



Toward a Mechanistic Understanding of Marine Invertebrate Behavior at Elevated CO₂

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Elevated carbon dioxide (CO₂) levels can alter ecologically important behaviors in a range of marine invertebrate taxa; however, a clear mechanistic understanding of these behavioral changes is lacking. The majority of mechanistic research on the behavioral effects of elevated CO₂ has been done in fish, focusing on disrupted functioning of the GABA_A receptor (a ligand-gated ion channel, LGIC). Yet, elevated CO₂ could induce behavioral alterations through a range of mechanisms that disturb different components of the neurobiological pathway that produces behavior, including disrupted sensation, altered behavioral choices and disturbed LGIC-mediated neurotransmission. Here, we review the potential mechanisms by which elevated CO₂ may affect marine invertebrate behaviors. Marine invertebrate acid–base physiology and pharmacology is discussed in relation to altered GABA_A receptor functioning. Alternative mechanisms for behavioral change at elevated CO₂ are considered and important topics for future research have been identified. A mechanistic understanding will be important to determine why there is variability in elevated CO₂-induced behavioral alterations across marine invertebrate taxa, why some, but not other, behaviors are affected within a species and to identify which marine invertebrates will be most vulnerable to rising CO₂ levels.

Keywords: marine invertebrate, behavior, carbon dioxide, ocean acidification, GABA, ligand-gated ion channel, mechanisms, GABA_A receptor

INTRODUCTION

Human activity is resulting in unprecedented amounts of carbon dioxide (CO₂) being released into the atmosphere. Since the Industrial Revolution, atmospheric CO₂ levels have increased by over 45%, from approximately 280 ppm (Joos and Spahni, 2008) to over 410 ppm today (Dlugokencky and Tans, 2019), higher than any time in the past several million years (Masson-Delmotte et al., 2013). In the worst case scenario, following the business-as-usual representative concentration pathway (RCP) 8.5, atmospheric CO₂ levels will increase to over 900 ppm by the end of this century. Even if substantial efforts are made to curb global CO₂ emissions to keep warming below 2°C, atmospheric CO₂ levels will still likely exceed 600 ppm by 2100 (Betts and McNeall, 2018). The ocean has absorbed 20–30% of anthropogenic CO₂ emissions since the mid-1980s (Bindoff et al., 2019), causing a reduction in seawater pH referred to as ocean acidification. Furthermore, CO₂ in the surface ocean is increasing at the same rate as in the atmosphere (Bindoff et al., 2019); therefore, marine organisms will need to cope with higher CO₂ levels as well as declining seawater pH. Finally, due to a decrease in the ocean's buffering capacity as CO₂ content rises, natural CO₂ fluctuations

in the ocean are projected to amplify dramatically at future higher CO₂ levels (Shaw et al., 2013; McNeil and Sasse, 2016). Natural diel (Hofmann et al., 2011; Santos et al., 2011; Shaw et al., 2012) and seasonal CO₂ fluctuations (McNeil et al., 2007; Feely et al., 2008) will be amplified by up to three times in the future (McNeil and Sasse, 2016; Gallego et al., 2018), meaning that marine organisms will experience elevated CO₂ levels for certain periods of time (daily or seasonally) much earlier than predictions based on atmospheric CO₂ alone.

Elevated CO₂ has been found to affect a range of processes in marine organisms, including altering calcification (Ries et al., 2009; Kroeker et al., 2013), growth and survival (Fabry et al., 2008; Kurihara et al., 2008), and behavior (Briffa et al., 2012; Clements and Hunt, 2015; Nagelkerken and Munday, 2015). Projected future CO₂ levels were first found to alter animal behavior in orange clownfish *Amphiprion percula* larvae reared in a partial pressure of CO₂ (pCO₂) of ~1,050 μatm (Munday et al., 2009). In laboratory experiments, the olfactory discriminatory abilities of 11-day-old clownfish larvae were tested in a two-channel flume. Most strikingly, larvae reared in control seawater (~390 μatm pCO₂) avoided the side of the flume with chemical cues from pungent tree leaves compared to the seawater control side. However, larvae reared in elevated CO₂ spent nearly all their time in the side with these odors. Larval clownfish reared in ~1,050 μatm CO₂ were also unable to discriminate between the odor of parents and non-parents, whereas control larvae avoided the odor of their parents (Munday et al., 2009). Elevated CO₂ has since been found to affect a variety of behavioral traits in a wide spectrum of fishes, including tropical and temperate reef species, eels, salmon and sharks (Munday et al., 2019). Behavioral alterations at elevated CO₂ have also been demonstrated in a variety of marine invertebrates, including cnidaria, polychaetes, echinoderms, arthropods and mollusks, from a range of environments, including the intertidal zone, coastal and offshore waters and the deep-sea (Clements and Hunt, 2015; Nagelkerken and Munday, 2015; Wang and Wang, 2019). Marine invertebrates exhibit alterations in a range of behaviors at elevated CO₂, including activity levels (Rosa and Seibel, 2008; Ellis et al., 2009; Spady et al., 2014), feeding rates (Saba et al., 2012; Vargas et al., 2014), settlement and metamorphosis behaviors (Albright et al., 2010; Doropoulos et al., 2012; Guo et al., 2015), burrowing behaviors (Green et al., 2013; Clements and Hunt, 2014), shelter selection (de la Haye et al., 2011), predatory behaviors (behaviors related to finding and eating prey) (Kim et al., 2015; Queirós et al., 2015; Spady et al., 2018) and predator avoidance (Bibby et al., 2007; Manríquez et al., 2013, 2014a; Spady et al., 2014; Watson et al., 2014). Behavioral categorization is often ambiguous as one behavior may actually include multiple behaviors or decision-making processes. For example, predator avoidance behaviors include multiple decisions such as mode of avoidance (including crypticity versus escape), flight-initiation distance and mode of escape (Lima and Dill, 1990). In this review, we use the behavioral category that was reported in the corresponding research paper.

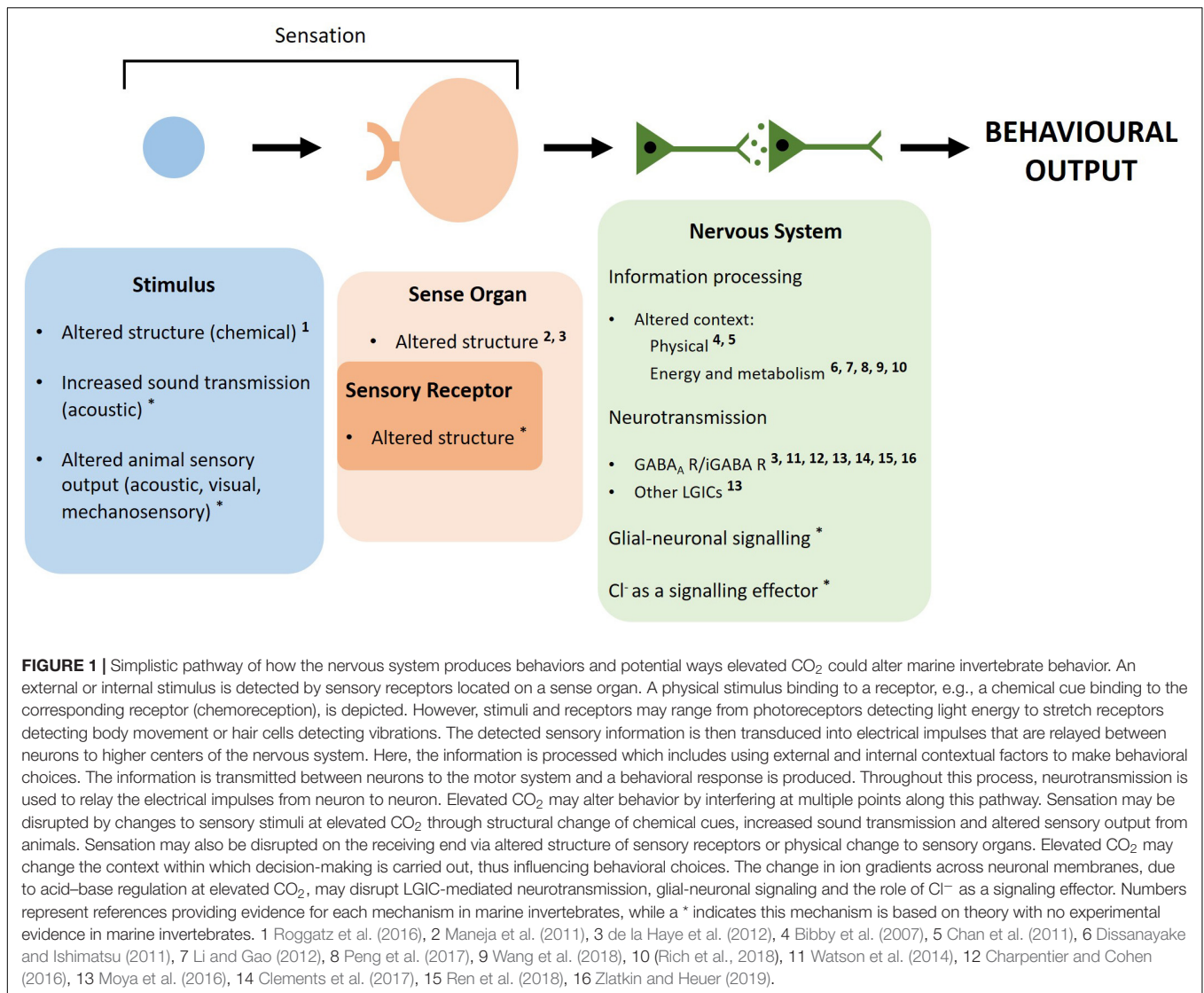
Since the review by Clements and Hunt (2015) at least 61 additional papers have assessed the impact of elevated CO₂ on marine invertebrate behaviors (**Supplementary Table S1**).

