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Cloning and characterisation of NMDA receptors in the Pacific oyster, *Crassostrea gigas* (Thunberg, 1793) in relation to metamorphosis and catecholamine synthesis

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ABSTRACT

Bivalve metamorphosis is a developmental transition from a free-living larva to a benthic juvenile (spat), regulated by a complex interaction of neurotransmitters and neurohormones such as L-DOPA and epinephrine (catecholamine). We recently suggested an *N*-Methyl-D-aspartate (NMDA) receptor pathway as an additional and previously unknown regulator of bivalve metamorphosis. To explore this theory further, we successfully induced metamorphosis in the Pacific oyster, *Crassostrea gigas*, by exposing competent larvae to L-DOPA, epinephrine, MK-801 and ifenprodil. Subsequently, we cloned three NMDA receptor subunits *CgNR1*, *CgNR2A* and *CgNR2B*, with sequence analysis suggesting successful assembly of functional NMDA receptor complexes and binding to natural occurring agonists and the channel blocker MK-801. NMDA receptor subunits are expressed in competent larvae, during metamorphosis and in spat, but this expression is neither self-regulated nor regulated by catecholamines. *In-situ* hybridisation of *CgNR1* in competent larvae identified NMDA receptor presence in the apical organ/cerebral ganglia area with a potential sensory function, and in the nervous network of the foot indicating an additional putative muscle regulatory function. Furthermore, phylogenetic analyses identified molluscan-specific gene expansions of key enzymes involved in catecholamine biosynthesis. However, exposure to MK-801 did not alter the expression of selected key enzymes, suggesting that NMDA receptors do not regulate the biosynthesis of catecholamines via gene expression.

1. Introduction

Metamorphosis in bivalve molluscs includes the loss of larval organs such as the velum, the development of adult gills, the production of adult shell and in species such as the Pacific oyster *Crassostrea gigas*, resorption of the larval foot (Kennedy et al., 1996). Neuroactive pathways appear to be the key regulators of this essential life event with various neurotransmitters and neuro-hormones inducing metamorphosis in many oyster, clam, mussel and scallop species (Joyce and Vogeler, 2018). We recently successfully induced metamorphosis in two oyster and two clam species by exposing competent larvae to two selective vertebrate *N*-Methyl-D-aspartate (NMDA) receptor antagonists, the channel blocker MK-801 and a non-competitive negative allosteric NMDA receptor modulator ifenprodil, which was the first evidence that an NMDA receptor pathway is also involved in regulating bivalve metamorphosis (Vogeler et al., 2018, 2019). Ligand-gated ion channel NMDA receptors are members of the glutamate receptor family, allowing the flow of positively charged ions such as Ca^{2+}, Na^{+}, K^{+} through synaptic cell membranes (for review see (Traynelis et al., 2010)). NMDA receptors are hetero-tetramer ion channels formed by two heterodimers, each consisting of a subunit GluN1 (NR1) combined with an additional subunit, either GluN2 (NR2) or GluN3 (NR3). Opening of NMDA receptor channel requires binding of a co-agonist such as glycine or serine, and an agonist, typically glutamate, to the ligand binding domains in the subunits NR1 and NR2 or NR3, respectively. Unique to NMDA receptors is a voltage dependent block inside the channel pore by extracellular Mg^{2+} ions, which for successful ion gating requires dislodgment, usually caused by depolarisation of the cell membrane through rapid activation of nearby

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AMPA receptors, another type of glutamate receptor located in the cell membrane. The resulting inflow of Ca^{2+} after opening of the NMDA receptor channel leads to an increase in intracellular calcium concentration used for subsequent intracellular signalling and downstream responses, such as activation of enzymes, gene regulation or other cell-specific responses. NMDA receptors have not yet been studied in any bivalve species, but cloning of NMDA receptor subunits in several invertebrates (Ultsch et al., 1993; Xia et al., 2005; Zannat et al., 2006; Brockie and Maricq, 2003; Grey et al., 2009; Huang et al., 2015; Ha et al., 2006), together with additional characterisation of NMDA receptors through immunohistochemistry, electrophysiological or pharmacological analysis (Dale and Kandel, 1993; Di Cosmo et al., 2004; Moroz et al., 1993; Pfeiffer-Linn and Glantz, 1991; Cattaert and Birman, 2001; Gallus et al., 2010; Xia et al., 2009; Hepp et al., 2013; Rosenegger and Lukowiak, 2010; Miyashita et al., 2012; Lima et al., 2003; Si et al., 2004; Burrell and Sahley, 2004), provides evidence for conserved and functional NMDA receptor homologs in invertebrates.

In relation to the NMDA receptor involved in regulation of metamorphosis, information on the underlying pathways in invertebrates is limited. In insects, NMDA receptors are engaged in the production of juvenile hormones, which regulate insect metamorphosis and reproduction (Chiang et al., 2002; Geister et al., 2008), suggesting that NMDA receptors are potentially responsible for the production of metamorphosis-regulating hormones. In 1990, Coon and Bonar (Bonar et al., 1990; Coon et al., 1990) proposed that in oysters and other bivalves, catecholamines such as epinephrine (EPI) and norepinephrine (NE) as well as dopamine (DA), including its precursor L-DOPA, act as neurotransmitters and neuro-hormones, and regulate metamorphosis by potentially interacting with a cascade of dopaminergic and adrenergic pathways. Dopamine potentially binds to DA receptors, initiating the production or release of NE and/or EPI, which in turn can bind to adrenergic receptors inducing morphological changes required for metamorphosis. Although this theory has not been proven unambiguously for bivalve species, additional evidence supported the theory of catecholamine involvement in bivalve metamorphosis. Upregulation of DA receptors and activation of adrenergic receptors were observed in late larval stages of different oyster species (Yang et al., 2012; Qin et al., 2012). Catecholamines are synthesised in catecholamine secreting cells and derived from the amino acid tyrosine (Fig. 1), which is either directly digested from the diet or derived from phenylalanine. The synthesis of DA, NE and EPI requires enzymes such as tyrosine hydroxylase (TH), dopa decarboxylase (DDC), dopamine-β-hydroxylase (DβH) and phenylethanolamine-N-methyltransferase (PNMT). Tyrosine is also involved in the biosynthesis of other neurotransmitters such as the monoamines, tyramine and octopamine (OA), two primary neurotransmitters in many invertebrate species, but with increasing importance in vertebrate models (Roeder, 2005; Farooqui and Akhlaq, 2016). Tyramine and OA biosynthesis is closely related to catecholamine synthesis with two key enzymes, tyrosine decarboxylase (TDC) and tyrosine β-hydroxylase (TβH). Catecholamine secreting cells have been identified in different bivalve larval stages and seem to reach their maximum presence in competent pediveliger larvae in all key tissues, such as vellum, foot, mouth, apical tuft and pedal, visceral and apical ganglia (Croll et al., 1997; Voronezhskaya et al., 2008). Two catecholamine synthesising enzyme homologs, DDC and DβH, have been identified in the scallop *Chlamys (Azumapecten) farreri,* wherein they emerged ventrolaterally and in the pedal ganglion in late veliger larvae (Zhou et al., 2011a, 2012). Expressions for these enzymes increased from veliger to pediveliger larvae, suggesting an importance of these enzymes during late larval development/competence for metamorphosis. A homolog to the *DBH* gene that expresses DβH has also been identified in the razor clam *Sinonovacula constricta* and metamorphosis was significantly inhibited by DβH inhibitor (Li et al., 2020). Actual catecholamine quantifications in bivalve larvae are limited, but differences in the presence of catecholamines in larvae of different bivalve species suggests variable catecholamine requirements for larval metamorphosis. In *C. gigas* and *Pecten maximus,* NE concentrations stay low throughout larval stages and in early spat, but significantly increase in oysters just before metamorphosis (Coon and Bonar, 1986; Cann-Moisan et al., 2002). Dopamine is abundant in much higher concentrations than NE, and rapidly increases in scallops just prior to metamorphosis, with a decrease to relatively low levels two weeks after settlement. Examples of potential cross-talk between NMDA receptors and catecholamine pathways can be found in vertebrate models. NMDA receptors are involved in the release of catecholamines in various regions of vertebrate brains (Fink et al., 1989; Tsuda, 2004; Hagino et al., 2010; Morari et al., 1996; Pampillo et al., 2002; Adams et al., 2002), with responses being tissue specific (Pampillo et al., 2002). NMDA receptors are also known to directly interact with the dopaminergic pathway through physical interaction with dopamine receptors, through second messenger signalling or potentiation of receptor responses (Cepeda et al., 2009). Furthermore, adrenergic receptors have been shown to alter NMDA receptor currents (Liu et al., 2006; O'Dell et al., 2015).

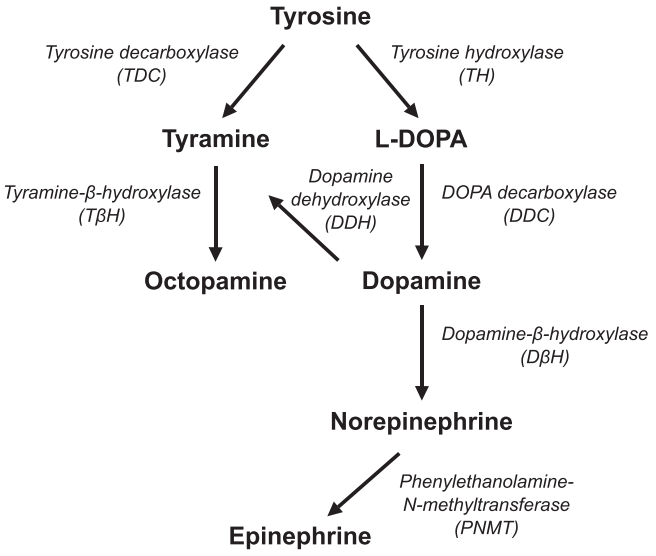

Fig. 1. Biosynthesis of catecholamines (dopamine, norepinephrine and epinephrine) and monoamines (tyramine and octopamine) from tyrosine including catalysing enzymes.

