Olfactory sensitivity to changes in environmental [Ca^{2+}] in the freshwater teleost *Carassius auratus*: an olfactory role for the Ca^{2+}-sensing receptor?

P. C. Hubbard[1,*], P. M. Ingleton[2], L. A. Bendell[2], E. N. Barata[1,3] and A. V. M. Canário[1]

[1]*Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8000-810 Faro, Portugal,*
[2]*Division of Genomic Medicine, Academic Unit of Endocrinology, University of Sheffield, The Medical School, Beech Hill Road, Sheffield S10 2TN, UK* and [3]*Departamento de Biologia, Universidade de Évora, Apartado 94, 7001 Évora Codex, Portugal*

*Author for correspondence (e-mail: phubbard@ualg.pt)

Summary

Olfactory sensitivity to changes in environmental Ca^{2+} has been demonstrated in two teleost species; a salmonid (*Oncorhynchus nerka*) and a marine/estuarine perciform (*Sparus aurata*). To assess whether this phenomenon is restricted to species that normally experience large fluctuations in external ion concentrations (e.g. moving from sea water to fresh water) or is present in a much wider range of species, we investigated olfactory Ca^{2+} sensitivity in the goldfish (*Carassius auratus*), which is a stenohaline, non-migratory freshwater cyprinid. Extracellular recording from the olfactory bulb *in vivo* by electroencephalogram (EEG) demonstrated that the olfactory system is acutely sensitive to changes in external Ca^{2+} within the range that this species is likely to encounter in the wild (0.05–3 mmol l^{-1}). The olfactory system responded to increases in external calcium with increasing bulbar activity in a manner that fitted a conventional Hill plot with an apparent EC$_{50}$ of 0.9±0.3 mmol l^{-1} (close to both ambient and plasma free [Ca^{2+}]) and an apparent Hill coefficient of 1.1±0.3 (means ± S.E.M., *N*=6). Thresholds of detection were below 50 μmol l^{-1}. Some olfactory sensitivity to changes in external [Na$^+$] was also recorded, but with a much higher threshold of detection (3.7 mmol l^{-1}). The olfactory system of goldfish was much less sensitive to changes in [Mg^{2+}] and [K$^+$]. Preliminary data suggest that Ca^{2+} and Mg^{2+} are detected by the same mechanism, although with a much higher affinity for Ca^{2+}. Olfactory sensitivity to Na$^+$ may warn freshwater fish that they are reaching the limit of their osmotic tolerance when in an estuarine environment. Olfaction of serine, a potent odorant in fish, was not dependent on the presence of external Ca^{2+} or Na$^+$. Finally, the teleost Ca^{2+}-sensing receptor (Ca-SR) was shown to be highly expressed in a subpopulation of olfactory receptor neurones by both immunocytochemistry and *in situ* hybridisation. The olfactory sensitivity to Ca^{2+} (and Mg^{2+}) is therefore likely to be mediated by the Ca-SR. We suggest that olfactory Ca^{2+} sensitivity is a widespread phenomenon in teleosts and may have an input into the physiological mechanisms regulating internal calcium homeostasis.

Key words: Ca^{2+}, olfaction, fish, freshwater, Ca^{2+}-sensing receptor, electroencephalogram, immunocytochemistry, *in situ* hybridisation, goldfish, *Carassius auratus*.

Introduction

Maintenance of a constant plasma-free Ca^{2+} concentration is crucial to vertebrates. In contrast to terrestrial vertebrates, whose main source of calcium is dietary, fish are continually surrounded by an external source of calcium; the water in which they live. The calcium content of water, however, can vary from vastly in excess of physiological needs (e.g. sea water) to nearly zero (soft fresh water). Although the ability of fish to estimate the levels of calcium in the environment is poorly understood, two species have previously been shown to have olfactory sensitivity to changes in environmental [Ca^{2+}]; the sockeye salmon *Oncorhynchus nerka* (Bodznick, 1978) and the gilthead sea-bream *Sparus aurata* (Hubbard et al., 2000).

The authors proposed that these fish may use this sensory information in identifying natal rivers and/or to activate appropriate physiological mechanisms to maintain a constant circulating [Ca^{2+}] in the face of potentially large changes in external [Ca^{2+}]. These species are either migratory (from sea water to fresh water; sockeye salmon) or regularly penetrate estuaries to feed (sea-bream) and, therefore, might have evolved olfactory Ca^{2+}-sensitivity under these rather specific circumstances. In order to establish whether this phenomenon is restricted to migratory and/or euryhaline species or is more widespread amongst teleosts, the current study examined the olfactory sensitivity to environmental Ca^{2+} in the goldfish

(*Carassius auratus*); a stenohaline freshwater, non-migratory cyprinid.

C. auratus typically inhabits land-locked still waters or the slow-moving lower reaches of rivers. It is possible, therefore, that it might, on occasion, come into contact with dilute sea water (e.g. in the upper reaches of estuaries) but the main source of variation in the calcium content of its environment is likely to be the source of the water (i.e. the underlying geology) or, possibly, human activity (Douglas et al., 1996). We hypothesise that any olfactory sensitivity to calcium might therefore be involved in activation and/or regulation of the appropriate physiological responses to elevations or decreases in environmental Ca^{2+}, rather than solely the recognition of home waters, and so would be widespread among teleosts. Furthermore, as we previously proposed that the olfactory Ca^{2+} sensitivity might be mediated by a subpopulation of olfactory receptor neurones expressing the Ca^{2+}-sensing receptor (Ca-SR) (Hubbard et al., 2000), the presence of this receptor within the olfactory epithelium was assessed by immunocytochemistry and *in situ* hybridisation. Finally, to examine whether olfactory sensitivity to inorganic cations is restricted to Ca^{2+}, we also investigated the response to changes in Na^+, K^+ and Mg^{2+}, the other major inorganic cations present in freshwater environments.

Materials and methods

Experimental animals

Adult goldfish *Carassius auratus* L. of both sexes (fork length = 130–167 mm; mass = 51–105 g; gonadosomatic index = 0.9–9.8%) were obtained from a local supplier more than a year before use, maintained outside in 1000 l tanks under semi-natural conditions (i.e. under natural photoperiod and temperature) and fed once or twice a day (depending on temperature) with commercial fish food (TetraPond Pondsticks; Melle, Germany). Experiments took place between August and October in 2000 and 2001.

Recording the electroencephalogram (EEG) from the olfactory bulb

Goldfish were anaesthetized by immersion in water containing $100\,\mathrm{ml\,l^{-1}}$ 3-aminobenzoic acid ethyl ester (MS222; Sigma) and immobilized by intramuscular injection of $1\,\mathrm{mg\,kg^{-1}}$ of the neuromuscular blocker gallamine triethiodide (Sigma) in 0.9% saline. The fish were then held in a padded V-clamp, and water containing $50\,\mathrm{mg\,l^{-1}}$ MS222 was pumped over the gills *via* a tube placed in the mouth (flow rate approximately $100\,\mathrm{ml\,min^{-1}\,100\,g^{-1}}$ body mass). The skull on top of the head from between the eyes to immediately posterior to the nares was removed using a small circular saw. Local anaesthetic (3% tetracaine in 0.9% saline; Sigma) was applied to the wound with a small paintbrush. Fat and connective tissue were then cleared to reveal the olfactory bulbs. The EEG was recorded by placing paired silver wire electrodes 0.25 mm in diameter, teflon-insulated right up to the tip (Clark Electromedical Ltd, Reading, UK), with slight pressure on the

dorso-lateral surface of the olfactory bulb of the rosette being irrigated. The fish was earthed *via* a metal pin inserted into the skin of the head, near the wound. The electrodes were connected to an a.c. preamplifier (NL100AK Digitimer Ltd, Welwyn Garden City, UK) and the signal was amplified (×20,000; NL104, Digitimer Ltd), filtered (2–500 Hz; NL125, Digitimer Ltd) and integrated (time constant 1 s; NL703, Digitimer Ltd). Both direct and integrated signals were digitised and recorded using a DigiData 1200 and a PC running Axoscope 1.1 (Axon Instruments, Inc., Foster City, USA).

