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Evidence for glutamatergic mechanisms in the vagal sensory pathway initiating cardiorespiratory reflexes in the shorthorn sculpin *Myoxocephalus scorpius*

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Summary

Glutamate is a major neurotransmitter of chemoreceptor and baroreceptor afferent pathways in mammals and therefore plays a central role in the development of cardiorespiratory reflexes. In fish, the gills are the major sites of these receptors, and, consequently, the terminal field (sensory area) of their afferents (glossopharyngus and vagus) in the medulla must be an important site for the integration of chemoreceptor and baroreceptor signals. This investigation explored whether fish have glutamatergic mechanisms in the vagal sensory area (Xs) that could be involved in the generation of cardiorespiratory reflexes.

The locations of the vagal sensory and motor (Xm) areas in the medulla were established by the orthograde and retrograde axonal transport of the neural tract tracer Fast Blue following its injection into the ganglion nodosum. Glutamate was then microinjected into identified sites within the Xs in an attempt to mimic chemoreceptor- and baroreceptor-induced reflexes commonly observed in fish. By necessity, the brain injections were performed on anaesthetised animals that were fixed by 'eye bars' in a recirculating water system. Blood pressure and heart rate were measured using an arterial cannula positioned in the afferent branchial artery of the 3rd gill arch, and ventilation was measured by impedance probes sutured onto the operculum.

Unilateral injection of glutamate (40–100 nl, 10 mmol l^{-1}) into the Xs caused marked cardiorespiratory changes. Injection (0.1–0.3 mm deep) in different rostrocaudal, medial-lateral positions induced a bradycardia, either increased or decreased blood pressure, ventilation frequency and amplitude and, sometimes, an initial apnea. Often these responses occurred simultaneously in various different combinations but, occasionally, they appeared singly, suggesting specific projections into the Xs for each cardiorespiratory variable and local determination of the modality of the response. Response patterns related to chemoreceptor reflex activation were predominantly located rostral of obex, whereas patterns related to baroreceptor reflex activation were more caudal, around obex.

The glutamate-induced bradycardia was *N*-methyl-D-aspartate (NMDA) receptor dependent and atropine sensitive. Taken together, our data provide evidence that glutamate is a putative player in the central integration of chemoreceptor and baroreceptor information in fish.

Key words: fish, vagus, reflex control, ventilation, bradycardia, blood pressure, glutamate, NMDA, chemoreceptor, baroreceptor.

Introduction

Without exception, vertebrates, in order to maintain homeostasis, regulate blood pressure and arterial gas concentrations by a variety of feedback loops, such as those that originate in peripheral chemoreceptors and baroreceptors. These receptors send afferent information into the central nervous system, which results in modulation of the activity in neurones generating respiratory rhythms and those controlling the cardiovascular system. While substantial knowledge exists regarding the central integration of activity in cardiorespiratory sensory afferents in mammals (Spyer, 1990; Talman, 1997; Van Giersbergen et al., 1992), nothing is known in fish, a separate, somewhat more primitive, group of vertebrates.

In mammals, the nucleus of the solitary tract (NTS) is the primary synaptic relay in the brainstem, where afferent information from visceral receptors is integrated. The NTS has been subdivided into different subnuclei based on its cytoarchitecture and the afferent and efferent connections of the neurons within it. The medial and lateral commissural subnucleus of the NTS has been shown to be the primary site of termination of cardiovascular afferent fibres, receiving inputs from carotid chemoreceptors, arterial baroreceptors and cardiopulmonary receptors (Loewy, 1990; Van Giersbergen et al., 1992). Glutamate, an excitatory amino acid (EAA), is the strongest candidate for the neurotransmitter released by these afferents (Ohtake et al., 1998; Talman, 1997).

Information regarding the location of sensory areas in the medulla important for control of the cardiorespiratory system in fish is sparse, and information about the nature of their

neurotransmitters and receptors is essentially lacking. It is documented that the gills are a major site for chemo- and baroreceptors, with their afferent nerves travelling in cranial nerves IX and X (Burleson et al., 1992). In the medulla, the areas of termination of afferent sensory fibres (Xs) are located dorsally and laterally above the sulcus limitans of His, whereas the motor area (Xm) is located ventral and lateral to the sulcus (Meek and Nieuwenhuys, 1998). Although in most teleosts a clear NTS is absent, the visceral sensory area forms a continuous column dorso-laterally on either side of the 4th ventricle in the medulla, into which viscerosensory fibres of nerves VII, IX and X terminate in a rostrocaudally ordered fashion (Meek and Nieuwenhuys, 1998).

Recently, it has been shown that EAAs are the neurotransmitters in taste pathways in goldfish (*Carassius auratus*; Smeraski et al., 1998), and immunohistochemistry has shown that glutamate is present in the nodose ganglia and vagal afferents in the shorthorn sculpin *Myoxocephalus scorpius* (J. Turesson and L. Sundin, manuscript submitted). Taken together, these results suggest that EAAs might also be the neurotransmitters in the general visceral sensory pathways conveying information from chemo- and baroreceptors *via* vagal and glossopharyngeal nerves.

A first step on the way to establish if glutamate is a functional neurotransmitter in the central processing of baroreceptor and oxygen chemoreceptor information in fish is to determine whether addition of glutamate into the vagal portion of the visceral sensory column elicits cardiorespiratory responses similar to the reflexes activated by stimulation of peripheral chemo- and baroreceptors. Therefore, the primary aim of this paper was to examine whether microinjection of glutamate, sometimes followed by appropriate antagonists, into different sites of the vagal sensory area (the terminal field of vagal afferent fibres characterised as the NTS in mammals) activates cardiorespiratory responses that mimic chemo- and baroreceptor reflexes. If clear responses were obtained, then glutamate could perhaps be used as a ‘mapping tool’. Accordingly, a second aim was to elucidate whether there was a distinguishable separation of areas in which different responses were elicited that might reflect a topographical arrangement of the central projection of receptor afferents. As knowledge of the central projections of the vagal afferent and efferent fibres in the medulla of the shorthorn sculpin is a prerequisite for reasonably accurate microinjections into the Xs, the initial aim of this study was to locate the Xs and Xm columns in this species, using a neuroanatomical technique.