To explore whether NMDA receptors are involved in bivalve metamorphosis, we cloned three NMDA receptor subunits for the first time in the Pacific oyster, *C. gigas*. Metamorphosis in this bivalve species was successfully induced by NMDA receptor inhibitors, MK-801 and ifenprodil as previously reported (Vogeler et al., 2018, 2019). Additional localisation of NMDA receptor subunit NR1 in Pacific oyster larvae, as well as expression patterns of all identified subunits across different developmental stages, were utilised to predict potential functions of NMDA receptors in oyster larval development. A potential link between catecholamine pathways and NMDA receptors was assessed by NMDA receptor expression after exposure to L-DOPA, EPI, MK-801 and ifenprodil in competent larvae and spat. Furthermore, the gene expression of enzymes involved in catecholamine synthesis, such as homologs to vertebrate TH, DDC and DβH, were investigated in larvae and spat after exposure to the aforementioned compounds. Given that NMDA receptor pathways have been largely overlooked in invertebrate developmental biology research, these findings provide fundamental information about the role of previously unexplored NMDA receptors and its potential link with the catecholamine pathways during Pacific oyster metamorphosis.

2. Methods

2.1. Cloning NMDA receptor subunits

Oligonucleotide primers for sequencing and cloning of the full length of NMDA receptor subunits CgNR1, CgNR2A and CgNR2B were designed with Primer Blast at NCBI (Ye et al., 2012) based on genomic data for *C. gigas* (updated for Annotation Release 101 (Zhang et al., 2012)) for each gene (GenBank: XM_020069512 (*CgNR1*), XM_011421368 (*CgNR2A*), XM_011456200 (*CgNR2B*). Total RNA was extracted from an approximate equal mixture of Pacific oyster, *Crassostrea gigas*, larvae (9 days post fertilisation (dpf) & 15 dpf) and spat (~30 mg) using the RNeasy Mini Kit (Qiagen). Larvae and spat were obtained from a previous rearing event described in Vogeler et al. (2018). DNA was digested using TURBO DNase (Ambion) and the RNA was reverse transcribed following the manufacture's protocol of the using SuperScript III Reverse Transcriptase (Invitrogen) as per the manufacturer's instructions. Amplicons of full NMDA receptor sequence were obtained by RT-PCR using the Platinum Green Hot Start PCR system (Invitrogen) under the following conditions: 94 °C for 2 min, thirty-five cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 4 min, and a final extension at 72 °C for 5 min. The products were, purified using a QIAquick PCR Purification kit (Qiagen) and cloned into pCR 2.1-TOPO TA vector (Invitrogen). Vectors were purified using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) and were subsequently sequenced by AGRF (Adelaide, Australia) using full length and internal primers (Supplementary File 1).

The obtained coding sequence (CDS) for all subunits (*CgNR1* GenBank: MT419891, *CgNR2A* GenBank: MT419892, *CgNR2B* GenBank: MT419893) were aligned to their associated genomic DNA sequence available for identification of intron/exon structure.

2.2. Protein analysis and phylogeny

The deduced amino acid sequences for each NMDA receptor subunit were annotated using the Conserved Domain Database at NCBI (Marchler-Bauer et al., 2017) and SMART (Letunic and Bork, 2017). Phylogeny of the NMDA receptor subunits were inferred using Maximum Likelihood, Bayesian Inference and distance Neighbor-joining methods. Full sequences of all NMDA receptor subunits were aligned with NMDA receptor subunit homologs of species across various phyla (Supplementary File 2) using the default parameters in MUSCLE implemented in Seaview v4.6.2 (Gouy et al., 2010). All gaps were removed. Models for protein evolution were selected by Protest v2.4 (Abascal et al., 2005). Maximum Likelihood phylogenetic tree was constructed using PHYML v3.1 (Guindon et al., 2010) with an LG matrix plus optimised invariable sites (+I) and gamma distributed rate heterogeneity among sites (+G) and 1000 bootstrap replicates. The Bayesian Inference tree was calculated using MrBayes v3.2.2 (Ronquist and Huelsenbeck, 2003) with the LG + I + G model. Four randomly started simultaneous Markov chains were running for 5 million generations with chains sampled every 100 generations and a burn-in of 5000 trees. The distance Neighbor-joining analysis was conducted in Seaview v4.6.2 using default characteristics and branch support was measured by bootstrap analysis with 1000 replicates. The trees were visualised in FigTree v1.4.3 (FigTree. FigTree V1.4.3 2, 2017).

Additional phylogenetic analyses of protein sequences for potential catecholamine synthesising genes (full TH and DDC; partial for DβH protein sequences) were conducted as described above using only Maximum Likelihood method (Supplementary File 3).

2.3. Animal husbandry and metamorphosis assay

Pacific oysters, *C. gigas,* were cultured at the South Australian Research and Development Institute in Adelaide, South Australia. Larvae for *C. gigas* were derived from nine family lines, reared at 24 °C and fed with an algal mixture of *Tisochrysis lutea*, *Pavlova lutheri*, *Chaetoceros calcitrans* and *Chaetoceros muelleri*. The seawater was filtered to 1 μm prior to usage and maintained at 24.5 ± 0.5 °C, with salinity and pH of 36.5 ± 0.5 ppt and 8.2 ± 0.1, respectively.

The metamorphosis assay followed the protocol as previously described in (Vogeler et al., 2018, 2019). Approximately 250–320 competent pediveliger larvae (18dpf, >236 μm) were placed per glass shell vial and fed with the algal mixture. Larvae were considered to be competent for metamorphosis when shrinking of velum, active crawling on the bottom and visible eye-spot were observed. The larvae were treated with the following neuro-active compounds at final concentrations in a total volume of 2.5 ml filtered seawater (FSW): epinephrine hydrochloride (EPI; obtained from Sigma-Aldrich) at 10^{-4} M, Levodopa (L-DOPA, obtained Santa Cruz Biotechnology) at 10^{-5} M based on Bonar et al. (1990), and (+)-MK 801 maleate (MK-801) at 10^{-4} M and Ifenprodil (+)-tartrate salt (ifenprodil) at 10^{-6} M based on Vogeler et al., 2018, 2019, both compounds obtained from Selleckchem. Memantine hydrochloride, a selective NMDA receptor channel blocker, from MERCK was used at final concentrations 10^{-4} M – 10^{-6} M. Stock solutions at 10^{-2} M were prepared by dissolving compounds in autoclaved sterile Milli-Q dH2O and working solutions (10x concentrate of final concentration) were prepared with FSW prior to experiments. After 1 h exposure for EPI and 3 h exposure for remining treatments, chemicals were removed by pipetting and 10 ml FSW including algae mixture was added to each vial. A control group of larvae were treated for 3 h with the same amount of sterile MilliQ water used in stock solutions before exchanging the water with 10 ml FSW including algae mixture. The larvae were either sampled for further analysis or kept in the vials for 24 h and then assessed under an inverted microscope. Early spat, as well as live and dead individuals were counted, with spat counted as being post-metamorphosis when adult shells and gill bars were clearly visible. Across all experiments, mortality was low (2.4 ± 0.6%) and did not differ significantly between treatments. Per sample point, including spat collection 24 h post treatment, nine biological replicates each with 200 larvae, were performed, of which three vials were combined to one sample. Three replicates for each treatment and the control group were assessed after 24 h for metamorphosis success.

2.4. Gene expression analysis

For the gene expression study, *C. gigas* larvae of several developmental stages and after exposure to metamorphosis inducers were sampled and preserved in RNAlater (Sigma-Aldrich). The following developmental stages were sampled: unfertilised eggs, 2–4 cell stage (1 h post fertilisation (hpf), blastula (3 hpf), gastrula (6 hpf), trochophore larvae (12 hpf), D-shelled larvae (24 hpf), early veliger (5 dpf), mid-veliger (9 dpf), late veliger (14 dpf), pediveliger larvae (16 dpf) and competent pediveliger larvae (17 dpf & 18 dpf). Larvae exposed to EPI, L-DOPA, MK-801 and ifenprodil were sampled 4 h post exposure start (hpe) and 6 hpe. Spat of each treatment were sampled 24 hpe and spat without exposure to an external inducer were taken daily from rearing tanks until a sufficient amount for further analysis were sampled. Furthermore, tissue sample from different adult organs (derived from three males and three females, originating from a hatchery Isle of Mull Oysters Ltd in Scotland) were also taken: heart, mantle margin, mid mantle, mantle ventral of adductor muscle, digestive tract, adductor muscle (white and translucent tissues), gills, labial pulps, haemolymph, male and female gonads.

The gene expressions of the NMDA receptor subunit CgNR1, CgNR2A and CgNR2B were analysed for all samples. Gene expressions of enzyme homologs potentially involved in catecholamine synthesis such as TH (*CgTH*; GenBank: XM_011442697), two DDCs (*CgDDC1* and *CgDDC2*; GenBank: XM_011444936 and XM_011419081) and DβH (*CgDBH*; GenBank: XM_011431297), were assessed in spat, veliger and pediveliger larvae as well as treated larvae. The elongation factor-1 α, ribosomal protein S18, ribosomal protein L7 were chosen as reference genes as previously described (Vogeler et al., 2016, 2017).

