© 2022. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2022) 225, jeb243112. doi:10.1242/jeb.243112

SHORT COMMUNICATION

GABA receptors in the olfactory epithelium of the gilthead seabream (*Sparus aurata*)

Rita A. Costa, Zélia Velez and Peter C. Hubbard*

ABSTRACT

Exposure to high P_{CO_2}/low pH seawater induces behavioural alterations in fish; a possible explanation for this is a reversal of Cl^-/HCO_3^- currents through $GABA_A$ receptors (the $GABA_A$ receptor theory). However, the main evidence for this is that gabazine, a $GABA_A$ receptor antagonist, reverses these effects when applied to the water, assuming that exposure to systems other than the CNS would be without effect. Here, we show the expression of both metabotropic and ionotropic GABA receptors, and the presence of $GABA_A$ receptor protein, in the olfactory epithelium of gilthead seabream. Furthermore, exposure of the olfactory epithelium to muscimol (a specific $GABA_A$ receptor agonist) increases or decreases the apparent olfactory sensitivity to some odorants. Thus, although the exact function of $GABA_A$ receptors in the olfactory epithelium is not yet clear, this may complicate the interpretation of studies wherein water-borne gabazine is used to reverse the effects of high CO_2 levels on olfactory-driven behaviour in fish.

KEY WORDS: Ocean acidification, Olfaction, $GABA_A$ receptors, Gabazine

INTRODUCTION

Since the Industrial Revolution, the concentration of carbon dioxide (CO_2) in the atmosphere has increased. The ocean absorbs about 30% of the CO_2 released into the atmosphere; water and CO_2 combine to form carbonic acid (H_2CO_3), a weak acid that dissociates into protons (H^+) and bicarbonate ions (HCO_3^-) and causes a reduction in seawater pH: ocean acidification. As atmospheric CO_2 has increased from pre-industrial (280 ppm) to present day values (~400 ppm), equilibration with the ocean has led to a corresponding pH decline of 0.1 units, with a further fall of 0.77 units predicted by the year 2300 (Doney et al., 2009). Elevated concentrations of CO_2 in seawater can disrupt sensory systems in marine fish, including olfaction (Dixson et al., 2010; Munday et al., 2010), hearing (Simpson et al., 2011) and vision (Ferrari et al., 2012b), and have also been implicated in cognitive function, such as changes in lateralization (Domenici et al., 2012) and learning (Ferrari et al., 2012a). Disruption to cognitive function suggests that ocean acidification affects not only individual sensory systems but also central neuronal processing (Wisenden, 2012). The most accepted explanation for this is the '$GABA_A$ receptor theory' (Nilsson et al., 2012); a reversal of ionic flux through neuronal γ-aminobutyric acid type A receptors ($GABA_A$Rs), owing to alterations of $[Cl^-]$ and/or $[HCO_3^-]$, causes depolarization (i.e. excitation) rather than hyperpolarization (i.e. inhibition) (Nilsson et al., 2012). The main empirical evidence for this is that gabazine, a $GABA_A$ receptor antagonist, reverses the effects of high CO_2 (e.g. Hamilton et al., 2014; Lai et al., 2015; Watson et al., 2014). However, an additional mechanism by which ocean acidification could affect olfactory-driven behaviour is through direct effects on the peripheral olfactory system. Acute exposure to high P_{CO_2}/low pH water decreases olfactory nerve responses to some odorants in seabream (Velez et al., 2019) and sea bass (Porteus et al., 2018). It was proposed that increased $[H^+]$ may change the protonation of odorants; this, in turn, may reduce the receptor–ligand binding affinity and, thus, reduce olfactory sensitivity (Velez et al., 2019). Therefore, animals must be closer to an odorant source to detect it (Porteus et al., 2018). Although the $GABA_A$ hypothesis can explain mal-adaptive changes in behaviour, it lacks comprehensive empirical demonstration. Furthermore, the use of gabazine has been confined to water-borne exposure, assuming that exposure to systems other than the CNS would be without effect, disregarding the possible presence of $GABA_A$ receptors in sensory systems (Chivers et al., 2014; Chung et al., 2014; Hamilton et al., 2014). However, a subset of rat taste bud cells contains GABA and GABA transporter subtype 3, suggesting that GABAergic transmission may be modulating taste (Cao et al., 2009; Obata et al., 1997). Therefore, we investigated the possible involvement of GABAergic innervation in the peripheral olfactory system. Our aim was to evaluate the presence of $GABA_A$ receptors in the olfactory epithelium of a marine teleost, the gilthead seabream (*Sparus aurata*).

