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N-methyl-D-aspartate receptors mediate chemoreflexes in the shorthorn sculpin *Myoxocephalus scorpius*

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Summary

Glutamate microinjected into the vagal sensory area in the medulla produces cardiorespiratory responses mimicking oxygen chemoreflexes in fish. Here we directly investigate whether these reflexes are dependent on the ionotropic *N*-methyl-D-aspartate (NMDA) glutamate receptor.

Fish were equipped with opercular, branchial and snout cannulae for measurements of cardiorespiratory parameters and drug injections. Oxygen chemoreceptor reflexes were evoked by rapid hypoxia, NaCN added into the blood (internal, 0.3 ml, 50 μg ml^{-1}) and the mouth (external, 0.5 ml, 1 mg ml^{-1}), before and after systemic administration of the NMDA receptor antagonist MK801 (3 mg kg^{-1}).

Hypoxia produced an MK801-sensitive increase in blood pressure and ventilation frequency, whereas the marked bradycardia and the increased ventilation amplitude were NMDA receptor-independent. The fish appeared more responsive to externally applied cyanide, but the injections and MK801 treatment did not distinguish whether external or internal oxygen receptors were differently involved in the hypoxic responses.

In addition, using single-labelling immunohistochemistry on sections from the medulla and ganglion nodosum, the presence of glutamate and NMDA receptors in the vagal oxygen chemoreceptor pathway was established.

In conclusion, these results suggest that NMDA receptors are putative central control mechanisms that process oxygen chemoreceptor information in fish.

Key words: Fish, hypoxia, NaCN, cardiovascular, respiration, nucleus tractus solitarius, glutamate, *N*-methyl-D-aspartate (NMDA) receptor.

Introduction

When fish are exposed to environmental hypoxia, a host of cardiorespiratory reflexes are elicited to correctly balance oxygen uptake with metabolic demand. Generally the cardiovascular reflexes consist of a marked bradycardia (Randall and Smith, 1967; Burleson and Smatresk, 1990) and an increase in vascular resistance (Holeton and Randall, 1967; Sundin and Nilsson, 1997), which may (depending on the severity of the bradycardia) result in an elevated blood pressure. The respiratory responses consist of an increase in ventilation amplitude and frequency (Hughes and Shelton, 1962; Saunders and Sutterlin, 1971; Sundin et al., 1999). The majority of the oxygen chemoreceptors that elicit these reflexes are situated in the gills, and are innervated by glossopharyngeal (IX) and vagal (X) nerves (for a review, see Sundin and Nilsson, 2002). From recordings in the IXth cranial nerve of afferent activity from single receptors in the isolated first gill arch of yellow fin tuna (Milsom and Brill, 1986) and rainbow trout (Burleson and Milsom, 1993) it was shown that the oxygen chemoreceptors can be divided into different groups, depending on their orientation: receptors that sense the oxygen levels in the environment (external O_2-receptors), or the blood (internal O_2-receptors), or receptors that monitor both media. These different groups of oxygen chemoreceptors may elicit different cardiorespiratory reflexes (Milsom, 1996; Milsom et al., 2002; Sundin et al., 1999).

In fish, the afferent signals from the branchial oxygen receptors terminate in the dorsomedial part of the medulla oblongata, in a bilateral, elongated structure along both sides of the fourth ventricle named the nucleus tractus solitarius (NTS). In the sculpin the vagal portion of the NTS, the vagal sensory area (Xs), has recently been defined and it stretches in a rostrocaudal direction from 2.0 mm rostral to obex to 0.5 mm caudal of obex (Sundin et al., 2003).

In the NTS the first integrative step of sensory input takes place (for a review, see van Geirsbergen et al., 1992), and the excitatory amino acid glutamate is probably the neurotransmitter being released by most of these sensory terminals (Saha et al., 1995; Sykes et al., 1997; Mizusawa et al., 1994; Perrone, 1981). It has also been established that an ionotropic *N*-methyl-D-aspartate (NMDA) glutamate receptor, found within NTS on both interneurons and afferent terminals (Aicher et al., 1999), is involved in the central mediation of

oxygen chemoreceptor inputs and the subsequent development of chemoreflexes in mammals (Aylwin et al., 1997; Haibara et al., 1995; Lin et al., 1996; Mizusawa et al., 1994; Ohtake et al., 1998).

While there exists some knowledge regarding hypoxia-induced cardiorespiratory reflexes and their control *via* the autonomic nervous system in fish, much less is known about the sensory system, particularly the central mechanisms involved in the processing of oxygen chemoreceptor information. As a first step towards establishing glutamate as an important transmitter in cardiorespiratory control in fish, Sundin et al. (2003) showed that glutamate, microinjected into the rostral end of the Xs in the shorthorn sculpin, could evoke cardiorespiratory responses that mimicked oxygen chemoreceptor-activated chemoreflexes. To continue that study, the first aim of this paper was to determine whether glutamate, *via* the NMDA receptor, is involved in the development of oxygen chemoreflexes in fish. The second aim was to establish the location of glutamate and NMDA receptors in the vagal sensory system, comprising vagal fibres and cell bodies in the ganglion nodosum, and the Xs. In addition, we sought to distinguish whether the activation of both external and/or internal oxygen chemoreceptors utilize NMDA receptors to produce cardiorespiratory reflexes.

