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Short communication

First report of a putative involvement of the NMDA pathway in Pacific oyster (*Crassostrea gigas*) development: Effect of NMDA receptor ligands on oyster metamorphosis with implications for bivalve hatchery management

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ABSTRACT

In bivalve aquaculture, the use of neurotransmitters such as epinephrine (a catecholamine) to induce settlement and metamorphosis in hatcheries is a common practice in some species, but the actual neuroendocrine pathways involved in bivalve metamorphosis are not well understood. In vertebrates, the *N*-methyl-D-aspartate (NMDA) receptor, a ligand-binding, ion-channel transmembrane receptor, is known to regulate the production and release of catecholamine, but the role of NMDA receptors has not been explored in relation to bivalve metamorphosis. In this paper we investigate the effect of known NMDA receptor interacting compounds on metamorphosis in the Pacific oyster *Crassostrea gigas*. Our results demonstrate that ifenprodil and MK-801 - specific antagonists to the NMDA receptor - affect metamorphic processes in Pacific oysters, with up to 50% increase in spat production after 3 h exposure, thus indicating a relationship between the NMDA pathway activation and oyster metamorphosis. In addition, metamorphosis was induced by the application of chlorpromazine, a non-selective antagonist to the NMDA receptor. These findings indicate a putative regulatory function of the NMDA pathway in Pacific oyster metamorphosis, providing a potential new direction for the development of new and better inducers for metamorphosis in cultivated bivalve species, particularly in cases wherein catecholamines cannot be applied effectively for hatchery applications.

1. Introduction

Three decades ago, epinephrine (EPI), a catecholamine and neuromodulator, otherwise known as adrenaline, was shown to produce non-attached spat (juveniles) in various oyster species (Coon et al., 1985, 1986; Shpigel et al., 1989). Since that time, the commercial use of neurotransmitters and neurohormones such as EPI to induce metamorphosis has become a common practice in many bivalve hatcheries (Helm et al., 2004; Lucas and Southgate, 2012). The successful use of chemicals such as EPI indicates that the induction and regulation of larval metamorphosis involves neurological pathways, many of which display similarities to those of vertebrates. The effectiveness of metamorphic induction however, is highly variable among bivalve species. Current knowledge about neuroendocrine function in bivalves is primarily based on empirical research for hatchery applications demonstrating endogenous induction using neurochemicals (see review Joyce and Vogeler, 2018); alternately, our knowledge is based on vertebrate models, which may not adequately explain the interaction and regulation of neurological pathways, signal transmission, and gene expression processes in bivalves. In 1990, Bonar, Coon and colleagues (Bonar et al., 1990; Coon et al., 1990) first proposed a theory to explain settlement and metamorphosis induction in oyster species based on two distinct, serial-signalling pathways. The first pathway controls typical and reversible settlement behaviours in oysters (e.g. eye-spotted pediveliger larvae swimming with extended foot, sinking to the bottom of tanks, and actively crawling and searching for acceptable settlement substrate), followed by attachment (cementation) to the surface of hard substrates. Settlement behaviour and attachment are hypothesised to be regulated by a dopaminergic pathway, during which the neurotransmitter dopamine (DA) interacts with dopamine receptors (DR) to initiate settlement activity. When formulating this theory, also hypothesised that, during the settlement process, norepinephrine (NE) and EPI are released to activate a secondary, adrenergic pathway, thereby triggering metamorphosis through interaction with adrenergic

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receptors (Bonar et al., 1990; Coon et al., 1990). These interpretations that the non-reversible cementation of oysters to a settlement surface was part of the dopaminergic pathway are consistent with the findings that levodopa (L-DOPA), the precursor of DA, induces attachment of oyster larvae. Exposure to exogenous EPI, on the other hand, results in cultchless (single seed) spat by bypassing the attachment process (Bonar et al., 1990; Coon et al., 1985, 1986; Coon et al., 1990; Mesías-Gansbiller et al., 2013; Murthy et al., 1999; Shpigel et al., 1989; Teh et al., 2012). Although a compelling theory, Coon and Bonar's working hypothesis has never been confirmed. Since their original work, a wide range of neurotransmitters, including catecholamines such as EPI, NE and L-DOPA as well as serotonin, acetylcholine, γ-aminobutyric acid (GABA) and other neuroactive compounds, have been tested empirically to optimise applications in different bivalve species. However, the published results of such experiments reveal that the effects of such chemicals on settlement and metamorphosis induction are often species-dependent and can vary for unknown reasons, even in different trials involving the same species (see detailed review in Joyce and Vogeler (2018)). Such empirical evidence has never elucidated the actual pathways involved, and to date, there remains a lack of understanding about how the individual neuroactive compounds and their associated receptors regulate metamorphosis, how these pathways interact, and whether or not a universal pathway exists to regulate metamorphosis in all bivalve species.