Materials and methods

Animals

Shorthorn sculpins *Myoxocephalus scorpius* L. weighing 172±18 g were caught on the Swedish West Coast by a local fisherman. They were given at least 3 days to recover from the effect of capture in holding tanks at 10°C and normal day/night length. All animal experiments have been approved by the local ethical committee in Gothenburg (No. 299/99).

Experimental preparation

On the day of surgery, the fish were anaesthetized in seawater containing 100 mg l^{-1} MS 222 (ethyl m-amino benzoate; Sigma; 10°C) until breathing movements ceased.. They were transferred to a surgical table where the gills were continuously irrigated with cooled, recycled water containing anaesthetic (40–50 mg l^{-1} MS 222, 10°C).

Topography of the vagal sensory and motor columns

The nodose ganglion was located by tracing the exposed branchial nerves centrally. Exposure was *via* a small incision (approximately 1 cm) made in the epithelium at the dorsal end of the 4th gill arch where it meets the roof of the opercular cavity, the operculum having been reflected forward. Using a 25 µl Hamilton syringe equipped with a 27-gauge hypodermic needle, 5–10 µl of Fast Blue (Sigma), as a 2% solution in polyethylene glycol, was injected through the nerve sheath into the ganglion. When visual observation confirmed that the ganglion had turned yellowish in appearance, the needle was withdrawn and the puncture was closed with tissue glue. The incision was sutured and the fish was tagged, then returned to holding tanks for 7–10 days to allow axonal transport (orthograde and retrograde) of the tracer into the projections of the vagus, in the medulla. Each fish was then sacrificed by an overdose of MS 222 and heparin (0.2 ml, 5000 IU) injected into the caudal vein. The fish were exanguinated by perfusion with physiological saline (0.9% NaCl) using a ventral aortic cannula connected to a peristaltic pump. After 10–15 min, when the gills had turned white, the saline was switched to 4% formaldehyde solution and the fish were perfused for a further 15 min. The brain was then carefully dissected from the skull and placed in 4% formaldehyde in 0.1 mol l^{-1} phosphate-buffered saline (PBS; pH 7.3) for at least 4–5 h at 4°C. Each brain was then rinsed for 30 min in PBS and stored in PBS containing 30% sucrose as a cryoprotectant. Finally, it was quick-frozen in iso-pentane cooled in liquid nitrogen and mounted on the stage of a cryostat. Serial, transverse sections, 20 µm thick, were cut, transferred directly to gelatine-coated glass slides and left to air-dry overnight. The sections were coverslipped with glycerol mounting media and viewed under a fluorescence microscope (BX60, Olympus) connected to a digital video camera. Pictures were frozen on a TV monitor and captured by computer using the Micro Image software (Micro Image, Gothenburg, Sweden). To visualize the general histology of the labelled sections, some were stained for Nissl substance.

Microinjection

The day before the experiment, the third afferent branchial artery on the left side was cannulated (PE 50 tipped with a PE 10) according to the procedures described for Atlantic cod (Axelsson and Fritsche, 1994). This cannula was used to measure ventral aortic blood pressure (P_{VA}) and heart rate (f_H) and for the administration of drugs. Measurements of ventilation frequency (f_V) and amplitude (V_{AMP}) were made using impedance probes, fastened with suture thread stitched through each operculum.

On the day of the experiment, the fish was again anaesthetised (100 mg l^{-1} MS 222) and lowered into a plastic box placed between the steel bars of a modified stereotaxic frame (model SN-2N; Narishige Instruments, Tokyo, Japan). It was fixed in position with eye bars and also, initially, with a mouthpiece through which re-circulating respiratory water (40–50 mg l^{-1} MS 222) flowed. As the animal started to breath spontaneously, the mouthpiece was withdrawn to deliver water approximately 2 cm in front of the snout. Using a dremel tool and vacuum suction, the skull was carefully opened (incision approximately 1.5 cm long) to expose the whole length of the medulla from the middle portion of the cerebellum to the first pair of the spinal nerves. The fish rested in a horizontal position on a height-adjustable platform inside the box. A standpipe controlled the water level, which was adjusted to cover the gills yet allowed the medulla to be uncovered.

Drugs were delivered into specific locations in the medulla from a single-barrel microinjection pipette (tip size 10–15 μm). Movements of the pipette were controlled by a micromanipulator (SM15 equipped with a base SM-15M). Injection volumes of 40–100 nl were delivered over a period of ≤1 s by applying a pulse of pressurized N_2 using a pressure injector (model PLI-100; Harvard Medical Systems, Holliston, MA, USA). The volume of drug delivery was controlled by changing the injection pressure, and the actual volume of the injection was determined by viewing the movement of the fluid meniscus in the barrel of the pipette, which was of known internal diameter, using a microscope (×50 magnification) equipped with a calibrated eyepiece micrometer.

The cannula was connected to a pressure transducer, the signal was amplified (4Champ; Somedic AB, Sollentuna, Sweden) and the leads from the impedance probes were connected to an impedance converter (model 2991; UFI, Morro Bay, CA, USA). The cardiorespiratory variables were continuously recorded to paper (recorder model 3701, LR 8100; Yokogawa, Tokyo, Japan), and the data were collected online, *via* data-acquisition software (Labview version 5.0; National Instruments, Solna, Sweden) onto a computer. Sampling frequency was 20 Hz, and mean values were subsequently created at 10 s intervals. From the pulsed blood pressure and ventilation signals, f_H and f_V were derived using a Labview-based calculation program. The injection signal from the PLI-100 pressure injector was also sampled, which allowed exact timing of the injection with the cardiorespiratory responses.

