Invasion of muscimol in dorsomedial hypothalamus and stress-induced Fos expression in paraventricular nucleus

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Received 16 December 1999; accepted in final form 12 December 2000

Injection of muscimol in dorsomedial hypothalamus and stress-induced Fos expression in paraventricular nucleus. Am J Physiol Regulatory Integrative Comp Physiol 280: R1276–R1284, 2001.—Prior microinjection of the GABA_A-receptor agonist muscimol into the dorsomedial hypothalamus (DMH) in conscious rats attenuates the increases in heart rate, blood pressure, and circulating adrenocorticotropic hormone seen in air stress. Here, we examined the effect of similar treatment on air stress- or hemorrhage-induced Fos expression in the paraventricular nucleus (PVN). Muscimol (80 pmol/100 nl per side) or saline (100 nl per side) was microinjected bilaterally into the DMH in conscious rats before either air stress, an emotional or neurogenic stressor, or graded hemorrhage, a physiological stressor. Each stressor evoked a characteristic pattern of Fos expression in the parvocellular and magnocellular PVN after saline. Injection of muscimol into the DMH suppressed Fos expression in the PVN associated with air stress but not with hemorrhage. Injection of muscimol at sites anterior to the DMH and closer to the PVN had no effect on Fos expression in the PVN after air stress. Thus activation of neurons in the DMH is necessary for excitation of neurons in the PVN during air stress but not during hemorrhage.

adrenocorticotrophic hormone; hemorrhage; heart rate; y-aminobutyric acid

DESPITE ITS RELEVANCE TO A variety of clinical disorders, much remains unknown about the exact neural mechanisms and circuitry responsible for generating the response to stress. In recent years, however, the notion that different neural circuits may be involved in the response to different classes of stressors has found support. Thus a distinction has been proposed between the pathways responding to emotional or neurogenic stressors, which have been termed exteroceptive, and to systemic or physiological stressors, referred to as interoceptive (see Refs. 9, 20, and 34). A neural substrate common to both is the hypothalamic paraventricular nucleus (PVN). Neurons concentrated in one subregion of this nucleus are believed to mediate the stimulation of the adrenal cortex that is evoked by a wide variety of interoceptive and exteroceptive stressors. These neurons project to the median eminence where they release corticotropin-releasing hormone (CRH) into the hypothalamic portal system through which the hormone reaches its principal site of action at the adenohypophysis. Here, CRH is thought to function as the primary secretagogue for the systemic release of adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal cortex. This sequence of events, activation of this hypothalamic-pituitary-adrenal (HPA) axis, is thought to represent a defining feature of the mammalian response to stress (see Ref. 31).

Thus mobilization of the HPA axis is one component of the complex pattern of hemodynamic, behavioral, and neuroendocrine changes that occurs in mammals in response to emotional stress. Consequently, models for the central mechanisms involved in the response to such stressors invariably include a key role for the hypothalamus and, within this region, for the PVN. However, whereas the PVN plays a central role in exteroceptive stress-induced activation of the HPA axis, recent evidence suggests that neurons in the nearby dorsomedial hypothalamus (DMH) may represent a higher order center responsible for integrating a broader range of physiological changes associated with this class of stress. In rats, chemical stimulation of neurons in the region of the DMH not only increases plasma ACTH (1, 12), but also elicits other physiological and behavioral responses characteristically seen during emotional stress (1, 25). Conversely, local neuronal inhibition by prior microinjection of the GABA_A-receptor agonist muscimol into the DMH attenuates the increases in heart rate, arterial pressure, and plasma ACTH seen in air stress, a paradigm for emotional stress (29, 30). Interestingly, similar injection of muscimol into the PVN reduces the increments in plasma ACTH but fails to affect the accompanying tachycardia or pressor responses in this paradigm (29, 30). Because GABA_A-receptor agonists are inhibitory to virtually all mature mammalian neurons (11), this finding confirms a role for neurons in the PVN in the stimulation of the HPA axis seen in this model but argues against a role for neurons in this region in air

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stress-induced increases in heart rate and blood pressure.