Research has continued to focus on mollusks, arthropods and echinoderms, however, cnidarian settlement and metamorphosis (Foster et al., 2015; Olsen et al., 2015; Viyakarn et al., 2015; Fabricius et al., 2017; Yuan et al., 2018), the settlement behavior and swimming activity of a bryozoan (Pecquet et al., 2017), and settlement of an annelid (Nelson et al., 2020) have also been studied. In addition to continuing to assess the impact of elevated CO₂ on the range of behaviors previously studied (above), a few new behaviors have also been investigated. For example, the first study assessing the effect of elevated CO₂ on marine invertebrate reproductive behavior was recently published (Borges et al., 2018). Exposure of male amphipods *Gammarus locusta* to elevated CO₂ (800 μatm pCO₂) for two generations disrupted the chemosensory detection of potential mates (Borges et al., 2018). A light/dark test on swimming crabs *Portunus trituberculatus* exposed to control (485 μatm pCO₂) or elevated (750 μatm and 1,500 μatm pCO₂) CO₂ was the first to assess the impact of elevated CO₂ on anxiety-like behavior in a marine invertebrate. Crabs exposed to elevated CO₂ levels spent significantly more time in the dark zone (Ren et al., 2018).

There appears to be large variability in behavioral responses to elevated CO₂; across taxonomic groups, the same behavior can respond differently to elevated CO₂, and within a species, some behaviors but not others can be affected (Nagelkerken and Munday, 2015) (**Supplementary Table S1**). As the phylogenetic variation among invertebrate taxa is enormous, it may account for a large component of the variability in behavioral responses across taxonomic groups; some taxa may be more tolerant to elevated CO₂ than others. At the same time, various behaviors are likely associated with different processes, such as specific circuits in the nervous system. These processes may be affected differently by elevated CO₂, accounting for the effects of elevated CO₂ on some, but not all, behaviors within a species. It must also be noted that variability may be due to differences in experimental techniques and conditions.

Animal behavior is, to a large extent, the functional output of the nervous system (Simmons and Young, 1999); therefore, behavioral changes induced by elevated CO₂ are likely caused by neurobiological mechanisms. In the nervous system, simplistically, the pathway that produces behavior involves sensory receptors that detect environmental stimuli (e.g., chemical cues, light waves) and internal stimuli (e.g., spatial orientation of the body). The received information is transduced into electrical impulses and neurotransmission relays these electrical impulses between neurons. Neurotransmission must be rapid to produce timely behavioral responses, and this is achieved via ligand-gated ion channel (LGIC) mediated neurotransmission (Dent, 2010). LGICs are transmembrane protein complexes that, upon binding of a specific neurotransmitter, allow ion flow which results in excitation or inhibition of neuronal firing depending on the ion charge and direction of flow (Tovar and Westbrook, 2012). When the sensory information arrives at higher centers of the nervous system, this information is processed and a behavioral output is produced (Blom, 1978; Kreher et al., 2008) (**Figure 1**).

Elevated CO₂ could induce behavioral alterations through a range of mechanisms in the nervous system that disturb different



components of the pathway that produces behavior [Briffa et al. (2012), as described in **Figure 1**]. (1) Sensation may be disrupted via changes to sensory stimuli at elevated CO₂, such as structural alteration of chemical cues, disturbed transmission of acoustic cues and altered sensory output from animals experiencing behavioral changes (Roggatz et al., 2016; Nagelkerken et al., 2019). (2) Alternatively, elevated CO₂ may disrupt sensation via physical change to sensory organs or structural alteration of sensory receptors (Maneja et al., 2011; Briffa et al., 2012; Bignami et al., 2013). (3) Morphological and respiratory changes at elevated CO₂ may alter the context within which decision making is carried out, influencing behavioral responses (Bibby et al., 2007; Chan et al., 2011; Peng et al., 2017; Rich et al., 2018). (4) Elevated CO₂ may induce change in ion gradients across neuronal membranes, due to acid–base regulation to prevent acidosis at elevated CO₂, which may disrupt LGIC-mediated neurotransmission via the γ -aminobutyric acid (GABA) type A receptor (GABA_A R) (Nilsson et al., 2012). These mechanisms

are not necessarily mutually exclusive and may interact to alter behavior at elevated CO₂.

Despite the growth in literature demonstrating elevated CO₂-induced behavioral alterations in marine invertebrates, the mechanisms responsible for these behavioral alterations are still poorly understood. Due to the diversity of invertebrate nervous and neurobiological systems, it is likely that a suite of different processes underlie these behavioral changes. Here, we discuss the potential mechanisms by which elevated CO₂ may alter marine invertebrate behavior; (1) disturbed sensation, (2) altered context within which behavioral choices are made, and (3) disrupted LGIC-mediated neurotransmission. Since the prominent hypothesis for altered LGIC-mediated transmission is the GABA hypothesis proposed in fish by Nilsson et al. (2012), here we discuss evidence for the GABA hypothesis in marine invertebrates and propose research to expand our understanding of the GABA hypothesis in marine invertebrates. We demonstrate that the effects of altered GABA_A receptor

function are likely to be widespread, including non-behavioral effects. Finally, we identify other neurobiological mechanisms that should be affected if the GABA hypothesis is correct, propose alternative neurobiological mechanisms by which behavior could be altered by elevated CO₂ and suggest techniques to be utilized for future study of elevated CO₂-induced behavioral alterations.

MECHANISMS FOR ELEVATED CO₂-INDUCED BEHAVIORAL CHANGES

Altered Sensory Stimuli at Elevated CO₂

Elevated CO₂ may influence behavior by altering an animal's ability to sense the environment (Briffa et al., 2012; Draper and Weissburg, 2019). A range of sensory stimuli may be disrupted at elevated CO₂ via differing mechanisms, thereby affecting associated behaviors (Figure 1). Structural alteration of chemical cues at elevated CO₂ may affect chemoreception – the detection of chemical cues by binding to sensory receptors, e.g., odor molecules binding to olfactory receptors (Tierney and Atema, 1988). Impaired chemo-responsive behavior was first shown to be due to structural alteration of the chemical cue at low pH in a freshwater system (Brown et al., 2002) and the same mechanism has since been demonstrated in a marine invertebrate; the shore crab *Carcinus maenas*. Near-future pH levels altered the structure and charge of signaling molecules that mediate egg ventilation behavior in the shore crab *C. maenas* and the threshold of signaling molecule concentration required to induce egg ventilation behavior in this species increased when tested at pH 7.7 compared to pH 8.1 (Roggatz et al., 2016). Receptor alteration, such as change in ionization state, could also conceivably occur at low pH disrupting chemoreception (Tierney and Atema, 1988), and may be an additional explanation for behavioral changes observed in the shore crab (Roggatz et al., 2016). However, to date receptor structure has never been directly tested at different CO₂ levels in conjunction with a behavioral assay.