MATERIALS AND METHODS

Fish maintenance

Animal maintenance and experimentation were carried out in certified experimental facilities and followed Portuguese national legislation (DL 113/2013) under a 'group-1' licence by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal. Gilthead seabream, *Sparus aurata* Linnaeus 1758 (207±32 g, 22.3±0.8 cm, means±s.e.m.), were obtained from a commercial supplier (Maresa - Mariscos de Esteros, SA, Huelva, Spain) and maintained at the experimental station of Ramalhete (University of Algarve, Portugal) in 1000 l tanks with continuously running natural seawater, under natural photoperiod and temperature and fed daily with commercial pellets (Sparos, Olhão, Portugal).

Analysis of gene expression by reverse transcription–quantitative PCR (RT-qPCR)

RT-qPCR was used to analyse the mRNA expression of target genes in the olfactory epithelium of seabream (n=5). Seabream were anaesthetised with phenoxy-ethanol in seawater (1:10,000), decapitated and pithed, and the olfactory epithelia disected out.

Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.

*Author for correspondence (phubbard@ualg.pt)

R.A.C., 0000-0002-6975-7576; Z.V., 0000-0003-2761-4048; P.C.H., 0000-0002-3007-4647

Received 1 July 2021; Accepted 6 January 2022

Total RNA was extracted from olfactory epithelium samples fixed in RNAlater® (Sigma-Aldrich) using the E.Z.N.A® Total RNA Kit I (R6834, Omega) and RNA extracts were column purified and treated with DNase using the E.Z.N.A® RNase-Free DNase Set I kit (E1091, Omega) according to the manufacturer's instructions. RNA quality and concentration were assessed with a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific) and its integrity verified by electrophoresis in a 2% agarose gel. DNA-free total RNA (500 ng) was used for cDNA synthesis as previously described (Costa et al., 2017). Briefly, cDNA was synthetized in a 20 µl reaction volume containing 100 mmol l^{-1} random hexamers (Specanalítica), 100 U of RevertAid Reverse Transcriptase (ThermoFisher Scientific), 8 U of NZY Ribonuclease Inhibitor (nzytech) and 100 mmol l^{-1} of nucleotides (nzytech). Specific PCR primers were designed for gilthead seabream *gabra1* (accession no. XM_030437596; forward: CATGACCACACTCAGTATCAG, reverse: CTTCTCTGGAACCACACTTT, 184 bp amplicon, T_a=58°C) and *gabbr2* (accession no. XM_030412068; forward: GGCGGTGGTTATCAGTTT, reverse: TCTTCGTTCCCAAGTT-CATC, 177 bp amplicon, T_a=60°C) transcripts using the PrimerQuest Tool (IDT Integrated DNA Technologies, https://www.idtdna.com). Duplicate 10 µl reactions of 1× SsoFast-Evagreen Supermix (BioRad) containing 5 ng cDNA and 300 nmol l^{-1} of forward and reverse primers were used. The PCR products were sequenced and run on a 2% (w/v) agarose gel to confirm amplicon identity and size, respectively. Transcripts were quantified in a StepOnePlus thermocycler (Applied Biosystems) using the standard-curve method (software StepOne™ Real Time) as previously described (Costa et al., 2017). The standard curve was generated using serial dilutions of specific PCR products for each gene (obtained using the same species and tissue and primers as for RT-qPCR analysis). A final melting curve was performed between 60 and 95°C and produced a single product dissociation curve for each gene. Efficiencies of standard curves were 88% and 93% for *gabra1* and *gabbr2*, respectively, with R^2=1. Relative expression was estimated using the geometric mean of *18 s* (Vieira et al., 2011) and *ef1α* (Pinto et al., 2016) expression, which did not vary significantly (P>0.05) between samples.