Several lines of evidence indicate that activation of CRH-containing neurons in the parvocellular PVN may occur in air stress through an excitatory synaptic input from neurons in the DMH. The DMH projects heavily to the parvocellular region of the PVN where CRH-containing neurons that project to the median eminence are concentrated (33), and electrophysiological data indicate that at least some of these projections provide excitatory input to their target neurons (3). Either swim or foot-shock stress provokes marked induction of Fos, a widely accepted marker for neuronal activation (8), in neurons in the DMH that project to the PVN (6, 14). However, it has recently been reported that, whereas air-puff startle, an exteroceptive stressor known to provoke powerful stimulation of ACTH secretions (7), does not (34).

Thus neurons in the DMH appear to signal activation of hypophysiotropic neurons in the PVN as well as various other components of the response to air stress, an exteroceptive stressor, but may play little part in the excitation of these same neurons that occurs in hemorrhage, a classic physiological or interoceptive stressor known to provoke powerful stimulation of ACTH secretion (7).

**MATERIALS AND METHODS**

All procedures involving rats that were employed in this study adhered to National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine.

**Surgical procedures.** Male Sprague-Dawley rats (275–300 g) that were individually housed and allowed food and water ad libitum were used in this study. The surgical procedures were performed as previously described (29, 30). In brief, rats were anesthetized with pentobarbital sodium (50 mg/kg ip) to allow the bilateral placement of guide cannulas (26 gauge, Plastics One, Roanoke, VA) at coordinates that correspond to the region of the DMH according to the atlas of Paxinos and Watson (18). The coordinates used were (from bregma) −1.2 mm posterior, 0.5 mm lateral, and 8.3 mm ventral, with the incisor bar set to 5 mm above the interaural plane and the angle of the cannula at 10° from sagittal. The animals were allowed to recover for 4 days and were then implanted with femoral arterial lines to allow monitoring of blood pressure and heart rate. The arterial lines were filled with heparinized saline and sealed with a stylet. The lines were routed subcutaneously to the nape of the neck, exteriorized, and held in place with a leather harness. The rats were allowed to recover for at least 2 days before the beginning of the experiment.

**Experimental protocols.** The rats were divided into four groups, such that each rat received bilateral microinjections of either saline vehicle (100 nl per side) or muscimol (80 pmol per side) before being subjected to either air stress or hemorrhage. Saline vehicle or muscimol was microinjected through a 28-gauge injector attached to a Hamilton syringe pump over 30 s.

Air-stress trials were conducted as previously described (29, 30). Briefly, the rats were placed in a Plexiglas tube sufficiently restrictive to prevent animals from turning around, and a continuous stream of air (40 l/min) was directed at the animal’s face for 20 min. Heart rate and blood pressure were monitored for at least 20 min before air stress or until a stable baseline was attained and for the duration of the 20-min period of air stress.

The hemorrhage protocol employed was adapted from the method of Slimmer and Blair (27). This protocol provides for a slow graded hemorrhage by first removing 3.2 ml/kg body wt of blood from the rat via the arterial line at t = 0, and the subsequent withdrawal of additional aliquots of 3.2 ml/kg at t = 2, 6, 10, 15, and 20 min. Thus a total blood loss of 19.2 ml/kg body wt was induced over a 20-min time course.

**Immunohistochemistry.** In all cases, rats were killed 90 min after the beginning of the stress or hemorrhage paradigm. The rats were deeply anesthetized with pentobarbital sodium (60 mg/kg ip) and transcardially perfused with 100 ml PBS followed by 100 ml of 4% paraformaldehyde in PBS. The brain was rapidly removed and postfixed for 90 min in 4% paraformaldehyde. The brain was then transferred to 30% sucrose until saturated. After quick freezing, serial 30-um sections were taken through the hypothalamus. Alternate sections were either stained with neutral red or processed for Fos-like immunoreactivity. The neutral red-stained sections were used to aid in neuroanatomical definition as well as to verify cannula placement (see Fig. 1 for schematic of cannula placements).