Given the small size of the fish, the relatively large recording electrodes and the overall anatomy of the olfactory system, the entire surface of the bulb was not accessible for recording. Accordingly, no attempt was made to map the olfactory bulb for its Ca^{2+} sensitivity. Instead, in an attempt to standardise the recording position between experiments, the electrodes were placed on the part of inner dorso-lateral surface of the bulb giving the strongest response to $10^{-5}\,\mathrm{mol\,l^{-1}}$ L-serine. Experiments took place in an air-conditioned laboratory in an effort to maintain the temperature as close as possible to the temperature of the water in which the fish were kept (15–25°C, depending on time of year). Water $[Ca^{2+}]$ was assayed colorimetrically (Sigma kit 587-M).

Stimulus solutions and delivery

Unless stated otherwise, all stimuli were made using artificial fresh water (AFW), Ca^{2+}-free AFW or Na^+-free AFW of the following composition: AFW = $100\,\mathrm{\mu mol\,l^{-1}}$ $CaCl_2$, $100\,\mathrm{\mu mol\,l^{-1}}$ NaCl and $100\,\mathrm{\mu mol\,l^{-1}}$ $NaHCO_3$ in distilled water (pH 6.2); Ca^{2+}-free AFW = $100\,\mathrm{\mu mol\,l^{-1}}$ NaCl, $100\,\mathrm{\mu mol\,l^{-1}}$ $NaHCO_3$ and $200\,\mathrm{\mu mol\,l^{-1}}$ choline chloride in distilled water (pH 6.9); Na^+-free AFW = $100\,\mathrm{\mu mol\,l^{-1}}$ $CaCl_2$ and $200\,\mathrm{\mu mol\,l^{-1}}$ choline chloride in distilled water (pH 5.7). No attempt was made to adjust the pH of the AFWs. This was in order to keep Na^+ and Cl^- concentrations constant and to avoid the introduction of buffers, the olfactory potencies of which have not been assessed. Ca^{2+} concentration was varied by addition of $1\,\mathrm{mol\,l^{-1}}$ $CaCl_2$ to Ca^{2+}-free AFW; Na^+ concentration was varied by dilution of $1\,\mathrm{mol\,l^{-1}}$ NaCl in Na^+-free AFW, and variations of Mg^{2+} and K^+ concentration were made by dilution of $1\,\mathrm{mol\,l^{-1}}$ $MgCl_2$ and KCl, respectively, in AFW. A constant flow of AFW (or Ca^{2+}-free AFW or Na^+-free AFW, as appropriate) was delivered to the olfactory epithelium *via* a glass tube (flow rate $6\,\mathrm{ml\,min^{-1}}$) under gravity. Stimulus solutions were introduced into this flow *via* a computer-controlled three-way solenoid valve. To ensure that the stimulus flow reached the olfactory rosette, the small flap of skin covering the nostril was cut away, and the end of the tube was placed immediately above the olfactory raphe.

Treatment of data

To treat the EEG responses to changes in external $[Ca^{2+}]$ in a quantitative manner, the amplitude *A* of the integrated responses was measured (mV). Data from a single experiment were normalised to the amplitude of the EEG response to a change in $[Ca^{2+}]$ from nominally zero (Ca^{2+}-free AFW) to

$100\,\mathrm{mmol\,l^{-1}}$ Ca^{2+}. These data were then fitted to a conventional three-parameter Hill equation using SigmaPlot 2000 (SPSS Science):

$$A = A_{max}[Ca^{2+}]^n/(EC_{50}{}^n + [Ca^{2+}]^n), \qquad (1)$$

where A is the normalised, integrated response amplitude, A_{max} is the maximal normalised response amplitude (i.e. 1), n is the Hill coefficient, $[Ca^{2+}]$ is the Ca^{2+} concentration (in $\mathrm{mmol\,l^{-1}}$), and EC_{50} is the $[Ca^{2+}]$ giving a half-maximal response.

To account for the variation in absolute amplitude of the integrated EEG response among fish (when comparing the sensitivity to different cations), the amplitude of the integrated response was normalised to that of $10^{-5}\,\mathrm{mol\,l^{-1}}$ L-serine in AFW. The amplitudes of integrated responses to $10^{-5}\,\mathrm{mol\,l^{-1}}$ L-serine in AFW, Ca^{2+}-free AFW and Na^+-free AFW were measured (in mV) and compared directly using a Student's t-test for paired data. $P<0.05$ was taken to represent statistical significance.

In situ *hybridisation of Ca-SR gene expression*

Goldfish were anaesthetised by immersion in water containing $100\,\mathrm{mg\,l^{-1}}$ MS222. The entire olfactory system (epithelium, nerve, bulb, tract and telencephalon) was dissected out from both sides and immediately fixed in freshly prepared sublimated Bouin–Hollande fixative (Kraicer et al., 1967). Fixed tissues were then dehydrated through a graded series of alcohols, cleared in xylene and embedded in paraffin wax. Sections were cut at $5\,\mu\mathrm{m}$ and mounted on 3-aminopropyltriethoxysilane (APES)-coated slides.

The method of *in situ* hybridisation was the same as that used for detecting sea-bream parathyroid-hormone-related protein (PTHrP) and is described in detail by Flanagan et al. (2000). Briefly, oligonucleotide probes (33-mer), positions 316–349, 591–623 and 2712–2737, were chosen from the proposed extracellular domain of the sea-bream Ca-SR receptor (GenBank accession number AJ289717) and end-labelled with digoxygenin (DIG; Gibco-BRL) using terminal transferase in a $20\,\mu\mathrm{l}$ reaction volume containing $12\,\mu\mathrm{l}$ 0.02% diethylpyrocarbonate in deionised water (DEPC water), $4\,\mu\mathrm{l}$ of 5× terminal deoxynucleotidyl transferase (TDT) buffer (Gibco-BRL), $1\,\mu\mathrm{l}$ ($1\,\mu\mathrm{g}$) of probe, $1\,\mu\mathrm{l}$ of DIG-11-UTP (25 nmol) and $2\,\mu\mathrm{l}$ of terminal transferase (Gibco-BRL). The mixture was incubated at $37\,^{\circ}\mathrm{C}$ overnight, and the reaction was stopped by addition of $2\,\mu\mathrm{l}$ of $100\,\mathrm{mmol\,l^{-1}}$ EDTA. Labelled probe was purified using a $2\,\mathrm{cm}$ column of G25 Sephadex™ soaked in elution buffer ($0.1\times$ standard saline citrate + 0.1% sodium dodecyl sulphate). Ten successive portions of $200\,\mu\mathrm{l}$ elution buffer were added to and collected from the column. To detect the fractions with labelled probe, $1\,\mu\mathrm{l}$ from each sample was spotted onto nitrocellulose paper pre-soaked in DEPC water and 20× standard saline citrate (SSC; $150\,\mathrm{mmol\,l^{-1}}$ NaCl, $15\,\mathrm{mmol\,l^{-1}}$ sodium citrate) and blotted dry. The samples were fixed by baking for $1\,\mathrm{h}$ at $80\,^{\circ}\mathrm{C}$ or by ultraviolet irradiation. The dried nitrocellulose paper was re-wetted with buffer 1 ($12.1\,\mathrm{g\,l^{-1}}$ Tris, $8.7\,\mathrm{g\,l^{-1}}$ NaCl in distilled water, pH 7.5).