Experimental protocols

In three fish, efforts were made to use a decerebrate and spinalectomized preparation to avoid any potential influence of anaesthesia on central reflex mechanisms. However, that approach was abandoned because these fish bled substantially, displayed low ventral aortic blood pressures (0.9–1.3 kPa) and never started spontaneous breathing. Instead of decerebration, light anaesthesia (40–50 mg l^{-1} MS 222) was used, as it permits a smaller hole in the skull to be made and leaves the spinal cord intact, maintaining sympathetic outflow to the vessels. This approach significantly improved the blood pressure (2.0–3.8 kPa) in the animals, who now also started to breathe spontaneously. These blood pressures are comparable with those in an unanaesthetised and free-swimming sculpin (Fritsche, 1990).

Preliminary trials using 0.1 mmol l^{-1} and 1.0 mmol l^{-1} of glutamate were employed to determine a concentration that would give clear and distinctive responses. The concentration (10 mmol l^{-1}) and volume range (40–100 nl) chosen are comparable with those commonly used for microinjections into the medulla of rats (Canesin et al., 2000; Dhruva et al., 1998; Le Galloudec et al., 1989).

General protocol

The general protocol for the experiments was as follows. When stable cardiorespiratory parameters were established, usually around 40–60 min after securing the fish in the stereotaxic frame, unilateral microinjections of glutamate were made sequentially into different discrete areas of the Xs column along the medulla. When a response was elicited, the following injection was postponed (between 10 min and 60 min) until stable parameters were again established. The microinjection pipette was advanced through the sensory area (as determined by the nerve tracing) in steps of 0.1 mm down to a depth of 0.3 mm along a rostrocaudal direction from 2.0 mm rostral to –1.0 mm caudal of obex, in steps of 0.5 mm. Lateral coordinates applied were 0.3 mm, 0.5 mm and 0.7 mm lateral to the midline. Each animal was subjected to 20–50 different injections, although not every coordinate received an injection in each animal and the order of injection sites varied among animals. When a clear and concise response was elicited, the pipette was raised, rinsed and vacuum loaded with the vehicle (0.9% NaCl) then lowered again to the same depth, and a control injection with the same or a larger injection volume was performed. To control for tachyphylaxis and possible mechanical damage of an injection, repetitive injections of glutamate in exactly the same area at 10 min intervals were performed in at least one site in each animal, and glutamate was sometimes re-injected into the area where a previous vehicle control had been carried out.

Cardiovascular mechanisms

In five animals, at the end of the above-described general protocol, a site that had previously elicited a distinct bradycardia was again injected before an intra-arterial injection of the agonist atropine (1 mg kg^{-1}). After 20 min, the agonist injection was repeated at the same site.

For experiments using the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801, the general protocol was as follows. Having obtained a cardiac response to unilateral microinjection of 40–100 nl of glutamate, the pipette was raised, rinsed then vacuum loaded with the antagonist (3 mg ml^{-1}), which was injected (40–100 nl) at the same depth. The agonist was then reloaded and the injection repeated. The

