

REVIEW

Acid–base physiology, neurobiology and behaviour in relation to CO₂-induced ocean acidification

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ABSTRACT

Experimental exposure to ocean and freshwater acidification affects the behaviour of multiple aquatic organisms in laboratory tests. One proposed cause involves an imbalance in plasma chloride and bicarbonate ion concentrations as a result of acid–base regulation, causing the reversal of ionic fluxes through GABA_A receptors, which leads to altered neuronal function. This model is exclusively based on differential effects of the GABA_A receptor antagonist gabazine on control animals and those exposed to elevated CO₂. However, direct measurements of actual chloride and bicarbonate concentrations in neurons and their extracellular fluids and of GABA_A receptor properties in aquatic organisms are largely lacking. Similarly, very little is known about potential compensatory mechanisms, and about alternative mechanisms that might lead to ocean acidification-induced behavioural changes. This article reviews the current knowledge on acid–base physiology, neurobiology, pharmacology and behaviour in relation to marine CO₂-induced acidification, and identifies important topics for future research that will help us to understand the potential effects of predicted levels of aquatic acidification on organisms.

KEY WORDS: Cerebrospinal fluid, Choroid plexus, Climate change, GABA, Gabazine, Ocean acidification

Introduction

Carbon dioxide (CO₂) is continuously generated by the geological processes of weathering and volcanic activity, as well as by anthropogenic activities such as the burning of fossil fuels and cement production. Additionally, CO₂ is a substrate for photosynthesis and an end product of aerobic respiration, two of the most essential biological processes in nature. In aquatic ecosystems, CO₂ levels can vary locally owing to biological activity, upwelling and CO₂ vents, and also globally owing to atmospheric and oceanographic processes such as winds, currents and gas exchange with the atmosphere. Since the onset of the industrial revolution, anthropogenic activities have induced a steep elevation in atmospheric CO₂ levels and have also increased average CO₂ levels in surface ocean waters (Sabine et al., 2004). Because CO₂ hydration produces H⁺ that decreases seawater pH, this phenomenon has been termed ‘ocean acidification’ (OA) (Caldeira and Wickett, 2003).

The initial concern regarding OA was that the additional H⁺ would react with CO₃^{2−} in seawater and the CaCO₃ in exoskeletons

of certain marine organisms, impairing calcification (Kleypas and Langdon, 2006). However, subsequent laboratory experiments on marine fish exposed to values of pH/CO₂ predicted for the end of this century and beyond revealed impacts on additional organisms and processes, such as embryonic and larval development (Frommel et al., 2012, 2016; Rossi et al., 2015), otolith growth (Checkley et al., 2009; Munday et al., 2011), reproduction (Miller et al., 2013), metabolic rate (Couturier et al., 2013; Rummer et al., 2013; Enzor et al., 2013) and behaviour (Allan et al., 2013; Dixon et al., 2010; Domenici et al., 2012; Hamilton et al., 2013; Munday et al., 2009, 2010, 2016; Rossi et al., 2015); however, it is worth noting that these effects are highly variable and depend on the species, experimental conditions, parameters analyzed and experimental techniques used. Most of these effects were proposed to be due to acid–base (A–B) regulatory processes, leading to altered ionic concentrations, energy expenditure and allocation; however, the specific molecular and cellular mechanisms remain largely unexplored.

OA-induced alteration of behaviour represents a unique case for which a specific mechanism has been proposed and tested: it is thought to result from a reversal of ionic flux through neuronal gamma-aminobutyric acid type A receptors (GABA_ARs) owing to an alteration of [Cl[−]] and/or [HCO₃[−]] that may result from A–B regulatory responses. In this Review, we assess the supporting data and assumptions of the ‘GABA_AR model’, and propose specific topics that, in our opinion, require further experimentation in order for this model to be validated, refuted or expanded. We begin by reviewing the original experiments that found an OA-induced alteration of fish behaviour and proposed alteration of GABA_AR as a cause. We then discuss basic aspects of neurobiology and acid–base regulation relevant for the GABA_AR model, and propose essential experiments that must be conducted to support, expand or refute the GABA_AR model. Finally, we identify additional neurobiological mechanisms that should be affected, assuming the GABA_AR model is correct.

Discovery of OA-induced behavioural alteration

In 2009, Munday and colleagues reported that orange clownfish (*Amphiprion percula*) larvae reared in seawater with a partial pressure of CO₂ (*P*_{CO₂}) of ~1050 μatm (pH 7.80) in the laboratory demonstrated an altered response to settlement olfactory cues administered in a 2-min, two-channel choice flume test (Munday et al., 2009). While larvae reared in control seawater (*P*_{CO₂} ~440 μatm; pH 8.15) completely avoided the side of the water flume with olfactory cues from pungent tree leaves and from parents, larvae reared in elevated *P*_{CO₂} spent almost all of their time in the side with those odours. The responses to cues from other tree leaves, grass and anemone were also impaired, although less dramatically. Interestingly, larvae reared in a much higher *P*_{CO₂}, 1700 μatm (pH 7.60), did not respond to any of the cues presented, suggesting that there are additional effects at higher *P*_{CO₂}. Larvae

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List of symbols and abbreviations

A–B	acid–base
BIF	brain interstitial fluid
CSF	cerebrospinal fluid
E_{GABA}	reversal potential for GABA _A R
E_{ion}	equilibrium potential
GABA	gamma-aminobutyric acid
GABA _A R	GABA _A receptor
KCC2	K ⁺ /2Cl [−] cotransporter 2
NKCC1	Na ⁺ /K/2Cl [−] cotransporter 1
OA	ocean acidification
P_{CO_2}	partial pressure of CO ₂
V_{m}	membrane potential

exposed to elevated CO₂ had the same external appearance and nasal cavity morphology as control fish, ruling out developmental effects and damage to olfactory organs.

Another study conducted concurrently by the same group reported that the detection of predator olfactory cues was also altered by acidified seawater in the same species (Dixson et al., 2010). In this case, the choices in the two-channel choice flume were untreated seawater and seawater with olfactory cues from predator or non-predator fish. There were no differences in choice between newly hatched larvae reared in control and acidified seawater; both groups preferred untreated seawater over seawater with any fish olfactory cues, and both preferred cues from non-predator over predator fish. However, settlement-stage larvae (11 days post-hatch) reared in CO₂-acidified seawater did show important significant differences compared with controls: they preferred predator cues to untreated seawater, and demonstrated no preference between cues from predator and non-predator fish.

A third paper (Munday et al., 2010) reported that the onset timing of olfactory and behavioural alteration in clownfish larvae was dependent on the magnitude of the CO₂ elevation, taking approximately 4 days after exposure to 700 μatm CO₂, but only 2 days following exposure to 860 μatm CO₂. Furthermore, it reported three other important findings: (1) the OA effects on clownfish larvae were reversible after exposure to control seawater for 2 days; (2) the effects were also evident in wild-caught *Pomacentrus wardi* damselfish larvae; and (3) when released back to the reef, *P. wardi* settlement-stage larvae exposed to elevated CO₂ in the laboratory experienced significantly higher mortality compared with larvae exposed to control CO₂ (Munday et al., 2010). These three pioneer studies suggested that future OA could have important deleterious consequences for fish populations, and also hinted at a CO₂ dose-dependent, reversible mechanism that required some time to take effect. A few years later, another seminal paper (Nilsson et al., 2012) proposed that the mechanism behind these behavioural disruptions related to changes in [Cl[−]] and [HCO₃[−]] in blood plasma and neurons as a result of A–B regulation, which caused an alteration of neuronal GABA_AR function and downstream effects on fish behaviour.

The GABA_AR model

The concepts behind the elegant GABA_AR model are grounded on decades of basic research on fish A–B regulation and mammalian neurobiology. Firstly, exposure to elevated CO₂ in the environment is known to result in a proportional increase in CO₂ in the blood, a condition known as hypercapnia (Table 1). Hypercapnic aquatic organisms typically restore blood A–B balance by upregulating active H⁺ excretion and accumulating HCO₃[−] in physiological fluids (reviewed in Evans et al., 2005; Larsen et al., 2014). In fish

Glossary

Anxiogenic

Anxiety-causing.

Anxiolytic

Anxiety-reducing.