Materials and methods

Animals

Shorthorn sculpins *Myoxocephalus scorpius* L., body mass 121–205 g, were used in the experiments. The fish, caught outside the Swedish West Coast, were placed in tanks with recirculating seawater (10°C) and allowed to recover for at least 2 days prior to use. All animal experiments were approved by the local ethical committee in Gothenburg (permit no. 299/99).

Immunohistochemical experiments

The presence of glutamate and NMDA receptors in vagal nerve fibres and sensory cell bodies in ganglion nodosum and the Xs was established using single-labelling immunohistochemistry (antisera listed in Table 1).

Ten animals were killed with an overdose of MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt, 300–400 mg l^{-1} seawater; Sigma), and the medulla together with the ganglion nodosum were removed and fixed in 4% formaldehyde overnight. After fixation the tissues were rinsed in phosphate buffer (PBS, 0.9% NaCl) for 30 min and then cryoprotected with a PBS-sucrose solution (0.9% NaCl, 30% sucrose) overnight. The tissues were embedded in mounting medium (Tissue-teck, Sakusa, Zoeterwoude, Netherlands) and frozen in isopentane cooled in liquid nitrogen. Cross sections of the medulla and planar sections comprising the ganglion nodosum (12 μm thick) were cut on a cryostat (Cryo-Star HM 560 M. Microm, Walldorf, Germany) and mounted on gelatine-coated slides. Immunostaining was performed by incubating the slides with primary antiserum against glutamate and the NMDAR1 subunit of the NMDA receptor (Table 1) for 24 h at room temperature. Excess primary antiserum was removed by rinsing in PBS (2.0% NaCl) for 3×10 min. The slides were then incubated with the secondary antiserum DaR-CY3 or DaR-FITC (Table 1) for 60 min. After an additional rinse in PBS (2.0% NaCl) for 3×10 min, coverslips were placed over carbonate-buffered glycerol on the slides. Finally, the slides were examined using a fluorescence microscope (Olympus BX 60, Olympus Optical Co. Ltd., Tokyo, Japan). Photographs were taken using a Nikon digital camera DMX 1200. In order to reduce unspecific staining the glass slides were pre-incubated with normal donkey serum (NDS) before the primary antibody incubation was begun.

To visualize the general histology of the labelled sections, some were stained for Nissl substance.

Specificity controls of antisera

To control the specificity of the glutamate primary antiserum, an absorption test was conducted according to the protocols given in Ottersen et al. (1986). Primary antiserum against glutamate was pre-incubated with glutaraldehyde-conjugated glutamate (10^{-2} mol l^{-1}) for 20 h at 4°C. This mixture was added to glass slides with sections from both the medulla and ganglion nodosum. The specificity of the secondary antiserum was assessed by adding a ‘blank’ solution (without the primary antiserum) to the slides. Both the absorption and blank tests were performed in parallel with positive controls.

Since the NMDAR1 antigen could not be obtained from

Table 1. *Primary and secondary antisera used in the immunohistochemical experiments*

	Code	Host	Dilution	Source
Primary antiserum raised against				
Glutamate	G6642	Rabbit	1:400	Sigma
NMDAR1*	AB-1516	Rabbit	1:50	Chemicon
Secondary antiserum raised against				
DaR-FITC, rabbit IgG	711-095-152	Donkey	1:100	Jackson
DaR-CY3, rabbit IgG	711-165-152	Donkey	1:800	Jackson

Addresses of supplies: Sigma (St Louis, USA), Chemicon (Temecula, USA), Jackson (West Grove, USA).
*Subunit of the NMDA receptor.

Chemicon Inc., only the blank test was performed. However, according to the producer Chemicon Inc. and Flynn et al. (1999), this antibody is very specific and does not crossreact with any other glutamate receptor subunits in fish.

Cardiorespiratory experiments

Surgical procedure

The fish (N=7) were anaesthetised in MS222 (100 mg l^{-1} seawater) until spontaneous breathing stopped, and then transferred to an operating table where cooled (10°C) aerated seawater containing MS222 (50 mg l^{-1} seawater) was passed over the gills throughout surgery. A polyethylene cannula (PE50), tipped with a thinner cannula (PE10), was inserted into the afferent branchial artery of the third gill arch according to the method described by Axelsson and Fritsche (1994). The cannula, filled with heparinized (100 i.u. ml^{-1}) 0.9% NaCl, was used for ventral aortic pressure measurements (P_{VA}), heart rate (f_H) and intra-arterial administrations of drugs. A second cannula (PE90) was inserted through the snout for drug administration into the respiratory water. Finally, for measurements of ventilation amplitude (V_{AMP}) and frequency (f_V), a third cannula (PE90) was inserted into the branchial operculum. After surgery the fish were transferred to an experimental chamber, with a slow water flow (approx. 1 l min^{-1}), and allowed to recover for 24 h before the experiments started.

