Cardiac effects of hypocretin-1 in nucleus ambiguus

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Ciriello, John, and Cleusa V. R. de Oliveira. Cardiac effects of hypocretin-1 in nucleus ambiguus. Am J Physiol Regul Integr Comp Physiol 284: R1611–R1620, 2003.—Although recent studies have reported hypocretin 1 (hcrt-1)-like-immunoreactivity (ir) within the region of the nucleus ambiguus (Amb) in the caudal brain stem, the function of hcrt-1 in the Amb on cardiovascular function is not known. Three series of experiments were done in male Wistar rats to investigate the effects of microinjections of hcrt-1 into Amb on heart rate (HR), mean arterial pressure (MAP), and the arterial baroreceptor reflex. In the first series, a detailed mapping of the distribution of hcrt-1- and hcrt-1 receptor (hcrtR-1)-like-ir was obtained of the Amb region. Although hcrt-1-like- and hcrtR-1-like-ir were found throughout the rostrocaudal extent of the Amb and adjacent ventrolateral medullary reticular formation, most of the hcrtR-1-like-ir was observed in the area just ventral to the compact formation of Amb, in the region of the external formation of the nucleus (Ambe). In the second series, the Amb region that contained hcrt-1 and hcrtR-1-ir was explored for sites that elicited changes in HR and MAP in urethane and α-chloralose-anesthetized rats. Microinjections of hcrt-1 (0.5–2.5 pmol) into the Amb elicited a dose-related decrease in HR, with little or no direct change in MAP. The small decreases in MAP were found to be secondary to the HR changes. The largest bradycardia responses were elicited from sites in the Ambe. Administration (iv) of the muscarinic receptor antagonist atropine methyl bromide or ipsilateral vagotomy abolished the HR response, indicating that the HR response was due to activation of vagal cardiomotor neurons. In the final series, microinjections of hcrt-1 into the Amb significantly potentiated the reflex bradycardia elicited by activation of the baroreflex as a result of the increased MAP after the intravenous injection of phenylephrine. These data suggest that hcrt-1 in the Amb activates neuronal systems that alter the excitability of central circuits that reflexly control the circulation through the activation of vagal preganglionic cardioinhibitory neurons.

heart rate; blood pressure; baroreceptor reflex; vagus nerve

THE NUCLEUS AMBIGUUS (Amb) is known to play a pivotal role in the reflex regulation of heart rate (HR) (9, 28). Focal electrical or chemical stimulation of the Amb region has been shown to elicit decreases in HR mediated by the activation of vagal cardiomotor neurons (8, 9, 31). In addition, electrophysiological and neuroanatomical tract tracing studies have shown that the Amb region is the medullary site of origin of vagal preganglionic cardioinhibitory axons (8, 9, 31, 32, 35, 36, 41, 47). Furthermore, activation of arterial baroreceptors has been demonstrated to evoke a powerful excitation of these vagal cardioinhibitory neurons (8, 31). Although a considerable amount of experimental work has been done to investigate the functional properties and the connectivity of Amb neurons (9, 28), less attention has been directed toward determining the functional significance of chemically specific axons and axon terminals innervating Amb neurons that function as components of the parasympathetic nervous system controlling the heart.

Recent studies have implicated the hypocretin neuropeptides in the neuronal control of the cardiovascular system (7, 12, 15, 17, 30, 39, 40). Hypocretin-1 (hcrt-1, orexin-A) and hypocretin-2 (hcrt-2, orexin B) (38, 42) form a family of neuropeptides recently identified exclusively within lateral and perifornical hypothalamic neurons (19, 34, 37, 38, 49). These peptides are derived from the same 130-amino acid prepro-hypocretin molecule by proteolytic cleavage (38). Hcrt-1 is a 33-amino acid peptide with an NH₂-terminal pyrroglutamyl residue and COOH-terminal amide, whereas hcrt-2 is a 28-amino acid peptide with a COOH-terminal amide (38). Although hcrt-2 has been reported to have 46% similarity with hcrt-1 (38) and both peptides bind and activate G protein-coupled receptors (13, 14, 38), hcrt-1 appears to exert a more potent effect when administered centrally on a variety of physiological variables (12, 14, 37, 49).

