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An *In Vitro* Brainstem-Heart Preparation of the Neonatal Rat with Intact Right Vagus Nerve

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Abstract: An *in vitro* brainstem preparation of the neonatal rat with intact right vagal (X) innervation of the right atrium, and intact medullary roots of the left X and glossopharyngeal (IX) nerves for stimulation, was developed. The preparation was continuously superfused with artificial CSF at 25°C. The electrical activity of the right atrium was recorded to determine the heart rate. Applications of atropine or propranolol to the superfusate did not alter the heart rate. Electrical stimulation (0.5 ms pulse, 20 Hz) of the left IX and X afferents elicited a reduction in the heart rate from 70.3±13.2 to 50.6±13.2 beats/min (mean±SD, $p<0.05$), which was abolished after division of the right X or application of atropine to the superfusing solution. A similar reflex bradycardia was seen in a preparation with intact left vagal-right atrium innervation during right IX and X afferent stimulation. Cervical spinal cord transection affected neither the baseline heart rate nor the magnitude of the reflex bradycardia. Longitudinal sectioning of the medulla oblongata in the mid-line down to the level of the posterior inferior cerebellar artery abolished the heart rate response. After bilateral cervical vagotomies, electrical stimulation (0.5 ms pulse, 20 Hz, up to 100 μA) of the ventrolateral medulla oblongata, lateral funiculus at C_2 or intermediate nucleus of the spinal cord at Th_{1-4} did not affect the heart rate. These results indicate that the functions in the lower brainstem are preserved in this preparation, at least in regard to the generation of reflex bradycardia. The results also suggest that the laterality of cardiac vagal innervation and sympathetic innervation will develop during the postnatal period. This preparation may be useful for the study of the central neuronal network controlling the heart rate. [Japanese Journal of Physiology, 47, 443–448, 1997]

Key words: *in vitro* brainstem-heart preparation, neonatal rat, autonomic nervous control of the heart, reflex bradycardia.

It is well established that activation of the X or IX afferent fibers arising from various cardiopulmonary and pharyngo-laryngeal areas elicits a reflex bradycardia [1–3]. The neural networks for this reflex including afferent pathways to the medulla, medullary integration, and efferent outflows to the heart have been clarified by many previous studies using intact adult animals [1–3]. However, further study on the detailed components of the reflex pathway becomes technically difficult and less examined because of external influences such as age, level of anesthesia, and mechanical interference due to respiratory and/or cardiac movement. It is true that isolated organ system preparations or tissue cell culture techniques can exclude these influences, but a major drawback of using an isolated preparation is that the analysis of the system function is inadequate. To overcome this shortcoming, an isolated preparation approach which uses multiple organ systems and includes some of the major control system components has been successfully developed.

The brainstem-spinal cord preparation obtained from neonatal rat has been widely used in experiments analyzing motor control, particularly of respiration [4, 5]. We adopted this preparation for the study of auto-

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nomic cardiovascular control and developed an *in vitro* brainstem-heart (right atrium) preparation with the right vagus nerve intact. We confirmed the viability of this preparation and analyzed the heart rate response evoked by electrical stimulation of the contralateral IX and X afferents. Our results support the hypothesis that, in neonatal rat, vagal inhibitory outflow plays a dominant role in reflex bradycardia induced by stimulation of the IX and/or X afferents.

MATERIALS AND METHODS

General. Twenty-six neonatal rats (Wistar strain; 0–4 d old) were used. The animals were deeply anesthetized with ether and decerebrated at the mid-collicular level. After dorsal laminectomy and transection of the body just below the diaphragm, animals were transferred into a dissecting chamber at 10–20°C. Under a dissecting microscope, the brainstem and cervical cord were isolated within 3 min, and the left IX and X nerves were dissected intracranially. To improve oxygenation, the ventral surface of the medulla oblongata was widely exposed. The thorax and its contents, except the right atrium, and tissues surrounding the right X nerve and cardiac nerve plexus were removed. The right cervical and thoracic vagus were carefully separated from the trachea and surrounding connective tissues. The preparation included the lower brainstem (medulla and caudal pons, rostral end just above the roots of the VIth cranial nerve), spinal cord (caudal end at C_6), intact right vagus nerve, and right atrium (Fig. 1A). The preparation was mounted in a recording chamber (volume, 2 ml) and continuously superfused at a rate of 5–10 ml/min with an artificial CSF (aCSF: mM composition; NaCl, 125; KCl, 4.0; NaH_2PO_4, 0.5; $CaCl_2$, 2.0; $MgSO_4$, 1.0; $NaHCO_3$, 26; glucose, 30). The solution was equilibrated with 2% CO_2 and 98% O_2 at 25–26°C and had a pH of 7.8. A rather higher pH value of aCSF was used because of the low temperature [6]. The preparation was left stabilized for at least 30 min.