Equipment

Branchial and opercular cannulae were connected to pressure transducers previously calibrated against a static water column. The ventilation and P_{VA} signals were amplified using a Grass low-level d.c. (model 7P122B, Quincy, USA) and continuously sampled at 20 Hz using a data acquisition software program (Labview version 5.0, National Instruments, Austin, USA). V_{AMP}, f_V and f_H were calculated from these signals using a Labview-based calculation program. Mean values were created at 10 s intervals for both internal and external NaCN exposure, and a representative value from the resting period and the maximum response, respectively, were chosen for each animal and plotted as histograms. For the hypoxia exposure experiments, the mean values were created at 5 s intervals, and to reduce the large set of data every third value was selected and plotted on the graphs.

Experimental protocols

The experiments started with control injections of seawater (0.8 ml) into the snout cannula and saline solution (0.9% NaCl, 0.3 ml) into the arterial cannula to ensure that the vehicle in itself had no effect. The control injections were followed by stimulating both external oxygen chemoreceptors, by NaCN injections into the respired water (0.5 ml, 1.0 mg ml^{-1}), and internal receptors, by NaCN injections into the afferent branchial artery (0.3 ml, 50 μg ml^{-1}), respectively. In addition, the fish were also subjected to a 10 min hypoxic period. The three types of chemoreceptor stimulation were performed randomly and the fish were allowed to recover to pre-exposure values. Different NaCN concentrations were assessed to determine the lowest concentration that gave clear responses for each animal, and then the same concentration was used after MK801 treatment. The chosen concentrations lie within range of previous studies (Burleson and Smatresk, 1990; Sundin et al., 1999, 2000). The hypoxic period started when nitrogen bubbling of the respiratory water was turned on, resulting in a P_{O_2} drop from 19 kPa to 5 kPa within the 10 min exposure period. The fish were left to recover until the cardiorespiratory variables had stabilized to pre-hypoxic levels, usually after 10–15 min. The NMDA receptor antagonist MK801 (3.0 mg kg^{-1}) was systemically injected, and after 30 min when the cardiorespiratory parameters had stabilized, the pre-MK801 protocol was repeated. Prior pilot experiments showed that the MK801 dose used provided a good blockade for 1–2 h.

Drugs

Sodium cyanide and Dizocilpine [5R,10S]-[+]-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine, maleate salt (MK801) were obtained from Sigma (St Louis, USA) and dissolved in 0.9% NaCl solution or seawater.

Statistical analysis

The results are presented as means ± S.E.M. (a value of P<0.05 was considered significant) and were statistically analysed by performing paired Student's t-tests comparing the maximum response for each cardiorespiratory parameter with the resting values directly before the treatment. The effect of MK801 on the O_2 receptor-activated responses was determined by comparing the change (maximum response value – resting value) for each measured variable in both untreated (control) and MK801 treated fish.

To establish whether MK801 affected the cardiorespiratory resting values, averages were plotted for each fish as a mean of values from three time points: the pre-hypoxia, pre-internal exposure (NaCN) and pre-external exposure (NaCN) values. These averages were then used to calculate representative mean resting values for each cardiorespiratory parameter. Resting values before and after MK801 treatment were compared with each other and evaluated for statistical significant differences.

Table 2. *Summary of the immunohistochemical results*

Antigen	Structure	Vagal sensory area	Ganglion nodosum
NMDAR1*	Nerve fibre	++	–
	Sensory cell body		–
	Interneuron	++	
Glutamate	Nerve fibre	+++	++
	Sensory cell body		++

*Subunit of the NMDA receptor.

–, absent intensity; +, weak intensity; ++, moderate intensity; +++, strong intensity.

