Extrabranchial chemoreceptors involved in respiratory reflexes in the neotropical fish *Colossoma macropomum* (the tambaqui)

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Summary

In a previous study, complete denervation of the gills in the tambaqui *Colossoma macropomum* did not eliminate the increase in breathing amplitude seen during exposure of this species to hypoxia. The present study was designed to examine other sites of putative O_2-sensitive receptors that could be involved in this reflex action. Superfusion of the exposed brain of decerebrate, spinalectomized fish did not reveal the presence of central chemoreceptors responsive to hyperoxic, hypoxic, hypercarbic, acidic or alkaline solutions. Subsequent central transection of cranial nerve IX and X, removing not only all innervation of the gills but also sensory input from the lateral-line, cardiac and visceral branches of the vagus nerve, did not eliminate the increase in breathing amplitude that remained following peripheral gill denervation alone. Administration of exogenous catecholamines (10 and 100 nmol kg^{-1} adrenaline) to fish with intact brains and minimal surgical preparation reduced both respiratory frequency and amplitude, suggesting that humoral release of adrenaline also could not be responsible for the increase in breathing amplitude that remained following gill denervation. Denervation of the mandibular branches of cranial nerve V and the opercular and palatine branches of cranial nerve VII in gill-denervated fish (either peripheral gill denervation or central section of cranial nerves IX and X), however, did eliminate the response. Thus, our data suggest that hypoxic and hyperoxic ventilatory responses as well as ventilatory responses to internal and external injections of NaCN in the tambaqui arise from O_2-sensitive receptors in the orobranchial cavity innervated by cranial nerves V and VII and O_2-sensitive receptors on the gills innervated by cranial nerves IX and X.

Our results also revealed the presence of receptors in the gills that account for all of the increase in ventilation amplitude and part of the increase in ventilation frequency during hyperoxic hypercarbia, a group or groups of receptors, which may be external to the orobranchial cavity (but not in the central nervous system), that contribute to the increase in ventilation frequency seen in response to hyperoxic hypercarbia and the possible presence of CO_2-sensitive receptors that inhibit ventilation frequency, possibly in the olfactory epithelium.

Key words: fish, tambaqui, *Colossoma macropomum*, chemoreceptor, hypoxia, hypercapnia, ventilation, gills, orobranchial cavity, cranial nerve, catecholamine.

Introduction

The tambaqui (*Colossoma macropomum*) is a hypoxia-tolerant neotropical fish found throughout the Amazon basin. This species responds to progressive hypoxia by increasing both the frequency and amplitude of gill ventilation and by slowing the heart (Rantin and Kalinin, 1996; Sundin et al., 2000). Slowing of the heart is accompanied by an increase in systemic vascular resistance, which maintains arterial blood pressure relatively constant (Sundin et al., 2000). Tambaqui are also one of a small, unique group of water-breathing species that employ *aiú*, or aquatic surface respiration, as a strategy to reduce the impact of severe aquatic hypoxia (Val and Almeida-

Val, 1995). Under these conditions, they come to the surface, where they skim the relatively O_2-rich surface film, which they pump across the surface of the gills where gas exchange takes place in the usual fashion. To facilitate this, in tambaqui, the lower lip swells to form a funnel that directs the surface water into the mouth and over the gills while the mouth contains a flap-like valve that appears to act to maintain a steady, laminar flow of surface water over the gills while the mouth is held agape (Val and Almeida-Val, 1995; Sundin et al., 2000). The lower lip is not involved in gas exchange *per se*, but serves as a mechanical structure to enhance skimming of the water

surface. At such times, tambaqui also exhibit a reduction in levels of erythrocytic ATP and GTP, an increased dependence on anaerobic metabolism and increased haematocrit (Val, 1993; Almeida-Val et al., 1993). These biochemical and physiological adaptations serve to buffer the negative effects of the low O_2 concentrations in the water.

In a previous study (Sundin et al., 2000), we showed that the decrease in heart rate and increase in breathing frequency exhibited by tambaqui exposed to environmental hypoxia were reflexly elicited by the stimulation of receptors located exclusively within the gills. The receptors responsible for elevating systemic vascular resistance, breathing amplitude and swelling of the inferior lip and that induced aquatic surface respiration during hypoxia, however, were located, at least in part, at some site outside the gills. Extrabranchial receptors have also been implicated in the increase in ventilation amplitude associated with hypoxia in tench (*Tinca tinca*), sea raven (*Hemitripterus americanus*) and traira (*Hoplias malabaricus*) (Hughes and Shelton, 1962; Saunders and Sutterlin, 1971; Sundin et al., 1999), but not channel catfish (*Ictalurus punctatus*) and longnose gar (*Lepisosteus osseus*) (Smatresk, 1989; Burleson and Smatresk, 1990). Several potential sites have been suggested for extrabranchial oxygen receptors including the orobranchial cavity (innervated by cranial nerves V and VII) (Hughes and Shelton, 1962; Butler et al., 1977), the central nervous system (Satchell, 1961; Saunders and Sutterlin, 1971) and the heart and ventral aorta (innervated by visceral branches of the vagus nerve) (Smatresk et al., 1986). It has also been suggested that hypoxia could produce cardiorespiratory responses by its action on adrenal chromaffin cells, giving rise to the systemic release of catecholamines (Randall and Taylor, 1991).

The goal of the present study was to examine each of these possible sites for the presence of extrabranchial receptors that reflexly contribute to the increase in respiratory amplitude during hypoxia in the tambaqui.

Materials and methods

Experimental animals

For these experiments, we used juvenile tambaqui (*Colossoma macropomum* Cuvier) (0.25–0.7 kg, $N=50$) obtained from CEPTA (Tropical Fish Research Centre)/ IBAMA in Pirassununga SP, Brazil, and transported to the Federal University of São Carlos. These fish were third- or fourth-generation descendants of native tambaqui taken from the Amazon in 1993. Animals were maintained outdoors in fibreglass aquaria supplied with aerated City of São Carlos tap water. Temperature was maintained at 25 °C, and the animals were exposed to a natural photoperiod. Fish were fed *ad libitum* every second day, and experiments were performed between February and April.

Animal preparation

All animals were anaesthetised in an aqueous solution of benzocaine (100 mg l^{-1}) predissolved in 2 ml of 70 % ethanol.