Blood–brain barrier

Series of membranes that separate brain interstitial fluid and cerebrospinal fluid from blood. It is composed of endothelial cells, astrocyte end feet and a thick fibrous basement membrane, and is highly selectively permeable to molecules.

Choroid plexus

Branching network of capillaries and epithelial cells located in each of the brain ventricles (four in humans). The choroid plexus secretes the cerebrospinal fluid.

Equilibrium potential (E_{ion})

The membrane potential at which there is no net movement of an ion through a permeable ion channel.

GABAergic

GABA-releasing.

Glutamatergic

Glutamate-releasing.

Membrane potential (V_{m})

The voltage difference between the intracellular and extracellular compartments.

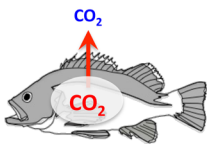
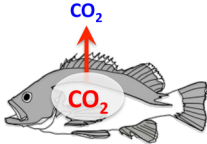
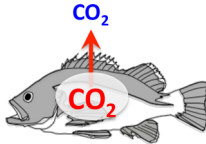
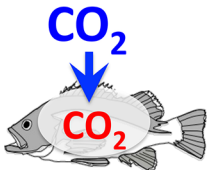
GABA_AR reversal potential (E_{GABA})

The membrane potential at which there is no net movement of Cl[−] and HCO₃[−] ions through GABA_ARs.

exposed to high CO₂ levels (>10,000 μatm) in both freshwater and seawater, the increased [HCO₃[−]] in blood plasma is associated with decreased [Cl[−]], at a ratio close to 1:1 (Heisler, 1988; Larsen et al., 1997; Toews et al., 1983) (Table 1). Secondly, it is known that ion flow through GABA_ARs can reverse – during development, the mammalian fetal brain experiences an increase in intracellular [Cl[−]] that reverses the (normally inward) Cl[−] flux through GABA_ARs – and GABA_ARs also display some permeability to HCO₃[−] (Bormann et al., 1987; Inomata et al., 1986). Under GABA-hyperactivating conditions, altered Cl[−] and HCO₃[−] gradients can reverse net ionic fluxes through GABA_ARs, turning their function from inhibitory to excitatory (Staley et al., 1995; Stein and Nicoll, 2003), thus affecting neuronal action potential generation and multiple neuronal functions (see Basic neurobiology considerations). Because OA is a mild case of hypercapnia, Nilsson et al. (2012) reasoned that it would result in slight HCO₃[−] accumulation and Cl[−] loss in extracellular and intracellular fluids in a manner consistent with the reversal of GABA_ARs from inhibitory to excitatory (Fig. 1). To test this hypothesis, they pre-treated control and CO₂-exposed clownfish with gabazine (an arylaminopyridazine derivative also known as SR-95531), a specific GABA_AR antagonist (Heaulme et al., 1986), and examined their response to olfactory predator cues (Nilsson et al., 2012). As predicted, gabazine restored predator avoidance in fish exposed to elevated CO₂, and it did not have any effect on control fish, which was interpreted as evidence of ‘reversal’ of the effect on GABA_AR currents. The term ‘reversal’ will be used throughout this Review; however, it is important to clarify that this is not necessarily a full reversal of function, as GABA_ARs can still be inhibitory at depolarized potentials (see Basic neurobiology considerations). This has also been referred to as a ‘switch’ in GABA_AR action in other studies (see Tyzio et al., 2006).

Nilsson et al. (2012) sparked a flurry of studies in which gabazine was applied to a variety of animals that were exposed to elevated CO₂ and subjected to diverse tests (Table 2). For the most part,

Table 1. Carbon dioxide fluxes between fish and seawater under control and elevated carbon dioxide levels

	Control	OA I	OA II	Extreme hypercapnia
Seawater				
P_{CO_2} (μatm)	~400	~1000	~2000	>10,000
pH	~8.00	~7.80	~7.60	<6.90
$[\text{H}^+]$ (nmol l^{-1})	~10	~16	~25	>126
				
Blood				
P_{CO_2} (μatm)	~3000	~3600	~4600	>13,000
pH	~7.80	~7.80	~7.80	~7.75
$[\text{H}^+]$ (nmol l^{-1})	~16	~16	~16	~18
$[\text{Cl}^-]$ (mmol l^{-1})	140–200	140–200?	140–200?	120–180?
$[\text{HCO}_3^-]$ (mmol l^{-1})	~5	~7	~10	~25

Under control conditions, constant metabolic CO_2 production in fish cells elevates P_{CO_2} in fish blood plasma to levels that are more than sevenfold higher compared with those of seawater (and CO_2 levels are much higher in intracellular and interstitial fluids than in plasma). The excess CO_2 is excreted by diffusion to seawater when blood passes through the gills. Elevated CO_2 under ocean acidification (OA I and OA II), reduces the CO_2 gradient into seawater, resulting in an elevation in blood plasma P_{CO_2} of the same magnitude (respiratory acidosis). Alteration of blood plasma pH is prevented by H^+ secretion and HCO_3^- accumulation through currently unidentified mechanisms that have been proposed to result in a reduction of $[\text{Cl}^-]$ equimolar to HCO_3^- accumulation. However, this putative decrease in blood plasma $[\text{Cl}^-]$ has not been detected in any of the three published studies that have measured it (indicated by question marks; see 'Ionic and A–B regulation considerations' for details). Under extreme hypercapnia, external P_{CO_2} is elevated to levels that are more than threefold higher than those of fish fluids under control conditions, >25-fold higher than control seawater P_{CO_2} levels, and more than threefold higher than those observed under the most extreme OA scenario. This extreme condition results in a reversal of the CO_2 diffusion gradient and, as a result, CO_2 diffuses from seawater into the fish, causing a very pronounced respiratory acidosis. It is clear that under this condition, compensatory HCO_3^- accumulation in blood plasma is associated with equimolar Cl^- loss. However, while this condition may be relevant for aquaculture, it is not necessarily relevant for OA. Note that the branchial cellular mechanisms responsible for these effects are not known (see Fig. 3). The size of the font for CO_2 represents predicted differences in partial pressures (blue, seawater; red, blood), and the size of the arrows represents the diffusion gradient between seawater and fish blood (not to scale).

gabazine reversed the effects of elevated CO_2 (reviewed in Heuer and Grosell, 2014; Nilsson and Lefevre, 2016). One of those studies using gabazine was our own (Hamilton et al., 2013); working on the California splitnose rockfish (*Sebastes diploproa*), we found increased anxiety-like behaviour in fish exposed to ~1100 μatm CO_2 (pH 7.75) for 7 days and subjected to the light/dark preference test (Hamilton et al., 2013). This test is routinely used in biomedical research to assess GABA_A function in rodents (Bourin and Hascoët, 2003), and has also been validated for fish (Maximino et al., 2010a,b; Holcombe et al., 2013). Briefly, an animal is placed in the middle of an arena in which half of the walls are white and half are black, and time spent in the different areas of the arena is recorded, ideally using automated motion-tracking software. This test presents the animal with a conflict between exploring a new environment and avoiding the unfamiliar. Because administration of anxiogenic drugs (see Glossary) increases the time spent in the dark area of the arena, and anxiolytic drugs (see Glossary) increase the time spent in the light area (Bourin and Hascoët, 2003), this test is said to assess 'anxiety-like' behaviour. GABA_A antagonists such as gabazine are potent anxiogenics. Thus, the light/dark preference test is an indication of anxiety-like behaviour and GABA_A function. This test is not necessarily relevant for animal performance in the wild; however, this point does not matter for the purpose of studying GABA_A function.

In our experiments, we found that rockfish exposed to elevated CO_2 spent more time in the dark area of the light/dark arena. But how can we know that this represented increased anxiety-like behaviour for a rockfish? The key was that gabazine increased the time that control fish spent in the dark area, thus causing them to behave like CO_2 -exposed rockfish, but it did not affect CO_2 -exposed rockfish (Hamilton et al., 2013). In other words, CO_2 exposure likely induced neuronal hyperexcitability, which resulted in increased anxiety-like

behaviour, an effect that was mimicked by blocking GABA_A Rs with gabazine in control fish. We concluded that gabazine did not have any effect on CO_2 -exposed rockfish in this test because their anxiety levels had already reached a maximum. In addition, these results have three important, often overlooked and misinterpreted implications: (1) gabazine can have anxiogenic effects on control fish; (2) gabazine does not necessarily reverse the phenotype resulting from elevated CO_2 exposure; instead, its effect depends on the specific experimental conditions/tests; and (3) blocking of GABA_A Rs with gabazine presumably increases excitatory neuronal activity in fish with normal GABA_A function. Thus, gabazine could help to counteract any condition that reduces neuronal excitability, regardless of that condition being originally caused by impaired GABA_A function (see Basic neurobiology considerations).

