Modulation of Sympathetic and Somatomotor Function by the Ventromedial Medulla

Malcolm W. Nason, Jr. and Peggy Mason
Committee on Neurobiology and Department of Neurobiology, Pharmacology and Physiology, University of Chicago, Chicago, Illinois 60637
Submitted 29 January 2004; accepted in final form 13 February 2004

Nason Jr., Malcolm W. and Peggy Mason. Modulation of sympathetic and somatomotor function by the ventromedial medulla. J Neurophysiol 92: 510–522, 2004. First published February 18, 2004; 10.1152/jn.00089.2004. The ventromedial medulla is implicated in a variety of functions including nociceptive and cardiovascular modulation and the control of thermoregulation. To determine whether single microinjections into the ventromedial medulla elicit changes in one or multiple functional systems, the GABA\(_A\) receptor antagonist bicuculline was microinjected (70 nl, 5–50 ng) into the ventromedial medulla of lightly anesthetized rats, and cardiovascular, respiratory, and nociceptive measures were recorded. Bicuculline microinjection into either the midline raphe or the laterally adjacent reticular nucleus simultaneously increased interscapular brown adipose tissue temperature, heart rate, blood pressure, expired \(\text{CO}_2\), and respiration rate and elicited shivering. Bicuculline microinjection also decreased the noxious stimulus-evoked changes in heart rate and blood pressure, decreased the frequency of heat-evoked sighs, and suppressed the cortical desynchronization evoked by noxious stimulation. Although bicuculline suppressed the motor withdrawal evoked by noxious tail heat, it enhanced the motor withdrawal evoked by noxious paw heat, evidence for specifically patterned nociceptive modulation. Saline microinjections into midline or lateral sites had no effect on any measured variable. All bicuculline microinjections, midline or lateral, evoked the same set of physiological effects, consistent with the lack of a topographical organization within the ventromedial medulla. Furthermore, as predicted by the isodendritic morphology of cells in the ventromedial medulla, midline bicuculline microinjection increased the number of c-fos immunoreactive cells in both midline raphe and lateral reticular nuclei. In summary, 70-nl microinjections into ventromedial medulla activate cells in multiple nuclei and elicit increases in sympathetic and somatomotor tone and a novel pattern of nociceptive modulation.

INTRODUCTION

The ventromedial medulla (VMM), which includes raphe magnus (RM), raphe pallidus (RP), and the adjacent nucleus reticularis magnocellularis (NRMC), has been implicated in nociceptive and cardiorespiratory modulation in the pre-motor control of cold defense (Mason 2001; Morrison 2001a). However, studies directed at understanding VMM’s role in each of these functions have focused on pharmacological or electrical activation of different regions within the ventromedial medulla. For instance, nociceptive modulatory studies have focused on the effect of microstimulation of and microinjection into RM and NRMC (Drower and Hammond 1988; Zhuo and Gebhart 1990, 1992a, 1997), whereas the role of VMM in cold defense has been tested using microinjection into RP (Morrison et al. 1999). Since neurons throughout VMM have large dendritic arbors that cross cytoarchitectonic boundaries (Edwards et al. 1987; Fox et al. 1976; Gao and Mason 1997; Leontovich and Zhukova 1963; Maciewicz et al. 1984; Mason et al. 1990; Newman 1985; Potrebic and Mason 1993; Ramon-Moliner and Nauta 1966; Valverde 1961), it is unlikely that a microinjection into any single nucleus in VMM will affect neurons confined to that nucleus. Furthermore, with few exceptions, experiments examining VMM’s role in nociceptive modulation have not recorded thermoregulatory measures and experiments examining VMM control of cold defense have not recorded dependent variables related to nociceptive sensitivity. Thus it is not clear whether microinjections into individual ventromedial medullary nuclei elicit changes in autonomic control or nociceptive modulation or simultaneously affect both functions. Therefore in these experiments, the effects of microinjections into midline and lateral regions of VMM on somatomotor, cardiorespiratory, and thermoregulatory function were studied.

A wealth of data implicates the VMM in nociceptive modulation. Electrical or chemical stimulation in the VMM modulates the cellular and motor reactions to noxious stimulation (Dostrovsky et al. 1982; Jensen and Yaksh 1986; Satoh et al. 1983; Zhuo and Gebhart 1990, 1992a, 1997; Zorman et al. 1981). RM and NRMC cells receive afferents from the periaqueductal gray matter (PAG), a site that evokes descending opioid analgesia, and project to the superficial dorsal horn of the spinal cord, where nociceptive sensory afferents synapse (Abols and Basbaum 1981; Fields et al. 1995; Urban and Smith 1994; Vanegas et al. 1984). Lesioning or inactivating the VMM greatly attenuates the antinociceptive effects of systemic opioids, opioid injection into PAG, or PAG stimulation (Behbehani and Fields 1979; Chung et al. 1987; Proudfit 1980; Proudfit and Anderson 1975; Sandkuhler and Gebhart 1984; Urban and Smith 1994). These findings suggest that, when activated by rostral regions, RM and NRMC cells modulate nociceptive dorsal horn cells. While activation of RM and NRMC cells typically suppresses the nociceptive responses of dorsal horn cells and the withdrawal movements evoked by noxious stimulation, facilitation may also result (Zhuo and Gebhart 1990, 1992a, 1997). Both the suppression and the facilitation of spinal nociceptive transmission are due to excitation, and not disinhibition, of medullary neurons (Behbehani and Fields 1979; Kaplan and Fields 1991).
Strong lines of evidence also implicate RP in the descending control of thermoregulatory cold defense. Bicuculline microinjection into RP elicits nonshivering thermogenesis in the interscapular brown adipose tissue (BAT) (Morrison et al., 1999), whereas muscimol microinjection into RP blocks the BAT activation evoked by cooling the hypothalamus or forebrain injection of prostaglandin E2 (Morrison 2001b, 2003; Nakamura et al. 2002). Pseudorabies virus injections into most sympathetic nervous system targets tested, including BAT, consistently reveal retrograde trans-synaptic labeling of RM, NRMC, and RP neurons (Bamshad et al. 1999; Cano et al. 2003; Strack et al. 1989a,b). VMM neurons, both serotonergic and nonserotonergic, respond to nonnoxious changes in skin temperature (Dickenson 1977; Rathner et al. 2001; Young and Dawson 1987), and c-fos labeling in the VMM has been reported after cold challenge (Cano et al. 2003; Martinez et al. 2001; Morrison et al. 1999). VMM neurons project to the sympathetic intermediolateral cell column, where targeted cells include preganglionic sympathetic neurons (Bacon et al. 1990; Basbaum et al. 1978; Henry and Calaresu 1974; Holstege and Kuypers 1982), further evidence that VMM cells could control BAT activation. However, neurons throughout VMM also project to the superficial dorsal horn, where thermoreceptors as well as nociceptors terminate. Moreover, electrical stimulation in RM modulates the responses of thermoreceptive neurons in the dorsal horn (Sato 1993), raising the possibility that VMM modulates thermoregulation via effects on thermoreceptive pathways that either ascend to the hypothalamus or serve as afferent input to local preganglionic sympathetic neurons.