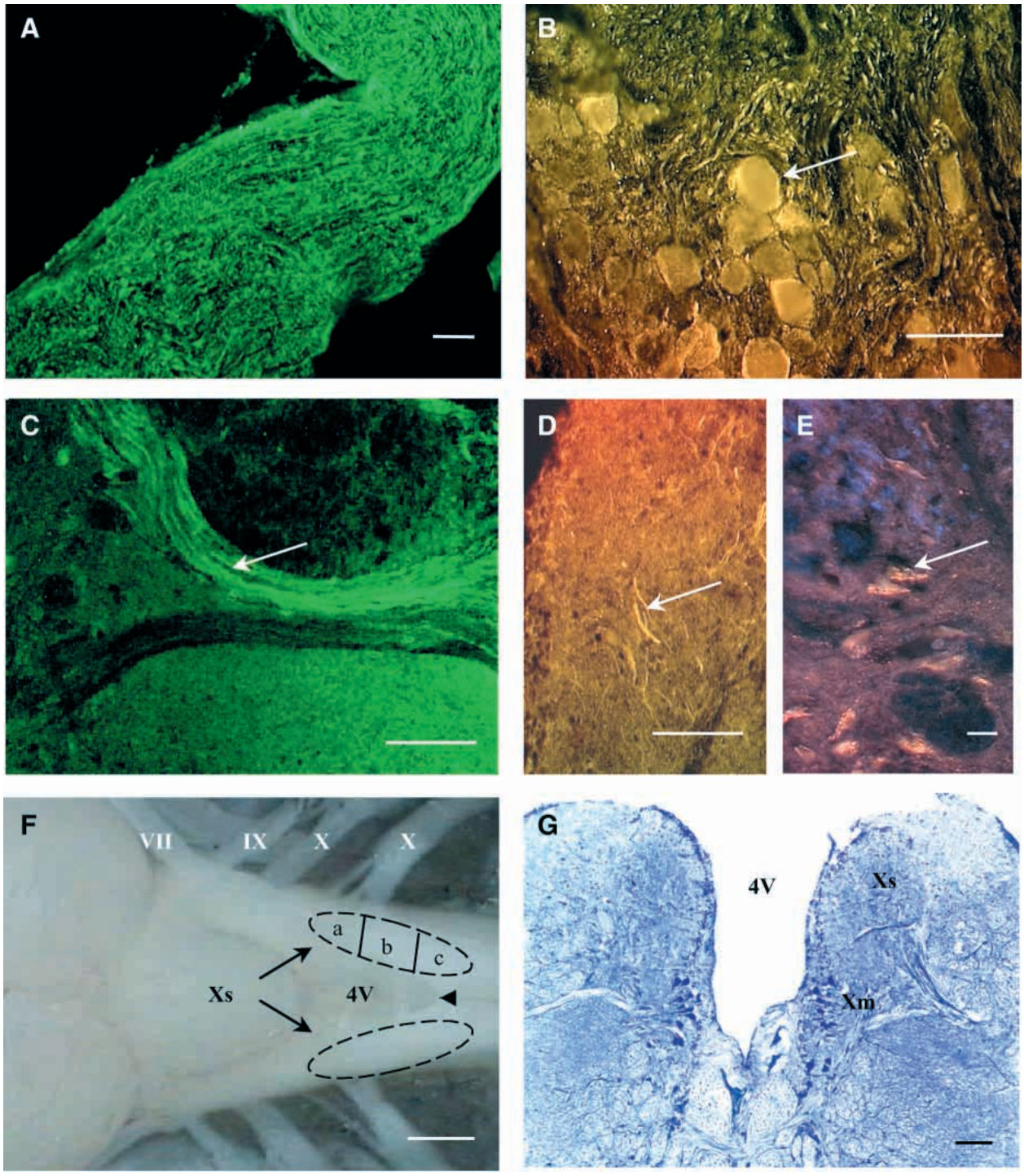

Fig. 1. Micrographs showing planar and cross sections of the ganglion nodosum and the medulla, immunostained with glutamate or NMDAR1 antisera. (A) Glutamate-like immunoreactivity (IR) in vagal nerve fibres proximal to the ganglion nodosum. (B) Glutamate-like IR in numerous cell bodies (arrow) within the ganglion nodosum. (C) Glutamate-like IR in almost all vagal nerve fibres (arrow) in the medulla projecting toward the vagal sensory area (Xs). (D,E) NMDAR1-like IR in fibres (arrow, D) and cell bodies (arrow, E) in the intermediate Xs (see F). (F) Dorsal view of the sculpin hindbrain. VII, facial nerve; IX, glossopharyngeal nerve; X, vagus nerve (two major branches); ovals indicate the positions of the Xs columns on each side of the medulla (a, rostral; b, intermediate; c, caudal portion of the Xs). Arrowhead points towards the caudal end of the fourth ventricle (4V), the obex. (G) Nissl stained cross section through the intermediate Xs (indicated in F) showing the precise location of the Xs and the vagal motor area (Xm) in the medulla. Scale bars, 1 mm (F), 100 μm (A,C,G), 50 μm (B,D) and 10 μm (E).

Results

Immunohistochemical experiments

The immunohistochemical results are summarized in Table 2; they disclose glutamate-like immunoreactivity (IR) in fibres and cell bodies in the whole vagal afferent pathway, including the ganglion nodosum, while NMDAR1-like IR was identified on fibres and interneurons within the Xs only.

Glutamate-like immunoreactivity

Vagal fibres proximal to the ganglion nodosum displayed strong immunoreactive intensity for glutamate (Fig. 1A). Accordingly up to 50% of the cell bodies (approx. diameter 30–40 μm) and their axons in the ganglion nodosum stained positive for glutamate (Fig. 1B). Fig. 1C shows that only the portion of the vagal nerve trunk containing the sensory fibres, but not motor fibres, displays glutamate-like IR.

NMDAR1-like immunoreactivity

NMDAR1-like IR was found in fibres throughout the vagal sensory column (Fig. 1D), while small interneurons (approx. diameter 5–8 μm) positive for NMDAR1 were only found rostral to obex (Fig. 1E).