Additional mechanistic support for the GABA_A model was provided by a second set of experiments using the GABA_A agonist muscimol in a 'shelter test' (Hamilton et al., 2013). Unlike the commonly used anxiolytic drugs benzodiazepines and barbiturates, which bind to GABA_A regulatory sites, muscimol binds to the same site as GABA and therefore activates GABA_A Rs independently of GABA release from presynaptic neurons (Frølund et al., 2002) (Fig. 1). Thus, if the GABA_A model is valid, muscimol should increase anxiety levels of CO_2 -exposed fish, but decrease it in control fish. However, the light/dark test already demonstrated a ceiling level of anxiety in CO_2 -exposed fish, so the potential effect of muscimol would not be detectable. To overcome this limitation, we used the shelter test, which is based on the propensity of juvenile rockfish to stay near kelp paddies during the juvenile life stage. Unlike the light/dark test, there were no differences in baseline behaviour between control and CO_2 -exposed fish in the shelter test, as both groups of fish spent similar amounts of time in close proximity to the shelter. However, with muscimol

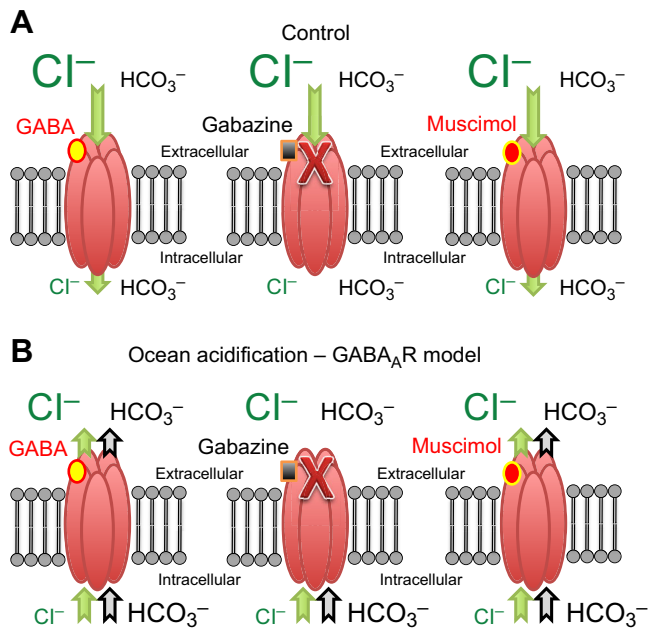


Fig. 1. Theoretical GABA_A receptor (GABA_AR) model. (A) GABA_AR is an ion channel formed by five subunits. It is permeable to Cl⁻ ions and, to a lesser extent, HCO₃⁻ ions. Under control conditions, the concentration of extracellular Cl⁻ is greater than intracellular Cl⁻. Left: when the neurotransmitter GABA binds to GABA_AR, Cl⁻ ions will move into the neuron causing hyperpolarization. Middle: gabazine, a GABA_AR antagonist, binds to the same site as GABA and prevents the ion channel from opening, thus no net movement of Cl⁻ or HCO₃⁻ ions will take place. Right: muscimol is a GABA_AR agonist and binds to the same site as GABA, causing opening of the ion channel, allowing Cl⁻ to enter the neuron and causing hyperpolarization even in the absence of GABA. (B) Left: under elevated CO₂, Nilsson et al. (2012) first proposed that the increase in HCO₃⁻ would be accompanied by a decrease in Cl⁻ in extra- and intracellular compartments. The result of this is a 'reversal' of GABA-activated currents, with Cl⁻ ions moving out of the neuron, causing depolarization instead of the hyperpolarization seen under control conditions. Middle: similar to control conditions, gabazine blocks any movement of ions through the GABA_AR; however, during ocean acidification (OA) it prevents depolarization. Right: muscimol, acting like GABA, would also cause depolarization of the neuron during OA (as opposed to hyperpolarization under control conditions). The size of the font for Cl⁻ and HCO₃⁻ represents predicted differences in concentrations (not to scale).

application we did observe a difference in preference for the shelter, with control fish spending more time away from the shelter exploring the arena relative to the CO₂-exposed fish, who spent more time near the shelter, consistent with the GABA_AR reversal proposed by Nilsson et al. (2012).

Although the effects of elevated CO₂ exposure on certain fish species and behavioural tests under laboratory conditions are clear and there is also strong experimental support for the GABA_AR model, there are several aspects of the normal biology of aquatic organisms that must be resolved. Specifically: (1) what are the actual [HCO₃⁻], [Cl⁻] and pH levels in the relevant intra- and extracellular fluids; (2) do OA-relevant CO₂ levels cause significant alterations of those parameters; and (3) what is the membrane potential (V_m ; see Glossary) of neurons from marine organisms and what are the GABA_AR permeabilities for Cl⁻ and HCO₃⁻, and how do these neurons regulate their intracellular pH? After these aspects are resolved for animals exposed to current CO₂ conditions, the next questions should be: (4) are there any species-specific mechanisms that might determine species-specific responses to OA; (5) could neuronal mechanisms other than GABA_AR, either related to GABA

or other neurotransmitters, be affected by exposure to OA-like conditions; and (6) are there compensatory mechanisms that act over longer time scales, and when P_{CO_2} slowly rises over generational times?

Basic neurobiology considerations

To understand the potential effects of OA on fish neurobiology, it is essential to understand some fundamental properties of neuronal functioning. Here, we review basic neurobiology concepts, mostly based on mammalian research, and identify key aspects relevant for the GABA_AR model.

What are membrane, equilibrium and reversal potentials?

The membrane potential (V_m) is essential for neuronal function. The value of V_m is determined by intracellular negatively charged proteins and active transport by the Na⁺/K⁺-ATPase, which maintains much higher [K⁺] and much lower [Na⁺] inside cells relative to the extracellular fluid. At rest, V_m is between -70 and -80 mV in larger glutamatergic (see Glossary) pyramidal neurons, and around -60 mV in smaller GABAergic (see Glossary) interneurons (Lee et al., 2013). V_m is modeled by the Goldman equation:

$$V_m = \frac{RT}{F} \ln \frac{P_K[K^+]_e + P_{Na}[Na^+]_e + P_{Cl}[Cl^-]_i}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_e}, \quad (1)$$

where R is the gas constant (8.315 J K⁻¹ mol⁻¹), T is the temperature in degrees Kelvin, F is the Faraday constant (96,485 Coulombs mol⁻¹), P is the permeability of each anion across the membrane, and [ion]_e and [ion]_i refer to the extra- and intracellular concentrations of each ion, respectively.

V_m is based on two important principles: (1) ions move along a concentration gradient from high to low; and (2) ions move along an electrical gradient, with opposite charges attracting and similar charges repelling. For example, because of active transport by the Na⁺/K⁺-ATPase, [K⁺] is much greater inside a neuron than outside, so K⁺ has a tendency to leave the neuron along the concentration gradient. However, the resting V_m is negative, so the electrical gradient opposes the concentration gradient. At a specific V_m , these two forces are equal and there will be zero net K⁺ movement in or out of the neuron. This is termed the 'equilibrium potential' for K⁺, or E_K , and the equilibrium potential of an ion (E_{ion}) can be calculated, if the relevant extra- and intracellular concentrations are known, using the Nernst equation:

$$E_{ion} = \frac{RT}{zF} \ln \frac{[ion]_e}{[ion]_i}, \quad (2)$$

where z is the valence of the ion.