A surgical level of anaesthesia was achieved in approximately 5 min. During surgery, the gills were ventilated with a second solution of benzocaine (50 mg l^{-1}) gassed with air. Impedance electrodes were sutured to each operculum to monitor the breath-by-breath displacement of the operculum and to measure ventilation rate (fv) and an index of ventilation amplitude (V_{AMP}). Using a Dremel tool, a hole was drilled through the snout between the nostrils, and a flared cannula (PE 160) was fed from inside the mouth out through the hole and was secured with a cuff. This allowed administration of NaCN solutions into the buccal cavity in order to stimulate O_2 chemoreceptors throughout the orobranchial cavity monitoring the respiratory water.

Series I (denervation studies, unanaesthetized fish)

In one group of fish, following the general preparation, the operculum was reflected forward, and a small incision (approximately 1.5 cm) was made in the epithelium at the dorsal end of the gill arches where they meet the roof of the opercular cavity. This permitted access to cranial nerves IX (glossopharyngeal) and X (vagus) innervating the gill arches (Fig. 1). The branchial nerves to all gill arches were carefully dissected free of connective tissue and cut with fine iris scissors (gill-denervated, GD, group, $N=9$). In all cases, the cardiac and visceral branches of the vagus were left intact. Tambaqui do not have a pseudobranch, so this produced complete gill denervation. In a control group (control, C, $N=9$), all nerves were exposed but left intact. In a third group (gills and orobranchial cavity denervated, GOD, $N=8$), following gill denervation, the opercular and palatine branches of cranial nerve VII and all mandibular branches of cranial nerve V innervating the orobranchial cavity were also sectioned (Fig. 2). This removed sensory information arising from the mouth and buccal cavity. We left two small branches of cranial nerve VII intact, and these were sufficient to produce opercular movements that could be monitored as an indication of the frequency and amplitude of ventilation. The opercular branches of cranial nerve VII innervating the floor of the mouth were accessed where they course over the inner surface of the operculum, the palatine branches of cranial nerve VII were accessed through a midline incision in the roof of the mouth and the mandibular branches of cranial nerve V innervating the roof of the mouth were accessed bilaterally by rotating the eyes and sectioning the nerves, where they coursed over the back of the orbit, through a small incision in the top of the conjunctiva.

All animals in series I were allowed to recover from surgery, and experiments were performed the following day. All denervations were confirmed *post mortem* by autopsy, and all data presented here are from fish for which complete denervations could be confirmed.

Series II (denervation studies, decerebrate fish)

In this series, following the general preparation, the fish were placed in a Perspex chamber filled with flowing water and secured into a stereotaxic apparatus. A mouthpiece fitted with a ventilation tube was sewn into the mouth, and the fish was
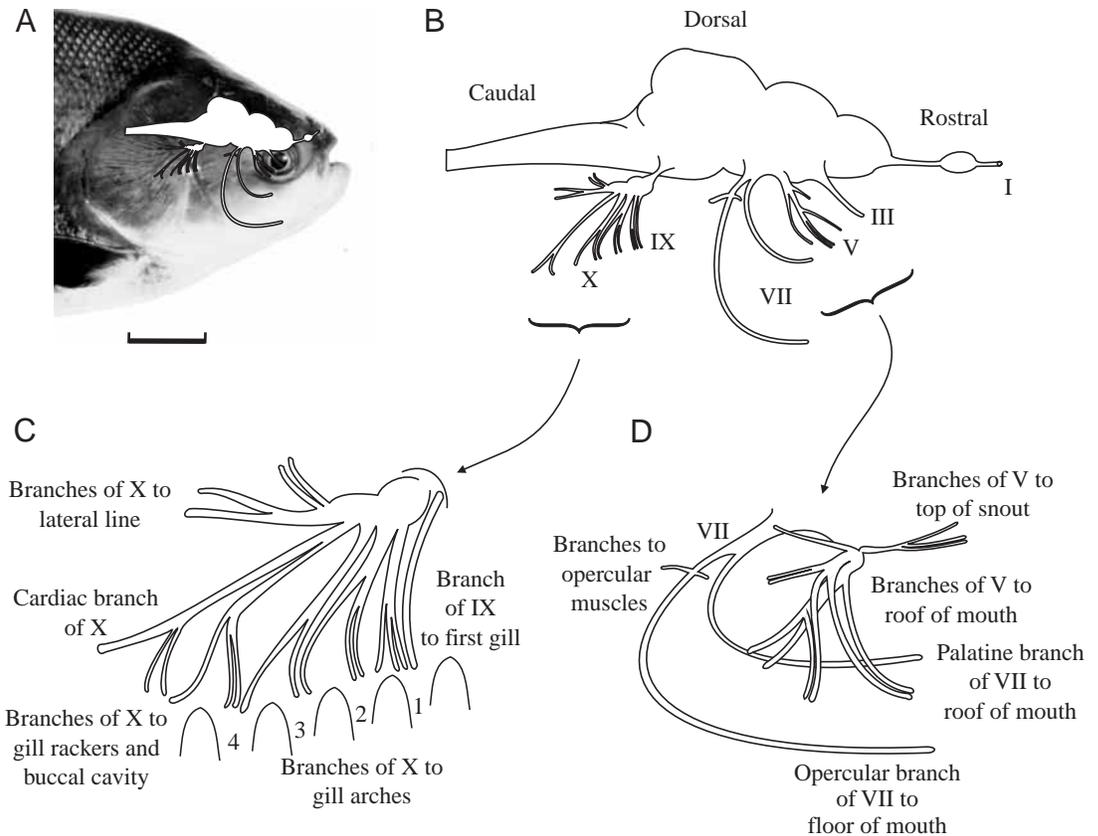
Fig. 1. Schematic diagram showing the cranial nerve roots relevant to this study. (A) The location of the nerves relative to the external anatomy of the fish. Scale bar, 5 cm. (B) An enlargement showing the origin of various cranial nerve roots. (C,D) Details of the branches of cranial nerves V, VII, IX and X.

placed on artificial ventilation. The fish was secured in such a way that the gills and operculum were submerged in the water in the box while the top of the head was above the water. Using a Dremel tool, the top of the cranium was removed and the brain exposed. The adipose material surrounding the brain was removed by suction, the forebrain aspirated (decerebration) and the rostral space so produced was packed with cotton. The spinal cord was then severed (spinalization) slightly caudal to the second spinal nerve to prevent the animal from moving. In the control group (DC, $N=12$), once the animal was decerebrate and spinalized, anaesthesia was discontinued and the animal was ventilated with aerated water. In nine of these animals (decerebrate, gills denervated, DGD group) following the control experimental run, cranial nerves IX and X were then denervated within the cranial cavity where they exit the medulla. This produced a total central denervation of these nerves, denervating not only the branchial branches to the gills but also all the visceral and lateral-line branches of the vagus nerve. In a final group of nine fish (decerebrate, gills and orobranchial cavity denervated, DGOD group), these procedures were repeated in animals in which the opercular and palatine branches of cranial nerve VII and the mandibular branches of cranial nerve V had just been sectioned peripherally as described in series I.