Specificity controls

The absorption and blank tests were negative, confirming the specificity of the primary and secondary antisera used.

Cardiorespiratory experiments

External and internal vehicle (control) injections occasionally produced startle reflexes, such as a transient bradycardia and apnea. In such cases the injections were repeated until the animals were accustomed and no further responses were observed.

Respiratory responses

Hypoxia significantly increased both f_V and V_{AMP} (Fig. 2A,B). While there was a short delay before the onset of the frequency response (at P_{O_2} approx. 10 kPa), the amplitude immediately started to increase upon commencement of the hypoxic period. MK801 treatment abolished the hypoxia-induced f_V increase but did not significantly affect the hypoxia-induced increase in V_{AMP}.

Similarly, external and internal NaCN injections significantly increased f_V; however, only the external injection significantly increased V_{AMP} (Fig. 3). MK801 also abolished the NaCN-induced frequency increase, although the comparison between internally injected control and MK801-treated fish did not reveal a significant difference. The NaCN induced increase in V_{AMP} was not affected by MK801 pre-treatment.

Cardiovascular responses

Hypoxia, external and internal NaCN injections decreased

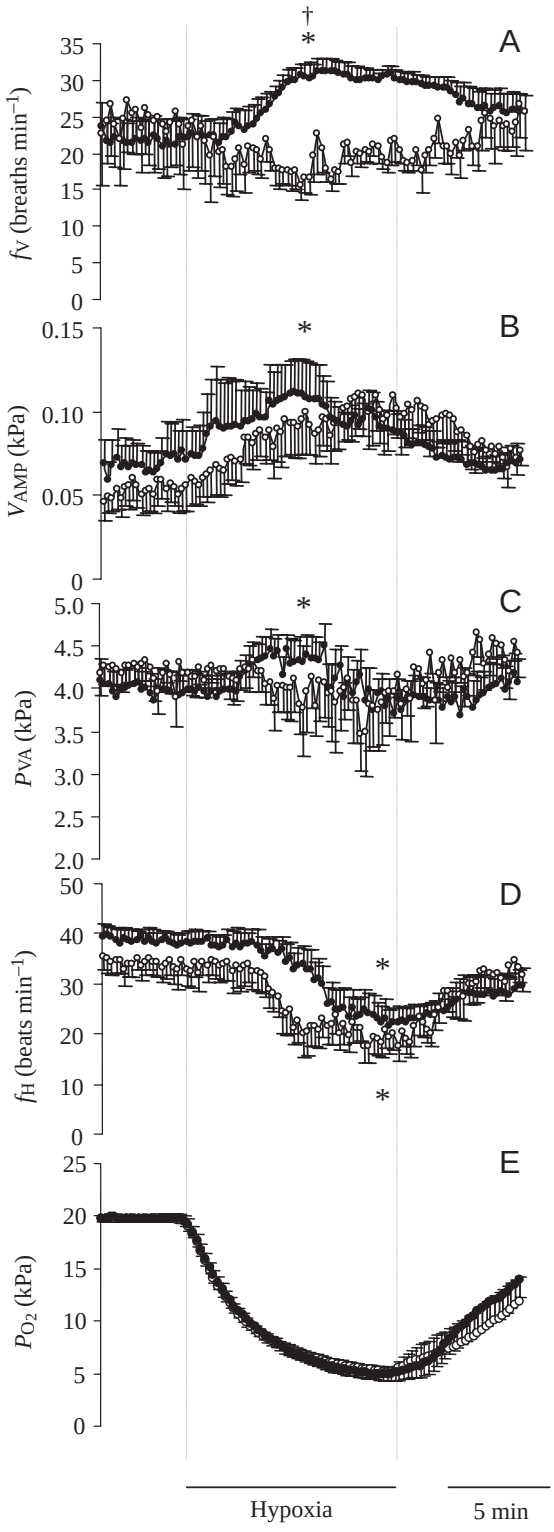

Fig. 2. Summary of hypoxia-elicited cardiorespiratory reflexes. Effects on ventilation rate (A), ventilation amplitude (B), ventral aortic pressure (C) and heart rate (D) in control fish (filled circles) and in MK801 treated fish (open circles). (E) Changes in partial oxygen tension (P_{O_2}) in the respiratory water during hypoxia. Values shown are means + S.E.M. (N=7). *A statistically significantly difference (P<0.05) from resting values; †statistically significant difference (P<0.05) between control and MK801 treated fish. For abbreviations, see Table 3.

