Cardiac Effects of Hypocretin-1 in Nucleus Ambiguus

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Although recent studies have reported hypocretin 1 (hcrt-1) like-immunoreactivity (ir) within the region of the nucleus ambiguus (Amb) in the caudal brainstem, 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 was 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 alpha-chloralose anaesthetized rats. Microinjections of hcrt-1 (0.5 - 2.5 pmol) into the Ambe 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 Ambe significantly potentiated the reflex bradycardia elicited by activation of the baroreflex as a result of the increased MAP following the iv injection of phenylephrine. These data suggest that hcrt-1 in the Ambe activates neuronal systems that alter the excitability of central circuits that reflexly control the circulation,
through the activation of vagal preganglionic cardioinhibitory neurons.

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 towards 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, 48). 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 N-terminal pyrogutalmyl residue and C-terminal amide, while hcrt-2 is a 28 amino acid peptide with a C-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, 13, 37, 48).

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, intracisternal injections of hcrt-1 have been reported to elicit a dose dependant increase in MAP and 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 have recently demonstrated that discrete injections of hcrt-1 into sites within the 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, brainstem and spinal cord structures (12, 13, 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 brainstem, 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 labelled fibers within this brainstem 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 hcrTR-1 on HR and MAP was investigated in the anaesthetized 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 hcrTR-1. Finally, the effect of microinjections of hcrTR-1 into Amb on the reflex HR response to the activation of arterial baroreceptors was also investigated in the anaesthetized rat.

**METHODS AND MATERIALS**

**General Procedure:** Experiments were done in adult male Wistar rats (250-350 g; Charles River Canada Inc., St. Constant, Canada). All animals were housed under controlled conditions with a 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 sodium pentobarbital anaesthesia (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 brainstem at 40 µm were cut in a cryostat (-17 °C; model 5030, Bright Instrument Co. Ltd., Huntington, England). For each animal, 1 in every 3 sections of the brainstem was processed immunohistochemically as previously described (11) for hcrTR-1-like-ir. An adjacent
section was processed for hcrtR-1-like-ir. In brief, brainstem sections were placed into normal goat serum (Vector Laboratories, Inc., 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 antiserum to hcrt-1 (affinity purified rabbit polyclonal anti-orexin-A; Alpha Diagnostic Intl. Inc., San Antonio, TX; Cat # OXA11-A, Lot # 305960A; Refs. 14, 38) or to the hcrtR-1 (affinity purified rabbit polyclonal anti-orexin receptor-1; Alpha Diagnostic Intl. Inc., San Antonio, TX; Cat # OX1R11-A1-A, Lot # 297980A) diluted 1:2000 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 Co., 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 rinsing the tissue in PBS, sections were mounted onto gelatinized glass slides, dried and cover-glassed. Adjacent brainstem 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 labelling 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 brainstem sections in primary hcrt-1
or hcrtR-1 antisera that had been preadsorbed with an excess of hcrt-1 or hcrtR-1 peptide (Cat. # OXA11-P or OX1R11-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 brainstem sections.

**Microinjections into Amb:** On the day of the experiments, the animals were anaesthetized with urethane (1.5 g/kg, ip; n = 16) or alpha-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 anaesthetics were used to determine whether the type of anaesthetic 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-C; American Hospital Supply Corp., 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. 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 79G).

The head of the animal was placed in a Kopf stereotaxic frame and bent downwards 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-barrelled 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 -14.0; lateral to the mid-line, 1.6 to 2.4; 
ventral to the dorsal surface, 2.4 to 3.2 ) where dense hcart-1- and hcartR-1-like- ir were observed in 
the immunohistochemical study. The microinjection of hcart-1 (0.5, 1.0 and 2.5 pmol; Phoenix 
Pharmaceuticals, Inc., 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 hcart-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 hcart-
1 in Amb:** To determine which components of the autonomic nervous system were involved in 
mediating the cardiovascular responses elicited by hcart-1 in the Amb, the cardiovascular responses 
elicited by the microinjections of hcart-1 (2.5 pmol) were re-tested after the iv injection of the 
muscarinic receptor blocker atropine methyl bromide (2 mg/kg, n=4) in urethane anaesthetized 
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 hcart-1 (2.5 pmol) were re-tested after ipsilateral vagotomy. The vagus nerve was identified 
following a mid-line incision in the neck and isolated from surrounding tissues. A silk thread was then 
placed around the nerve for the later identification of the nerve. Following the recovery of the HR
response to an injection of hcr-1 into the Ambe region, the nerve ipsilateral to the injection site was cut. Thirty min after the transection of the vagus nerve, the Ambe site was re-injected with hcr-1. Only one site in each animal was tested in these studies.