All animals were left for 2 h to stabilize before experiments began. Again, all denervations were confirmed *post mortem* by autopsy.

Series III (central perfusion studies)

Fish used in this study ($N=6$) were prepared in an identical fashion to the control fish in series II.

Series IV (pharmacology studies)

In this series ($N=6$), following the general preparation, a cannula (PE50) was inserted into the ventral aorta through the afferent branchial artery of the third gill arch on one side. These fish were then allowed to recover from anaesthesia, and experiments were performed the following day. No nerves were denervated in this group.

Experimental protocols
Series I (denervation studies, unanaesthetized fish)

In this group, animals were placed into individual cylindrical tubes housed within larger experimental tanks (approximately 80 l) with free-flowing aerated water. This prevented the fish from swimming to the surface to perform aquatic surface respiration. Mesh covered the ends of the cylindrical tubes to allow rapid equilibration between the tubes and the larger tanks. A large slit on the top of the tubes permitted leads and cannulae to exit the tank. The tank was covered to maintain a dark and quiet environment for the fish. After at least 24 h of recovery from surgery, the fish were first subjected to a series of external (into the snout cannula) injections of water (control) and NaCN (1 ml of 1 or 2 mg ml^{-1} in water). In each case, the cannula was flushed with 1.0 ml of water to ensure complete

Control
(C)

IX

V   III

X   VII

I

Gill-denervated
(GD)

X

V   III

VII

I

Gill- and orobranchial-
cavity-denervated
(GOD)

X

V   III

VII

I

Decerebrate, spinalectomized,
vagotomized, gill- and
orobranchial-cavity
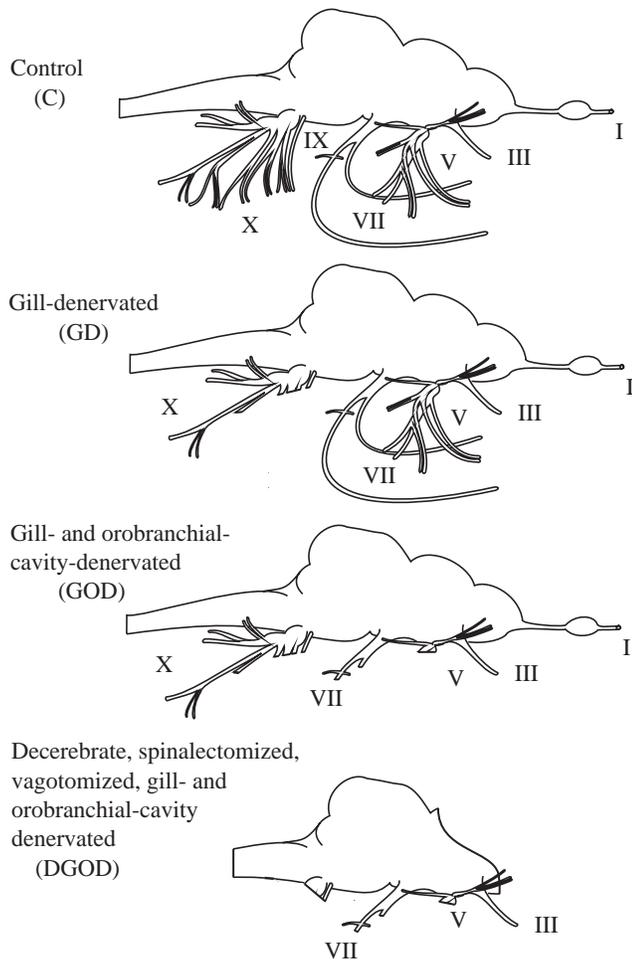denervated
(DGOD)

V   III

VII

Fig. 2. Schematic diagram illustrating the different transections performed in various groups of fish in the different series of experiments. Control fish had all their cranial nerves intact (both series I and II). In the gill-denervated group, all branches of cranial nerves IX and X to the gills were sectioned where they enter the gills (series I). For the gill- and orobranchial-cavity-denervated group, all branches of cranial nerve V to the roof of the mouth as well as the palatine branches of VII and the opercular branches of VII to the floor of the mouth were also denervated (series I). In series II, the fish were decerebrate and spinalectomized, cranial nerves IX and X were transected where they originated from the brain within the cranium and cranial nerves V and VII were sectioned as in series I.

drug delivery. After each injection, respiratory variables were recorded for 3–5 min.

The animals were next subjected to abrupt, progressive environmental hypoxia produced by shutting off the airflow and gassing the tank holding the fish with nitrogen. The water P_{O_2} was lowered from an air-saturated level of 18.6 kPa (140 mmHg; 25 °C) to 1.3 kPa (10 mmHg) over approximately 10 min. At this point, the nitrogen flow was halted, airflow was restored and the water P_{O_2} was gradually returned to normoxic levels.

Animals were then subjected to progressive environmental hyperoxia produced by shutting off the airflow and gassing

the tank holding the fish with 100 % O_2. The water P_{O_2} was increased from an air-saturated level of 18.6 kPa (140 mmHg; 25 °C) to 80 kPa (approximately 600 mmHg) over approximately 10 min. At this point, the oxygen flow was halted, airflow was restored and the water P_{O_2} gradually returned to normoxic levels.

Finally, the C and GD groups were subjected to abrupt, progressive environmental hyperoxic hypercarbia by gassing the tank with a mixture of 5 % CO_2 in 100 % O_2. We had determined in a previous study (Sundin et al., 2000) that exposure to 5 % CO_2 was necessary to produce a reliable respiratory stimulus in this species. With this level of CO_2, the water pH fell from approximately 7.0 to 5.0 over 10 min. The animals were then returned to normocapnic conditions, and cardiorespiratory variables were monitored until the water pH returned to at least 6.5. As this procedure failed to stimulate ventilation in the GD group, it was not repeated in the GOD group.