To examine the laterality of the efferent vagal output, the right IX and X roots were stimulated in 4 preparations with an intact left X innervation to the right atrium. To examine the integrity of the cardiac sympathetic innervation and its contribution to the reflex bradycardia, the preparation included lower brainstem, spinal cord with the caudal end of Th_{12}, right atrium, and cervical and thoracic sympathetic ganglia and their surrounding tissues ($n=3$). In these experiments, special care was taken not to damage the cardiac sympathetic fibers innervating the right atrium. Spinalization between C_1 and C_2 or right vagotomy was performed in 3 experiments. In 2 experiments, longitudinal sectioning of the medulla oblongata at the mid-line from the rostral end of the preparation to the level of the anterior inferior cerebellar artery and further caudal to the level of posterior inferior cerebellar artery were performed using a microscissor (Fig. 1B).

Pharmacological test. The following pharmacological agents (i.e., adrenaline, propranolol, and at-

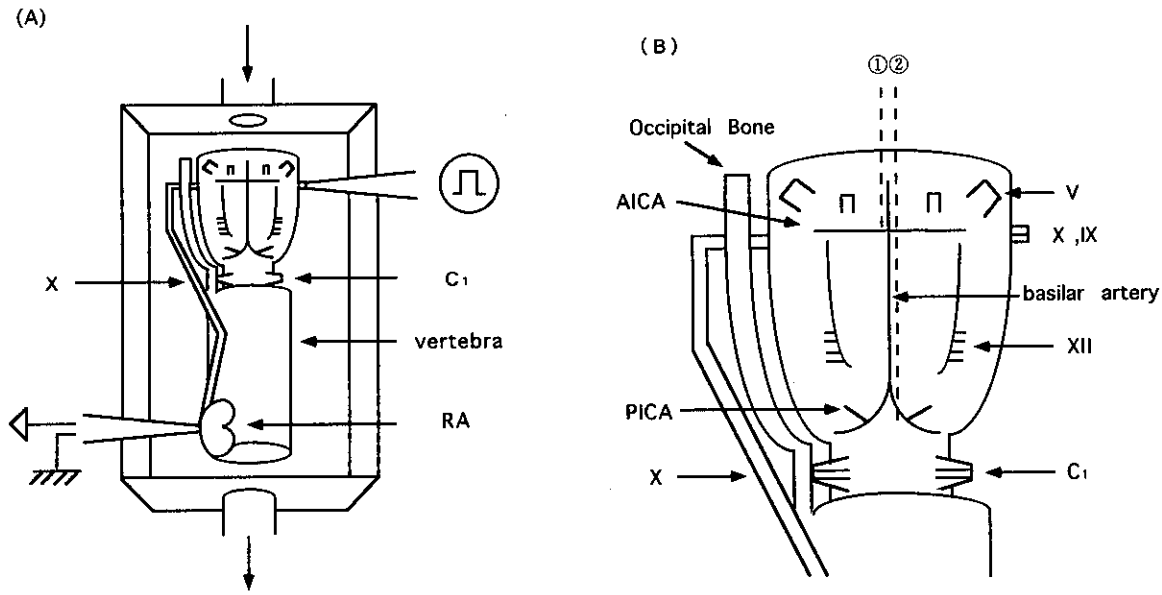

Fig. 1. (A) Schematic representation of the experimental setup. The left vagus and glossopharyngeal nerve roots were stimulated (upper right of A) and the electrical activity of the right atrium was recorded (lower left) by suction electrodes. **(B) Longitudinal sectioning of medulla oblongata along the mid-line at the level of anterior (①) and posterior (②) inferior artery.**

ropine sulfate) were added to the aCSF in the superfusion chamber to examine the contribution of autonomic nervous influence on the baseline electrical activity of the right atrium (HR_{RA}). Approximate concentrations of these agents were estimated to be 2.0 μM each in the superfusion chamber. The effect on the HR_{RA} was determined at least 5 min after adding the drug.