Light microscopy immunocytochemistry

Seabream were anaesthetised with phenoxy-ethanol (1:10,000) in seawater, decapitated and pithed and the olfactory rosettes were dissected out and fixed in 4% paraformaldehyde and embedded in low-melting point paraffin wax (Histoseck). Serial 5 µm sections were obtained and mounted on poly-L-lysine (Sigma-Aldrich) coated glass slides. To visualize the presence of $GABA_A$ receptors in the olfactory epithelium, dewaxed and rehydrated tissue sections were blocked with a Tris-carrageenan-Triton X-100 (TCT) solution containing 4% sheep serum (Sigma-Aldrich) and then incubated overnight at 4°C with a 1/100 dilution in TCT of the primary antibody 62-3G1 deposited in the Developmental Studies Hybridoma Bank (DSHB) by Angel L. De Blas (DSHB Hybridoma Product 62-3G1). This antibody recognizes both the β2 and β3 subunits of $GABA_A$ receptors. Tissue sections were then incubated in a 1/200 dilution in phosphate-buffered saline (PBS) with 0.1% Triton X-100 (PBST) of the secondary antibody anti-mouse IgG (whole-molecule)-peroxidase antibody produced in rabbit (A9044, Sigma-Aldrich) for 1 h at room temperature. Colour was developed using 3,3′-diaminobenzidine (DAB; D12384, Sigma-Aldrich) as the chromogen in a solution containing 0.05% DAB, 0.015% H_2O_2 (H1009, Sigma-Aldrich) in 0.15 mol l^{-1} PBS pH 7.2 for 10 min at room temperature. The reaction was stopped by washing sections for 5 min in the same buffer. Sections were then dehydrated, cleared in xylene and mounted in dibutylphthalate polystyrene xylene (DPX; Merck, Sigma-Aldrich). Control reactions in which the primary antisera were omitted from the staining procedure were negative. Stained sections were analysed using a microscope (Leica DM2000) coupled to a digital camera (Leica DFC480) and linked to a computer for digital image analysis.

Electrophysiology

To investigate the role of $GABA_A$ receptors in the olfactory epithelium, olfactory sensitivity to amino acids was tested in the presence and absence of GABA, gabazine (allosteric inhibitor of the $GABA_A$ receptor) or muscimol ($GABA_A$ receptor agonist). Gabazine was from Merk Millipore; muscimol and GABA were from Sigma-Aldrich.

Firstly, the olfactory sensitivity of fish to each drug was evaluated at 10 µmol l^{-1} (the concentration of gabazine used in many behavioural studies). Olfactory sensitivity to amino acids was then tested, in the absence and then the presence of GABA, gabazine or muscimol (only one drug tested on each fish). The olfactory epithelium was superfused for 10 min with seawater containing the drug prior to recording responses to odorants. All odorants tested during this stage were prepared in the presence of the drug at the same concentration as the water superfusing the olfactory epithelium. After testing, the olfactory epithelium was washed out for 15 min with seawater. In all cases, the amplitude of the response returned to control levels. The percentage response was calculated by dividing the amplitude of the olfactory response in the presence of the drug by that in its absence.

Olfactory nerve recording

Seabream were anaesthetized in aerated natural seawater containing 300 mg l^{-1} MS222 (ethyl-3-aminobenzoate methane sulphonate salt, Sigma-Aldrich) until responses to tail pinch had stopped; an intramuscular injection of the neuromuscular blocker gallamine triethiodide (Sigma-Aldrich; 10 mg kg^{-1} in 0.9% NaCl) was then given. Fish were then placed in a padded V-support and the gills irrigated with aerated natural seawater containing 150 mg l^{-1} MS222.

The olfactory rosette was exposed by cutting the skin and connective tissue overlying the nasal cavity. The nostril was constantly irrigated with charcoal-filtered sea water (without anaesthetic) under gravity (flow rate: 6 ml min^{-1}) via a glass tube. Test solutions were delivered to the tube irrigating the nasal cavity via a computer-operated three-way solenoid valve for 4 s. The olfactory nerve was exposed by removal of the skin, connective tissue and overlying bone. Olfactory nerve activity was recorded using tungsten microelectrodes (0.1 MΩ, World Precision Instruments) as previously described (Hubbard and Velez, 2020). The electrodes were placed in the olfactory nerve in a position that gave maximal response to 10^{-3} mol l^{-1} L-serine, usually lateral and close to the olfactory bulb. Fish were connected to earth via a copper wire inserted in the flank. The raw signal was amplified (20,000×; AC pre-amplifier, Neurolog NL104, Digitimer Ltd, Welwyn Garden City, UK), filtered (high pass: 200 Hz, low pass: 3000 Hz; Neurolog NL125, Digitimer Ltd) and integrated (time constant 1 s; Neurolog NL703, Digitimer Ltd). Raw and integrated signals were digitized (Digidata 1440A, Molecular Devices, San Jose, CA, USA) and recorded on a PC running AxoScope™ software (version 10.6, Molecular Devices).