Non-specific antibody binding was blocked by incubation in 1 ml of 3% bovine serum albumin (BSA) in buffer 1 for 30 min at room temperature ($20\,^{\circ}\mathrm{C}$) before incubation with antiserum to DIG, diluted 1:500 in BSA/buffer 1 solution, for 30 min at room temperature. The nitrocellulose membrane was then washed five times for 3 min each in buffer 1, with gentle rocking. Finally, the paper was equilibrated with buffer 2 ($12.1\,\mathrm{g\,l^{-1}}$ Tris, $5.84\,\mathrm{g\,l^{-1}}$ NaCl, $10.16\,\mathrm{g\,l^{-1}}$ $MgCl_2$, pH 9.5) for 5 min. Colour was then developed in a solution of buffer 2 containing $45\,\mu\mathrm{l}$ of Nitroblue Tetrazolium salt (NBT) and $35\,\mu\mathrm{l}$ of 5-bromo-4-chloro-3-indoylphosphate *p*-toluidine salt (BCIP) at room temperature together with a trace of levamisole. The samples containing labelled probe were then pooled, divided into $10\,\mu\mathrm{l}$ portions and lyophilized for storage at $-20\,^{\circ}\mathrm{C}$.

Hybridisation procedure

Sections were de-waxed and rehydrated through a graded series of alcohols diluted with DEPC water. For pre-hybridisation, slides were immersed in 25% deionized formamide in 3× SSC for 60 min. Samples of hybridisation ($20\,\mu\mathrm{l}$ of mixture per section) were prepared, each containing $1\,\mu\mathrm{l}$ of labelled probe. Sections were covered with glass coverslips and incubated overnight at room temperature. Coverslips were removed by soaking in 4× SSC, and the slides were then washed twice in 3× SSC for 15 min. Further more-stringent washes followed: 1× SSC for 5 min, then 0.1× SSC for 5 min. Hybridised probe was detected using anti-DIG/alkaline-phosphatase-conjugated serum (Dako, Denmark) at a dilution of 1:500 in blocking solution for 2 h after blocking non-specific reactions with a 2% solution of dried milk in buffer 1 for 30 min. After washing in buffer 1 followed by buffer 2, colour was developed by addition of a solution of NBT/BCIP and levamisole in buffer 2. Slides were incubated overnight at room temperature in a dark chamber. After washing in deionised water and running tapwater, the sections were mounted in glycerogel.

Immunocytochemistry of the calcium-sensing receptor

An antiserum was prepared (in rabbits) to an amino acid sequence from the deduced amino acid sequence of the extracellular domain of the Ca^{2+}-sensing receptor, common to both puffer fish (*Fugu rubripes*; GenBank accession number AB008857) and sea-bream (*Sparus aurata*; GenBank accession number AJ289717); positions 106–115 of the mature protein. This oligopeptide was conjugated to bovine thyroglobulin and injected into rabbits, as previously described for the generation of antibodies to rat prolactin receptors (Nevalainen et al., 1996). The resulting antiserum has previously been used to demonstrate the presence of the Ca-SR in tissues of the flounder (*Platichthys flesus*) and puffer fish (Ingleton et al., 1999).

Sections of olfactory tissues were prepared, as above, and subjected to immunocytochemistry using a method based on that of Sternberger (1974). Briefly, sections were dewaxed in xylene, rehydrated through a graded series of alcohols and washed in running tapwater. Non-specific peroxidase activity was destroyed by immersion in phosphate-buffered saline

(PBS), pH 7.4, containing 25% methanol and 1% hydrogen peroxide, and non-specific binding sites were blocked with 4% normal swine serum in 1% BSA in 100 mmol l⁻¹ PBS, pH 7.4. Reaction with specific antiserum occurred overnight at 4°C, followed by detection of bound antiserum with swine anti-rabbit serum (Dako, Denmark) at room temperature, then incubation with peroxidase/anti-peroxidase reagent. Finally, colour was developed by immersion in diaminobenzidine/hydrogen peroxide solution in Tris/HCl, pH 7.6. Control reactions used normal rabbit serum instead of the specific primary antiserum.

Results

Olfactory EEG responses to changes in external [Ca^{2+}]

The olfactory system of goldfish responded to increases in [Ca^{2+}] of the water flowing over the olfactory epithelium with large-amplitude increases in bulbar activity (Fig. 1A). This activity was concentration-dependent up to a maximum of approximately 10 mmol l⁻¹ Ca^{2+} and could be fitted with a conventional Hill equation (Fig. 1B), giving an apparent EC_{50} of 0.9±0.3 mmol l⁻¹ and Hill coefficient of 1.1±0.3 (mean ± S.E.M., N=6). Although data from individual fish were variable, giving EC_{50} values from 0.2 to 2 mmol l⁻¹, the fit to the curve in each case was good (r^2=0.81–0.96), suggesting a true biological variability in sensitivity between individual fish. The increase in bulbar activity was short-lived, however, returning to near baseline levels within the period of stimulus delivery (10 s). Similarly, the threshold of detection was variable among fish, some fish giving large-amplitude responses to 50 μmol l⁻¹ Ca^{2+}, while others barely responded to this concentration.

Preliminary experiments showed the olfactory system of the goldfish to be insensitive to increases in choline chloride concentration (up to 10 mmol l⁻¹), indicating that the bulbar activity is due to changes in [Ca^{2+}], rather than accompanying
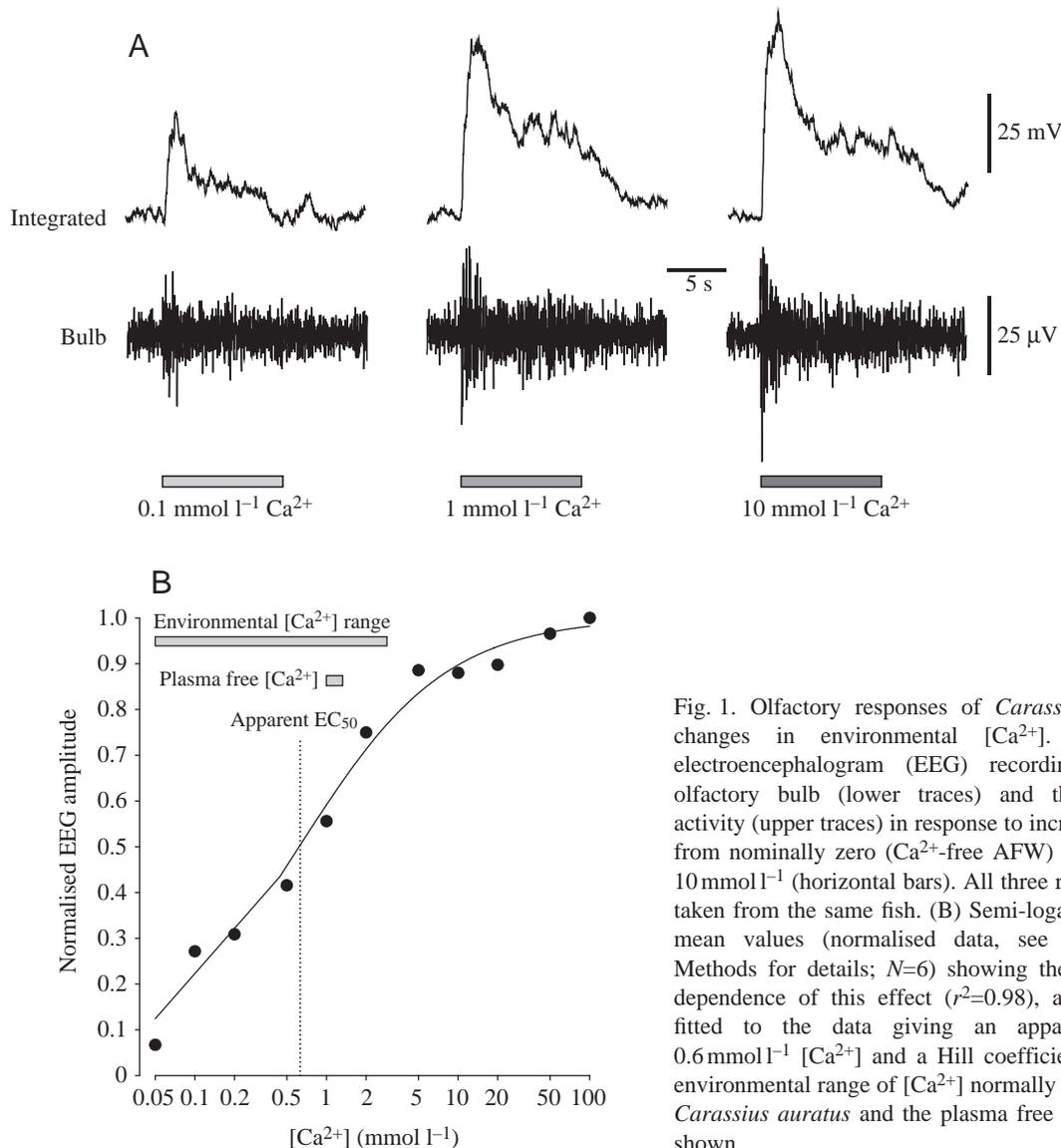