Intracerebroventricular injections of hcrt-1 have been shown to elicit an increase in renal sympathetic activity and catecholamine release and a long-lasting increase in mean arterial pressure (MAP) (30, 40). Similarly, intracerebral injections of hcrt-1 have been reported to elicit a dose-dependent increase in MAP and heart rate (HR) (7), effects suggested to be mediated by the activation of the rostral ventrolateral medulla (17), the location of sympathetic premotor neurons (10). Additionally, it has been shown that intrathecal injections of hcrt-1 into the thoracolumbar cord elicit increases in MAP and HR, effects suggested to be mediated by activation of sympathetic preganglionic neurons (2). Finally, we recently demonstrated that discrete injections of hcrt-1 into sites within the nu-...

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nucleus of the solitary tract that receive cardiovascular afferent projections elicit a depressor and bradycardia response (15).

Hypothalamic hcrT-1 neurons have been shown to contribute to an extensive innervation of forebrain, brain stem, and spinal cord structures (12, 14, 37, 42, 48, 49). Similarly, mRNA for the hcrT-1 receptor (hcrTR-1) has been found throughout the different levels of the neuraxis (29). Within the brain stem, axonal processes immunoreactive to hcrT-1 have been demonstrated within the ventral medulla, including the region containing the Amb (12, 23, 37, 42, 48). However, a detailed mapping of hcrT-1-labeled fibers within this brain stem cardiovascular region is not available.

The presence of hcrT-1 immunoreactivity (ir) in the Amb region suggests that hcrT-1 may be associated with central pathways controlling vagal outflow to the heart. Therefore, the present study was done to investigate the effect of microinjections of hcrT-1 into the Amb on HR, MAP, and the baroreceptor reflex. In the first series of experiments, to determine the area in which hcrT-1 microinjections were to be made, a mapping of the distribution of hcrT-1-like-ir and of the distribution of hcrTR-1-like-ir in the Amb region was made in the male Wistar rat. In the second series, the effect of microinjection of hcrT-1 on HR and MAP was investigated in the anesthetized rat. Additionally, studies were done to investigate the components of the autonomic nervous system that mediated the circulatory effects to activation of Amb neurons by hcrT-1. Finally, the effect of microinjections of hcrT-1 into Amb on the reflex HR response to the activation of arterial baroreceptors was also investigated in the anesthetized rat.

METHODS AND MATERIALS

General procedure. Experiments were done in adult male Wistar rats (250–350 g; Charles River Canada, St. Constant, Canada). All animals were housed under controlled conditions with a 12:12-h light/dark cycle. Food and water were available to all animals ad libitum. All experimental procedures were done in accordance with the guidelines on the use and care of laboratory animals as set by the Canadian Council on Animal Care and approved by the Animal Care Committee at The University of Western Ontario.