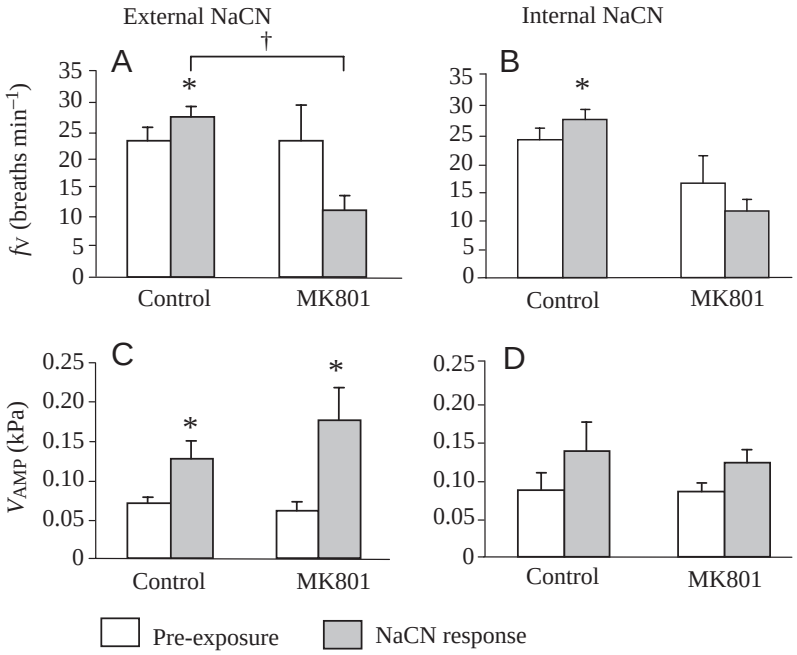

Fig. 3. Summary of NaCN-induced respiratory reflexes. Effects of external NaCN injections into the respiratory water (A,C) or internal NaCN injections into the afferent branchial artery (B,D) on ventilation frequency and amplitude in control and MK801-treated fish (MK801) (means + S.E.M.; N=7). Open bars show the pre-exposure values; grey bars, the maximal response to NaCN. *A statistically significantly difference (P<0.05) from pre-exposed values; †statistically significant difference (P<0.05) between control and MK801-treated fish. For abbreviations, see Table 3.

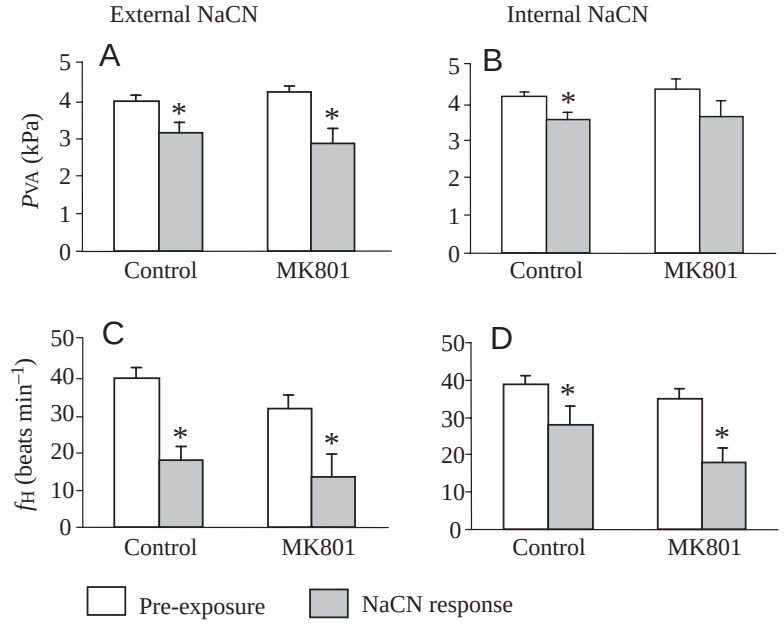

Fig. 4. Summary of NaCN-induced cardiovascular reflexes. Effects on ventral aortic pressure and heart rate after NaCN injections either externally into the respiratory water (A,C) or internally into the afferent branchial artery (B,D) in control and MK801-treated fish (MK801) (means + S.E.M.; N=7). Open bars show the pre-exposure values; grey bars, the maximal response to NaCN. *A statistically significantly difference (P<0.05) from pre-exposure values. For abbreviations, see Table 3.

f_H (Figs 2, 4). The threshold for the response was at a water P_{O_2} of approx. 8 kPa (reached after approx. 5 min) before the heart rate started to drop. The bradycardia was MK801-insensitive.

Before the onset of the hypoxia-induced bradycardia, P_{VA} showed an initial significant increase (at a P_{O_2} of 10 kPa, after approximately 3 min). As soon as the bradycardia commenced P_{VA} declined to baseline values (Fig. 2). The P_{VA} followed the heart rate responses seen during external and internal NaCN injections, and thus decreased significantly (Fig. 4). The only clear effect of MK801 treatment on the P_{VA} responses was the absence of the initial increase during hypoxia (Fig. 2).