Changes in ocean chemistry associated with rising CO₂ levels will directly affect acoustic cues, potentially altering auditory driven behaviors. Sound absorption, in the low frequency range of ~0.01–10 kHz, is reduced by decreasing pH due to shifts in the chemical reactions of sound absorbing compounds (e.g., magnesium sulfate, boric acid and carbonate ions) in seawater (Hester et al., 2008). Sound absorption (decibels per kilometer) is predicted to decrease by over 20% and almost 40% with a pH drop from 8.1 to 7.95 and 7.8, respectively (Hester et al., 2008). Thus, as CO₂ levels rise, transmission of low-frequency sounds will increase and ecologically relevant acoustic cues, within this affected frequency range, will be transmitted further at elevated CO₂ levels. For example, compared to off reef-locations, oyster reefs have higher acoustic energy levels within the frequency range of 1.5–20 kHz. This acoustic signature of reefs is used as a settlement cue by larval oysters (Lillis et al., 2013). As CO₂ levels rise, oyster larvae may thus be able to detect appropriate settlement habitats from greater distances, however, it remains unknown whether the magnitude

of change is sufficient to be of biological relevance. Elevated CO₂ will also increase the transmission of abiotic sounds produced naturally (e.g., waves, raindrops) and by human activity (e.g., shipping, sonar and construction) (Ilyina et al., 2010). This will create a noisier environment in which it is harder for marine invertebrates to detect ecologically relevant sounds, such as those used for communication (Popper et al., 2001; Buscaino et al., 2011) as well as navigation and habitat selection for settlement (Jeffs et al., 2003; Stanley et al., 2009; Vermeij et al., 2010; Lillis et al., 2013). In a coral reef fish, predatory behavior decreased when exposed to boat noise or elevated CO₂ (925 μatm pCO₂), however, there was no additive effect when these stressors co-occurred (McCormick et al., 2018). Studies in marine invertebrates to determine how increased transmission of biologically relevant cues and background noise will interact as CO₂ levels rise, and if this will be biologically relevant will be important.

Behavioral changes induced by elevated CO₂ may alter the sensory output of an animal, affecting whether and how this animal is sensed by other animals (Draper and Weissburg, 2019). For example, increased activity of a prey animal could enhance how much or how often sound, visual and mechanosensory cues are produced, strengthening predatory sensory detection of the prey and increasing the chance of predation (Draper and Weissburg, 2019). Elevated CO₂ reduced the intensity and frequency of snaps produced by snapping shrimp (Rossi et al., 2016). As these snaps are commonly present in the soundscapes used by marine invertebrate larvae as settlement cues (Stanley et al., 2009; Vermeij et al., 2010; Lillis et al., 2013), altered snapping behavior of snapping shrimp at elevated CO₂ may in turn alter marine invertebrate settlement behavior.

Physical Change of Sensory Organs

Sensation may also be disrupted by physical change of sensory organs at elevated CO₂. Due to lower saturation states of seawater with respect to calcium carbonate at elevated CO₂, animals can have difficulty maintaining calcium carbonate structures (Orr et al., 2005) which may damage sensory organ structures (Briffa et al., 2012). Alternatively, active acid–base regulation to maintain a steady internal pH may alter the concentrations of ions that are fundamental for the formation of calcified sensory organs (Grosell, 2019). For example, many marine invertebrates use statocysts, which contain mineralized statoliths, to detect gravity to maintain orientation (Cohen, 1960; Clarke, 1978; Spangenberg, 1986), as well as vibrational stimuli for hearing in cephalopods (Mooney et al., 2010). Statocysts are also involved in motor programs that underlie hunting behavior in mollusks (Levi et al., 2004). Statolith size was reduced and morphology altered in cephalopods exposed to ~1,300 μatm (Zakroff et al., 2019), 2,200 μatm (Kaplan et al., 2013), and 4,000 μatm (Maneja et al., 2011) pCO₂. Abalone exposed to ~700 and ~1,000 μatm pCO₂ also exhibited decreased statolith size compared to control conditions (Manríquez et al., 2014b). Conversely, statolith size was increased, and chemical composition altered, in squid exposed to 850 and 1,500 μatm pCO₂ (Lacoue-Labarthe et al., 2011). Cuttlefish exposed to

4,000 $\mu\text{atm } p\text{CO}_2$ exhibited reduced statolith calcification, altered statolith microstructure and decreased prey capture efficiency compared to squid in control conditions (700 $\mu\text{atm } p\text{CO}_2$) (Maneja et al., 2011). Furthermore, computer modeling showed that an increased statolith mass, similar to that seen in the otoliths of fish exposed to 2,500 $\mu\text{atm } p\text{CO}_2$, would alter cephalopod hearing below 10 Hz (Zhang et al., 2015). However, squid exposed to $\sim 1,300 \mu\text{atm } p\text{CO}_2$ had smaller statoliths with an altered morphology (Zakroff et al., 2019) but no impairment in swimming orientation (Zakroff et al., 2018). Therefore, elevated CO₂-induced alteration of statoliths may disturb hearing but not gravity detection in cephalopods, impacting auditory-driven behavioral outputs but not the ability to maintain orientation. Decapod crustaceans possess calcified antennules, housing chemoreceptors, which are used for long range chemoreception. Rapid antennule flicking is used to gather chemical cue information, much in the way sniffing increases our ability to determine smells (Schmitt and Ache, 1979; Koehl, 2005). Hermit crabs with disrupted chemo-sensory responses at extremely high levels of CO₂ (*c.* >12,000+ $\mu\text{atm } p\text{CO}_2$) showed no damage to their antennules (de la Haye et al., 2012), suggesting that other mechanisms must be responsible for the observed response.

Altered Behavioral Choices

The physiological and ecological context, including external factors (e.g., presence of predators or temperature) and internal factors (e.g., hunger or reproductive state) can influence behavioral choices (Palmer and Kristan, 2011). Elevated CO₂ may alter both external and internal factors, changing contextual modulation of behavioral choice and resulting in altered behavioral output. Physical changes induced by elevated CO₂ may alter an animal's behavioral choice. For example, predator-induced shell thickening observed in control periwinkles *Littorina littorea* did not occur in periwinkles exposed to extremely high levels of CO₂ (*c.* >12,000+ μatm). However, predator avoidance behavior increased at elevated CO₂ conditions, compared to control, which suggests behavioral compensation for the lack of morphological defense at extremely high levels of CO₂ (Bibby et al., 2007). In another example, the swimming performance of larval sand dollars *Dendraster excentricus* was maintained at elevated CO₂ ($\sim 1,000 \mu\text{atm } p\text{CO}_2$) despite impaired arm and body morphology, likely due to a behavioral change in ciliary beat patterns (Chan et al., 2011). By contrast, both predator cue-induced byssal thread production and protective clustering behavior was decreased in mussels exposed to elevated CO₂ (1,100 $\mu\text{atm } p\text{CO}_2$) (Kong et al., 2019), indicating no behavioral compensation for the lack of morphological defense at elevated CO₂.

The influence of elevated CO₂ on respiration, energy turnover and mode of metabolism (Pörtner et al., 2004) may also alter behavioral choice. Depressed metabolism at elevated CO₂ levels may reduce energy production, reducing the energy available to meet other demands and constraining performance of some behaviors. For example, metabolic scope and swimming ability were reduced in shrimp exposed to $\sim 1,000 \mu\text{atm } p\text{CO}_2$ (Dissanayake and Ishimatsu, 2011) and oxygen consumption

rate and digging depth were decreased in razor clams exposed to 1,900 and 3,000 $\mu\text{atm } p\text{CO}_2$ (Peng et al., 2017). Increased metabolism can indicate an increased energy demand at elevated CO₂ and may decrease the energy available for other costly processes. For example, crabs exposed to 1,200 and 2,300 $\mu\text{atm } p\text{CO}_2$ exhibited an increased metabolic rate but a decreased feeding rate (Wang et al., 2018). Alternatively, organisms may alter behaviors to meet the high energy demand. For example, respiration and feeding rate were increased in a copepod exposed to 1,000 $\mu\text{atm } p\text{CO}_2$ (Li and Gao, 2012) and a sea urchin exposed to 1,300 $\mu\text{atm } p\text{CO}_2$ (Rich et al., 2018). However, other studies show a change in metabolism with no associated behavioral change at 750 and 1,200 $\mu\text{atm } p\text{CO}_2$ in an echinoderm (Carey et al., 2016) and at 1,500 $\mu\text{atm } p\text{CO}_2$ in a mollusk (Benítez et al., 2018), or no metabolic change but altered behavior at 960 $\mu\text{atm } p\text{CO}_2$ in a mollusk (Watson et al., 2014) and at 1,000, 2,000, and 3,000 $\mu\text{atm } p\text{CO}_2$ in a crustacean (Menu-Courey et al., 2018). Therefore, it seems that altered metabolism in elevated CO₂ may be responsible for some instances of altered behaviors, but not others.

Altered Functioning of the GABA_A Receptor

Elevated CO₂ has been found to alter a range of behaviors across different sensory modalities, as well as behaviors that involve higher order processing, such as decision making (de la Haye et al., 2011) and anxiety-like behaviors (Ren et al., 2018). This suggests that not only sensory detection, but also other neuronal processes are altered by elevated CO₂. Neurotransmission is crucial to all components of the pathway producing behavior, from relaying electrical signals from sensory receptors to (and between) higher-order neurons for information processing, to motor neurons for the production of a behavioral response. Therefore, altered neurotransmission at elevated CO₂ may underlie a variety of behavioral disturbances.

