Identification of barosensitive neurons in the mediobasal forebrain using juxtacellular labeling

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Kirouac, Gilbert J., and Quentin J. Pittman. Identification of barosensitive neurons in the mediobasal forebrain using juxtacellular labeling. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1766–R1771, 1999.—Previous investigations suggest a possible role in cardiovascular regulation for neurons of the mediobasal forebrain. The present study was designed to determine the location and morphology of basal forebrain neurons that respond to acute changes in arterial blood pressure. Extracellular recordings of single units were done in α-chloralose- or urethane-anesthetized rats. The effect of cardiovascular pressor (phenylephrine, 1–2 µg/kg iv) and depressor (sodium nitroprusside, 0.5–1 µg/kg iv) events on the discharge rates of units was determined. Some of the neurons tested were subsequently filled with biocytin using the juxtacellular method. Brain sections were processed using the avidin-biotin complex reaction to reveal a Golgi-like appearance of the neuron. Of 32 neurons located in the horizontal limb of the diagonal band of Broca (hDB), 13 (41%) were found to be excited by depressor events. Barosensitive biocytin-labeled cells were located in all regions of the hDB and had small- to medium-sized cell bodies with sparse and simple dendritic morphology. Only 2 of 47 neurons tested in the region of the olfactory tubercle, islands of Calleja (IC), and ventral pallidum responded to changes in arterial blood pressure. The results of the present investigation suggest a role in the regulation of cardiovascular function for neurons of the hDB. The findings also suggest that most neurons in the olfactory tubercle, including the IC complex, do not respond to acute changes in arterial blood pressure.

diagonal band of Broca; baroreceptors; cardiovascular regulation; electrophysiology; biocytin

THE MEDIOBASAL FOREBRAIN may be involved in the regulation of the cardiovascular system. For example, the protein Fos was expressed in neurons of the horizontal limb of the diagonal band of Broca (hDB) and of the islands of Calleja (IC) following a decrease in arterial pressure (AP) (8, 17). Single-unit studies indicated that neurons recorded in the olfactory tubercle and region of the IC responded to both depressor and pressor changes in the circulation (2). Chemical stimulation with the excitatory amino acid L-glutamate of sites within the hDB and around the IC caused cardiovascular depressor responses (2, 14). These studies suggest that neurons in mediobasal forebrain receive information about the level of AP in the circulatory system and may be involved in cardiovascular control or baroreflexes.

The basal forebrain contains several nuclei that are identified based on their projection patterns and neurochemical identity (10). However, the boundaries between the different nuclei are not clearly demarcated, and cells believed to be functionally or anatomically related may be intermixed with a variety of other cell types. For example, the olfactory tubercle contains the granule cell clusters of the IC along with a combination of pallidal and striatal types of neurons (6, 19). The ventral pallidum extends ventrally into the polymorph layer of the olfactory tubercle, whereas clusters of striatal-type cells form the striatal cell bridges that connect the polymorph layer of the olfactory tubercle with the nucleus accumbens and caudate nucleus (6, 19). Mixed within these different cellular elements are fibers and neurons of the hDB (19). Traditional extracellular recording techniques allow an estimation of the location of the recorded neuron and provide no anatomic information about the cell type recorded. The present investigation takes advantage of the recent development of the electrophysiological juxtacellular labeling technique that labels the recorded neuron with biocytin (23) to identify the precise location and morphology of barosensitive neurons in the mediobasal forebrain.

METHODS

Animal surgery and preparation. Experiments were conducted on 15 male Sprague-Dawley rats (300–450 g) according to guidelines of the Canadian Council on Animal Care. Rats were anesthetized with urethane (1.4–1.6 g/kg ip) or with intravenous administrations of α-chloralose (60 mg/kg after induction of anesthesia with administration of 0.3 ml/100 g ip of equithesin, supplemented by additional doses of 30 mg/kg of α-chloralose approximately every 1–2 h). Polyethylene catheters were inserted into the femoral artery and vein for the recording of AP and the administration of drugs, respectively. Rats anesthetized with urethan were allowed to breathe normally. The trachea was cannulated in rats anesthetized with α-chloralose, and the animal was paralyzed with pancuronium bromide (Pavulon, Organon Canada, Toronto, ON; 1 mg/kg iv initially and additional doses of 0.5 mg/kg when necessary) and artificially ventilated with 100% oxygen. Rectal temperature was monitored and maintained at 35–37°C with a heating pad.