Series II (denervation studies, decerebrate fish)

In this series, fish were left in the stereotaxic apparatus following surgery and artificially ventilated with well-aerated water for at least 2 h. The animals were then exposed to the same protocol used in series I with one small difference. In this series of experiments, a separate reservoir of water was pre-gassed with each gas mixture so that the transition from one gas to another was immediate. Animals were left on each experimental gas mixture for at least 10 min before being returned to aerated water. In this study, all groups (DC, DGD and DGOD) were exposed to all gas mixtures.

Series III (central perfusion studies)

In this series, all fish were artificially ventilated with well-aerated water throughout. The cranial cavity was initially superfused with well-aerated saline (0.9 % NaCl) buffered to pH 7.8 by the addition of a small amount of CO_2. Flow was maintained by gravity at a rate of 5 ml min^{-1} from a series of pre-gassed reservoirs. After a 15 min control period, the superfusate was switched to a hypoxic (1.3 kPa or 10 mmHg P_{O_2}), a hyperoxic (80 kPa or approximately 600 mmHg P_{O_2}), a hypercapnic (5 kPa or 38 mmHg P_{CO_2}), an acidic (pH 7.2) or an alkaline (pH 8.4) solution for 15 min. The hypoxic, hyperoxic and hypercapnic solutions were all buffered to pH 7.8, while the acidic and alkaline solutions were gassed with air. Test solutions were presented at random with a 15 min period of superfusion with well-aerated saline between each.

Series IV (pharmacology studies)

In this series of experiments, animals were maintained in cylindrical tubes housed within larger experimental tanks with free-flowing, aerated water as described for series I. The protocol began with an injection of 0.3 ml of saline (0.9 % NaCl) into the ventral aorta to determine whether the vehicle would cause any change in ventilation. Adrenaline was then injected in boluses of 1, 10 and 100 nmol kg^{-1}, and the catheter was flushed with

0.3 ml of saline following each injection. The animals were next subjected to the same abrupt, progressive environmental hypoxia protocol described for series I [water P_{O_2} reduced from an air-saturated level of 18.6 kPa (140 mmHg; 25 °C) to 1.3 kPa (10 mmHg) over approximately 10 min]. The beta-blocker sotalol was then administered (3 mg kg^{-1}), and the highest dose of adrenaline (100 nmol kg^{-1}) and the hypoxia run were repeated to examine the effects of beta-blockade on the adrenaline- and the hypoxia-induced ventilatory response.

Data analysis

In all experiments, the opercular impedance leads were connected to an impedance converter to measure f_V (breaths min^{-1}) and V_{AMP} (arbitrary units). The partial pressure of oxygen in the water (Pw_{O_2}) was monitored continuously (± 0.1 mmHg) with an oxygen electrode (FAC 001 O_2 electrode and FAC 204A oxygen analyser) supplied, *via* a siphon, with a steady flow of water from each experimental chamber. The electrodes were calibrated with solutions of sodium bisulphate in borax ($P_{O_2}=0$ kPa) and air-equilibrated water ($P_{O_2}=18.6$ kPa; 25 °C). Water pH was continuously measured with a pH electrode calibrated with standard solutions.

For each gas mixture (hypoxia, hyperoxia and hyperoxic hypercarbia), respiratory variables were analysed for a 1 min control period immediately prior to the run, for the final minute of the run and after 30 min of recovery. During the NaCN injection experiments, data were analysed for a 30 s control period immediately prior to an injection of saline or NaCN and at 10 s intervals for the first minute post-injection. During the second and third minute post-injection, data were analysed for a 30 s period each minute. Maximum responses are reported. For the adrenaline injections, data were analysed for a 30 s control period immediately prior to an injection of saline or adrenaline, at 30 s and at 1, 2, 3 and 4 min post-injection.

f_V is reported in absolute values. Since V_{AMP} was measured in arbitrary units, V_{AMP} and total ventilation ($V_{TOT}=V_{AMP}f_V$) are reported as the percentage change from the control or starting value.

Statistical analyses

The data are reported as the mean ± 1 standard error of the mean (S.E.M.). Data were compared using one-way repeated-measure analysis of variance (ANOVA) to test for the significance of changes in response to each stimulus. If significant differences ($P<0.05$) were found, a Dunnett's multiple-comparison test was used as a *post hoc* test. To evaluate the effects of selective denervations on the responses to the different treatments, a two-way repeated-measures ANOVA was used. For series IV, paired comparisons were made using the Wilcoxon rank sum test.

Results
Series I and series II

The only significant effect of the experimental manipulations on resting beathing frequency under normoxic
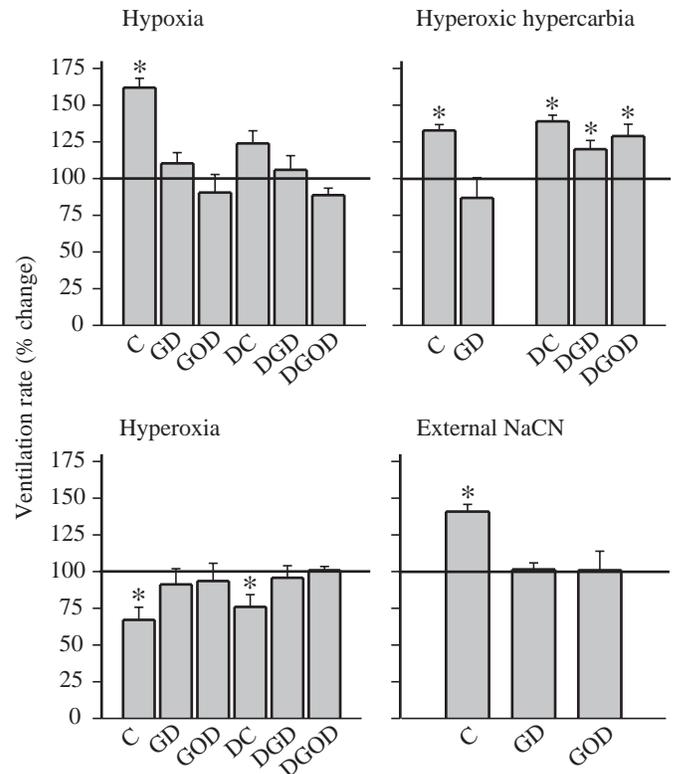