The prominent hypothesis for altered neurotransmission at elevated CO₂ is the GABA hypothesis, proposed to occur in fish (Nilsson et al., 2012; Heuer and Grosell, 2014) and also suggested to apply to marine invertebrates (Watson et al., 2014). In vertebrates, GABA acts on the GABA type A receptor (GABA_A R), a LGIC permeable to chloride (Cl⁻) and bicarbonate (HCO₃⁻) ions, as the main inhibitory neurotransmitter in the central nervous system (DeFeudis, 1975; Bormann et al., 1987). Under normal conditions, binding of GABA opens the GABA_A R channel allowing a net influx of negative charge resulting in hyperpolarization and inhibition of neuronal firing. Nilsson et al. (2012) proposed that the change in HCO₃⁻ and Cl⁻ gradients across the neuronal membrane, due to acid-base regulation at increased CO₂ levels, results in a net efflux of negative charge from the GABA_A R upon GABA binding (Figure 2A). This could cause depolarization and excitation of neurons, thereby altering behavioral responses. Pharmacological studies administering GABA_A R antagonists and agonists (Nilsson et al., 2012; Hamilton et al., 2013; Chivers et al., 2014; Chung et al., 2014), and measurements of brain ion gradients (Heuer et al., 2016) have supported this hypothesis in fish.

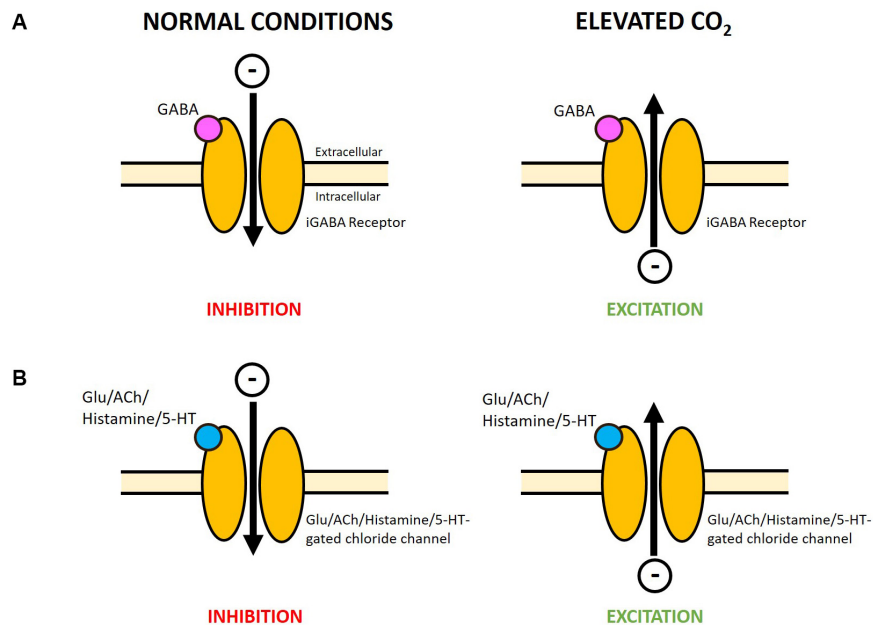


FIGURE 2 | Potential reversal in function of varying LGICs at elevated CO₂ in marine invertebrates. **(A)** A change in HCO₃⁻ and Cl⁻ gradients across the neuronal membrane, due to acid–base regulation at elevated CO₂, was proposed to reverse the net flow of negative charge through the GABA_A R (Nilsson et al., 2012) and likely applies to the marine invertebrate iGABA R. Under normal conditions the net influx of negative charge is primarily carried by Cl⁻, while at elevated CO₂ the net efflux of negative charge may be primarily carried by HCO₃⁻ (see Heuer et al., 2019 for a detailed explanation). **(B)** A range of invertebrates also possess glutamate, acetylcholine, histamine and serotonin-gated chloride channels. The flow of Cl⁻ through these channels may be similarly altered at elevated levels of CO₂. Research is needed to determine whether these receptors are also permeable to HCO₃⁻ which could also contribute to the reversal of the net movement of negative charge. A net influx of negative charge will result in hyperpolarization and inhibition of neuronal firing, while a net efflux of negative charge will cause depolarization and excitation. Glu, glutamate; ACh, acetylcholine; 5-HT, serotonin.

THE GABA HYPOTHESIS IN MARINE INVERTEBRATES

GABA is the main inhibitory neurotransmitter in the invertebrate peripheral and central nervous systems (Lummis, 1990; Lunt, 1991), acting on the ionotropic GABA receptor (iGABA R), which is also permeable to Cl⁻ and HCO₃⁻ ions (Kaila and Voipio, 1987). Thus, the GABA hypothesis likely applies to marine invertebrates. The GABA hypothesis has been tested in marine arthropods and mollusks using pharmacological studies (Watson et al., 2014; Charpentier and Cohen, 2016; Clements et al., 2017), measurement of ion concentrations (de la Haye et al., 2012; Charpentier and Cohen, 2016) and molecular studies (Moya et al., 2016; Ren et al., 2018) (**Table 1**).

One method of assessing the GABA hypothesis involves administering the GABA_A R antagonist gabazine (SR-95531) (Heulme et al., 1986). If GABA_A R functioning is altered at elevated CO₂, gabazine administration should reverse elevated CO₂-induced behavioral alterations by inhibiting channel opening and thus blocking the altered ion flow (Nilsson et al., 2012). Indeed, impaired escape behavior caused by exposure to 961 μatm pCO₂ was restored to control levels by gabazine in the jumping conch snail *Gibberulus gibbosus* (Watson et al., 2014). In the soft shell clam *Mya arenaria*, burrowing behaviors altered by CO₂ sediment levels representing present day variation were restored by gabazine (Clements et al., 2017). By contrast,

in Asian shore crab larvae *Hemigrapsus sanguineus*, the loss of chemical cue-induced photosensitive behavior at elevated CO₂ conditions (1,380 μatm pCO₂) was not restored by gabazine (Charpentier and Cohen, 2016). These contrasting results initially appear to suggest that the GABA hypothesis applies to some, but not other, marine invertebrate taxa. However, crustacean iGABA Rs are commonly insensitive to gabazine (El Manira and Clarac, 1991; Jackel et al., 1994; Pearstein et al., 1996; Barry, 2002), meaning that gabazine may be inadequate for testing the GABA hypothesis in crustaceans.

It is interesting to note that the action of gabazine differed across control animals; gabazine significantly altered the behavior of control crab larvae (Charpentier and Cohen, 2016), had a non-significant trend of altering the behavior of control snails (Watson et al., 2014), and did not alter control clam burrowing behavior (Clements et al., 2017). As gabazine also blocks ion flow under normal conditions, preventing inhibition, over-excitation and behavioral alterations should occur in control animals. As gabazine does not appear to affect crustacean iGABA Rs, the behavioral change observed in crabs held at control CO₂ levels may be through the action of gabazine on a different pathway, such as a different receptor type. Characterizing the pharmacology of gabazine in the studied species and using a range of GABA_A R drugs will be important to confirm the GABA hypothesis is actually being tested (see the section “Pharmacological Considerations”).

TABLE 1 | Summary of publications that have mechanistically tested whether LGIC-mediated neurotransmission is altered in marine invertebrates at elevated CO₂.