The entrance of a cation or the exit of an anion shifts the V_m of the neuron to a more positive value, which is known as 'depolarization'. Conversely, the exit of a cation or the entrance of an anion shifts V_m to a more negative value, known as 'hyperpolarization'. If V_m is depolarized above a certain value ('threshold'), an action potential is triggered, and this electrical signal is propagated along the neuron's axon and neurotransmitters are released at the presynaptic terminal. By contrast, if V_m is hyperpolarized, a neuron is farther from threshold and less likely to generate an action potential. Neuronal excitation is largely regulated by the neurotransmitter glutamate, which is released from presynaptic terminals of axons, diffuses across the synapse and binds to postsynaptic glutamate receptors on dendrites of the target neuron. This leads to Na⁺ or Na⁺ and Ca²⁺ entry (depending on the receptor to which glutamate binds) that

Table 2. Summary of publications that used gabazine in studies on aquatic acidification

Publication	Organism	CO ₂ level, exposure time	CO ₂ effect	Gabazine effect on control group	Gabazine effects on OA group
Nilsson et al. (2012)	Larval clownfish (<i>Amphiprion percula</i>) Larval damselfish (<i>Neopomacentrus azysron</i>)	~450 and ~900 µatm, 11 days (clownfish), 4 days (damselfish)	Loss of olfactory discrimination (two-choice olfactory flume test); reduced behavioural lateralization (detour test)	No effect	Restored olfactory discrimination, restored behavioural lateralization
Watson et al. (2014)	Conch snail (<i>Gibberulus gibbosus</i>)	~400 and ~960 µatm, 5–7 days	Reduced jumping response to a predator	No effect	Restored antipredator jumping
Hamilton et al. (2013)	Juvenile Californian rockfish (<i>Sebastes diploproa</i>)	~480 and ~1125 µatm, 7 days	Increased anxiety-like behaviour (light/dark preference test)	Increased anxiety-like behaviour	No effect (OA group was at ceiling levels)
Chivers et al. (2014)	Juvenile ambon damselfish (<i>Pomacentrus amboinensis</i>)	~440 and ~990 µatm, 4 days	Impaired learning of the antipredator response (feeding strike and line crossing tests); decreased survival in the wild (releasing fish back to the reef)	No effect	Restored predator cue learning; increased survival in the wild
Chung et al. (2014)	Small spiny damselfish (<i>Acanthochromis polyacanthus</i>)	~466 and ~944 µatm, 6–7 days	Reduced retinal reaction to fast visual stimuli (critical flicker fusion threshold electroretinogram)	No effect	Restored retinal reaction speed
Ou et al. (2015)	Larval pink salmon (<i>Oncorhynchus gorbuscha</i>) (exposed to elevated CO ₂ since embryo)	~450 and ~2000 µatm (freshwater), 70 days	Reduced anxiety-like behaviour (novel approach test)	Increased anxiety-like behaviour (but close to ceiling levels)	Increased anxiety-like behaviour to same levels as controls treated with gabazine
Lai et al. (2015)	Adult three-spined stickleback (<i>Gasterosteus aculeatus</i>)	~440 and 990 µatm, 40–50 days	Reduced behavioural lateralization (detour test)	No effect	Restored behavioural lateralization
Munday et al. (2016)	Larval clownfish (<i>A. percula</i>)	~500 and ~1050 µatm, 4 days	Reduced predatory cue response (two-choice olfactory flume test/predatory cue, startle stimulus)	No effect	Restored olfactory discrimination (startle stimulus not tested with gabazine)
Lopes et al. (2016)	Larval sand smelt (<i>Aterina presbyter</i>)	~540 and ~2080 µatm, 7 and 21 days	Shift in absolute behavioural lateralization (detour test)	No effect	Partially restored behavioural lateralization
Regan et al. (2016)	Juvenile catfish (<i>Pangasianodon hypophthalmus</i>) ^a	Normally hypercapnic fish were kept at high CO ₂ (controls: ~30,600 µatm) or brought to normocapnic levels (experimental: ~400 µatm), (freshwater) 7–14 days	Increased activity (open field test); increased time near novel object (novel object test); no effects on predator response or sociality	No effect	Decreased locomotor activity in all tests; decreased time near novel object; no effect on predator or sociality tests

Animals were exposed to 4 mg l⁻¹ (13.9 µmol l⁻¹) gabazine for 30 min in a small container, except in Chung et al. (2014) (4 mg l⁻¹ gabazine, flow rate of 0.1 l min⁻¹ for 5–20 min) and Regan et al. (2016) (4 and 8 mg l⁻¹, gabazine).

^aOA group in this study is lowered CO₂.

depolarizes V_m , and therefore is excitatory. To prevent excessive excitation, the neurotransmitter GABA acts on postsynaptic GABA_ARs, leading to Cl⁻ entry that hyperpolarizes V_m , and therefore is inhibitory. Proper neurological functioning depends on the balanced activity of glutamatergic and GABAergic synapses (Hille, 2001; Levinthal and Hamilton, 2015; Mele et al., 2016). However, things become more complicated because whether an ion enters or leaves the neuron and the resulting magnitude of the V_m change induced depends on the ion permeability and on the relationship between V_m and E_{ion} . For anions such as Cl⁻ and HCO₃⁻, if E_{anion} is more negative than V_m , the anion would flow into the cell, hyperpolarizing V_m towards E_{anion} and making an action potential less likely. By contrast, if E_{anion} is more positive than V_m and a permeable channel opens, the anion will flow out of the cell, depolarizing V_m and therefore making an action potential

more likely. The larger the difference between V_m and E_{ion} , the larger the ion flux, and therefore the larger the depolarizing or hyperpolarizing effect.

So what happens if the channel that opens is permeable to both Cl⁻ and HCO₃⁻, as is the case for GABA_R? Using a combination of Eqns 1 and 2 (Hille, 2001), we can calculate the reversal potential for GABA_AR (E_{GABA} ; see Glossary), which depends on the relative permeability to Cl⁻ and HCO₃⁻, and the concentration of each anion in the brain interstitial fluid (BIF) and neuron cytoplasm:

$$E_{GABA} = \frac{RT}{F} \ln \frac{P_{Cl}[Cl^-]_e + P_{HCO_3}[HCO_3^-]_e}{P_{Cl}[Cl^-]_i + P_{HCO_3}[HCO_3^-]_i} \quad (3)$$

The classic (and correct) method to empirically measure E_{GABA} is with sharp electrodes that impale the cell body of the neuron. A

GABA_AR response is evoked by applying GABA or by injecting a current to manipulate V_m and stimulate an inhibitory postsynaptic potential, followed by anion substitutions and additional recordings to observe GABA_AR-dependent changes in V_m and estimate the permeability of each anion. Whole-cell intracellular recordings and cell-attached recordings (Verheugen et al., 1999) have also been used to calculate E_{GABA} . Under normal conditions, E_{GABA} is more negative than V_m , and therefore it is hyperpolarizing and inhibitory. Using these methods, Kaila et al. (1989) found that increasing $[\text{HCO}_3^-]_i$ (by elevating intracellular pH) depolarized E_{GABA} , and Bormann et al. (1987) reported that E_{GABA} was depolarized 56 mV for every 10-fold change in $[\text{Cl}^-]_i$.

It is clear that under specific circumstances, the Cl^- flux through GABA_ARs is inhibitory rather than excitatory. The degree of the change in polarity of GABA_ARs depends on how far the E_{GABA} moves away from V_m . However, caution should be taken when applying the Goldman equation to neurons from a live animal, because *in vivo* ionic permeabilities and concentration ratios are not constant (Nicholson, 1980; Sandblom and Eisenman, 1967). Furthermore, because each parameter is correlated with the others, the Goldman equation requires knowledge of V_m and actual ion concentrations in the same neuron (or in a group of the same neuron type), and using parameters from different neurons or neuron groups will most likely give misleading results. Thus, calculating E_{GABA} using the Goldman equation is ideal for isolated systems, such as brain slices, cultured neurons or channels in oocytes.