Effects of MK801 on resting parameters

MK801 did not significantly change any resting level of the measured parameters (Table 3), but the treatment caused major changes in the breathing patterns of the animals. From being continuous with occasional apneas occurring in conjunction with spontaneous bradycardia, four types of breathing pattern evolved almost immediately or up to 60 min after the MK801 treatment before stabilisation; these were regular continuous breathing but at an increased frequency (two animals, Fig. 5B), frequency cycling (two animals, Fig. 5C), episodic breathing (two animals, Fig. 5D) and breathing with decreased ventilation amplitude and frequency (one animal, not shown).

Discussion

This study reveals for the first time that central glutamatergic mechanisms are involved in chemoreflex activation of cardiorespiratory reflexes in fish. The shorthorn sculpin displayed the typical cardiorespiratory responses commonly observed in other species, i.e increase in respiration (frequency and amplitude), bradycardia and an initial elevation in blood pressure. We then tried to reveal whether the elicited reflexes utilize NMDA receptors for the central transmission of oxygen receptor information. Most significantly, we found that the hypoxia-induced elevation in f_V was NMDA-receptor dependent, whereas the amplitude response was independent. Similarily Ohtake et al. (1998) showed that NMDA receptors only mediate the peripheral chemoreceptor afferent input responsible for an increased f_V in rats. Other authors, however, have presented evidence that only the V_{AMP} response is NMDA-receptor dependent (Ang et al., 1992; Lin et al., 1996; Mizusawa et al., 1994). The involvement of NMDA receptors in mediating ventilatory responses is thus

Table 3. *Mean resting values of cardiovascular and ventilatory variables before (control) and after MK801 treatment*

Variable	Control	MK801
P_{VA} (kPa)	4.0±0.2	4.2±0.2
f_H (beats min^{-1})	38.6±2.4	33.8±2.7
f_V (breaths min^{-1})	22.9±1.8	21.8±5.5
V_{AMP} (kPa)	0.075±0.015	0.066±0.007

P_{VA}, ventral aortic pressure; f_H, heart rate; f_V, ventilation frequency; V_{AMP}, ventilation amplitude.

Values are means ± S.E.M. (N=7).

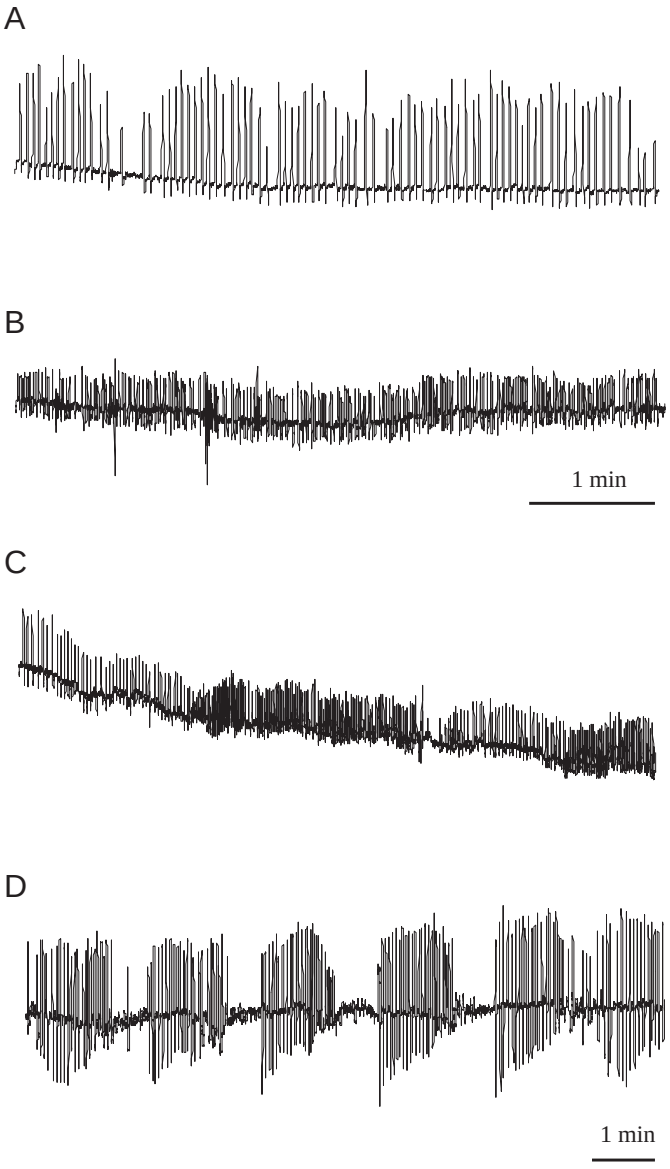

Fig. 5. Original traces showing different types of breathing patterns occurring after treatment with MK801. (A) Normal breathing before MK801 treatment and (B) increased frequency after MK801 treatment. (C) Frequency cycling after MK801; (D) Episodic breathing after MK801.

ambiguous, but our findings do show that glutamate is also a putative player for the development of respiratory reflexes in more ancient animal groups. The observation that MK801 could not significantly alter the V_{AMP} response to any chemoreceptor stimuli applied suggests the possibility of a non-NMDA-receptor chemoreflex pathway.