Species and life stage	pCO ₂ (μatm) and exposure time	CO ₂ behavioral effect	Mechanistic test	Outcome of mechanistic test	References
Pharmacological studies					
<i>Hemigrapsus sanguineus</i> Asian shore crab	Control: 461 Treatment: 1,380	Lost predator chemical-cue induced photosensitive behavior	Gabazine (0.1, 1, and 10 μM for 1–3 h)	<u>Control</u> : 1 and 10 μM gabazine loss of chemical cue-induced photosensitive behavior. 0.1 μM gabazine no effect. <u>CO₂ treatment</u> : 10 μM gabazine no effect	Charpentier and Cohen (2016)
(Third stage larvae)	12 h				
<i>Gibberulus gibberulus gibbosus</i> Jumping conch snail	Control: 405 Treatment: 961	Reduced jumping response to a predator	Gabazine (4 mg/L for 30 min)	<u>Control</u> : Gabazine had a non-significant trend of decreasing number of jumps <u>CO₂ treatment</u> : Gabazine restored number of jumps to control levels	Watson et al. (2014)
(Adult)	5–7 days				
<i>Mya arenaria</i> Soft shell clam	Control: 1,480 Treatment: 9,532 (in sediment porewater)	Decreased proportion of clams burrowing	Gabazine (5 mg/L for 30 min)	<u>Control</u> : No effect <u>CO₂ treatment</u> : Gabazine increased proportion of clams burrowing to control levels	Clements et al. (2017)
(Juvenile)	Clams placed on sediment surface and allowed to burrow for 20 min.				
Studies measuring ion concentration					
<i>Hemigrapsus sanguineus</i> Asian shore crab	Control: 461 Treatment: 1,380	Lost predator chemical cue-induced photosensitive behavior	Extracellular [Cl ⁻] Extracellular osmolality	No difference between control and treatment Increased at treatment compared to control	Charpentier and Cohen (2016)
(Third stage larvae for [Cl ⁻], stage 1 larvae for osmolality)	12 h				
<i>Pagurus bernhardus</i> Hermit crab	Control: 373 Treatment: 12,061	Decreased antennular flicking rates, longer to locate odor and less time in contact with odor	Hemolymph [Cl ⁻]	Increased at treatment compared to control	de la Haye et al. (2012)
(Life stage not stated)	5 days				
<i>Aplysia californica</i> California sea hare	Control: 400 Treatment: 1,200 and 3,000	No effect on self-righting behavior, decreased time of tail withdrawal reflex	Hemolymph [HCO ₃ ⁻]	Increased in both treatments compared to control	Zlatkin and Heuer (2019)
(Adult)	4–11 days				
Molecular studies					
<i>Heliconoides inflatus</i> Mediterranean pteropod	Control: 382 and 410 Treatment: 617 and 720	Not tested	Whole body transcriptomic analysis	Upregulated transcripts at elevated CO ₂ : - 1 GABA _A R subunit - 1 glycine R subunit - 14 transcripts of acetylcholine R subunits (1 specified as nicotinic) - 1 glutamate R subunit - 1 glutamate transporter - 1 voltage-gated potassium channel Downregulated transcripts at elevated CO ₂ : - 1 voltage-dependent calcium channel subunit	Moya et al. (2016)
(Life stage not stated)	3 days				
<i>Portunus trituberculatus</i> Swimming crab	Control: 485 Treatment: 750 and 1,500	Shoal average speed significantly higher compared to control: - 3 and 6 h (750 μatm pCO ₂) - 6 and 12 h (1,500 μatm pCO ₂)	Real-time PCR of the gene encoding the GABA _A R associated-protein from whole crabs	mRNA levels upregulated compared to control: - 6 h (750 μatm pCO ₂) - 3 h (1,500 μatm pCO ₂)	Ren et al. (2018)
(Phase I juvenile)	0, 3, 6, 12, 24, 48, and 72 h				

Mechanistic support for the GABA hypothesis in marine invertebrates also comes from recent studies indicating changes in ion concentration and altered behavior in the same species at elevated CO₂ (Table 1). Hermit crabs *Pagurus bernhardus* exhibited impaired chemosensory responses to a food odor and increased hemolymph Cl⁻ concentration ([Cl⁻]) at extremely high (12,061 μatm pCO₂) compared to control (373 μatm pCO₂) conditions (de la Haye et al., 2012). Asian shore crab larvae had altered chemical cue-induced photosensitive behavior and increased extracellular osmolality, but similar extracellular [Cl⁻] at elevated CO₂ (1,380 μatm pCO₂) compared to controls (461 μatm pCO₂) (Charpentier and Cohen, 2016). However, the [Cl⁻] measurements were very close to the limit of detection, which may be why no difference was observed. The increased extracellular osmolality was suggested to be due to an increase in HCO₃⁻ concentration ([HCO₃⁻]), however, [HCO₃⁻] was not directly measured. In a more recent study, sea hares *Aplysia californica* exposed to elevated CO₂ (1,200 and 3,000 μatm pCO₂) showed a reduced antipredator response and increased hemolymph [HCO₃⁻] compared to control (400 μatm pCO₂) (Zlatkin and Heuer, 2019). Together, these studies support the hypothesis of altered [HCO₃⁻] and [Cl⁻] underlying altered iGABA R function and behavioral change at elevated CO₂.

Molecular studies also provide support for the GABA hypothesis in marine invertebrates (Table 1). Transcriptomic analysis of the Mediterranean pteropod *Heliconoides inflatus* exposed to elevated CO₂ (617–720 μatm pCO₂) for 3 days showed upregulation of the transcript encoding a GABA_A R subunit (Moya et al., 2016). However, RNA was extracted from the whole animal, potentially masking differential expression within the nervous system, and behavioral assays were not carried out. Ren et al. (2018) isolated the gene encoding the GABA_A R associated-protein (GABARAP) in the crab *Portunus trituberculatus*. Real-time PCR found that GABARAP mRNA levels were significantly upregulated by 4.34-fold after 6 h at 750 μatm pCO₂ and by 2.89-fold after 3 h at 1,500 μatm pCO₂, compared to control (485 μatm pCO₂). Showing a similar trend, average speed of the crab shoal's movement was significantly higher after 3 and 6 h at 750 μatm pCO₂, and six and 12 h at 1,500 μatm pCO₂, compared to control. The increase in the GABARAP gene was suggested to assist more GABA_A Rs to cluster on neuronal membranes, which may exaggerate the impaired function of GABA_A Rs at elevated CO₂ and lead to the altered behavior (Ren et al., 2018).

iGABA R Subtypes and Variability in Elevated CO₂-Induced Behavioral Alterations

Inter- and intra-species variation in the behavioral effects of elevated CO₂ may be due to the presence and variability of iGABA R subtypes in invertebrates. The ion permeable pore of iGABA Rs is made up of five subunits (Olsen and Sieghart, 2008). There is large variation in gene structure and the number of genes encoding iGABA R subunits between invertebrate species. For example, 5 GABA R-like genes have been found in the sea-squirt *Ciona intestinalis*, 12 in the fruit fly *Drosophila*

melanogaster and 39 in the roundworm *Caenorhabditis elegans* (Tsang et al., 2006). Differing subunit composition forms iGABA R subtypes (Olsen and Sieghart, 2008) which vary in a range of functional properties including GABA binding affinity, and Cl⁻ and HCO₃⁻ permeability (Lee and Maguire, 2014). Differences in iGABA R subunits may account for the variability in behavioral alterations at elevated CO₂ observed between invertebrate species. Furthermore, subunit composition can vary between regions in the nervous system and cell types (Lee and Maguire, 2014). As different behaviors are driven by different nervous system regions, this may explain why some behaviors, but not others, are disrupted by elevated CO₂ within a species.

Pharmacological Considerations

Studies using gabazine have provided a useful starting point to understand the mechanisms underlying behavioral alterations at elevated CO₂ in marine invertebrates. However, the pharmacological profile of invertebrate iGABA Rs differs from that of vertebrate GABA_A Rs (Rauh et al., 1990), and have not been characterized as extensively as in vertebrates. The majority of invertebrate research has been in non-marine invertebrates, with invertebrate iGABA R pharmacology being best studied in insects due to their potential target for insecticides (Hosie et al., 1995; Bloomquist, 2003). Gabazine inhibits a cloned planthopper iGABA R subunit expressed in a cell line (Narusuye et al., 2007) and two cloned fruit fly iGABA R subunits expressed in *Xenopus laevis* oocytes (Hosie and Sattelle, 1996). Gabazine also inhibits iGABA Rs in native neurons of locusts (Janssen et al., 2010) and moths (Satoh et al., 2005), but not in cockroaches (Aydar and Beadle, 1999). The few studies in other diverse invertebrates are conflicting; gabazine inhibits iGABA Rs in a freshwater hydrozoan (Concas et al., 1998), only weakly antagonizes GABA responses in a terrestrial nematode (Duittoz and Martin, 1991) and has no effect on crustaceans, including a freshwater crayfish (El Manira and Clarac, 1991; Pearstein et al., 1996) and a marine lobster (Jackel et al., 1994). These non-marine examples are more taxonomically relevant to marine invertebrates than comparisons with evolutionarily divergent marine vertebrate taxa. They indicate the wide variability in invertebrate iGABA R responses to gabazine. As iGABA R pharmacology can differ by subunit composition (Lee and Maguire, 2014; Sieghart, 2015) and there is large variation in subunit genes between invertebrates (Tsang et al., 2006) it will be useful to characterize the pharmacology of gabazine on iGABA Rs in the studied marine invertebrate species.

