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Nitric oxide synthase in the glossopharyngeal and vagal afferent pathway of a teleost, *Takifugu niphobles*

The branchial vascular innervation

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Abstract To examine the presence of nitric oxide synthase (NOS) in the sensory system of the glossopharyngeal and vagus nerves of teleosts, nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) activity and immunoreactivity for NOS were examined in the puffer fish *Takifugu niphobles*. The nitrergic sensory neurons were located in the ganglia of both the glossopharyngeal and the vagal nerves. In the vagal ganglion, positive neurons were found in the subpopulations for the branchial rami and the coelomic visceral ramus, but not for the posterior ramus or the lateral line ramus. In the medulla, nitrergic afferent terminals were found in the glossopharyngeal lobe, the vagal lobe, and the commissural nucleus. In the gill structure, the nitrergic nerve fibers were seen in the nerve bundles running along the efferent branchial artery of all three gill arches. These fibers appeared to terminate in the proximal portion of the efferent filament arteries of three gill arches. On the other hand, autonomic neurons innervating the gill arches were unstained. These results suggest that nitrergic sensory neurons in the glossopharyngeal and vagal ganglia project their peripheral processes through the branchial rami to a specific portion of the branchial arteries, and they might play a role in baroreception of this fish. A possible role for nitric oxide (NO) in baroreception is also discussed.

Key words Nitric oxide · Visceral sensory system · Baroreceptor · Gill arch · Medulla oblongata · Tetraodontiformes · *Takifugu niphobles* (Teleostei)

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Introduction

The glossopharyngeal (NIX) and vagus (NX) nerves constitute a main visceral sensory component throughout vertebrates from fish to mammals. Immunoreactivity for nitric oxide synthase (NOS) and nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) activity, which is considered to be a histochemical marker for catalytic enzyme activity for NOS in the nervous system (Dawson et al. 1991; Hope et al. 1991; Blottner et al. 1995), has been found in the primary sensory neurons of NIX and NX in mammals (Aimi et al. 1991; Forster and Southam 1993; Alm et al. 1995; Fischer et al. 1996; Ichikawa and Helke 1996; Kadota et al. 1996; Zhuo et al. 1997). It has also been found in the solitary nucleus or its homologue in various mammals (Vincent and Kimura 1992; Forster and Southam 1993; Ruggiero et al. 1996; Krowicki et al. 1997), in the chicken, *Gallus domesticus* (for birds, Brüning 1993), and in the turtle, *Pseudemys scripta elegans* (for reptiles, Brüning et al. 1994), suggesting that nitric oxide (NO) might act as a neuronal messenger in the visceral sensory system of NIX and NX in all amniotes. In mammals, nitrergic afferent innervation by NIX and NX was demonstrated in the trachea (Fischer et al. 1996), the carotid body (Wang et al. 1993; Höhler et al. 1994), the carotid sinus (Höhler et al. 1994), and the basilar artery (Kadota et al. 1996). But the gastrointestinal canal, especially the esophagus and the stomach, also appears to have nitrergic vagal afferent innervation (Forster and Southam 1993; Ruggiero et al. 1996; Krowicki et al. 1997).

Fishes, which possess gills as their respiratory organs, have different cranial nerve composition from amniotes. In the dogfish *Triakis scyllia* (Elasmobranchs), NADPHd activity was found in the primary sensory neurons of NX, suggesting that NO might act as a neuronal messenger in the primary visceral sensory system in a wide variety of vertebrates from fishes to mammals (Funakoshi et al. 1997). But in *T. scyllia*, a positive reaction was found almost only in neurons projecting to the coelomic organs, but not in neurons projecting to the gills

(Funakoshi et al. 1997). In teleosts, on the other hand, dense concentrations of NOS-immunoreactive and NADPHd-positive nerve terminals were found in the vagal lobe of the Atlantic salmon, *Salmo salar* (Holmqvist et al. 1994), suggesting that NOS might be present in the visceral sensory pathway to the gills. Therefore, to examine the precise distribution of NOS in the visceral sensory system of teleosts, we examined NADPHd activity and immunoreactivity for NOS in the ganglia of NIX and NX, and their central and peripheral projections to the gill arches of a teleost, the puffer fish *Takifugu niphobles*.

Materials and methods

We used seven puffer fish, *Takifugu niphobles* (Tetraodontiformes), captured and maintained in a 700-l aquarium. The fish were anesthetized with 0.01% tricaine methanesulfonate (MS-222) in water and perfused transcardially with heparinized saline, followed by a solution containing 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH 7.4). NIX, NX, and the gill structures were removed en bloc, postfixed immediately in the same solution for 5–6 h at 4°C, and infiltrated overnight with 30% sucrose in 0.1 M PBS at 4°C for cryoprotection. The specimens were cut serially into 30-µm cross sections on a cryostat and thaw-mounted on glass slides. For NADPHd staining, the sections were postfixed for 15 min and then incubated in 0.1 M PB containing 0.25% Triton-X 100 for 5 min. Then, the sections were incubated in 0.1 M PB containing 0.25% Triton-X 100, 0.5 mg/ml β-NADPH (Sigma, St. Louis, MO, USA), and 0.2 mg/ml nitroblue tetrazolium (Sigma) for 2 h at 37°C.

