

# Mediation of Vagal Cardioinhibitory Responses by Glutamatergic Receptors in the Caudal Medulla of Turtles

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## Abstract

Our previous studies showed that electrical stimulation of the nuclei ambiguus (NA) or dorsomotor nuclei of the vagus (DMV) complex in the brain stem of spontaneously breathing pond turtles (*Cyclemys faviomarginata*), anesthetized with chloralose (4 mg/100 g) and urethane (40 mg/100 g), produced a marked slowing or even cessation of the heart rate, and resulted in an immediate fall of blood pressure. Results of the present study further demonstrated that the cardioinhibitory responses could also be elicited by microinjection of monosodium glutamate (0.2-20 nl, 50 mM) into the NA/DMV complex in turtles. A two-barrel glass micropipette held in a manipulator was connected to a pneumatic pressure pump for microinjection. The glutamate-induced cardioinhibitory responses could be significantly reduced in a dose-dependent manner by pretreatment with AP-5 (a NMDA receptor antagonist, at 1-8 nmole) or CNQX (a non-NMDA receptor antagonist; at 0.1-0.8 nmole) 20 min before glutamate administration. Histochemical verification by injecting horseradish peroxidase into the cervical vagus nerves revealed that retrogradely labeled glutamatergic neurons in the NA/DMV complex were observed. These results suggest that glutamatergic receptors in the caudal medulla may mediate vagal cardioinhibitory responses in the turtle.

**Key Words:** nuclei of the ambiguous, dorsomotor nuclei of the vagus, bradycardia, glutamate, N-methyl-D-aspartate, 6-cyano-7-nitroquinoxaline, 2,3-dione, D-2-amino-5-phosphonovalerate

## Introduction

In mammals, cardiac arrest and resultant anoxia following vagal stimulation last for only a short period before resumption of heart beat (3). However, this vagal escape phenomenon in response to continuous vagal stimulation has been observed to occur for more than an hour in the turtle (6, 10). In order to explore the important role of vagal bradycardia mechanism in the brain stem of turtles, cardiovascular parameters of spontaneously breathing pond turtles (*Cyclemys*

*faviomarginata*), anesthetized with chloralose and urethane, were examined during exploratory electrical stimulation of the brain stem (7). Stimulation of areas of the nucleus ambiguus (NA), solitary and dorsomotor nuclei of the vagus (DMV) in the caudal medulla produced a marked slowing or even cessation of heart beat, and, thus, resulted in an immediate fall of blood pressure even down to zero. This cardioinhibitory response may depend on the integrity of the vagus nerves. The problem is that electrical stimulation may excite both neural perikarya and fibers of passage.

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Whether this cardioinhibition is caused by electric stimulation delivery to the NA and DMV remains to be determined. To our knowledge, it is unknown that the caudal medulla of turtles contains neural perikarya to mediate cardioinhibitory responses.

The present study included three aims. First, we attempted to ascertain whether cardioinhibition produced by electrical stimulation of the caudal medulla could be mimicked by microinjection of glutamate (Glu) which, unlike electrical stimulation, excites neural perikarya (2). Second, horseradish peroxidase (HRP) was injected into the cervical vagus nerve to detect the retrogradely labeled neurons in the caudal medulla. Third, we investigated whether cardioinhibition elicited by Glu, if any, could be antagonized by a N-methyl-D-aspartate (NMDA) receptor antagonist, D-2-amino-5-phosphonovalerate (AP-5), and/or by a non-NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (4).

## Materials and Methods

### *Animal Preparation*

Adult turtles (*Ocadia sinensis*) of either sex, weighing 1.0-1.5 kg, were used. In all cases, anesthesia was achieved by immersion of the animal in an ice bath for about 1 h for sedation followed by an injection of sodium pentobarbital (15-25 mg/kg) through a cannula in the right jugular vein. Details of the experimental procedure have previously been described (7). Briefly, these steps included cannulation of the right cervical artery for monitoring systemic arterial pressure (SAP) and mean SAP (MSAP) via a pressure transducer, and heart rate (HR) was monitored by a tachography that was triggered by the SAP wave. The SAP, MSAP and HR data were simultaneously recorded on a polygraph (TA11, Gould Valley View, OH, USA). Tracheal intubation was applied for artificial ventilation and end-tidal CO<sub>2</sub> concentration was maintained at 3.5-5% by adjusting the frequency and volume of the ventilator. The body temperature was maintained at 25°C by using a heating lamp.