It is also important to note that antagonists are commonly not completely specific. For example, in insects gabazine partially and bicuculline (a GABA_A R antagonist) fully inhibits locust nicotinic acetylcholine receptors (Jackson et al., 2002), and the GABA_A R antagonist bicuculline inhibits and the GABA_A R agonist muscimol activates a model of insect GABA-gated cation channels (Gisselmann et al., 2004). Less research has studied the off-target effects of GABA drugs in marine invertebrates, though bicuculline and picrotoxin both inhibit acetylcholine-gated chloride channels in the sea hare *Aplysia californica* (Yarowsky and Carpenter, 1978). To ensure the low affinity, alternative effects of drugs do not occur, careful consideration

of concentration administered must be made. Using a range of GABA_A R drugs with differing side effects will provide further evidence for or against the role of iGABA Rs in behavioral alterations at elevated CO₂.

Marine Invertebrate Acid–Base Regulatory Mechanisms and the GABA Hypothesis

Acid–base regulatory mechanisms in marine invertebrates indicate that extra- and intra-cellular [HCO₃[−]] and [Cl[−]] will alter at elevated CO₂, providing theoretical support for the GABA hypothesis. Such mechanisms have best been studied in crustaceans [see reviews by Henry and Wheatly (1992) and Wheatly and Henry (1992)]. The primary mechanism to maintain extracellular pH (pH_e) is via ion exchange with the external water environment (Cameron, 1985; Wheatly and Henry, 1992; Pörtner et al., 1998), including HCO₃[−] influx in exchange for Cl[−] efflux (Truchot, 1983; Wheatly and Henry, 1992). Indeed, in all marine invertebrates studied so far, [HCO₃[−]]_e in the blood/hemolymph increases upon exposure to elevated seawater CO₂. Exposure to pCO₂ of 15 and 30 mm Hg (~2,000 and 4,000 μatm pCO₂) increased hemolymph [HCO₃[−]] and decreased hemolymph [Cl[−]] compared to control conditions in the blue crab *Callinectes sapidus* (Cameron and Iwama, 1987). At a pCO₂ of 45 mm Hg (~6,000 μatm pCO₂) in the same species, hemolymph [HCO₃[−]] also increased, however, hemolymph [Cl[−]] increased (Cameron and Iwama, 1987), and 12,061 μatm pCO₂ increased hemolymph [Cl[−]] compared to control (373 μatm pCO₂) in the hermit crab *Pagurus bernhardus* (de la Haye et al., 2012). In palemonid shrimps exposure to 0.3 kPa CO₂ (~3,000 μatm pCO₂) decreased hemolymph [Cl[−]] in the high shore *Palaemonidae elegans*, but increased hemolymph [Cl[−]] in the subtidal *Palaemonidae serratus* (Dissanayake et al., 2010). Thus, changes in both [HCO₃[−]]_e and [Cl[−]]_e at elevated CO₂ support the role of compensatory acid–base regulation as a key part of the GABA hypothesis, although studies at CO₂ levels more relevant to future scenarios, such as ~1,000 μatm CO₂, would be valuable.

Intracellular pH (pH_i) is also regulated via ion exchange (Walsh and Milligan, 1989), including Na⁺ dependent Cl[−]/HCO₃[−] exchange (Roos and Boron, 1981), as seen in muscle fibers of the sipunculid worm (Pörtner et al., 2000), crayfish (Galler and Moser, 1986) and barnacle (Boron, 1977; Boron et al., 1981), as well as in crayfish neurons (Moody, 1981) and the squid giant axon (Russell and Boron, 1976; Boron and Russell, 1983). This suggests that [HCO₃[−]]_i increases and [Cl[−]]_i decreases in order to maintain pH_i. To date, neural [HCO₃[−]]_i has not been measured in a marine invertebrate exposed to elevated CO₂. However, in the sipunculid worm *Sipunculus nudus* muscle [HCO₃[−]]_i significantly increased over 96 h in 1% CO₂ (~10,000 μatm pCO₂) in air (Pörtner et al., 1998). A net efflux of Cl[−] is observed from the squid giant axon at a pH_i of 6.5 reached by intracellular acid administration (Boron and Russell, 1983), and in crayfish isolated abdominal ganglia the resting [Cl[−]]_i (35 mM) decreased by 3–5 mM when exposed to Ringer's solution equilibrated with 5% CO₂ (~10,000 μatm

pCO₂) (Moody, 1981). Therefore, changes in [HCO₃[−]]_i and [Cl[−]]_i occur in marine invertebrates exposed to extremely high levels of CO₂. Again, studies using CO₂ levels more relevant to future scenarios will be important to understand the theory underlying the GABA hypothesis.

It is unknown whether the above changes in [Cl[−]]_{i/e} and [HCO₃[−]]_{i/e} are sufficient to alter iGABA R functioning. For elevated CO₂ to disrupt GABA functioning, it is not simply altered [HCO₃[−]] and [Cl[−]], but a difference in ion gradients across neuronal membranes that will alter ion flow through the iGABA R, i.e., [Cl[−]]_i and [HCO₃[−]]_i within neurons must change by a different amount to [Cl[−]]_e and [HCO₃[−]]_e present in the fluid bathing the neurons (Nilsson and Lefevre, 2016). A useful way to determine whether the changes in [HCO₃[−]] and [Cl[−]] can alter ion flow through the iGABA R is by determining the GABA reversal potential (E_{GABA}) and comparing it to the neuronal resting membrane potential (see Tresguerres and Hamilton (2017) and Heuer et al. (2019) for a detailed explanation). This approach has previously been employed to demonstrate that altered [Cl[−]] and [HCO₃[−]] at elevated CO₂ could change GABA_A R function in the spiny damselfish *Acanthochromis polyacanthus* (Heuer et al., 2016).

Calculating E_{GABA} requires knowledge of the HCO₃[−]/Cl[−] permeability ratio of the iGABA R, and [HCO₃[−]]_i, [Cl[−]]_i, [HCO₃[−]]_e and [Cl[−]]_e at both control and elevated CO₂ conditions. The HCO₃[−]/Cl[−] permeability ratio is estimated to be between 0.2 and 0.6 in crayfish muscle fibers (Kaila and Voipio, 1987; Kaila et al., 1989; Farrant and Kaila, 2007), indicating that the iGABA R is more permeable to Cl[−] than it is to HCO₃[−]. However, this permeability ratio is unknown for other invertebrates. It is important to ensure ion concentration measurements are taken in the correct fluids. In most marine invertebrates no blood brain barrier is present (Cserr and Bundgaard, 1984) and measuring extracellular ion concentration in the hemolymph may be adequate. However, structural organization of nervous tissue may provide some regulation of the neuronal microenvironment (Cserr and Bundgaard, 1984). Cephalopods have a blood brain barrier separating the blood from the brain (Cserr and Bundgaard, 1984) and extracellular measurements should be made in the brain interstitial fluid that bathes the neurons. It is also vital intracellular measurements are done on neuronal cytoplasm, as pH_i regulatory mechanisms can differ between different cell types (Wheatly and Henry, 1992). Studies measuring these parameters and calculating E_{GABA} in a marine invertebrate exposed to elevated CO₂ will be useful to understand if altered [Cl[−]] and [HCO₃[−]] could change iGABA R function.

The Effects of Altered iGABA Receptor Functioning

Altered iGABA R functioning is likely to disrupt behaviors due to the role of iGABA R-mediated neurotransmission in invertebrate sensation and a range of behavioral outputs. In mollusks, GABA is present in the olfactory, chemoreceptive (Nezlin and Voronezhskaya, 1997; Ito et al., 2001; Kobayashi et al., 2008),

nociceptive (Kavaliers et al., 1999), visual and vestibular (Yamoah and Kuzirian, 1994) systems. iGABA R signaling mediates molluscan nociception (Kavaliers et al., 1999) and visual-vestibular interaction (Alkon et al., 1993), while mollusk photoreceptors respond to iGABA R signaling (Yamoah and Kuzirian, 1994). iGABA R-mediated signaling is important for feeding and prey-capture behaviors in mollusks (Arshavsky et al., 1993; Norekian and Satterlie, 1993; Romanova et al., 1996; Jing et al., 2003; Norekian and Malyshev, 2005) and a cnidarian (Pierobon et al., 1995, 2004; Concas et al., 1998), and GABAergic neurons are associated with effectors of feeding in a sea urchin (Bisgrove and Burke, 1987). iGABA R signaling mediates swimming of larval sea urchins (Kato et al., 2013), the righting response in a sea urchin (Shelley et al., 2019) and locomotion of a mollusk (Romanova et al., 1996). GABA mediates settling and metamorphosis, including associated behavioral changes in a range of mollusks (Morse et al., 1979; Morse et al., 1980; García-Lavandeira et al., 2005; Stewart et al., 2011; Biscocho et al., 2018), an echinoderm (Pearce and Scheibling, 1990) and a urochordate (Danqing et al., 2006). GABA is thought to mimic ligands from the environment (Morse et al., 1979) which may be detected by the iGABA R (Stewart et al., 2011) to initiate settlement and metamorphosis. Internal iGABA R mediated neurotransmission is also suggested to regulate metamorphosis (Biscocho et al., 2018). Thus altered iGABA R function will likely affect a variety of behaviors in a range of marine invertebrates.