Rats were placed in a stereotaxic frame, and a burr hole was made above the forebrain. Regions of the mediobasal forebrain were systematically explored for spontaneously active units 0–1.5 mm anterior to bregma, 0.5–2.5 mm lateral to the midline, and 7.0–9.0 mm ventral to the surface of the brain. A minimum distance of 0.5 mm was placed between each pipette tract. Glass micropipettes (tip diameter 1–2.5 µm) containing 0.5 M solution of potassium acetate or sodium chloride plus 1.7% biocytin (Sigma, St. Louis, MO) were used for recording and labeling of cells.
Data acquisition and analysis. Arterial blood pressure was recorded using a Statham P23 XL pressure transducer and Gould transducer amplifier (model 13–4615–50), and heart rate was measured with a Gould Electrocardiography/Biotechnical amplifier (model 13–4615–65), which was triggered by the pressure pulse. Mean AP was defined as the diastolic pressure plus one-third of the pulse pressure. Single unit activity was amplified (×10) through a multipurpose microelectrode amplifier (Axoclamp 2A, Axon Instruments, Foster City, CA), which was further amplified (×100; Neurolog) and filtered (Neurolog NL126; filtered at 500 Hz to 5 kHz). The signal was displayed on an oscilloscope, and the spikes were discriminated using a Neurolog NL200 spike trigger discriminator. The electronic signals for AP, heart rate, and unit activity were digitized using a Cambridge Electronic 1401 Plus Interface Design (CED; Cambridge, UK), and the data were captured and analyzed on a computer (CED Spike2 data capture and analysis software).

The neuronal discharge rate was recorded during acute pressor and depressor events in the circulation. The neuronal discharge rate during changes in AP was compared with the baseline discharge rate before the administration of pressor or depressor agents. A mean of at least 30% above or below the mean baseline discharge rate for at least five consecutive bins (1-s bin width) was considered a significant response (13, 15). In addition, a response was considered to be due to the direct effect of changes in AP if 1) the changes in discharge rate and AP coincided, 2) the response was reproducible, and 3) the response was induced by depressor responses of ≤50 mmHg, which are not of sufficient magnitude to compromise cerebral blood flow (1) but sufficient to cause a reflex tachycardia. The shape and amplitude of the spike were carefully observed to eliminate the possibility that changes in activity were due to movement artifacts. Results are expressed as means ± SE.

Experimental protocol. Spontaneously active units were tested for their responses to pressor and depressor events in the circulation produced by the intravenous administration of phenylephrine (1.0–2.0 µg/kg; Sigma) or sodium nitroprusside (0.5–1.0 µg/kg; Sigma). Units that responded to acute changes in AP were filled with biocytin using the single-cell juxtacellular procedure according to a previously described method (20, 23, 26). Briefly, depolarizing pulses of anodal current (1–10 nA, 200-ms duration, 50% duty cycle) for periods of 1–7 min were applied under continuous electrophysiological monitoring of cell firing rate. Current intensity was increased to enthrall the firing rate of the neuron with the current pulse. The intensity of the current was then adjusted to maintain the entrainment and was terminated when there were indications that the neuron was being damaged by the electrical current (spike broadening and increased discharge rate during the off period). Attempts to label neurons were done on both sides of the brain. A distance of at least 0.5 mm between each attempt was used when there was more than one attempt unilaterally.

Histology. After a survival period ranging from 30 min to 6 h, rats were perfused transcardially with saline (0.9% NaCl, 200–300 ml) followed by 750 ml of a fixative containing 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was removed and postfixed for 1 h in the same fixative. Horizontal sections (100 µm) of the basal forebrain were cut using a vibrating microtome, and sections were collected in PBS (0.1 M, pH 7.4) and washed (3 × 10 min) in PBS. The sections were then incubated in avidin-biotin-peroxidase complex (Vectastain ABC Elite, Vector Laboratories, Burlingame, CA) PBS solution containing 0.1% Triton X-100 for 18–24 h. Sections were rinsed in PBS (3 × 10 min) and Tris buffer saline (TBS, 0.05 M, pH 7.6, 1 × 10 min) and reacted in TBS containing diaminobenzidine, hydrogen peroxide, and nicked ammonium sulfate (substrate kit for peroxidase, Vector Laboratories) followed by several rinses in PBS (4 × 10 min). Sections were mounted on gelatin-coated slides, allowed to dry overnight, dehydrated in alcohol, cleared in xylene, and placed under a coverslip without staining.