### *HRP-Tetramethyl-Benzidine Method*

In three turtles anesthetized with sodium pentobarbital (15-25 mg/kg, i.v.), a 20% aqueous solution of HRP (Sigma VI, Sigma Chemical, Saint Louis, MO, USA) was injected into the cervical vagal nerves to trace the location(s) of the soma. After a survival period of 36-48 h, each animal was perfused transcardially first with physiological saline at room temperature, followed by 1.25% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer at pH 7.3 at room temperature, and finally with 10%

sucrose in the same buffer but at 4°C. After the perfusion, the brain was removed and placed in a sucrose-buffer solution at 4°C for 12-24 h, and then a section of 50-μm thickness was made. The sections were incubated according to the tetramethyl benzidine (TMB) procedure of Mesulam (9). Some sections were then counterstained with cresyl violet.

### *Drug Administration*

The head of each animal was positioned in a David-Kopf stereotoxic apparatus with a modified mouthpiece. The dorsal surface of the brain stem was exposed and the obex was used as the reference point. A two-barrel glass micropipette (outside tip diameter of 1.2 mm), one filled with Glu (50 mM, pH8.0 in 0.6 % saline; Sigma) and the other filled with AP-5 (1-8 nmole; Sigma) and/or CNQX (0.1-0.8 nmole; Sigma), was connected to a pneumatic pressure pump (PPS-2, PPM-2, Medical System Corp., Great Neck, NY, USA) for microinjection. The agent was mixed with 1% pontamine sky blue, and the microinjection volume was measured by monitoring the movement of the fluid meniscus in the micropipette through a stereomicroscope (13).

### *Experimental Protocols*

In experiment 1, the microinjection of various volumes (0.2-20 nl) of Glu (glu, 50 mM) into the NA and DMV was performed in 6 animals and the responses of SAP, MSAP and HR to the Glu microinjection was evaluated at a time interval of 0, 20 and 60 min. In this study, we were able to determine the optimal concentration of Glu producing the highest effect of cardioinhibition.

In experiment 2, decreases in SAP, MSAP and HR induced by Glu microinjection (50 mM in 20 nl) into the NA/DMV complex in 6 turtles at 0, 20 and 60 min were evaluated after pretreatment of microinjection of AP-5 (1-8 nmoles) to examine whether Glu-induced cardioinhibition was mediated through NMDA receptors.

In experiments 3, diminishes of SAP, MSAP and HR elicited by Glu microinjection (50 mM in 20 nl) into the NA/DMV complex in 6 turtles were again performed at the same interval of 0, 20 and 60 min before and after a pretreatment of microinjection of CNQX (0.1-0.8 nmoles). This experiment was performed to determine if non-NMDA receptors might be involved in cardioinhibition caused by Glu.

In experiment 4, injection of HRP into cervical vagus nerves was performed to trace whether the retrogradely labeled neurons could be observed in the right NA/DMN complex of turtle brainstem. This study was attempted to localize the areas that produced cardioinhibition in the caudal medulla.