Neural processes other than behavior may also be affected by altered iGABA R functioning. In vertebrates, GABA can act as a trophic factor (a molecule supporting cell survival) through the GABA_A R, influencing cell proliferation, migration and differentiation (Owens and Kriegstein, 2002; Sernagor et al., 2010). In a fish, the three-spined stickleback *Gasterosteus aculeatus*, genes involved in neurogenesis and neuroplasticity were upregulated after exposure to ~1,000 μatm pCO₂ compared to control (~330 μatm pCO₂) (Lai et al., 2017). Similarly, GABA induces cellular differentiation and proliferation in abalone larvae (Morse et al., 1980). Thus, elevated CO₂ may alter neurogenesis in marine invertebrates.

GABA can also have effects in non-neural tissue, playing an important role in the vertebrate immune system (Barragan et al., 2015; Wu et al., 2017). Invertebrate GABA also appears to play an immunomodulatory role. The iGABA R associated protein is implicated in the immune response of the abalone (Bai et al., 2012), GABA in the immune response of an oyster (Li et al., 2016a) and mussel (Nguyen et al., 2018), and a homolog of the glutamic acid decarboxylase (a rate limiting enzyme in GABA production) in immune regulation of an oyster (Li et al., 2016b). Thus, altered iGABA R functioning at elevated CO₂ may have widespread effects.

Cross-talk between neurotransmitter receptors may also result in widespread effects of altered iGABA R functioning. Different types of neurotransmitters can be co-released from the same nerve terminal, resulting in simultaneous activation of their specific receptors, co-localized at the same post-synaptic site. This simultaneous activation can result in cross-talk between the receptors, modulating signal transmission. For example,

negative cross-talk occurs when two different neurotransmitters simultaneously bind to their specific receptors, resulting in a current smaller than the sum of the currents of these two neurotransmitters acting separately (Li et al., 2003). Cross-talk involving GABA_A Rs is well documented in mammals. For example, GABA_A R activation suppresses the function of a dopamine receptor (de la Mora et al., 1997) and negative cross-talk occurs in both directions between GABA_A Rs and glycine Rs (Trombley et al., 1999; Li et al., 2003) and GABA_A Rs and the adenosine-triphosphate R P2X (Karanjia et al., 2006; Toulmé et al., 2007). Neurotransmitter cross-talk is yet to be studied in an invertebrate, but co-release of neurotransmitters occurs in the marine invertebrate nervous system, such as proctolin and GABA in crabs (Blitz et al., 1999), and dopamine and GABA in the sea hare *Aplysia californica* (Díaz-Ríos et al., 2002; Díaz-Ríos and Miller, 2005; Svensson et al., 2014). Furthermore, GABA has been found to post-synaptically increase dopamine currents in the sea hare *Aplysia californica* (Svensson et al., 2014). If cross-talk is present between invertebrate iGABA Rs and other neurotransmitter Rs, altered functioning of the iGABA R at elevated CO₂ may alter cross-talk mechanisms. Thus, altered iGABA R function at elevated CO₂ would not only alter the GABAergic pathway, but other pathways as well.

ALTERNATIVE MECHANISMS FOR BEHAVIORAL CHANGE AT ELEVATED CO₂

If elevated CO₂ does alter HCO₃⁻ and Cl⁻ gradients across neuronal membranes, it is likely that functioning of LGICs other than the iGABA/GABA_A R, that are also permeable to these ions, will also be disrupted (**Figure 2**). In vertebrates, altered glycine receptor functioning at elevated CO₂ has been suggested due to its similarity to the GABA_A R (Tresguerres and Hamilton, 2017). Many invertebrates lack glycine receptors (Tsang et al., 2006); however, invertebrates possess a larger variety of LGICs than vertebrates (Dent, 2010). Glutamate-gated chloride channels have been found in mollusks (Kehoe and Vulvius, 2000; Kehoe et al., 2009) and crustaceans (Marder and Paupardin-Tritsch, 1978), and are suggested to be the invertebrate equivalent of vertebrate glycine Rs (Vassilatis et al., 1997; Kehoe and Vulvius, 2000). There is also evidence for acetylcholine-gated chloride channels in mollusks (Kehoe, 1972). Moya et al. (2016) found a range of nervous system transcripts differentially expressed at elevated CO₂ in the pteropod *Heliconoides inflatus*, including genes encoding for the LGICs (and associated proteins) of cholinergic, GABAergic, glutamatergic and glycinergic-like synapses (Moya et al., 2016) (**Table 1**). Thus, altered [Cl⁻] can conceivably disrupt functioning of a range of LGICs (**Figure 2**). Furthermore, taxa specific differences in the presence of specific LGICs (Dent, 2010) may explain the variability of the effects of elevated CO₂ on marine invertebrate behavior.

Elevated CO₂ may not only disrupt neurotransmission, but also neuronal-glial and glial-glial signaling. Glia are non-neuronal cells present in the vertebrate and invertebrate nervous

system (Pentreath, 1989; Laming et al., 2000). Initially thought to be restricted to supporting neurons, the role of glia is now understood to include active participation in nervous system functioning, thus contributing to behavior (Laming et al., 2000; Jackson and Haydon, 2008). Like many other cell types, glial cells regulate pH_i via ion exchange, including HCO₃⁻/Cl⁻ exchange (Deitmer and Rose, 1996). GABA_A Rs are present in vertebrate glial cells (Butt and Jennings, 1994; Fraser et al., 1994). Less research has been carried out on invertebrate glia, with no research on the presence of iGABA Rs on marine invertebrate glial cells. However, leech glial cells reportedly respond to GABA [unpublished work reported in Deitmer and Rose (1996)]. If marine invertebrates are found to express iGABA Rs, elevated CO₂ may also affect information processing through glial cells. Furthermore, fluxes in H⁺ ions have been found to contribute to neuron-glia signaling (Deitmer and Rose, 1996; Laming et al., 2000), which may be disrupted by exposure to elevated CO₂ (and the resultant increase in H⁺ ions).

Changes in [Cl⁻] due to acid–base regulatory mechanisms at elevated CO₂ may affect LGIC-mediated neurotransmission through a different mechanism, as well as having alternative effects on the nervous system. Cl⁻ has a role as a signaling effector, with changes in [Cl⁻]_i affecting a range of processes including gene expression, protein activity and cell proliferation (Valdivieso and Santa-Coloma, 2019). Mammalian work has supported the role of Cl⁻ as a signaling effector in the nervous system, including [Cl⁻]_i regulation of GABA_A R expression (Succol et al., 2012) and growth of neuronal processes (Nakajima and Marunaka, 2016). The role of Cl⁻ as a signaling anion has also been observed in bacterial cells, suggesting a conserved function (Valdivieso and Santa-Coloma, 2019). Thus, invertebrate Cl⁻ is also likely to act as a signaling effector. If altered [Cl⁻]_i has similar effects in marine invertebrates, elevated CO₂ may impact nervous system functioning not only by altered iGABA R function but also changes in iGABA R expression, as well as altered growth of neuronal projections. Thus, altered [Cl⁻]_i at elevated CO₂ may have effects additional to altered LGIC function, having widespread consequences.

