMDA or AMPA into the same region of the DMH (Soltis and DiMicco, 1991a, 1992). Thus, disinhibition or stimulation of neurons in the DMH—an area usually included in the "hypothalamic defense area"—

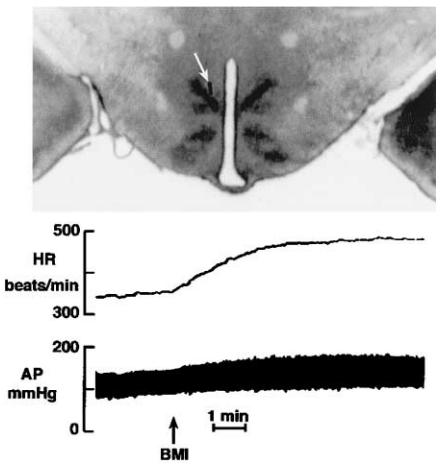

Fig. 1. Cardiovascular effect of chemical stimulation of the DMH in a urethane-anesthetized rat. Top: Photomicrograph of neutral red-stained coronal section of rat brain (contrast enhanced to emphasize regions of high neuronal density) depicting dye-marked microinjection site in the DMH (arrow). Bottom: Direct recording of heart rate (HR) and arterial pressure (AP) illustrating response to microinjection of BMI 20 pmol/50 nl (arrow) at site in the DMH indicated above.

evoked a pattern of cardiovascular changes that closely resembled the classic defense reaction.

In the case of both the GABA receptor antagonists and the excitatory amino acids, the most responsive sites seemed to be restricted to a region consisting of the dorsomedial hypothalamic nucleus itself—particularly at anterior–posterior levels where a clear zona compacta was evident—and immediately adjoining areas lateral and dorsal to it. Injection at sites more lateral, dorsal or posterior to this region evoke progressively smaller cardiovascular changes. However, as indicated above, microinjection of KA, NMDA or BMI into the PVN, located less than a millimeter anterior to the DMH, had been reported to produce marked cardiovascular changes, the salient feature of which was usually sympathetically mediated cardiac stimulation. Such changes seemed to provide evidence in support of the appealing concept of a role for the PVN in autonomic function as suggested by numerous anatomical studies. However, the doses employed in these studies ranged from 5 to 5000 pmol for KA, 50 to 100 pmol for NMDA and 50 to 200 pmol for BMI. In contrast, microinjection of the same agents into the DMH produced similar effects at doses orders of magnitude lower—only 0.1–5 pmol for KA, 1–10 pmol for NMDA and 2–10 pmol for BMI (Soltis and DiMicco, 1991a,b, 1992). These findings suggested that microinjection of excitatory amino acids or BMI into the PVN at the relatively high doses employed in these studies might produce cardiovascular effects as a consequence of diffusion to neurons in the nearby DMH which appeared to be exquisitely sensitive to these agents.

To examine this issue more closely, we compared the effects microinjection of relatively low doses of BMI (10 pmol), KA (0.5 pmol) and NMDA (5 pmol) at sites in the DMH, in the PVN or in the area between the two regions in anesthetized and conscious rats (DeNovellis et al., 1995). For each of the three agents, the same pattern emerged. In conscious rats, marked and immediate tachycardia accompanied by a modest pressor response appeared after injection into the DMH while lesser effects were observed after identical injection into the intermediate area (i.e., anterior to the DMH and closer to the PVN), and the smallest response was seen after microinjection directly into the region of the PVN. Furthermore, in anesthetized rats where similar results were obtained, the exact time at which heart rate began to increase could be more precisely determined. The latency from injection to onset of tachycardia was shortest after injection of BMI into the DMH (5 ± 1 s) and significantly longer after injection into the PVN (66 ± 18 s)—and intermediate after injection at sites between the two regions (39 ± 17 s). The longer times to onset are consistent with additional time required for diffusion of BMI to a single site of action in the DMH from these progressively anterior injection sites. Taken together, the findings of this study provide compelling evidence that the principal site of action mediating the tachycardia seen after microinjection of excitatory amino acids or BMI into any of these areas of the medial hypothalamus is likely to be the DMH and not the PVN. Accordingly, it seems possible if not likely that the tachycardia—and even the pressor effects—reported after injection of GABA antagonists or excitatory amino acids into the PVN may be attributed in large part to spread or diffusion of these agents to the DMH.

These cardiovascular changes resulting from chemical stimulation of the DMH were accompanied in our studies by other effects characteristic of the defense reaction and typically seen in stress. Respiratory rate increased in anesthetized rats (DiMicco and Abshire, 1987), and in conscious rats we observed locomotor stimulation suggesting "escape behavior" (Shekhar and DiMicco, 1987), an effect reported by others (DiScala et al., 1984; Brandao et al., 1986). This subjective impression was borne out by subsequent behavioral analysis: Microinjection of BMI selectively augmented avoidance responding in an approach/avoidance schedule and produced experimental "anxiety" in a punished responding paradigm (Shekhar et al., 1987, 1990). Conversely, injection of the $GABA_A$ receptor agonist muscimol (see below) produced an "anxiolytic" effect (Shekhar et al., 1990). The striking similarity between the effects of disinhibition of the DMH in rats and the response to acute emotional stress in this species led us to speculate that the same mechanism was involved in both phenomena. Thus, we hypothesized that the the pattern of physiologic changes seen in stress resulted from activation of the neurons in the DMH that were excited by local $GABA_A$ receptor blockade or glutamate ionotropic receptor stimulation in our microinjection studies. If so, then we reasoned that inhibition of neuronal activity in this region would reduce or block stress-induced cardiovascular changes.