Recording and stimulation. The electrical activity of the right atrium was recorded extracellularly with a glass suction electrode containing aCSF, amplified 10–50 times and filtered at 100 Hz–3 kHz. The electrical activity of the right atrium was integrated (TC=0.2 s) to reduce the stimulus artifacts, and instantaneous heart rate (HR_{RA}) was calculated with an analogue computer. To activate afferent fibers in the IX and X nerves, another suction electrode was applied to the bundle of IX and X nerve roots at the left medullary surface. Both nerves were stimulated simultaneously because the roots of the IX and X nerves could not be distinguished. After suprathreshold stimulation (20 Hz, 0.5 ms duration, 5–40 V) for 10 s, the changes in the HR_{RA} were monitored for at least 30 min.

In rats older than 3 d, the reflex bradycardia was sometimes absent or lost early during the experiment, although spontaneous respiratory muscle movements and the inspiratory inhibitory (Hering-Breuer) reflex induced by IX and X stimulation were preserved (figure not shown).

Using a glass microelectrode (1 M NaCl; resistance, 1–3 MΩ), the cardiac sympathetic pathway projecting to the right atrium was electrically stimulated (0.5 ms, 20 Hz, up to 100 μA) for 10 s at the rostral ventrolateral medulla, dorsolateral funiculus at C_2 or intermediate nucleus at Th_{1-4}.

All values are expressed as mean and standard deviation (SD). Student's *t*-test was used for statistical comparisons, and a *p* value less than 0.05 was considered significant.

RESULTS

Baseline heart rate

In 0–3 d old rats, the stable electrical activity of the right atrium and consistent reflex bradycardia in response to the stimulation of contralateral IX and X nerves were recorded for a period more than 5 h. The baseline HR_{RA} was 70.3±12.9 beats/min (n=10, temp. 27°C). The baseline HR_{RA} was not affected by the application of atropine sulfate or propranolol, but increased with the application of adrenaline.

Electrical stimulation of the rostral ventrolateral medulla, dorsolateral funiculus or intermediate nucleus did not elicit any change in the baseline HR_{RA} (n=3, p>0.1), although a short-lasting increase in respiratory frequency (for 1–3 min) was induced by stimulation of the rostral ventrolateral medulla (Fig. 2).

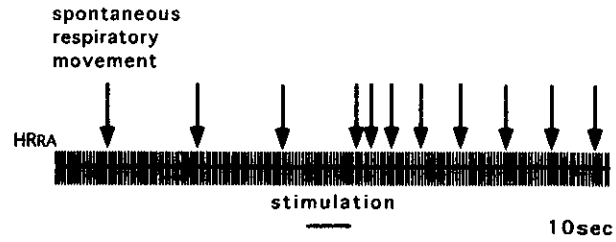

Fig. 2. The effects of right rostral ventrolateral medulla stimulation on baseline heart rate and respiratory frequency. Arrows indicate each inspiratory burst.

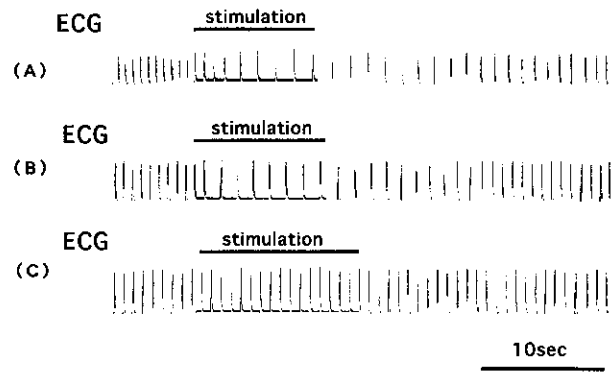

Fig. 3. Tracings of electrical activity recorded from the right atrium showing the reflex bradycardia due to stimulation of the contralateral glossopharyngeal and vagus nerves (A), which was not affected by splitting the medulla to the level of the anterior inferior cerebellar artery (B) but was abolished by extending the split to the level of the posterior inferior cerebellar artery (C).