Primers were designed with an amplicon size ranging from 155 bp to

205 bp (Supplementary File 1). Specificity of primer pairs for each gene was verified by sequencing. The sequencing was conducted by Eurofins MWG Operon (Germany). Total RNA was extracted in triplicates from all developmental staged (~ 40 mg larvae), exposed larvae (~ 600 larvae), spat (100–600 larvae depending on metamorphosis success) and adult tissues using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich) following the manufacturer's protocol. Genomic DNA was removed with RQ1 RNase-Free DNase (Promega) and one μg of total RNA was transcribed to cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using oligo $(dT)_{18}$ primers following the manufacturer's protocol. Quantitative PCR was performed using the Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific) and the reactions were run on a 384 well plate PCR thermal cycler Light Cycler 480 Instrument II (Roche). Three biological replicates for each sample per gene were run in duplicates on a single 384-well plate with 0.5 μl cDNA per 10 μl reaction. The final $MgCl_2$, annealing temperature and primer concentrations were adjusted for primer optimisation (Supplementary File 1). qPCR conditions were as followed: 95 °C for 10 min, 45 cycles of 95 °C for 15 s, 60–62 °C (primer pair dependent) for 30 s and 72 °C for 30 s. A melt curve was run at the end at 65–95 °C with a temperature transition rate of 0.05 °C. A non-template control and a cDNA dilution series for each primer pair were analysed in parallel to assess primer efficiency (standard curve) and exclude contamination. Primer efficiency and relative gene expression were based on a modified comparative Ct model as described in (Vogeler et al., 2016).

2.5. In-situ hybridisation (ISH)

Pediveliger larvae fixed in the PaxGene Tissue system (PreAnalytix) were washed twice in 0.01 M phosphate buffered saline (1xPBS) for 10 min, then decalcified with 10% EDTA in 1xPBS for 5 h and washed two times with 1xPBS for 15 min, and fixed in 100% ethanol overnight at −20 °C. Approximately 50 larvae were equally dispersed in 0.5 ml molten 3% agar and the hardened agar was then sliced into 5 mm thick discs. Larvae-agar discs were dehydrated using an ethanol dilution series, washed twice with isopropanol and xylene and embedded in paraffin wax. Larvae samples were sectioned to 5 μm thickness with a microtome and mounted on Plus + Frost positively-charged microscope glass slides (Solmedia).

Riboprobes synthesis and ISH protocol were adapted from Kvamme et al. (2004). Specific riboprobes were designed from the cDNA sequences of *CgNR1* using a 323 bp fragment as template. Antisense riboprobes were produced by *CgNR1* specific forward primer (5′-TGCAACTGGGACAAGAACGA-3′) and a reverse primer with a T7 antisense extension (5′- TAATACGACTCACTATAGGGTGCAACTGGG ACAAGAACGA-3′) by PCR using the MyTaq PCR Mix (Bioline) at 95 °C for 1 min, 35 cycles of 95 °C for 15 s, 64 °C for 15 s and 72 °C for 30 s with a final extension of 2 min at 72 °C. For the sense riboprobes, used as non-specific binding control in the ISH assay, the *CgNR1* specific forward primer with a T7 sense extension and the reverse primer were used during the PCR. Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen) and Digoxigenin (DIG) labelling of the riboprobes was performed by the incorporation of modified nucleotides UTP-DIG from the DIG RNA labelling kit (Roche) using an RNA T7 polymerase. Microslides with sectioned larvae were washed twice in xylene for 10 min, rehydrated in an ethanol series (95%, 70% and 50% ethanol for 1 min) and washed in 0.3 M saline-sodium citrate buffer (2xSSC) for 1 min. Sections were treated with 10 μg/ml proteinase K in 100 mM Tris/50 mM EDTA buffer at 37 °C for 5 min, post-fixed with ice-cold 4% paraformaldehyde in 1xPBS for 5 min and washed twice in 1xPBS for 2 min. The sections were then covered with a pre-hybridisation mix (5xSSC, 50% formamide in nuclease free water) for 10 min at 37 °C before being incubated in the hybridisation mix (10% dextran sulphate, 5xSSC, 50% formamide, 250 μg/ml yeast tRNA, 500 μg/ml herring sperm DNA, 5x Denhardts Solution, 1% blocking solution) with either antisense or sense riboprobes (0.3–0.8 ng/μl) or no riboprobes (negative control) overnight for 16 h at 60 °C in the dark. Samples were washed twice with 2xSSC for 30 min, 50% formamide in 2xSSC solution for 30 min at 65 °C, and washed again twice with 2xSSC for 10 min at 37 °C. The DIG Wash and Blocking set as well as the DIG Nucleic Acid Detection Kit (Roche) were used to for additional washing and immunological detection. Sections were washed in 1x washing buffer for 5 min, 1x blocking solution for 30 min, incubated with anti-DIG antibody coupled to alkaline phosphatase (150 mU/ml) in 1x blocking solution for 2 h and washed twice with 1× washing buffer. For detection of alkaline phosphatase activity, sections were washed with 1× detection buffer for 5 min and incubated with SIGMAFAST Fast Red TR/Napthol AS-MX (Sigma-Aldrich) substrate for 30 min. The reaction was stopped by a brief rinse with nuclease-free water and slides were mounted using Vectamount (Vector Laboratories). Sections were examined and photographed using an epi-fluorescent Arcturus XT Laser Capture Microdissection system (ThermoFisher Scientific) built on a Nikon Eclipse Ti-E microscope with blue B-2A, green G-2A and triple-band DAPI-FITC-Texas Red excitation filters and a QImaging MicroPublisher Color RTV-5.0 CCD Camera (QImaging Corp.) for imaging. Tissue specific staining in the non-specific binding controls (sense riboprobes) or negative controls (no riboprobes) were not observed (Supplementary File 4).

An additional conventional hematoxylin and eosin H&E staining of a section (sequential to the sections used in ISH) was performed for anatomy identification.

2.6. Data analysis

Percentages of larvae completing metamorphosis were calculated based on the total number of oysters in each vial. 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 gene expression patterns of all genes were analysed with a one-way ANOVA followed by multiple pairwise comparisons using a Tukey's Honestly Significant Difference Test. All statistical tests were run using the R software v3.5.1 (R Core Team. R, 2017) and the probability level of 0.05 was chosen as being significant.

3. Results

3.1. Cloning, protein analysis and phylogeny of NMDA receptor subunits

Three NMDA receptor subunit homologs were identified by a tBLASTn search of the *C. gigas* genome using protein sequences of known homologs to NR1, NR2 and NR3 of different vertebrate and invertebrate species (Supplementary File 2). All three subunits were successfully cloned and subsequent phylogenetic analysis (Fig. 2) of the deduced amino acid sequences identified one homolog, CgNR1, to the NMDA receptor subunit 1 and two isotypes of the NMDA receptor subunit 2, hereafter named CgNR2A and CgNR2B. The NR1 subunit homolog *CgNR1* coding sequence is 2883 bp long and comparison to the genome sequences showed that the CDS comprises of 17 exons encoding for a 960 aa long protein. The protein sequence identity of CgNR1 to its two closest homologs, AcNR1 and LsNR1, is 53%, but lower to the human homolog HsNR1 with only 44%. The CDS of the two NR2 homologs, *CgNR2A* and *CgNR2B*, are 3159 bp and 3237 bp long, respectively, and both CDS are generated by 16 exons. The protein sequences of CgNR2A (1052 aa) and CgNR2B (1078 aa) show a sequence identity of 42%, and phylogenetic analysis as well as locus identification in the genome verifies these homologs as different isotypes of the subunit NR2. However, CgNR2A and CgNR2B are not direct orthologs to the NR2A and NR2B subunit types, respectively, as seen for vertebrate species, and indicate a unique gene duplication event of this subunit. Sequence identities of CgNR2A and CgNR2B to the different human isotypes HsNR2A-HsNR2D range between 17% and 20%. A homolog to the NMDA receptor subunit 3 has not been cloned, however, a new assembly of the *C. gigas* oyster genome was