Regulation of GABA_AR

In mammals, GABA_ARs are made up of five subunits that form the pore that allows anions across the cell membrane (Fig. 1). However, the number of potential combinations of subunits is astronomically high because there are 19 known homologous subunit gene products: six α , three β , three γ , three ρ , and one each of the ϵ , θ , δ and π subunits (Sigel and Steinmann, 2012). To date, 26 GABA_ARs with different subunit compositions have been identified (Olsen and Sieghart, 2008). The distinct subunits that make up the pentameric complex are important because subunit composition dictates binding affinity for GABA, pharmacological agonists and antagonists, channel kinetics, cellular localization and Cl^- and HCO_3^- permeability, among other functional properties (Lee and Maguire, 2014). Multiple GABA_AR subunits are also present in invertebrates (Martyniuk et al., 2007) and fish (Biggs et al., 2013; Martyniuk et al., 2007), but their relation to GABA_AR function is much less studied. To our knowledge, only two papers so far have studied GABA_AR mRNA isoform abundance in brains from OA-exposed fish (Lai et al., 2015; Schunter et al., 2016), and neither study found any major changes compared with control fish. However, changes in protein abundance, changes in specific neuron types and differential responses in OA-tolerant fish cannot be ruled out.

Another mechanism known to affect GABA_AR function is changes in $[\text{Cl}^-]_i$ resulting from the balance in activity of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter 1 (NKCC1), which moves Cl^- into the neuron, and the $\text{K}^+/\text{2Cl}^-$ cotransporter 2 (KCC2), which moves Cl^- out of the neuron. For example, in neurons from developing embryos, KCC2 is expressed at low levels (Banke and McBain, 2006; Ben-Ari, 2002). As a result, NKCC1 activity prevails over KCC2 activity, resulting in higher $[\text{Cl}^-]_i$ (Ito, 2016), a resting V_m 10–20 mV more positive than in adult neurons (Khazipov et al., 2015), Cl^- flux out of the neuron through GABA_ARs or glycine receptors, and excitatory V_m depolarization (Banke and McBain, 2006; Ben-Ari, 2002; Ito, 2016). A similar effect is seen in ventral spinal cord motoneurons from KCC2 knockout mice (Hübner et al., 2001), in

which GABA depolarized neurons ~21.1 mV more than in controls, consistent with increased $[\text{Cl}^-]_i$. This leads to an important question relevant to the GABA_AR model: if exposure to elevated CO_2 induces GABA_AR reversal because of altered Cl^- and/or HCO_3^- gradients across neuronal membranes, could neurons downregulate NKCC1 and/or upregulate KCC2 to increase $[\text{Cl}^-]_i$ and thereby restore normal GABA_AR inhibitory function? GABA_AR function can be modulated by multiple other dynamic and long-term translational and posttranslational regulatory mechanisms – too many to describe here (the interested reader is directed to Mele et al., 2016). If the GABA_AR model is correct, regulation of GABA_AR function may explain the fact that some studies show no effect of elevated CO_2 on fish behaviour (Jutfelt and Hedgärde, 2013, 2015; Lonthair et al., 2017). However, this hypothesis should be experimentally tested.

Pharmacological considerations

Gabazine is a competitive GABA_AR antagonist (Heaulme et al., 1986), meaning it binds to the same active site as GABA (Fig. 1) and prevents channel opening. With the exception of our previous study that used the GABA_AR agonist muscimol (Hamilton et al., 2013; discussed above), experiments using gabazine have provided the only mechanistic evidence in support of the GABA_AR model thus far. Table 2 details the studies that have, to date, used gabazine on fish and invertebrates exposed to elevated CO_2 . In most cases, gabazine reversed the effect of elevated CO_2 and restored normal behaviour. Gabazine also significantly alters the behaviour of control rockfish (Hamilton et al., 2013) and pink salmon (Ou et al., 2015). However, no other study found any effect of gabazine on behaviour (Chivers et al., 2014; Lai et al., 2015; Lopes et al., 2016; Munday et al., 2016; Nilsson et al., 2012; Watson et al., 2014) or visual processing (Chung et al., 2014) in control fish. This is an interesting point because gabazine should also affect normal inhibitory GABA regulation, inducing neuronal excitation; in fact, one of the features precluding the use of gabazine as a therapeutic agent in humans is the risk of convulsions resulting from inappropriate neuronal excitation (Enna and Bowery, 1997). A similar effect was seen in intact zebrafish (*Danio rerio*) brains, where gabazine increased the spontaneous firing rate of olfactory neurons, altered the dynamics of odour-induced firing, blocked fast oscillatory synchronization of local neurons and induced epileptiform activity (Tabor et al., 2008).

To confirm that the effects of gabazine on CO_2 -exposed aquatic animals are indeed due to alteration of GABA_ARs, we suggest that it will be important to test the effects of other GABA antagonists not structurally related to gabazine, such as bicuculline or picrotoxin (Johnston, 2013), or an agonist such as muscimol (Andrews and Johnston, 1979). A final consideration is that gabazine causes a net increase in excitation of the nervous system, and it would counteract any other mechanism that decreases excitatory activity, not only GABA_AR reversal. For example, a theoretical OA-induced decrease in glutamate release could be ‘restored’ by gabazine, and this could be erroneously interpreted as GABA_AR reversal. The GABA_AR model does seem to fit with principles of basic neuroscience, but it must be validated with more than the use of gabazine and behavioural studies.

Ionic and A-B regulation considerations

Theoretical models of the effects of OA on fish GABA_ARs using the Goldman equation typically consider P_{CO_2} , $[\text{HCO}_3^-]$, pH and $[\text{Cl}^-]$ in blood plasma and whole brain as the extra- and intracellular parameters, respectively (Heuer and Grosell, 2014; Heuer et al.,

2016; Nilsson and Lefevre, 2016; Regan et al., 2016). However, only a few studies have measured these parameters in the blood plasma of fish exposed to OA-relevant conditions (i.e. P_{CO_2} between 600 and 2000 μatm). The pattern that emerged was complete regulation of blood plasma pH and a 2–5 mmol l^{-1} increase in blood plasma $[\text{HCO}_3^-]$ (Ern and Esbaugh, 2016; Esbaugh et al., 2016, 2012; Green and Jutfelt, 2014; Heinrich et al., 2014; Heuer et al., 2016; Strobel et al., 2012) (Table 1). Only three studies measured blood plasma $[\text{Cl}^-]$, but none of them found significant changes after exposure to OA-like conditions (Esbaugh et al., 2016; Green and Jutfelt, 2014; Heinrich et al., 2014). The lack of change in plasma $[\text{Cl}^-]$ in marine fish could be due to the difficulty in accurately measuring small changes in $[\text{Cl}^-]$ compared with ‘background’ control levels of 140–200 mmol l^{-1} (see Genz et al., 2008; Erlacher-Reid et al., 2011; Esbaugh et al., 2016; Lin et al., 2003; Toews et al., 1983; reviewed in Evans et al., 2005). However, it is also possible that the large range of reported values of $[\text{Cl}^-]$ reflect species-, life stage- or metabolism-specific differences. Surprisingly, very little is known about the molecular and cellular mechanisms of A–B regulation in marine bony fish. The little we do know is largely derived from whole-animal experiments that

exposed adult fish to extreme hypercapnia, and is based on marine elasmobranchs or on freshwater fish models which, because of different ionic conditions in the surrounding water, are not relevant for marine fish. Most relevant to the GABA_AR model, the tight link between Cl^- loss and HCO_3^- accumulation has so far only been demonstrated for adult fish exposed to P_{CO_2} levels $\geq 10,000 \mu\text{atm}$ (most notably, Toews et al., 1983), which is much higher than OA-relevant P_{CO_2} levels. Therefore, different compensatory mechanisms during OA-relevant conditions cannot be ruled out. For example, a combination of buffering, H^+ secretion and Cl^- -independent HCO_3^- accumulation would result in elevated plasma $[\text{HCO}_3^-]$ without associated Cl^- loss (Table 1, Fig. 2C). Another common misconception is that elevated P_{CO_2} during OA will diffuse inside fish and elevate blood CO_2 . However, as explained in detail in Melzner et al. (2009), values of P_{CO_2} in fish internal fluids are several-fold higher than seawater P_{CO_2} , even for the most extreme case of OA (Table 1). Thus, although OA indeed results in elevated blood P_{CO_2} , it does so by reducing the diffusive rate of metabolic P_{CO_2} excretion to seawater (Fig. 2). Far from a technicality, this affects the magnitude of the A–B disturbance, and potentially also the regulatory mechanisms involved (Table 1, Fig. 2).