Immunohistochemistry. Under pentobarbital sodium anesthesia (65 mg/kg ip; MTC Pharmaceuticals, Cambridge, ON, Canada) animals (n = 5) were perfused transcardially with 500 ml of 0.9% physiological saline followed by 500 ml of Zamboni’s fixative containing 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2–7.4 and 15% saturated picric acid at 4°C (11). The brains were removed and stored in a 10% sucrose-PBS solution overnight. Frozen, serial transverse sections of the brain stem at 40 μm were cut in a cryostat (−17°C; model 5030, Bright Instrument, Huntington, UK). For each animal, one in every three sections of the brain stem was processed immunohistochemically as previously described (11) for hcrT-1-like-ir. An adjacent section was processed for hcrTR-1-like-ir. In brief, brain stem sections were placed into normal goat serum (Vector Laboratories, Burlingame, CA) diluted 1:50 with PBS containing 0.3% Triton X-100 for 30 min. The sections were then rinsed in PBS and placed into primary antisera to hcrT-1 (affinity purified rabbit polyclonal anti-orexin-A; Alpha Diagnostic International, San Antonio, TX; Cat. #OX11-A, Lot #305960A: Refs. 13 and 38) or to the hcrTR-1 (affinity purified rabbit polyclonal anti-orexin receptor-1; Alpha Diagnostic International; Cat. #OXIR11-A1-A, Lot #297980A) diluted 1:2,000 in PBS-0.3% Triton X-100 at 4°C. After 72 h the sections were rinsed in PBS and placed for 30 min into goat biotinylated anti-rabbit IgG (Vector Laboratories) diluted 1:500 in PBS-0.3% Triton X-100. After a rinse in PBS, the sections were placed into a solution of methanol and hydrogen peroxide (29:1) for 30 min. The sections were then rinsed in PBS and placed into an avidin-biotin complex reagent (Vectastain ABC Elite Kit) in PBS-0.3% Triton X-100 for 75 min and then washed again in acetate buffer at pH 5.5. The peroxidase contained in the ABC reagent was visualized by placing the sections into a solution of 0.006% hydrogen peroxide and 0.02% 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical, St. Louis, MO) in PBS for 20 min or in 0.05% DAB, 0.05% hydrogen peroxide, and 0.01% nickel ammonium sulfate in acetate buffer for 15–20 min. After tissues were rinsed in PBS, sections were mounted onto gelatinized glass slides, dried, and placed under a cover glass. Adjacent brain stem sections to those processed for hcrT-1- or hcrTR-1-like-ir were stained with either Neutral red or thionine for the identification of cytoarchitectonic boundaries. Analysis was done using bright- and dark-field microscopy. The location of hcrT-1- or hcrTR-1-like-ir labeling was mapped onto camera lucida projection drawings of the Amb for each experimental case.

Controls for hcrT-1- or hcrTR-1-like-ir included placing brain stem sections in primary hcrT-1 or hcrTR-1 antisera that had been preadsorbed with an excess of hcrT-1 or hcrTR-1 peptide (Cat. #OX11-P or OXIR11-P, respectively; Alpha Diagnostic) or sections in which the reaction of the tissue with the primary antisera was omitted (11). Under these conditions, no hcrT-1- or hcrTR-1-like-ir was demonstrated in the brain stem sections.

Microinjections into Amb. On the day of the experiments, the animals were anesthetized with urethane (1.5 g/kg ip; n = 16) or α-chloralose (80 mg/kg iv initially and supplemented by additional doses of 10–20 mg/kg every 1–2 h; n = 10) after induction with equithesin (0.3 ml/100 g ip). Different anesthetics were used to determine whether the type of anesthetic altered the cardiovascular responses (5, 6) to microinjections of hcrT-1 into Amb.

The trachea was cannulated, and the animals were artificially ventilated using a small rodent ventilator (Harvard Apparatus; model 683) with a mixture of room air and 95% of O2. Body temperature was maintained at 36–37°C by a heating pad (model K-20; American Hospital Supply, Cincinnati, OH). Polyethylene catheters-50 (Clay Adams, Parsippany, NJ) were inserted into the femoral artery and vein for the recording of the arterial pressure and the administration of drugs, respectively. Arterial pressure (AP) was recorded via a Statham pressure transducer (model P23 XL), and a Grass tachograph (model 7P4K) triggered by the AP pulse was used to monitor HR. AP, MAP, and HR were recorded continuously on a Grass polygraph (model 78G).

The head of the animal was placed in a Kopf stereotaxic frame and bent downward at a 45° angle to the horizontal meridian. The dorsal surface of the medulla was exposed by partial occipital craniotomy. The dura was cut and reflected laterally, and the caudal floor of the fourth ventricle was exposed by gently removing the vermis of the cerebellum by suction. The nervous tissue was kept moist by physiological saline throughout the experiment.
Single-or double-barreled glass micropipettes (tip diameter, 20–35 μm) were pulled from 5 μl Socorex capillary tubing (Mississauga, ON, Canada). Micropipettes were placed stereotaxically into the Amb region (rostrocaudal to bregma, −13.0 to −14.6; lateral to the midline, 1.6 to 2.4; ventral to the dorsal surface, 2.4 to 3.2) where dense hcrt-1- and hcrtR-1-like-ir were observed in the immunohistochemical study. The microinjection of hcrt-1 (0.5, 1.0, and 2.5 pmol; Phoenix Pharmaceuticals, Mountain View, CA) in 0.9% saline into the Amb region was done by the application of pressurized nitrogen pulses controlled by a picospritzer (General Valve, Fairfield, NJ). The injected volume was measured by direct observation of the fluid meniscus in the micropipette by using a microscope fitted with an ocular micrometer that allowed a 1 nl resolution. Three to four sites on each side of the Amb in each animal were tested for the effects of hcrt-1 on AP and HR. Control injections of similar volumes (20 nl) of the vehicle saline into these Amb sites were shown not to elicit cardiovascular responses.