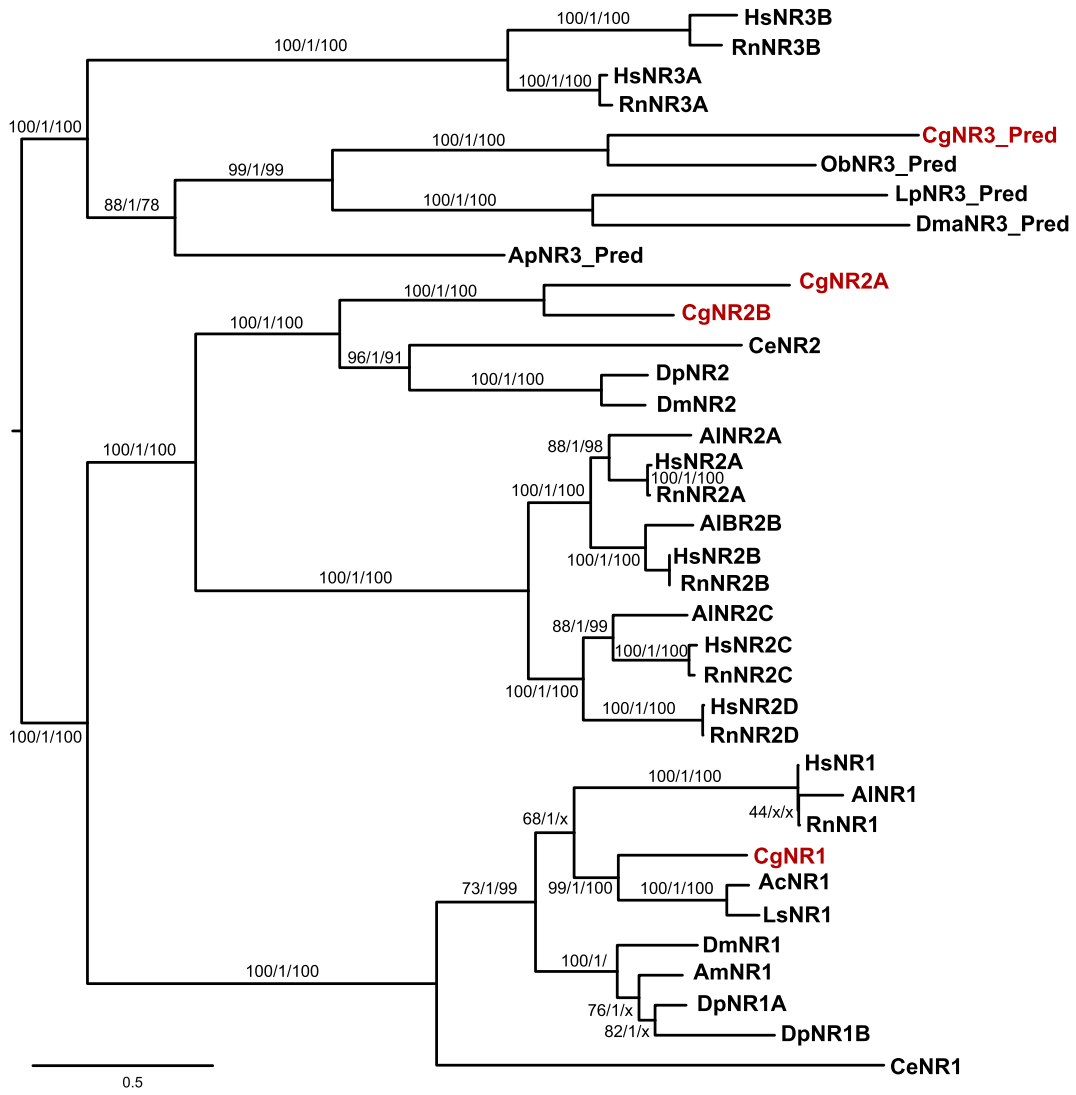

Fig. 2. Phylogenetic relationship of NMDA receptor subunits in *Crassostrea gigas* among various phyla. The alignment was constructed using the full amino acid sequences with all gaps removed and the phylogenetic relationship was constructed by Maximum Likelihood (ML), Bayesian Inference (BI) and Neighbor-joining (NJ) analyses. Maximum Likelihood bootstrap support values (percentage of 1000 boot straps), Bayesian posterior probabilities and NJ support values (percentage of 1000 boot straps) are provided above the nodes separated by slash. X indicates the node obtained from the BI and NJ analyses, which were different from that obtained by ML method. Ac: *Aplysia californica*; Am: *Apis mellifera carnica*; Al: *Apteronotus leptorhynchus*; Ap: *Acanthaster planci*; Ce: *Caenorhabditis elegans*; Cg: *Crassostrea gigas*; Dm: *Drosophila melanogaster*; Dma: *Daphnia magna*; Dp: *Diploptera punctata*; Hs: *Homo sapiens*; Lp: *Limulus polyphemus;* Ls: *Lymnaea stagnalis*; Op: *Octopus bimaculoides;* Rn: *Rattus norvegicus*. Pred: predicted protein.

updated in August 2019 (ASM29789v2 (Zhang et al., 2012)) after all experiments were completed. This new assembly release suggests a potential NR3 homolog in *C. gigas* (XP_011413969) supported by our phylogenetic analysis using additional predicted invertebrate NR3 homologs (Greer et al., 2017; Ramos-Vicente et al., 2018). While no isoforms have been identified in this study for any of the cloned receptor subunits, searches of the NCBI database revealed potential isoforms: a potential isoform for CgNR2A with an eleven amino acid insertion in a non-conserved region between exon 1 and exon 2 (XP_011419668) and a putative isoform for CgNR2B (XP_019930458), which shows a 36 aa sequence difference caused by a shift in exon 10.

All three identified proteins contain the general domain structures and transmembrane helixes (TM) for glutamate receptor subunits including an N-terminal domain (NTD), a bilobed ligand binding domain (LBD) formed by two domains S1 and S2, a pore forming transmembrane domain formed by four TMs with TM1, TM3 and TM4 and a pore forming segment TM2 that is not crossing the membrane, and an intracellular C-terminal domain (Figs. 3 and 4). The pore regions are highly conserved, in particular for CgNR1, and all subunits contain the SYTANLAAF motif in TM3, crucial for ion channel gating (Jones et al., 2002; Sobolevsky et al., 2007; Chang and Kuo, 2008). CgNR1 contains a potential RQR retention signal sequences (RSS) for endoplasmic reticulum retentions, as well as a potential PDZ binding motif for PDZ protein-protein interaction as previously described for other NR1 homologs (Xia et al., 2005; Zannat et al., 2006; Ha et al., 2006; Standley et al., 2000; Brockie et al., 2001). Putative dimerization sites for heterodimer and tetramer formation between CgNR1 and CgNR2 subunits were also identified by conserved domain search with additional conserved residues in CgNR1 and CgNR2A/CgNR2B based on a in depth analysis of the crystal structure of a rat heterodimer NR1/NR2A complex (Furukawa et al., 2005).

CgNR1 and the two CgNR2 subunits also contain most of the conserved residues for a predicted binding to co-agonists and agonists such as glycine to CgNR1 and glutamate and NMDA to CgNR2A and CgNR2B. An aspartic acid residue in CgNR2A (D766) and CgNR2B (D783) is also present, which indicates the specificity of these subunits to NMDA and classifies them not only as glutamate receptors but as NMDA

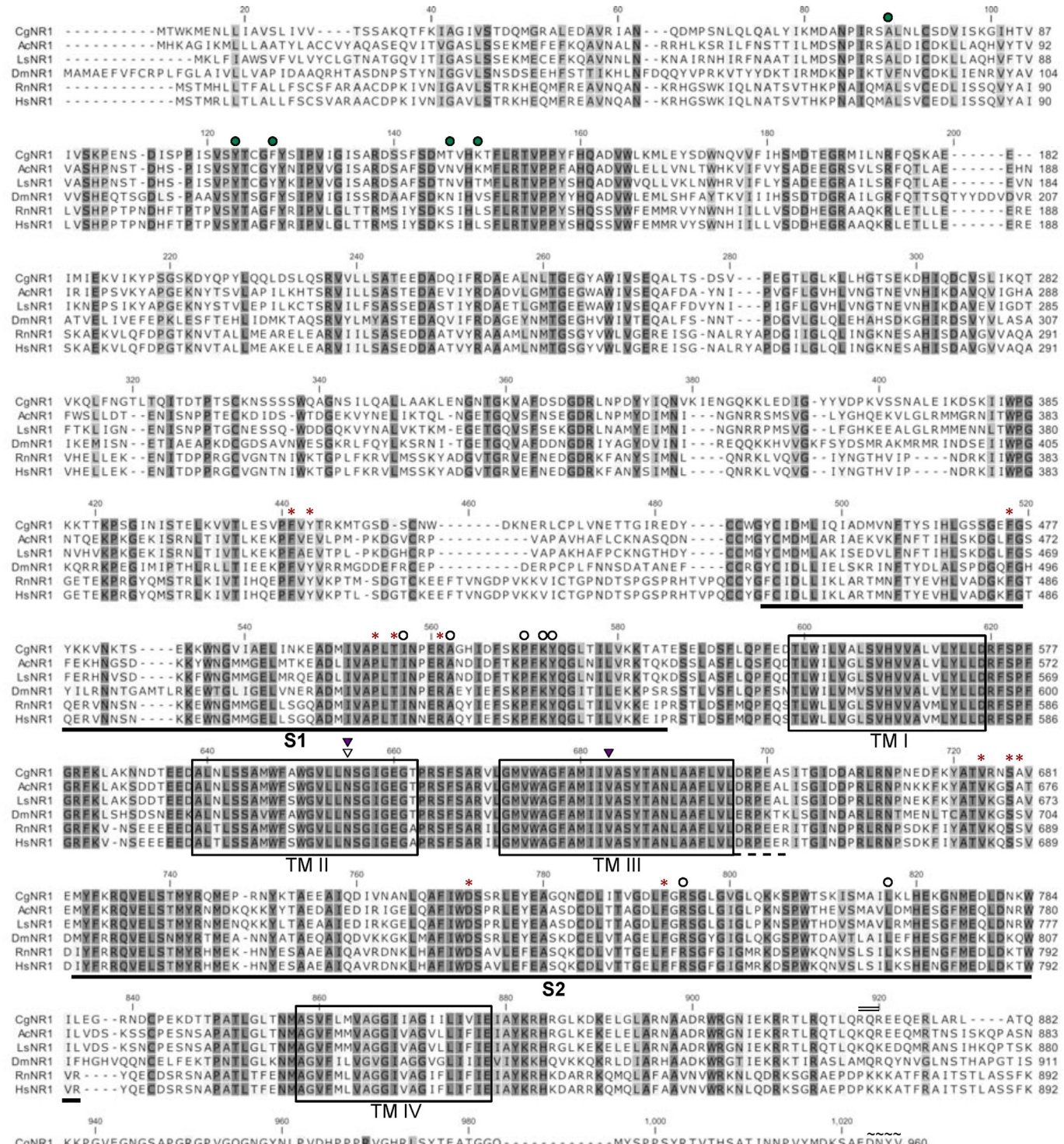

Fig. 3. Alignment of NMDA receptor NR1 subunits. Full NR1 amino acid sequences from *Crassostrea gigas* (GenBank ID pending, sequences submitted to NCBI), *Aplysia californica* (AAO62106), *Lymnaea stagnalis* (AAT40576), *Drosophila melanogaster* (Q24418), *Rattus norvegicus* (P35439) and *Homo sapiens* (Q05586). The ligand domains S1 and S2 are underlined; transmembrane helixes TMs are marked by open boxes; potential binding sites are marked with open circles ○ for dimerization, stars × for glycine, open triangles Δ for Mg^{2+} and Ca^{2+}, underscored dotted line for DPREER motif, purple closed triangles ▼ for MK-801, and closed circles ● for ifenprodil, equal sign for RSS motif, tilde ~ for PDZ binding domain.