The role of the VMM in cardiorespiratory function is not well understood, but there are strong hints that such a role exists. Both depressor and pressor responses to electrical stimulation of the medullary raphe, sometimes accompanied by bradycardia or tachycardia, have been reported (Adair et al. 1977; Blessing and Nalivaiko 2000; Henry and Calaresu 1974; McCall 1984; McCall and Harris 1987; Yen et al. 1983). Bicuculline microinjections into the midline raphe result in sympathetically mediated increases in heart rate and blood pressure (Cao and Morrison 2003). Inactivation of the medullary raphe by muscimol microdialysis greatly reduces the respiratory response to increased CO₂ (Messier et al. 2002). Both serotonergic and nonserotonergic neurons in the medullary raphe contribute to the respiratory reflex evoked by elevated CO₂ (Messier et al. 2004; Nattie et al. 2004). Finally, microinjection of a substance P antagonist causes respiratory arrest and death (G. F. Gebhart, personal communication; Aimone and Gebhart 1986).

To determine whether microinjection into single sites of the VMM elicits single or multiple modulatory changes, the GABAₐ receptor antagonist bicuculline was microinjected into the midline raphe and into the lateral portion of NRMC, and the effects on nociception, BAT temperature, and cardiorespiratory physiology were simultaneously examined. By using c-fos immunoreactivity as a gross marker for neuronal activity (Morgan and Curran 1991; Wang and Redgrave 1997), the anatomical extent of the neurons excited by microinjections was estimated. The results demonstrate that small (70 nl) microinjections activate neurons in a broad area of the medullary raphe and adjacent reticular formation and elicit sympathoexcitatory and somatomotor effects, including a novel pattern of nociceptive modulation.

**METHODS**

**Surgical preparation**

The methods for all experiments were approved by the University of Chicago Institutional Animal Care and Use Committee and conformed to the guidelines of National Institutes of Health and the International Association for the Study of Pain.

Male Sprague-Dawley rats (250–450 g; n = 110; Charles River, Portage, MI) were pretreated with atropine sulfate (40 μg in 0.1 ml ip) and anesthetized with halothane. Rats were placed on a heated (36°C) water blanket and covered with a plastic blanket for the duration of the experiment. A Y-tube was inserted into the trachea through which humidified halothane (2%) in oxygen was administered. Expired [CO₂] was measured through a 23-gauge, blunted needle placed at the end of the Y tube. A catheter filled with heparinized (10 IU/ml) saline was inserted into the right femoral artery to record blood pressure. To record the EEG, two stainless steel screws with attached wire leads were affixed to the lateral skull and coated with dental cement. Stainless steel electrodes (Grass, Warwick, RI) were inserted bilaterally into the thorax to record the EKG and into the paraspinal muscles to record the EMG during tail withdrawal movements. Needle electrodes were also placed through the skin of the upper thigh into multiple muscles, including the quadriceps and hamstring, to record the EMG from paw withdrawal movements. BAT activation evoked by cooling the hypothalamus or forebrain injection of prostaglandin E2 (Morrison 2001b, 2003; Nattie et al. 2004). Finally, microinjections activate neurons in a broad area of the medullary raphe and adjacent reticular formation and elicit sympa-thoexcitatory and somatomotor effects, including a novel pattern of nociceptive modulation.

**Data collection**

Data were collected onto a Pentium III PC using a Power 1401 A/D converter (CED, Cambridge, UK) and analyzed using Spike 4.0 (CED). Halothane concentration and expired [CO₂] were monitored by a Datex Capnomac (Tewksbury, MA). EEG, blood pressure, and EKG were collected at 250 Hz. EMG signals were rectified, integrated, and collected at 1.2 kHz, while temperature (stimulus, core, and BAT) and [CO₂] were collected at 100 Hz.

**Protocol**

Peltier thermodes were held at a constant temperature of 32°C by computer-driven Peltier controllers (Yale Instrumentation Laboratory, New Haven, CT). Noxious thermal stimulation was applied to the hindpaw and the tail alternately with 5-min intervals between stimulus site trials. Each stimulus consisted of a 3-s ramp from 32°C to a plateau of 56°C for 3 (hind paw) or 4.5 s (tail), followed by a 2- to 6-s return to 32°C. The animal’s tail or paw was strapped on to the thermode throughout baseline and stimulus conditions. There were three to five trials at each stimulus site prior to microinjection.