Sections were examined for biocytin-labeled cells, and the location of the labeled cells along with the position of the micropipette tract was mapped on drawings of the rat brain (22). The approximate location of nonlabeled cells was determined using the detailed record of the stereotaxic coordinates along with the location of the pipette tract and biocytin-labeled cells. Biocytin histochemistry with the ABC Elite kit provided sufficient background staining to delineate basal forebrain nuclei. Biocytin-labeled neurons were examined at high magnification to determine the cytoarchitecture and dimensions of the labeled cells. Some cells were traced using a camera lucida and scanned in Corel PhotoPaint to produce drawings of biocytin-labeled neurons. The photomicrographs were acquired by a light microscope (Zeiss, Germany) equipped with a digital camera (Quantix System, Photometrics, Tucson, AZ), and viewed on a computer screen by using the program V for Windows (Quantix System) and printed using an ink-jet printer (Epson Stylus Color 600).

RESULTS

A total of 84 neurons in the basal forebrain were tested for their responses to pressor and depressor events in the circulation. Rats anesthetized with urethane had a mean AP of 88 ± 3 mmHg and a heart rate of 366 ± 15 beats/min (n = 5), whereas rats anesthetized with α-chloralose had a mean AP of 112 ± 5 mmHg and a heart rate of 391 ± 6 beats/min (n = 10). Figure 1 shows the location of neurons in the basal forebrain that responded to changes in AP. Of the 84 neurons tested, 32 were located in the hDB of which 13 (41%) responded to depressor responses with an increase in discharge rate. There was no statistical difference in the baseline activity of responsive (discharge rate 5.6 ± 1.6 spikes/s) and nonresponsive (discharge rate 9.3 ± 2.1 spikes/s) neurons in the hDB. Depressor events caused an increase from the baseline discharge rate of 5.6 ± 1.5 to a discharge rate of 11.98 ± 3.2 spikes/s (n = 13) for a similar period of time prior to and during the depressor event. Two of the neurons that responded to depressor events also responded to pressor events with an increase in discharge rate. Of five neurons tested in the vertical limb of the diagonal band of Broca (vDB), two responded with excitation to depressor events and one neuron responded to pressor events. From a total of 30 neurons tested in the region of the olfactory tubercle (vDB), only one neuron (discharge rate 3.3 spikes/s) responded with excitation to an acute decrease in AP. Only one of 17 neurons (discharge rate 7.5 ± 2.9 spikes/s) located in the ventral pallidum responded with excitation to depressor events in the circulation. There was no significant difference in the baseline discharge rates of neurons located in the hDB (9.0 ± 1.8 spikes/s), vDB (8.4 ± 3.6), dfactory tubercle (6.5 ± 1.3), and ventral pallidum (7.3 ± 2.6).

An attempt to label 20 neurons in the diagonal band of Broca with the juxtacellular application of biocytin resulted in the successful labeling of 12 neurons (60%...
success rate), 9 of which were barosensitive neurons. Figure 2 shows an example of a barosensitive neuron in the hDB labeled with biocytin. Attempts at labeling neurons in the ventral pallidum and olfactory tubercle resulted in the labeling of 10 of 19 neurons (53% success rate). Figure 3 shows examples of nonresponsive neurons in the ventral pallidum and olfactory tubercle that were successfully labeled with biocytin. Of the labeled neurons, only one nonresponsive neuron was found in a granule cell cluster of the IC. Labeled neurons were scattered within different regions of the ventral pallidum (4 of 5 attempts) and olfactory tubercle (6 of 14 attempts).

Labeled neurons in the hDB that responded to depressor responses had cell body dimensions that ranged from small (45.5 µm²) to large (300 µm²) diameter. The majority of these neurons were medium sized (50–100 µm², discharge rate 7.1 ± 2.7 µm², n = 6), two were large (>100 µm², discharge rate 4.8 ± 3.7 spikes/s, n = 2), and one was small (<50 µm², 3.6 spikes/s). All the barosensitive cells in the hDB had regular discharge rates. Most of the labeled neurons had elongated cell bodies with short aspiny primary dendrites (2–4 primary dendrites, dendritic field extending 52.5 ± 10.3 µm, n = 9) and few secondary aspiny dendrites (Fig. 4).

In a few cases (n = 3) an axon containing varicose terminals could be followed for a short distance as it projected toward the ventral surface of the hDB (Fig. 2). Labeled neurons in the ventral pallidum were large cells (100–200 µm², n = 4) with aspiny dendrites. Labeled neurons in the olfactory tubercle were composed of a mixed population of small granule-like cells (<50 µm², n = 3), large cells (100–200 µm², n = 2) with aspiny dendrites, and one large striatal-type cell (187.5 µm²) with spiny and aspiny dendrites (Fig. 3).