This was, indeed, shown to be the case in studies employing muscimol, an agent found to be inhibitory to virtually all mammalian neurons by virtue of its $GABA_A$ receptor agonist properties. Microinjection of muscimol has come to be a standard technique to achieve acute reversible suppression of neuronal activity in a discrete region of the brain (for recent examples, see Hoshi et al., 2000; Waitzman et al., 2000; Martin et al., 2000; Schieber, 2000; Clayton and Williams, 2000). In our initial experiments, bilateral microinjection of muscimol (88 pmol/250 nl) at hypothalamic sites where injection of BMI elicited marked tachycardia under anesthesia failed to influence baroreflex-induced tachycardia but abolished the increases in heart rate normally seen in an air stress paradigm (Lisa et al., 1989; see Fig. 2). Identical injection of muscimol in the same rats under unstressed conditions lowered heart rate by only 30 beats/min at 10 min—a time point in the air stress trial at which heart rate was elevated by more than 130 beats/min after saline but only 13 beats/min after muscimol. This modest effect may have resulted from muscimol-induced suppression of a low but significant level of activity in the DMH owing to the minimal stress of the laboratory setting. Most importantly, however, the small baseline effect cannot account for the dramatic reduction of the tachycardia seen under conditions

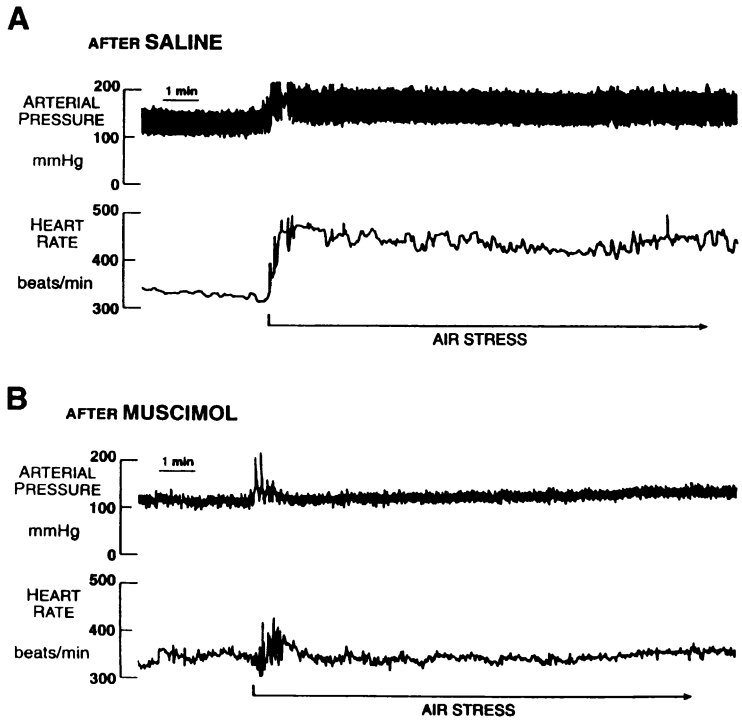

Fig. 2. Original recordings of arterial pressure (recorded from the femoral artery) and heart rate in two different rats illustrating typical cardiovascular responses to air jet stress (applied during period indicated by arrow) after bilateral microinjection of (A) vehicle (100 nl saline) or (B) muscimol (80 pmol/side) into the DMH. (Reproduced from Morin et al., 2001.)

of air stress. These results suggested that the activity of neurons somewhere in this region played a critical role in the sympathetically mediated cardiac stimulation seen in this stress paradigm.

In a subsequent study that paralleled our experiments employing chemical stimulation, we better defined the location of these neurons, particularly with regard to the DMH versus the PVN (Stotz-Potter et al., 1996a). In random order and on different days, muscimol 80 pmol/100 nl and saline vehicle were microinjected bilaterally into the region of the DMH, the PVN and the area between the two regions just prior to air stress (Fig. 3). As in our previous study, injection of muscimol into the DMH nearly abolished stress-induced increases in both heart rate and arterial pressure. Identical treatment at sites between the DMH and the PVN reduced stress-associated cardiovascular changes by approximately half, while injection into the immediate vicinity of the PVN had no effect. That such treatment was sufficient to inhibit neurons in the parvocellular PVN was clear from the results of another study in which plasma ACTH was also measured (Stotz-Potter et al., 1996b). Microinjection of muscimol into the PVN markedly reduced the associated elevation in plasma ACTH (Fig. 4), indicating that neurons in the parvocellular PVN were effectively inhibited, but again failed to influence air stress-induced increases in heart rate or arterial pressure. Thus, our results suggest that activity of neurons in the PVN plays no discernible role in the generation of air stress-associated tachycardia or pressor responses. They also clearly demonstrate that the cardiovascular effects of microinjecting these agents into the DMH in our studies are not a consequence of spread or diffusion to the nearby PVN. Instead, our findings highlight the possibility that interventions that have targeted the PVN and have been shown to either mimic (see above) or suppress (Callahan et al., 1992; Morris et al., 1995) stress-induced cardiovascular changes may have actually done so by affecting the DMH.