After baseline recording, a borosilicate glass micropipette (~25 μm tip diam) was introduced into the midline (L 0.0 mm) or lateral (L 1.0 mm) portions of the VMM (P 2.0–2.6 mm from interaural 0; V 9.5 mm from the cerebellar surface). There were no changes in heart rate, blood pressure, EMG, or EEG on introduction of the injection pipette into either locus. A picospritzer (WPI, Sarasota, FL) was used to inject saline or 5, 15, or 50 ng of bicuculline methiodide (Sigma, St. Louis,
MO) in 70 nl of phosphate buffered saline or Dubelco’s NES, (both pH 7.4). The dose of 15 ng used by Morrison et al. (1999) formed the starting point for our study. Semi-log doses were chosen on either side of that starting point. The injection lasted 30–100 s and was monitored using a calibrated reticule. Trials resumed 60–100 s after drug injection. In the case of some injections, both saline and bicuculline, changes in blood pressure or respiration rate occurred during the injection itself; these injections were not analyzed further. Additional injections were made after a minimum of 1.5 h, including a 20- to 30-min rest interval (i.e. between the stimulus trains) and after all baseline measures had returned to preinjection levels or had remained constant for ≥30 min. No animal received >4 injections. Animals were occasionally supplemented with 5 ml of 10% buffered dextrose sc at the beginning of the rest period, ≥30–45 min before the time of the next microinjection. At the end of recording, 35–70 nl of 1% Fast green (Sigma) was injected at the stereotaxic coordinates of the last microinjection.

Animals were overdosed with halothane and perfused transcardially with phosphate buffered saline, followed by 10% formalin, and immersed in 30% sucrose in 0.1 M PBS until equilibration. Brains were sectioned (50 μm) in the coronal plane, and marked injection sites were visualized and plotted on standard sections. The raphe nuclei were considered to include a region 400 μm wide centered on the raphe nuclei. A small region between the pyramids with densely packed, small cells was considered to be RP. RM was considered to be the raphe region stretching from the dorsal boundary of RP to a point 1.5 mm dorsal to the ventral surface of the brain. In the case of animals that received multiple injections, the locations of unmarked injections were determined by their distances from labeled sites with known stereotaxic coordinates.

Analysis

Systolic blood pressure was computed from the peaks in the recorded blood pressure from each heartbeat and converted by linear interpolation to an array of 10 samples/s. Similarly, heart rate was calculated from the sampled EKG as the reciprocal of the interval between sequential heartbeats and converted by linear interpolation into an array of 10 samples/s. Resting blood pressure and heart rate values were calculated as the average measure during the 30 s before each noxious thermal stimulus. Blood pressure and heart rate values are expressed in millimeters of mercury (mmHg) and beats per minute (bpm), respectively.

Noxious stimulation typically elicited changes in blood pressure and heart rate. For these measures, stimulus-evoked changes were calculated as the difference of the maximum value during the post-stimulus period (+30 s) minus the resting value. The tachycardia evoked by paw heat was somewhat variable and was observed in most, but not all (66/79), animals. Heart rate was lable, fluctuating in seemingly random fashion around a mean. Because of this variation, changes in heart rate <2 SD from resting levels were considered insignificant. In 13 animals, heart rate changed by less than this criterion and was considered insignificant; these animals were not used in further analysis of the heat-evoked tachycardia.

EEG power spectra with 1,024 points were created by summing the power in the frequency range of 0–4 Hz for 30-s epochs before and after the stimulus. This low frequency power typically decreases in response to noxious stimulation (Grahn and Heller 1989), and the percentage decrease was used as a measure of cortical “desynchronization.” In some animals (n = 6), the EEG was never sufficiently synchronized for a noxious heat-evoked desynchronization to occur; EEG recordings from these animals were not further analyzed.

Since [CO₂] was obtained at some distance from the trachea, all values were considered as relative, rather than absolute, measures and were normalized to the average preinjection resting [CO₂] for each experiment. Resting respiratory rate was calculated as the reciprocal of the interval between the [CO₂] peaks for the 30 s before the noxious stimulus. Noxious stimulation elicited an enhanced expiration that we have termed a sigh (see below). Sighs were stereotyped, and therefore the incidence of sighs was compared before and after drug or saline microinjection. Animals without sighs (n = 4) and animals whose sighs occurred outside the time scope of our analysis (n = 6; within 30 min of injection) were omitted from analysis.

All EMG signals were full-wave rectified. A 30-s integral of the EMG prior to each noxious stimulus (ending at the beginning of the stimulus ramp) was used as a measure of resting motor tone. The sections were determined, off-line, by visual inspection of the difference between the integrated EMG activity for 30 s before and 30 s beginning at the start of the plateau phase of the stimulus and including the 3- (paw) or 4.5-s (tail) plateau. All responses were normalized to the mean response prior to injection. In experiments exhibiting significantly increased EMG baseline activity (see below), the maximum EMG activity following noxious stimulation was used as the assessment of the response before and after injection. Normally, when EMG signals exceeded 5 V, the amplifier gain was reduced. Unfortunately, in several experiments (such as that shown in Fig. 5), this was overlooked, resulting in the truncation of some EMG values and causing some EMG data to be underestimated. Resting EMG values from experiments exhibiting increased EMG baseline activity were removed from the general population and compared separately. Withdrawal latencies were determined, off-line, by visual inspection of the 5- (foot) and 10-s (tail) records following the noxious stimulus. In cases where no tail withdrawal occurred, a latency of 10 s was assigned to the trial (Carstens and Wilson 1993). All heat stimuli to the paw evoked a motor withdrawal response that was observed by eye as well as recorded electromyographically.

c-fos experiments

To estimate the number and distribution of cells that were activated by microinjection, a group of rats (n = 25) received microinjections as above and were processed for c-fos immunoreactivity (Wang and Redgrave 1997). Animals were anesthetized with halothane and placed in a nose cone delivering oxygenated halothane. A craniotomy was completed as described above. To estimate the physiological effects of the microinjections, EKG and core temperature were recorded. However, additional physiological measures were not recorded to reduce the activation of c-fos due to surgical procedures. Anesthetic was lowered to 1% and animals were allowed to equilibrate for 1 h. Microinjections (70 nl) of 5, 15, or 50 ng bicuculline or saline were made as described above. Either fluorescent beads (Molecular Probes, Eugene, OR) or Fast green was added to the injectate. Anesthetic was lowered to 1% and animals were allowed to equilibrate for 1 h. Microinjections (70 nl) of 5, 15, or 50 ng bicuculline or saline were made as described above. Either fluorescent beads (Molecular Probes, Eugene, OR) or Fast green was added to the injectate. After a 1-h rest period, animals were killed, perfused, and postfixed for 4–12 h.