All integrated response amplitudes were normalized to the amplitude of the integrated response to 10^{-3} mol l^{-1} L-serine (the

Journal of Experimental Biology (2022) 225, jeb243112. doi:10.1242/jeb.243112

'standard'). Responses to the standard were recorded regularly at the beginning and end of each group of samples (every 3–5 samples) throughout the recording session. Each stimulus was applied for 4 s, with at least 1 min between odorants to allow complete recovery of the receptors (Hubbard and Velez, 2020).

Data and statistical analysis

All statistical analyses of electrophysiological results were carried out on normalized data. Differences between olfactory responses in the presence and absence of each drug were analysed using Student's *t*-test for paired data (log-transformed). All the analyses were performed in GraphPad Prism 9.0.2 (134) for Mac OS X (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). The significance cut-off was set at $P<0.05$ and data are presented as means±s.e.m., unless otherwise stated.

RESULTS AND DISCUSSION

The mRNA expression of *gabra1* and *gabbr2* subunits of GABA receptors was quantified in the olfactory epithelium of gilthead seabream (Fig. 1A) suggesting the presence of both ionotropic and metabotropic GABA receptors. The presence of cells with $GABA_A$ receptors in the olfactory epithelium was evaluated by immunohistochemistry using an antibody (62-3G1, DSHB) against type A receptors and revealed positive immunostaining in small rounded cell bodies (Fig. 1B) along the apical side of the olfactory lamella.

Seabream responded to all drugs (GABA, gabazine and muscimol) at 10 µmol l^{-1} (Fig. 2A) although not as strongly as to other amino acid odorants, such as L-serine. However, exposure of the epithelium to 10 µmol l^{-1} GABA (Fig. 2B) and 10^{-5} mol l^{-1} gabazine (Fig. 2C) did not affect the olfactory response to any of the amino acid odorants. In the presence of 10^{-5} mol l^{-1} muscimol, the olfactory response to L-glutamic acid was significantly higher (t=7.08; P=0.006), and that to L-serine (t=4.46; P=0.021) and L-cysteine (t=3.43; P=0.041) were lower (Fig. 2C,D).

The current study clearly shows the expression of both ionotropic and metabotropic GABA receptors in the olfactory epithelium of seabream. In addition, immunohistochemistry showed the presence of the β2 and/or β3 subunits of the $GABA_A$ receptor in the olfactory epithelium. The near-apical position of the staining within the epithelium suggests that the $GABA_A$ receptor is expressed in olfactory receptor neurones, possibly the microvillous cells (Hamdani and Døving, 2007; Hansen et al., 2004); however, the exact site of expression remains to be determined experimentally. In an attempt to evaluate the modulatory effect of GABA receptors, the response to a range of amino acids in the presence and absence of GABA, gabazine and muscimol was compared. GABA, the endogenous ligand of GABA receptors, binds to both metabotropic and ionotropic receptors. Conversely, gabazine is a specific and potent allosteric modulator of $GABA_A$ receptors, where it acts as an allosteric inhibitor of channel opening (Ueno et al., 1997). Muscimol is a naturally occurring specific agonist of $GABA_A$ receptors (Johnston, 2014). Therefore, the effect of gabazine was expected to be opposite to that of GABA and muscimol. However, neither GABA nor gabazine altered olfactory responses to the amino acid odorants, whereas muscimol increased olfactory nerve response to L-glutamic acid but decreased it to L-serine and L-cysteine. The difference between the effects of GABA and muscimol (agonists) may be because GABA activates both metabotropic and ionotropic receptors, while muscimol activates only $GABA_A$ receptors. Alternatively, the penetration of the two compounds into the epithelium may be different. The lack of effect of gabazine could be because GABA is not released in the olfactory epithelium under experimental conditions (e.g. under anaesthesia); thus, $GABA_A$ receptors are not activated and, therefore, exposure of the olfactory epithelium to a $GABA_A$ receptor inhibitor has no effect.