Effect of administration of muscarinic receptor blocker on the MAP and HR responses to hcrt-1 in Amb. To determine which components of the autonomic nervous system were involved in mediating the cardiovascular responses elicited by hcrt-1 in the Amb, the cardiovascular responses elicited by the microinjections of hcrt-1 (2.5 pmol) were retested after the intravenous injection of the muscarinic receptor blocker atropine methyl bromide (2 mg/kg, n = 4) in urethane-anesthetized animals. In an additional series of experiments (n = 6) to further support the finding that the cardiac responses were due to vagal activation, the cardiovascular responses elicited by the microinjections of hcrt-1 (2.5 pmol) were retested after ipsilateral vagotomy. The vagus nerve was identified following a midline incision in the neck and isolated from surrounding tissues. A silk thread was then placed around the nerve for later identification. After the recovery of the HR response to an injection of hcrt-1 into the Amb region, the nerve ipsilateral to the injection site was cut. Thirty minutes after the transection of the vagus nerve, the Amb site was reinjected with hcrt-1. Only one site in each animal was tested in these studies.

Activation of the baroreceptor reflex. To determine whether hcrt-1 exerted an effect on the baroreceptor reflex, the effect of microinjections of hcrt-1 (2.5 pmol) into Amb on the reflex bradycardia elicited by the increase in MAP to an intravenous injection of phenylephrine (Phe; 2, 3, or 4 μg/kg) was tested in 11 animals. The dose of 2.5 pmol was chosen in these studies to maximize the number of neurons exposed to a sufficient concentration of hcrt-1 for activation within the small injection area. Injections of Phe were made 5 min before (Control) and 0.5, 2.5, and 5.0 min after the microinjections of hcrt-1 into Amb. In each animal (n = 9), injections of 2, 3, or 4 μg/kg of Phe (in 0.05–0.1 ml saline) were made while testing only one site in each side of the Amb.

Histological verification of injection sites. At the end of all experiments, the micropipette was withdrawn from the last site of hcrt-1 microinjection, emptied of the hcrt-1 solution without removing it from the stereotaxic frame, filled with Pontamine sky blue in 0.9% saline, and lowered back stereotaxically to the same site in the Amb at which a 20-nl
microinjection of the dye was made to mark the injection site. Injections of the Pontamine sky blue dye did not elicit cardiovascular responses at the site at which the hcrt-1 previously elicited a bradycardia response. The animals were perfused with 50 ml of 0.9% saline solution followed by 50 ml of 10% formalin. The brains were postfixed in the 10% buffered formalin solution for 2–4 days. Frozen, transverse sections of the brain stem were cut in a cryostat at 50 μm, mounted on glass slides, and stained with Neutral red. Stimulation sites were determined by extrapolation along the pipette tracts in each animal from the center of the marked injection site. All stimulation sites were mapped on projection drawings of transverse sections of the rat brain stem for each animal and later plotted on a standard set drawings of sections of the ventral medulla modified from a rat stereotaxic atlas (43).

**Data analysis.** Means ± SE were calculated for the magnitude of the peak changes in MAP and HR to hcrt-1 injections. A response was defined as a change in MAP or HR of >5 mmHg or 10 beats/min, respectively. Comparisons of the changes in MAP or HR before and after the administration of the muscarinic blocking agent were made using an analysis of variance for repeated measures followed by a Bonferroni post hoc test. The effects of hcrt-1 injections on the baroreceptor reflex were analyzed by using a regression analysis, and statistical comparisons among the slopes of the lines were made using an analysis of variance followed by Dunnett’s multiple comparison test. In all cases, a P value of <0.05 was taken to indicate statistical significance.