The medulla was cut transversely into 40-μm sections. Sections containing the microinjection site were identified by the presence of fluorescent beads or Fast green. Typically 8–10 sections distributed around the central site were processed for c-fos immunocytochemistry. In brief, sheep anti-c-fos antibody (Cambridge Research Biochemicals, Cheshire, UK) was preabsorbed with rat liver powder at 4°C for 24 h in 0.9% phosphate buffered saline containing 1% normal goat serum (NGS) and 0.3% Triton X-100 (termed “buffer”) below). Tissue was incubated for 30 min in 50% ethanol and washed with buffer three times at room temperature. Sections were incubated in 3% NGS in buffer for 30 min. Sections were transferred directly into sheep anti-c-fos antibody (1:2000) at 4°C for 48 h. After incubation in the primary antibody, sections were washed in buffer and placed in biotinylated rabbit anti-sheep antibody (Vector Labs, Burlingame, CA) at 1:250 dilution for 2 h at room temperature. Tissue was washed and transferred to 1:1,000 ExtraAvidin HRP (Sigma) for 2 h. Finally, sections were washed and processed with di-aminobenzidine as previously described (Mason and Fields 1989).

All c-fos immunoreactive cells in the VMM, medial to the facial nucleus and ventral to the medial longitudinal fasciculus, were plotted
(Neurolucida, Colchester, VT) using a 20× objective (Nikon). In addition, c-fos immunoreactive cells in the dorsal cochlear nucleus that were consistently labeled in each animal, as has been noted before (Erickson and Millhorn 1994), were also plotted. The median number of neurons labeled per section in each animal and at each site was compared across experimental groups.

Statistical analyses were completed using Excel 9.0 (Microsoft Corp., Redmond, WA) and SigmaStat 2.0 (SPSS Corp, Chicago, IL). Drug effects were compared using two-way repeated measures ANOVAs and the Student-Newman-Keuls post hoc test. A P value ≤ 0.05 was considered significant.

RESULTS

None of the recorded physiological measures changed after saline microinjection into either RM or NRMC. In contrast, microinjections of bicuculline, at every dose tested and at both sites, produced similar multiple physiological effects. Since there were virtually no dose- or site-dependent effects, all data from animals injected with bicuculline were combined, and will be described. Drug-evoked changes in resting physiological measures are considered first followed by a description of drug-evoked changes in the motor and autonomic responses to noxious heat stimulation.

Drug-evoked changes in resting variables

BAT temperature, blood pressure, heart rate, respiration rate, EMG magnitude, and EEG delta frequency power were analyzed for 30 s prior to each noxious stimulus trial. These measures comprise the resting variables. Prior to microinjection, none of the resting variables analyzed differed between the experimental and control groups. Furthermore, none of the resting variables changed after injection of saline into either RM or NRMC (Fig. 1).

BAT temperature began to increase within 5 min of bicuculline microinjection (Fig. 2) and had increased significantly by an average of 1.3 ± 0.2°C 20 min after bicuculline microinjection. BAT temperature remained elevated for another 20 min before slowly returning to baseline values. A significant increase of 34.3 ± 0.3% in expired [CO₂] occurred during the rise in BAT temperature (n = 32). Bicuculline elevated core body temperature significantly, but in contrast to its effects on BAT temperature, evoked a small increase of only 0.3 ± 0.1°C after a long delay (n = 40).

Injection of bicuculline significantly elevated blood pressure by 23.7 ± 2.0 mmHg (n = 56) and increased heart rate by 92.4 ± 6.5 bpm (n = 53), and respiration rate by 0.4 ± 0.1 breaths/s (n = 32) for 20–30 min regardless of injection site (Fig. 2). Increases in heart rate were sometimes accompanied by alternating strong and weak heartbeats within the same cardiac rhythm, a phenomenon known as pulsus alternans (Fig. 3; Lab and Seed 1993). Pulsus alternans occurred in 20–30% of the animals that received bicuculline but was never observed in saline treated animals (0/26).

As shown in Fig. 2, medullary microinjections of all three doses of bicuculline elicited tonic increases in resting EMG activity (5/12 low, 4/24 mid-, and 12/20 high dose), averaging 7.0 ± 2.9 times baseline levels. The increases in the baseline EMG activity of paraspinal muscles and limb muscles were not significantly different. Such increases in EMG activity were not observed in any of the 26 saline-treated animals (Figs. 1 and 4). This increase in resting EMG activity was observed in all muscles recorded and may represent a shivering response (Fuller et al. 1975). Although the animal often visibly shivered, a rigorous distinction between shivering and high muscle tone could not be made as hardware rectification of the EMG signal prevented testing of the spectral content of the EMG signal. Microinjections that evoked an increase in resting EMG activity were more likely to also evoke pulsus alternans (10/22) than those that did not (7/57; χ², P = 0.001).

Resting EEG delta activity did not change after injection of bicuculline into either RM or NRMC.

Somatomotor responses to noxious heat

Paw heat evoked significant withdrawal movements that were unaffected by saline microinjection (Fig. 4). Injections of bicuculline into either RM or NRMC significantly increased...
the magnitude of noxious paw heat-evoked withdrawals by an average of 2.3- to 2.9-fold (Figs. 4 and 5). No change in paw withdrawal latency (3.2 ± 0.2 s before, 3.5 ± 0.3 s after) accompanied this change in withdrawal strength (Fig. 6).

Our observation that bicuculline microinjection elicited an enhancement of the noxious heat-evoked paw withdrawal appeared to be at odds with the previously established suppression of tail flick by bicuculline microinjection into RM (Drower and Hammond 1988). Therefore in most experiments, tail heat and paw heat stimulation trials were presented alternately. In contrast to its effects on paw heat-evoked withdrawals and in agreement with published reports, bicuculline, into either RM or NRMC, significantly attenuated the tail withdrawal from noxious heat (Fig. 5). Bicuculline significantly decreased the magnitude of tail withdrawal movements to 28 ± 10% of baseline values and increased the withdrawal latency from 6.8 ± 0.4 to 9.4 ± 0.4 s (Fig. 5).