(caption on next page)

Fig. 4. Alignment of NMDA receptor NR2 subunits. NR2 amino acid sequences from *Crassostrea gigas* CgNR2A (GenBank ID pending, sequences submitted to NCBI) and CgNR2B (GenBank ID pending, sequences submitted to NCBI), *Drosophila melanogaster* DmNR2 (AAT40461), and *Homo sapiens* NR2A (Q12879), NR2B (Q13224), NR2C (Q14957), and NR2D (O15399). The ligand domains S1 and S2 are underlined; transmembrane helixes TMs are marked by open boxes; potential binding sites are marked with open circles ○ for dimerization, stars × for glutamate including circled star for NMDA specificity, open triangles Δ for Mg^{2+} and Ca^{2+}, closed triangles ▼ for MK-801, and green closed circles ● for ifenprodil. Far ends of receptor's C-terminals are not shown.

receptor subunits in particular. In vertebrate NMDA receptor, NMDA and glutamate mostly share the same residues for binding (Laube et al., 2004). However, a few differences in the amino acids were observed including an alanine at position A680 in CgNR1 instead of a serine as seen in vertebrates and drosophila, as well as a cysteine or tryptophan instead of a histidine at in CgNR2A (C521) and CgNR2B (W538), and a glutamine or lysine instead of an threonine in CgNR2A (Q551) and CgNR2B (K568).

The asparagine residues for potential binding of Mg^{2+} for blockage of the ion channel and successful Ca^{2+} flux (Burnashev et al., 1992; Dingledine et al., 1999; Wollmuth et al., 1998) are conserved in CgNR1 (N608), but less so in CgnR2A (G649) and CgNR2B (G666). Another motif in the NR1 subunit near the end of TM3, the DRPEER motif predicted to be important for Ca^{2+} flux by providing an external Ca^{2+} binding site (Watanabe et al., 2002; Karakas et al., 2011), is also relatively conserved in CgNR1 (DRPEAS). The binding site for the NMDA receptor channel blocker, MK-801, based on a crystal structure analysis in *Xenopus laevis* NR1-NR2B receptor complex (Song et al., 2018), are also predicted in all *C. gigas* subunits. While CgNR1 residues, N608 and V636, are conserved, two of the three residues in the CgNR2 subunits, however, were different from the asparagine residues in vertebrate NR2 subunits with a glycine at G649 and G666 and alanine amino acid at A650 and

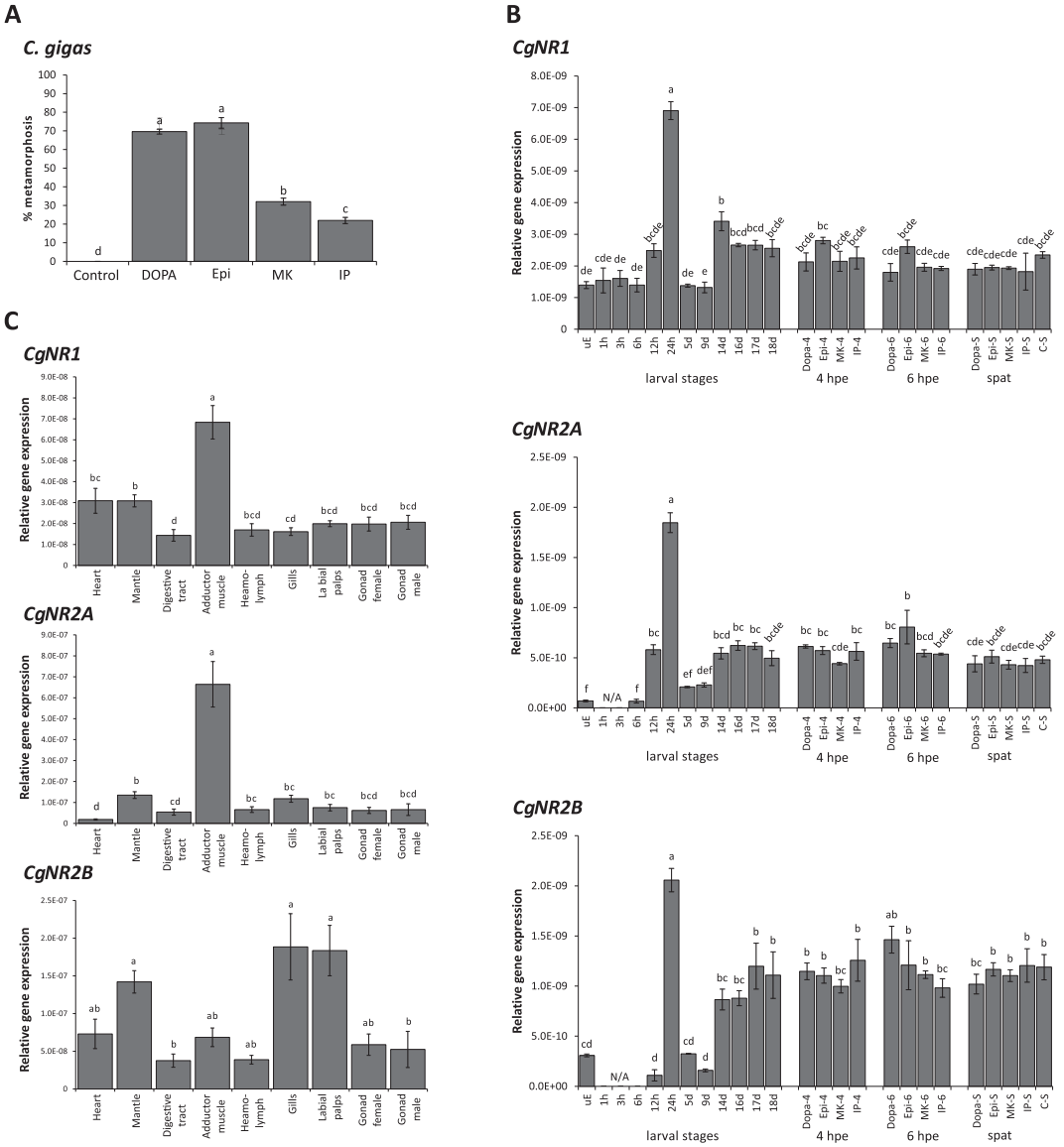

Fig. 5. Effect of metamorphosis inducers on competent Pacific oyster *Crassostrea gigas* larvae and gene expression patterns of NMDA receptor subunits *CgNR1*, *CgNR2A* and *CgNR2B* in different larval stages and adult tissues. **a)** Effect of L-DOPA (10^{-5} M), EPI (10^{-4} M), MK-801 (MK, 10^{-4} M) and ifenprodil (IP, 10^{-6} M) on metamorphosis success in competent *C. gigas* larvae after 24 h post start exposure (hpe). **b & c)** Relative gene expression of *C. gigas* NMDA receptor subunits: **b)** across different larvae stages, after exposure of the aforementioned compounds 4 hpe and 6 hpe, and in spat (S); C: control spat, uE: unfertilised eggs; h: hours post fertilisation; d: days post fertilisation. **c)** in different adult tissue types. Error bars represent the standard error, different lower-case letters represent significant difference ($p = 0.05$); N/A: expression below detection limit.

A667 in CgNR2A and CgNR2B, respectively. Assumption on binding ability of *C. gigas* NMDA receptors to ifenprodil is more difficult given that most of the predicted binding sites are positioned in the NTD, which is one of the least conserved domains across the NMDA receptor subunits. The predicted binding sites for vertebrate NR1-NR2B complexes (Karakas et al., 2011; Burger et al., 2012; Stroebel et al., 2016; Fjelldal et al., 2019) vary for most positions from the residues in *C. gigas* subunits CgNR2A and CgNR2B (Fig. 4).

3.2. Metamorphosis, NMDA receptor expression and location

Exposures to L-DOPA, EPI, MK-801 and ifenprodil significantly increased the metamorphosis success in competent Pacific oyster larvae after 24 h (Fig. 5a) with L-DOPA and EPI having the highest effect on metamorphosis induction of 69.6 ± 1.3% and 74.2 ± 2.8%, respectively. The effect of MK-801 and ifenprodil were significantly lower with 32.0 ± 1.9% and 21.9 ± 1.7%, respectively. No metamorphosis was observed in the control group after 24 h, which was expected given that no external metamorphosis cue (no treatment) or adequate settling surface was provided for competent larvae to undergo metamorphosis. Additional exposure experiments using the channel blocker memantine did not induce metamorphosis at the tested concentration at 10^{-6} M to 10^{-4} M and no metamorphosis was observed in the control nor in exposed larvae.

The gene expression patterns of the three NMDA receptor subunits in different larval stages, as well as in treated competent larvae and spat, are shown in Fig. 5b. In relation to the reference genes, *CgNR1* was overall more highly expressed than *CgNR2A* and *CgNR2B* for all tested time points. Expression of all subunits did not change significantly over the first 6 h of development and was partially undetectable in both *CgNR2* subunits. In trochophores 12 hpf all subunits expression increased slightly and peaked at their highest expression level measured across all samples in D-shelled larvae after 24 hpf. All expressions decreased significantly in 5 dpf and 9 dpf larvae, but amplified again at late veliger stage. Interestingly, NMDA receptor subunits expression did not vary considerably over the course of later larval development stages throughout the first 6 hpe or in spat at 24 hpe. The different treatments did not affect the expression of the different NMDA receptor subunits.