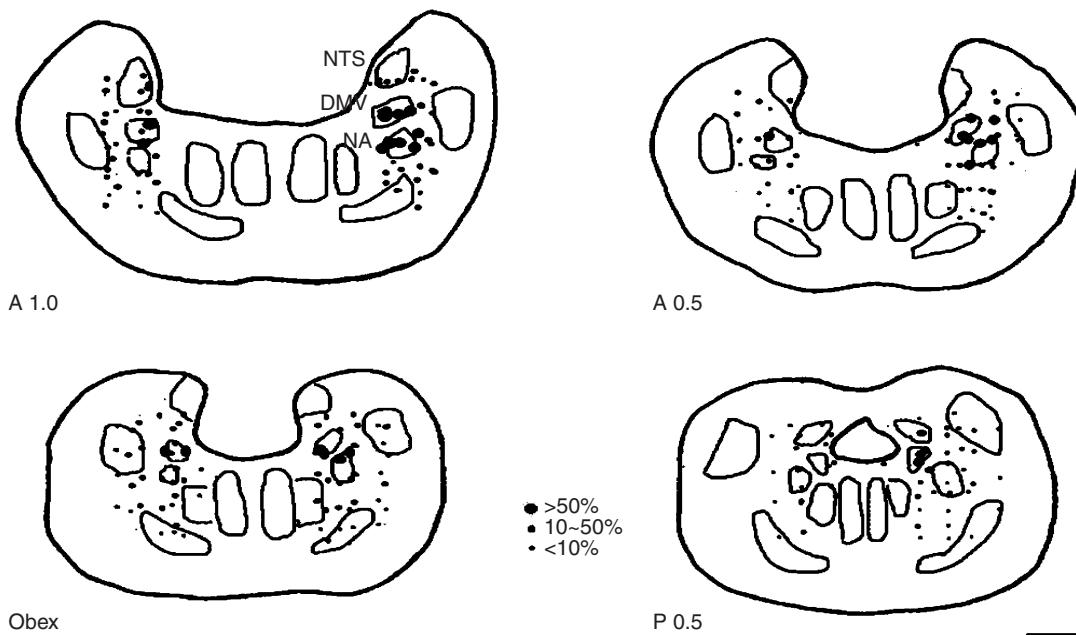


Fig. 1. Line drawings of transverse sections through turtle caudal medulla illustrating the location and relative bradycardia responses to microinjection of glutamate (Glu). Dots of large, medium and small sizes denote the Glu-induced bradycardia changes of > 50%, 10-50% and < 10%, respectively. Sites of chemical stimulation in this figure are indicated as solid dots in the diagram. The Glu-induced bradycardia responses in the right medulla ( $-30 \pm 10$  bpm) were significantly greater than those of the left medulla ( $-19 \pm 2$  bpm). Scale bar, 1 mm.

#### Data and Statistical Analysis

Data were analyzed from the polygraph tracings. All data were expressed as means  $\pm$  standard error of mean (SEM) and were then analyzed by a repeated measurements of analysis of variance (ANOVA) followed by a posteriori test of modified *t*-test with the SPSS software (version 10, spss statistics, IBM company, Chicago, IL, USA) for multiple comparisons between means. A  $P < 0.05$  denotes statistical significance.

#### Results

##### Mapping the Bradycardia Sites in the Turtle Brain Stem by Chemical Stimulation

The anesthetized turtles ( $N = 13$ ), breathing spontaneously at room temperature, had an average MSAP of  $25.8 \pm 1.1$  mmHg (= 133.3 Pa) and HR of  $23.9 \pm 1.2$  bpm (beat/min). As shown in Fig. 1, microinjections of Glu (50 mM in 20 nl) were made into 23 sites on each side of the brain stem. The microinjection sites were localized at a range of 1 mm (A1.0) and 0.5 mm (A0.5) anterior to, and 0.5 mm (P0.5) posterior to, the obex and with a depth between 2 and 3 mm. The microinjection points within both the NA and DMV as shown in Fig. 1 produced a significant decrease in HR and, thus, had a higher sensitivity

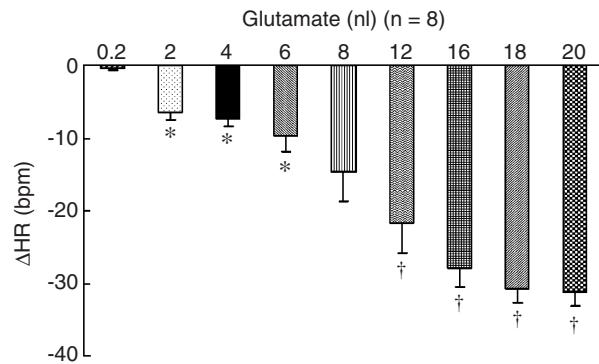


Fig. 2. Mean values ( $\pm$  SEM) of the decreases in heart rate (HR) with Glu (50 mM in 0.2-20 nl) microinjection into the NA/DMV complex of 8 turtles. \* $P < 0.05$  in comparison with the Glu 0.2 nl group; † $P < 0.05$  in comparison with the Glu 6 nl group.