Cardiorespiratory and cortical responses to noxious heat

Both paw and tail heat elicited transient changes in blood pressure, heart rate, respiration, and EEG delta frequency
power (Figs. 4 and 5A). However, noxious paw heat evoked larger and more consistent responses than did tail heat. Therefore only the responses to paw heat were analyzed in detail and are reported below.

As seen in Figs. 4 and 5A, noxious paw heat elicited a pressor response, tachycardia, change in respiratory pattern (see following text), and decrease in EEG delta power. These reactions were transient with all physiological measures returning to baseline within 30 s. None of the analyzed responses to paw heat changed after injection of saline into either RM or NRM C (Figs. 1 and 4).

Bicuculline microinjection suppressed cardiorespiratory and cortical reactions to noxious paw heat (Figs. 2 and 5). Paw heat evoked a mean pressor response of $22.5 \pm 0.5$ mmHg ($n = 56$), which decreased by 50–100% (mean: $72.3 \pm 5.5\%$) after bicuculline injection. Noxious paw heat evoked a mean tachycardia of $28.1 \pm 2.2$ bpm ($n = 53$) that was significantly reduced by 28–100% (mean, $44.4 \pm 7.8\%$) after bicuculline injection. The reduction in pressor and tachycardic reactions to paw heat appeared within 90 s after injection and remained constant until $\geq 25$ min after injection. While cardiovascular reactions evoked by noxious paw heat were suppressed, they were still evident in most animals after bicuculline microinjection (Figs. 2 and 7). Therefore the suppression of noxious heat-evoked tachycardia and pressor reactions is at least partially a depression and is not entirely attributable to a “ceiling effect.”

Paw heat elicited a consistent and stereotyped respiratory response (Figs. 4 and 5A). This consisted of a long (0.5 s, 2 times baseline duration) inspiration that preceded an enhanced expiration, of normal duration, that peaked at two times baseline [CO$_2$] levels. This expiration was followed by 2–3 s of shallow, rapid breaths, sometimes interspersed with pauses. This sequence was termed a “sigh” (Issa and Porostocky 1993) and was marked by the maximum [CO$_2$] level recorded during the enhanced expiration. Sighs appeared in all but four animals, but never occurred in response to every noxious heat within an experiment. Sighs were typically most pronounced for the first noxious stimulation of the experiment and remained consistent in size ($\sim 90\%$ of maximum) and latency thereafter. Sigh incidence did not change in saline-treated rats ($n = 13$), averaging 0.58/stimulus before injection and 0.48/stimulus afterward. In contrast, bicuculline injected at either site significantly decreased the incidence of sighs ($n = 28$) from 0.64/stimulus to 0.04/stimulus ($t$-test for proportions, $P < 0.001$).

Paw heat significantly decreased EEG delta activity by $60.2 \pm 0.2\%$ ($n = 53$), a phenomenon that we refer to as cortical “desynchronization” (Figs. 4 and 5A). After injection

**Fig. 4.** Noxious paw heat (bottom trace marked stimulus) evokes a tachycardia (HR), a pressor response (BP), changes in respiration including a sigh (expired [CO$_2$]), a cortical desynchronization (EEG), and a motor withdrawal (Paw EMG) both before (A) and after (B) saline microinjection. Time scale in A applies to A and B. Traces are found at a more compressed time scale in Fig. 1.

**Fig. 5.** Tachycardia, pressor response, changes in respiration, and cortical desynchronization evoked by noxious paw heat (A) are entirely blocked (the former 3) or attenuated (the latter reaction) by bicuculline microinjection (B). Motor withdrawal evoked by noxious paw heat (A) is much larger after bicuculline microinjection (B). Also note that there are changes in the resting blood pressure and respiration rate after bicuculline microinjection. Time scale in A applies to A and B. Traces are found at a more compressed time scale in Fig. 2.
of bicuculline \( (n = 36) \) into RM or NRMC, the evoked desynchronization was significantly attenuated such that paw heat evoked only a 35.8 \( \pm \) 2.9% reduction in EEG delta activity (Fig. 5B).

Injection sites

As shown in Fig. 8, midline microinjection sites were concentrated within RM, on the midline, and dorsal to RP. Lateral microinjection sites were located in NRMC, just dorsal to the lateral edge of the pyramid. Injection sites were labeled by the co-injectate, Fast green, which stained an ovoid area with the longer axis oriented dorso-ventrally. The stained area was centered on the midline and reached approximately to each medial edge of the pyramids and was readily apparent for \( \sim 300-350 \) \( \mu \)m in the rostro-caudal plane.

Distribution of neurons affected by injections

To delineate the area within which neurons were influenced by drug microinjections, rats that were minimally prepared (see Methods) received microinjections of 5, 15, or 50 ng of bicuculline or saline into the midline. Fast green or fluorescent beads labeled a small region on the midline, presumably the center of the injection. The area containing c-fos immunoreactive cells always included this region and neither had any effect on staining patterns. An average of 15.6 \( \pm \) 4.3 c-fos positive cells were observed after saline microinjections \( (n = 5) \); Fig. 9A). In contrast, after midline microinjections of bicuculline, at every dose, a significantly greater number of c-fos labeled cells was observed \( (n = 20) \); average for all 3 doses: 88.2 \( \pm \) 9.3; Fig. 9, B–E). There were no differences between the numbers of cells labeled after each dose of bicuculline. It should be noted that the area delineated by the c-fos positive stained cells always exceeded the area demarcated by the Fast green or bead co-injectate. In anesthetized rats, neurons in the dorsal cochlear nucleus (DCN) contain c-fos immunoreactivity constitutively (Erickson and Millhorn 1994). Therefore the number of c-fos labeled DCN cells was used as a control for any variation in histological processing. The number of c-fos labeled cells in the DCN was not different in rats receiving saline or bicuculline (Fig. 9F). There were significantly fewer labeled DCN neurons in rats that received 50 ng bicuculline than in rats that received 5 ng bicuculline. It is therefore possible that the number of VMM neurons labeled with c-fos immunoreactivity in the former group is underestimated.