Expressions of all subunits were also assessed in different adult tissues (Fig. 5c). The different mantle tissues separately sampled, including mantle margin, mid mantle and mantle ventral of the adductor muscle, did not show significant differences for any subunit and were pooled (Supplementary File 5). Furthermore, significant differences between male and female were not observed for any tissue type and were also pooled except for gonads, which also did not show significant differences. The expressions of *CgNR1* and *CgNR2A* were relatively low in most adult tissue types except for the adductor muscle, in which both subunits were significantly higher expressed when compared to the other tissues. The expression for *CgNR2B*, however, showed a different pattern: this was, in fact, expressed at low level in adductor muscle, but expressed at higher levels in mantle, gills and labial palps.

In-situ hybridisation was used to localise the gene expression of the *CgNR1* subunit, which was present in competent oyster larvae in areas where the apical organ is located, and in the foot (Fig. 6). Differences in location of *CgNR1* expression, however, did not vary when either competent larvae, or competent larvae exposed to different metamorphosis inducers 4 hpe or 6 hpe were assessed. A sagittal section of whole competent larva shows (Fig. 6a) a clear staining of the *CgNR1* subunit transcripts near the apical organ's location. This is confirmed by a transverse section (Fig. 6b) of a larva 6 hpe to EPI, which displays the location of *CgNR1* transcripts at the base of the retracted velum, where the apical organ is usually located. Clear identification of the nervous structures in the apical region was impractical in the current study. However, previous studies on the nervous system in *Crassostrea virginica* (Ellis and Kempf, 2011) and *C. gigas* (Yurchenko et al., 2018, 2019) have

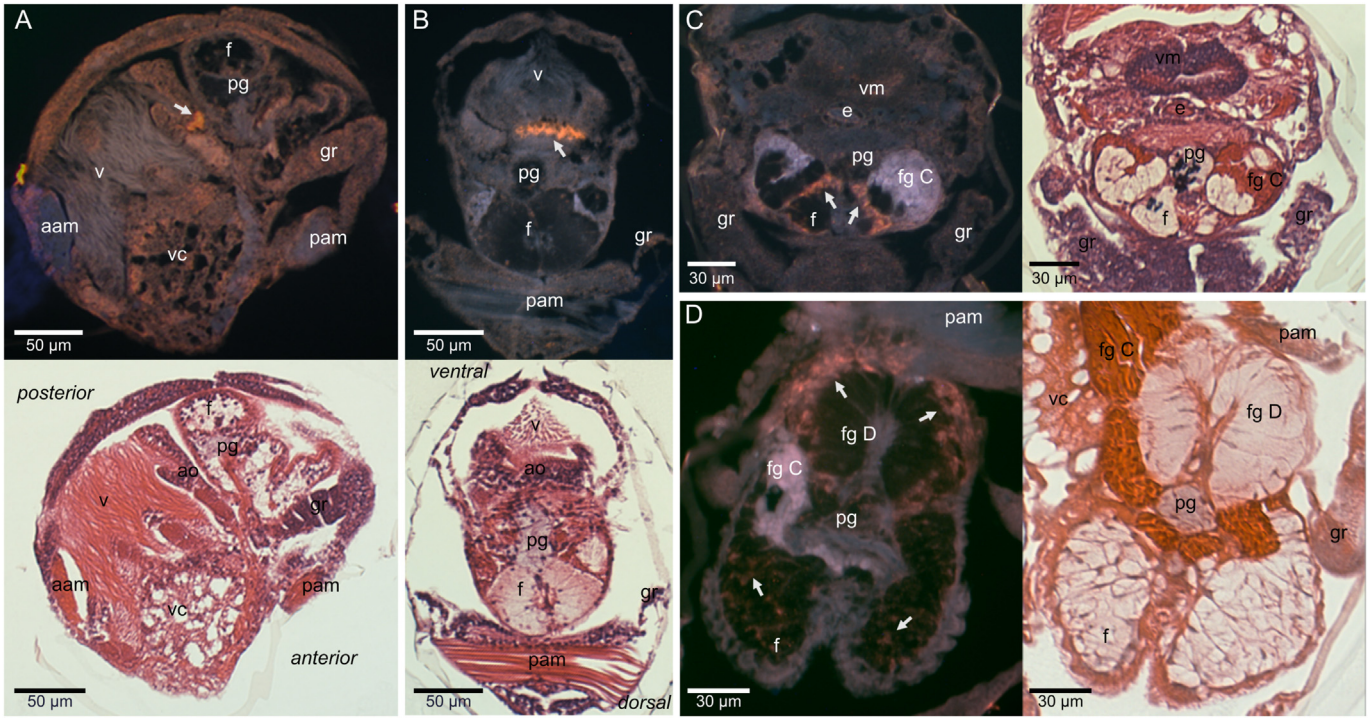

Fig. 6. *In-situ* hybridisation of competent Pacific oyster larvae sections using digoxigenin labelled riboprobes for *CgNR1* subunit (orange staining) with a triple-band DAPI-FITC-Texas Red excitation filter. Each fluorescent *in-situ* hybridisation section is accompanied with a sequential section stained with H&E technique. **a)** Sagittal section of whole competent larvae 18 days post fertilisation (dpf) before exposure. **b)** Transverse section of whole larvae 6 h post exposure start (hpe) with EPI. **c)** Transverse-frontal section of partial larvae 18 dpf before exposure. **d)** Frontal section of the foot area of an 18 dpf larvae before exposure. White arrows: successful *CgNR1* ISH labelling. aam: anterior adductor muscle; ao: apical organ; e: oesophagus; f: foot; fg C: foot glands C; fg D: foot glands D; gr: gill rudiments; pam: posterior adductor muscle; pg: pedal ganglion v: vellum with cilia; vc: visceral cavity; vm: vellum membrane.

shown that the apical organ partially consists of an apical ganglion tightly located between two cerebro-pleural ganglia connected with a cerebral commissure. *CgNR1* transcripts were also detected in the foot (Fig. 6c&d), penetrating the whole foot likely through a nerve network starting from the base of the foot towards the ciliated tip (Fig. 6d). *CgNR1* was not expressed either in the pedal ganglia or in one of the foot glands C (fg C), here seen as part of the foot structure in white with the fluorescent triple-band filter or pink in the H&E stained sections. This foot glands C together with another foot glands group D (fg D) are proposed as key glands during cementation process in *C. gigas* (Foulon et al., 2018) and other oyster species (Cranfield, 1973; Elston, 1980). Additionally, no evidence could be found that *CgNR1* subunit is highly expressed near the visceral ganglia, which should be located ventral of the posterior adductor muscle in the transverse sections based on previous work on the nervous system in *C. virginica* pediveliger larvae (Ellis and Kempf, 2011).

3.3. Effect of metamorphosis inducers on catecholamine biosynthesis enzyme expression

Several genes translating to proteins potentially involved in catecholamine biosynthesis have been identified by a BLASTp search of the *C. gigas* genome using protein sequences of human homologs (Fig. 7) including a single TH homolog, three DDC homologs and seven DβH or DβH-like homologs. A phylogenetic analysis of the protein sequences shows that the three DDC homologs group in three clusters of which CgDDC1 and CgDDC2 are direct homologs to the single vertebrate DDC homologs. A reverse BLASTp search using the CgDDC to identify potential homologs in other molluscs suggest that the expansion of DDC1 and DDC2 are also present in other bivalve species such as *Crassostrea virginica* and *Mizuhopecten yessoensis*. A third group of potential DDC homologs has been identified including the *C. farreri* homolog CfDDC (Zhou et al., 2012), but it seems that this group is more closely related to TDCs in *Drosophila* (Cole et al., 2005) than to human DDC. Several DβH or DβH-like homologs have been identified in the *C. gigas* genome. Only one homolog, however, CgDβH groups together with vertebrate DβH or invertebrate TβH or DβH homologs. The other six identified oyster homologs are clustered together in two distinct groups, hereafter named of DβH-like and TβH/DβH-like due to lack of any closer homologs: three CgDβH-like homologs closely related to the identified *C. farreri* CfDβH and *S. constricta*. ScDβH, and three CgTβH/DβH-like not closely related to any cloned DBH- or TBH-like homolog. This gene expansion seems to apply to many molluscan species. It is noteworthy, that no homolog to the human PNMT protein, the enzyme that converts NE to EPI, could be identified in the *C. gigas* genome.

For gene expression analysis, homologs of the enzymes involved in catecholamine biosynthesis closest related to the human homologs have been selected. These included *CgTH*, *CgDDC1*, *CgDDC2* and *CgDBH*. All genes were expressed in all tested larval stages. In general, significant differences between life stages as well as between treated animals after 6 hpe and in spat were mostly not detected for *CgTH*, *CgDDC2* and *CgDBH*. Significant differences in expressions, however, were most dominantly seen in larvae after 4 hpe. At this sampling point, while the expression pattern for these three genes in larvae treated with EPI and MK-801 did not differ from each other or from competent larvae, L-DOPA and ifenprodil significantly induced the expression of *CgTH*, *CgDDC2* and *CgDBH*. Additionally, *CgDDC2* shows an increasing trend in expression in spat compared to the late larval stage. Finally, *CgDDC1* seems to not be involved in late larval development or metamorphosis given its low expression in these life stages, but it seems to be important in mid-veliger development and potentially earlier development.