Our study clearly shows the transcription and presence of $GABA_A$ receptors in the olfactory epithelium of seabream. As far as we are aware, only one other study has identified these receptors in the olfactory epithelium of an aquatic vertebrate: *Xenopus* tadpoles (Kaeser et al., 2011). Furthermore, in the seabream, the activation of these receptors alters the olfactory response to some – but not all – odorants, in the current case increasing sensitivity to L-glutamate and decreasing it to L-serine and L-cysteine. We hypothesize that, similar to taste receptors (Huang et al., 2011), GABA receptors may modulate the olfactory response at a peripheral level. In taste buds, GABA acts as an inhibitory transmitter released during taste stimulation, acting on both $GABA_A$ and $GABA_B$ receptors (Dvoryanchikov et al., 2011). Furthermore, acid stimulation seems to elicit GABA release from mouse taste cells (Huang et al., 2011). Although the exact function of $GABA_A$ receptors in the olfactory epithelium is not yet clear, it may complicate the interpretation of

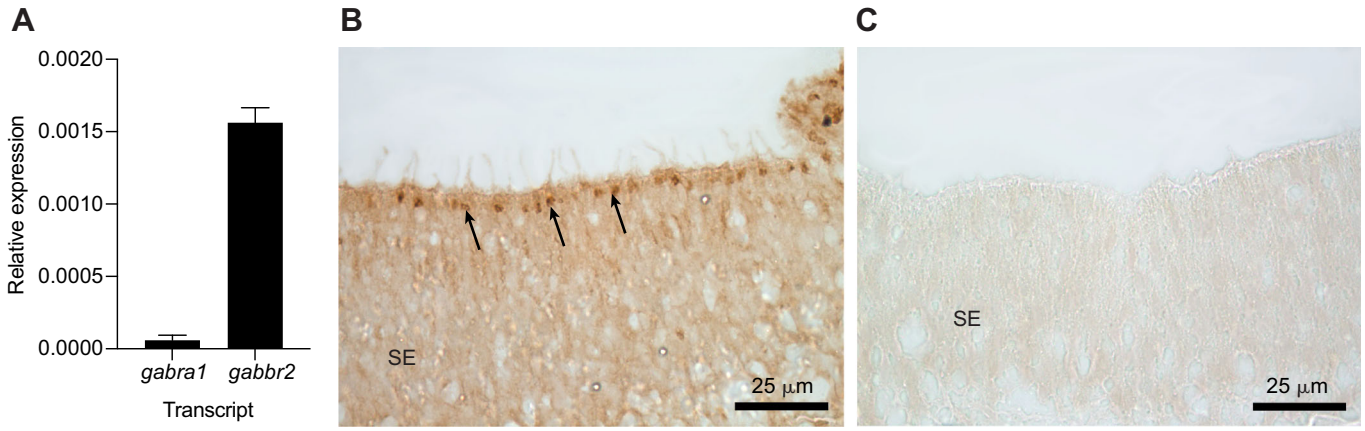

Fig. 1. GABA receptor expression in seabream olfactory epithelium. (A) Relative expression of *gabra1* and *gabbr2* in the olfactory epithelium of seabream (n=5). Data are presented as means+s.e.m. Relative expression was estimated using the geometric mean of *18S* and *ef1α* expression as reference genes. (B) Immunoreactivity of $GABA_A$ receptor positive cells in the olfactory epithelium of seabream. The antibody used recognizes the β2 and β3 subunits of $GABA_A$ receptors. Arrowheads indicate cells staining positively with the antibody 62-3G1 (DSHB). (C) Negative control, where the primary antibody was omitted from the staining procedure. SE, sensory epithelium.