For immunohistochemistry for NOS, the sections were incubated in the fixative for 10 min and washed in several changes of 0.3% Triton X-100 in 0.1 M PBS (PBST). Then the sections were incubated with 10% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.2% sodium azide in PBST for 1 h at room temperature. After rinsing in PBST, the sections were incubated for 48 h at 4°C with the rabbit polyclonal antibody raised against a C-terminal fragment of rat cerebellar NOS (1:1500; Euro-Diagnostica, Malmö, Sweden), diluted in a solution of 1% NGS, 0.2% BSA, and 0.2% sodium azide in PBST. This antibody is highly specific to neuronal NOS (Alm et al. 1993) and effective even in the nervous tissue of teleosts (Holmqvist et al. 1994; Östholm et al. 1994; Holmqvist and Ekström 1997). Next, the sections were incubated for 2 h with anti-rabbit IgG (1:100; Cappel, Aurora, Ohio, USA), at room temperature, and for an additional 2 h with peroxidase-anti peroxidase complex (1:200; Jackson, West Grove, Pa., USA). The peroxidase reactivity was demonstrated with 3,3´-diaminobenzidine (Sigma). The specificity of the staining was tested by omission of the antibody.

To examine the colocalization of NADPHd activity and NOS immunoreactivity, the sections were first incubated for 48 h at 4°C with the antibody against neuronal NOS (1:1000; Euro-Diagnostica), diluted in a solution of 1% NGS, 0.2% BSA, and 0.2% sodium azide in PBST. Then the sections were incubated for 2 h with rhodamine-conjugated goat anti-rabbit IgG (1:100; Cappel) at room temperature. Slides were mounted in glycerine-PBS and examined with an epifluorescence microscopy (Leica, Welzlar, Germany). After rinsing in PBST, the sections were processed for NADPHd staining.

For a control study, a series of sections were stained with hematoxylin and eosin (H&E).

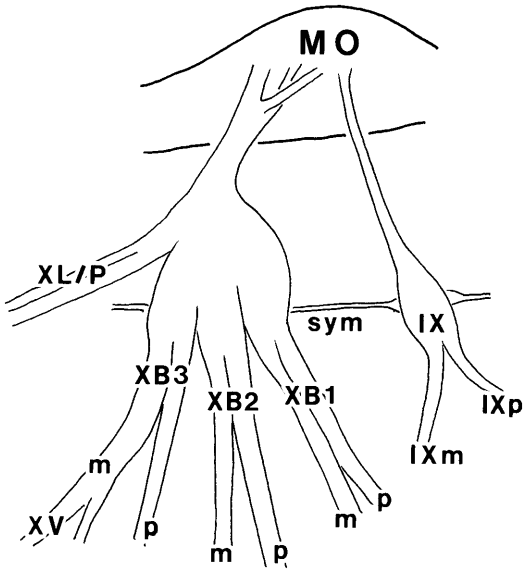


Fig. 1 Lateral view of the glossopharyngeal and vagal sensory ganglia and nerves on the right side. Each is also divided into a protrematic branch (*p*) and a metatrematic branch (*m*) (*MO* medulla oblongata, *IX* glossopharyngeal nerve, *IXp* protrematic branch of glossopharyngeal nerve, *IXm* metatrematic branch of glossopharyngeal nerve, *XB1–3* first to third branchial rami of vagus nerve, *XV* visceral ramus of vagus nerve, which is a continuation of XB3m, *XL/P* posterior ramus and lateral line ramus of vagus nerve, *sym* sympathetic trunk)

Results

Anatomy of the glossopharyngeal and vagus nerves

NIX is the branchial nerve that innervates the oral plate, the pharynx, and the first gill arch. NX has three branchial nerves, named the first, second, and third branchial rami (NXB1–3), supplying fibers to the pharynx, the heart, the coelomic viscera, and three gill arches of this fish. Both NIX and NXB1–3 branch into the protrematic nerve and the metatrematic nerve (Fig. 1). The metatrematic ramus of NIX (NIXm) and the protrematic ramus of NXB1 (NXB1p) innervate the first gill arch. The metatrematic branch of NXB1 (NXB1m) and the protrematic branch of NXB2 (NXB2p) innervate the second gill arch, and NXB2m and NXB3p innervate the third gill arch. NXB3m is the visceral nerve that branches to the pharyngeal ramus and the visceral ramus (NXV) that enters the coelomic cavity to innervate the gastrointestinal organs. In addition to the branchial nerves, NX has a posterior ramus (NXP) that innervates the operculum, the pectoral fin muscles, and the adjacent skin, and a lateral line ramus (NXL) that innervates the lateral line organs. The vagal sensory ganglion located in the proximal part of the vagal nerve trunk is clearly divided into five parts corresponding to five rami of NX. Thus there are three ganglia for the branchial rami, one for the NXP, and one for the NXL. The ganglion for NXB3 is incompletely separated into a branchial part that projects to NXB3p and a visceral part that projects to NXB3m.