in response to Glu stimulation as compared with the other microinjection points outside the NA/DMV complex. In addition, microinjection of Glu (50 mM) into the NA/DMV complex of the right medulla oblongata produced a much higher degree of bradycardia than those of the left medulla ( $-30 \pm 1$  bpm vs.  $-19 \pm 2$  bpm). Microinjection of Glu into the NA/DMV complex of the right medulla with a dose range of 0.2-20 nl could produce a dose-dependent bradycardia and hypotension as is shown in both Figs. 2 and 3.

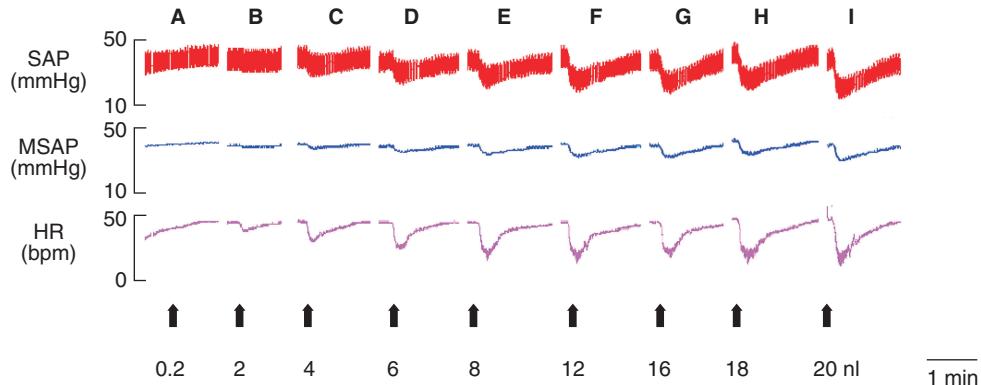


Fig. 3. Microinjection of Glu (50 mM in 0.2-20 nl of Glu) into the NA/DMV complex of a turtle brain producing decreases in systemic arterial pressure (SAP), mean SAP (MSAP) and heart rate (HR).

#### *Attenuation of Glutamate-Induced Bradycardia by Pretreatment with AP-5*

To determine whether Glu-induced bradycardia could be attenuated by pretreatment of AP-5, an NMDA receptor antagonist, we selected the dose of Glu that produced the highest decrease of HR in the following study. Fig. 4 summarizes the bradycardia response to microinjection of Glu (50 mM) into NA/DMV complex in 5 turtles 20 min after pretreatment of AP-5 microinjection (1-8 nmoles) and in 5 turtles 60 min after AP-5 microinjection (1-8 nmoles), as well as in 5 turtles 20 min after vehicle injection. It is evident that the Glu-induced bradycardia was attenuated by pretreatment of AP-5 microinjection 20 min before Glu but not by AP-5 pretreatment 60 min before Glu microinjection. A typical example of AP-5 producing attenuation of bradycardia response to Glu microinjection is depicted in Fig. 5.

#### *Attenuation of Glutamate-Induced Bradycardia by Pretreatment with CNQX*

Figure 6 summarizes the bradycardia response to microinjection of Glu (50 mM) into the NA/DMV complex in 4 turtles 20 min after CNQX injection (0.1-0.8 nmoles), in 4 turtles 60 min after CNQX injection, and in 4 turtles 20 min after vehicle injection. Again, the Glu-induced bradycardia was attenuated by CNQX pretreatment 20-60 min before Glu injection in a dose-dependent manner. A typical example of CNQX producing attenuation of bradycardia response to Glu microinjection is depicted in Fig. 7.