In animals that received bicuculline microinjections, c-fos immunoreactive neurons were concentrated on the midline, labeling neurons in both RP and RM (Fig. 9, B–D). However, c-fos immunoreactive neurons were present, at a lower density, bilaterally throughout the NRMC. Scattered labeling was

---

**FIG. 6.** A: magnitude of motor withdrawal from noxious paw heat increased after bicuculline microinjection, but withdrawal from noxious tail heat decreased. B: latency to withdrawal from noxious paw heat did not change after bicuculline microinjection, whereas the latency to tail withdrawal increased. Since there were no dose- or site-dependent effects, data from all bicuculline microinjections were grouped together. *Significant differences \( (P < 0.01) \).

**FIG. 7.** Noxious stimulation of the paw (\( \triangledown \)) evoked a tachycardia, pressor reaction, and sometimes a sigh (\( \star \) above \([CO_2]\) trace). After 15 ng bicuculline (dashed line marked by \( \Theta \) in top trace), heat-evoked reactions were reduced but not abolished. The noxious-evoked tachycardia observed after 15 ng bicuculline peaked below the maximum baseline achieved after microinjection of 50 ng bicuculline (dashed line marked by \( * \) in top trace).
sometimes observed in nucleus reticularis paragigantocellularis pars lateralis and the parapyramidal region lateral to the pyramids (data not shown).

In rats that were processed for c-fos immunoreactivity, heart rate was monitored using transcutaneous needle electrodes to measure the physiological effect of each microinjection. The greater number of c-fos immunoreactive cells in bicuculline-treated rats was associated with a significant increase in heart rate (Fig. 9G; average for all 3 doses: 85.3 ± 7.5 bpm, n = 17) that was similar to that observed in rats that were fully instrumented (82.9 ± 5.6 bpm). Heart rate did not change after saline injections (n = 5) in either minimally (Fig. 9G) or fully (Fig. 1) instrumented rats.

**DISCUSSION**

This study demonstrates that microinjection of the GABA<sub>A</sub> receptor antagonist, bicuculline, into either the medullary midline raphe or adjacent reticular region, simultaneously elicits thermoregulatory, cardiorespiratory, somatomotor, and nociceptive modulatory effects. Bicuculline microinjection depressed most reactions to cutaneous noxious heat, including the somatomotor withdrawal to tail heat as well as cardiorespiratory changes and cortical desynchronization evoked by paw heat. However, contrary to these suppressive effects, bicuculline microinjection markedly enhanced the motor withdrawal evoked by noxious paw heat. This finding is strong evidence that RM/NRMC differentially modulates multiple circuits engaged by noxious stimulation, an idea supported by previous reports (Jasmin et al. 1997; Mason et al. 2001). Moreover, the simultaneously observed opposing effects of bicuculline microinjection on tail flick and paw withdrawal are unequivocal evidence for a form of nociceptive modulation that is somatopic, rather than diffuse.

After bicuculline microinjection into either RM or NRMC, the autonomic and somatomotor state was consistent with the physiology of an “excited” animal. Heart rate, blood pressure, and BAT temperature were all elevated. Respiration rate and expired [CO<sub>2</sub>] increased and shivering was elicited in many cases. However, this excited physiology was not accompanied by changes in resting cortical synchrony. Thus there is no evidence that bicuculline’s effects on the body’s physiology were secondary to an evoked cortical arousal. Instead, bicu-

![Figure 8](http://jn.physiology.org/)

**FIG. 8.** Microinjection sites were concentrated within RM or NRMC, just dorsal to the lateral edge of the pyramid. •, saline microinjection sites; ▲, 50 ng bicuculline microinjection sites. Photomicrographs of the ventromedial medulla. These sites are representative of all microinjection sites. Interaural level of each photomicrograph is listed to the right.

![Figure 9](http://jn.physiology.org/)

**FIG. 9.** c-fos–labeled cells throughout ventromedial medulla (VMM) are observed after microinjection of bicuculline (B–D), but not saline (A) into the midline raphe. A–D: c-fos immunoreactive cells from animals that received saline or bicuculline. Each montage includes the cells present on 1 section for each of 5 animals randomly selected from every treatment group. Within each montage, the cells from different animals are shown in different colors. Contours show the outline of the medullary section and are shown in the same color as the cells contained within. E: average number of c-fos–labeled cells in VMM per section for each treatment group. F: average number of c-fos–labeled cells in the dorsal cochlear nucleus per section for each treatment group. G: average change in heart rate observed after microinjection for each treatment group.
bicuculline microinjection may increase sympathetic and somatomotor outflow more directly via descending projections from VMM to the spinal cord.

Methods: bicuculline microinjection

Bicuculline is a competitive GABA_A receptor antagonist and as such blocks transmission through that receptor, thereby disinhibiting neurons. Like opioid agonists, bicuculline is thought to produce antinociception when microinjected into RM or NRMC through the selective disinhibition of one class of RM neurons, the nociceptive-inhibitory OFF cells (Heinricher et al. 1991). However, when \( \geq 50 \) ng bicuculline is injected into RM, other RM cell types, including nociceptive-facilitatory ON cells, are also excited (Heinricher and Tortorici 1994). This may occur because bicuculline nonselectively activates neurons through a block of small-conductance calcium-activated potassium channels (SK channels) (Grunnet et al. 2001; Khawaled et al. 1999), resulting in depolarization and an increase in impedance. Since SK channels are present within the medullary raphe (Stocker and Pedarzani 2000), the bicuculline microinjections in this study likely increased the discharge of multiple RM neuronal types, including serotonergic cells (Bagdy et al. 2000; Levine and Jacobs 1992).