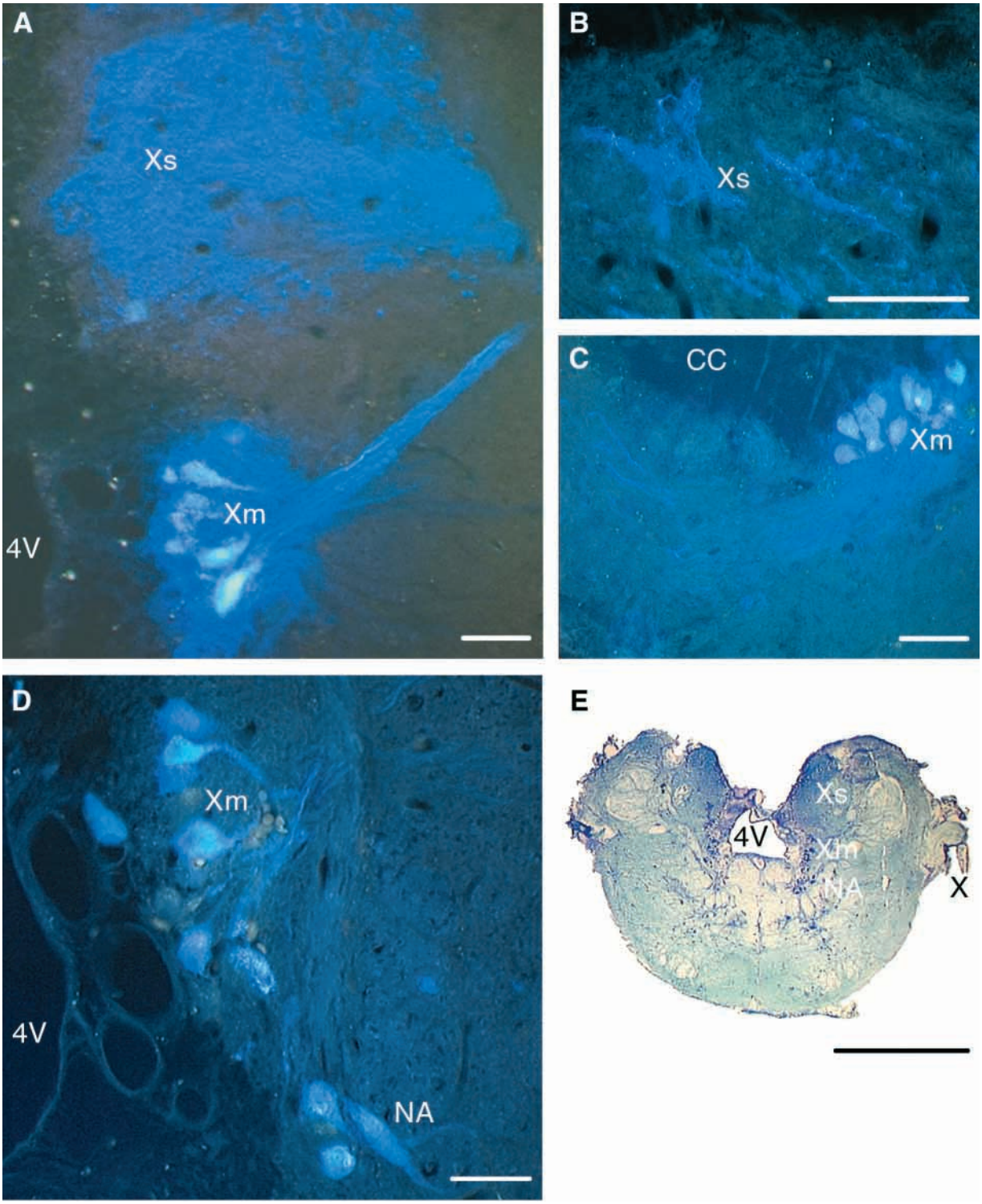

Fig. 1. Micrographs demonstrating the termination of the vagal projections into the medulla marked by the axonal transport of the tracer Fast Blue from an injection site in the nodose ganglion. (A) Transverse section (TS) taken 0.6 mm rostral to obex, showing the vagal sensory (Xs) and motor (Xm) areas, dorso-lateral to the fourth ventricle (4V). (B) TS taken 0.12 mm caudal to obex, showing commissural sensory fibres. (C) TS taken 0.08 mm caudal to obex, showing commissural fibres beneath the central canal (CC) of motor origin. (D) TS taken 0.3 mm rostral of obex to show the nucleus ambiguus (NA), situated ventro-laterally to the Xm. (E) Nissl-stained section from the same animal as in A, 580 µm rostral to obex. White scale bars represent 50 µm and the black scale bar represents 1 mm. Note that the Xs and Xm areas are clearly separate.

time between the application of the antagonist and the agonist was 5–10 min. To further control for the specificity of the blockade, the pipette was lowered 0.1 mm beyond or moved 0.5 mm in a sagittal direction from the MK-801 saturated area, and the agonist injection was repeated.

Drugs

Monosodium L-glutamate, dizocilpine (5R, 10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) and atropine were obtained from Sigma and dissolved in 0.9% NaCl.

Statistical analyses

Comparison of the cardiovascular effects before and after glutamate was made using a paired *t*-test. The same test was used for the comparison of the cardiovascular effects of glutamate before *versus* after MK-801, and before *versus* after atropine. Differences were considered significant at $P<0.05$. All values are means ± S.E.M.

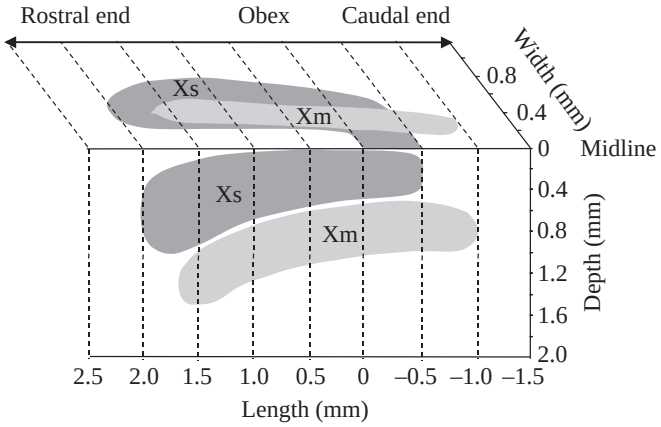

Fig. 2. The coordinates of the vagal sensory (Xs) and motor (Xm) columns in the brainstem of the sculpin derived from the results of the neural tract tracing. The areas are plotted and drawn in both the horizontal and the sagittal planes. The dorsal rostrocaudal surface of the medulla, its midline and obex are all set to zero.

Results

Nerve tracing

A time period of 7–10 days (at 10°C) was sufficient to complete ortho- and retrograde axonal transport of Fast Blue from the nodose ganglia to the medulla (approximately 0.5–1.0 cm) in *M. scorpius*. In four animals, both the sensory (Xs) and the motor (Xm) columns contained the tracer, whereas in one animal only the motor column stained blue. The sensory nucleus and the motor nucleus of the Xth cranial nerve form two continuous columns parallel with and dorso-lateral to the fourth ventricle (4V) along the length of the medulla (Figs 1, 2). Even though both the motor and sensory columns were heavily marked with fluorescent tracer, the columns were clearly separated along their lengths (Fig. 1A). The sensory column is positioned slightly rostral to the motor column, and the total length of the two columns that fluoresce is 2500–2800 μm in each fish (Fig. 2).

Retrograde labelling with Fast Blue also identified cells in the nucleus ambiguus (NA) of the vagal motor column. This nucleus consists of a relatively small number of neurones located ventro-laterally with respect to the Xm and separated from it by a tract of nerve fibres (Fig. 1D).

The anterior ends of the two columns are located approximately 1.5–2.0 mm rostral of obex, and the posterior end of both columns stretches to 1 mm caudal of obex. At this caudal extremity, commissural fibres (Fig. 1B) cross above the central canal, to constitute the commissural nucleus of the Xs, while fibres crossing beneath are of motor origin (Fig. 1C).