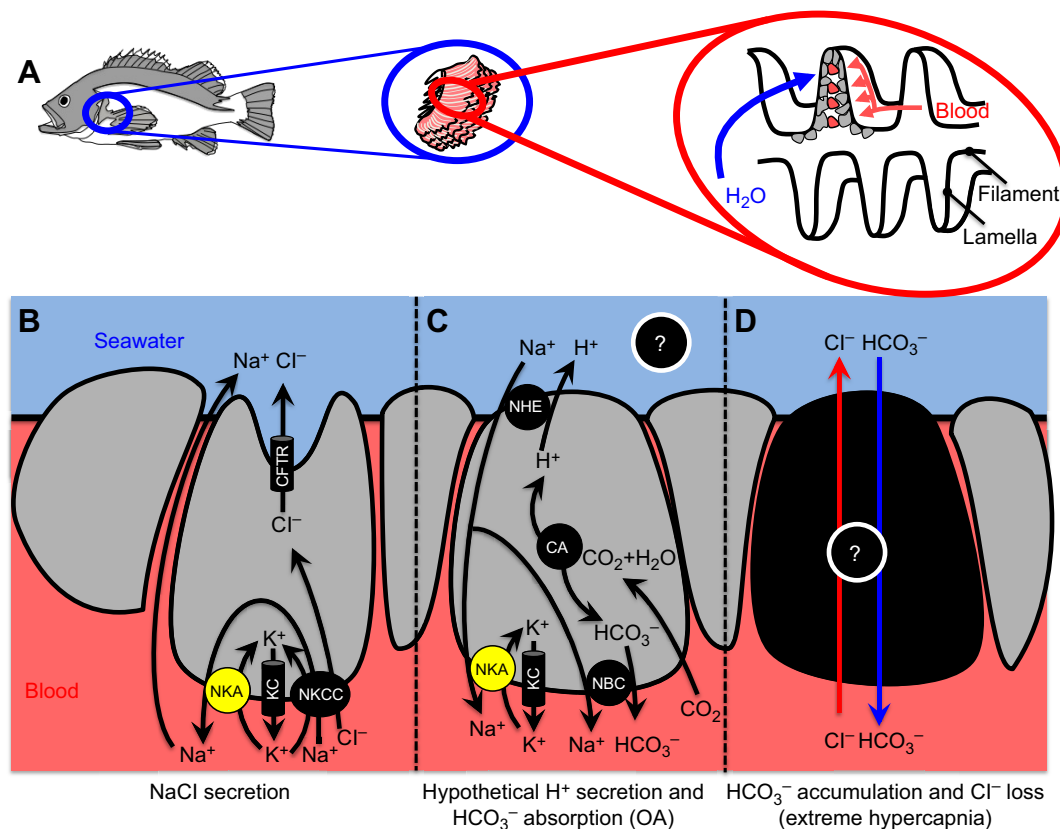


Fig. 2. Cellular mechanisms for blood ionic regulation in gills from marine bony fish. (A) General anatomy of the fish gill. Gills are composed of filaments and lamellae; blood capillaries (red) run in counter-current fashion to seawater (which facilitates gas and ionic exchange), and specialized gill epithelial cells called ionocytes (grey) are responsible for NaCl and acid–base regulation. (B) Model for NaCl secretion in gill ionocytes from marine fish (formerly known as ‘chloride cells’). NaCl -secreting ionocytes represent the majority of ionocytes in marine fish gills. NKA energizes transcellular Cl^- excretion, and the transepithelial potential drives paracellular Na^+ secretion, resulting in net NaCl secretion (reviewed in Zadunaisky, 1996). (C) Potential model for H^+ secretion and HCO_3^- absorption in marine gill ionocytes. Notice that (1) CO_2 enters the ionocyte from the blood, and (2) CA hydrates CO_2 into H^+ (which is secreted to seawater in exchange for Na^+ via NHEs) and HCO_3^- (which is transported to blood together with Na^+ via NBCs). This model is based on acid-secreting ionocytes from mammalian kidney and marine elasmobranch gill; however, it has not been confirmed in marine bony fish gill ionocytes (as represented by question mark). It is also not known whether these putative ionocytes are the same as ‘chloride cells’ or are a different cell type. (D) Extreme hypercapnia causes HCO_3^- accumulation and Cl^- loss considered by the GABA_AR model. This model is based on whole-animal fluxes from fish experiencing extreme hypercapnia (Fig. 1); however, the cellular mechanisms are not known (question mark). Similarly, it is not known whether this model is relevant for OA. CA, carbonic anhydrase; CFTR, cystic fibrosis transmembrane regulator; KC, K⁺ channel; NBC, Na⁺-HCO₃⁻ cotransporter; NHE, Na⁺/H⁺ exchanger; NKA, Na⁺/K⁺-ATPase; NKCC, Na⁺/K⁺/2Cl⁻ cotransporter.

An urgent topic of future research is elucidating common, species-specific and life-stage-specific mechanisms of A–B regulation in the skin, gill and choroid plexus (see Glossary) of fish exposed to control and OA-relevant CO_2 levels. Complicating this quest, marine fish gills are constantly secreting NaCl to maintain blood ionic balance. This process uses enzymes and ion-transporting proteins that may overlap with A–B balance (most notably, the Na^+/K^+ -ATPase) (Fig. 2B). Because ionoregulation is, by far, more energy demanding than A–B regulation, it imposes baseline protein levels against which detecting putative changes in enzyme activity and mRNA and protein abundance in response to OA becomes challenging. Another intriguing question is whether the robust ionoregulatory mechanisms of marine fish in gill, skin, kidney and intestine (reviewed in Larsen et al., 2014), which can maintain blood plasma $[\text{NaCl}] \sim 200 \text{ mmol l}^{-1}$ lower than seawater, can handle the few additional millimoles of Cl^- expected to result from blood A–B regulation during OA exposure.

To our knowledge, only Heuer et al. (2016) have estimated A–B parameters in the brains of fish exposed to OA-relevant P_{CO_2} levels. They reported elevated pH and $[\text{HCO}_3^-]$ in brain homogenates of spiny damselfish exposed to $1900 \mu\text{atm}$ (pH 7.60) for 4 days. These values were taken as representative of neuronal cytoplasm; $[\text{Cl}^-]$ in blood or brain was not measured. When plasma and whole brain $[\text{HCO}_3^-]$ values were plugged into the E_{GABA} (Goldman) equation together with theoretical values from intra- and extracellular $[\text{Cl}^-]$ and theoretical GABA_{A} Cl^- and HCO_3^- permeability ratios, the results generally supported the GABA_{A} model. However, these results are not without potential issues, amongst which is the very high P_{CO_2} of $\sim 16,000 \mu\text{atm}$ that was estimated for whole brains from control fish (which was surprisingly unchanged in OA-exposed

fish) as well as the theoretical intracellular $[\text{Cl}^-]$ of 8 mmol l^{-1} , which was also considered to not change in OA-exposed fish. Another interesting point is that CO_2 has been reported to impair olfactory predator discrimination at levels as low as $700 \mu\text{atm}$ (Munday et al., 2010), which are very unlikely to induce changes in $[\text{HCO}_3^-]$ and $[\text{Cl}^-]$ large enough to cause significant alteration of E_{GABA} . Is it possible that the mechanisms that induce behavioural alteration at mildly increased CO_2 levels ($\geq 700 \mu\text{atm}$) are different from those acting at higher CO_2 levels ($\sim 1900 \mu\text{atm}$)? This might explain why larvae reared at $1700 \mu\text{atm}$ (pH 7.60) did not respond to any olfactory cues (Munday et al., 2009). Similarly, the effects seen in different behavioural tests might be caused by different mechanisms.