Microinjection of an excitatory (or disinhibitory) drug is a method that has been used extensively to determine the function of a group of activated cells in the complex mammalian CNS. However, there is no direct evidence that microinjections mimic physiological events. The heterogeneous group of cells activated by microinjections may never be collectively engaged by normal physiological events. Furthermore, microinjections cannot tell us the extent of patterned activity in affected neurons or whether individual neurons contribute to one or more of the observed effects. Nonetheless, microinjections remain the best available technique to elucidate the full range of outputs evoked by the mass activation of the neurons affected.

Anatomical locus

As mentioned in the Introduction, studies examining nociceptive responsiveness have focused on injections into RM, whereas studies that measure thermoregulatory variables have employed injections into the most ventral region of the raphe, the RP. However, this distinction is likely spurious as the dendrites of cells located on the midline at the level of the facial nucleus extend both dorsally and laterally (Edwards et al. 1987; Fox et al. 1976; Gao and Mason 1997; Leonovich and Zhukova 1963; Maciewicz et al. 1984; Mason et al. 1990; Newman 1985; Potrebic and Mason 1993; Ramon-Molinero and Nauta 1966; Valverde 1961). Furthermore, cells in the adjacent reticular nuclei extend dendrites medially into all parts of the midline raphe and often reach the contralateral reticular nucleus. The spread of radioactive bicuculline is approximated well by the spread of Fast green (Yoshida et al. 1991). Since the latter was limited to a small sphere in this study, it is unlikely that diffusion can account for the distribution of c-fos immunoreactive cells. Therefore even the small volumes (\( \leq 70 \) nl) and concentrations injected by us and others (Morrison et al. 1999; Samuels et al. 2002) would be expected to affect cells in the ventral and dorsal portions of the midline as well as cells located more laterally, not because of drug diffusion, but because of the dendritic morphology of the cells in the region. Our observations that midline microinjections of bicuculline, but not saline, stimulate c-fos immunoreactivity in neurons throughout raphe and NRMC bilaterally are in agreement with this prediction.

Our original intention was to use small microinjections to locally excite cells and thereby test whether single ventromedial medullary nuclei were uni- or multi-functional. However, we observed that even using very small injections with a limited diffusion of \( \leq 300 \) \( \mu \)m, cells in a large area, including cells belonging to three to four nuclei, were “activated,” as assessed by c-fos immunoreactivity. These cells represent the bicuculline-responsive neurons with either somata or dendrites located within the immediate diffusion zone of the microinjection site. Since our methods closely mirrored those of Morrison and utilized smaller volumes than those used by others, the microinjections made by these other groups are likely to have also activated cells within multiple cytoarchitectonically bounded VMM nuclei. This is likely but not tested, because no previous study attempted to assess the distribution of activated cells.

Since we were ultimately unable to activate neurons restricted to a single VMM nucleus, an exclusive and unique correspondence between a single nucleus and a single “function” remains a formal possibility. However, leaving aside the conceptual ambiguity in what constitutes or defines a single “function,” the extreme heterogeneity of neurons contained within each of the VMM nuclei with respect to neurochemistry, electrophysiology, connectivity, and morphology makes this idea unlikely. The best evidence that at least a subgroup of neurons within a single VMM nucleus share a common function is the finding that a cold (4°C) challenge elicits c-fos immunoreactivity in cells in RP, suggesting that neurons in RP, and not those in NRMC and RM, produce thermoregulatory effects (Cano et al. 2003; Martinez et al. 2001; Morrison et al. 1999). However, even in the limited fields of view that are illustrated in these papers, scattered c-fos immunoreactive cells are evident in RM and NRMC as well as RP. Further, exposure to a milder cold temperature (12°C) sufficient to evoke an increase in BAT temperature (Sakurada and Shido 1993) does not result in the induction of c-fos immunoreactivity anywhere in the brain (C. B. Saper and K. Kano, personal communication). Thus the activation of VMM neurons in response to a 4°C challenge may represent these cells’ involvement in something other than, or in addition to, cold defense, such as the reaction to stress (Zaretsky et al. 2003). Finally, we observed in the present experiments that injections into the lateral portions of NRMC, where very few RP cells extend dendrites, elicited the same increases in BAT temperature as did midline injections. Thus it is unlikely that the neurons involved in cold defense are restricted to RP. Instead it is likely that the heterogeneous neurons distributed throughout the cytoarchitectonic structures within VMM have multiple functions including the modulation of cardiorespiratory, thermoregulatory, and nociceptive function as demonstrated here.

Sympathetic mediation of autonomic effects

Bicuculline microinjections caused an increase in BAT temperature at short latency that was almost an order of magnitude...
larger than the evoked increase in core temperature. The observed elevation in BAT temperature is therefore unlikely to be secondary to a change in core temperature or hormonal status. More likely, this temperature elevation represents an increase in sympathetic outflow since the BAT is only innervated by the sympathetic system (Bamshad et al. 1999). Such an increase has been demonstrated after bicuculline microinjection into either RM or RP (Morrison et al. 1999).

It has been proposed that medullary raphe activation elicits an increase in BAT temperature through the direct activation of preganglionic sympathetic neurons (Morrison 2001a; Morrison et al. 1999). The primary evidence that VMM neurons are premotorneurons is that the labeling of VMM cells after pseudo-rabies injections into BAT tissue occurs at a time that suggests a retrograde trisynaptic connection (BAT to postganglionic, postganglionic to preganglionic, preganglionic to raphe). However, this data may be misleading because most spinal neurons infected with PRV at short survival times (thought to suggest a disynaptic connection) are in fact interneurons rather than preganglionic motoneurons (Nadelhaft and Vera 1995, 1996; Vera and Nadelhaft 2000). Thus the VMM neurons labeled shortly thereafter would be presynaptic to interneurons rather than to motoneurons. Furthermore, the variety of VMM’s visceral and sympathetic targets suggests a general modulatory rather than a specific driving effect on autonomic function (Marson 1997; Nadelhaft and Vera 1995, 1996, 2001; Vizzard et al. 1995). A simple alternative to the premotorneuron model is that VMM activation elicits an increase in BAT temperature by modulating the excitability of preganglionic sympathetic neurons. This could occur through connections to premotorneurons in the intermediate gray or via projections from VMM to thermoreceptive cells in the superficial dorsal horn (Sato 1993) that are themselves presynaptic to preganglionic sympathetic neurons (Cabot 1996).