Motoneurons, probably belonging to the nucleus ambiguus, in a ventrolateral position to the motor column were observed from obex to 0.9 mm rostral of obex. Observations of Fast Blue-filled neurons in this position were not common, probably resulting from incomplete staining and a vague and disperse nucleus in this species.

Microinjections

Control injections

As the dorsal sensory and the ventral motor columns are located rather close to each other, adjacent at depths of 0.4–0.5 mm from the dorsal surface of the medulla, the results reported here are restricted to injections made down to 0.3 mm.

To control for non-specific pressure and volume effects of the injections, the vehicle (0.9% NaCl) alone was delivered into the same sites (equal or larger volume) where glutamate had previously elicited a response. The vehicle produced small insignificant blood pressure increases only in one fish. In addition, two fish displayed bradycardia, a concomitant blood pressure decrease and a short apnea when accidentally large volumes (150–300 nl) were injected. When smaller injection volumes were applied at the same sites no responses were evoked.

Repeated injections of glutamate into the same site at 10 min intervals did not decrease the responsiveness of the animal. Hence, the repetition of a glutamate injection into a vehicle-applied site always produced a response.

Responses to injection of glutamate

Dependent on the injection site in the Xs, glutamate elicited decreases in heart rate (f_H) and either increases or decreases in ventral aortic blood pressure (P_{VA}), ventilation frequency (f_V) and amplitude (V_{AMP}). A tachycardia was never observed. Occasionally, an injection elicited a transient apnea. The coordinates and responses for each injection in all animals are summarised in Fig. 3. Sometimes, an injection elicited a response in a single cardiorespiratory parameter (Fig. 4) or, at other times, in two or three or all of them (Figs 5, 6). Blood pressure and the ventilatory responses could be biphasic, often reflecting the relationship between f_H and P_{VA} on the one hand and f_V and V_{AMP} on the other hand. In clear cases, such as a bradycardia-induced drop in P_{VA} (Fig. 7), this depressor event

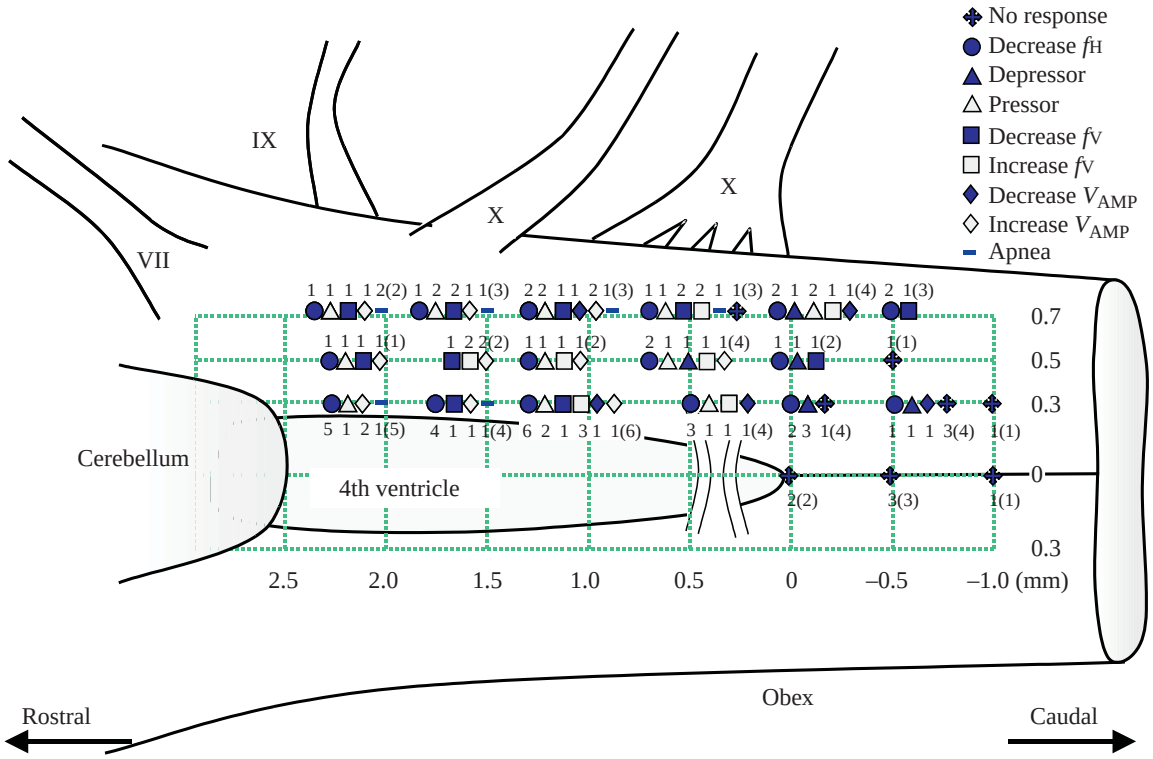

Fig. 3. A schematic drawing of the medulla showing the responses to glutamate injection at each coordinate. Numbers in brackets denote the total number of animals injected at that coordinate. The other numbers above and beneath the symbols at a specific coordinate represent the number of animals that have shown that particular response. Filled symbols depict a decrease, whereas open symbols depict an increase. Note the differences in distribution of the pressor and depressor responses, as well as the excitatory ventilatory responses. Heart rate (f_H), ventilation frequency (f_V), ventilation amplitude (V_{AMP}).