Fig. 1. Olfactory responses of *Carassius auratus* to changes in environmental [Ca^{2+}]. (A) Typical electroencephalogram (EEG) recordings from the olfactory bulb (lower traces) and their integrated activity (upper traces) in response to increases in [Ca^{2+}] from nominally zero (Ca^{2+}-free AFW) to 0.1, 1.0 and 10 mmol l⁻¹ (horizontal bars). All three recordings were taken from the same fish. (B) Semi-logarithmic plot of mean values (normalised data, see Materials and Methods for details; N=6) showing the concentration dependence of this effect (r^2=0.98), and a Hill plot fitted to the data giving an apparent EC_{50} of 0.6 mmol l⁻¹ [Ca^{2+}] and a Hill coefficient of 0.8. The environmental range of [Ca^{2+}] normally encountered by *Carassius auratus* and the plasma free [Ca^{2+}] are also shown.

A

Integrated

25 mV

5 s

Bulb

125 µV

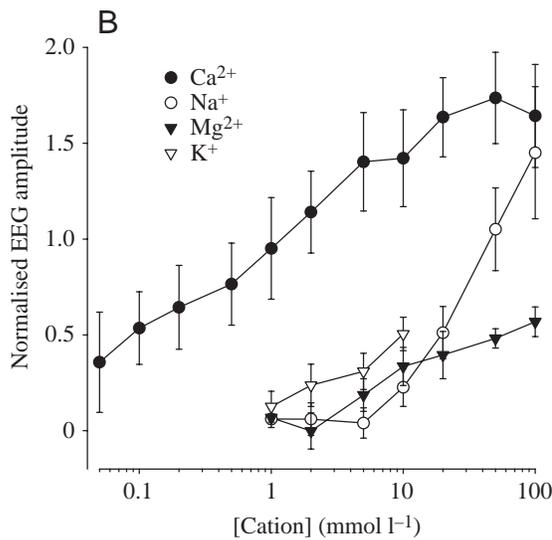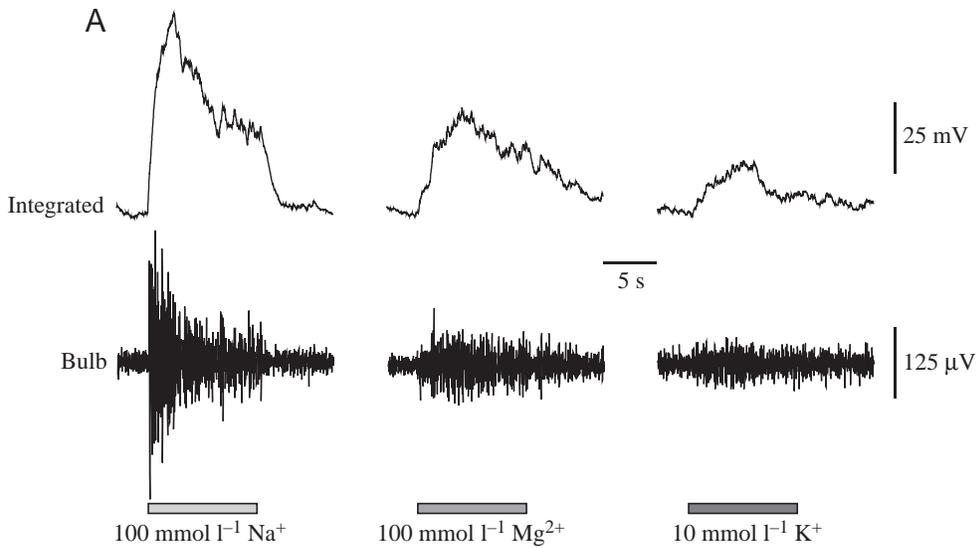100 mmol l⁻¹ Na⁺   100 mmol l⁻¹ Mg²⁺   10 mmol l⁻¹ K⁺

B

Fig. 2. Olfactory responses of *Carassius auratus* to changes in environmental [Na⁺], [Mg²⁺] and [K⁺]. (A) Typical electroencephalogram (EEG) recordings from the olfactory bulb (lower traces) and their integrated activity (upper traces) in response to 100 mmol l⁻¹ Na⁺ (in Na⁺-free AFW), 100 mmol l⁻¹ Mg²⁺ in AFW and 10 mmol l⁻¹ K⁺ in AFW (horizontal bars). All three recordings were taken from the same fish. (B) Semi-logarithmic plot of pooled, normalised (to the response to 10⁻⁵ mol l⁻¹ L-serine in AFW) data of integrated EEG amplitude (means ± S.E.M.; N=5–6) in response to changes in [Ca²⁺] (filled circles), [Mg²⁺] (filled triangles), [Na⁺] (open circles) and [K⁺] (open triangles). Note that only the responses to Na⁺ approach the amplitude of those evoked by Ca²⁺, and then only at high concentrations.

Integrated

10 mV

5 s

Bulb

50 µV

Ca²⁺-free AFW   Ca²⁺-free AFW plus 100 mmol l⁻¹ Mg²⁺
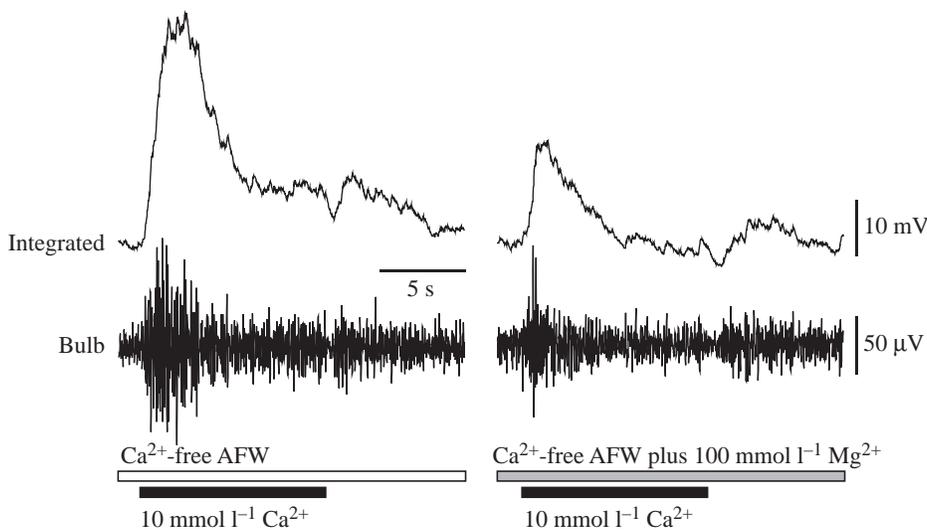
10 mmol l⁻¹ Ca²⁺   10 mmol l⁻¹ Ca²⁺

Fig. 3. Effects of cross adaptation to Mg²⁺ on olfactory responses to Ca²⁺ in *Carassius auratus*. Two consecutive olfactory electroencephalogram (EEG) responses (lower traces) and their integrated activities (upper traces) to 10 mmol l⁻¹ Ca²⁺ (black horizontal bars) in the absence (open horizontal bar) and presence (shaded horizontal bar) of 100 mmol l⁻¹ Mg²⁺. Note that the response to calcium is bluntened, but not abolished, in the presence of excess Mg²⁺.

changes in $[Cl^-]$. This also demonstrates that the olfactory system is insensitive to choline (used to replace Ca^{2+} and Na^+), small changes in pH and/or changes in osmolality *per se*. The $[Ca^{2+}]$ of the water in which the fish were kept ranged from 0.7 to $1.2 \, \mathrm{mmol \, l^{-1}}$.