4. Discussion

The Pacific oyster possesses three NMDA receptor subunits, CgNR1 a homolog to the NMDA receptor subunit NR1, and two homologs to the NMDA receptor subunit 2, CgNR2A and CgNR2B. While NMDA receptor subunits have been previously reported in other invertebrate species, including several insect species (Ultsch et al., 1993; Xia et al., 2005; Zannat et al., 2006; Huang et al., 2015), gastropods (Ha et al., 2006), nematodes (Brockie and Maricq, 2003) and annelids (Grey et al., 2009), this is the first report of NMDA receptor subunits in a bivalve species. CgNR1 seems to be conserved along with other members of the NR1 subunit. Given that this subunit is essential for most NMDA receptor complexes, conservation within this group is not surprising. Furthermore, the phylogenetic analysis has shown that CgNR2A and CgNR2B are not direct orthologs to the vertebrate NR2A and NR2B subtypes, but instead originate from a separate duplication event of a paralogous CgNR2.

All three cloned *C. gigas* subunits contain the essential domains and conserved sequences to form functional NMDA receptor complexes including the unique asparagine residue for NMDA binding in both CgNR2 S2 domains. As ligand-gated and voltage-dependent ion channel receptors, NMDA receptors are dependent on agonists and co-agonists to engage in the required conformational changes to open the channel for successful ion-flux. The *C. gigas* NMDA receptor subunits are conserved in most of the residues for binding to the co-agonist glycine with its NR1 subunit, and to glutamate and NMDA with its NR2 subunits based on vertebrate NMDA receptor models for glycine (Kuryatov et al., 1994; Furukawa and Gouaux, 2003; Yu and Lau, 2018), glutamate and NMDA binding (Laube et al., 2004; Yu and Lau, 2018; Bonaccorso et al., 2011; Anson et al., 1998; Chen et al., 2005) with few exceptions that could potentially impact the binding affinity of these ligands to the oyster NMDA receptor. However, the serine residue in human NR1, where CgNR1 possesses an alanine A680, seems to briefly interact with the glycine after the ligand is already partially bound (Yu and Lau, 2018) and might not be significant for CgNR1. Substitution of the histidine and threonine in the NR2B subunit of vertebrate NR1-NR2B complexes to alanine residues decreased the binding affinity for glutamate (Laube et al., 2004). Although CgNR2A and CgNR2B possess different residues at these positions, similar differences have been observed in the *Drosophila* DmNR2 homolog and electrophysiological profiles of DmNR1 and DmNR2 co-expressed in *Xenopus* oocytes showed selective activation of *Drosophila* NMDA receptors by NMDA, L-aspartate, glycine and glutamate (Xia et al., 2005). Together with various reports on the effects of NMDA, glutamate and glycine on electrical activity of neurons and muscle fibres in different invertebrate species, including *Drosophila* and gastropods (Dale and Kandel, 1993; Moroz et al., 1993; Pfeiffer-Linn and Glantz, 1991; Cattaert and Birman, 2001; Miyashita et al., 2012; Lima et al., 2003; Burrell and Sahley, 2004; Brockie et al., 2001; Dyakonova and Dyakonova, 2010), it can be assumed that *C. gigas* NMDA receptor complexes are able to be activated by glycine, glutamate and NMDA to some extent. Nevertheless, the presence of two different NR2 subunits in *C. gigas* with distinct protein sequences opens the possibility for subunit-specific biophysical, signalling and pharmacological properties as seen for vertebrate NMDA receptor subunit 2 members. For instance, the four vertebrate NR2 subunits NR2A-NR2D display unique agonists potencies, opening probabilities and activation/deactivation time courses (Erreger et al., 2007; Yuan et al., 2009). The observed differences in gene expressions of *CgNR2A* and *CgNR2B* are further indicators that these two CgNR2 subunits might have distinct properties, such specific NMDA receptor complexes are needed in different tissue types such as in the adductor mussel (CgNR2A) and mantle, gill and labial palps (CgNR2B) based on the tissue function.

Inactivated vertebrate NMDA receptors are additionally blocked by a Mg^{2+} ion, which binds to asparagine residues in the channel-forming TM2 segments of each of the NMDA receptor subunits (Burnashev et al., 1992; Dingledine et al., 1999; Wollmuth et al., 1998). The asparagine residues in CgNR2A (G649) and CgNR2B (G666) are not conserved, which has also been observed in other identified invertebrate NR2 subunits (Xia et al., 2005; Brockie and Maricq, 2003; Huang et al., 2015). While studies on *Drosophila* NMDA receptors have shown that the asparagine residue in the DmNR1 subunit is sufficient for Mg^{2+} blockage

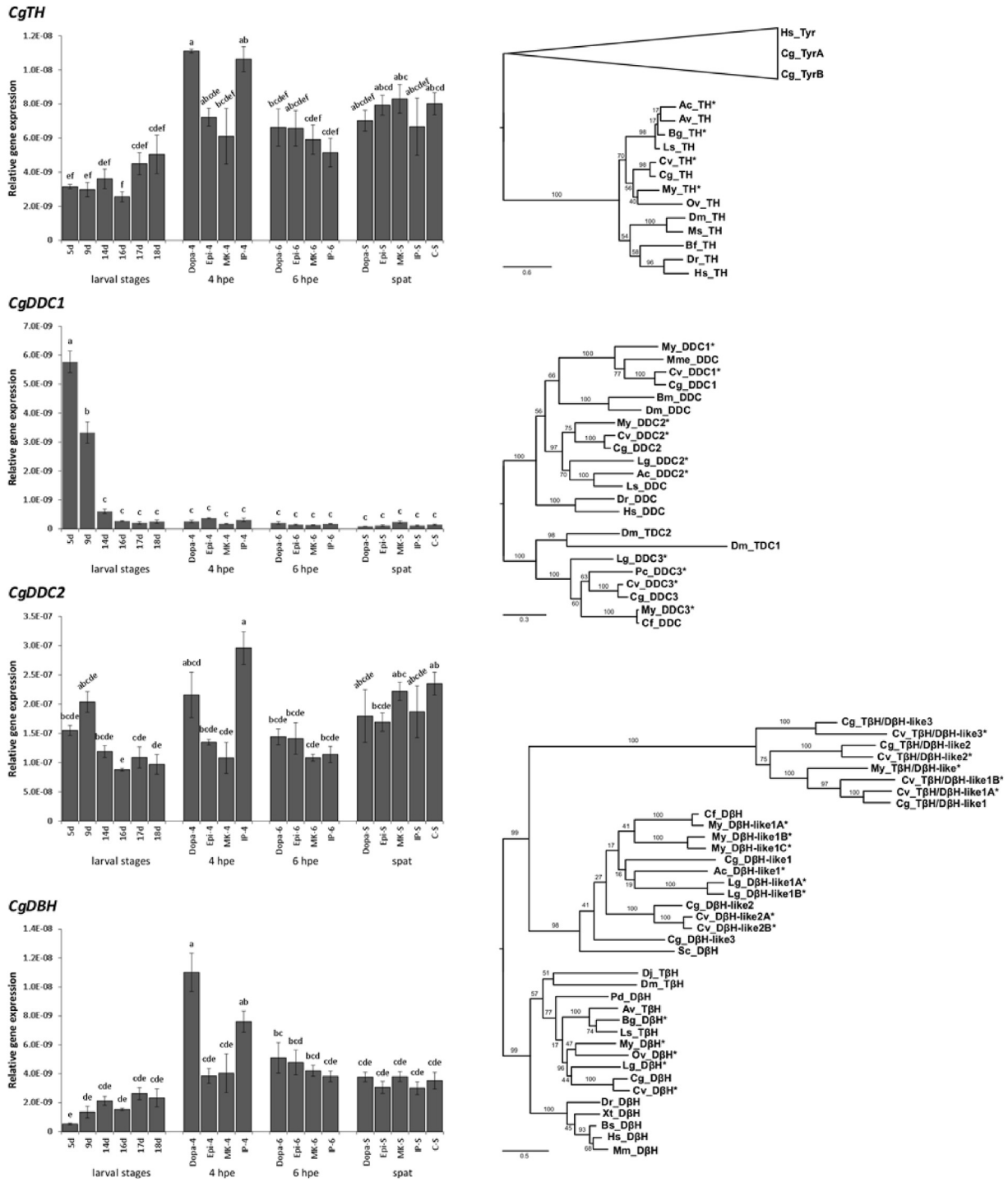

Fig. 7. Relative gene expression of *C. gigas CgTH*, *CgDDC1*, *CgDDC2* and *CgDBH* across different larvae stages, competent larvae after 4 h post exposure start (hpe) and 6 hpe of exposure to L-DOPA (10^{-5} M), EPI (10^{-4} M), MK-801 (MK; 10^{-4} M) and ifenprodil (IP, 10^{-6} M) and in spat 24 hpe. d: days post fertilisation. Error bars represent the standard error, different lower-case letters represent significant difference ($p = 0.05$). Maximum likelihood phylogenetic analysis of the amino acid sequence of CgTH, CgDDCs and CgDβH homologs by BLASTp search of the *C. gigas* genome protein annotation release 101. Accession numbers and detailed tree information are provided in Supplementary File 3. TH: tyrosine hydroxylase; DDC: DOPA decarboxylase; DβH or *DBH*: DOPA-β-hydroxylase; Tyr: tyrosinase; TβH: tyrosine-β-hydroxylase; TDC: tyrosine decarboxylase*: identified predicted molluscs proteins based on reverse BLASTp using identified CgTH, CgDDCs and CgDβH amino acid sequences. Ac: *Aplysia californica*; Av: *Ambigolimax valentianus*; Bf: *Branchiostoma floridae*; Bg: *Biomphalaria glabrata*; Bm: *Bombyx mori*; Bs: *Bos taurus*; Cf: *Chlamys farreri*; Cg: *Crassostrea gigas*; Cv: *Crassostrea virginica*; Dm: *Drosophila melanogaster*; Dj: *Dugesia japonica*; Dr: *Danio rerio*; Hs: *Homo sapiens*; Lg: *Lottia gigantea*; Ls: *Lymnaea stagnalis*; Mme: *Meretrix*; Mm: *Mus musculus*; Ms: *Manduca sexta*; My: *Mizuhopecten yessoensis*; Ov: *Octopus vulgaris*; Pc: *Pomacea canaliculate*; Pd: *Platynereis dumerilii*; Sc: *Sinonovacula constricta*; Xt: *Xenopus tropicalis.*

(Miyashita et al., 2012), alterations in the asparagine in NR2 subunits still potentially leads to a less sensitive Mg^{2+} block with lower blockage efficiency reported for *Drosophila* and other invertebrate species (Xia et al., 2005; Dale and Kandel, 1993; Pfeiffer-Linn and Glantz, 1991; Miyashita et al., 2012; Lima et al., 2003; Chiang et al., 2002), and even absent for some species (Moroz et al., 1993; Brockie et al., 2001). This suggests that invertebrate NMDA receptors may have evolved to be less sensitive to Mg^{2+} blockage. Although the reason for this is speculative, for marine species the insensitivity to Mg^{2+} could be a potential adaptation to extremely high Mg^{2+} concentrations in the marine environments (Ha et al., 2006).