**Activation of the Baroreceptor Reflex:** To determine whether hcr-1 exerted an effect on the baroreceptor reflex, the effect of microinjections of hcr-1 (2.5 pmol) into Amb on the reflex bradycardia elicited by the increase in MAP to an iv 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 hcr-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 hcr-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 hcr-1 microinjection, emptied of the hcr-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 hcr-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 post-fixed in the 10% buffered formalin solution for 2-4 days. Frozen, transverse sections of the brainstem 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 centre of the marked injection site. All stimulation sites were mapped on projection drawings of
transverse sections of the rat brainstem 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 ± standard error of the means 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 greater than 5 mmHg or 10 bpm, 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 Bonferonni post-hoc test. The effects of hcrt-1 injections on the baroreceptor reflex were analysed 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 Figures 1 - 2. Scattered hcrt-1 labelled 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), labelled 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 labelled 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 labelled axons in the Ambe region were found to contain spine-like 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, last two sections in figure).

Relatively moderate punctate hcrtR-1-like-ir was also observed scattered throughout the Amb region. Within the Ambc, the punctate reaction product was primarily observed in association with pericarya (Figs. 3b, and 3d). 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 Ambc was found scattered throughout the neuropil of the caudal aspects of the nucleus (Figs. 1, 3b and 3d).

**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 3 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 - 2).

In the anaesthetized rat, baseline HR and MAP were found to be 400 ± 5.6 bpm 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 - 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 - 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 Ambc site. As shown by the responses to hcrt-1 injections into Ambc, 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 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 iv. 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 iv administration 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 hcrt-1 into the Ambe. As summarized in Figure 7, ipsilateral cervical vagotomy abolished the bradycardia response to hcrt-1 injections into the Ambe.

**Effect of hcrt-1 Injections Into Amb on the BR:** To investigate whether activation of Amb neurons by hcrt-1 altered the HR component of the baroreflex, changes in systemic blood pressure were used to activate arterial baroreceptors following the injection of hcrt-1 into the Ambe. As shown in the representative experiment in Figure 8a and the summarized data from 9 experiments in Figure 8b, activation of Ambe neurons by hcrt-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 about 0.5 min (Fig. 8) after the hcrt-1 injection into Ambe. By 5 min after the hcrt-1 injection into the Ambe, the potentiated reflex HR response had returned to control values (Fig. 8).

**DISCUSSION**

Lateral hypothalamic and perifornical hypothalamic hcrt 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, 13, 34, 37, 38, 48, 49). Within the brainstem, hcrt-1 ir has been previously observed within the ventral medullary reticular formation, including the region of Amb, (12, 13, 34, 37, 48). These areas of the brainstem 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 has demonstrated the existence of hcrtR mRNA within the region of the ventral medulla (29). Microinjections of hcrt-1 into the rostral ventrolateral medulla has been reported to elicit increases in MAP and HR (7). This study has now demonstrated that activation of Amb neurons by hcrt-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 labelled axons and presumptive axonal terminals are found within the Amb region is consistent with earlier observations (12, 13, 34, 37, 48). This study has also demonstrated that hcrt-1 labelling 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 hcrtr-1 may be involved in the regulation of vagal cardiomotor neurons, it should be kept in mind that within the Ambe 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 hcrtr-1 labelled axons compared to the Ambe is of some interest. However, it should be noted that vagal preganglionic neurons in the Ambc have a dendritic arborisation that extends far beyond the nucleus, although most is dorsal to the nucleus (3). Therefore, as hcrtr-1 axons were found within the areas immediately surrounding Ambc, it would not be unexpected to find that hcrtr-1 exerts an effect on Ambc neurons, similar to that on neurons within the Ambe. This would not be an unexpected finding as hcrtr-1 is known to be involved in ingestive behaviours (16, 24, 27), of which the motor and autonomic aspects are partially under the control of Amb neurons.

The distribution of punctate reaction product associated with the labelling of the hcrtrR-1 was consistent with the distribution of hcrtr-1 labelled axons and presumptive axonal terminals in the Amb region. However, in a recent study using in situ hybridization (29), no evidence of hcrtrR-1 mRNA was observed within the Amb region, although a small amount mRNA of the hcrtrR-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 hcrtrR-1. As previously suggested by Marcus et al (29), their data using the expression of mRNA to the hcrtrR-1 may be limited to the localization of hcrtrR-1 found heavily concentrated on pericarya and not in the neuropil. Only a small number of neurons were found within the Ambc that contained the hcrtrR-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 have also demonstrated in this study that microinjections of hcrt-1 into the Ambe elicit 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 Ambe region. 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 following the microinjection of the larger doses of hcrt-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 hcrt-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 hcrt-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, hcrt-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 hcrt-1 does not
exert an effect on caudal ventrolateral medullary neurons that control the cardiovascular system, at least not at the dosages of hcrt-1 used in these studies. This latter argument also applies to the rostral ventrolateral medulla, as some of the region encompassed by the Amb overlaps this sympathoexcitatory area. However, the possibility can not 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 hcrt-1 to activate neurons within these sites and elicit cardiovascular responses.