EEG responses to changes in [Na$^+$], [K$^+$] and [Mg^{2+}]

The olfactory system of goldfish was also sensitive to changes in $[Na^+]$ and, to a much lesser extent, $[K^+]$ and $[Mg^{2+}]$, provoking increases in bulbar activity in a concentration-dependent manner (Fig. 2A). However, neither the amplitude of the responses nor the sensitivity were as great as that for Ca^{2+} (Fig. 2B). Only Na^+ evoked responses approaching the magnitude of those of Ca^{2+}, and then only at much higher concentrations ($10-100 \, \mathrm{mmol \, l^{-1}}$). The threshold of detection (calculated by linear regression) was $3.7 \, \mathrm{mmol \, l^{-1}}$ for Na^+. Furthermore, there was no apparent plateau of the magnitude of the response, even at or above the maximal concentrations of these ions that goldfish are likely to encounter in their natural environment. Continuous exposure of the olfactory epithelium to $100 \, \mathrm{mmol \, l^{-1}}$ $MgCl_2$ severely attenuated the response to $10 \, \mathrm{mmol \, l^{-1}}$ Ca^{2+} (Fig. 3), suggesting that these two ions are acting *via* the same cellular mechanism, although the affinity for Mg^{2+} is at least one order of magnitude less than that for Ca^{2+}.

Effect of the absence of Ca^{2+} and Na$^+$ on EEG responses to L-serine

The absence of Ca^{2+} in the water flowing over the olfactory epithelium did not reduce the amplitude of the EEG response evoked by $10^{-5} \, \mathrm{mol \, l^{-1}}$ L-serine (Fig. 4A). In fact, the response to serine was significantly larger in the absence of external Ca^{2+} (Fig. 4A,B). Similarly, the amplitude of the EEG response appeared to increase in the absence of Na^+ (Fig. 4C) but the increase failed to reach statistical significance. These data discount the possibility that the observed responses to changes in $[Ca^{2+}]$ and $[Na^+]$ are merely due to non-specific effects on the olfaction of other odorants.

Comparison of EEG responses to cations with those to serine

A comparison of the bulbar responses to changes in ion concentrations with that of $10^{-5} \, \mathrm{mol \, l^{-1}}$ L-serine shows that the rapid increase in activity followed by a period of accommodation, with the activity returning to near pre-stimulus levels within the 10 s period of stimulus delivery is common to all responses (compare examples in Figs 1–3 with the response to $10^{-5} \, \mathrm{mol \, l^{-1}}$ L-serine shown in Fig. 4). In general, the responses to $\geq 1 \, \mathrm{mmol \, l^{-1}}$ Ca^{2+} were higher in amplitude than those to $10^{-5} \, \mathrm{mol \, l^{-1}}$ L-serine, whilst those to the other cations were always lower in amplitude, with the exception of $100 \, \mathrm{mmol \, l^{-1}}$ Na^+.
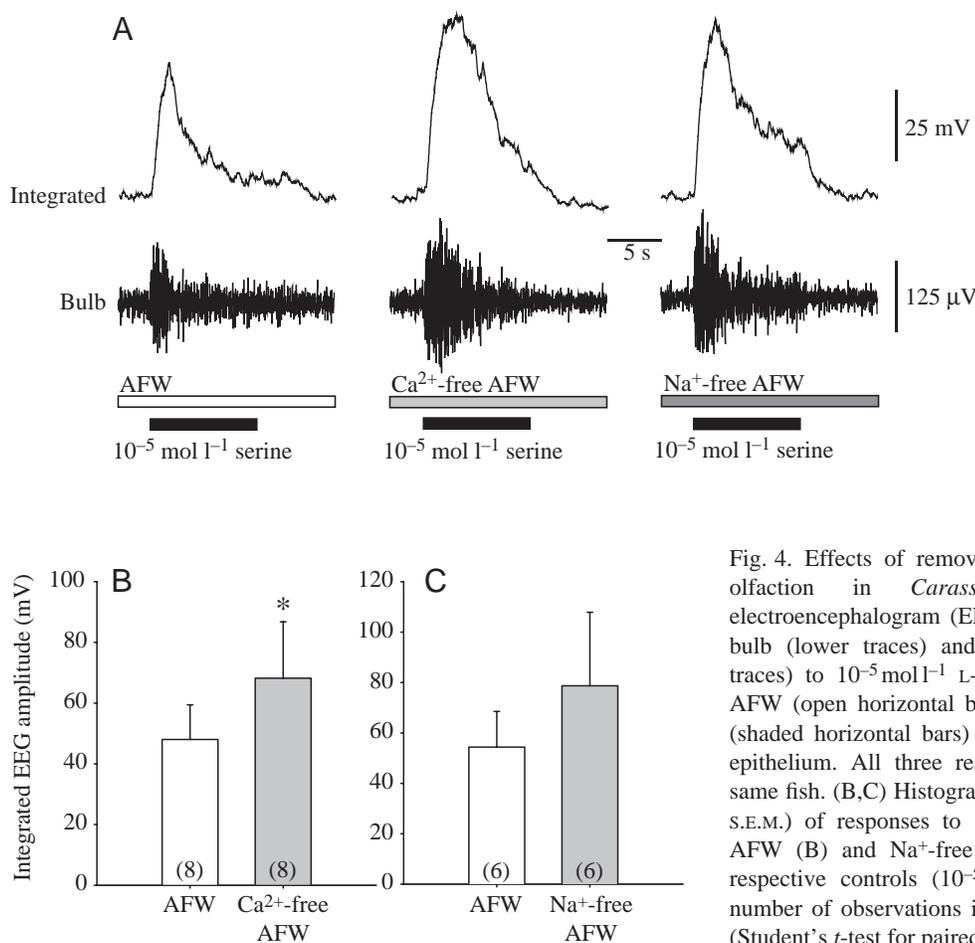

Fig. 4. Effects of removal of external Ca^{2+} and Na^+ on olfaction in *Carassius auratus*. (A) Typical electroencephalogram (EEG) responses from the olfactory bulb (lower traces) and their integrated activity (upper traces) to $10^{-5} \, \mathrm{mol \, l^{-1}}$ L-serine (black horizontal bars) in AFW (open horizontal bar), to Ca^{2+}-free and to Na^+-free (shaded horizontal bars) AFW flowing over the olfactory epithelium. All three responses were recorded from the same fish. (B,C) Histograms showing pooled data (means + S.E.M.) of responses to $10^{-5} \, \mathrm{mol \, l^{-1}}$ L-serine in Ca^{2+}-free AFW (B) and Na^+-free AFW (C) compared with their respective controls ($10^{-5} \, \mathrm{mol \, l^{-1}}$ L-serine in AFW). The number of observations is shown in parentheses. *$P<0.05$ (Student's *t*-test for paired data).

Expression of the Ca-SR in the olfactory epithelium

Immunocytochemistry of Ca^{2+}-sensing receptors in the olfactory rosette is shown in Fig. 5A. The sensory epithelium of the goldfish is multilayered, with a surface zone within which isolated receptor cells react strongly with the antiserum to Ca-SR. Ca-SR-positive cells are most prevalent in the surface zone, but they also occur in the basal zone and apparently migrating between the layers. Some nerve axons also contained Ca-SR protein (data not shown). The results of the *in situ* hybridisation with Ca-SR oligoprobes are shown in Fig. 5C. The principal site of Ca-SR gene expression is cells of the basal layer of the sensory epithelium. However, multiple cell layers of the integument epithelium, which is contiguous with the olfactory epithelium, hybridised abundantly with the Ca-SR probe.