Muscimol, when employed in careful microinjection protocols, represents a powerful pharmacologic tool for establishing the location of the specific neurons involved in a particular function by virtue of its ability to inhibit virtually all neurons. However, for this very reason, the results of such studies provide little insight into the specific physiologic mechanisms that may regulate the activity of the relevant neurons. In our experiments, an important role for tonic inhibition mediated by endogenous GABA was clearly implied not by the effect of muscimol but by the ability of local $GABA_A$ receptor blockade to activate this critical population of neurons. Consistent with this implication, inhibiting local GABA uptake in the DMH was shown to elevate extracellular GABA levels in the DMH and markedly suppress air stress-induced cardiovascular changes (Anderson and DiMicco, 1990). In addition to interventions targeting GABAergic mechanisms in the DMH, we found that microinjection of kynurenate, a non-selective antagonist of ionotropic glutamate receptors, also suppressed air stress-induced cardiovascular changes (Soltis and DiMicco, 1992). Significant suppression was also

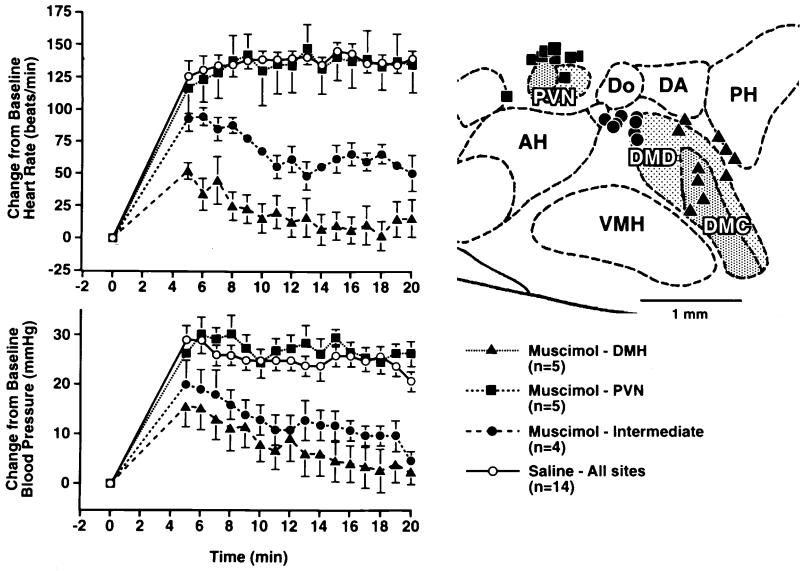

Fig. 3. Comparison of cardiovascular responses to air jet stress (left) immediately after bilateral microinjection of muscimol (80 pmol/100 nl/side) or saline vehicle into the DMH, the PVN or the intermediate area between the two regions. Each rat was subjected to air stress from $t=0-20$ min immediately after treatment with saline and muscimol in staggered order on alternate days. Response after saline injection in all areas was equivalent and so has been pooled for all 14 rats. Right: Approximate location of all injection sites shown in parasaggital schematic adapted from Paxinos and Watson, 1997. All injection sites were 200–900 μm from the third ventricle. AH, anterior hypothalamus; DA, dorsal hypothalamic area; DMC, dorsomedial hypothalamic nucleus, compact; DMD, dorsomedial hypothalamic nucleus, diffuse; Do, dorsal hypothalamic nucleus; PH, posterior hypothalamic area; PVN, paraventricular hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus. (Adapted from Stotz-Potter et al., 1996a; Copyright 1996 by the Society for Neuroscience.)

apparent after local microinjection of 2-amino-5-phosphopentanoic acid (AP5) or 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) at doses that were shown to block selectively at NMDA and non-NMDA receptors, respectively (Soltis and DiMicco, 1992). Thus, our results seemed to support (a) the existence of a critical population of hypothalamic neurons in the DMH that are responsible for generating stress-induced tachycardia and (b) important roles for synaptic activity at local $GABA_A$ and glutamate ionotropic receptors in regulating their activity. While $GABA_B$ and metabotropic glutamate receptors in this region also have the potential to influence heart rate (DiMicco and Monroe, 1996, 1998), their roles, if any, in stress-induced cardiovascular changes have yet to be evaluated.

Until recently, little was known about the efferent pathways through which relevant neurons in the DMH might provoke cardiovascular changes. However, Fontes et al. (2001) have now demonstrated that the increases in blood pressure resulting from microinjection of BMI into the DMH can be blocked by microinjection of muscimol into the rostral ventrolateral medulla (RVLM). Neurons in the RVLM are thought to provide tonic excitatory drive to spinal sympathetic preganglionic neurons responsible for vasomotor tone (Reis et al., 1989). Accordingly, a retrograde tracer injected into the RVLM labeled numerous neurons in the region of the DMH, both in the dorsal aspect of the dorsomedial hypothalamic nucleus and outside the boundaries of the nucleus itself, in the adjacent area dorsal and lateral to it (Fonte et al., 2001). Thus, a direct projection from neurons in the region of the DMH to the RVLM seems likely to play a role in the pressor response seen after chemical stimulation of the DMH—and perhaps also in the increases in blood pressure seen in emotional stress.