Given the current deficit of knowledge regarding pathways involved in bivalve metamorphosis, approaching the issue from a new direction is potentially the key to furthering our current understanding of developmental processes. Considerable research on *N*-methyl-D-aspartate (NMDA) receptors exists for vertebrate models, but has never previously been explored in bivalves. The NMDA pathway has implications in vertebrate catecholamine release and production, and we believe that it could provide the missing link required to explain the interaction that Coon and Bonar proposed between dopaminergic and adrenergic pathways involved in oyster metamorphosis. The NMDA receptor is a ligand-gated, ion-channel receptor that allows positively-charged ions (Ca^{2+}, Na^{+}, K^{+}) to flow through the cell membrane of post-synaptic sites, which can lead to intracellular signalling through second-messenger and downstream gene regulation. Opening the ion channel of the NMDA receptor requires not only activation by an agonist (e.g. NMDA or glutamate) and a co-agonist (e.g. glycine or D-serine), but also the depolarisation of the cell membrane to dislodge the Mg^{2+} ion from the ion pore (Blanke and Van Dongen, 2009). In vertebrates, the NMDA pathway is often involved in regulating the release of catecholamines (DA, EPI and NE), such as occurs in chromaffin cells of rats (Gonzalez et al., 1998), or in the rat *medulla oblongata*, where L-glutamate increases the NE concentrations, while MK-801, a highly selective NMDA receptor channel blocker, functions as an inhibitor (Fink et al., 1989). Similarly, phencyclidine, a non-competitive NMDA receptor antagonist, has been shown to increase extracellular levels of DA in mice by binding specifically to one of the NMDA receptor subunits (Hagino et al., 2010). NMDA perfusion of rat *striata nigra* results in the release of DA, glutamate, and GABA that can be reversed by the NMDA antagonist MK-801 (Morari et al., 1996). D-aspartate (D-asp), a precursor of NMDA, has also been shown to inhibit DA release through interaction with NMDA receptors in the hypothalamus of rats (Pampillo et al., 2002). Research on specific NMDA pathways in bivalves is still largely unexplored, but a recent highly relevant study by Uda et al. (2016) has shown that D-aspartate racemase is highly expressed in oyster pediveliger larvae and spat, and that oysters are able to convert L-aspartate (L-asp) to D-asp. The concentrations of L-asp, D-asp, NMDA, and NMLA (the L-form of NMDA) and D-asp racemase have also been measured in adult tissue of various bivalve species (Okuma et al., 1998; Shibata et al., 2001; Shibata et al., 2003; Tarui et al., 2003), thus confirming the presence of these amino acids and their derivatives in bivalves. The presence of functional NMDA receptors has also been reported in gastropods (Ha et al., 2006), and given their close evolutionary relationship with bivalves, it is not an improbable hypothesis that NMDA receptors are also found in bivalves.

To test the hypothesis of NMDA receptor involvement in bivalve metamorphosis, we chose the Pacific oyster (*Crassostrea gigas)*, the most commonly-cultured oyster worldwide, as a model species based on existing knowledge regarding behavioural and morphological changes during settlement and metamorphosis, as well as the predictable success of EPI induction of non-attached ("single-seed") spat. The well-developed genomic data for this species also provides an opportunity for us to complete further additional molecular analysis of downstream gene regulation. Based on the data reported in this paper, we provide preliminary evidence that the NMDA pathway is involved in regulating metamorphosis of *C. gigas*. Such speculation is based on the fact that exposure to several NMDA receptor antagonists resulted in the induction of metamorphosis for Pacific oyster larvae. Although we provide herein only a report of preliminary evidence, such a theory has not been previously explored and we believe is likely also relevant in other bivalve species, an area that we are also currently testing, as it is of considerable interest to the aquaculture industry, largely because this knowledge can be exploited to identify neuroactive compounds for species in which no effective inducer such as EPI has been identified.

2. Methods

2.1. Oysters and chemical reagents

The Pacific oyster (*C. gigas*) larvae used in this study were derived from four family lines reared at the South Australian Research and Development Institute in Adelaide, South Australia. The seawater was filtered to 1 μm prior to usage and maintained at 24.5 ± 0.5 °C, with salinity and pH of 34.5 ± 0.5 ppt and 7.8 ± 0.1, respectively. The larvae were fed with an algal mixture of *Tisochrysis lutea* (T-Iso), *Pavlova lutheri*, *Chaetoceros calcitrans* and *Chaetoceros muelleri*. Ifenprodil (+)-tartrate salt, chlorpromazine hydrochloride, (+)-MK 801 maleate, and *N*-Methyl-D-aspartic acid (NMDA) were purchased from Alomone Labs, and (±)-epinephrine hydrochloride, glutamic acid (glutamate) and γ-Aminobutyric acid (GABA) were obtained from Sigma-Aldrich. Stock solutions (10^{-2} M) for each chemical treatment were prepared by dissolving compound in sterile MilliQ water.

2.2. Metamorphosis assay

In this study, pediveliger larvae were considered to be competent for metamorphosis when they were observed crawling on the bottom of the tank and possessed visible eyespots (18–20 days post fertilisation (dpf), > 236 μm size). Approximately 80–110 competent pediveliger larvae were placed in each glass shell vial (outside diameter x height: 29 × 94 mm) with 1.5 ml filtered seawater (FSW). The vials were chilled at 4 °C for 15 min, rewarmed to room temperature for another 15 min, and then fed with the algal mixture to ensure maximal larval activity prior to chemical exposure. The larvae were treated with specific concentrations (10^{-4} M to 10^{-8} M) of neurotransmitters prepared as solutions dissolved in filtered seawater (10× concentrated) and dosed to larvae within a total volume of 2.5 ml FSW: single exposures with EPI for 1 h; ifenprodil, chlorpromazine, MK-801, NMDA, GABA, or glutamate for 3 h, and co-exposures with MK-801 & glutamate, ifenprodil & NMDA, and MK-801 & GABA for 3 h. Controls were treated for 3 h with the same amount of sterile MilliQ water used in stock solutions. After treatments, chemicals were removed by pipetting, and 10 ml FSW was added to each vial. The larvae were kept in vials for 72 h with the addition of 10 ml FSW every 24 h and fed daily with the algal mixture during the experimental period. Larvae were assessed at 24 h, 48 h, and 72 h under an inverted microscope. Early spat, as well as live and dead larvae, were counted; individuals with adult shells and gill bars were considered spat; whereas, the larvae that had no distinct organ structure or no cilia movement on key organs such as velum, gut, and foot