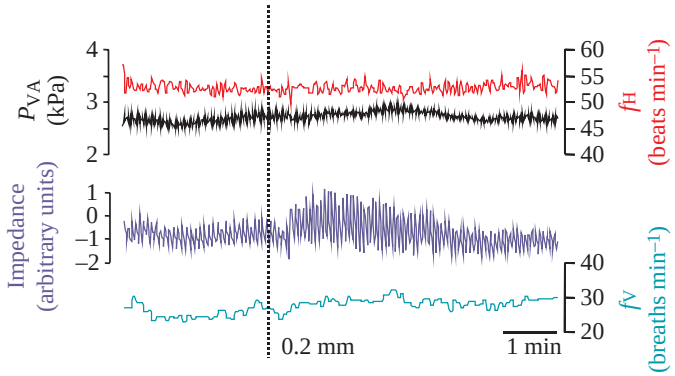

Fig. 4. Responses to an injection of 40 nl glutamate (10^{-2} mol l^{-1}) 1.5 mm rostral of obex and 0.5 mm lateral to midline at 0.2 mm depth. Note the specific increase in the ventilation amplitude (V_{AMP}) only (divided vertical line denotes time of injection). Heart rate (f_H), ventilation frequency (f_V), ventral aortic blood pressure (P_{VA}).

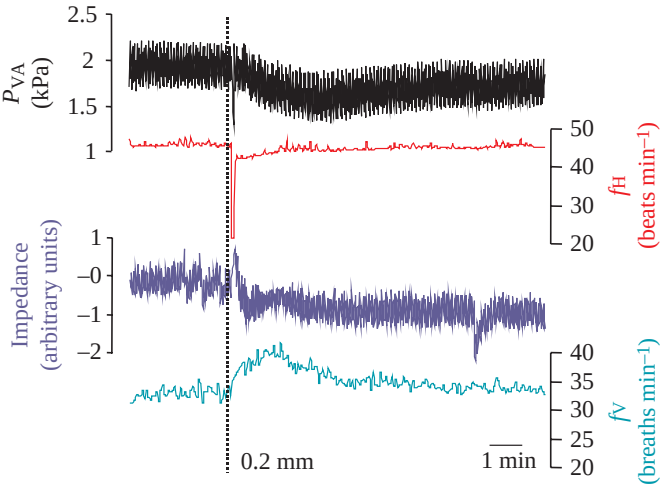

Fig. 5. Responses to an injection of 80 nl glutamate 0.5 mm rostral of obex and 0.5 mm lateral to midline at 0.2 mm depth, showing both a ventilatory and a definitive depressor response with a transient bradycardia. Heart rate (f_H), ventilation frequency (f_V), ventral aortic blood pressure (P_{VA}).

was excluded from the summarised data in Fig. 3. Only depressor responses that were apparently independent of a change in f_H are included (e.g. Fig. 5). The convention adopted with respect to ventilatory parameters is that reciprocal changes in frequency and amplitude are recorded as the appropriate excitatory response. Thus, an increase in amplitude that led to a decreased frequency or a marked increase in frequency that resulted in reduced amplitude have been recorded as an increase in either variable.

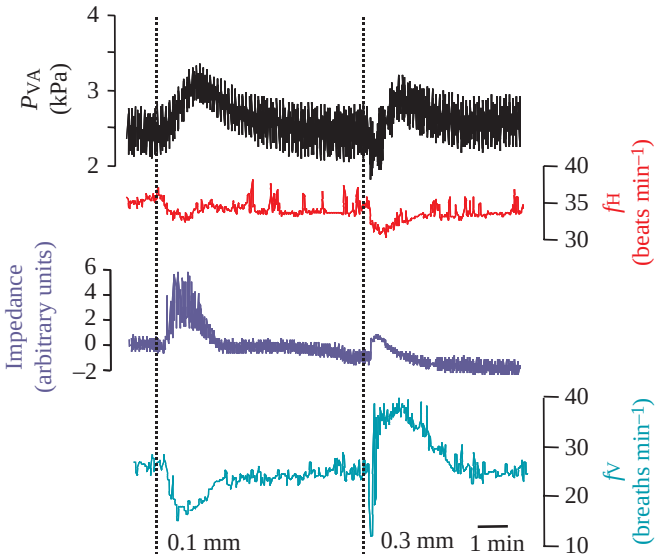

Fig. 6. Responses to successive injections of 40 nl glutamate 1.0 mm rostral to obex and 0.5 mm lateral to midline at 0.1 mm and then at 0.3 mm depths showing a pressor response. Note the marked changes in the ventilatory responses arising from advancing the pipette just 0.2 mm.

Mapping of the distribution of specific responses

Some salient features of these seemingly complex patterns of response summarised in Fig. 3 are:

(1) Caudal of obex: no responses to injection in the midline but a bradycardia and reduction in P_{VA}, f_V and V_{AMP} following injection into more lateral sites, 0.5 mm caudal of obex.

(2) At obex: a bradycardia plus specific pressor/depressor responses, including a definitive depressor area 0.3 mm lateral to obex. Some sites induced increases in f_V.

(3) Rostral (0.5 mm) of obex: bradycardia plus pressor/depressor effects; increases in f_V and V_{AMP}.

(4) Rostral (1.0 mm) of obex: bradycardia; pressor responses but no depressor responses rostral of this level; both f_V and V_{AMP} increased or decreased.

(5) Rostral (1.5 mm) of obex: bradycardia; pressor responses; only increases in V_{AMP}, increases or decreases in f_V.

(6) Rostral (2.0 mm) of obex: bradycardia; pressor responses; increases in V_{AMP}, independent decreases in f_V.