Interestingly, although microinjection of muscimol into the RVLM virtually abolished DMH-induced increases in blood pressure in the study of Fontes et al. (2001), it failed to influence the associated tachycardia. Thus, while the RVLM appears to mediate DMH-induced pressor effects, this region seems to play little part in generating the tachycardia that represents the salient feature of the cardiovascular response to both stimulation of the DMH and emotional stress in rats. Fontes and colleagues instead proposed that a projection from the DMH to the midbrain periaqueductal grey (PAG) could mediate these cardiac sympathetic effects. Because the PAG receives input from the DMH (Ter Horst and Luiten, 1986; Thompson et al., 1996) and has been proposed as a relay between forebrain centers involved in emotional processing and cardiac sympathetic pathways (Farkas et al., 1998), this is an attractive hypothesis.

Another site of potential interest with regard to the pathway originating in the DMH that may mediate stress-induced tachycardia is the raphe pallidus in the brainstem. We have confirmed and extended the original observations of Morrison and coworkers (1999) and found that disinhibition of the region of the raphe pallidus by local microinjection of BMI provokes marked tachycardia and modest increases in arterial pressure—a pattern closely resembling the cardiovascular response elicited by chemical stimulation of the DMH (Samuels et al., 2002). The raphe

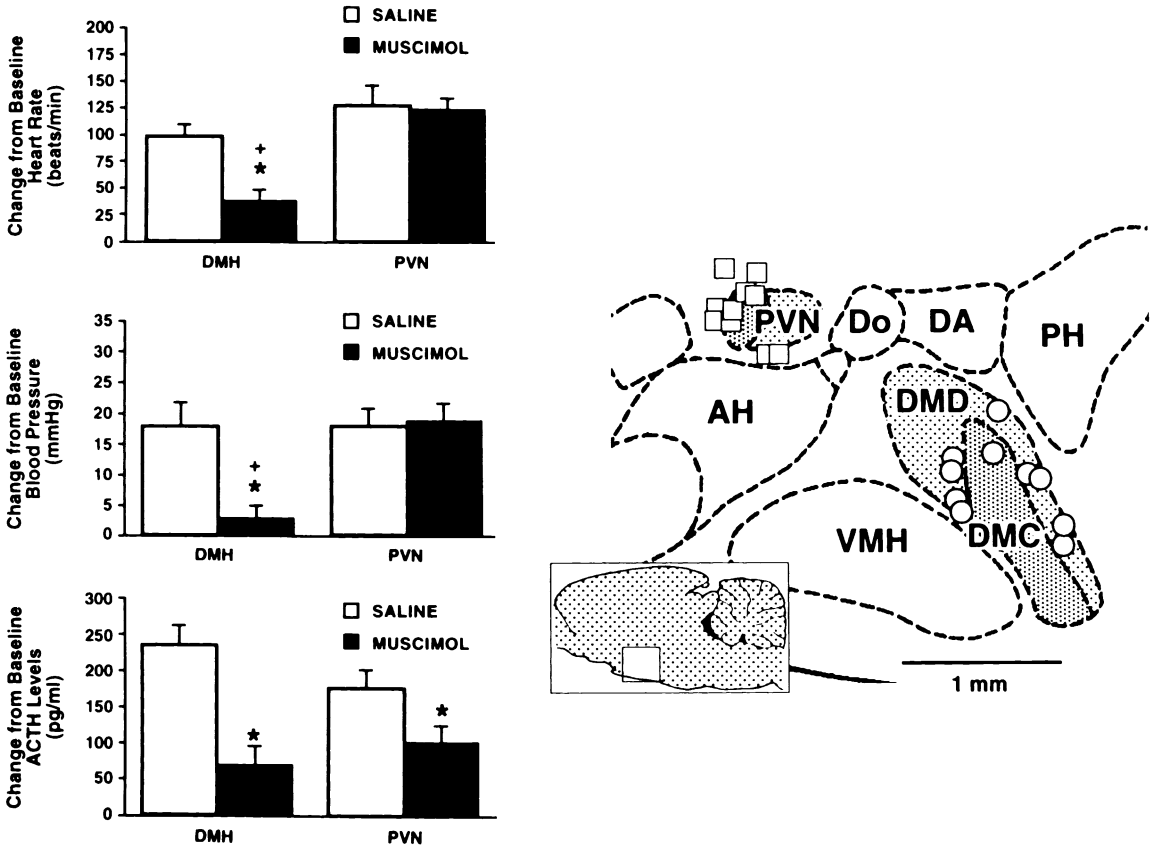

Fig. 4. Effect of microinjection of muscimol into the DMH versus the PVN on air stress-induced increases in heart rate, blood pressure and plasma ACTH. Left: Mean changes (±S.E.M.) from baseline heart rate and blood pressure (averaged over 10 min of air stress), and from baseline plasma ACTH levels (from sample taken after 10 min of air stress) after bilateral microinjection of muscimol (80 pmol/100 nl/side) or saline vehicle into the region of the DMH ($n=5$) or the PVN ($n=5$). * Significantly different from response after injection of saline by paired t test ($P<.05$). $^{+}$ Significantly different from response after injection of muscimol in PVN by two-way ANOVA ($P<.05$). Right: Schematic parasagittal section of mediobasal diencephalon (see inset) adapted from the atlas of Paxinos and Watson (1986) illustrating bilateral injection sites corresponding to data at left. All injection sites within 900 μm of third ventricle. For abbreviations, see legend, Fig. 3. (Reprinted from Stotz-Potter et al., Effect of microinjection of muscimol into the dorsomedial or paraventricular hypothalamic nucleus on air stress-induced neuroendocrine and cardiovascular changes in rats, Brain Res 742, pp. 219–224, copyright 1996, with permission from Excerpta Medica.)