The actual fluids that should be considered in order to confirm the GABA_{A} model are the cerebrospinal fluid (CSF) or BIF (representing the extracellular fluid) and the neuron cytoplasm (representing the intracellular fluid) (Fig. 3, Table 3). This is an important point, because CSF and BIF have different ionic and A–B composition compared with blood plasma (reviewed in Damkier et al., 2013; Syková and Nicholson, 2008), neurons have different composition compared with whole brain homogenate, and the Goldman equation is extremely sensitive to even minute differences in the parameters used. In fact, a tiny difference in one of the parameters can change the calculation of GABA_{A} equilibrium potential from hyperpolarizing to depolarizing in relation to V_m , and minute differences in various parameters can compensate for (or potentiate) each other's effects on equilibrium potential (see Basic neurobiology considerations). Furthermore, at least in humans, the brain is composed of similar numbers of neurons and glial cells (Azevedo et al., 2009), so measurements on brain homogenates

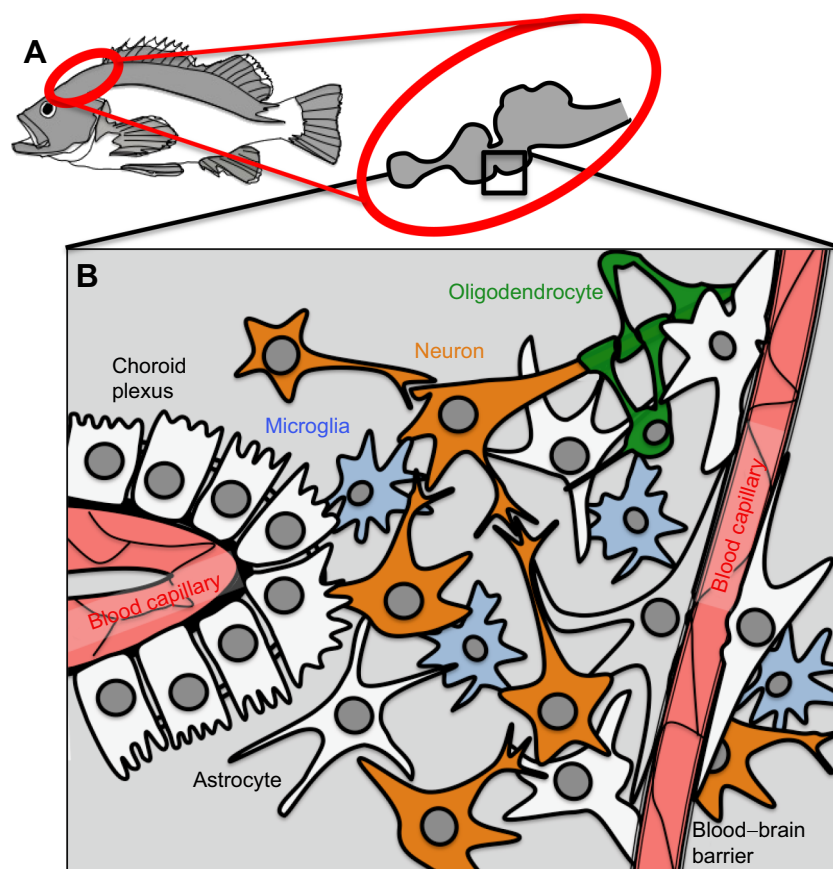


Fig. 3. Acid–base parameters and cell composition of fish brain. (A) The brain of vertebrate animals is separated from blood plasma by the blood–brain barrier and the choroid plexus. (B) Several cell types are present in the brain, including neurons, astrocytes, microglia and oligodendrocytes. Blood capillaries are surrounded by the blood–brain barrier (represented as thick edges in the capillary on the right), or by choroid plexus (capillary in the left). Those structures restrict and regulate the exchange of substances between the cerebrospinal fluid (CSF) and the brain interstitial fluid (BIF) and blood. As a result, the ionic composition and acid–base (A–B) status of CSF and BIF are different from those of blood.

Table 3. Representative ion and acid-base parameters in blood of marine fish, and theoretical values for cerebrospinal fluid (CSF), brain interstitial fluid (BIF) and neurons

	Blood	CSF/BIF	Neuron
P_{CO_2} (μatm)	~3000	>3000?	?
pH	~7.80	~7.55?	~7.2?
$[\text{H}^+]$ (nmol l^{-1})	~16	~28?	~50
$[\text{Cl}^-]$ (mmol l^{-1})	140–200	>140–200?	6–80?
$[\text{HCO}_3^-]$ (mmol l^{-1})	~5	>5?	?

The question marks for CSF, BIF and neurons represent lack of data. In the GABA_AR model and the Goldman equation, CSF and BIF (and not blood) should be used as extracellular fluid, and neuron cytoplasm as intracellular fluid.

provide an average estimation of the two (plus contaminating CSF and BIF). Additionally, the choroid plexus, blood–brain barrier (see Glossary) and astrocytes tightly regulate the ionic and A–B composition of CSF and BIF (reviewed in Damkier et al., 2013; Syková and Nicholson, 2008). In fact, the chief evolutionary selective pressure favouring the evolution of the blood–brain barrier is believed to be the maintenance of a stable ionic environment to promote neuronal function (Abbott, 1992; Cserr and Bundgaard, 1984). This is a very important point for the GABA_AR model, because fish experience large fluctuations in blood plasma $[\text{NaCl}]$ and $[\text{HCO}_3^-]$, for example, during migration between freshwater and seawater (e.g. Maxime et al., 1990; reviewed in Evans et al., 2005), in the postprandial period (Buckling et al., 2009; Cooper and Wilson, 2008; Wood and Buckling, 2011; Wood et al., 2005) and upon temperature changes (reviewed in Heisler, 1988). If fish were unable to maintain a stable ionic composition of the CSF and BIF and the GABA_AR model was correct, these animals would regularly experience olfactory disturbances such as lack of predator odour discrimination, with the subsequent obvious negative impact on survival.

The few available studies on fish CSF are on elasmobranchs, and have reported higher $[\text{Na}^+]$, $[\text{Cl}^-]$, $[\text{HCO}_3^-]$ and P_{CO_2} , and lower pH and protein-buffering capacity compared with blood (Maren, 1972, 1977; Smith et al., 1929; Wood et al., 1990). At least in the skate *Raja ocellata*, active aerobic metabolism by brain cells results in CSF/BIF having P_{CO_2} levels that are approximately threefold higher compared with blood plasma, and ~0.25 pH units more acidic (Wood et al., 1990). In addition, the blood–brain barrier of *R. ocellata* is highly permeable to CO_2 (Wood et al., 1990), which is advantageous for CO_2 diffusion to blood plasma down its partial pressure gradient. However, the blood–brain barrier is also permeable to inwardly directed CO_2 diffusion, and exposure to environmental hypercapnia (~10,000 μatm) induces a proportional increase in CSF P_{CO_2} (Wood et al., 1990). Similar to blood plasma, the initial drop in CSF P_{CO_2} is compensated by HCO_3^- accumulation. Can this process result in changes in $[\text{Cl}^-]$ and $[\text{HCO}_3^-]$ in the CSF consistent with the GABA_AR model? This question can only be answered by performing experiments to elucidate fish CSF and BIF composition and regulatory mechanisms, which should consider the following points: (1) the high P_{CO_2} and low pH in the CSF imply that OA-relevant P_{CO_2} elevations will induce smaller A–B disturbances in CSF compared with blood plasma; (2) in mammals, HCO_3^- secretion into the CSF and BIF takes place by both Cl^- - and Na^+ -dependent mechanisms (Damkier et al., 2013; Hladky and Barrand, 2016), implying that HCO_3^- accumulation does not necessarily mean a reciprocal reduction in $[\text{Cl}^-]$; and (3) inhibition of choroid plexus carbonic anhydrase significantly reduces both HCO_3^- and Cl^- transport into the CSF, implying that a vast majority of secreted

HCO_3^- originates from aerobic CO_2 production in choroid plexus cells, and that there is some degree of $\text{HCO}_3^-/\text{Cl}^-$ co-transport (Maren, 1988; Maren and Broder, 1970).

Because the GABA_AR model also implies intracellular HCO_3^- accumulation in exchange for Cl^- (Nilsson et al., 2012; Nilsson and Lefevre, 2016), the neuronal mechanism for intracellular pH regulation is a final point to consider. To our knowledge this has not been studied in fish neurons, but mammalian neurons typically regulate their intracellular pH by a combination of Na^+/H^+ exchangers, $\text{Na}^+/\text{HCO}_3^-$ co-transporters, Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchangers and $\text{Cl}^-/\text{HCO}_3^-$ exchangers (reviewed in Ruffin et al., 2014). If we assume that fish have similar mechanisms of ion transport, a linear inverse correlation between HCO_3^- and Cl^- concentrations is unlikely to occur in fish, because Na^+ would compensate for at least part of the excess negative charge resulting from HCO_3^- accumulation.