Discussion

Olfactory sensitivity to environmental Ca^{2+}

The present study clearly shows that the olfactory system of the goldfish is highly sensitive to changes in external $[Ca^{2+}]$ well within the range likely to be encountered in its natural habitat. Previous studies demonstrating olfactory sensitivity in teleosts to changes in environmental $[Ca^{2+}]$ have used marine (Hubbard et al., 2000) or euryhaline, migratory (Bodznik, 1978) species. The goldfish is a non-migratory stenohaline freshwater cyprinid that cannot tolerate water of much greater osmotic strength than that of its plasma. For these reasons, olfactory sensitivity to Ca^{2+} is unlikely to be of use in identification of natal rivers (Bodznik, 1978) or to forewarn the fish that it may be approaching the limit of its osmotic tolerance (Hubbard et al., 2000). We therefore suggest that olfactory
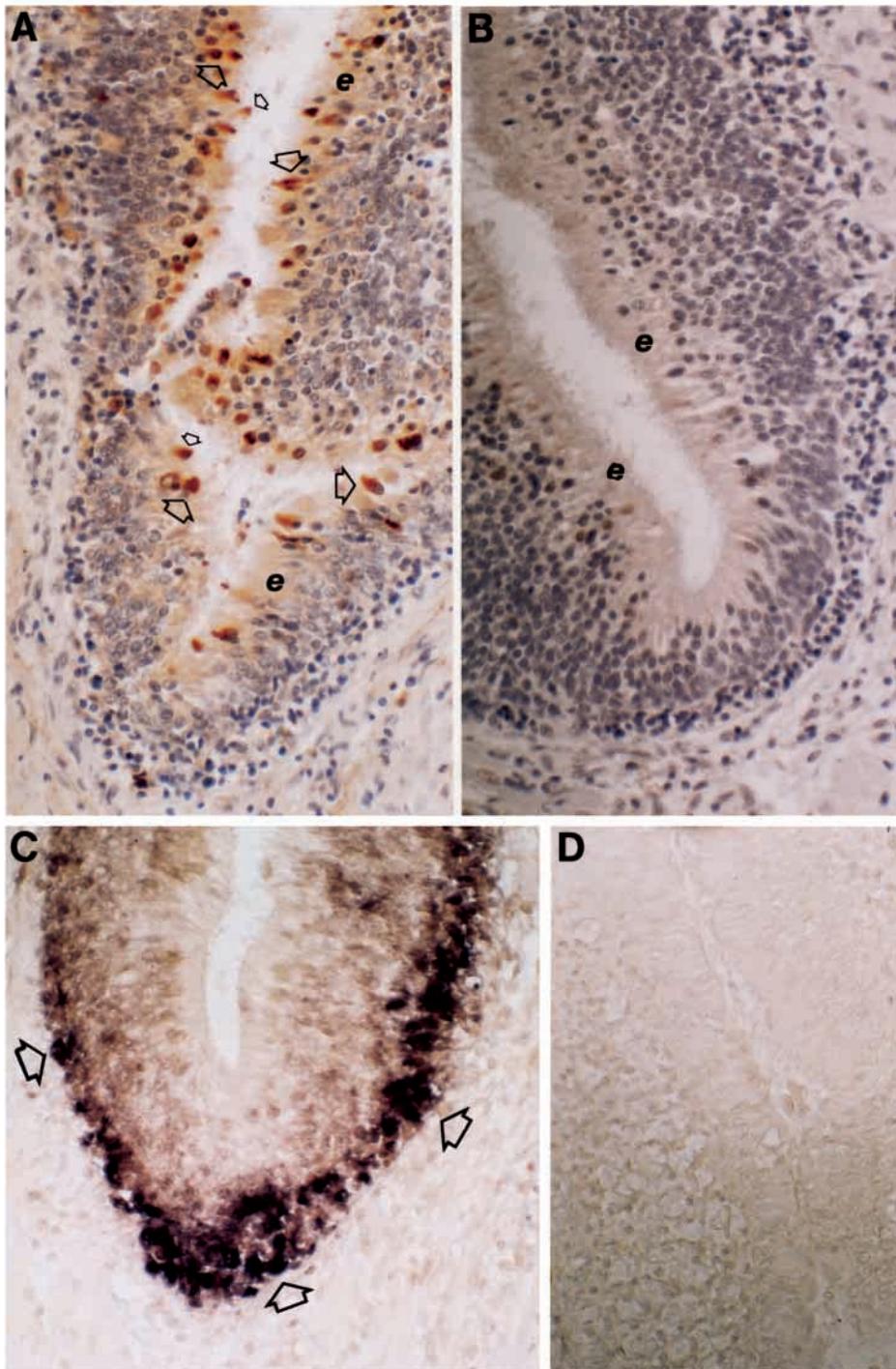

Fig. 5. Ca^{2+}-sensing receptors (Ca-SR) in goldfish olfactory epithelium. (A) Immunocytochemistry with rabbit antiserum to an epitope of the external domain of puffer fish Ca-SR. There is a relatively low concentration of receptors in the cells of the outermost layer of the epithelium (*e*) of a primary lamella with isolated, apparently migratory receptor-type cells (some indicated by open arows) staining more intensely, indicating a much higher receptor content. Magnification ×350. (B) Negative control reaction in which the specific primary antibody was replaced by normal rabbit serum. No reaction was detected in any cells of the olfactory epithelium. Magnification ×350. (C) *In situ* hybridisation of Ca-SR with a DIG-labelled oligonucleotide probe, showing abundant hybridisation in the basal layer of the olfactory epithelium (open arrows). Magnification ×350. (D) A negative control *in situ* hybridisation in a similar area of epithelium to that in C; the specific oligoprobe was omitted from the reagents. There was no reaction in any olfactory tissue cells. Magnification ×350.

sensitivity is a more general phenomenon in teleosts and is probably involved in internal calcium homeostasis.

The apparent EC_{50} of the olfactory response to changes in environmental $[Ca^{2+}]$ was $0.9\,mmol\,l^{-1}$, very close both to the plasma free $[Ca^{2+}]$ of cyprinids (1.0–$1.5\,mmol\,l^{-1}$; Chakraborti and Mukherjee, 1995) and to the water $[Ca^{2+}]$ in which they were kept (0.7–$1.2\,mmol\,l^{-1}$). This is also close to, but below, the IC_{50} of $1.7\,mmol\,l^{-1}$ of the olfactory response to changes in $[Ca^{2+}]$ in the sea-bream (Hubbard et al., 2000), although the response was in the opposite direction (responding to decreases, rather than increases, of $[Ca^{2+}]$). These differences are perhaps to be expected of a marine species *versus* a fresh water species (i.e. the former is usually surrounded by high levels of Ca^{2+}, the latter by low levels).

In common with the EEGs recorded from the olfactory bulbs of the sockeye salmon (Bodznik, 1978), the EEG response of the goldfish to changes in $[Ca^{2+}]$ was phasic, giving a large-amplitude response at the onset of the stimulus, which accommodated rapidly over time, reaching near-baseline levels within $10\,s$. This is in contrast with the responses to changes in $[Ca^{2+}]$ seen in the marine sea-bream (Hubbard et al., 2000), which showed little accommodation over time (up to $100\,s$). This suggested that sea-bream continually monitor external $[Ca^{2+}]$ as a proxy for salinity. However, these responses were recorded from the olfactory nerve and reflect activity of the axons of the olfactory receptor neurones, rather than activity in the olfactory bulb, the 'first-order processing centre' of olfactory information (Christensen and White, 2000).

It is unlikely that this discrepancy reflects a marked difference in the rate of accommodation of Ca^{2+}-sensitive olfactory receptor neurones between marine and freshwater teleosts, but is more probably a function of the site from which the signal was recorded (Christensen and White, 2000). For anatomical reasons, recording from the olfactory nerve is much more difficult in the goldfish (the bulbs directly underlie the olfactory rosettes, and the nerve is merely as long as the width of the skull separating the two). However, single-unit (mitral cell) responses recorded by Bodznick (1978) from the olfactory bulb of the sockeye salmon did not show any such rapid accommodation; indeed, some units showed inhibition of activity in response to increases in $[Ca^{2+}]$.