pallidus receives direct projections from neurons in the dorsal hypothalamic area immediately adjacent to the dorsomedial hypothalamic nucleus (Hosoya et al., 1989; Hermann et al., 1997) and projects heavily to the specific levels of the spinal cord known to give rise to cardiac sympathetic innervation in rats (Miura and Kitamura, 1979) and cats (Miura et al., 1983). More recently, the raphe pallidus was identified as one of relatively few brain regions doubly labeled by transsynaptic retrograde infection from two different pseudorabies viruses placed in the stellate ganglion and the adrenal gland (Jansen et al., 1995). Both swim and restraint stress evoked marked increases in Fos-positive neurons in the raphe pallidus (Cullinan et al., 1995), and we have recently confirmed a similar induction in this region in our air stress paradigm (Zaretskaia and DiMicco, unpublished observations). Thus, neurons in the raphe pallidus, a region where chemical stimulation provokes marked sympathetically mediated tachycardia, project to both of the circuits most closely associated with the sympathetic response to emotional stress (i.e., the heart and the adrenal medulla) and are activated in several experimental stress paradigms.

4. The DMH and stress-induced neuroendocrine changes

Activation of the HPA axis represents a defining feature of the response to stress in mammals. As discussed above, the rationale for the notion that the PVN represents the principal hypothalamic site for integration of the autonomic and endocrine response to stress has its historical origin in the acknowledged role of neurons in this nucleus in the mobilization of adrenal corticosteroids. However, several lines of evidence point to the possibility that neurons in the DMH play a crucial role in activation of the neurons in the PVN that are responsible for recruitment of the HPA axis in some forms of stress. According to PHA-L anterograde tracing studies (Ter Horst and Luiten, 1986, 1987; Thompson et al., 1996), the PVN represents a primary target for DMH efferents. In vitro studies in a hypothalamic slice

preparation identified the DMH as a region that provides glutamate-mediated excitatory input to neurons in the parvocellular PVN (Boudaba et al., 1997). Accordingly, microinjection of BMI into the DMH—but *not* into the PVN—increased plasma ACTH and corticosterone in pentobarbital-anesthetized rats (Keim and Shekhar, 1996).

Our recent results have confirmed that activation of neurons in the DMH elevates circulating levels of ACTH in conscious rats (Bailey and DiMicco, 2001). Microinjection of BMI 10 pmol or KA 1 or 3 pmol into the DMH provoked marked increases in plasma ACTH that accompanied and were correlated with the usual tachycardia as described above. In contrast, similar treatment in the PVN caused no significant increase in ACTH. This latter finding was somewhat surprising, given that: (1) KA, owing to its ability to stimulate ionotropic glutamate receptors, is generally considered to be excitatory to all mammalian neurons including those in the PVN (van den Pol et al., 1990), (2) neurons in the hypophysiotropic subregion of the PVN, including those containing CRH, appear to express relatively high levels of ionotropic glutamate receptors (Aubry et al., 1996; van den Pol et al., 1994; Herman et al., 2000) and (3) microinjection of glutamate itself into the PVN has been reported to increase plasma levels of ACTH in anesthetized rats (Darlington et al., 1989). One possible explanation for our negative finding is that microinjection of KA excited local GABAergic interneurons resulting in $GABA_A$ receptor-mediated inhibition of parvocellular hypophysiotropic neurons in the PVN (Roland and Sawchenko, 1993). However, plasma ACTH was also unaffected when the GABA antagonist BMI was coinjected into the PVN along with KA in pilot experiments (Bailey and DiMicco, unpublished observations). A second possibility that should be considered whenever microinjection of excitatory amino acids yields unexpected negative findings is that of depolarizing blockade (Lipski et al., 1988). However, the relatively low dose of KA employed makes this possibility unlikely. Whatever the explanation, these results argue against the PVN as the site of action for the activation of the HPA axis seen after microinjection of both BMI and KA into the DMH in these experiments. Thus, our findings supported the earlier results of Keim and Shekhar (1996) and, together with the work of others, indicate that activation of neurons in the region of the DMH can excite the HPA axis through a direct projection to the parvocellular PVN.