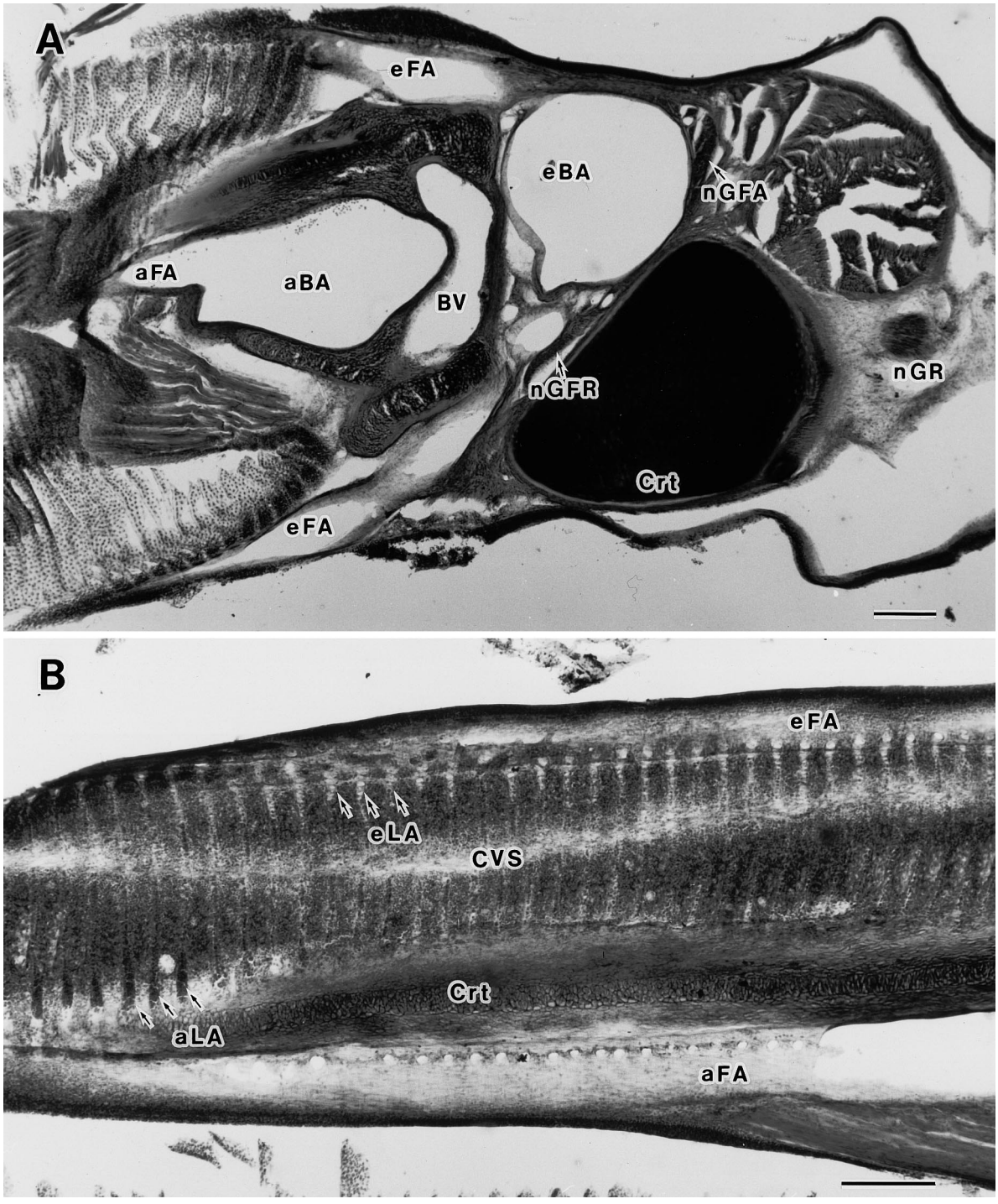


Fig. 2A,B H&E staining of the gill filament. **A** Proximal part of the gill filament (*aBA* afferent branchial artery, *aFA* afferent filament artery, *eBA* efferent branchial artery, *eFA* efferent filament artery, *BV* branchial vein, *nGR* gill raker nerve, *nGFA* anterior gill filament nerve, *nGFR* posterior gill filament nerve, *Crt* cartilage). **B** Distal part of the gill filament (*aFA* afferent filament artery, *eFA* efferent filament artery, *CVS* central venous sinus, *aLA* afferent lamellar arteriole, *eLA* efferent lamellar arteriole, *Crt* cartilage). *Bars* 100 µm

Anatomy and innervation of the gill vasculature

There are three gill arches in *T. niphobles*. Each contains two rows of gill rakers on the inner edge and two rows of gill filaments on the outer edge. As in all other teleosts (Laurent and Dunel 1976; Laurent 1984; Donald 1987), the gill vasculature consists of the arterioarterial pathway, which mediates gas exchange in the gill lamellae and continues to the cranial and systemic circulation, and the arteriovenous pathway, which provides perfusion and drainage of the gill structures to mediate nutritive and