The Ca^{2+}-sensitivity varied among fish, some giving strong responses to Ca^{2+} concentrations as low as $10\,\mu mol\,l^{-1}$. This is reflected by the variation in the calculated EC_{50} of the response (0.2–$2\,mmol\,l^{-1}$). However, the sigmoidal nature of the concentration/response curve makes the threshold of detection difficult to predict. Bodznick (1978) obtained some olfactory EEG responses from sockeye salmon to Ca^{2+} concentrations as low as $1\,\mu mol\,l^{-1}$. It is likely that the olfactory sensitivity to Ca^{2+} is influenced by the prevailing external Ca^{2+} concentration (approx. $1.0\,mmol\,l^{-1}$ in this case) and/or the internal Ca^{2+} status of the fish, and this may explain the variability in the calculated EC_{50} of the response. Previously, we have shown that removal of calcium from artificial sea water causes a

dramatic leftward shift of the concentration/response curve of the olfactory Ca^{2+} response in sea-bream (Hubbard et al., 2000). Changes in the pH of the water perfusing the olfactory epithelium have been shown to alter the magnitude of recorded response (e.g. Moore, 1994). It is possible, therefore, that the variation observed in Ca^{2+} responses in the present study is due, in part, to small differences in the pH of the AFW. The influence of external and internal Ca^{2+} status on olfactory sensitivity to Ca^{2+} in goldfish is currently under investigation.

The role of the Ca-SR in olfactory Ca^{2+} sensitivity

The present study clearly demonstrates the presence of the Ca^{2+}-sensing receptor (Ca-SR) in a subpopulation of olfactory receptor neurones in the goldfish in terms of both mature protein (Fig. 5A) and mRNA (Fig. 5C). The Ca-SR is also expressed in a subpopulation of olfactory receptor neurones of sea-bream (P. M. Ingleton, unpublished observations). This is consistent with our hypothesis that the Ca-SR is responsible for mediating the olfactory sensitivity to Ca^{2+} (Hubbard et al., 2000). Further support for this is provided by the close match between the IC_{50} of parathyroid hormone release from mammalian parathyroid glands ($1.2\,mmol\,l^{-1}$ Ca^{2+}; Brown, 1991), which is mediated by the mammalian Ca-SR (Brown et al., 1993), and the EC_{50} of the goldfish olfactory response ($0.9\,mmol\,l^{-1}$ Ca^{2+}).

Although the mature Ca-SR protein is mainly present in a subpopulation of the olfactory receptor neurones (Fig. 5A), the mRNA is most abundant in the basal layer (Fig. 5C). This phenomenon (i.e. message and protein differentially distributed between associated cell types) has also been described for transforming growth factor β (TGFβ) in developing mouse skin (Lehnert and Akhurst, 1988) and heart (Akhurst et al., 1990) and for parathyroid-hormone-related protein (PTHrP) in the frog pituitary gland (Danks et al., 1997). Here, gene expression of PTHrP was detected in the pars intermedia, but the cells of the pars distalis contained the protein. There is an obvious parallel between the situation of TGFβ in the developing mouse skin and Ca-SR in the goldfish olfactory system in that the olfactory epithelium is a specialised region contiguous with the fish integument. The Ca-SR-containing receptor neurones appear to be a renewable population of cells, probably neuroepithelial, which function during passage through the layers of epithelial cells of the primary lamellae and are then extruded to the exterior (Bertmar, 1982).

The Ca-SR is a member of the G-protein-coupled seven-transmembrane-domain receptor superfamily (Brown et al., 1993) and is expressed in many mammalian tissues involved with Ca^{2+} homeostasis, including the kidney (Riccardi et al., 1995) and stomach (Cheng et al., 1999). Although we have yet to demonstrate unequivocally that those olfactory receptor neurones expressing the Ca-SR are the same as those that are responding to changes in external $[Ca^{2+}]$, it seems highly probable that the Ca-SR also has an olfactory role, at least in fish. However, the fish Ca-SR, if it is responsible for the

olfactory Ca^{2+} sensitivity, is functionally slightly different from its mammalian counterpart. The amplitude of EEG responses to Mg^{2+} was much lower than that to Ca^{2+} (Fig. 2), and cross-adaptation with $100\,mmol\,l^{-1}$ $MgCl_2$ significantly attenuated the response to $10\,mmol\,l^{-1}$ Ca^{2+}, but failed to eliminate it (Fig. 3). These preliminary data suggest that the affinity of the fish Ca-SR is at least 10 times higher for Ca^{2+} than for Mg^{2+}. By comparison, the mammalian Ca-SR has an affinity for Ca^{2+} only three times greater than that for Mg^{2+} (Brown et al., 1993).

Olfactory sensitivity to Na^+, K^+ and Mg^{2+}

The olfactory system of the goldfish proved to be much less sensitive to changes in $[Na^+]$, $[K^+]$ and $[Mg^{2+}]$, with minimal responses to K^+ (even up to $10\,mmol\,l^{-1}$) and Mg^{2+} (up to $100\,mmol\,l^{-1}$) but with strong responses to Na^+, albeit at relatively high concentrations ($10–100\,mmol\,l^{-1}$). In fact, the pattern of responsiveness to these ions was very similar to that reported by Bodznick (1978), who recorded EEGs from the sockeye salmon. As previously suggested, Mg^{2+}-sensitivity might be mediated by the Ca-SR. However, given the relative sensitivities of the olfactory response to $[Ca^{2+}]$ and $[Mg^{2+}]$ in fresh water fish (Bodznick, 1978; present study) and the concentrations of either ion they are likely to encounter in freshwater environments, the Mg^{2+} response is unlikely to be of any physiological consequence.

The small-amplitude EEG responses to K^+ may be explained as a non-specific depolarisation of olfactory receptor neurones caused by increases in external $[K^+]$ (which was why sensitivity to $[K^+]$ above $10\,mmol\,l^{-1}$ was not tested); fresh water teleosts are unlikely to encounter a $[K^+]$ above $1.0\,mmol\,l^{-1}$. This provides further circumstantial evidence that it is the cation that is important in the olfactory responses to different salts (Bodznick, 1978) and suggests that the olfactory system of goldfish is insensitive to normal environmental levels of K^+. The observation that the olfactory epithelium of goldfish is sensitive to relatively high concentrations of Na^+ is not novel; Hara and Gorbman (1967) used $50\,mmol\,l^{-1}$ NaCl as an olfactory stimulant in a study of the electrophysiological characteristics of the olfactory system. However, these authors did not address the potential physiological significance of this phenomenon. It is possible that the relatively high threshold of detection of this ion ($3.7\,mmol\,l^{-1}$) means that this sensitivity is not involved in routine monitoring of Na^+, but may serve as an 'early warning' signal that the goldfish is approaching the limits of its osmoregulatory capacity; goldfish cannot tolerate full-strength sea water. As an inhabitant of the lower reaches of slow-flowing rivers, however, it could possibly stray into an estuarine environment. Some cyprinids readily penetrate brackish environments (Muus and Dahlstrom, 1967), and goldfish can survive in 30% sea water ($[Na^+]=140\,mmol\,l^{-1}$; e.g. Fenwick, 1975) or even slightly hyperosmotic sea water ($[Na^+]=190\,mmol\,l^{-1}$) if adapted gradually (Lahlou et al., 1969).

The mechanism(s) of olfactory Na^+-sensitivity was not investigated directly. Nevertheless, in light of the relatively high $[Na^+]$ required to elicit a response, and the apparently non-saturable nature of the concentration/response curve, we envisage that the response to Na^+ is likely to be channel-mediated rather than receptor-mediated. It is not clear whether this is a true olfactory response to changes in $[Na^+]$ or a non-specific, physico-chemical effect of the changes in Na^+ distribution across the exposed microvilli and/or cilia of olfactory receptor neurones. However, the goldfish was able to respond to the conventional fish odorant L-serine in the absence of external Na^+. Therefore, the observed responses may be due to a specialised subpopulation of 'Na^+-sensitive' olfactory receptor neurones in a manner similar to that we propose for Ca^{2+}-sensitive olfactory neurones. This hypothesis awaits direct investigation.