The Fos antibody used in this study was obtained first from Cambridge Research and then from Genosys (sheep anti-Pan Fos #OA1184). The optimum dilution was determined to be 1:5,000 in preliminary experiments. In brief, sections were incubated in PBS containing blocking serum and 0.5% Triton X-100 for 1 h. Sections were then incubated with the Fos antibody at 4°C overnight. Visualization of the Fos-like immunoreactivity was performed with a Vectastain ABC Elite Kit (Vector Labs, Burlingame, CA) using the standard protocol supplied with the kit. Nickel-intensified diaminobenzidine was used as the chromagen to yield a gray-black precipitation product. After visualization of the Fos-like immunoreactivity, the sections were mounted on gelatin-coated glass slides and allowed to dry. The sections were then dehydrated and placed on a coverslip.

Fos-positive cells were quantitated in six alternate sections corresponding to the main body of the PVN [i.e., from bregma, approximately −1.8 through −2.2 mm according to the atlas of Paxinos and Watson (18)] by an observer blinded as to treatment. Neurons were counted with the Microcomputer Imaging Device program from Imaging Research (Ontario, Canada) using the automated target-detection feature (both size and density criteria defined). Separate counts were made for parvocellular and magnocellular subdivisions of the nucleus, which could be distinguished and easily demarcated by examination of adjacent counterstained sections. Data were organized according to cannula placement assessed from examination of neutral red-stained sections representing microinjection sites.

A total of 58 rats entered the protocol, 24 of which met the criteria for inclusion in one of the study groups. Criterion for inclusion as an experiment in which microinjections accurately targeted the DMH was that both injection sites were in or within 300 µm of the main body of the DMH itself (defined as the region where a clear zona compacta was evident; Fig. 1, B and C). These included 1) four rats receiving bilateral saline injections followed by air stress, 2) five rats receiving bilateral muscimol injections followed by air stress, 3) four...
After saline vehicle was injected into the DMH, air stress resulted in an immediate and sustained increase in heart rate (+78 ± 10 beats/min) and blood pressure (+21 ± 8 mmHg; see Figs. 2A and 3). However, prior bilateral microinjection of muscimol into the DMH virtually abolished the cardiovascular response to air stress (heart rate, +9 ± 4 beats/min; blood pressure, +3 ± 2 mmHg; Figs. 2B and 3) as previously reported (29, 30). In three rats in which guide cannulas had been misplaced and were determined to be bilaterally symmetrically placed anterior to the DMH according to our criteria, cardiovascular responses to air stress were similar to those seen after saline (+112 ± 35 beats/min and +13 ± 2 mmHg). Data on heart rate and Fos expression for three such rats in which injection sites were judged to be equidistant or closer to the PVN (relative to injection sites in the DMH; see Fig. 1) are shown in Figs. 3 and 5, respectively.

Control rats that received no injections and were subjected to neither stress nor hemorrhage exhibited few Fos-positive neurons in the main body of the PVN (mean = 23 ± 3 neurons/rat). In contrast, Fos-like immunoreactivity was markedly enhanced throughout the main body of the PVN (mean = 708 ± 47 neurons/rat), but particularly in the parvocellular region in rats subjected to air stress after microinjection of saline into the DMH (Figs. 4 and 5). In rats in which muscimol was microinjected into the DMH before stress, the increase in Fos-positive neurons was significantly attenuated for the entire PVN (mean = 187 ± 28 neurons/rat) and in both parvocellular (by 75%) and magnocellular (by 53%) subdivisions of the nucleus (Figs. 4 and 5). Identical injections of muscimol in animals in which guide cannulas had been misplaced into areas adjacent to the DMH, including areas located between the DMH and PVN, did not affect the induction of Fos-like immunoreactivity in either region of the PVN in this paradigm (Fig. 5).

Injection sites in the DMH in hemorrhaged rats were distributed similarly to those in rats subjected to air stress (see Fig. 1, B and C). Hemorrhage provoked modest increases in heart rate that were equivalent in saline- and muscimol-pretreated rats (Fig. 3). The pattern of Fos-like immunoreactivity evoked by hemorrhage differed from that seen after air stress (Fig. 4). Thus a larger proportion of the total number of Fos-positive neurons in these rats was located in adjacent or closer to the PVN.