**RESULTS**

**Hcrt-1 and hcrtR-1-like-ir within the Amb region.** The distribution of hcrt-1-and hcrtR-1-like-ir in the region of the Amb region is summarized on Figs. 1 and 2. Scattered hcrt-1-labeled axons and presumptive axonal terminals were observed in and around the region of the Amb, throughout its rostrocaudal extent (Figs. 1–3A). In the compact formation of Amb (Ambc), labeled axons were found mainly along its lateral borders, appearing more to encircle the Ambc than to enter this component of the nucleus. Few axons were found to course through the Ambc. On the other hand, hcrt-1-labeled axons were found throughout the external formation of Amb (Ambe), especially ventral to the Ambc. This region of the caudal Ambe overlaps the caudal ventrolateral medulla. Many of these hcrt-1-labeled axons in the Ambe region were found to contain spinelike processes (Fig. 3C). It was also observed that the reticular formation, just dorsal to the Ambc, appeared to receive a moderately dense innervation by hcrt-1 axons (Fig. 1). Rostrally, the Amb area appeared to receive a less dense hcrt-1 innervation, as with the adjacent rostral ventrolateral medullary reticular formation (Fig. 2, E and F).

Relatively moderate punctate hcrtR-1-like-ir was also observed scattered throughout the Amb region. Within the Ambc, the punctate reaction product was

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**Fig. 2.** Series of projection drawings of transverse sections of the ventral medulla through the region of the rostral Amb of the rat showing the location of hcrt-1-labeled axons (A–C) and the distribution of punctate reaction product (small dots) representing the hcrtR-1 (D–F). Note that the density of hcrt-1 and hcrtR-1 immunoreactivity was less throughout the rostral Amb area compared with the caudal Amb areas. However, the hcrt-1 axons and hcrtR-1 labeling was still localized mostly within the Ambe. Note also the little amount of hcrt-1 labeling found with the LPGi and the rostral ventrolateral medullary region. Gi, nucleus gigantocellularis; GiA, nucleus gigantocellularis pars alpha; LPGi, nucleus paragigantocellularis lateralis. Calibration mark, 1 mm.
primarily observed in association with perikarya (Fig. 3, B and D). A small number of Ambc neurons containing the hcrTR-1-like-ir were found scattered throughout the rostrocaudal extent of the Amb region. In contrast, most of the punctate hcrTR-1-like-ir in the Ambe was found scattered throughout the neuropil of the caudal aspects of the nucleus (Figs. 1 and 3, B and D).

**Cardiovascular effects of microinjections of hcrT-1 into Amb.** To determine the effect of hcrT-1 in the Amb on the MAP and HR, hcrT-1 was microinjected at three different doses into histologically verified sites within the Amb region (Fig. 4) where hcrT-1- and hcrTR-1-like-ir were observed in the previous study (Figs. 1 and 2).

In the anesthetized rat, baseline HR and MAP were found to be 400 ± 5.6 beats/min and 100.8 ± 3.2 mmHg, respectively. Microinjections of hcrT-1 (0.5, 1.0, and 2.5 pmol) into the Amb region elicited a dose-related bradycardia response (Figs. 5 and 6), with an associated decrease in MAP observed only at the higher dose of hcrT-1 (Fig. 5). The maximal amplitude of the bradycardia response was also observed at this higher dosage of 2.5 pmol (Figs. 5 and 6). The mean duration of the bradycardia response was 83.6 ± 26 s. Figure 5 shows a representative experiment at which different doses of hcrT-1 were injected into the same Ambe site. As shown by the responses to hcrT-1 injections into Ambe, the peak of the bradycardia response was reached within 5–10 s after the injection (Fig. 5).