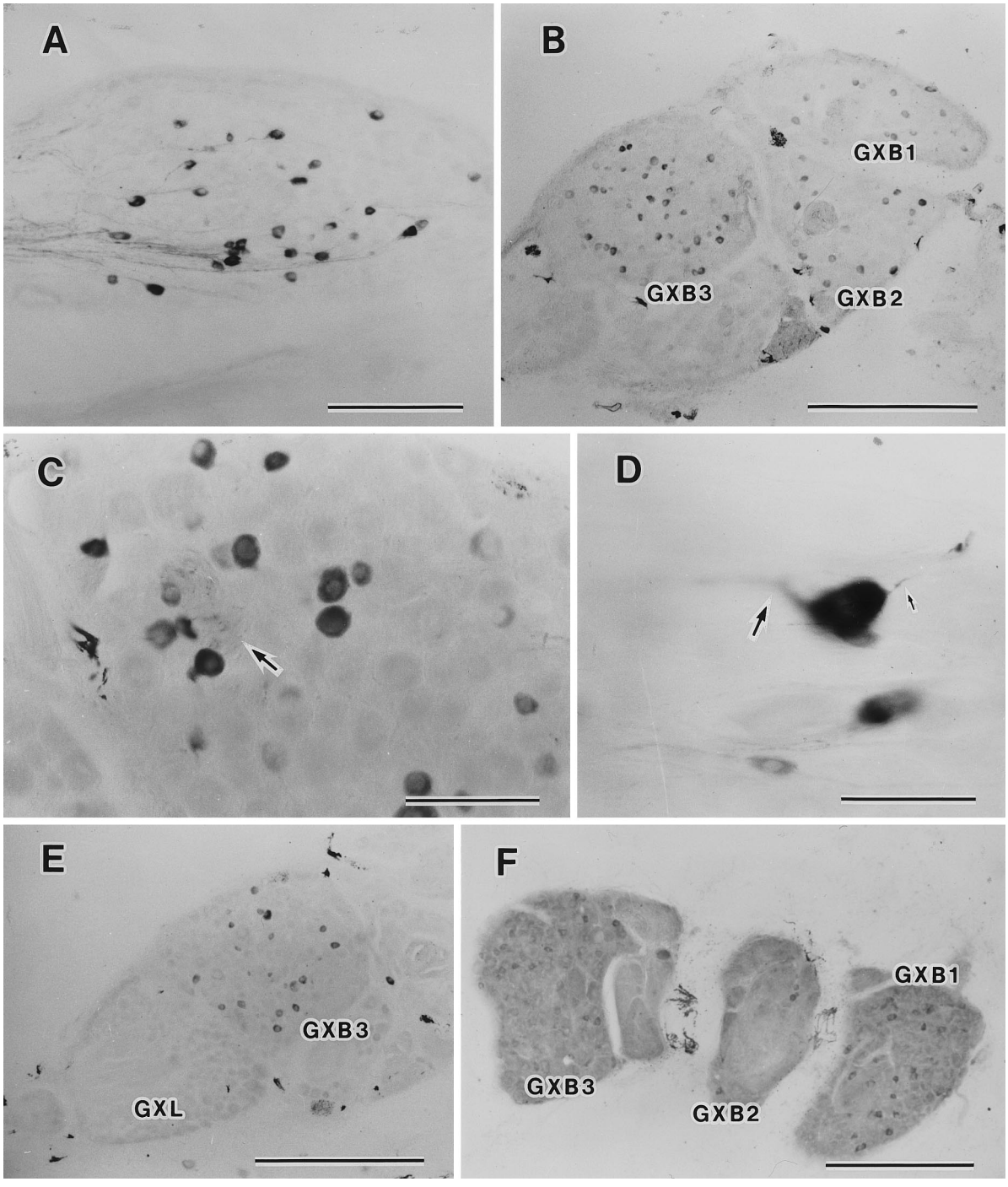


Fig. 3 **A** Nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd)-positive neurons scattered in the glossopharyngeal ganglion (GIX). **B** NADPHd-positive neurons were seen in the vagal ganglia of the first to third branchial rami: GXB1 (*right*), GXB2 (*middle*), and GXB3 (*left*). **C** NADPHd activity was found predominantly in small neurons in the GXB2. The nerve bundle of the GXB2 (*arrow*) was also stained. **D** Higher magnification of a NADPHd-positive neuron, with a central (*small arrow*) and a peripheral (*large arrow*) process. **E** NADPHd-positive neurons were seen in the *GXB3*, but not in the the ganglia of the posterior ramus (*GXL*). **F** NOS-immunoreactive neurons were seen in the in the GXB1 (*right*), GXB2 (*middle*), and GXB3 (*left*). *Bars* 200 µm **(A);** 500 µm **(B,E,F);** 100 µm **(C);** 50 µm **(D)**

osmoregulatory functions. The arterioarterial pathway includes the afferent branchial artery, the afferent filament artery, the afferent lamellar arteriole, the capillaries in each lamella, the efferent lamellar arteriole, the efferent filament artery, and the efferent branchial artery. The arteriovenous pathway arising from both the efferent branchial artery and the efferent filament artery includes small arteries in each lamella, the central venous sinus, and the branchial veins (Fig. 2).

In each gill arch, there are three major nerve bundles: the gill raker nerve, the anterior gill filament nerve, and the posterior gill filament nerve. The last two course