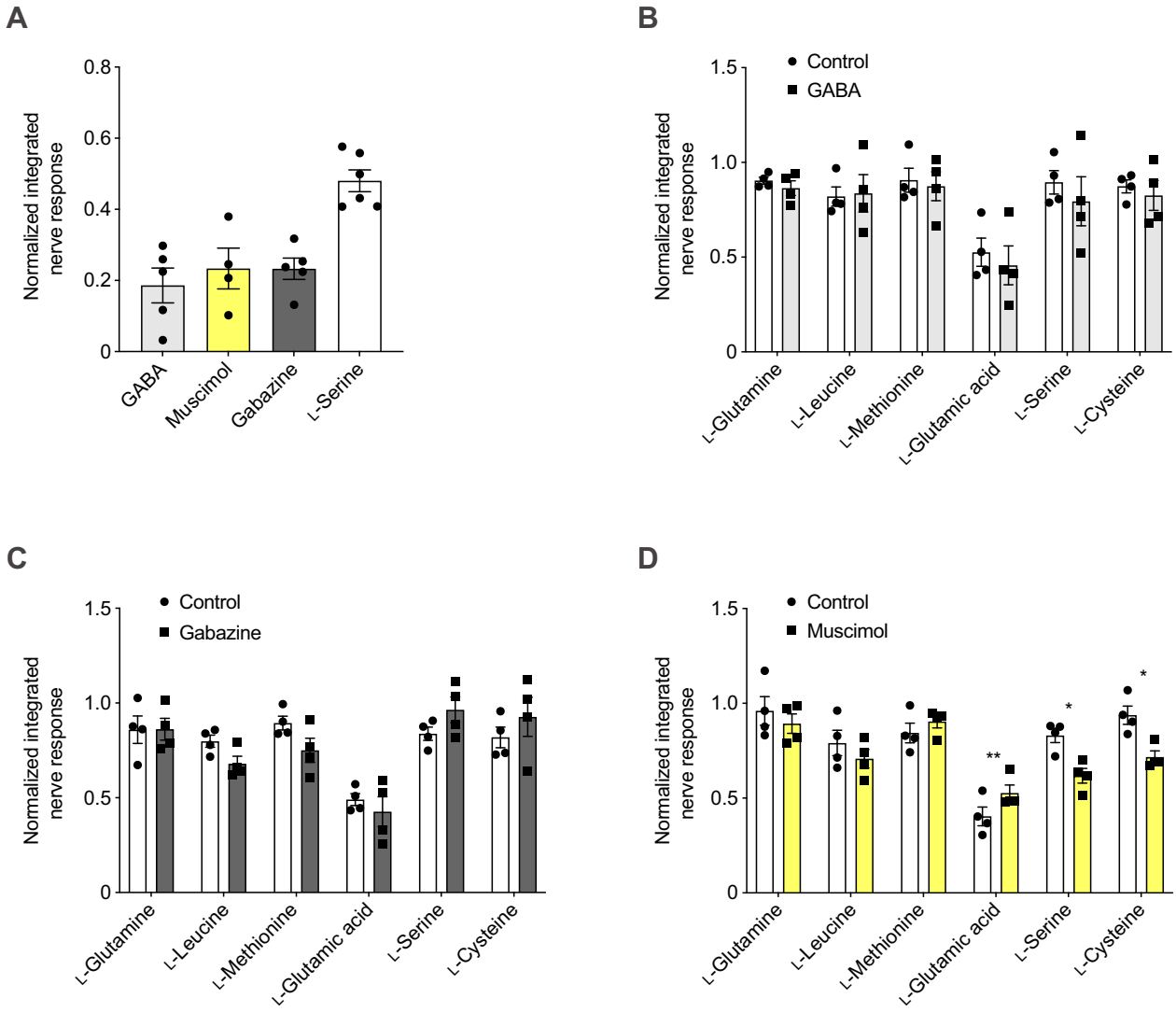

Fig. 2. Seabream olfactory nerve responses to GABA, GABA receptor agonist/antagonist and amino acid odorants. (A) Normalized olfactory nerve responses to 10^{-5} mol l^{-1} GABA (n=5), muscimol (n=4), gabazine (n=5) and L-serine (n=6). (B–D) Normalized olfactory nerve responses to 10^{-4} mol l^{-1} L-glutamine, L-leucine, L-methionine, L-glutamic acid, L-serine and L-cysteine with the olfactory epithelium conditioned with control seawater and seawater containing (B)10^{-5} mol l^{-1} GABA (n=4), (C) 10^{-5} mol l^{-1} gabazine (n=4) and (D) 10^{-5} mol l^{-1} muscimol (n=4). Statistical significance was verified using Student's t-test for paired data (log-transformed) and values are shown as means±s.e.m.; *P<0.05, **P<0.01.

those studies wherein water-borne gabazine is used to reverse the effects of high CO_2 levels on olfactory-driven behaviour in fish as it could alter the perception of a given odour. Nevertheless, the effect of stimulating these receptors in the olfactory epithelium on fish behaviour awaits investigation.

Acknowledgements
The monoclonal antibody 62-3G1 developed by Angel L. De Blas was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology (Iowa City, IA 52242, USA).

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: R.A.C., Z.V., P.C.H.; Investigation: R.A.C., Z.V., P.C.H.; Writing - original draft: R.A.C., Z.V., P.C.H.; Writing - review & editing: R.A.C., Z.V., P.C.H.; Funding acquisition: Z.V., P.C.H.