Fish are the most diverse group of vertebrate animals and occupy ecological niches with very diverse ionic, A–B and temperature conditions. Therefore, species-specific mechanisms of CSF, BIF and neuronal A–B regulation could very well explain differential sensitivity to OA in a GABA_AR-dependent manner. In addition, the GABA_AR model has also been proposed to explain the effects of OA on the behaviour of a few marine invertebrates (Rossi et al., 2016; Watson et al., 2014). With the exception of cephalopods, marine invertebrates lack a blood–brain barrier, which suggests a lesser ability to regulate the micro-environment around neurons compared with mammals (Cserr and Bundgaard, 1984). However, the nervous central ganglia of marine invertebrates possess other structural adaptations that isolate neuronal processes from the hemolymph (Cserr and Bundgaard, 1984). In addition, $[\text{Cl}^-]$ in the hemolymph of marine invertebrates is similar to seawater at ~560 mmol l^{-1} (Larsen et al., 2014), which raises the question of whether the potential reduction in hemolymph $[\text{Cl}^-]$ in response to OA would be large enough to induce a reversal of GABA_AR.

Other potential effects of OA on neuronal function

If exposure to OA-relevant elevations in P_{CO_2} indeed changes $[\text{HCO}_3^-]$ and $[\text{Cl}^-]$ in brain intra- and extracellular fluids, this would not just affect the function of central GABA_ARs; other neurobiological aspects that could be affected include glycine receptors, neuronal network dynamics, certain K^+ channels, astrocyte–neuron metabolic communication and CO_2 -sensing peripheral neurons, to name a few. The implications of OA-associated effects on these features are discussed below.

Glycine receptors

Glycine receptors are abundant inhibitory ligand-gated channels in the mammalian spinal cord, the brainstem, and the forebrain hippocampus and prefrontal cortex (Legendre, 2001; Salling and Harrison, 2014). Upon binding of the neurotransmitter glycine, these channels open, allowing Cl^- to flow according to the reversal potential for glycine receptors (E_{glycine}) and V_m as explained above. As for GABA_ARs, activation of glycine receptors under normal conditions hyperpolarizes the neuron via Cl^- influx, and in neurons of the immature nervous system, activation of glycine receptors depolarizes the neuron owing to Cl^- efflux (Avila et al., 2013). Also similar to GABA_ARs, glycine receptors are permeable to HCO_3^- (Bormann et al., 1987). Thus, if OA-like conditions affect GABA_ARs according to the GABA_AR model, glycine receptors should undoubtedly also be affected. Because glycine receptors are involved in generating motor patterns for locomotion and spinal reflex actions (Avila et al., 2013), future research should investigate

OA-induced changes in reversal potential in these receptors as well as the potential physiological and behavioural implications. Furthermore, if both E_{GABA} and E_{glycine} are reversed during OA and no compensatory mechanism exists, their effects on neuronal hyperexcitation would be additive.

Network dynamics

Mammalian cortical neurons fluctuate between a resting hyperpolarized ‘down state’ and another ‘up state’ that is more depolarized, can last up to hundreds of milliseconds, and are close to the action potential threshold (Wilson, 2008). In a cellular network, neurons move between these two states in a rhythmic manner and, although the exact function is unknown, this is thought to help synchronize distant neuronal populations (Hahn et al., 2006) and maintain attention and memory formation (Holcman and Tsodyks, 2006). This important feature of neurons is relevant to the function of voltage and ligand-gated ion channels and whether they will reach the threshold for action potential generation. For example, when neurons are in a down state, the reversal potential for Cl^- can be higher than the membrane potential, so the opening of GABA_A Rs can be excitatory in nature; however, in an up state, GABA_A R regains its inhibitory action (Plenz, 2003). Intracellular recordings in larval zebrafish demonstrate that Purkinje neurons also fluctuate between up states, when they fire bursts of action potentials, and down states, when only short bursts of action potentials occur with AMPA receptor activation (Sengupta and Thirumalai, 2015). Taken together, a shift in E_{GABA} would have a prominent effect when the neuron is in the down state but not in the up state. As we learn more about the down and up states of neurons during different behaviours (e.g. mobile versus immobile, exploring, searching for food, under different stress levels), we will hopefully be able to discern under which circumstances an OA-induced change in E_{GABA} may affect neuronal dynamics, and how these dynamics might be affected by the behavioural state of the organism.

Other potential effects of HCO_3^-

In addition to potential reversal of E_{GABA} and E_{glycine} , the elevation in intra- and extracellular $[\text{HCO}_3^-]$ during OA predicted by the GABA_A R model could affect other ion channels and signalling pathways. For example, changes in $[\text{HCO}_3^-]$ could modulate K^+ channels in neurons (Jones et al., 2014; Kaila et al., 1997) and astrocytes (Ma et al., 2012), or affect metabolic coupling between these two cell types (Choi et al., 2012). A potential role for such effects in OA-induced behavioural alterations remains to be explored.

CO_2 -sensing peripheral neurons

The GABA_A R model proposes an OA-induced alteration of neuronal function in the brain; however, it is possible that peripheral neurons are additionally, or alternatively, affected. Because CO_2 -sensing peripheral neurons are directly exposed to seawater and not immersed in BIF and CSF, the ionic considerations that may result in neuronal malfunction would be different compared with brain neurons. For example, seawater acidification can reduce the sensitivity of chemosensing neurons in the barbels of Japanese sea catfish *Plotosus japonicus* (Caprio et al., 2014). Although the magnitude of the seawater acidification in that study was within the range of OA, a caveat was that acidification was experimentally achieved by addition of HCl instead of CO_2 .

Another example of peripheral CO_2 -sensing neurons is the neuroepithelial cells present in the gills of adult zebrafish. V_m of these cells becomes gradually depolarized when exposed to increasing P_{CO_2} from control levels to 0.5% CO_2 (~5000 μatm) (Qin et al., 2010), which encompasses the OA-relevant P_{CO_2} range.

Zebrafish neuroepithelial cells have been proposed to regulate important cardiorespiratory processes such as increasing ventilatory amplitude in adult zebrafish (Vulesevic et al., 2006) and tachycardia in 7-day-old larval zebrafish (Miller et al., 2014). Interestingly, the ventilatory response was blunted in zebrafish chronically exposed to hypercapnia (Vulesevic et al., 2006), suggesting potential acclimation. And in zebrafish larvae, the tachycardia response to hypercapnia was absent in 7-day-old larval zebrafish (Miller et al., 2014), showing life-stage-specific differences. Thus, as reported in freshwater zebrafish, OA-relevant CO_2 disturbances could potentially affect CO_2 -sensing neurons in marine fish and trigger physiological responses, which could be compensatory or maladaptive. However, this type of neuron has not been described in any marine fish to date, and their CO_2 -sensing mechanisms and sensitivity may well differ from those of zebrafish because of the different A–B properties of seawater and freshwater.

Conclusions

While short exposure to OA-relevant elevations in CO_2 induces clear alterations in neurobiology and behaviour in aquatic organisms, the mechanisms behind these effects are not clear. To date, the only mechanistic explanation is an alteration of GABA_A R function caused by putative changes in $[\text{Cl}^-]$ and $[\text{HCO}_3^-]$ in intra- and/or extracellular fluids. At this point, we would like to highlight the importance of basic science research, without which the identification of this putative mechanism would have been impossible, or at least severely delayed. Indeed, the GABA_A R model is based on over a century of basic research on A–B and ionic regulation, both at the comparative and human health levels, as well as decades of research that perfected the behavioural techniques that allowed us to identify effects caused by OA.

When OA was identified as a potential problem for marine animals and the deleterious effects on fish behaviour were noticed, the GABA_A R mechanistic model was proposed and experimentally tested relatively shortly after. Similarly, more basic science research is required to confirm, expand or refute the GABA_A R model. Together with long-term acclimation and trans-generational studies, mechanistic information on physiological processes potentially affected by OA will allow us to identify species- and life-stage-specific differences and responses to OA that could determine the trite ‘winners and losers’ of this process. Some of the relevant avenues of research have been highlighted throughout this Review, which we hope will serve as frame of reference for future work.

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Competing interests

The authors declare no competing or financial interests.

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