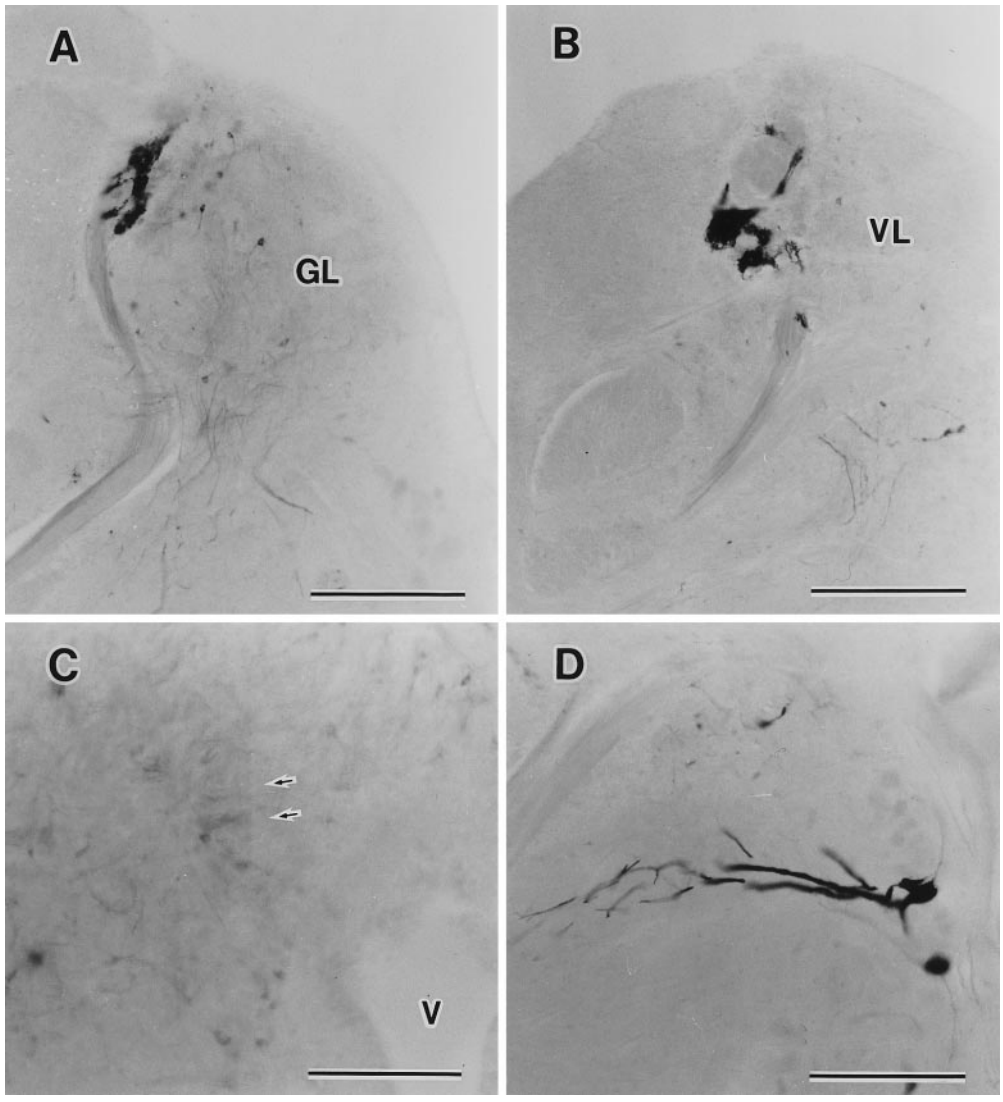


Fig. 4 **A** NADPHd-positive nerve fibers of the glossopharyngeal nerve terminated in the glossopharyngeal lobe (*GL*). **B** NADPHd-positive nerve fibers of the vagus nerve terminated in the vagal lobe (*VL*). **C** NADPHd-positive nerve terminals (*arrows*) in the commissural nucleus (*V* ventricle). **D** Some neurons in the caudal portion of the vagal motor cell column were NADPHd-positive. *Bars* 200 µm **(A,B,D)**; 100 µm **(C)**

along the efferent branchial artery and ramify to run along the efferent filament artery that branches from the efferent branchial artery. The anterior gill filament nerve appears to originate from the metatrematic ramus and the posterior gill filament nerve from the protrematic ramus of the branchial nerve.

NADPHd activity and NOS immunoreactivity in the glossopharyngeal and vagal sensory ganglia

NADPHd-positive neurons were found in the glossopharyngeal ganglion (GIX) and in the vagal ganglia of the first to third branchial rami (GXB1–3), but not in the ganglia of the posterior ramus (GXP) or the lateral line ramus (GXL; Fig. 3A–C,E). In the GXB1–3, a total of 18.5% of neurons were NADPHd-positive. The proportion of positive neurons was almost equal among these three ganglia, but activity was weaker in the visceral part of the GXB3 than in any other part of the vagal ganglion. Most of the positive neurons were small to medium, 10–24 µm (mean 16.4 µm) in diameter, and bipolar, with well-stained processes (Fig. 3D). NADPHd-positive nerve fibers issuing from the ganglia were also seen peripherally in all of the branchial rami including NXV, and centrally in the nerve roots.

Many NOS-immunoreactive neurons were also seen in both the GIX and GXB1–3, but not in the GXP and GXL (Fig. 3F). Most NOS-immunoreactive neurons in the ganglia showed NADPHd activity and vice versa; but a small population of immunopositive neurons showed only faint staining of NADPHd, and some NADPHd-positive neurons showed only weak NOS immunoreactivity (Fig. 4C,D).

NADPH-diaphorase activity and NOS immunoreactivity in the medulla oblongata

In the medulla, NADPHd-positive nerve fibers were found in the nerve fascicles of the branchial rami of NIX and NX. These nerve fibers formed a dense NADPHd-