In contrast to the efficient blockade of the respiratory frequency response, the chemoreceptor elicited bradycardia was MK801-insensitive. Interestingly, when a bradycardia was evoked instead by microinjection of glutamate into the Xs of the sculpin (Sundin et al., 2003), it was NMDA-receptor dependent. Similarly, a glutamate-induced bradycardia in rats could also be blocked by NMDA receptor antagonists (Canesin et al., 2000; Colombari et al., 1997), while the chemoreceptor-elicited bradycardia has been shown to be both sensitive (Haibara et al., 1995) and insensitive (Ohtake et al., 1998) to NMDA receptor blockade. Apparently there might exist compensatory mechanisms that override the NMDA-receptor-dependent response in order to maintain the chemoreflex bradycardia (as seen in this study), even without the presence of functional NMDA receptors.

The hypertension produced by hypoxia in fish is mediated *via* α-adrenoceptors (Fritsche and Nilsson, 1990). In this study the initial hypertension was abolished when the animals were pre-treated with MK801, which suggests that the activation of the sympathetic component of the vascular reflex is mediated by NMDA receptors. Evidence that also supports a role for glutamate in the transmission of vascular responses during hypoxia in fish is that microinjection of glutamate into the rostral Xs of the sculpin can produce pressor effects (Sundin et al., 2003).

Corroborating our physiological experiments is the identification of glutamate in nerve fibres throughout the Xs, the vagal nerve trunk, and in a large fraction of the cell bodies within the ganglion nodosum. These findings correspond well with those obtained in mammals (Saha et al., 1995; Schaffar et al., 1997; Sykes et al., 1997), thus showing that glutamate is present in fish vagal sensory pathways. Further implicating the presence of glutamate in sensory pathways is the finding of NMDA receptors on interneurons and nerve fibres within the Xs (this study), NTS in rats (Aicher et al., 1999; Lin and Talman, 2000; Ohtake et al., 2000) and cats (Ambalavanar et al., 1998). Although we have identified the presence of NMDA receptors in the sculpin sensory pathways, it should be recognized that these receptors are found in the majority of glutamatergic synapses in the vertebrate brain (for a review, see Colquhoun and Sakmann, 1998) and that a systemic administration of MK801 will affect all available receptors.

There are several reports that fish possess both internal and external oxygen receptors (Burleson and Milsom, 1993; Milsom and Brill, 1986) and that they may elicit different cardiorespiratory responses (Milsom 1996; Milsom et al., 2002; Sundin et al., 1999), so we used injection of NaCN into the respiratory water and intra-arterially to determine whether the sculpin showed any differences in their cardiorespiratory

reflexes depending on the type of oxygen chemoreceptor group being stimulated. Overall there was no difference between the external (water) and internal (blood) NaCN-elicited cardiorespiratory responses other than that NaCN applied to the respired water significantly increased the ventilation amplitude and produced a more marked bradycardia. Even though MK801 significantly blocked the externally but not the internally elicited increase in f_V, there was no persuasive evidence for separate central transmission pathways of cardiorespiratory reflexes for the two oxygen receptor groups, because in the presence of the antagonist internally applied NaCN could not significantly increase f_V.

Although MK801 treatment did not significantly change any of the cardiorespiratory mean resting values, it clearly changed the breathing pattern in the sculpin. From being quite regular and continuous, the animals displayed more irregular patterns, including frequency cycling and episodic breathing. Breathing in vertebrates originates from a central respiratory pattern generator, which is dependent on numerous afferent inputs for initiation of breathing (Feldman et al., 1990; Smatresk, 1990). Removal of the afferent input by selective denervation in the neotropical fish tambaqui gave rise to similar breathing patterns (as seen in this study), such as frequency cycling (denervation of the whole oro-branchial cavity) or episodic breathing (denervation of only the branchial nerves) (Reid et al., in press). Consequently, afferent information to the central respiratory pattern generator is important for maintaining normal breathing in fish. As MK801 produced similar irregular breathing patterns in the sculpin and, in addition, alters breathing patterns in a similar fashion in mammals (Ling et al., 1994; Connelly et al., 1992; Harris and Milsom, 2001), it is likely that NMDA receptors are involved in regulating the respiratory rhythms in all vertebrates. The effect of MK801 on breathing patterns in fish are in keeping with previous findings on isolated lamprey brain preparations, which showed that ionotropic glutamate receptors participate in the central respiratory network (Bongianni et al., 1999).

In conclusion, this study is the first to show that the excitatory amino acid glutamate, present in the vagal afferent pathway, is involved in central processing of oxygen chemoreceptor information in fish. The most significant observation is that only the increase in f_V and initial hypoxic hypertension are NMDA-receptor dependent, while the elevated V_{AMP} and the bradycardic responses are not. Furthermore, NMDA receptors were identified on both nerve fibres and interneurons within the Xs, so the glutamate-NMDA receptor mechanism for regulation of oxygen chemoreceptor reflexes might be present in all vertebrates.

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