In summary, certain main features emerge with regard to each recorded variable: a bradycardia and specific pressor responses were induced by injections at most reactive sites, both caudal and rostral of obex; depressor responses were obtained at and immediately (0.5 mm) caudal or rostral of obex; f_V was increased by injection into some sites at and up to 1.5 mm rostral of obex, while a decrease in f_V accompanied injection into sites just caudal (0.5 mm) and 2.0 mm rostral of obex; V_{AMP} was increased by injection into most areas rostral of obex and decreased by injections just caudal (0.5 mm) and rostral (1.0 mm) of obex.

It is clear from these data that, while there is some evidence for rostrocaudal distribution of projections from specific receptor-mediated responses, a reflex bradycardia is induced by injection of glutamate into most sites either side of obex. The induced bradycardia sometimes resulted in cardiac arrest, in one case for up to 4 min (Fig. 7).

Cardiovascular mechanisms

Along the fourth ventricle at the medial (0.3 mm lateral) injection sites, glutamate always induced a bradycardia that sometimes was very marked, in one extreme case without a heart beat for up to 4 min (Fig. 7). In five animals, the non-competitive antagonist of NMDA receptors, MK-801, was injected into bradycardia-inductive sites and the glutamate injection was repeated. MK-801 abolished the rapidly glutamate-induced bradycardia (Figs 7, 8). The bradycardia was also blocked by a systemic injection of atropine (Fig. 9).

Discussion

The neuroanatomical study revealed that the sculpin had vagal sensory (Xs) and motor (Xm) columns similar in all respects to those described in other teleost fish (Burleson et al., 1992; Meek and Nieuwenhuys, 1998). As such, it is not new information but

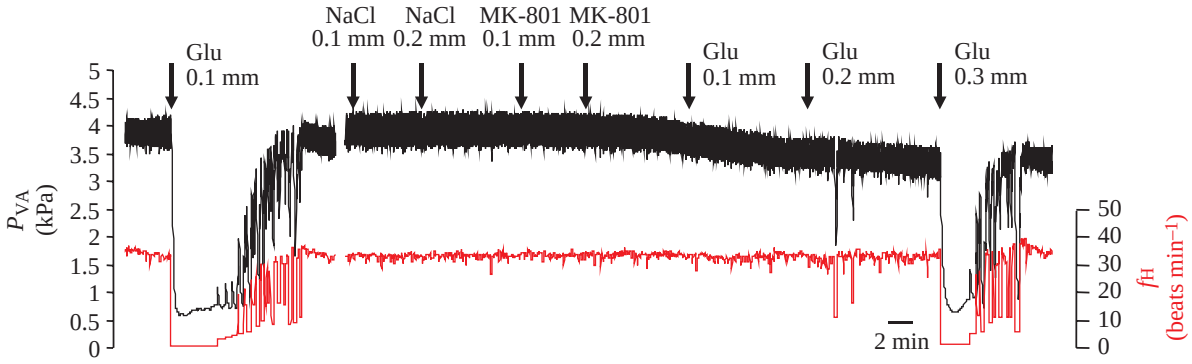

Fig. 7. Changes in ventral aortic blood pressure (P_{VA}) and heart frequency (f_H) arising from sequential injections of glutamate, vehicle (NaCl) and *N*-methyl-L-aspartate (NMDA) receptor antagonist MK-801 at 1.0 mm rostral of obex and 0.3 mm lateral to the midline. The arrows indicate the time of injection, and the numbers (in mm) indicate the depth of the injections. Note the rapid and the marked bradycardia. Also observe the absence of responses to NaCl and the preciseness of the MK-801 blockade. Injection volume ranged between 40 nl and 100 nl.

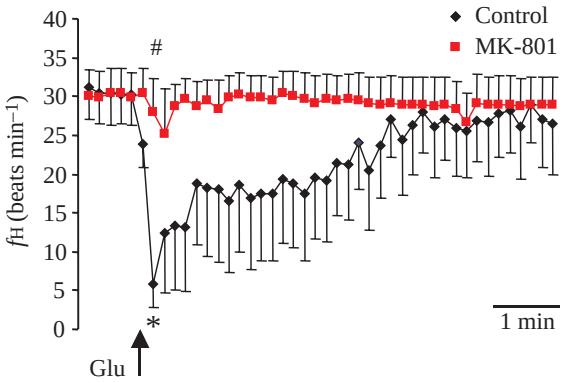

Fig. 8. Changes in mean (±S.E.M.) heart rate (f_H) following medullary injections of glutamate (40–100 nl) in five animals. A marked bradycardia was blocked by prior application of MK-801 in the same sites. * indicates significant changes from pre-injection values at $P<0.01$, and # indicates a significant difference in the same time point before and after injection of MK-801 at $P<0.01$.

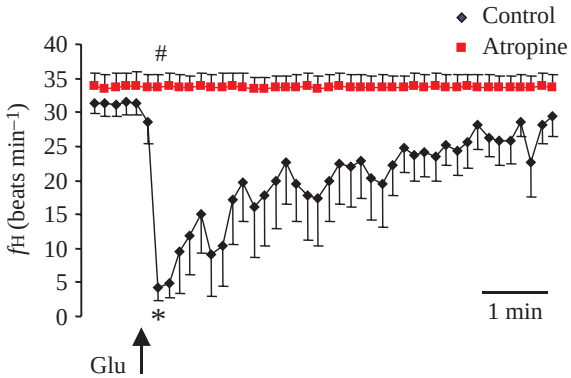

Fig. 9. Changes in mean (±S.E.M.) heart rate (f_H) following medullary injections of glutamate (40–100 nl) in five animals. A marked bradycardia was blocked by prior systemic injection of atropine. * indicates significant changes from pre-injection values at $P<0.0001$, and # indicates a significant difference in the same time point before and after injection of atropine at $P<0.001$.

