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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

Susanne Vogeler[a], Penny Miller-Ezzy[b], Xiaoxu Li[b], Gary H. Wikfors[c], Alyssa Joyce[a,*]

[a] Department of Marine Science, University of Gothenburg, Gothenburg, Sweden
[b] South Australian Research and Development Institute, Primary Industries and Regions (PIRSA) Aquatic Sciences Centre, West Beach, SA, Australia
[c] NOAA Fisheries Service (NMFS) Northeast Fisheries Science Center, Milford, CT 06460, USA

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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

* Corresponding author.
E-mail address: alyssa.joyce@gu.se (A. Joyce).

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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

were counted as dead. Dead spat were not observed in any treatments, and therefore are not represented in the data. For each experiment, there were four biological replicates. Experiments using single chemical exposures, as opposed to co-exposures, were repeated with competent larvae of different ages post fertilisation but from the same biological families (same spawning event).

2.3. Data analysis

Percentage of larvae completing metamorphosis and larval mortality were calculated based on the total number of oysters in each vial, and statistical tests were run using R software (The R-Project). The non-parametric Kruskal–Wallis H-test was applied to analyse the effects between different treatments, followed by pairwise comparison using a generalisation of the Dunnett's T3 method to trimmed means (Wilcox, 2016). The probability level of 0.05 was chosen as being significant in all statistical tests.

3. Results

Ifenprodil, MK-801, and chlorpromazine induced metamorphosis in competent Pacific oyster larvae (Fig. 1). In general, significant responses were observed at the optimal concentrations of all three chemicals 24 h post-exposure, whereas, a further increase in cultchless spat production ($p < 0.05$) until 72 h post treatment occurred only in the larvae (20 dpf) exposed to 10^{-5} M ifenprodil or 10^{-6} M chlorpromazine. Larvae of different ages (18 to 20 dpf) showed similar responses to the same chemical treatments (Fig. 1). In the ifenprodil treatments, the most effective concentration was 10^{-6} M, resulting in 41 ± 5.2% −44 ± 2.2% metamorphosis at 72 h, which is significantly higher than induction obtained at other concentrations. The spat induced at this concentration exhibited healthy adult shell growth and normal organ development, including distinct gill bars. Ifenprodil concentrations of 10^{-4} M were unfavourable for inducing metamorphosis and may have been subsequently lethal, as at 24 h larvae were immobile on the bottom of the vial, and detached velums were observed swimming in the vials. Metamorphosis was also achieved with MK-801 at the high concentrations tested10^{-3}M and 10^{-4} M (41 ± 4.7% −51 ± 4.7%), although the spat induced at 10^{-3} M seemed less active and less vital than at 10^{-4} M, based on observed organ and gill bar movements. Free-swimming, detached velums were also observed in these treatments. No effect on metamorphosis was observed at other MK-801 concentrations. Chlorpromazine treatments displayed a pattern similar to ifenprodil, with 10^{-6} M being the most effective concentration and producing the highest metamorphosis percentages of 56 ± 2.2% −59 ± 2.4% (Fig. 1).

In this study, 10^{-4} M epinephrine served as a positive control and resulted in 82 ± 3.0% −92 ± 2.2% metamorphosis across the experiments at 72 h post treatment. Spat displayed normal adult shell growth and gill bars as well as the loss of larval organs such as velum, foot, and eyespot. The high rate of spat production after EPI treatment verified that the larvae were competent for metamorphosis. Without the application of an exogenous inducer in the controls, however, only limited larval metamorphosis occurred naturally (< 6 ± 2.5%; Fig. 1).

The NMDA receptor agonists glutamate and NMDA, or the inhibitory neurotransmitter GABA, did not affect spat production at most of the concentrations when co-applied with MK-801 or ifenprodil (Fig. 2), except for one treatment with MK-801 and 10^{-4} M glutamate wherein metamorphosis was reduced by approximately 18% in comparison with the MK-801 treatment. Single treatments with glutamate, NMDA, or GABA resulted in metamorphosis similar to controls at all concentrations (10^{-4} M-10^{-6} M) tested (data not shown).