Increases in blood pressure and heart rate were evoked by bicuculline microinjection into the midline raphe or the more laterally located NRMC, a finding consistent with others (Morrison 1999; Morrison et al. 1999). Increases in heart rate and blood pressure elicited by midline raphe injections of bicuculline are independent of hormonal release and are sympathetically mediated (Cao and Morrison 2003). Pseudorabies virus injections into a large number of sympathetic nervous system targets including the stellate ganglion (which provides sympathetic innervation of the heart and BAT) consistently reveal labeling in RM, RP, and NRMC (Jansen et al. 1995a,b; Smith et al. 1998; Strack et al. 1989a). Thus anatomical and physiological evidence strongly supports sympathetic mediation of cardiovascular changes evoked by bicuculline into RM/RP or NRMC.

**Somatomotor effects**

Bicuculline, but not saline, microinjections evoked tachypnea and increased [CO₂] levels, and often caused shivering. Direct projections from the medulla to phrenic motoneurons and to the premotor neurons in the ventral respiratory group may contribute to these bicuculline-induced changes in respiration (Gao and Mason 1997, 2001; Hosogai et al. 1998; Mason and Fields 1989). The observed tachypnea could also be secondary to the increase in metabolic rate produced by both elevated brown adipose tissue activity and shivering. Since virus injection into somatic muscle labels VMM neurons at short survival times (Kerman et al. 2003), VMM projections to the ventral horn may be important in eliciting shivering.

**Nociceptive modulatory effects**

Consistent with previous results that activation of the VMM by either bicuculline or excitatory amino acid microinjection reliably blocks tail flick (Drower and Hammond 1988; Heinricher and Tortori 1994; Heinricher et al. 1991; Jensen and Yaskh 1984; Satoh et al. 1983; Zhuo and Gebhart 1992b), we observed that the tail flick was suppressed by bicuculline administration into either the medullary raphe or NRMC. Our findings that the cardiovascular, respiratory, and cortical responses to noxious paw heat were also attenuated after bicuculline microinjection are novel evidence that multiple reactions to noxious stimulation are suppressed by medullary raphe and reticular neurons. It is unlikely that the sympathoexcitatory effects of bicuculline microinjection into VMM underlie the decreases in noxious-evoked cardiorespiratory responses because paw heat still evoked increases in blood pressure and heart rate, even when baseline blood pressure and heart rate were significantly elevated (Fig. 7). Instead our results strongly suggest that VMM exerts its nociceptive modulatory effects on an early stage of central sensory processing, perhaps even at the first synapse between nociceptors and secondary sensory neurons in the dorsal horn. The attenuation of the cortical desynchronization evoked by paw heat may also be due to an inhibition of nociceptive neurons in the dorsal horn, particularly those that project to the forebrain and are involved in evoking arousal in response to noxious inputs.

Although inhibition of most reactions to noxious stimulation can be explained by an inhibitory effect early in the nociceptive transmission pathway, the enhancement of the motor withdrawal from paw heat cannot be explained by this mechanism. Our finding that bicuculline microinjection into VMM facilitated the motor response elicited by noxious paw heat was unexpected, because brain stem mediated antinociception has previously been described as affecting the entire body (Fardin et al. 1984a,b; Oliveras et al. 1974, 1978). However, there is some precedence for tail reactions being suppressed and hindpaw reactions facilitated by the same or similar manipulations. Bicuculline microinjection into RM in awake rats suppresses the tail flick while decreasing the latency to a paw paddle reaction evoked by heat (Drower and Hammond 1988). Furthermore, hot water immersion of the hindpaw blocks the noxious heat-evoked tail flick while tail immersion decreases paw withdrawal latency (Morgan et al. 1994). The full expression of both of these effects—tail flick inhibition and paw withdrawal facilitation—is attenuated after transection of the spinal cord, indicating supraspinal, possibly medullary, involvement in such differential modulation.

The opposing effects of VMM bicuculline on paw and tail withdrawals could be explained if nociceptive-inhibiting VMM cells act earlier in the dorsal horn than do nociceptive-facilitating VMM cells. In essence, this allows VMM’s descending inhibitory influence to act as a gate and VMM’s descending facilitation to control the gain of spinal nociceptive circuits. As discussed above, bicuculline microinjection is likely to have increased the discharge of both
nociceptive-inhibiting and facilitating neurons in VMM. If nociceptive-inhibiting cells project to and modulate early sensory neurons, incoming nociceptive information would be largely blocked when they are active. The simultaneous activation of nociceptive-facilitating cells would enhance motor reactions only to the extent that a nociceptive signal has leaked through the inhibitory gate. Finally, it is interesting to speculate how these alternate modulatory mechanisms for nociceptive inhibition and facilitation would affect tail and paw withdrawals, motor programs that are supported by qualitatively different spinal circuits. The tail flick is an all-or-none reaction that results from a low-redundancy convergence from nociceptors to motoneurons (Carstens and Ansley 1993; Carstens and Wilson 1993; Levine et al. 1980). Thus when both inhibitory and facilitatory outputs of VMM are activated, the initial inhibition will sufficiently reduce incoming tail nociceptive signals such that descending facilitation has nothing to act on. In contrast, the paw withdrawal circuit, which exhibits both divergence and convergence, produces graded motor reactions. In this case, VMM activation is likely to evoke incomplete inhibition, leaving a substantial paw nociceptive signal for descending facilitation to act on. In summary, the proposed mechanism may explain how VMM can specifically pattern nociceptive transmission, simultaneously inhibiting some reactions and facilitating others.

ACKNOWLEDGMENTS

The authors thank Dr. Keming Gao for help with the initial experiments, J. Streets for programming assistance, and Drs. Patrice Guyenet, Ron Harper, and Hayley Foo for comments on the manuscript.

GRANTS

M. W. Nason was supported by National Institute of General Medical Sciences Grant T32 GM-07839.

REFERENCES