Fig. 3. The effects on ventilation rate (percentage change from starting values) of graded hypoxia, hyperoxic hypercarbia, hyperoxia and injections of NaCN externally into the respiratory water. The data are shown as the mean + S.E.M. (*N*=8–12). In each graph, the different groups are C, control; GD, gills denervated; GOD, gills and orobranchial cavity denervated; DC, decerebrate control; DGD, decerebrate, gills denervated; DGOD, decerebrate, gills and orobranchial cavity denervated. Asterisks indicate values that are significantly different from resting values for fish within that group under normoxic normocapnic conditions (100 % value).

conditions was an increase in the decerebate control group (C, 60.4±4.1 breaths min^{-1}; GD, 56±4.6 breaths min^{-1}; GOD, 63.4±7.9 breaths min^{-1}; DC, 81.8±6.7 breaths min^{-1}; DGD, 58.0±5.7 breaths min^{-1}; DGOD, 51.8±3.0 breaths min^{-1}; *N*=8–12). Hypoxia and external NaCN produced a rapid increase in f_V, while hyperoxia produced a decrease in f_V in the control group (C) in series I (Fig. 3). Hypoxia and hyperoxia produced similar trends in the decerebrate control group (DC) in series II, but while the decrease in breathing frequency during hyperoxia was significant in this group, the increase in f_V during hypoxia was not. Following complete branchial denervation, neither hypoxia nor hyperoxia produced any significant changes in f_V in either group, and external NaCN no longer produced any significant increase in f_V in the non-decerebrate group. Further denervation of the orobranchial cavity eliminated virtually all of the small, non-significant changes that remained.

Breathing frequency also increased during exposure to hyperoxic hypercarbia in both C and DC groups (series I and II respectively). Interestingly, while this increase was
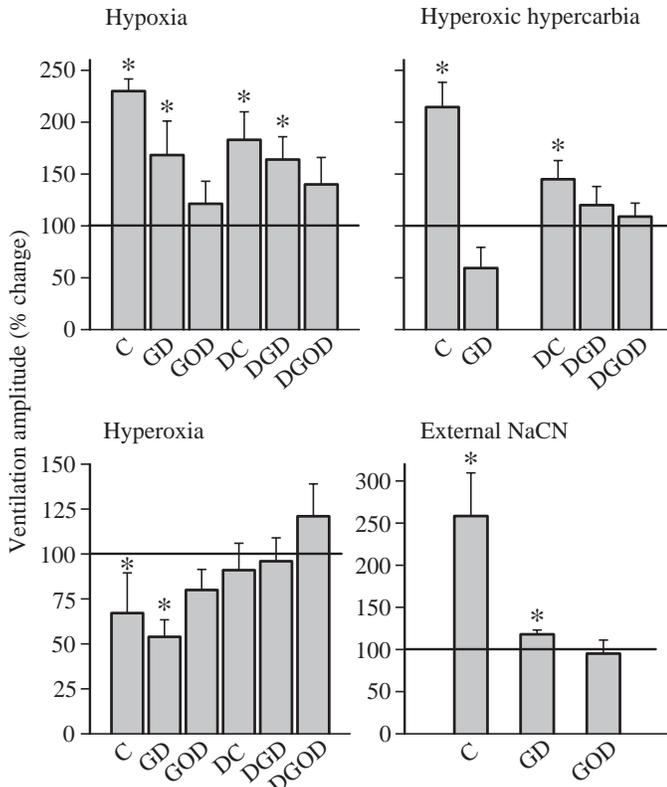
Fig. 4. The effects on ventilation amplitude (percentage change from starting values) of graded hypoxia, hyperoxic hypercarbia, hyperoxia and injections of NaCN externally into the respiratory water. The data are shown as the mean + S.E.M. (N=8–12). In each graph, the different groups are C, control; GD, gills denervated; GOD, gills and orobranchial cavity denervated; DC, decerebrate control; DGD, decerebrate, gills denervated; DGOD, decerebrate, gills and orobranchial cavity denervated. Asterisks indicate values that are significantly different from resting values for fish within that group under normoxic normocapnic conditions (100 % value).
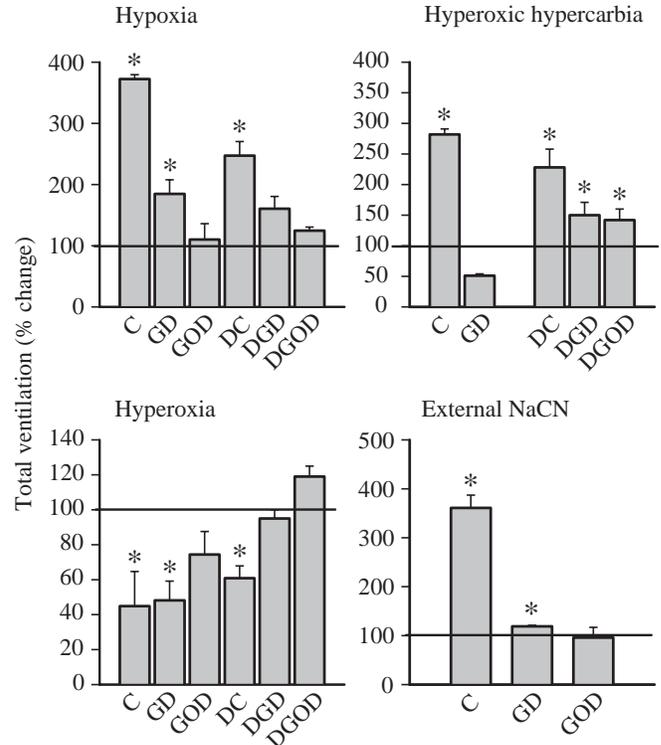

Fig. 5. The effects on total ventilation (percentage change from the starting values) of graded hypoxia, hyperoxic hypercarbia, hyperoxia and injections of NaCN externally into the respiratory water. In each graph, the different groups are C, control; GD, gills denervated; GOD, gills and orobranchial cavity denervated; DC, decerebrate control; DGD, decerebrate, gills denervated; DGOD, decerebrate, gills and orobranchial cavity denervated. The data are shown as the mean + S.E.M. (N=8–12). Asterisks indicate indicates values that are significantly different from resting values for fish within that group under normoxic, normocapnic conditions (100 % value).

eliminated following complete branchial denervation in fish with intact brains (GD), it was not eliminated in the DGD or the DGOD groups (Fig. 3).