4. Discussion

The NMDA receptor antagonists tested, ifenprodil and MK-801, successfully induced metamorphosis in the Pacific oyster with low toxicity at the most effective concentrations. Such results clearly

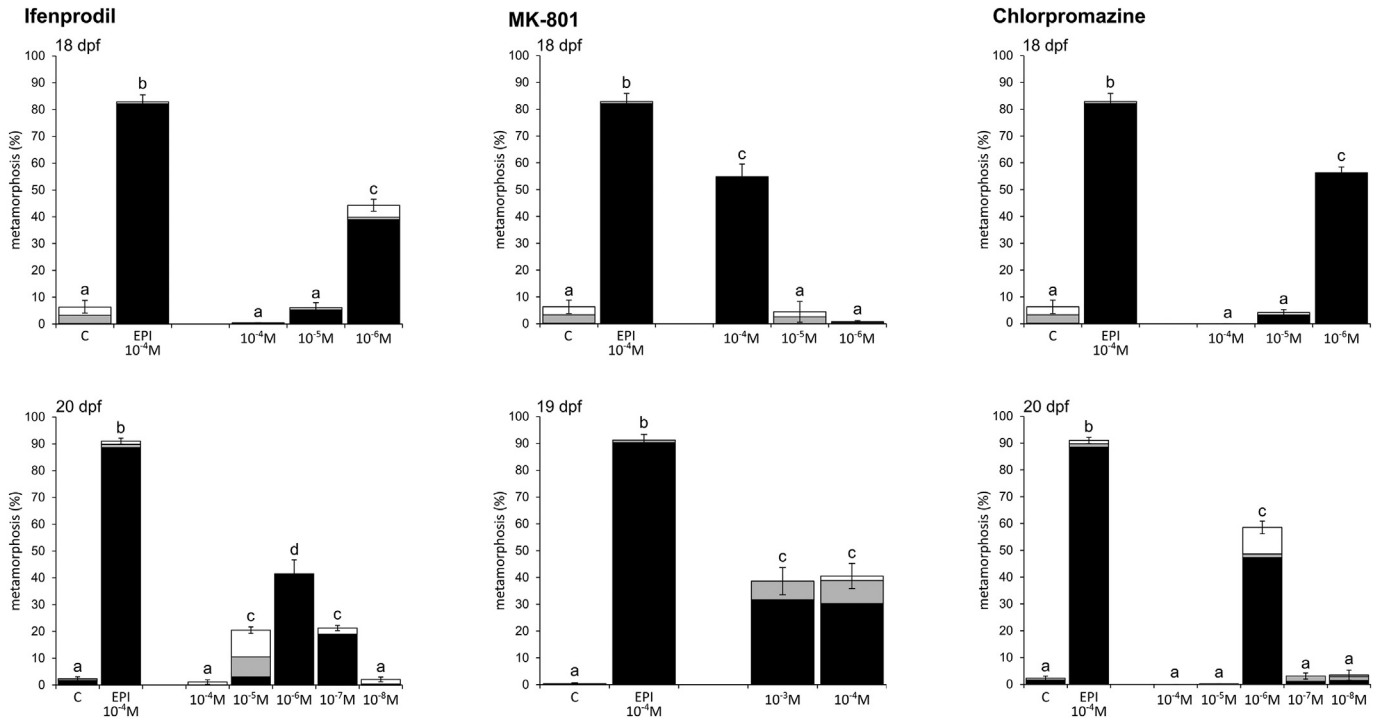

Fig. 1. Metamorphosis percentages of Pacific oyster (*Crassostrea gigas*) larvae after 3 h exposure to different concentrations of ifenprodil, MK-801 or chlorpromazine, with 1 h exposure to epinephrine (EPI, 10^{-4} M) and 3 h to non-chemical as positive and standard (C) controls, respectively. Data were collected at 24 h (black), 48 h (grey) and 72 h (white) post treatments. Error bars represent standard error of 72 h data. Different lower case letters above bars represent significant differences ($p < 0.05$). dpf: days post fertilisation.