Table 1. Baseline cardiovascular status for all treatment groups before intervention

<table>
<thead>
<tr>
<th>Group (treatment/site/stimulus)</th>
<th>Basal Heart Rate, beats/min</th>
<th>Basal Mean Arterial Pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/DMH/stress (n = 4)</td>
<td>366 ± 34</td>
<td>109 ± 6</td>
</tr>
<tr>
<td>Muscimol/DMH/stress (n = 5)</td>
<td>367 ± 37</td>
<td>109 ± 5</td>
</tr>
<tr>
<td>Muscimol/other/stress (n = 3)</td>
<td>353 ± 18</td>
<td>107 ± 2</td>
</tr>
<tr>
<td>Saline/DMH/hemorrhage (n = 4)</td>
<td>350 ± 20</td>
<td>108 ± 4</td>
</tr>
<tr>
<td>Muscimol/DMH/hemorrhage (n = 5)</td>
<td>397 ± 10</td>
<td>104 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, nos. of rats. DMH, dorsomedial hypothalamus.
Fig. 2. Tracings of original experimental records depicting effect of air stress (arrows) on arterial pressure and heart rate in 1 rat after bilateral microinjection of saline into the DMH (A; rat 1 in Fig. 1B) and in another rat after microinjection of muscimol 80 pmol/side into the DMH (B; rat 3 in Fig. 1B). Air-stress intervals were initiated within 2 min of microinjections.

Fig. 3. Mean (±SE) heart rate changes from baseline seen during 20 min of air stress and first 20 min of hemorrhage protocol after bilateral microinjection of either saline (Sal) or muscimol (Mus; 80 pmol/side). *Significantly different from other treatments in stressed rats by 1-way ANOVA and Dunnett’s test (P < 0.05).
positive neurons was found in the magnocellular subdivision of the PVN in hemorrhaged animals. Pretreatment with muscimol at sites in the DMH that were distributed similarly to those at which muscimol was injected in stressed rats had no significant effect on the number of Fos-positive cells in the hemorrhage paradigm compared with saline-treated animals (Figs. 4 and 5).

DISCUSSION

The immediate early gene product Fos is now widely employed as a marker for neuronal excitation. Quantitation of Fos expression allows for the elucidation of specific brain regions that may be activated under certain experimental conditions. Because interventions that activate neurons in the PVN reliably induce Fos expression in this nucleus, Fos immunocytochemistry has been used in functional studies of the PVN in a wide variety of settings (2, 17, 19). In this study, Fos expression was used to examine the activation of neurons in the PVN resulting from experimental air stress or graded hemorrhage, representing exteroceptive and interoceptive stressors, respectively, and to determine whether the expected increases might be attributable to activation of neurons in the nearby DMH.

Air stress, the experimental paradigm for neurogenic stress employed here, was developed as a means of provoking near-maximal increases in key physiological indexes of exteroceptive stress such as heart rate and plasma ACTH without the need for painful stimuli or excessive physical restraint. Nonetheless, air stress produces increases in circulating ACTH and marked tachycardia similar in magnitude to those reported in other widely used paradigms for emotional or neurogenic stress (29, 30). Here, as has been reported for such models as immobilization (17), foot shock (19), and air-puff startle (34), air stress induced a high level of expression of Fos in the PVN. Consistent with findings in other exteroceptive stress paradigms, most of the Fos-like immunoreactivity was localized in the parvocellular division of the nucleus, the region where CRH-containing neurons are primarily localized (22).
Air stress-induced increases in Fos expression in the PVN were dramatically reduced after injection of muscimol into the DMH, providing a neuroanatomical correlate for the ability of similar treatment to reduce the increases in plasma ACTH seen in this paradigm (29). Injection of muscimol into the PVN also suppressed the tachycardia associated with air stress, as was reported previously (29, 30). Microinjection of muscimol, a powerful inhibitor of the activity of virtually all mammalian neurons owing to its agonist activity at GABA_\text{A} receptors (11), has come to be a standard technique to achieve acute reversible inactivation of neurons in a discrete region of the brain (for recent examples, see Refs. 5, 10, 15, and 37). In this study, injection of muscimol into areas adjacent to the DMH, including areas closer to the PVN, failed to replicate the effect of microinjection into the DMH with respect either to the increases in heart rate (Fig. 3) or to the expression of Fos (Fig. 5) after air stress. Because of this, the effects of muscimol microinjected into the DMH cannot be attributed to this agent acting directly on neurons in the PVN after spread or diffusion to this region. Instead, neurons in the vicinity of the DMH appear to constitute the specific targets that mediate the effects of microinjected muscimol. Thus excitation of neurons in the region of the DMH, an area that provides a dense innervation of the parvocellular subregion (32, 33), appears to signal both the activation of neurons in the PVN and the increases in heart rate seen in air stress.