This study has also demonstrated that hcrt-1 activates a neuronal circuit that potentiates the reflex bradycardia to activation of arterial baroreceptors. This was not unexpected as the Amb contains the final vagal output neurons to the heart. These data suggest not only that hcrt-1 is altering the activity of Amb vagal neurons to incoming baroreceptor afferent inputs, but also as hcrt-1 neurons are found only within the lateral hypothalamus (12, 14, 23, 38, 48), these lateral hypothalamic hcrt-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 hcrt-1 may mediate the potentiation of the reflex vagal bradycardia to activation of baroreflex, it is possible that hcrt-1 either exerted an effect directly on the vagal cardiomotor neuron or it may have altered the release of a transmitter contained in afferents 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 (48). In addition, it has been reported that hcrt-1 may alter the release of glutamate from presynaptic terminals (26, 48). As it is known that afferents from the NTS to the Amb use glutamate as their putative neurotransmitter (22), the possibility exists that hcrt-1 potentiated the reflex vagal
bradycardia by increasing the release of glutamate from NTS neuron axonal terminals within Amb (26, 48).

**Perspectives:**

Previous studies investigating the effect of central administration of hcr-t-1 have shown that hcr-t-1 elicits sympathoexcitatory responses (2, 7, 30, 39, 40). Both intracerebroventricular and intracisternal injections of hcr-t-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-t-1 into this medullary region elicited an increase in MAP and HR (7). Furthermore, it has been shown that intrathecal injections of hcr-t-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 have recently shown that hcr-t-1 injections into the nucleus tractus solitarius elicits a sympathoinhibition and a vagal bradycardia (15). These findings taken together with the observations in this study that hcr-t-1 in Amb elicits a vagal bradycardia suggests that hcr-t-1 containing hypothalamic neurons are able to exert multiple and sometimes opposite functions with regards to controlling the circulation. This suggestion is not only supported by the observation that hcr-t-1 injections into the ventral medial medulla can either facilitate or inhibit muscle tone (33), but also by the finding that hcr-t-1 can influence the release of both excitatory and inhibitory neurotransmitters (48). Therefore, it is not unreasonable to suggest that under specific physiological states lateral hypothalamic hcr-t-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 hCrt-1 role in controlling the cardiovascular system, hCrt-1 has been implicated in variety of homeostatic mechanisms. Hcrt-1 injections into the brain have been shown to influence feeding (16, 24) and drinking (27) behaviours, 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 hCrt-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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Figure Legends

Figure 1:

Series of projection drawings of transverse sections of the ventral medulla through the region of the
caudal nucleus ambiguus (Amb) of the rat showing the location of hcr-t-1 labelled axons (a-c) and the
distribution of punctate reaction product (small dots) representing the hcrtR-1 (d-f). Note the dense
innervation by hcr-t-1 axons around the compact formation of Amb (Ambc) and within the external
formation of Amb (Amb e), an area which overlaps the caudal ventrolateral medulla (a-c). In addition,
ote note that dense hcrt-R-1 immunoreactivity was found in the same Amb regions. Numbers in each
section indicate the rostrocaudal coordinates from bregma. Abbreviations: 5sp, spinal trigeminal
nucleus; Ambc, nucleus ambiguus, compact formation; Ambe, nucleus ambiguus, external formation;
MdV, medullary reticular nucleus, ventral division; ION, inferior allover nucleus; LRN, lateral
reticular nucleus; Py, pyramidal tract. Calibration mark, 1 mm.

Figure 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 hcr-t-1 labelled axons (a-c) and the distribution of
punctate reaction product (small dots) representing the hcrtR-1 (d-f). Note that the density of hcr-t-1
and hcrtR-1 immunoreactivity was less throughout the rostral Amb area compared to the caudal Amb
areas. However, the hcr-t-1 axons and hcrtR-1 labelling was still localized mostly within the Ambe.
Note also the little amount of hcr-t-1 labelling found with the LPGi and the rostral ventrolateral
medullary region. Abbreviations: Gi, nucleus gigantocellularis; GiA, nucleus gigantocellularis pars
alpha; LPGi, nucleus paragigantocellularis lateralis. See Fig. 1 for additional abbreviations.
Calibration mark, 1 mm.