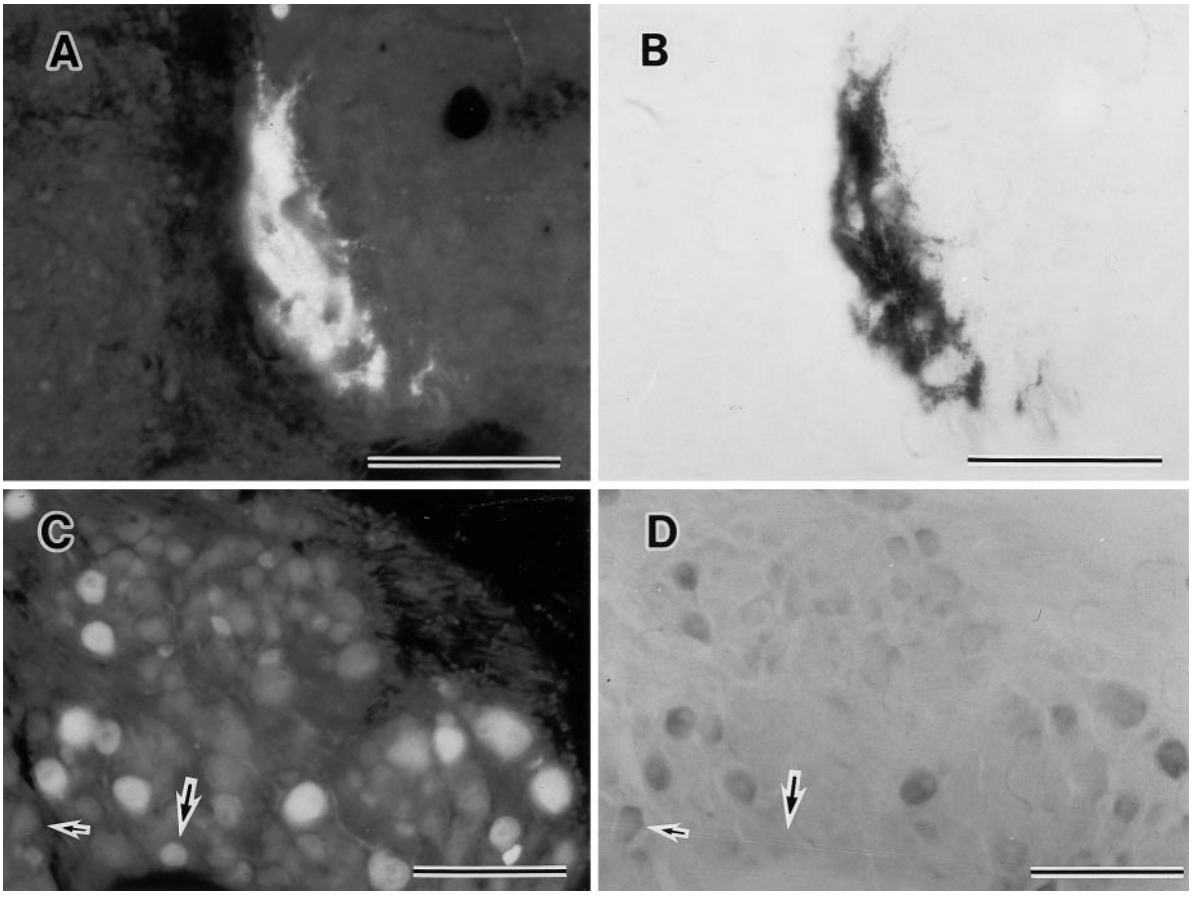


Fig. 5A–D Double staining using NOS immunohistochemistry and NADPHd histochemistry. **A** Rhodamine-labeled NOS-immunoreactive nerve terminals in the vagal lobe. **B** NADPHd-positive nerve terminals in the vagal lobe (the same section as in **A**). **C** Rhodamine-labeled NOS-immunoreactive neurons were scattered in the GXB3. **D** NADPHd staining in the same section as in **C**. Most of the NADPHd-positive neurons were also positive for NOS immunoreactivity. A neuron strongly positive for NOS but only weakly positive for NADPHd (*large arrow*), and a neuron weakly positive for NOS but strongly positive for NADPHd (*small arrow*) were visible. *Bars* 100 µm

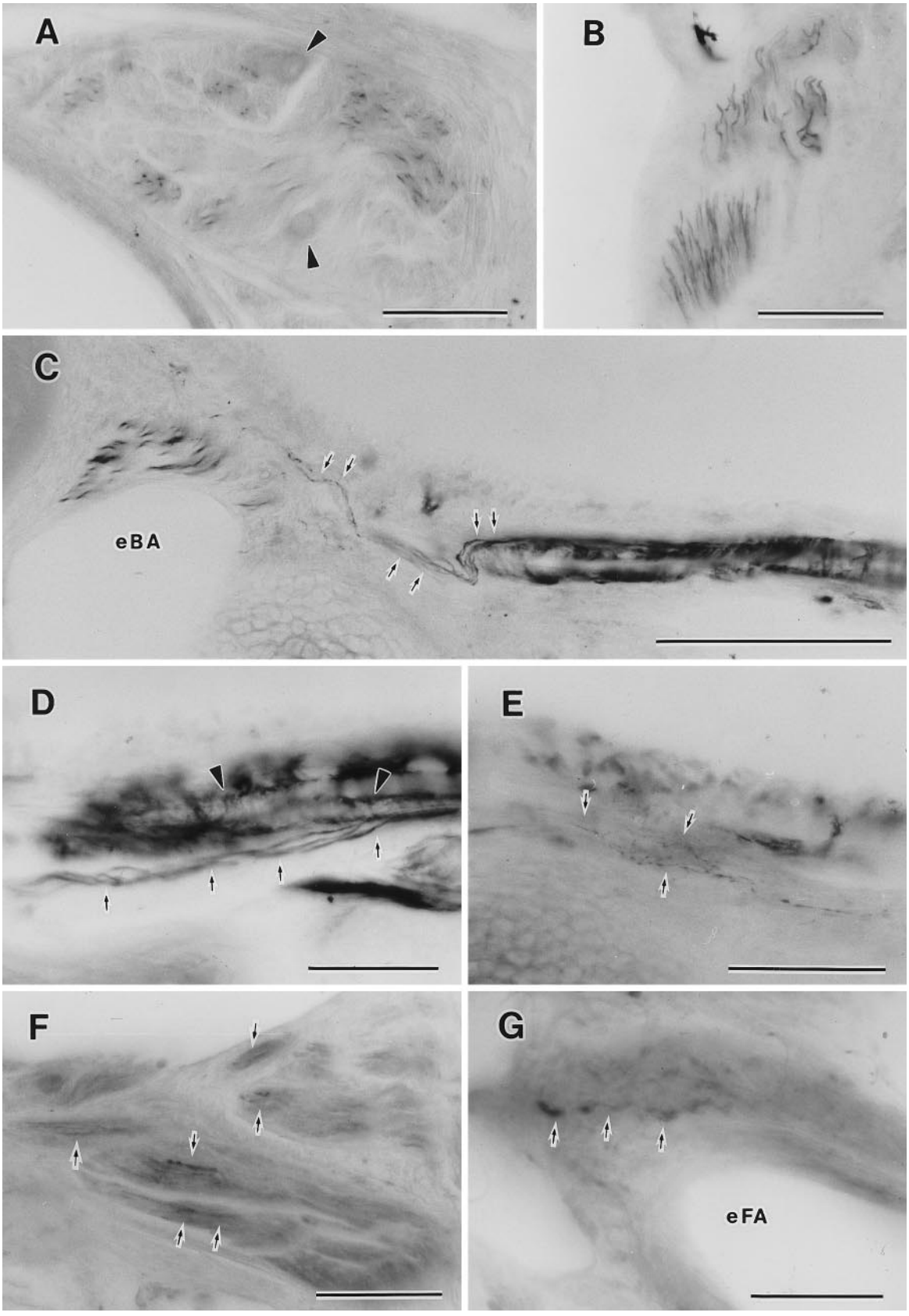
positive group of nerve terminals in the lateral portion of the glossopharyngeal lobe and the vagal lobe, in the form of a continuous visceral sensory column (Fig. 5A,B). Inside the glossopharyngeal lobe, some small intrinsic neurons were also stained (Fig. 5A). NADPHd-positive nerve fibers were also found in the nerve fascicles of the visceral ramus of NX. Some nerve terminals in the commissural nucleus were also NADPHd-positive (Fig. 5C). The neuronal cell bodies and axons in the caudal portion of the vagal motor nucleus were NADPHd-positive, whereas those in the rostral portion were NADPHd-negative (Fig. 5D).