Sites that elicited the largest decrease in HR were localized predominantly in and around the ventral aspects of the Amb, in the region corresponding to the Ambe (3) (Fig. 4). Injections of hcrT-1 into the Ambc and in areas immediately outside the Ambe region were found to elicit no or little cardiovascular responses. Control injections of the vehicle, 0.9% physiological saline into the same Ambe region did not elicit cardiovascular responses (Fig. 6). Figure 4 shows the location of histologically verified sites at which only microinjections of the 2.5-pmol dose of hcrT-1 were made into the Amb region.

**Autonomic nervous system components mediating hcrT-1 HR responses.** To investigate which peripheral components of the autonomic nervous system contributed to the cardiovascular responses elicited by microinjections of hcrT-1 (2.5 pmol) into the Ambe, the muscarinic receptor blocker atropine methyl bromide was administered intravenously. The bradycardia response elicited by hcrT-1 was abolished (Fig. 7) after systemic administration of atropine methyl bromide. The occasional MAP response observed at this higher dosage of hcrT-1 was also blocked after the intravenous adminis-

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Fig. 3. Bright-field photomicrographs showing hcrT-1 labeled axons (A and C), punctate reaction product representing hcrTR-1 (B and D) within the neuropil (open arrows; B and D) and associated with perikarya (filled arrows; B and D) in the Amb region. Note in A the scattered hcrT-1-labeled axons throughout the Amb region (open arrow points to labeled axon shown in C). B: punctate reaction product corresponding to the hcrTR-1 within both the Ambc and Ambe. Note that most of hcrTR-1 is found within the neuropil in the Ambe and associated with some cell bodies in the Ambc. Area outlined in B corresponds to that in D. C: an hcrT-1-labeled axon and spinelike processes on the axon (open arrows). D: hcrTR-1 labeling on cell bodies (filled arrows) and in the neuropil (open arrows) in the Ambe. Calibration mark in A represents 100 μm. Same calibration mark in A represents 50 μm in B and 25 μm in C and D.
tration of atropine methyl bromide. To further support the finding that the cardiac responses were the result of activation of vagal cardiomotor neurons, the effect of ipsilateral vagotomy was determined after injection of hCt-1 into the Amb. As summarized in Fig. 7, ipsilateral cervical vagotomy abolished the bradycardia response to hCt-1 injections into the Amb.

**Effect of hCt-1 injections into Amb on the BR.** To investigate whether activation of Amb neurons by hCt-1 altered the HR component of the baroreflex, changes in systemic blood pressure were used to activate arterial baroreceptors after the injection of hCt-1 into the Amb. As shown in the representative experiment in Fig. 8A and the summarized data from nine experiments in Fig. 8B, activation of Amb neurons by hCt-1 increased the magnitude of the reflex decrease in HR resulting from the activation of arterial baroreceptors. This potentiation of the HR response during activation of the baroreflex was evident at ~0.5 min (Fig. 8) after the hCt-1 injection into Amb. By 5 min after the hCt-1 injection into the Amb, the potentiated reflex HR response had returned to control values (Fig. 8).

**DISCUSSION**

Lateral hypothalamic and perifornical hypothalamic hCt containing neurons have been shown to contribute extensively to a number of neuronal systems throughout the brain involved in controlling a variety of homeostatic mechanisms (12, 14, 34, 37, 38, 48, 49). Within the brain stem, hCt-1-ir has been previously observed within the ventral medullary reticular formation, including the region of Amb (12, 14, 34, 37, 49). These areas of the brain stem are well known to be involved in the maintenance and reflex regulation of arterial pressure (9, 10). In addition, a recent in situ hybridization study demonstrated the existence of hCtR mRNA within the region of the ventral medulla (29). Microinjections of hCt-1 into the rostral ventrolateral medulla have been reported to elicit increases in MAP and HR (7). This study has now demonstrated that activation of Amb neurons by hCt-1 elicits a decrease in HR that is mediated by the activation of vagal preganglionic cardioinhibitory neurons and to potentiate the reflex decrease in HR to activation of the arterial baroreflex.
The finding in this study that hcrt-1-labeled axons and presumptive axonal terminals are found within the Amb region is consistent with earlier observations (12, 13, 34, 37, 49). This study also demonstrated that hcrt-1 labeling within the Amb area is primarily localized to the ventral aspects of the nucleus, that region of the nucleus previously shown to contain preganglionic parasympathetic neurons that innervate the heart (3, 35, 36, 41). Although this latter finding suggests that hcrt-1 may be involved in the regulation of vagal cardiomotor neurons, it should be kept in mind that within the Amb region, vagal preganglionic motoneurons are found that also innervate the pharynx, larynx, and esophagus (3). Furthermore, it is well known that the Amb region ventral to the Ambc contains secretomotor, bronchomotor, and respiratory neurons (18, 31, 32). The finding that Ambc neurons appeared to receive a less dense projection from hcrt-1-labeled axons compared with the Ambe is of some interest. However, it should be noted that vagal preganglionic neurons in the Ambc have a dendritic arborization that extends far beyond the nucleus, although most is dorsal to the nucleus (3). Therefore, because hcrt-1 axons were found within the areas immediately surrounding Ambc, it would not be unexpected to find that hcrt-1 exerts an effect on Ambc neurons, similar to that on neurons within the Ambe. This would not be an unexpected finding, because hcrt-1 is known to be involved in ingestive behaviors (16, 24, 27), of which the motor and autonomic aspects are partially under the control of Ambc neurons.