Hypoxia and external NaCN significantly elevated V_{AMP} in control and GD groups in series I, while hyperoxia reduced V_{AMP} in these same groups (Fig. 4). Hypoxia also produced an increase in V_{AMP} in the decerebrate control (DC) and DGD groups (series II), but the decrease in V_{AMP} seen during hyperoxia was not significant in any of the decerebrate groups. Following denervation of the gills and orobranchial cavity (GOD and DGOD groups in series I and II respectively), no significant changes in V_{AMP} were seen in response to any of these experimental treatments.

Ventilatory amplitude also increased during exposure to hyperoxic hypercarbia in both C and DC groups (Fig. 4), and these increases were eliminated by complete branchial denervation alone in both groups.

In series I, the effects of hypoxia, hyperoxia and NaCN on total ventilation (V_{TOT}) were reduced, but still significant,

following total branchial denervation but completely abolished following gill and orobranchial denervation (Fig. 5). In the decerebrate fish in series II, the responses to hypoxia and hyperoxia were smaller than those of the fish in series I and were eliminated by complete branchial denervation alone.

By contrast, while the increase seen in V_{TOT} during hyperoxic hypercarbia in series I was eliminated following complete branchial denervation, it was not eliminated even following complete denervation of the gills and orobranchial cavity in the decerebrate fish in series II (Fig. 5).

Series III

There were no effects of central perfusion of the decerebrate brain on f_V or V_{AMP} with any of the test solutions (Fig. 6).

Series IV

Adrenaline at 10 and 100 nmol kg^{-1} significantly decreased ventilation rate and amplitude and, hence, total ventilation in unanaesthetized, intact fish (Fig. 7). When sotalol was present, the decrease in frequency was abolished, indicating that beta-receptors were involved in the adrenaline-induced depression
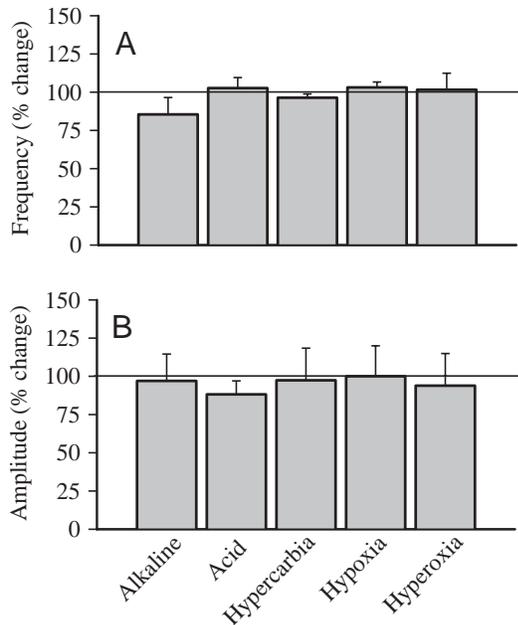
Fig. 6. The effects on breathing frequency and amplitude (percentage change from the starting values) of superfusing the exposed brain of decerebrate fish for 15 min with various solutions (see text for details). The data are shown as the mean + S.E.M. (N=6).

of respiratory frequency. The reductions in ventilation amplitude and total ventilation persisted following sotalol treatment, however, although they were delayed. The increase in breathing frequency during hypoxic exposure was also altered following sotalol treatment (Fig. 8). Breathing frequency now reached peak values at 5.3 kPa (40 mmHg) and then fell as $P\text{w}_{O_2}$ was reduced further; breathing frequency returned towards normoxic values more quickly during the recovery period. This is in contrast to the progressive increase in breathing frequency seen in control fish down to 1.3 kPa (10 mmHg) and the slow return of breathing frequency to control levels on recovery. The pattern of change in V_{AMP} on exposure to hypoxia was unaffected by pretreatment with sotalol.

Discussion

Critique of methods

To determine the possible sites of sensory input contributing to the hypoxic ventilatory response in the present experiments, it was necessary extensively to denervate cranial nerves that not only carry afferent input to the central nervous system, but that also carry the primary motor output to the respiratory muscles. This raises questions about both the health of the animals and the extent to which the fish could respond to respiratory stimuli.

We have previously shown that fish tolerate total gill denervation well. Indeed, it was the failure of such denervation to remove all the hypoxic ventilatory response that led to the present study. Our next step was to also remove any
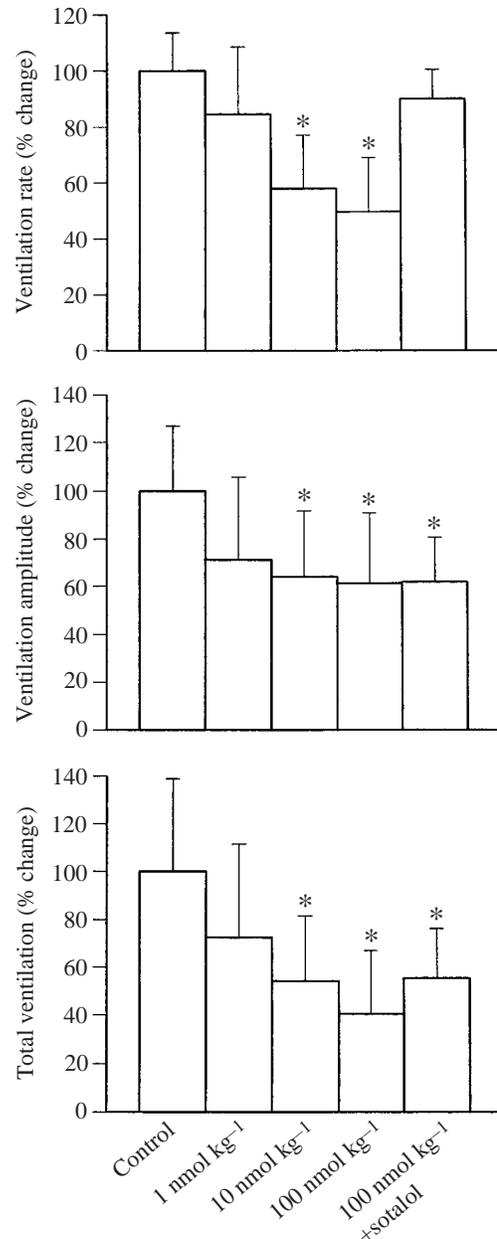