The distribution of the NOS-immunoreactive nerve fibers and terminals was identical to that of the NADPHd-positive ones (Fig. 4A,B).

NADPH-diaphorase activity and NOS immunoreactivity in the gill arch

In each gill arch, NADPHd-positive nerve fibers were found in the nerve bundles of the anterior gill filament nerve and the posterior gill filament nerve, but not in those of the gill raker nerve (Fig. 6A,B). NADPHd-positive nerve fibers in the anterior gill filament nerve and the posterior gill filament nerve branched to run along the efferent filament artery (Fig. 6C,E). NADPHd-positive fine varicose nerve terminals were found on the wall of the most proximal portion of the efferent filament ar-

Fig. 6 A NADPHd-positive nerve fibers in the nerve bundle of the anterior gill filament nerve of the second gill arch. Parasympathetic neurons (*arrowheads*) were NADPHd-negative. **B** NADPHd-positive nerve fibers in the nerve bundle of the posterior gill filament nerve of the second gill arch. **C** NADPHd-positive nerve fibers (*small arrows*) branched from the posterior gill filament nerve to course along the efferent filament artery (*eBA* efferent branchial artery). **D** Higher magnification of NADPHd-positive nerve fibers (*small arrows*) that run in the vicinity of the endothelial cell layer of the efferent filament artery. The endothelial cells and the elastica interna also showed strong NADPHd activity (*arrowheads*). **E** NADPHd-positive varicose nerve terminals (*small arrows*) on the most proximal portion of the efferent filament artery. **F** NOS-immunoreactive nerve fibers (*small arrows*) in the nerve bundle of the anterior gill filament artery of the second gill arch. **G** NOS-immunoreactive varicose nerve terminals (*small arrows*) on the most proximal portion of the efferent filament artery (*eFA* efferent filament artery). *Bars* 100 µm **(A,B,F)**; 500 µm **(C)**; 200 µm **(D,E);** 50 µm **(G)** ▶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tery (Fig. 6D). NADPHd-positive nerve fibers could be traced along the wall of the proximal portion of the efferent filament artery, but the precise morphology of the terminals was not clear, because the other vascular structures such as endothelial cells and the elastica interna of the efferent filament artery also showed strong NADPHd activity (Fig. 6E). On the other hand, only a few NADPHd-positive nerve fibers were found around the efferent branchial artery, afferent branchial artery, the afferent filament artery, the afferent lamellar arteriole, the efferent lamellar arteriole, the central venous sinus, and the branchial vein.

Distribution of NOS-immunoreactive nerve fibers in the gill arch was the same as that of NADPHd-positive nerve fibers; thus they were seen in the nerve bundles of the anterior gill filament nerve and the posterior gill filament nerve, but not in the gill raker nerve (Fig. 6F). NOS immunoreactivity was also seen in the varicose nerve terminals on the proximal portion of the efferent artery (Fig. 6G). In the distal portion of the efferent filament arteries, where NADPHd-positive structures were found in the endothelial layer, no NOS-immunoreactive structures were seen.

The parasympathetic neurons, located in the nerve bundles at the levels of the branching points of the efferent filament arteries from the efferent branchial artery, were not stained by either NADPHd histochemistry or NOS immunohistochemistry (Fig. 6A). Neurons in the glossopharyngeal and vagal sympathetic ganglia were also devoid of NADPHd activity and NOS immunoreactivity.