Funding
This study received Portuguese national funds from Fundação para a Ciência e Tecnologia through project grants UIDB/04326/2020 and PTDC/BIA-BMA/30262/2017.

References
Cao, Y., Zhao, F. I., Kolli, T., Hivley, R. and Herness, S. (2009). GABA expression in the mammalian taste bud functions as a route of inhibitory cell-to-cell communication. *Proc. Natl. Acad. Sci. USA* **106**, 4006-4011. doi:10.1073/pnas.0808672106

Chivers, D. P., McCormick, M. I., Nilsson, G. E., Munday, P. L., Watson, S. A., Meekan, M. G., Mitchell, M. D., Corkill, K. C. and Ferrari, M. C. O. (2014). Impaired learning of predators and lower prey survival under elevated CO_2: a consequence of neurotransmitter interference. *Glob. Change Biol.* **20**, 515-522. doi:10.1111/gcb.12291

Chung, W. S., Marshall, N. J., Watson, S. A., Munday, P. L. and Nilsson, G. E. (2014). Ocean acidification slows retinal function in a damselfish through interference with $GABA_A$ receptors. *J. Exp. Biol.* **217**, 323-326. doi:10.1242/jeb.092478

Costa, R. A., Cardoso, J. C. R. and Power, D. M. (2017). Evolution of the angiopoietin-like gene family in teleosts and their role in skin regeneration. *BMC Evol. Biol.* **17**, 14. doi:10.1186/s12862-016-0859-x

Dixson, D. L., Munday, P. L. and Jones, G. P. (2010). Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. *Ecol. Lett.* **13**, 68-75. doi:10.1111/j.1461-0248.2009.01400.x

Domenici, P., Allan, B., McCormick, M. I. and Munday, P. L. (2012). Elevated carbon dioxide affects behavioural lateralization in a coral reef fish. *Biol. Lett.* **8**, 78-81. doi:10.1098/rsbl.2011.0591

Doney, S. C., Fabry, V. J., Feely, R. A. and Kleypas, J. A. (2009). Ocean acidification: the other CO_2 problem. *Annu. Rev. Mar. Sci.* **1**, 169-192. doi:10.1146/annurev.marine.010908.163834

Dvoryanchikov, G., Huang, Y. J. A., Barro-Soria, R., Chaudhari, N. and Roper, S. D. (2011). GABA, its receptors, and GABAergic inhibition in mouse taste buds. *J. Neurosci.* **31**, 5782-5791. doi:10.1523/JNEUROSCI.5559-10.2011

Ferrari, M. C. O., Manassa, R. P., Dixson, D. L., Munday, P. L., McCormick, M. I., Meekan, M. G., Sih, A. and Chivers, D. P. (2012a). Effects of ocean acidification on learning in coral reef fishes. *PLoS ONE* **7**, e31478. doi:10.1371/journal.pone.0031478

Ferrari, M. C. O., McCormick, M. I., Munday, P. L., Meekan, M. G., Dixson, D. L., Lonnstedt, O. and Chivers, D. P. (2012b). Effects of ocean acidification on visual risk assessment in coral reef fishes. *Funct. Ecol.* **26**, 553-558. doi:10.1111/j.1365-2435.2011.01951.x

Hamdani, E. H. and Døving, K. B. (2007). The functional organization of the fish olfactory system. *Prog. Neurobiol.* **82**, 80-86. doi:10.1016/j.pneurobio.2007.02.007

Hamilton, T. J., Holcombe, A. and Tresguerres, M. (2014). CO_2-induced ocean acidification increases anxiety in rockfish via alteration of $GABA_A$ receptor functioning. *Proc. R. Soc. B Biol. Sci.* **281**, 20132509. doi:10.1098/rspb.2013.2509

Hansen, A., Anderson, K. T. and Finger, T. E. (2004). Differential distribution of olfactory receptor neurons in goldfish: structural and molecular correlates. *J. Comp. Neurol.* **477**, 347-359. doi:10.1002/cne.20202

Huang, Y. J. A., Pereira, E. and Roper, S. D. (2011). Acid stimulation (sour taste) elicits GABA and serotonin release from mouse taste cells. *PLoS ONE* **6**, e25471. doi:10.1371/journal.pone.0025471