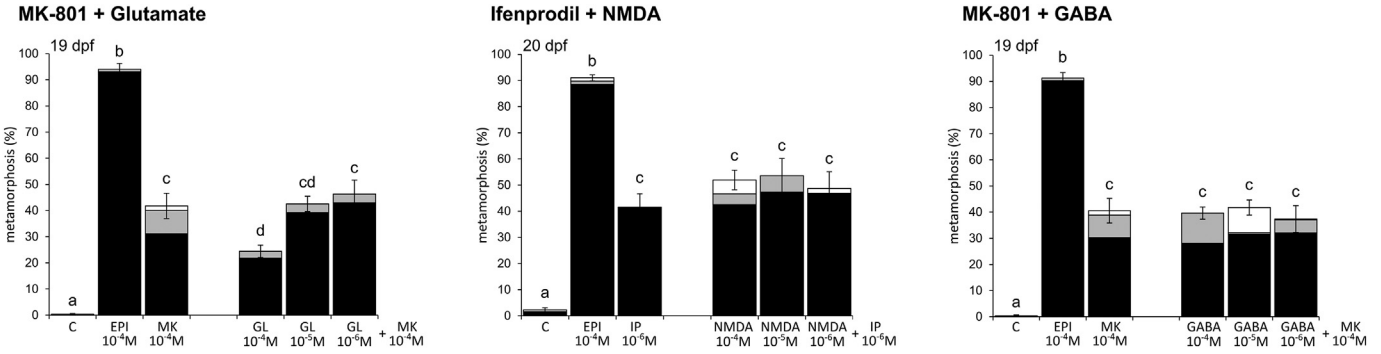

Fig. 2. Metamorphosis percentage of Pacific oyster (*Crassostrea gigas*) larvae after 3 h exposure to different concentrations of glutamate (GL), NMDA and GABA in co-exposure with MK-801 (MK; 10^{-4} M) or ifenprodil (IP; 10^{-6} M), with 3 h exposure of single treatment of MK-801and ifenprodil, and 1 h exposure to epinephrine (EPI, 10^{-4} M) and 3 h to non-chemical as positive and standard (C) controls, respectively. Data were collected at 24 h (black), 48 h (grey) and 72 h (white) post treatments. Error bars represent standard error of 72 h data. Different lower case letters above bars represent significant differences ($p < 0.05$). dpf: days post fertilisation.

indicate some form of regulatory function of NMDA receptors during oyster metamorphosis. Ifenprodil is a selective, non-competitive antagonist to the vertebrate NMDA receptor complex built of two heteromers with the subunits NR1 and NR2B (Williams, 2001). NMDA receptors are formed by heteromeric tetramers usually composed of two principal NR1 subunits and two subunits of various NR2 or NR3 types. MK-801, on the other hand, is a selective NMDA receptor channel blocker, which binds to the channel pore after the Mg^{2+} ion has been dislodged from the pore by depolarisation of the cell membrane (Huettner and Bean, 1988). Both compounds have, to our knowledge, never been tested before in bivalves, and the results presented suggest that the NMDA receptor is an important pathway that should be considered in more detail when attempting to explain neuroendocrine regulation of bivalve metamorphosis.

Contrary to expectations, however, the assumption that NMDA receptor agonists inhibit metamorphosis (as opposed to antagonists) in the Pacific oyster could not be confirmed unambiguously. Although glutamate, a natural agonist to NMDA receptors, inhibited metamorphosis to some extent at the highest concentration in the presence of MK-801 (metamorphosis inducer), glutamate also may interact with other iontrophic glutamate receptors, such as kainate and AMPA receptors, which both regulate synaptic transmission in vertebrates (Dingledine et al., 1999). The reduction in metamorphosis by glutamate could therefore be attributable to interaction of the agonist with other glutamate receptors. Furthermore, the chemical NMDA did not inhibit metamorphosis when co-applied with ifenprodil, although it would have been expected to act as an NMDA receptor selective partial-agonist, binding to the glutamate site. It is possible that NMDA cannot activate the receptor and thus induce the ion exchange through the channel pore in the presence of the non-competitive antagonist ifenprodil. Similarly, the agonistic effect of glutamate might be blocked by MK-801. Co-exposures are complicated, given that it is unknown exactly how long, or in what order to expose the larvae to suspected inducers and inhibitors. Negative effects observed were inconclusive, therefore, and it cannot be ruled out that compounds might not have been taken up or transported to their site of action during co-exposures because of competitive exclusion or other interactions at binding sites. A potential stress response to glutamate, which could inhibit metamorphosis, can also not be excluded.

In vertebrates, the GABAergic pathway involving GABA, a chief inhibitory neurotransmitter, is tied closely to the NMDA pathway. In particular, DA release can be regulated by NMDA receptors through GABA release, which either reduces or increases the extracellular levels of DA, depending on the neuronal region in the brain (Balla et al., 2009). Furthermore, GABA can have a role in the activation of NMDA receptors by polarisation, which leads to dislodgment of Mg^{2+} (Ben-Ari, 2014; Wang and Kriegstein, 2008), or GABAergic receptors can block an NMDA receptor-induced response (Paladini et al., 1999). In the present study, GABA neither increased nor decreased metamorphosis in Pacific oyster larvae in a single exposure or when co-exposed with an NMDA receptor antagonist such as MK-801. These results are consistent with previous research on *C. gigas* larvae which did not find an inducing effect on metamorphosis with single GABA exposure (Beiras and Widdows, 1995; Coon et al., 1985). Nevertheless, GABA shows a broad variety of effects on metamorphosis in various bivalve species, from broad, inducing effects to no effect whatsoever, even for species within the same genus (see (Joyce and Vogeler, 2018)). Accordingly, our preliminary results are not broad enough in scope – including multiple species – to determine whether or not GABA effects are linked to the NMDA pathway in all bivalves.