does serve the initial role of identifying injection sites within the Xs in this species. The present study is to be extended by further use of fluorescent markers to study the detailed topography of the vagal supply to specific target organs, such as the heart and branchial arches, including both the location of cell bodies and their processes. This will enable central injection and eventual recording from these specific sites to identify areas integrating cardiorespiratory reflexes and generating central interactions. In the present study, we show that glutamate injected into the vagal sensory column in the dorso-lateral medulla in fish elicits several cardiorespiratory responses, demonstrating that it may be an important neurotransmitter released by the afferents of baro- and chemoreceptors, as has been suggested for mammals (Schaffar et al., 1997; Sykes et al., 1997; Talman et al., 1980). The most ubiquitous response obtained was a bradycardia, which can be explained by the fact that this is a component of both the baroreceptor and chemoreceptor reflex responses in fish (Taylor et al., 1999). However, these separate reflexes may be distinguished by their accompanying changes in physiological variables, with the baroreflex leading to a reduction in blood pressure and the chemoreflex leading to an increase in both blood pressure and ventilation. Examination of these responses reveals some evidence of a topographic separation of the projections from these different reflexogenic areas. Our mapping showed that the cardiorespiratory responses characteristic of a chemoreflex (bradycardia, increased blood pressure, ventilation frequency and amplitude; Fritsche and Nilsson, 1993) are located rostral to obex, whereas responses typical of a baroreflex (a bradycardia accompanied by a decrease in blood pressure; Lutz and Wyman, 1932) are located at the level of obex or just caudal to it, i.e. in the commissural segment.

Interestingly, the specific location of the terminal field within the NTS is crucial for the production of respiratory or cardiovascular reflexes in mammals (Dhruva et al., 1998; Marchenko and Sapru, 2000). In mammals, both chemo- and baroafferents terminate in the commissural nucleus of the NTS (Loewy, 1990; Van Giersbergen et al., 1992). Within this restricted area, the baroreflexogenic field is located rostral to the chemoreflexogenic field (Dhruva et al., 1998). With the finding of a depressor area lateral to obex at the beginning of the commissural segment, our results seem similar to the location of depressor areas in the commissural nucleus in mammals. However, caudal to the depressor area, we found no evidence for a chemoreflexogenic zone. In fact, most of the injections in this region produced no responses at all. Instead, responses simulating chemoreflexes were elicited rostral of obex. This is consistent with the fact that most peripheral chemoreceptors have been described as being located on the gills of fish. The gill arches are innervated sequentially by the IXth glossopharyngeal nerve then the first four branches of the vagus. The fifth branch innervates the viscera, including the heart (Burleson et al., 1992; Taylor et al., 1999). Thus, chemoreceptor afferents will travel in the more rostral projections into the Xs from the branchial branches of the vagus nerve, which terminate rostral of obex (Taylor, 1992). Caudal of obex, at the commissural nucleus, the afferents of the most caudal root terminate. Thus, this structure only receives sensory information from visceral afferents rather than from the gill arches (Kanwal and Caprio, 1987; Lazar et al., 1992; Morita and Finger, 1987). The finding of a specific depressor site 0.3 mm lateral to obex substantiates that the barostatic reflex in fish, implicating changes in the resistance of the vessels, may project through the area innervating the heart (Taylor, 1992).

In addition to the distribution of reaction patterns simulating a specific reflex, injection of glutamate could sometimes elicit a unitary response such as an increase in respiratory amplitude or a decrease in blood pressure. This suggests specific areas in the Xs for reflex control of each cardiorespiratory variable. Identification of these ‘single’ responses may have been prejudiced by the extracellular injection technique. Although different response patterns

could be obtained with a pipette movement of just 0.1 mm, the spread of the injection solution will probably cause stimulation of many neighbouring neurons. With smaller injection volumes and even smaller steps during mapping, a better picture of this single response topography may evolve. Nevertheless, this single response topography may accord with the physiological evidence for more than one population of oxygen receptors in fish, which elicit different cardiorespiratory parameters dependent on their peripheral location (specific gill arch or extrabranchial) or orientation (monitoring respiratory water or blood oxygen levels) (Burleson and Smatresk, 1990; Smatresk et al., 1986; Sundin et al., 1999, 2000). If glutamate is, as in mammals (Schaffar et al., 1997; Sykes et al., 1997; Talman et al., 1980), the neurotransmitter released by the afferents of baro- and chemoreceptors in fish, there should be glutamate receptors on target neurons binding the EAA. Indeed, our results show that the non-competitive NMDA receptor antagonist MK-801 effectively blocked the glutamate-induced bradycardia. Similarly, NMDA receptors mediate a glutamate-induced bradycardia in rats (Canesin et al., 2000; Colombari et al., 1997). In addition, the data following systemic injection of atropine show that the bradycardic responses produced by microinjection of glutamate along the 0.3 mm lateral coordinates are due to parasympathetic neurotransmission, so that a glutamatergic mechanism for chemo- and baroreflex activation in fish seems likely. This is borne out by the demonstration that NMDA receptors in the NTS are involved in the bradycardic element of both the chemoreflex (Haibara et al., 1995) and the baroreflex in rats (Canesin et al., 2000).

In conclusion, glutamate applied to different areas in the Xs of the sculpin evoked responses simulating chemo- and baroreflexes. There was some evidence for a topographic separation of these two areas with a chemoreflexogenic zone rostral to a baroreflexogenic zone. The ubiquitous, glutamate-induced bradycardia depended on NMDA receptors in the sensory pathway and was of muscarinic cholinergic, and therefore vagal, origin. Evidence has thus been presented that glutamate may have been present as a key neurotransmitter in the reflex control of the cardiorespiratory system from early in vertebrate evolution. Thus, this work may provide a first step in establishing the fundamental central mechanisms for the processing of chemo- and baroreceptor signals in all vertebrates.

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