Discussion

The visceral sensory system of NIX and NX of teleosts is divided into an exteroceptive component, innervating the oropharyngeal and branchial structure via the branchial rami, and an interoceptive component, innervating the coelomic visceral organs via the visceral ramus (Kanwal and Caprio 1987). Our results suggest that NOS is present in both of the two visceral sensory systems of *T. niphobles*. In the vagal sensory ganglion of an elasmobranch fish, the dogfish *T. scyllia*, NADPHd activity was almost entirely confined to neurons of the coelomic organs and few positive neurons were seen in the glossopharyngeal ganglion or the branchial part of the vagal ganglion (Funakoshi et al. 1997). Therefore, nitrergic sensory innervation of the coelomic viscera is common in both teleosts and elasmobranchs and may have been retained in the evolutionary process up to mammals (Funakoshi et al. 1997), whereas that of the branchial structures might be specific only to teleosts. It is not known whether NOS is present in NIX and NX of amphibians, which also have gills in their larval stage.

In the present study, we found that nitrergic nerve terminals in the medulla were rostrocaudally divided into two parts, i.e., the lateral portion of the visceral sensory column and the commissural nucleus. In light of retrograde tracing studies in other teleosts (Kanwal and Caprio 1987; Morita and Finger 1987; Díaz-Regueira and Anadón 1992), the lateral portion of the visceral sensory column is probably the site of the branchial afferent termination, whereas the commissural nucleus is the site of the coelomic afferent termination.

A previous study reported considerable discrepancy between NOS-immunoreactive cells and NADPHd-positive cells in the vagal nerve cells in teleosts (Li and Furness 1993). The present results using a specific antibody against mammalian neuronal NOS showed that NOS immunoreactivity and NADPHd activity were almost identical in the glossopharyngeal and vagal ganglion cells, but some strongly NADPHd-positive neurons did not exhibit strong NOS immunoreactivity, and some strongly NOS-immunoreactive neurons showed only weak NADPHd activity. Such different levels of staining by two methods were reported also in the vagal autonomic neurons in the goldfish, from a study using a different antibody raised against mammalian neuronal NOS (Brüning et al. 1996). Different isoforms of neuronal NOS might be present in the teleost peripheral nervous tissue.

In the gill arch of *T. niphobles*, NADPHd activity and NOS immunoreactivity were shown in the nerve bundles of the anterior gill filament nerve and the posterior gill filament nerve. These nitrergic nerve fibers appeared to run exclusively to the efferent filament artery and not to other vessels. Varicose nerve terminals positive for both NADPHd activity and NOS immunoreactivity were found on the arterial walls of the proximal portion of the efferent filament arteries of all three gill arches. Previously, the gill vasculature of teleosts was reported to be innervated by the parasympathetic cholinergic nerves of the branchial nerves, the serotonergic parasympathetic nerves, the sympathetic adrenergic nerves, and the sensory nerves (Bailly and Dunel-Erb 1986; Dunel-Erb and Bailly 1986; Donald 1987, 1998; Bailly et al. 1989; Dunel-Erb et al. 1989; Nilsson and Holmgren 1992). In the cod, *Gadus morhua*, NADPHd activity was found in the parasympathetic postganglionic neurons in the branchial nerves of NIX and NX (Gibbins et al. 1995). However, in *T. niphobles*, no NADPHd activity or NOS immunoreactivity was present either in the parasympathetic postganglionic neurons of the branchial nerves or in the sympathetic postganglionic neurons innervating the gills. Therefore, the nitrergic nerve terminals on the efferent filament artery of *T. niphobles* must be peripheral processes of the sensory neurons of NIX and NX, which pass through the branchial rami.

Previously, some physiological studies have reported that the gills of teleosts have a baroreceptor function, as well as chemoreceptive, proprioceptive, and nociceptive functions (Nilsson 1984; Burleson and Milson 1993; Olson 1998). Baroreceptors are free nerve endings located in the arterial wall and activated by vascular stretch during increases in arterial pressure. In mammals, baroreceptors are located in the carotid sinus at the bifurcation of the carotid artery and in the aortic arch, and these are homologous to the branchial arteries of the first and

the second gill arches of fishes, respectively. Studies have shown that NADPHd activity and NOS immunoreactivity are present in the baroreceptive sensory nerve fibers innervating the carotid sinus in the rat (Höhler et al. 1994). The distribution of nitrergic afferent innervation of the branchial arteries, shown in the present study, is consistent with the physiological data from the rainbow trout, *Oncorhynchus mykiss*, in which baroreceptor activity was detected in all of the gill arches (Burleson and Milson 1993). Therefore, it is possible that the nitrergic sensory nerves innervating the efferent filament artery in *T. niphobles* function as baroreceptors, which transmit signals to the medullary visceral sensory column.

Many studies have examined the role of NO in the baroreceptor reflex system in mammals. Because the baroreflex responses were reduced by the administration of NOS inhibitors into the solitary nucleus, NO generated in the solitary nucleus has been postulated to be involved in modulation of the baroreceptor reflex in mammals (Lo et al. 1996, 1997). On the other hand, NO released from the peripheral sensory nerve terminals has been shown to mediate vasodilatation (Zheng et al. 1997; Merhi et al. 1998), and thus it is possible that vascular relaxation caused by NO released from the baroreceptor terminals might modulate sensitivity of the baroreceptors themselves. A recent in vitro study reported that NO functions as an autocrine modulator of the sodium channel in baroreceptive neurons (Li et al. 1998). Therefore, it is also possible that NO modulates baroreceptor sensitivity via the sodium channel. The present study demonstrated the presence of NOS in the baroreceptor sensory nerves in teleosts, which is a trait held in common with mammals. Thus, the role of NO as a sensory modulator of baroreceptors might have been retained in the evolutionary process from fish to mammals, despite the significant change in the branchiomeric organs and vasculature.

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