The positive effect of catecholamines, such as DA, NE and EPI, on metamorphosis in a wide range of bivalve species summarised in (Joyce and Vogeler, 2018) and previous theories suggested by Bonar, Coon, and colleagues (Bonar et al., 1990; Coon et al., 1990) point toward strong involvement of catecholamines in the induction and regulation of settlement, including attachment to surfaces, but also primarily in inducing – and altering - metamorphic processes. Although responses to exogenous induction may differ between species, it would fair to assume that many of the pathways are evolutionarily conserved among bivalve species. Consequently, the ability to fully explain induction pathways and to identify genes involved has the potential to develop dependable hatchery techniques for “setting” new species. Indeed, further work using molecular tools has the potential to finally elucidate relevant pathways and methods of activation, and can thus eliminate much of the trial-and-error testing that has occurred over the past thirty years since Coon and Bonar initially published their findings.

The potential that the NMDA pathway could be involved in regulating catecholamine release is an area worthy of further investigation. In vertebrates, the effects of NMDA receptors on catecholamine release vary between tissue types. For example, NMDA increases the release of DA in the *striata nigra* of rats (Morari et al., 1996). Similarly, the NMDA receptor, activated by D-asp, inhibits DA release in the hypothalamus, but D-asp has no effect on DA release in the posterior pituitary gland in rats (Pampillo et al., 2002). In the hippocampus of rats, in contrast, NMDA receptor activation results in a decrease in NE (Dazzi et al., 2011), but NE concentration increases after NMDA and glutamate exposure in mediobasal hypothalamus cells (Navarro et al., 1995). Such studies provide a compelling theory that NMDA pathways are linked to catecholamine release, but extrapolating such findings to bivalves based on vertebrate models is questionable given that receptors and mechanisms of action may not be conserved.

Nevertheless, although the mechanism of action of the NMDA pathway in bivalve metamorphosis is currently unknown, there are several other pathways which could be related to NMDA receptors. For

example, NMDA receptors and dopamine receptors (DRs) are known to interact with each other in vertebrates, through DR potentiation of NMDA responses, interactions through second messengers, or physical interactions between receptors (for detailed review see (Cepeda et al., 2009; Cepeda and Levine, 2012)). NMDA receptor activation can increase the recruitment of DRs belonging to the D_1-like family (Pei et al., 2004; Scott et al., 2002; Scott et al., 2006). D_1-DRs are hypothesised to be the primary active DRs of the dopaminergic pathways during bivalve metamorphosis (He et al., 2017; Pechenik et al., 2002). DRs conversely can increase NR1, NR2A, and NR2B proteins in synaptosomal membrane fractions (Dunah and Standaert, 2001) and can enhance or inhibit the NMDA receptor response (Cepeda et al., 1993; Cepeda and Levine, 1998; Flores-Hernandez et al., 2002; Seamans and Yang, 2004). NMDA receptors and DRs can also interact physically with various outcomes of inhibition or potentiation of NMDA receptor currents (Lee et al., 2002; Liu et al., 2006). Although the direct and indirect interactions between NMDA receptors and DRs are not understood fully in vertebrates, it still shows a potential for co-existing regulatory pathways which might be regulating bivalve metamorphosis.

The NMDA pathway can also be linked to nitric oxide (NO) production via a Ca^{2+}/calmodulin pathway, with NMDA receptors regulating the intracellular Ca^{2+} concentration. Ca^{2+} functions as a second messenger and increases the production of nitric oxide synthase (NOS), which catalyses L-arginine and NADPH to L-citrulline, NO, and NADP (Bredt, 2003; Schmidt et al., 1992). The Ca^{2+}/calmodulin pathway regulating NO production has been studied in gastropod species (Bodnárová et al., 2005; Tagliazucchi and Conte, 2005), and activation of an NMDA receptor increases NO production in adult *Lymnaea stagnalis*, which was blocked by MK-801 (Dyakonova and Dyakonova, 2010). In various gastropod species, it has been shown that NO inhibits metamorphosis, and NO concentration decreases in larvae closer to metamorphosis (Froggett and Leise, 1999; Pechenik et al., 2007; Pechenik et al., 2002), with *nNOS* gene expression also decreasing during the first 24 h of metamorphosis in the marine snail *Ilyanassa obsoleta* (Hens et al., 2006). It has been hypothesised that NO prevents programmed cell death (apoptosis), thereby inhibiting metamorphic changes (Leise et al., 2004). Hence, inhibition of NMDA receptors in competent bivalve larvae could lead to a reduction in NO, which ultimately promotes metamorphosis.