Figure 3:
Bright-field photomicrographs showing hcrt-1 labelled axons (a and c), punctate reaction product representing hcrtR-1 (b and d) within the neuropil (open arrows; b and d) and associated with pericarya (filled arrows; b and d) in the Amb region. Note in (a) the scattered hcrt-1 labelled axons throughout the Ambe region (open arrow points to labelled axon shown in (c)). (b) shows 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) shows an hcrt-1 labelled axon and spine-like processes on the axon (open arrows). (d) shows hcrtR-1 labelling on cell bodies (filled arrows) and in the neuropil (open arrows) in the Ambc. Refer to Fig. 1 for list of abbreviations. Calibration mark in (a) represents 100µm. The same calibration mark in (a) represents 50 µm in (b) and 25 µm in (c) and (d).

Figure 4:
(a), a series of drawings of transverse sections of the ventral medulla through the region of the Amb modified from a stereotaxic atlas of the rat brain (43) extending from approximately -13.3 to -13.7 mm caudal to bregma showing the location of sites (dots) that were microinjected with 2.5 pmol of hcrt-1. (●), sites eliciting decreases in heart rate (HR); (●), sites eliciting no changes in HR. Note that most of the sites that elicited decreases in HR were found within the ventral aspect of the Ambe. Note that injections into sites dorsal and lateral to the Ambe did not elicit HR responses. (b), a bright-field photomicrograph at approximately -13.7 caudal to bregma showing the location of a Pontamine sky blue deposit in the Ambe (open arrow) corresponding to a site at which hcrt-1 elicited a vagal bradycardia response. Refer to Fig. 1 for additional list of abbreviations. Calibration mark, 1 mm in
(a) and 0.5 mm in (b).

Figure 5:
Representative tracings of heart rate (HR) and arterial pressure (AP) responses elicited by microinjections of different amounts (0.5, 1.0 and 2.5 pmol) of hcrt-1 into a site in the Ambe. Note that hcrt-1 elicited a decrease in HR, with little associated change in AP, except for an occasional decrease at the higher dosages of hcrt-1. Arrows indicates time of microinjections. Calibration mark, 0.5 min.

Figure 6:
Bar chart showing effects of microinjections of different dosages of hcrt-1 (0.5, 1.0 and 2.5 pmol) and the vehicle saline into the Amb on mean arterial pressure (MAP; open bars) and heart rate (HR; cross-hatched bars). Note that hcrt-1 did not elicit significant changes in MAP at all dosages tested. On the other hand, hcrt-1 microinjections elicited a dose-related decrease in HR. Note that injections of similar volumes of the saline vehicle into these Amb sites did not elicit cardiovascular responses. Numbers in parentheses represent number of sites injected. Bars with the same letters are not significantly different from each other, whereas bars with different letters are significantly different (p < 0.05) from each other.

Figure 7:
Bar charts showing the effect of muscarinic receptor blockade (upper panel) or ipsilateral vagotomy (lower panel) on the heart rate (HR) and mean arterial pressure (MAP) responses to hcrt-1
microinjections into the Ambe. Note that both the intravenous administration of atropine methyl bromide or transection of the ipsilateral vagus completely blocked the HR response. *, p < 0.05 compared to before (control) value. n = the number of animals tested.

Figure 8:
(a), shows representative tracings of arterial pressure (AP) and heart rate (HR) responses elicited by iv injections of phenylephrine (solid arrows) before (control) and after the injection of hcrt-1 (2.5 pmol; open arrow) into the Ambe. Note that the magnitude of reflex bradycardia is potentiated from 0.5 to 2.5 min after hcrt-1 microinjections into Ambe, and returns to control levels at about 5 min after the hcrt-1 microinjection. Calibration mark, 1 min. (b), is a graph showing the effect of hcrt-1 microinjection (2.5 pmol) into the Ambe on the reflex bradycardia elicited by activation of arterial baroreceptors as a result of the increase in mean arterial pressure (MAP) after iv injections of phenylephrine. Note that the reflex vagal bradycardia is significantly potentiated at both 0.5 and 2.5 min (p < 0.05) after the microinjection of hcrt-1 into the Ambe. The reflex HR response returns to control values by 5.0 min after the hcrt-1 injection. n = number of responses used to calculate each point plotted on graph. All values are means ± S. E.. Control (○, 5 min before hcrt-1 injection, R² = 0.9808). Time after hcrt-1 microinjection into Ambe: 0.5 min (■, R² = 0.7691), 2.5 min (▲, R² = 0.8507), 5.0 min (×, R² = 0.7967).
HR (bpm)

AP (mmHg)

0.5 pmol

1.0 pmol

2.5 pmol

500

250

175

50

↑

↑

↑
Atropine Methyl Bromide

Before After

n= 4

Change in MAP (mmHg)

Change in HR (bpm)

MAP HR

Ipsilateral Vagotomy

Before After

n= 6

Change in MAP (mmHg)

Change in HR (bpm)