The relationship between muscimol-induced suppression of air stress-induced Fos expression in the PVN and the accompanying reduction in the associated tachycardia merits consideration. In addition to hypophysiotropic neurons, the parvocellular PVN includes neurons that project to the brain stem and spinal cord and thus are thought to be involved in autonomic function (21). Consequently, the PVN has been proposed as a single nucleus capable of integrating autonomic as well as endocrine components of the response to various stressors (31). In this study, no attempt was made to quantitate Fos expression specifically in the dorsal cap or ventral subregions of the parvocellular PVN, the areas where autonomic-projecting neurons are localized (21). However, Fig. 4, A and C, suggests that air stress provoked Fos expression throughout the parvocellular PVN and that this expression was suppressed in all these regions after microinjection of muscimol into the DMH. On the basis of these findings alone and the notion that these neurons in the PVN may mediate the autonomic sequelae associated with many forms of stress, the muscimol-induced suppression of air stress-induced Fos expression in these regions and the associated tachycardia could be viewed as causally related. However, in a previous study, microinjection of muscimol directly into the PVN itself significantly reduced only the increase in circulating ACTH without affecting these stress-associated cardiovascular changes (29). Unless they are mediated through a population of parvocellular neurons that is resistant to the inhibitory effect of GABA_\text{A} receptor agonists, the increases in heart rate and blood pressure seen in this model are unlikely to rely on...
activation of neurons in the PVN. These autonomic regions of the PVN may instead play a role in other effects of air stress that were not assessed in this and previous studies.

As with air stress, graded hemorrhage caused a striking increase in Fos expression in the PVN, but the distribution of Fos-positive neurons was somewhat different. Like air stress, hemorrhage resulted in Fos expression in the parvocellular PVN, an effect consistent with the marked increases in circulating ACTH seen under both conditions. However, hemorrhage induced more Fos expression in the magnocellular region of the PVN as has been reported by others (2, 24, 34). This difference reflects the greater stimulatory effect of hemorrhage on vasopressin-secreting neurons (28), which are known to be concentrated in this subregion of the nucleus (35, 36).

In contrast to its marked effect on air stress-induced Fos expression, microinjection of muscimol into the DMH before hemorrhage failed to alter the number of Fos-positive cells in either parvocellular or magnocellular subregions of the PVN. Two explanations might account for such a difference. First, the same population of neurons in the DMH is responsible for excitation of the PVN in air stress or hemorrhage, but in the latter case, the effect of a single microinjection of muscimol 90 min before death had waned sufficiently to allow for their activation and subsequent excitation and Fos expression in neurons in the PVN. Air stress was only imposed during the first 20 min after microinjection of muscimol, whereas hemorrhage could be viewed as a persistent stimulus throughout the 90-min period preceding death because shed blood was not replaced. Thus the duration of the effect of microinjected muscimol may have been sufficient to suppress neuronal activity in the DMH during the 20-min period of air stress but not throughout the entire 90-min period of hemorrhage-induced stimulation. However, data from this (see Fig. 2) and a previous study (30) indicate that increases in heart rate and arterial pressure remained maximally suppressed throughout the entire period of air stress after microinjection of muscimol into the DMH, indicating no evidence of loss of the drug’s effect. Therefore, the inhibitory effect of muscimol was likely to have persisted well beyond the initial 20 min. If so, the lack of a significant effect of muscimol on hemorrhage-induced Fos expression in the PVN in this study makes it highly unlikely that neurons in the DMH are involved, given the fact that at least 60–90 min seem to be required for maximal expression of Fos in response to a given stimulus. Thus the most reasonable explanation for the data is that activity of neurons in the DMH plays no role in the activation of the PVN seen in hemorrhage.