The distribution of punctate reaction product associated with the labeling of the hcrtR-1 was consistent with the distribution of hcrt-1-labeled axons and presumptive axonal terminals in the Amb region. However, in a recent study using in situ hybridization (29), no evidence of hcrtR-1 mRNA was observed within the Amb region, although a small amount mRNA of the hcrtR-2 was detected in the area of Amb. Although the reason for this discrepancy is not clear, it may be a result of the different methodological approaches used in these two studies to localize the hcrtR-1. As previously suggested by Marcus et al. (29), their data using the expression of mRNA to the hcrtR-1 may be limited to the localization of hcrtR-1 found heavily concentrated on perikarya and not in the neuropil. Only a small number of neurons was found within the Ambc that contained the hcrtR-1. In addition, the location of this punctate reaction product within the neuropil of the Ambe, as suggested previously (29), may be interpreted to indicate that hcrtR-1 in the Ambe may be localized to presynaptic axonal terminals found at some distance from the cell bodies that do not contain the receptor.

We also demonstrated in this study that microinjections of hcrt-1 into the Ambe elicited a dose-related bradycardia with little or no change in MAP. As injections of the vehicle into similar sites did not alter the HR indicates that the hcrt-1 was likely exerting an effect on hcrtR-1, a suggestion consistent with the observation of hcrtR-1 in the Amb area. This bradycardia response was shown to be mediated solely by the activation of vagal cardiomotor neurons as administration of the muscarinic receptor blocker atropine methyl...
bromide and ipsilateral vagotomy completely blocked the response. The small decrease in MAP observed on some occasions after the microinjection of the larger doses of hcr-1 into Amb sites was likely the result of a decrease in cardiac output due to the decreased HR. This suggestion is consistent with the observation of no MAP responses to injections of hcr-1 after the bradycardia response was blocked by atropine or after transection of the ipsilateral vagus nerve.