DISCUSSION

The present investigation demonstrates that 41% of spontaneously active neurons tested in the hDB respond to acute decreases in AP with an increase in discharge rate. Two of the 13 neurons were activated by both depressor and pressor responses in the circulation. A few spontaneously active neurons in the vDB were also found to respond to depressor events with excitation. Only a few (2 of 47) of the neurons located in the ventral pallidum, olfactory tubercle, and the IC responded to depressor events in the circulation. The use of the juxtacellular labeling technique to label the recorded neuron yielded a success rate of 56%, which resulted in a Golgi-like labeling of responsive neurons in

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**Fig. 1.** Drawings of transverse sections of mediobasal forebrain (A) modified from stereotaxic atlas (22), extending 8.5–9.5 rostral to intra-aural line showing location of single units that were tested for their response to depressor and pressor events in circulation (B). ○, responsive cells to either pressor or depressor events; ○, nonresponsive cells. AC, anterior commissure; CPU, caudate-putamen; IC, islands of Calleja; hDB, horizontal limb of diagonal band of Broca; MFB, medial forebrain bundle; MS, medial septal nucleus; NA, nucleus accumbens; OT, olfactory tubercle; PO, preoptic area; vDB, vertical limb of diagonal band of Broca; VP, ventral pallidum.

**Fig. 2.** Representative example of biocytin-labeled neuron in hDB that was activated by acute decreases in arterial pressure (AP). A: Photomicrograph of biocytin-labeled neuron (arrow) with single dendrite (not in plane of focus) and axon containing en passant varicosities directed toward ventral surface of brain. Electrode tract is seen immediately above filled soma. B: AP tracing and running ratemeter record showing that unit responded with excitation to depressor events (intravenous sodium nitroprusside) but not to pressor events (intravenous phenylephrine).
the hDB as well as nonresponsive neurons throughout the mediobasal forebrain. The majority of barosensitive neurons in the hDB had small- to medium-sized cell bodies with simple and sparse dendritic fields.

The possibility exists that the neurons were activated by movement artifacts or hypoxia caused by decreases in cerebral blood flow to the basal forebrain. However, the cerebral vasculature possesses an extensive autoregulatory capability within a perfusion range of 70–160 mmHg (21). The pressor and depressor responses in the present study (between 25 and 50 mmHg) were within this range and should not have compromised blood flow to the brain. It is also possible that significant hypoxia and hypercapnia resulting from the depressor events could influence the activity of peripheral and central chemoreceptors sensitive to pH and blood gases. Although neurons in the hDB may have responded to activation of peripheral and central chemoreceptors, this is unlikely in the present study because arterial chemoreceptors are only stimulated with changes in AP greater than 50 mmHg (1).

A previous study showed that neurons in the diagonal band of Broca antidromically activated by stimulation of the supraoptic nucleus responded with excitation to the activation of baroreceptors by acute pressor events in the circulation (11). These results seem to be somewhat at odds with the present investigation in which neurons in the hDB were primarily excited by depressor events. However, the present investigation tested spontaneously active units in the hDB, whereas the previous study tested mostly silent units in the vDB that were antidromically activated by stimulation of the supraoptic nucleus (11). Therefore, neurons in the vDB that project to the supraoptic nucleus may respond differently than the spontaneously active units recorded in the hDB.

The results of the present investigation are somewhat contradictory to those of a recent investigation showing that electrical stimulation of the aortic depressor nerve resulted in the expression of Fos protein in the hDB (17), which would indicate that neurons in the hDB are excited by pressor events. However, the expression of Fos in that study may have been due to the chronic hypotensive effects produced by activation of baroreflex pathways in the brain during the stimulation of the aortic depressor nerve (13). Another possibility is that stimulation of the aortic depressor nerve may have excited a different population of neurons within the hDB than the population of spontaneously active cells recorded in this study. In fact, a few neurons were found in this study to respond to pressor events in the circulation.

In two cases, a neuron was found to respond with excitation to both depressor and pressor events in the circulation. There is no information in the literature that we are aware of concerning barosensitive neurons responding with excitation to both depressor and pressor events. McKitrick et al. (17) reported a similar observation for some of their single-unit recordings in the olfactory tubercle. One could speculate that these neurons receive information about changes in AP irrespective of the direction and that these neurons may be involved in mechanisms that do not relate to the baroreflexes. For example, these neurons may be inte-

**Fig. 3.** Examples of biocytin-labeled neurons in the ventral pallidum (A) and olfactory tubercle (B) that did not respond to changes in AP.

**Fig. 4.** Camera lucida drawings of biocytin-labeled neurons in hDB that responded to depressor events in circulation. Arrows indicate location of axonal processes.
grating baroreceptor information with other physiological functions such as arousal.