Effects of afferent stimulation

A typical reflex bradycardia is shown in Fig. 3A. In response to stimulation of the contralateral IX and X nerves, HR_{RA} started to decrease within 2–5 beats and reached the minimum level at 7.5±1.9 s. The HR_{RA} decreased by 20.4±13.8 beats/min (p<0.05) (Fig. 4). After cessation of the stimulus, the HR_{RA} returned to the pre-stimulus level within 10–20 s (Fig. 4). This response was abolished by the addition of atropine to the superfusing solution (n=2). There was no significant difference in the magnitude of reduction in HR_{RA} between the left- and right-side IX and X nerve stimulation (Fig. 5).

Right vagotomy totally abolished the response (Fig. 6, circles), but the bradycardia could be elicited by direct stimulation of the peripheral cut end of the right

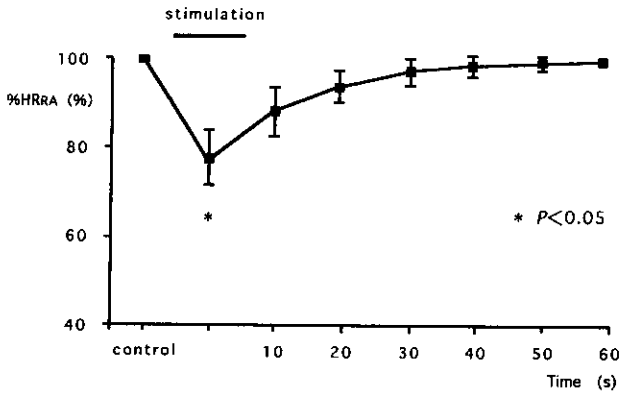

Fig. 4. Time course of reflex bradycardia given as percentage change of right atrial heart rate (HR_{RA}).

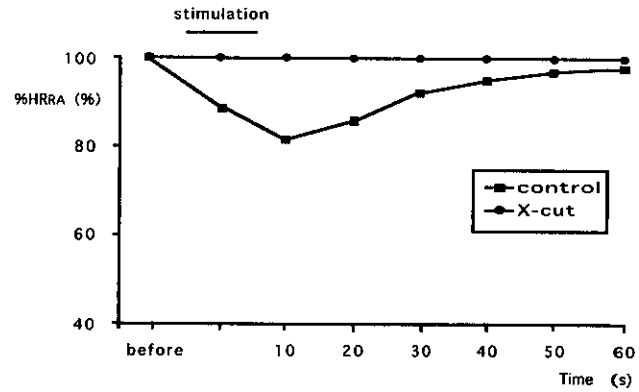

Fig. 7. Time course of reflex bradycardia given as a percentage of HR_{RA} in the preparation with intact cardiac sympathetic nervous system. Squares, reflex bradycardia due to stimulation of the contralateral vagus nerve; circles (R-cut), after right vagotomy.

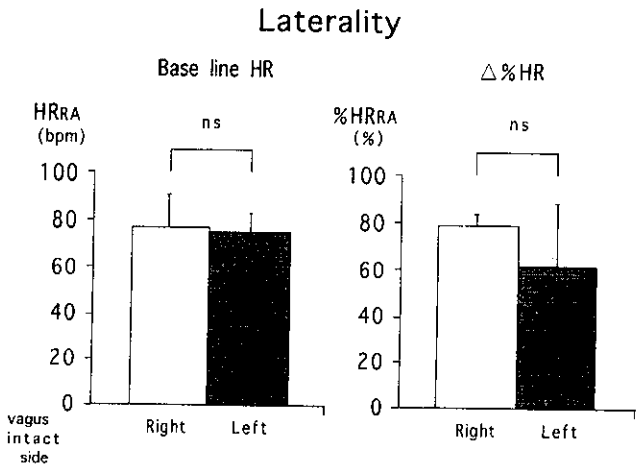

Fig. 5. Effects on baseline heart rate (left side) and magnitude of the reflex bradycardia as percentage of change in the heart rate (Δ%HR) during stimulation of the left- and right-side glossopharyngeal and/or vagus nerves.