A role for this pathway in the generation of stress-induced increases in plasma ACTH was suggested by two different double-labeling studies employing a retrograde tracer applied to the PVN and Fos expression as an indicator for neuronal excitation (Cullinan et al., 1996; Li and Sawchenko, 1998). In both studies, the DMH was identified as a major source of afferent input to the PVN that was activated in stress. Interestingly, however, opposite roles for the DMH in stress-induced HPA activation were inferred from these essentially similar results. Cullinan and colleagues (1996) speculated that the projection was inhibitory because their data suggested that the majority of these neurons contained glutamic acid decarboxylase (GAD), the enzyme responsible for the biosynthesis of the inhibitory neurotransmitter GABA in GABA-releasing neurons. Thus, they believed that the apparent activation of this pathway in stress noted in their study represented negative feedback modulation of HPA function. In contrast, Li and Sawchenko (1998) theorized that the projection originating in the DMH could represent a primary pathway through which neurons in the PVN might be activated under conditions of stress.

We assessed the role of neuronal activity in the DMH with respect to stress-induced activation of the HPA axis by microinjecting muscimol into the DMH or the PVN prior to air stress and examining plasma ACTH as well as cardiovascular function (Stotz-Potter et al., 1996b; Fig. 4). Once again, bilateral microinjection of muscimol (80 pmol/100 nl/side) into the DMH greatly attenuated the increases in heart rate and arterial pressure whereas similar treatment in the PVN had no discernible effect. As we had anticipated, air stress provoked marked increases in plasma ACTH, and these increases were significantly reduced by prior injection of muscimol into the PVN. This indicated that treatment with muscimol had, indeed, effectively inhibited neurons in the parvocellular PVN at the same time that stress-induced tachycardia and pressor responses were unaffected—an observation that further undermined a role for neurons in the PVN in air stress-induced cardiovascular changes. However, microinjection of muscimol into the DMH also suppressed the increases in plasma ACTH as well as the increases in heart rate and arterial pressure. Thus, neuronal activity in the DMH appears to play a crucial role in the activation of the HPA axis seen in this experimental paradigm for stress.

Functional neuroanatomical data using stress-induced Fos expression as an indicator also support the idea that activation of neurons in the PVN occurs in air stress and is signaled by excitation from the DMH (Morin et al., 2001). Rats implanted with guide cannulae in the DMH and arterial lines for monitoring heart rate and blood pressure received bilateral microinjections of either muscimol (80 pmol/100 nl/side) or saline (100 nl/side) just prior to being subjected to air stress for 20 min. Ninety minutes later, rats were sacrificed and the brains processed for Fos immunohistochemistry. Fos-positive neurons in the parvocellular and magnocellular subregions of the PVN were quantitated in each rat and found to be markedly increased relative to the number seen in control animals (Fig. 5). Treatment in the DMH with muscimol—in a manner shown previously to suppress air stress-induced increases in plasma ACTH (Stotz-Potter et al., 1996b)—dramatically reduced the number of Fos positive neurons in both subregions of the PVN. Similar injection of muscimol at sites anterior to the DMH and *closer* to the PVN failed to influence either the tachycardia or the increased Fos expression resulting from air stress, excluding the possibility that the effect of microinjection of muscimol into the DMH was a consequence of