#### *Localization of Retrogradely Labeled Neurons in Right NA/DMV Complex by Injection of HRP into the Jugular Vagal Nerve*

Figure 8 depicts the photomicrographs of the

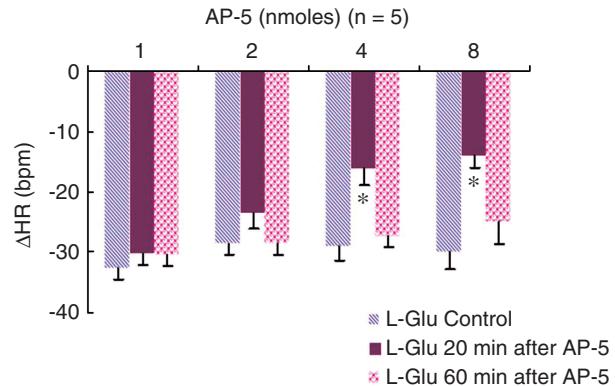


Fig. 4. Mean values ( $\pm$  SEM) of decreases in heart rate ( $\Delta$ HR) with Glu (50 mM in 20 nl) microinjection into the NA/DMV complex before and after AP-5 microinjection. Bradycardia induced by Glu microinjection (▨) was significantly reduced by 20-min pretreatment (■) but not by 60-min pretreatment (▨) of AP-5 microinjection. \* $P < 0.05$  in comparison with the vehicle controls. Animals observed ( $N = 5$ ).

retrogradely labeled neurons in the right NA/DMV complex caudal to obex after injecting HRP into the jugular vagal nerve. The results show that the location of the HRP-labeled neurons in the NA/DMV complex corresponds to the Glu-injected sites.

## Discussion

The main finding of the present study was that Glu-induced activation of neurons located in the NA/DMV complex of the turtle could produce a significant decrease both in HR and SAP. This bradycardia response could be substantially attenuated by NMDA and non-NMDA receptor antagonists suggesting that Glu ionotropic receptors may be involved in the vagal escape phenomenon in turtles. In our previous study (7), cardiovascular responses of spontaneously

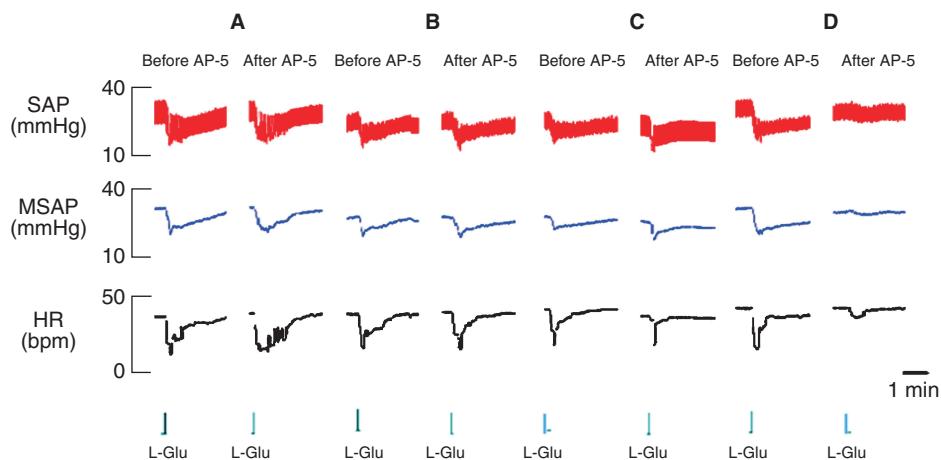


Fig. 5. Effects on SAP, MSAP, and HR after microinjection of Glu (50 mM in 20 nl) into DA/DMV complex of a turtle treated with microinjection of AP-5 [1 nmole (A), 2 nmoles (B), 4 nmoles (C), and 8 nmole (D)] 20 min before Glu injection.