The observation in this study that stimulation of the Ambe with hcr-1 elicited a decrease in HR is consistent with the earlier findings that electrical or chemical activation of Ambe neurons evoked a bradycardia response mediated exclusively by increased parasympathetic activity (8, 31, 32). Consistent with these earlier studies, hcr-1 injections into the Ambe region did not elicit responses in MAP. This latter finding is of some significance as the Ambe overlaps with neurons belonging to the caudal ventrolateral medulla, a sympathoinhibitory area (10). Therefore, this finding, along with the observation that the HR response was due to vagal activation only, suggests that hcr-1 does not exert an effect on caudal ventrolateral medullary neurons that control the cardiovascular system, at least not at the dosages of hcr-1 used in these studies. This latter argument also applies to the rostral ventrolateral medulla, as some of the region encompassed by the Ambe overlaps this sympathoexcitatory area. However, the possibility cannot be eliminated that circulatory effects were not elicited from either the caudal or rostral ventrolateral medulla as the small volumes used in these studies did not deliver a sufficient concentration of hcr-1 to activate neurons within these sites and elicit cardiovascular responses.
This study has also demonstrated that hcr-1 activates a neuronal circuit that potentiates the reflex bradycardia to activation of arterial baroreceptors. This was not unexpected because the Amb contains the final vagal output neurons to the heart. These data suggest not only that hcr-1 is altering the activity of Amb vagal neurons to incoming baroreceptor afferent inputs, but also as hcr-1 neurons are found only within the lateral hypothalamus (12, 13, 23, 38, 49), these lateral hypothalamic hcr-1 neurons are components of a neuronal circuit involved in the control of the baroreceptor reflex at the level of the final output vagal cardiomotor neuron (1, 20, 46). Although it was beyond the scope of this study to identify the neuronal mechanisms by which hcr-1 may mediate the potentiation of the reflex vagal bradycardia to activation of baroreflex, it is possible that hcr-1 either exerted an effect directly on the vagal cardiomotor neuron or it may have altered the release of a transmitter contained inafferents from the nucleus of the solitary tract (NTS) that relay the baroreflex information to Amb neurons. Hcrt-1 has been reported to exert both a presynaptic and postsynaptic effect on central neurons (49). In addition, there has been reported that hcr-1 may alter the release of glutamate from presynaptic terminals (26, 49). As it is known that afferents from the NTS to the Amb use glutamate as their putative neurotransmitter (22), the possibility exists that hcr-1 potentiated the reflex vagal bradycardia by increasing the release of glutamate from NTS neuron axonal terminals within Amb (26, 49).

**Perspectives**

Previous studies investigating the effect of central administration of hcr-1 have shown that hcr-1 elicits sympathoexcitatory responses (2, 7, 30, 39, 40). Both intracerebroventricular and intracisternal injections of hcr-1 have been shown to elicit increases in renal sympathetic activity and catecholamine release and a long-lasting increase in MAP (7, 30, 39, 40). These effects have been suggested to be mediated by the activation of sympathetic premotor neurons located in the rostral ventrolateral medulla (7, 10), as direct injections of hcr-1 into this medullary region elicited an increase in MAP and HR (7). Furthermore, it has been shown that intrathecal injections of hcr-1 into the thoracolumbar cord elicit increases in MAP and HR, effects suggested to be mediated by activation of preganglionic sympathetic neurons in the intermediolateral cell column (2). In contrast, we recently showed that hcr-1 injections into the NTS elicit a sympathoinhibition and a vagal bradycardia (15). These findings taken together with the observations in this study that hcr-1 in Amb elicits a vagal bradycardia suggest that hcr-1 containing hypothalamic neurons are able to exert multiple and sometimes opposite functions with regard to controlling the circulation. This suggestion is not only supported by the observation that hcr-1 injections into the ventral medial medulla can either facilitate or inhibit muscle tone (33), but also by the finding that hcr-1 can influence the release of both excitatory and inhibitory neurotransmitters (49). Therefore, it is not unreasonable to suggest that under specific physiological states lateral hypothalamic hcr-1 neurons may selectively alter sympathetic or parasympathetic tone. Such a role for hypothalamic neurons is consistent with an earlier finding that the direction of arterial pressure changes during hypothalamic stimulation is influenced by the level of the resting systemic arterial pressure (20).

In addition to the role of hcr-1 in controlling the cardiovascular system, hcr-1 has been implicated in a variety of homeostatic mechanisms. Hcr-1 injections into the brain have been shown to influence feeding (16, 24) and drinking (27) behaviors, sleep and wakefulness (21), arousal (26), analgesia (4), neuroendocrine control (25, 45), gastric acid secretion (44), motor movements (33), and temperature regulation (50). The Amb, because of its prominent role in the control of not only cardiovascular function but also gastrointestinal and respiratory function, is ideally located to mediate many of the effects observed during the central injections of hcr-1. Therefore, the activation of Amb vagal cardiomotor neurons and the increase in the reflex HR response to baroreflex activation may be important components of a central mechanism that functions to adjust the cardiovascular responses during the activation of different physiological mechanisms (4, 16, 21, 24–27, 33, 44, 45, 50).

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