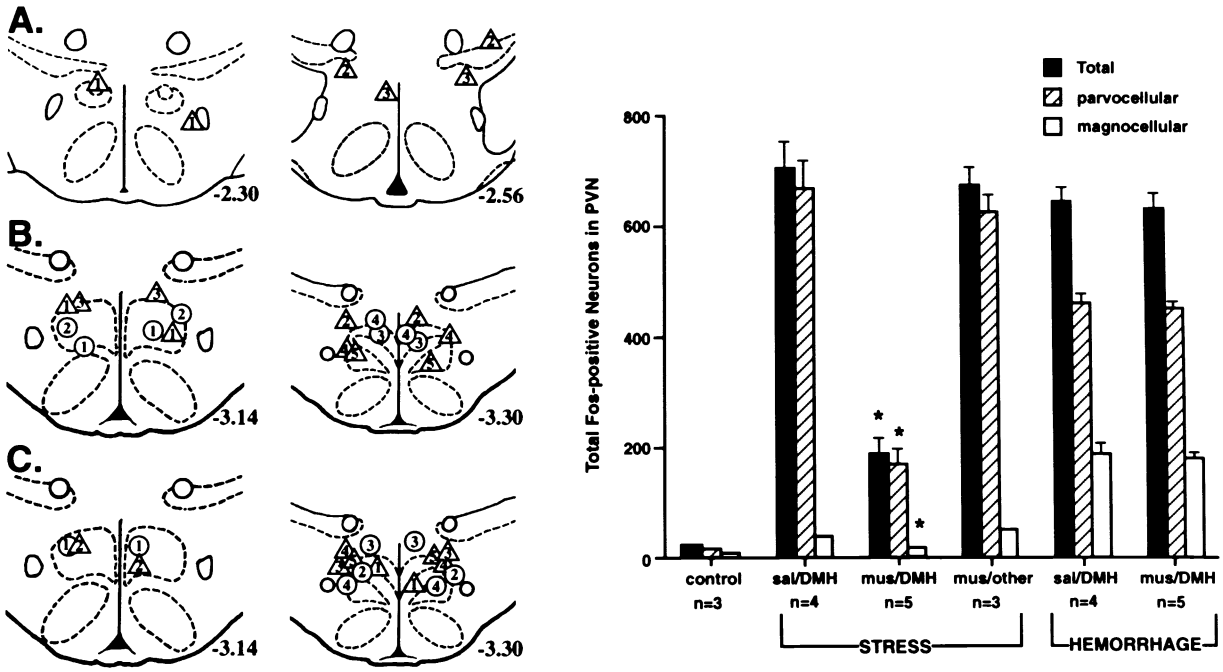

Fig. 5. Effect of prior bilateral microinjection of muscimol (80 pmol/100 nl) into the region of the DMH on increases in Fos expression in the PVN induced by air stress or hemorrhage. Left: Schematic coronal sections adapted from the atlas of Paxinos and Watson (1997) depicting approximate location of all injection sites for which data represented. Shown are paired bilateral injection sites located in the hypothalamus at sites anterior to the DMH (A; indicated by "other" at right) and at the level of the DMH (B and C). Rats were injected with either saline vehicle (circles) or muscimol (triangles) and then subjected to air stress (A or B) or hemorrhage (C). Right: Cell counts of Fos-positive neurons in main body of PVN expressed as total number and as number in parvocellular and magnocellular subregions of the nucleus in rats representing all treatment groups (sal = saline-treated; mus = muscimol-treated) and three control (unstressed/untreated) rats. Data plotted represent means (± S.E.M.) of total number of Fos-positive neurons in six alternate coronal sections representing the main body of the PVN for each individual rat. * Significantly different from corresponding values in saline-treated rats and in rats in which muscimol was microinjected at sites anterior to the DMH by one-way ANOVA and Dunnett's test, $P < .05$. (Reproduced from Morin et al., 2001.

spread or diffusion to the PVN itself. The data indicate that, like the sympathetically mediated tachycardia, activation of the PVN that occurs in air stress can be suppressed by inhibition of the DMH.

An intriguing aspect of this study involved parallel experiments in which the stressor employed was hemorrhage rather than air stress. Neurons in the PVN represent the final common pathway for activation of the HPA axis that occurs in response to a wide range of stressors. However, the concept that different afferent pathways to the PVN may mediate its activation in response to different modes of stress has become a subject of considerable interest (see Emmert and Herman, 1999; Sawchenko et al., 2000; Thivrikraman et al., 2000). A primary distinction has been proposed between emotional or neurogenic stressors, which have been termed exteroceptive, and stressors primarily of a physiological nature, referred to as interoceptive stressors. The air stress paradigm employed in our studies thus represents a classic exteroceptive or neurogenic stressor. Hemorrhage, on the other hand, is an interoceptive stressor known to provoke activation of CRH—and vasopressin-releasing neurons in the PVN and thus powerful induction of Fos in this nucleus (Darlington et al., 1992; Schreihofer et al., 1997; Thivrikraman et al., 2000). The ability of hemorrhage to provoke marked induction of Fos in the PVN was confirmed in our study. However, in stark contrast to its effect on air stress-induced Fos expression, microinjection of muscimol at identical sites in the DMH had no effect whatsoever on hemorrhage-induced Fos induction in the PVN in our study (Morin et al., 2001; Fig. 5). This failure to influence the similar changes evoked by hemorrhage suggests that neuronal activity in the DMH is not responsible for the activation of the PVN that occurs in this setting. Our findings are complemented by the previous results of Thivrikraman et al. (2000) who found that air puff startle provoked marked increase in Fos expression in the DMH, as has been reported previously in other pradigms for exteroceptive stress (Beck and Fibiger, 1995; Cullinan et al., 1996; Li and Sawchenko, 1998), while hemorrhage did not. Thus, neurons in the DMH are activated by exteroceptive stressors but not by the interoceptive stress of hemorrhage, and this activation appears to play a crucial role in recruitment of the HPA axis seen in the former paradigms but not the latter state.

5. Afferent pathways to the DMH

While evidence exists that points to efferent pathways mediating the increases in plasma ACTH and the tachycar-

dia as described above, much less is known about the afferent pathways to the DMH that might activate the neurons in this region that are relevant to these changes. The DMH receives input from a host of forebrain regions that might play such a role (Thompson and Swanson, 1998). Interestingly, while the PVN represents a major target for efferents from the region, neurons in the most anterior subregion of the PVN also project heavily to the DMH (Ter Horst and Luiten, 1987; Thompson and Swanson, 1998). In contrast to those in other subregions of the PVN, nothing is known about the potential role of these neurons in any facet of the response to stress. However, two other forebrain regions worthy of consideration with regard to afferent regulation of neurons in the DMH in stress are the amygdala and the median preoptic area.

The amygdala has a long history connected with stress and anxiety (for reviews, see Davis, 1997; Buijs and Van Eden, 2000). Chemical stimulation of the central amygdaloid nucleus with glutamate (Iwata et al., 1987) or the basolateral amygdala by local microinjection of BMI (Sanders and Shekhar, 1991) provokes marked increases in heart rate and moderate pressor responses resembling those seen in emotional stress. Most importantly, these cardiovascular effects of amygdalar stimulation with BMI are virtually abolished by prior injection of glutamate ionotropic receptor antagonists into the DMH (Soltis et al., 1998). This result suggests that these amygdalar-induced cardiovascular changes are mediated through glutamatergic excitation of neurons in the latter region and parallels our finding discussed above that similar blockade of ionotropic glutamate receptors in the DMH suppresses air stress-induced tachycardia (Soltis and DiMicco, 1992). Neurons in the amygdala appear to send few if any projections directly to the DMH (Thompson and Swanson, 1998), but project extensively to the adjacent lateral hypothalamus (Gray et al., 1989). Thus, cardiovascular changes generated from the amygdala, a structure closely linked with fear and anxiety, may be mediated by glutamatergic excitation of neurons in the DMH relayed via the lateral hypothalamus.