Muscimol-induced suppression of neuronal activity in the DMH also failed to influence the modest increases in heart rate associated with hemorrhage noted in this study. The usual response to moderate hemorrhage is thought to be a decrease in heart rate (23), but there have been reports of increases in dogs (16, 26) and mixed increases and decreases in rats (7). Because hemorrhage is likely to provoke hypotension, the baroreflex represents an appealing mechanism for these increases in heart rate (16). However, tachycardia in response to hemorrhage has also been reported in rats with chronic lesions of the nucleus of the solitary tract, thought to represent the central site where baroreceptor afferents terminate (24). Regardless of the mechanism involved in hemorrhage-induced tachycardia, its persistence, unchanged after microinjection of muscimol, indicates that as with hemorrhage-induced Fos expression in the PVN, neurons in the DMH are not likely to be involved.

Thus, although both hemorrhage and air stress provoke powerful activation of neurons in the PVN, different afferent pathways appear to mediate the effects of each stimulus. The notion that activation of the PVN may be signaled from the DMH in the response to exteroceptive stressors, but not in response to interoceptive stressors, finds several lines of support. When a retrograde tracer was applied to the PVN in rats subsequently subjected to foot shock or swim stress, two exteroceptive stress paradigms, and processed for both tracer and Fos expression, the most prominent population of double-labeled neurons in the hypothalamus was found in the DMH (6, 14). Therefore, neurons in the DMH appear to be the primary source of excitatory afferent input to the PVN from hypothalamic neurons that are activated in exteroceptive stress, and our data suggest that this activity is required for the activation of the PVN known to occur in this setting. Other laboratories have shown increased Fos expression in the PVN resulting from hypotensive and nonhypotensive hemorrhage (2, 24, 34), and it has been proposed that this activation may be signaled through innervation of the PVN from medullary autonomic sites that relay afferent signals from cardiovascular pressure or volume receptors (4, 28). Interestingly, transsection of ascending projections from the brain stem prevents Fos expression in the PVN in response to systemic administration of interleukin 1 but not that in response to foot shock (13), suggesting a parallel dichotomy for the mechanisms involved in these two modes of stress. Most recently, Fos expression was reported to be increased in the DMH in rats after air-puff startle but not after hemorrhage, suggesting that the DMH is activated in response to the former but not in response to the latter stressor (34). Whatever the afferent pathway may be, hemorrhage-induced activation of neurons in the PVN does not appear to involve the neurons in the DMH that are responsible for their excitation in air stress.

In summary, microinjection of the neuronal inhibitor muscimol into the region of the DMH, an area known to send excitatory projections to the parvocellular PVN, attenuates Fos expression in the latter region associated with air stress but not with hemorrhage. This finding, together with our previous demonstration that similar treatment also reduces the increases in plasma ACTH seen in air stress, indicates that activity of neurons in the region of the DMH plays a key role in the recruitment of the HPA axis under conditions of air
stress, an exteroceptive stressor, but not in response to hemorrhage, an interoceptive stressor.

**Perspectives**

The results of this study confirm and extend previous work that points to a key role for neuronal activity in the DMH in the integration of autonomic, neuroendocrine, and behavioral responses to air stress, a paradigm for exteroceptive stress. A key finding of this and previous studies is that both the increases in plasma ACTH and the tachycardia that represents a salient feature of the autonomic response seen in this setting were reduced by suppression of neuronal activity in the DMH. In contrast, similar inhibition of the PVN attenuated increases in circulating ACTH but not the autonomically mediated tachycardia and increased blood pressure (29, 30). Evidently, these neurons in the DMH represent a higher-order processing center relative to the PVN for the exteroceptive stress response. The determinants of the activity of these neurons, their neurochemical identity, and even their precise location in the region of the DMH remain unclear. Studies that would provide this information could fill an important gap in our understanding of the neural circuitry relevant to the integrated multisystem response to emotional stress in mammals, a response that appears to be highly conserved across diverse species.

This work was supported by United States Public Health Service Grant NS-19883.

**REFERENCES**