Involvement of external cations in olfactory signal transduction

In terrestrial vertebrates, the olfactory transduction pathway is highly dependent on the presence of external ions, principally Ca^{2+} (for a review, see Schild and Restrepo, 1998). However, these animals rely on the ion content of the olfactory mucus, which they are able to regulate tightly. Freshwater fish, in contrast, are constantly exposed to a highly dilute environment and are in constant danger of ion loss and osmotic uptake of water. The olfactory sensitivity to Ca^{2+} and Na^+ may therefore form part of a homeostatic mechanism that maintains the ionic composition of the mucous layer within certain limits to facilitate the olfactory transduction process despite very low environmental concentrations of these ions. Removal of Ca^{2+} from the water supplying the olfactory epithelium caused a temporary attenuation of the olfactory response to serine in the sea-bream (Hubbard et al., 2000), but no such effect was seen in the goldfish. In fact, a slight increase was seen, possibly due to the hyperexcitability of fish olfactory neurones in Ca^{2+}-free environments (Hille, 1992) previously demonstrated by chelation of external Ca^{2+} by citrate on the olfactory response to amino acids in the catfish (*Ictalurus punctatus*; Parker et al., 2000).

To summarise, acute olfactory sensitivity to changes in external $[Ca^{2+}]$ has been demonstrated in a freshwater teleost over a similar concentration range to that of marine/euryhaline species, although the direction of response was opposite to that of marine fish (responding to increases rather than decreases). The presence of the Ca-SR in a subpopulation of olfactory receptor neurones suggests this receptor as a possible mechanism for this sensitivity. Olfactory sensitivity to Ca^{2+} may prove to be a widespread phenomenon in teleosts, and this sensory input might be linked to Ca^{2+} homeostatic mechanisms.

References

Akhurst, R. J., Lehnert, S. A., Faissner, A. and Duffie, E. (1990). TGF beta in murine morphogenic processes: the early embryo and cardiogenesis. *Development* **108**, 645-656.

Bertmar, G. (1982). Structure and function of the olfactory mucosa of migrating baltic trout under environmental stresses, with special reference to water pollution. In *Chemoreception in Fishes, Developments in Aquaculture and Fisheries Science*, vol. 8 (ed. T. J. Hara), pp. 395-422. Amsterdam: Elsevier Scientific Publishing Company.

Bodznik, D. (1978). Calcium ion: An odorant for natural water discriminations and the migratory behavior of sockeye salmon. *J. Comp. Physiol. A* **127**, 157-166.

Brown, E. M. (1991). Extracellular Ca^{2+} sensing, regulation of parathyroid cell function, and role of Ca^{2+} and other ions as extracellular (first) messengers. *Physiol. Rev.* **71**, 371-411.

Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J. and Hebert, S. C. (1993). Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. *Nature* **366**, 575-580.

Chakraborti, P. and Mukherjee, D. (1995). Effects of prolactin and fish pituitary extract on plasma calcium levels in common carp, *Cyprinus carpio*. *Gen. Comp. Endocrinol.* **97**, 320-326.

Cheng, I., Qureshi, I., Chattopadhyay, N., Qureshi, A., Butters, R. R., Hall, A. E., Cima, R. R., Rogers, K. V., Hebert, S. C., Geibel, J. P., Brown, E. M. and Soybel, D. I. (1999). Expression of an extracellular calcium-sensing receptor in rat stomach. *Gastroenterology* **116**, 118-126.

Christensen, T. A. and White, J. (2000). Representation of olfactory information in the brain. In *The Neurobiology of Taste and Smell.* Second edition (ed. T. E. Finger, W. L. Silver and D. Restrepo), pp. 201-232. New York: Wiley-Liss.

Danks, J. A., McHale, J. C., Martin, T. J. and Ingleton, P. M. (1997). Parathyroid hormone-related protein in tissues of the emerging frog (*Rana temporaria*): immunohistochemistry and in situ hybridisation. *J. Anat.* **190**, 229-238.

Douglas, W. S., Grasso, S. S., Hutton, D. G. and Schroeder, K. R. (1996). Ionic imbalance as a source of toxicity in an estuarine effluent. *Arch. Environ. Contam. Toxicol.* **31**, 426-432.

Fenwick. J. C. (1975). Effect of partial ultimobranchialectomy on plasma calcium concentration and on some related parameters in goldfish (*Carassius auratus* L.) during acute transfer from fresh water to 30 per cent sea water. *Gen. Comp. Endocrinol.* **25**, 60-63.

Flanagan, J. A., Power, D. M., Bendell, L. A., Guerreiro, P. M., Fuentes, J., Clark, M. S., Canário, A. V. M., Danks, J. A., Brown, B. L. and Ingleton, P. M. (2000). Cloning of the cDNA for sea bream (*Sparus aurata*) parathyroid hormone-related protein. *Gen. Comp. Endocrinol.* **118**, 373-382.

Hara, T. J. and Gorbman, A. (1967). Electrophysiological studies of the olfactory system of the goldfish, *Carassius auratus* L. I. Modification of the electrical activity of the olfactory bulb by other central nervous structures. *Comp. Biochem. Physiol.* **21**, 185-200.

Hille, B. (1992). *Ionic Channels of Excitable Membranes.* Second edition. pp. 457-461. Sunderland, MA: Sinauer.

Hubbard, P. C., Barata, E. N. and Canario, A. V. M. (2000). Olfactory sensitivity to changes in environmental $[Ca^{2+}]$ in the marine teleost *Sparus aurata*. *J. Exp. Biol.* **203**, 3821-3829.

Ingleton, P. M., Hubbard, P. C., Danks, J. A., Elgar, G., Sandford, R. A. and Balment, R. J (1999). The calcium-sensing receptor in fishes. In *Calcium Metabolism: Comparative Endocrinology* (ed. J. Danks, C. Dacke, G. Flik and C. Gay), pp. 1-4. Bristol: BioScientifica Ltd.

Kraicer, J., Herlant, M. and Duclos, P. (1967). Changes in adenohypophyseal cytology and nucleic acid content in the rat 32 days after bilateral adrenalectomy and the chronic injection of cortisol. *Can. J. Physiol. Pharmacol.* **45**, 947-956.

Lahlou, B., Henderson, I. W. and Sawyer, W. H. (1969). Sodium exchanges in goldfish (*Carassius auratus* L.) adapted to a hypertonic saline solution. *Comp. Biochem. Physiol.* **28**, 1427-1433.

Lehnert, S. A. and Akhurst, R. J. (1988). Embryonic expression pattern of TGF beta type-1 RNA suggests both paracrine and autocrine mechanisms of action. *Development* **104**, 263-273.

Moore, A. (1994). An electrophysiological study on the effects of pH on olfaction in mature Atlantic salmon (*Salmo salar*) parr. *J. Fish Biol.* **45**, 493-502.

Muus, B. J. and Dahlstrom, P. (1967). *Collins Guide to the Freshwater Fishes of Britain and Europe*. London, UK: Collins. 222pp.

Nevalainen, M. T., Valve E. M., Ingleton, P. M. and Harkonen, P. L. (1996). Expression and hormone regulation of prolactin receptors in rat dorsal and lateral prostate. *Endocrinology* **137**, 3078-3088.

Parker, J. M., Chang, Q. and Caprio, J. (2000). Citrate enhances olfactory receptor responses and triggers oscillatory receptor activity in the channel catfish. *J. Neurophysiol.* **83**, 2676-2681.

Riccardi, D., Park, J., Lee, W.-S., Gamba, G., Brown, E. M. and Hebert, S. C. (1995). Cloning and functional expression of a rat kidney extracellular calcium/polyvalent cation-sensing receptor. *Proc. Natl. Acad. Sci. USA* **92**, 131-135.

Schild, D. and Restrepo, D. (1998). Transduction mechanisms in vertebrate olfactory receptor cells. *Physiol. Rev.* **78**, 429-466.

Sternberger, L. A. (1974). *Immunocytochemistry*. Englewood Cliffs, NJ: Prentice Hall.