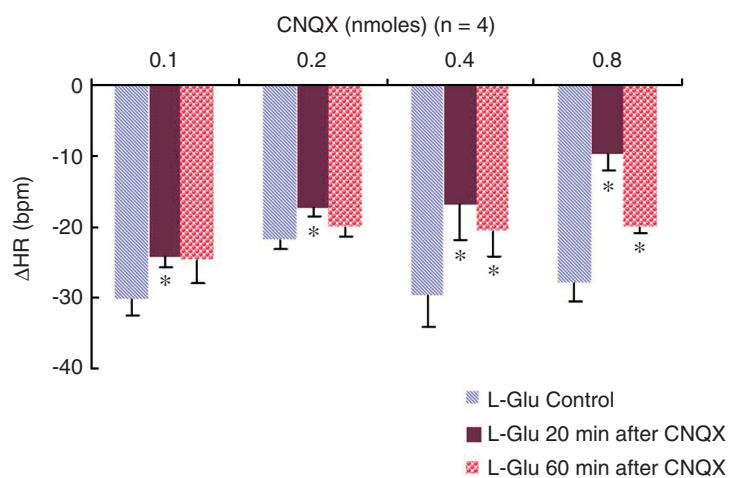


Fig. 6. Effects of microinjection of Glu (50 mM in 20 nl) into the NA/DMV complex on heart rate changes ( $\Delta$ HR) in 4 turtles treated with vehicle controls 20 min before Glu injection (□), in 4 turtles treated with CNQX (0.1-0.8 nmoles) 20 min before Glu injection (■), and in 4 turtles treated with CNQX (0.1-0.8 nmoles) 60 min before Glu injection (▨). The values are expressed as means  $\pm$  SEM of 5 animals per group. \* $P$  < 0.05 in comparison with vehicle controls.

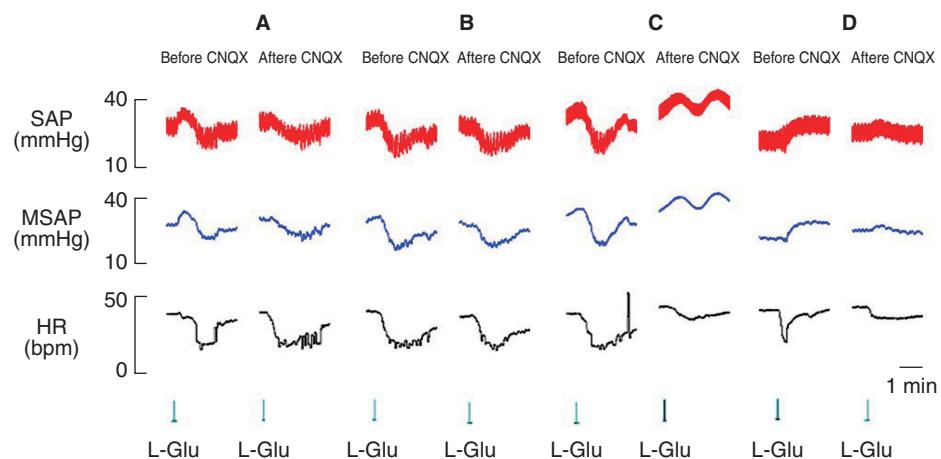


Fig. 7. Effects on SAP, MSAP, and HR after microinjection of Glu (50 mM in 20 nl) into the NA/DMV complex of a turtle treated with microinjection of CNQX [0.1 nmoles (A), 0.2 nmoles (B), 0.4 nmoles (C), and 0.8 nmoles (D)] 20 min before Glu injection.