In this study, the effect of chlorpromazine on larval metamorphosis was also tested, with inducing effects (up to 60% increase in metamorphosis). Chlorpromazine, although primarily known for its antagonistic properties to DRs (York, 1972), is recognised as a “dirty drug” because of its wide interaction with various receptors such as serotonin, histamine, adrenergic (Peroutka and Snyder, 1980) and muscarine acetylcholine (Snyder et al., 1974) receptors, among others. Chlorpromazine also can function as an antagonist for NMDA receptors by binding to the zinc binding site and blocking the ion channel (Barygin et al., 2017; Lidsky et al., 1997; Mokrushin, 2016; Żarnowska and Mozrzymas, 2001); yet the effect of chlorpromazine on NMDA receptors appears to be dose-dependent, with receptor activation at low concentrations and inhibition at high concentrations (Lidsky et al., 1997). Thus, the observed inducing effect of chlorpromazine might be partly a consequence of an interaction with the NMDA pathway, but also related to interactions with other neuroactive receptors. Notably, the present results appear contradictory to previous research on bivalve metamorphosis, wherein chlorpromazine has been shown to inhibit metamorphosis success in *C. gigas* (Coon and Bonar, 1987) and in various mussel species (He et al., 2017; Yang et al., 2014; Yang et al., 2011). In these studies, however, chlorpromazine was tested in co-exposures with EPI and not as a single treatment. It is possible that chlorpromazine, with its broad spectrum of activity, inhibits the positive effect of the co-exposure compound, for instance when co-exposed with an inducer such as EPI, by inhibiting the induction pathway (i.e. adrenergic receptors). Conversely, chlorpromazine has a positive effect on metamorphosis as a single treatment (i.e., acting as an NMDA receptor antagonist) given pathways are often sequential and require different temporal activation.

5. Conclusion

Many neuroendocrine compounds, including neurotransmitter and hormonal compounds, which regulate reproduction in humans and other vertebrates, are also found in bivalves, but the role of the NMDA receptor had not to our knowledge been previously explored in relation to bivalve metamorphosis. The exposure of competent larvae to two NMDA receptor antagonists, ifenprodil and MK-801 resulted in a significant induction of metamorphosis, thus supporting our theory that the NMDA pathway is intrinsically implicated in metamorphosis for this species. Chlorpromazine, a non-selective antagonist to a wide range of neuro-receptors including NMDA receptors, also demonstrated an inducing effect. In contrast, metamorphosis was not inhibited by co-exposure of NMDA receptor antagonists and agonists, such as NMDA and glutamate, nor by GABA (an inhibitory neurotransmitter potentially, but not necessarily directly linked to NMDA receptors).

The findings of the present study open up an exciting opportunity for new research related to neuroendocrine regulation of bivalve larval development. With full genomic data available for the Pacific oyster, and addition data available for other commercially important bivalve species such as the Yesso scallop (*Patinopecten yessoensis*) (Wang et al., 2017) and pearl oyster (*Pinctada fucata martensii*) (Du et al., 2017), in depth genetic analyses have become more feasible and will allow potential further research on NMDA pathways including receptor analysis and transcription, downstream gene regulations and potential interactions between NMDA pathways, catecholamine release and other neuroendocrine pathways (e.g. dopaminergic or adrenergic pathways) for bivalve species. Receptor binding assays may also provide essential information whether oyster NMDA receptors are able to interact with the putative receptor agonists and antagonists used in this study.

Productivity of the shellfish aquaculture industry is dependent on effective rearing of larvae and hence successful production of juveniles. In hatcheries, metamorphosis is often viewed as a bottleneck, given that it is a life stage marked by variable competence and often higher mortality - not all individuals within a population are able to complete the transition, or do so in a timely way. Indeed, metamorphosis is heavily dependent on water quality, seasonality, and overall larval health. Induction of metamorphosis by EPI in oyster species has been adopted by hatcheries to improve synchronicity of metamorphosis, but also to increase survival rates. EPI, however, does not work, or is only partially effective, in many other commercially-important species. Much of the empirical knowledge about exogenous neurotransmitter effectiveness for hatchery applications is based on trial and error, thus making it time consuming when developing new species for aquaculture. As such, a fundamental understanding of actual pathways regulating bivalve metamorphosis is commercially relevant, and our findings, as the first report of a putative involvement of NMDA pathway in regulating oyster metamorphosis, are important toward optimising hatchery productivity in this key area.

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