Only a few barosensitive cells were found in the region of the olfactory tubercle and the IC. Our investigation did not confirm the results of previous studies that demonstrated intense staining for the Fos protein in the IC of rats following long-term hypotension (1 h) due to stimulation of the aortic depressor nerve or to the administration of a vasodilating agent (8, 17). Such a prolonged stimulus could have activated neurons in the IC that were not activated during an acute depressor response (<1 min) as used in the present study. The present investigation is also at odds with a previous single-unit study (2) showing that 68% of the units in the olfactory tubercle in the region of the IC responded to pressor and depressor events. However, the doses of phenylephrine and sodium nitroprusside used in the study of Calaresu et al. (2) to test whether neurons in the IC received baroreceptor input were several magnitudes greater than in the present investigation. This may have resulted in the identification of false positives in the IC because of artifacts such as movement of the electrode, hypoxia, or hypercapnia. Support for this comes from a report showing expression of Fos in neurons located in the region of the olfactory tubercle and IC in rats made to breathe high concentrations of CO₂ (9). Indeed, it was recently proposed that the IC are involved in the regulation of blood flow to the ventral striatopallidal region. This is based on the observation that the IC surround and follow the arterioles that supply the ventral striatopallidal complex (18). In addition granule cells of the IC contain nitric oxide (18), which could be released in the vicinity of the arterioles where it could act as a vasodilator substance. Therefore, the IC may be involved in regulating blood flow to parts of the ventral striatopallidal region after prolonged hypotension as opposed to being involved in a long-loop baroreflex as suggested by Calaresu et al. (2).

As discussed previously, a study in rats under urethan anesthesia by Calaresu et al. (2) suggested that neurons in the IC were responsive to changes in AP levels. The experiments in the present investigation were conducted in rats under either α-chloralose or urethan to exclude the possibility that the response of neurons was dependent on the type of anesthesia. However, a role in cardiovascular regulation for neurons of the olfactory tubercle and IC cannot be entirely excluded because their responses to inputs from chemoreceptors or cardiopulmonary receptors were not examined in the present investigation.

The present investigation, which used the juxtacellular labeling technique to label the neuron under study, showed that neurons in the hDB were responsive to depressor events. It has been proposed that the labeled neuron is the same neuron that was recorded during the experiment (20, 23, 26). Several observations support this hypothesis. First, ejection of the same amount of current at a site where no neuron is active never resulted in the labeling of a neuron. Second, no labeling occurred in cases that neurons could not be entrained or were entrained for only a few seconds. Finally, labeled neurons were located at proper stereotaxic coordinates and in some instances the labeled neuron was found at the end of a pipette tract. There was only one occasion where two neurons were labeled during the same attempt. The identification of the actual cell recorded in this case could easily be inferred by the location of the cell relative to the pipette tract. Pinault (23) described some labeled neurons that had been filled by the retrograde transport of biocytin by damaged axons in the region of another labeled neuron.

The juxtacellular labeling technique is useful in demonstrating the precise location of a neuron as well as showing the cytoarchitecture of the cell. The present study shows that the majority of barosensitive neurons in the hDB had small- to medium-sized somata with short simple dendrites. The size and morphology of nonbarosensitive neurons in the mediobasal forebrain were variable with many neurons exhibiting characteristics of pallidal and striatal types of cells as described by other investigators using Golgi stain methods (6, 19). The significance of the observation that barosensitive neurons in the hDB are small neurons with sparse dendritic morphology is not known. However, it may be important in terms of how these neurons receive and integrate afferent inputs. Future investigation may be useful in defining the functional characteristics of these relatively small barosensitive neurons in the hDB.

Perspectives

The present investigation provides evidence that neurons in the hDB are excited by acute depressor events. However, the role of the hDB in cardiovascular regulation is poorly understood. It has been proposed that the hDB relays baroreceptor information to the lateral hypothalamus to modulate the activity of vasopressin-secreting neurons in the supraoptic nucleus (24). Tracing studies show that the hDB receives projections from the lateral hypothalamus, parabrachial nucleus, and the catecholaminergic neurons of the A1, A2, and A6 cell groups (12, 16, 25, 29, 30). The parabrachial nucleus and the catecholaminergic brainstem neurons relay baroreceptor information to various regions of forebrain (4, 27). The hDB in turn projects to a variety of regions involved in the regulation of the cardiovascular system, including the prelimbic cortex, bed nucleus of the stria terminalis, central nucleus of the amygdala, and most of the hypothalamus (3, 5, 7, 28). Stimulation of the hDB with the excitatory amino acid L-glutamate causes cardiovascular depressor responses (14). Based on this observation, we speculate that decreases in AP may activate neurons in the hDB that may regulate the level of sympathetic activity to the cardiovascular system.

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