Fig. 7. The effects of systemic injection of different doses of adrenaline and of 100 nmol kg^{-1} adrenaline after pretreatment with sotalol (3 mg kg^{-1}) on breathing frequency, breathing amplitude and total ventilation. The data are shown as the mean + S.E.M. (N=6). Asterisks indicate values that are significantly different from control values. All values were taken 30 s after injection except the 100 nmol kg^{-1} adrenaline+sotalol values, which were taken 120 s after injection.

contribution of sensory input arising from the heart and ventral aorta (innervated by visceral branches of the vagus nerve). To do this, we opened the cranium, decerebrated and spinalectomized the fish and made initial recordings with all cranial nerves intact. Cranial nerves IX and X were then sectioned centrally. This not only denervated the gills but also removed any sensory information coming from the cardiac,
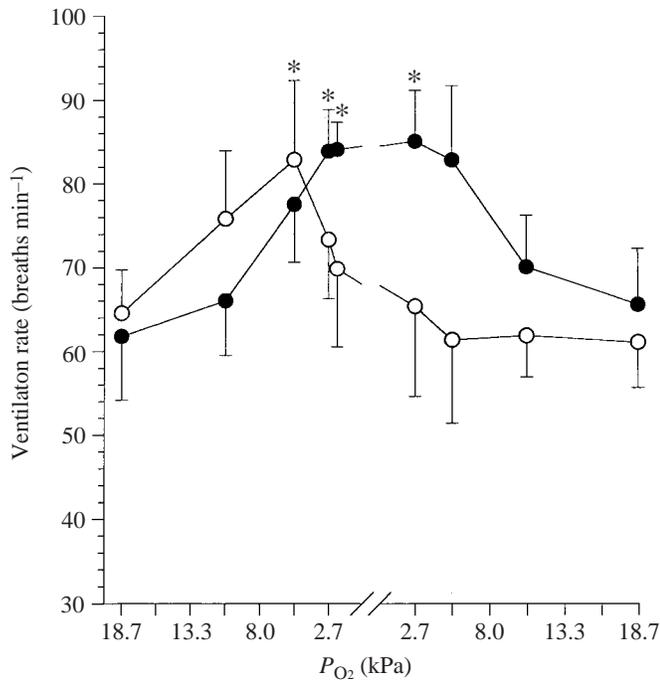
Fig. 8. The effects of graded hypoxia on breathing frequency before (filled circles) and after (open circles) pretreatment with sotalol. The data are shown as the mean ± S.E.M. (*N*=6). Asterisks indicate values that are significantly different from starting (18.7 kPa) values.

visceral and lateral-line branches of the vagus nerve. These had been left intact in our previous studies. The responses to hypoxia obtained from both these groups of fish (decerebrate, spinalectomized fish with all cranial nerves intact, DC, and decerebrate, spinalectomized fish with transections of cranial nerves IX and X, DGD) were extremely similar to the responses obtained from control fish (C) and from fish that had undergone peripheral denervation of the gills (GD) in both our previous (Sundin et al., 2000) and current experiments. This validated our approach.

To eliminate possible extrabranchial oxygen receptors in the orobranchial cavity (innervated by cranial nerves V and VII), we then transected the mandibular branches of cranial nerve V as well as both the opercular and palatine branches of cranial nerve VII. This removed sensory information arising from the mouth and buccal cavity. We left two small branches of cranial nerve VII intact on each side, which were sufficient to produce opercular movements that could be monitored as an indication of the frequency and amplitude of ventilation. These movements were small but appeared to be sufficient to ventilate the fish adequately under resting, normoxic conditions, despite the fact that under these conditions all other motor output to the respiratory muscles had been transected. The primary evidence for this was that the brain-intact fish (GOD group) recovered from surgery, could be run through a full protocol 24 h later and had mean breathing frequencies that did not differ from those of control fish (63.4±7.0 *versus* 60.6±4.1 breaths min⁻¹ in control fish).

While large decreases in arterial P_{O_2} have been shown to accompany bilateral gill denervation in the sea raven *Hemitripterus americanus* (Saunders and Sutterlin, 1971), the lack of any elevation in resting breathing frequency in the tambaqui suggests that blood gas levels must have been relatively normal in the fish in series I (although this could also have resulted from such offsetting factors as a reduced breathing frequency due to denervation coupled with an increased breathing frequency due to lower levels of arterial P_{O_2}). This was certainly not a concern for the fish in series II since they were artificially ventilated. Resting ventilation aside, the question remains, could these fish respond to respiratory stimuli in a normal fashion? There is no doubt that the ability to generate forceful respiratory movements was compromised in these fish, and the question remained, could they increase breathing amplitude or could they only increase breathing frequency? Could we distinguish whether the absence of an amplitude response to hypoxia or hypercarbia was because the sensory receptors involved in producing this response were removed or because the muscles capable of generating this response were denervated? This is an important question since, after this denervation, the only changes seen in breathing amplitude in response to hypoxia and hyperoxic hypercarbia were small and insignificant. The critical observation pertinent to this question comes from observation of the variation that occurred in individual recordings and the responses of fish to disturbance. This evidence suggests that all the fish were capable of at least doubling the amplitude of their opercular movements, implying that this experimental approach was also valid.

Responses to hypoxia, hyperoxia and NaCN

We have shown previously that total denervation of the gills in tambaqui eliminated the increases in breathing frequency seen during hypoxia and in response to internal injections of NaCN. However, it neither eliminated the increase in breathing frequency in response to external cyanide nor the increase in breathing amplitude that occurred in response to hypoxia or external injections of NaCN (Sundin et al., 2000). The results we obtained in the present study are consistent with this with the exception that, this time, total gill denervation did eliminate the frequency response to external cyanide. The results of the present study go on to show, however, that all remaining responses could be eliminated by further denervation of the orobranchial cavity. Not surprisingly, the results also show that the receptors involved in producing ventilatory adjustments of breathing frequency and amplitude in response to hyperoxia (not examined in earlier studies) are innervated just as are those involved in producing the responses to hypoxia. The responses of the fish in series I and II were very similar and, thus, our data do not support suggestions that O_2-sensitive receptors involved in respiratory responses may be situated in the heart and dorsal aorta, instead suggesting that such receptors may be confined to the gills and orobranchial cavity.