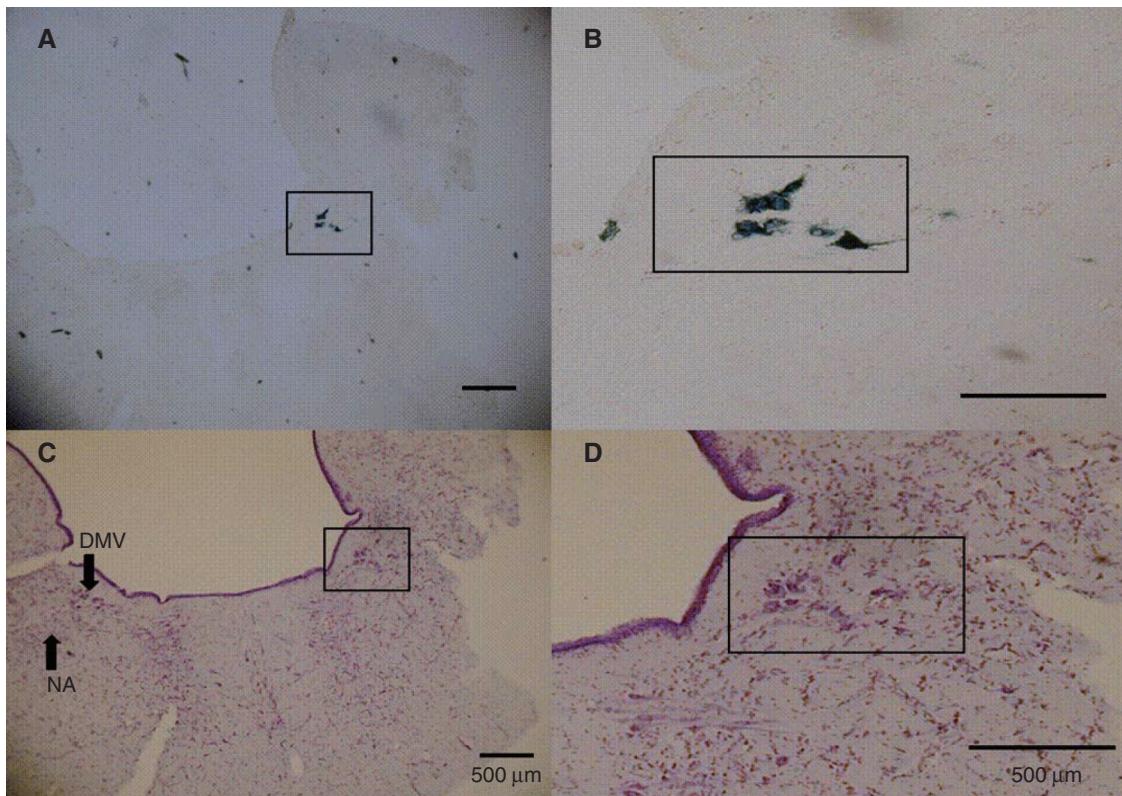


Fig. 8. Photomicrographs showing the retrogradely labeled neurons in the right NA/DMV complex by injecting HRP into the right jugular nerve (A). Magnified view of (A) is depicted in (B). Retrogradely labeled neurons in the right NA/DMV stained by cresyl violet are depicted in (C) and magnified in (D). Scale bar, 500  $\mu$ m.

breathing pond turtles to electrical stimulation of different parts of brain stem were performed. Upon stimulation, pressor (sympathetic), depressor (sympathetic inhibition), and bradycardia and hypotensive responses were elicited from various regions of the brain stem extending from the medulla oblongata to the hypothalamus. Stimulation of the caudal medulla including the NA and DMV produced a marked slowing or even cessation of the heart beat, and thus resulted in an immediate fall of the blood pressure even down to zero. This cardioinhibition depended largely on the integrity of the vagus nerves. The present study provides additional evidence to indicate that neurons in the NA/DMV complex of turtle brain stem may mediate this cardioinhibitory effect. Our current results show that microinjection of Glu into the NA/DMV complex produced cardioinhibitory responses in a dose-dependent way. Glu, unlike electrical stimulation, excites mainly neural perikarya, and not fibers of passage or axons. Furthermore, histological verification confirmed that retrogradely labeled neurons of the jugular vagus nerves was traced and localized in the NA/DMV complex which was the area receiving Glu microinjection. Specifically, the Glu-induced vagal cardioinhibition could be significantly antago-

nized by a pretreatment of non-NMDA receptor antagonist and/or a NMDA receptor antagonist. These results strongly indicate that Glu NMDA and non-NMDA receptors in the turtle caudal medulla may mediate vagal cardioinhibitory responses.