Most recently, an intriguing connection has been forged between the DMH and human panic disorder, and this work now points to the mPOA as a potential source of excitatory input to the DMH (Shekhar and Keim, 1997; Shekhar et al., 1996). The effects of $GABA_A$ receptor blockade in the DMH in rats closely resembles the clinical picture of panic disorder in patients. While little is understood about the specific neural substrates that play a role in panic disorder, a standard technique employed in its diagnosis has for many years been intravenous infusion of sodium lactate. This procedure provokes panic attacks characterized by intense anxiety as well as such typical autonomic effects as tachycardia in susceptible patients (but not in normal individuals) through mechanisms that remain unknown (see Cowley and Arana, 1990). Interestingly, infusion of sodium lactate evokes a similar response—including marked tachycardia—in rats in which GABAergic function in the DMH has been impaired pharmacologically by chronic inhibition of local GABA synthesis, but not in control rats (Shekhar and Keim, 1997; Shekhar et al., 1996). Thus, neurons in the DMH appear to be responsible both for air stress-induced behavioral and autonomic changes and for the similar effects seen in lactate-induced "panic attacks" in these rats.

A key anatomical substrate mediating the effect of sodium lactate in this model appears to be a particular region of the anteriorly located median preoptic area (mPOA). Retrograde tracing studies show that neurons in the mPOA adjacent to the organum vasculosum lamina terminalis (OVLT) represent the greatest single source of afferent input to the DMH (Thompson and Swanson, 1998; Cavanagh and DiMicco, unpublished observations). The OVLT, one of the few areas of the central nervous system unprotected by a blood–brain barrier, is a site at which a circulating substance such as sodium lactate might gain access to and influence activity in the brain, particularly in adjoining areas of the mPOA. Accordingly, (1) microinjection of tetrodotoxin into this area blocks the ability of systemic lactate to provoke tachycardia and panic-like behavior while injections placed dorsolaterally in more distal locations in the preoptic area failed to block these effects and (2) microinjection of sodium lactate directly into this area elicited increases in heart rate and behavioral changes similar to those seen after systemic infusion of doses more than 6000 times greater (Shekhar and Keim, 1997). These findings have obvious implications for both panic disorder and the mechanism for its diagnostic provocation by sodium lactate infusion in humans. However, they are also important in suggesting that the dense projection to the DMH from the mPOA adjacent to the OVLT has the potential to recruit those neurons in the DMH capable of generating the autonomically mediated changes seen in stress such as tachycardia.

Little information is available with regard to a potential role for the mPOA in the response to stress. However, increased Fos expression has been reported in the mPOA in response to a variety of stressors including foot shock, leg pinch, immobilization/restraint, swim stress and intraperitoneal injection of hypertonic saline (Senba et al., 1993; Larsen and Mikkelsen, 1995; Cullinan et al., 1995, Li and Sawchenko, 1998), suggesting that neurons in this region are excited in these diverse stress paradigms in rats. Chemical stimulation of the mPOA by local micronjection of a wide variety of agents has been reported to produce a pattern of cardiovascular changes that replicates that seen in exteroceptive stress in rats (Feuerstein et al., 1982; Diz and Jacobowitz, 1984a,b; Siren and Feuerstein, 1991; Osborne and Kurosawa, 1994; Szabo et al., 1998). Thus, chemical stimulation of the mPOA, a region that projects heavily to the DMH, produces marked increases in heart rate accompanied by modest elevations in arterial pressure and increases in plasma ACTH—the same pattern of changes seen after chemical stimulation of the DMH or in air stress

in our studies. The role of this pathway in the response to stress would seem to be a promising area for future study.

6. Summary and conclusion

Our work over the past decade provides compelling evidence that neurons in the region of the DMH, a region included in the ''hypothalamic defense area'' nearly thirty years ago, are responsible for coordination and integration of multiple physiological and behavioral responses to emotional or exteroceptive stressors. Support for this hypothesis derives from the results of studies that have effectively employed both careful microinjection protocols and complementary functional neuroanatomic techniques. Of particular importance, our data have ruled out the possibility that the effects observed might be attributable to spread or diffusion of injected agents to the PVN, long held to represent a logical site for integration of endocrine and autonomic responses to stress (Swanson and Sawchenko, 1980). Instead, spread or diffusion to the nearby DMH could account for some of the changes seen upon microinjection of neuroactive substances into the PVN. The challenges that lie ahead include (1) the identification of the specific neurons in the DMH that mediate these effects, a task that lies beyond the limits of resolution of current microinjection techniques, and (2) elucidation of the source and nature of afferent inputs that may trigger activation of these key neurons in the DMH under conditions of exteroceptive stress and the efferent ''downstream'' pathways involved in the generation of specific autonomic, endocrine and behavioral changes.

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