DIRECTIONS FOR FUTURE RESEARCH

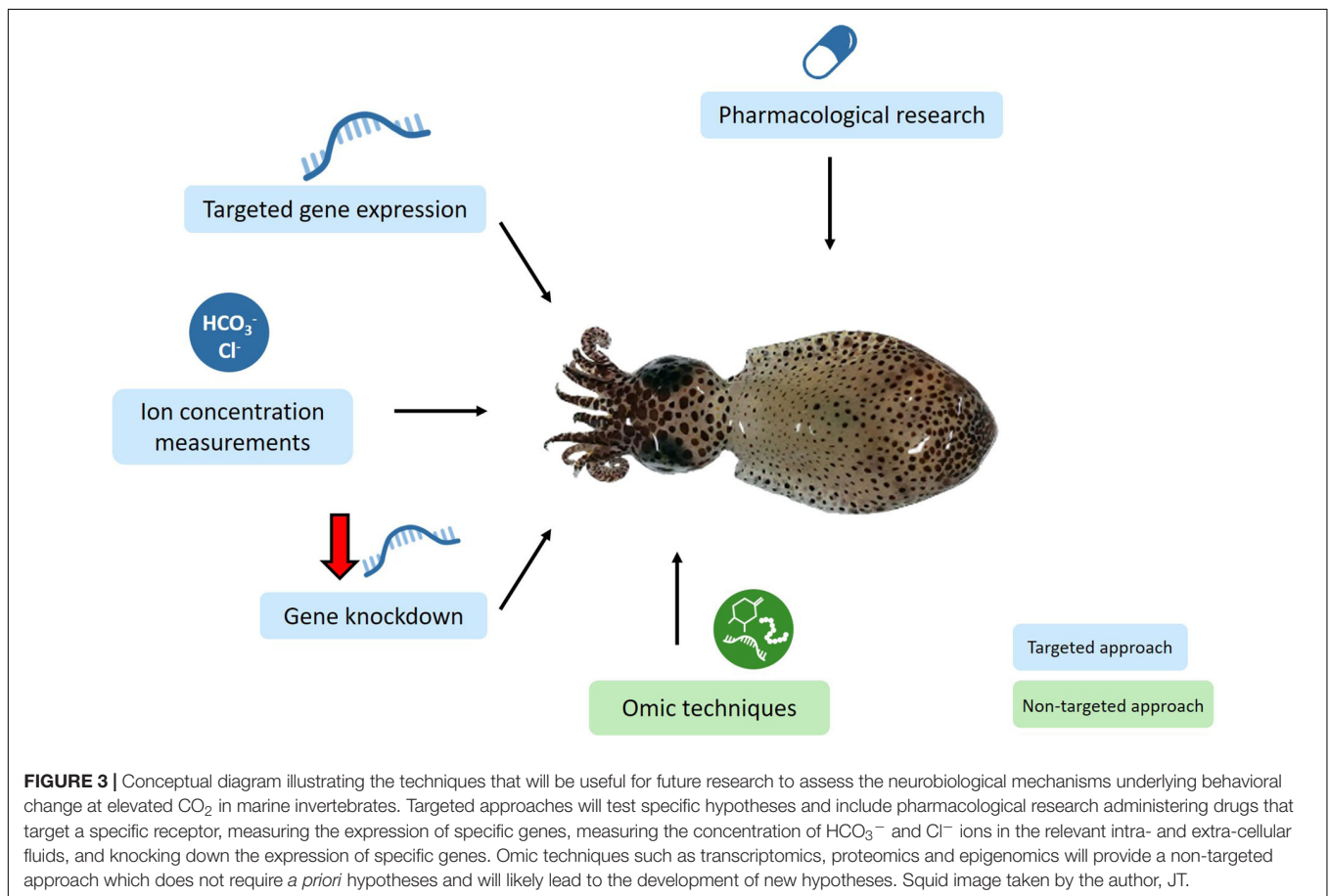
Mechanistic studies have used targeted approaches to assess the GABA hypotheses in marine invertebrates. These approaches will also be useful to assess potential alternative mechanisms by which elevated CO₂ may alter marine invertebrate behavior (Figure 3). The GABA hypothesis has been pharmacologically assessed by administering the GABA_A R antagonist gabazine to marine invertebrates (Watson et al., 2014; Clements et al., 2017). Likewise, the administration of pharmacological agents targeting different LGICs will be useful to assess whether altered functioning of alternative LGICs may underlie behavioral changes at elevated CO₂ conditions. Due to the diversity of invertebrates and the potential for off-target effects, it will be important to use a range of pharmacological agents,

particularly those shown to work in the invertebrate taxa being studied. Furthermore, pilot studies to determine the optimal drug concentration to use will be important. For example, Charpentier and Cohen (2016) tested three gabazine concentrations. Measurement of ion concentrations at control and elevated levels of CO₂ in conjunction with behavioral tests has been carried out in crabs (de la Haye et al., 2012; Charpentier and Cohen, 2016) and a mollusk (Zlatkin and Heuer, 2019). Future studies in other invertebrate taxa, with measurements made within the correct intra- and extra-cellular fluids, as well as determining the HCO₃⁻/Cl⁻ permeability ratio will be important to calculate E_{GABA} at elevated CO₂ to theoretically assess the GABA hypothesis. Real-time PCR, measuring the expression level of a specific gene, has been employed to assess the GABA hypothesis in a crab (Ren et al., 2018) and will also be useful to assess alternative mechanisms, e.g., measuring the expression of genes encoding for alternative LGIC subunits, and genes involved in neuronal growth and proliferation.

These targeted approaches, however, may leave potentially relevant information unexplored. Omic technologies, such as transcriptomics and proteomics, provide a non-targeted approach in which *a priori* hypotheses are not required (Figure 3). Thus, data from omic approaches could unveil patterns leading to the development of novel hypotheses. For example, transcriptomics and proteomics have already been employed in fish nervous tissue, providing support for the GABA hypothesis as well as new avenues to pursue (Schunter et al., 2016, 2018; Porteus et al., 2018; Williams et al., 2019).

In marine invertebrates exposed to control and elevated CO₂ levels, transcriptomic studies so far have analyzed the whole animal and have focused on pteropods (Koh et al., 2015; Maas et al., 2015; Moya et al., 2016; Thabet et al., 2017) and sea urchins (Todgham and Hofmann, 2008; Evans et al., 2013; Padilla-Gamiño et al., 2013; Clark et al., 2019). Likewise, proteomic studies have analyzed the whole body of tubeworm larvae (Mukherjee et al., 2013), oyster larvae (Dineshram et al., 2012; Dineshram et al., 2013), barnacle larvae (Wong et al., 2011), sea snail larvae (Di et al., 2019) and clam larvae (Timmins-Schiffman et al., 2019), and a metabolomics study analyzed the whole body of a crab (Trigg et al., 2019) exposed to control or elevated CO₂. To understand the neurobiological mechanisms underlying behavioral changes, rather than the general molecular response to elevated CO₂, it will be important to carry out omic techniques on the nervous tissue, as measurements at the whole body level may mask differential expression in the nervous system due to the heterogeneity and complexity of gene/protein expression. This is exemplified by Liu et al. (2019) who found region-specific regulation of neuropeptides in the nervous tissue of crabs *Callinectes sapidus* exposed to elevated CO₂.

Omic technologies will provide a powerful, holistic approach to explore neurobiological mechanisms underlying behavioral change, potentially leading to the development of novel hypotheses. However, omic approaches can only determine correlational, and not causative, links between expression and behavior. Gene knockdown, in which the expression of a specific



gene is reduced, will be a promising avenue for future research to determine a causative link between gene expression and behavioral change at elevated CO₂. Gene knockdown is yet to be used in elevated CO₂ behavioral research, however, gene knockdown of a heat shock protein assessed the stress tolerance of the white leg shrimp *Litopenaeus vannamei* to high CO₂ (Aishi et al., 2019).

CONCLUSION

There is large variability in the effects of elevated CO₂ on marine invertebrate behavior, which is likely due to the incredible diversity of marine invertebrates. Elevated CO₂ likely alters behavior via a range of mechanisms that disrupt the nervous system pathway producing behavior; from sensory input to behavioral output. These mechanisms are not necessarily mutually exclusive, and interactions between mechanisms may account for the diversity in responses. Many of these mechanisms are based on theory and lack solid experimental evidence. Mechanistic research addressing these gaps will be important, for example linking altered sensation at elevated CO₂ to behavioral change. Mechanistic fish research has focused on altered neurotransmission via disrupted GABA_A R functioning at elevated CO₂. The GABA hypothesis, as

well as altered functioning of other LGICs, likely applies to marine invertebrates. Further research into the ionic properties of the iGABA R and other LGICs, including whether they are also permeable to HCO₃⁻, and measuring intra- and extra-cellular ion levels in the relevant fluids at near-future CO₂ levels will be beneficial for advancing our understanding of this mechanism. The diversity of LGIC subtypes between invertebrate species, and even between nervous system regions, may explain the variability in behavioral responses. Investigating the presence of LGICs on invertebrate glial cells, other modes of neuronal-glial and glial-glial transmission, and the role of Cl⁻ as a signaling effector in invertebrates will help us understand the wider impact elevated CO₂ may have on nervous system functioning.

The interconnectivity of the nervous system, such as receptor cross-talk, suggests that even disruption of one component or pathway will have widespread effects. This will make understanding the neurobiological mechanisms underlying elevated CO₂-induced behavioral change extremely complex. Omics approaches will be useful in providing an untargeted, holistic approach to understand the response of the nervous system to elevated CO₂, provide support or opposition for proposed mechanisms, and likely provide new avenues to explore. Exploring the mechanisms underlying behavioral change at elevated CO₂ will help us to understand the variability in

behavioral responses to elevated CO₂ and predict which marine invertebrates are likely to be the most vulnerable to rising CO₂ levels.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the review. JT wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.00345/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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