In the turtle, the heart beat may stop for more than an hour in response to continuous vagal nerve stimulation (6, 10), which is quite different from mammals (1, 3). The sustained cardiac arrest induced by vagal stimulation was attributed to the low concentration of catecholamines in turtle's heart (5). Our previous study demonstrated that epinephrine given intravenously produced an increase of MSAP and increased force of cardiac contraction, and that reflex bradycardia induced by vagal stimulation in the turtle was, therefore, not observed (7). Taken together, these observations show that a very prominent vagally-mediated bradycardia can be evoked by the activation of neurons located in the turtle brain stem, specifically in the areas of the NA/DMV complex which may contribute to its well-known capacity for tolerating anoxia during diving.

Our current data demonstrated that the bradycardia induced by Glu microinjection into the right NA/DMV complex was predominant over the

activation of the left side ( $-30 \pm 1$  bpm vs.  $-19 \pm 2$  bpm). This is a very interesting data and correlates well with our previous study (7). In our previous experiments (7), the peripheral cut-end of the vagus nerve was stimulated electrically to study the escape phenomenon. It was found the cardiac arrest was prolonged when electric stimulation was delivered to the right vagus ( $30 \pm 2$  min) compared with the left vagus ( $13 \pm 1$  min). These observations strongly suggest that a lateralization of vagal escape may have existed in the caudal medulla of turtle's brain.

Data showing that microinjection of Glu into the NA and DMV of turtle's brain stem produced bradycardia may also indicate that there might be a neural pathway or input projecting to the NA/DMV complex to participate in the modulation of neuronal activity and/or HR. The NA/DMV complex in mammals is, indeed, the preganglionic parasympathetic neurons which have been demonstrated to be silent instead of being active in the *in vitro* and *in vivo* studies (12). Thus, it must be modulated or even activated by peripheral inputs, such as baroreceptors and chemoreceptors, and by central resources, e.g. rostral ventrolateral medulla and/or hypothalamus (8). Indeed, the tonic firing and reflex control of preganglionic parasympathetic cardiac neurons in the brain stem has been shown to modulate the normal HR and respiratory sinus arrhythmia present in healthy mammals (12). These neurons, like those in the turtles, are also primarily located in the NA and the DMV and believed to be dominant in the neural control of HR under normal conditions and to exert influence on the prognosis of many cardiovascular disorders such as sudden cardiac death, ventricular fibrillation and myocardial ischemia.

One of the important innervating vagal cardiac neurons is originated from the neurons of the solitary tract nucleus (NTS) in mammals (8). Neurons located in the NTS receive information of the peripheral inputs of baroreflex and chemoreflex and may then relay information via neural projection pathway to the other brain nuclei in the brain stem. This is, indeed, critical for efferent sympathetic and parasympathetic activities including the NA. In this regard, Wang *et al.* (12) have found that stimulation of the NTS may activate excitatory NMDA and non-NMDA receptor-mediated postsynaptic currents in cardiac vagal neurons in the NA. They further demonstrated that the NMDA antagonist AP-5 blocked the long-lasting component of the synaptic response and that most cardiac vagal neurons possessed both AMPA and kainite receptors which could be blocked by CNQX. This direct projection from the NTS to the NA having NMDA and non-NMDA receptors located in the postsynaptic NA neurons has also been demonstrated by Neff *et al.* (11). Wang *et al.* (12) have

suggest that this pathway, projecting from NTS to cardiac vagal neurons in the NA and/or DMV, may constitute cardioinhibition during increase in blood pressure in rats. Based on our present data and results of Wang *et al.* (12), turtles may have the same cardioinhibitory mechanisms common with mammals in such a way that Glu, released upon activation of the NTS or other nucleus in the central nervous system, may activate NMDA, AMPA and kainite receptors in cardiac vagal neurons located in the NA and/or DMV to modulate HR specifically to produce vagal escape during diving.

In summary, our data demonstrated that cardioinhibition could be elicited by microinjection of Glu into the NA and/or DMV in turtles. The cardioinhibition induced by Glu could be significantly reduced by pretreatment of AP-5 and CNQX. These results strongly suggest that Glu NMDA and non-NMDA receptors may have been activated by an unknown input pathway in the turtle under physiological conditions such as diving reflex.

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