The picture that emerges from this and our previous study (Sundin et al., 2000) is that the receptors involved in producing

the hypoxic ventilatory response are distributed throughout all the gill arches and the orobranchial cavity in the tambaqui. While the frequency response arises almost exclusively from the branchial receptors, a significant component of the amplitude response arises from extrabranchial sites within the orobranchial cavity. It has previously been shown in tench (*Tinca tinca*), sea raven (*Hemipterus americanus*) and traira (*Hoplias malabaricus*) that branchial denervation alone was insufficient to eliminate ventilatory responses to hypoxia (Hughes and Shelton, 1962; Saunders and Sutterlin, 1971; Sundin et al., 1999) while it was sufficient to do so in the channel catfish (*Ictalurus punctatus*) and the gar (*Lepisosteus osseus*) (Smatresk, 1989; Burleson and Smatresk, 1990). The biological significance of these species differences remains unclear, and it will also remain to be seen whether these former species in which the hypoxic ventilatory response was not eliminated by gill denervation alone share the same receptor distribution as the tambaqui.

Our data do not support a role for central O_2 chemoreceptors in fish, in agreement with several previous studies on other species (Kawasaki, 1980: Hedrick et al., 1991; McKenzie et al., 1991a,b). Attempts at central stimulation were without effect (series III). Our data also do not support a role for the hypoxic release of catecholamines into the circulation as a causal factor in the hypoxic ventilatory response (Randall and Taylor, 1991) since the exogenous application of catecholamines inhibited ventilation. This suggests that, if hypoxia becomes severe enough to cause a release of catecholamines from chromaffin tissue into the circulation, the net effect would be to depress ventilation. It appears that the exogenous adrenaline administered in the present study acted to reduce breathing frequency *via* beta-receptor activation while the depression of ventilation amplitude occurred, at least in part, by some other mechanism. Paradoxically, while beta-receptor blockade eliminated the frequency depression caused by exogenous adrenaline, it attenuated the frequency response to hypoxia, leading to a respiratory depression at levels of hypoxia below 5.3 kPa (40 mmHg). Such a depression is common during severe hypoxia in bimodal breathers (McKenzie et al., 1991a,b; Randall et al., 1980) but has not, to our knowledge, been investigated in water-breathing fish.

At present, this result is difficult to explain. Our data indicate that the entire frequency response arises from the gills and is eliminated by gill denervation (Sundin et al., 2000; present study, series I and II). It has previously been shown that O_2-sensitive chemoreceptors on the first gill arch of trout (*Oncorhynchus mykiss*) are not stimulated by catecholamines (Burleson and Milsom, 1995) and that exogenous catecholamines infused during hypoxia inhibit breathing in this species (Kinkead and Perry, 1991). Sotalol, however, which is a specific antagonist of beta-adrenoceptors, reduces the magnitude of the frequency response to hypoxia (series IV data), suggesting that such receptors are involved in the excitatory response. One possibility is that catecholamines may act as neurotransmitters at some central site in the pathway by which severe hypoxia stimulates ventilation. It has been

suggested that catecholamines stimulate breathing by central mechanisms in mammals (Eldridge et al., 1985) and fish (Randall and Taylor, 1991).

Whatever the situation in tambaqui, our data show that circulating catecholamines do not stimulate breathing. In previous studies, catecholamine infusion has been shown to increase or decrease ventilation frequency in fish depending on the species, the time of the year and the physiological state of the animal (for a review, see Randall and Perry, 1992). It has been argued both that circulating catecholamines do (Randall and Taylor, 1991) and that they do not (Perry et al., 1992) play a physiological role in regulating breathing, and our data support the latter view.

Responses to hyperoxic hypercarbia

Total denervation of the gills in fish with intact brains in our previous work eliminated the ventilatory response to hypercarbia both in this species (Sundin et al., 2000) and in the traira (Reid et al., 2000). Identical results were obtained again in this study with hyperoxic hypercarbia. The increases in breathing frequency, amplitude and total ventilation during hyperoxic hypercarbia in control fish (C) were eliminated in the GD fish, clearly showing the presence of specific CO_2/pH receptors within the gills. Indeed, total ventilation now decreased during hyperoxic hypercarbia compared with ventilation in normoxia, most likely an effect of the hyperoxia, which was not eliminated until the orobranchial cavity was also denervated (see section above on the responses to hypoxia and hyperoxia).

Following decerebration and spinalectomy, however, a significant increase in frequency was still present when fish with complete central transection of cranial nerves IX and X were exposed to hypercarbic water. Decerebration or spinalectomy appeared to have removed an inhibitory influence of hypercarbia, revealing another source of excitation arising from an extrabranchial site. Thus, breathing rate went from being slightly reduced in the GD fish to being significantly elevated in the DGD fish. One possible explanation of these results is that there are receptors present in the olfactory epithelium which, when stimulated by hypercarbia, inhibit ventilation. Such inhibition has been shown to be present in many air-breathing lower vertebrates (Ballam, 1985; Coates and Ballam, 1989; Coates et al., 1991). The removal of this inhibition by decerebration (cranial nerves 0, I and II would be transected) appears to reveal a secondary source of excitation. This additional excitation was not removed by further transections (DGOD fish). While the receptors responsible for this excitation may reside within the brainstem, addition of CO_2 to the mock cerebrospinal fluid, as well as superfusion of the brain with acidic or alkaline solutions, did not affect ventilation in our study (series III), just as it has not in other studies (Kawasaki, 1980; Hedrick et al., 1991; McKenzie et al., 1991a,b). Thus, if central receptors exist within the brainstem itself in tambaqui and if they contribute to the hypercapnic ventilatory response, they are not located near the surface of the brain, the typical location for

many central chemoreceptors in air-breathing vertebrates (Nattie, 2000). The response that remains is almost exclusively a frequency response, and it is also possible that it arises from receptors with afferent fibres in the two small opercular branches of cranial nerve VII that we left intact. Unfortunately, we could not test this in the present study because further denervation of these branches would have completely eliminated motor innervation of the respiratory muscles.

In summary, our data reveal the presence of O_2-sensitive receptors in the orobranchial cavity innervated by cranial nerves V and VII that are involved in reflex increases in breathing amplitude. Denervation of these and of the O_2-sensitive receptors on the gills is required to eliminate the hypoxic and hyperoxic ventilatory responses as well as all responses to internal and external injections of NaCN. In addition, the data confirm the presence of specific CO_2/pH receptors within the gills that mediate increases in ventilation amplitude and also reveal the presence of CO_2-sensitive receptors, probably in the olfactory epithelium, that inhibit ventilation frequency and a group(s) of extrabranchial receptors that contribute to increases in ventilation frequency in response to hyperoxic hypercarbia. The site(s) of these latter receptors remains to be determined.

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