Hubbard, P. and Velez, Z. (2020). Extracellular multi-unit recording from the olfactory nerve of teleosts. *J. Vis. Exp.* **164**, e60962. doi:10.3791/60962

Johnston, G. A. R. (2014). Muscimol as an ionotropic GABA receptor agonist. *Neurochem. Res.* **39**, 1942-1947. doi:10.1007/s11064-014-1245-y

Kaeser, G. E., Rabe, B. A. and Saha, M. S. (2011). Cloning and characterization of $GABA_A$ α subunits and $GABA_B$ subunits in *Xenopus laevis* during development. *Dev. Dyn.* **240**, 862-873. doi:10.1002/dvdy.22580

Lai, F., Jutfelt, F. and Nilsson, G. E. (2015). Altered neurotransmitter function in CO_2-exposed stickleback (*Gasterosteus aculeatus*): a temperate model species for ocean acidification research. *Conserv. Physiol.* **3**, cov018. doi:10.1093/conphys/cov018

Munday, P. L., Dixson, D. L., McCormick, M. I., Meekan, M., Ferrari, M. C. O. and Chivers, D. P. (2010). Replenishment of fish populations is threatened by ocean acidification. *Proc. Natl. Acad. Sci. USA* **107**, 12930-12934. doi:10.1073/pnas.1004519107

Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sorensen, C., Watson, S.-A. and Munday, P. L. (2012). Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nat. Clim. Chang.* **2**, 201-204. doi:10.1038/nclimate1352

Obata, H., Shimada, K., Sakai, N. and Saito, N. (1997). GABAergic neurotransmission in rat taste buds: immunocytochemical study for GABA and GABA transporter subtypes. *Mol. Brain Res.* **49**, 29-36. doi:10.1016/S0169-328X(97)00118-6

Pinto, P. I. S., Estêvão, M. D., Andrade, A., Santos, S. and Power, D. M. (2016). Tissue responsiveness to estradiol and genistein in the sea bass liver and scale. *J. Steroid Biochem. Mol. Biol* **158**, 127-137. doi:10.1016/j.jsbmb.2015.12.023

Porteus, C. S., Hubbard, P. C., Webster, T. M. U., van Aerie, R., Canário, A. V. M., Santos, E. M. and Wilson, R. W. (2018). Near-future CO_2 levels impair the olfactory system of a marine fish. *Nat. Clim. Chang* **8**, 737-743. doi:10.1038/s41558-018-0224-8

Simpson, S. D., Munday, P. L., Wittenrich, M. L., Manassa, R., Dixson, D. L., Gagliano, M. and Yan, H. Y. (2011). Ocean acidification erodes crucial auditory behaviour in a marine fish. *Biol. Lett.* **7**, 917-920. doi:10.1098/rsbl.2011.0293

Ueno, S., Bracamontes, J., Zorumski, C., Weiss, D. S. and Steinbach, J. H. (1997). Bicuculline and gabazine are allosteric inhibitors of channel opening of the $GABA_A$ receptor. *J. Neurosci.* **17**, 625-634. doi:10.1523/JNEUROSCI.17-02-00625.1997

Velez, Z., Roggatz, C. C., Benoit, D. M., Hardege, J. D. and Hubbard, P. C. (2019). Short- and medium-term exposure to ocean acidification reduces olfactory sensitivity in gilthead seabream. *Front. Physiol.* **10**, 731. doi:10.3389/fphys.2019.00731

Vieira, F. A., Gregório, S. F., Ferraresso, S., Thorne, M. A. S., Costa, R., Milan, M., Bargelloni, L., Clark, M. S., Canario, A. V. M. and Power, D. M. (2011). Skin healing and scale regeneration in fed and unfed sea bream, *Sparus auratus*. *BMC Genomics* **12**, 19. doi:10.1186/1471-2164-12-490

Watson, S. A., Lefevre, S., McCormick, M. I., Domenici, P., Nilsson, G. E. and Munday, P. L. (2014). Marine mollusc predator-escape behaviour altered by near-future carbon dioxide levels. *Proc. R. Soc. B Biol. Sci.* **281**, 9. doi:10.1098/rspb.2013.2377

Wisenden, B. D. (2012). Cognitive dysfunction and risk assessment by prey: predictable changes in global climate have unpredictable effects. *Funct. Ecol.* **26**, 551-552. doi:10.1111/j.1365-2435.2011.01956.x