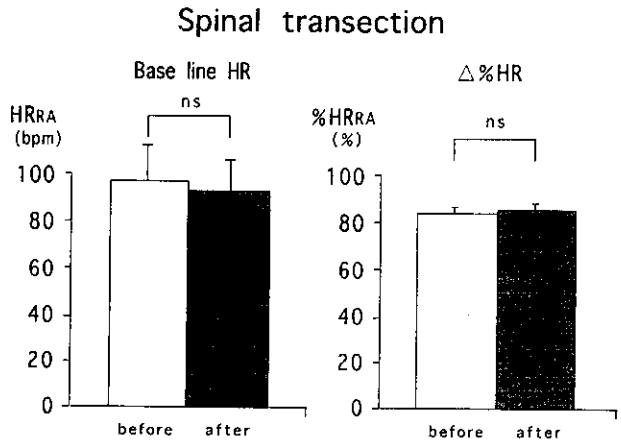

Fig. 8. Effects on baseline heart rate (left side) and magnitude of the reflex bradycardia as percentage of change in the heart rate (Δ%HR) during stimulation of glossopharyngeal and/or vagus nerves before and after spinal cord transection.

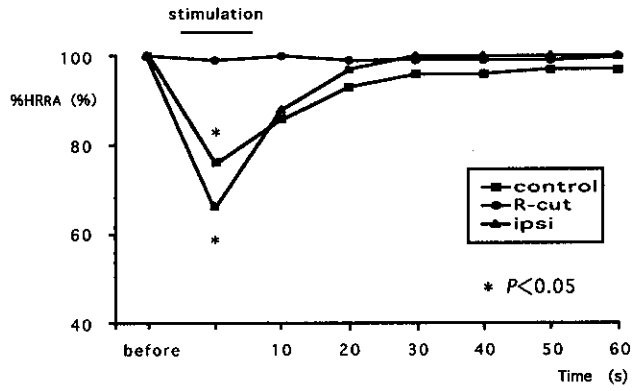

Fig. 6. Time course of reflex bradycardia given as a percentage of control right atrial heart rate (HR_{RA}). Squares, reflex bradycardia due to stimulation of the contralateral vagus nerve; circles (R-cut), after right vagotomy; triangles (ipsi), effect of stimulation of the ipsilateral (right) peripheral divided vagus.

X nerve (Fig. 6, triangles). The magnitude and time course of the response elicited by direct X stimulation were similar to those reflexly evoked (Fig. 6, squares). In the preparation including lower brainstem, spinal cord with the caudal end of Th_{12}, right atrium, and cervical and thoracic sympathetic ganglia and their surrounding tissues, right vagotomy also totally abolished the bradycardic response (Fig. 7).

Sectioning of the medulla oblongata, right vagus nerve or spinal cord

Longitudinal sectioning of the medulla oblongata at the mid-line from the rostral end of the preparation to the level of the anterior inferior cerebellar artery (Fig. 1B) neither affected the baseline HR_{RA} nor the reflex bradycardia in response to IX and X nerve stimulation (Fig. 3B). However, the reflex bradycardia was abol-

ished by further sectioning down to the level of posterior inferior cerebellar artery (Fig. 3C) without a change in the baseline HR_{RA}. Spinal cord transection at C_{1-2} did not alter the baseline HR_{RA} or the magnitude of the reflex bradycardia (Fig. 8) ($n=3$).

DISCUSSION

In this current study, we developed an *in vitro* lower brainstem-heart preparation with the intact right vagus nerve from neonatal rat. With this preparation, the activation of X and/or IX afferent stimulation induced reflex bradycardia.

In the interpretation of experiments with an *in vitro* organ preparation, various components included in the preparation must be addressed in terms of their contribution to system functions. Spinal transection, application of propranolol or atropine and right vagotomy did not change the baseline HR_{RA}. These findings suggest that neither sympathetic nor parasympathetic systems play a significant role in controlling the baseline heart rate. However, direct electrical stimulation of the right vagal efferents decreased the HR_{RA}. Therefore, the vagal efferent innervation, release of acetylcholine at the right atrial pacemaker cells and cholinegic receptors of pacemaker cell membrane were kept intact.

On the other hand, stimulation of the cardiac sympathetic efferent pathway did not alter the HR_{RA}, and spinal cord transection did not affect the magnitude of the bradycardic reflex. Since special care was taken not to damage the sympathetic innervation to the right atrium, it is likely that sympathetic efferent innervation to the heart is functionally silent in the neonatal stage. In this regard, Pappano has indicated delayed maturation of synaptic transmission in the cardiac sympathetic nervous system as compared to that in the cardiac vagal system [6]. Therefore, we considered it likely that, in neonatal rats, the cardiac sympathetic nervous system may not be functionally matured. Since the HR_{RA} was increased by the application of adrenaline, the sarcolemmal receptors and associated intracellular signal transduction to provoke tachycardia seemed to be kept intact. Repetitive stimulation of the contralateral IX and X nerves induced the reflex bradycardia, and the response was totally abolished by right vagotomy. This finding indicates that the neuronal network, including at least vagal cardiac motoneurons in the lower brainstem and their efferent outflow, were kept intact and activated during stimulation of the IX and X afferents.

The presence of spontaneous respiratory activity and an inspiratory inhibitory reflex due to IX-X stimulation lasting for a long period after isolation indicated the viability of the lower brainstem. Even in this condition, however, the reflex bradycardia was gradually attenuated much earlier than the respiratory response to IX-X stimulation. These differences in the susceptibility of two responses might be ascribed to the differences in the anatomical locations of neuronal substrates for respective responses. The vagal motoneurons controlling the heart rate may be located in a deeper medullary area [7], whereas the respiratory integrative and output neurons may be located in the ventral medullary areas close to the surface. Diffusion of O_2, CO_2, and nutrients to the motoneuron from the medullary surface may be insufficient in deeper areas.

Since the reflex bradycardia was completely abolished by right vagotomy, current spread effect through the surrounding tissues was not involved. The reflex bradycardia was lost after splitting the lower brainstem along the mid-line to the level of the posterior inferior cerebellar artery. In adult rats, some baroreceptor afferents of the IX and X nerves travel in the solitary tract, cross the mid-line at the most caudal level of the solitary tract, and synaptically activate interneurons in the contralateral commissural subnucleus of the nucleus tractus solitarius [8–11]. These interneurons project exclusively to the cardiac vagal motoneuron in the ipsilateral dorsal motor nucleus of the vagus and in the nucleus ambiguus [3]. In this study, the medullary split may have interrupted crossing baroreceptor afferents at the level between the anterior and posterior inferior cerebellar arteries, thereby abolishing the reflex bradycardia elicited by stimulation of the contralateral IX and X nerves.

It was difficult to specify the fibers stimulated to provoke the reflex bradycardia. We stimulated the IX and X afferents simultaneously because of the difficulty in distinguishing their medullary roots. Bradycardia is induced by the activation of various afferents conveyed in the IX or X nerves [1–3]. Baroreflex traffic is likely to be a major input to the circuit. Another possible input might be related to hypoxia (carotid chemoreceptor), which is an important factor controlling heart rate, especially in the neonatal period [12]. The activation of C-fibers from cardiopulmonary areas (i.e., the Bezold-Jarisch reflex) [13] might also have contributed to the bradycardiac response. We could not exclude the effects of stimulating X afferents arising from other visceral organs such as pharyngo-laryngeal areas or the gastro-intestinal system.

In various species [14] including the rat [15], it has been reported that stimulation of the right X nerve, but not the left one, elicits bradycardia. In our preparation, there was no such laterality in the magnitude of

the bradycardic response elicited by stimulation of the left or right IX and/or X nerves. The laterality of vagal innervation to the heart would be evolved during postnatal development.

The advantages of *in vitro* preparations for the study of central autonomic nervous control such as cardiovascular, respiratory, and gastro-intestinal functions have been emphasized in recent literature [16, 17]. These include the feasibility of neuropharmacological analysis of central autonomic control of heart rate. The pharmacological and anatomical organization of the cardiovascular "center" could be further explicated with this preparation using techniques such as microstimulation and direct recording from the brainstem neurons.

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