Differences in residues in the channel-forming TM2 segments in invertebrate's NR2 subunits might also affect binding to known-vertebrate channel blockers such as MK-801 and memantine. In the *C. gigas* NMDA receptor subunits, three of the five proposed key binding residues in vertebrate NR1-NR2B complexes (Song et al., 2018) are conserved, with two asparagine residues in the TM2 segment of both CgNR2 subunits replaced by glycine and alanine residues. These asparagine residues in NR2 subunits seem to be critical in vertebrate's NMDA receptor binding to MK-801, insomuch as substitution of these residues to glutamine showed marked negative effects on the block by MK-801 and memantine (Kashiwagi et al., 2002), thus suggesting a negative effect for the potential of an MK-801 blockage in *C. gigas*. However, one of these asparagine residues, N612 in human NR2A, is also responsible for Mg^{2+} blockage and is also not conserved in the Pacific oyster CgNR2A (G649) and CgNR2B (G666) or other invertebrate NR2 subunits. As Mg^{2+} blockage is still possible in most invertebrates and MK-801 is able to block the NMDA or glutamate selective responses on neurons and muscle fibres as well as abolish NMDA receptor-dependent processes (Cattaert and Birman, 2001; Miyashita et al., 2012; Si et al., 2004; Chiang et al., 2002; Brockie et al., 2001; Dyakonova and Dyakonova, 2010; Pedreira et al., 2002; Troncoso and Maldonado, 2002), the remaining three key conserved residues in CgNR1 and CgNR2 seem to be sufficient for successful MK-801 blockage. Memantine, another vertebrate NMDA receptor channel blocker, only binds to the asparagine residues in the TM2 segments in both vertebrate subunits without the additional binding to residues in the TM3 segments as proposed for MK-801 (Song et al., 2018). The single key remaining conserved residue N608 in CgNR1 might not be sufficient for memantine to successfully bind to the channel pore. This is supported by the inability of memantine to successfully induce metamorphosis in competent Pacific oyster larvae, similar to MK-801. However, memantine has been shown to have a weak effect on memory recall and memory loss in honey bees (Si et al., 2004) and *Drosophila* (Wang et al., 2012), which suggests that memantine might interact differently with insect NMDA receptors compared to oyster NMDA receptors. Furthermore, it is even more difficult to make predictions on a potential ifenprodil binding to CgNR2 subunits due to potential binding sites located in the highly variable NTD (Karakas et al., 2011; Burger et al., 2012; Stroebel et al., 2016; Fjelldal et al., 2019). Ifenprodil is highly selective to vertebrate NR2B subunits with a 400-fold less affinity to NR2A subunits and no potential binding to NR2C and NR2D (Williams, 1993, 2001). However, most of the CgNR2 subunit differences in the residues are unique and are not identical to those in human NR2A, NR2C or NR2D. Thus, binding to ifenprodil might or might not be possible in *C. gigas* NMDA receptors and could even vary between the two types of CgNR2 subunits. The observed positive effect of ifenprodil on bivalve metamorphosis (Vogeler et al., 2018, 2019) might also be a result of binding to non-NMDA receptors as additional antagonist for serotonin receptors, σ receptors and K^+ channels (Chenard et al., 1991; McCool and Lovinger, 1995; Hashimoto and London, 1995; Kobayashi et al., 2006). As previously suggested, ifenprodil might additionally inhibit bivalve adrenergic receptors (Vogeler et al., 2019) given that ifenprodil inhibits the inducing effect of EPI, a potential agonist of adrenergic receptors in bivalve species. Adrenergic receptors were the original target receptors for the design of ifenprodil in vertebrates (Chenard et al., 1991). However, metamorphosis was still significantly induced, even in larvae co-treated with ifenprodil and EPI, similar to the lowest level of induction for single dose ifenprodil, thus suggesting that ifenprodil, although inhibiting adrenergic receptors, induces metamorphosis via a different receptor pathway, potentially the NMDA receptor pathway. Additional analyses are required to elaborate on binding abilities of invertebrate NMDA receptors including electrophysical profiles of *C. gigas* and other invertebrate NMDA receptor subunits to MK-801, or less commonly studied compounds such as memantine and ifenprodil.

Regulations of *C. gigas* NMDA receptor gene expression seem to be neither self-regulated nor regulated by downstream pathways of L-DOPA or EPI, given that none of the tested compounds affected the expression levels of the different oyster NMDA receptor subunits. However, NMDA receptors might be regulated through additional mechanisms such as post-translational modifications, regulation by endoplasmic reticulum retention, including receptor subunit assembly, as well as transportation for additional control of NMDA receptor types incorporation into cell membranes (Traynelis et al., 2010; Standley et al., 2000; Horak et al., 2014). Furthermore, subunit selection for receptor complex assembly, as well as different splicing variations, may lead to alteration in assemblage success or ligand binding affinities that could alter the function and responsiveness of NMDA receptors (Xia et al., 2005; Zannat et al., 2006; Huang et al., 2015; Ha et al., 2006; Zukin and Bennett, 1995). These post-translational modification as well as receptor functions could also potential be affected by L-DOPA and EPI through currently unknown pathways. Further research is needed to explore the possibility of a catecholamine effect on NMDA receptors. Nevertheless, the significant increase in expression of all NR subunits in 24 hpf D-shelled larvae was likely caused by the development of the apical organ and cerebral ganglia, which are part of the nervous system development in trochophores to early veliger bivalve larvae (Ellis and Kempf, 2011; Yurchenko et al., 2018, 2019; Dyachuk et al., 2012; Rusk et al., 2017; Pavlicek et al., 2018). As the localisation of *CgNR1* expression in eyed pediveliger larvae has shown, NMDA receptors are highly present in the area of the apical organ/cerebral ganglia. The apical organ is a sensory organ suggested to identify environmental cues involved in metamorphosis in molluscan species (Voronezhskaya et al., 2004; Hadfield et al., 2000; Urrutia et al., 2004). NMDA receptor presence in this sensory organ may aid the detection and transformation of such exogenous cues for metamorphosis. Another important organ in pediveliger larvae is the foot that is used for locomotion while searching for adequate substrate to attach. While the larval foot in bivalves has also been suggested to sense for environmental cues (Foulon et al., 2018; Cranfield, 1973), NMDA receptors may function primarily in regulation of muscle contractions in the neuromuscular network of the foot. In vertebrates, NMDA receptors are responsible for contraction of smooth muscle by regulating intracellular calcium concentrations for muscle contraction and/or through a cascade of inducing nitric oxide and subsequence cGMP production for muscle relaxation (Colombo and Francolini, 2019). This theory is supported by the observed effects of MK-801 on competent larvae, which after exposure were immobile on the bottom of the vials. Additional research in other invertebrates has also demonstrated that MK-801 negatively affect locomotion and locomotor rhythm (Cattaert and Birman, 2001; Dyakonova and Dyakonova, 2010). Interestingly, ifenprodil had the opposite effect, where larvae motile activity such as swimming and extending the foot increased after exposure, which might be another indication that ifenprodil's main target was not the NMDA receptor in *C. gigas*. The high expression of NMDA receptor subunits *CgNR1* and *CgNR2A* might be explained by a potential presence of NMDA receptors in the closely located large visceral ganglion, which is ventrally adjacent of the adductor muscle, with a large adductor muscle nerve penetrating the muscle itself. The NMDA receptor might fulfil regulatory functions of muscle contractions in addition to its potential function in synaptic plasticity.

NMDA receptor presence in Pacific oyster larvae and their potential ability to bind to ligands such as MK-801 as effective synthetic exogeneous inducer of metamorphosis, strongly indicates that NMDA receptors

are part of the regulating processes governing the oyster transition from larvae to spat. Regulation of metamorphosis, however, does not seem to be controlled by a downstream response of NMDA receptors regulating catecholamine synthesis, given that MK-801 did not alter the gene expression of *CgTH*, *CgDDC2* or *CgDBH* homologs to enzymes involved in vertebrate catecholamine synthesis. Nevertheless, NMDA receptors might still affect the release of the catecholamines from synapses, or catecholamines could modify NMDA receptor plasticity. Further research is needed to explore this possibility.

Nonetheless, our data provides additional evidence for the importance of catecholamines during bivalve metamorphosis and the existing theories, which suggest that dopaminergic/adrenergic pathways are regulators of metamorphosis (Bonar et al., 1990; Coon et al., 1990). Catecholamine synthesis seems to mainly occur within the first few hours after onset of metamorphosis. The presence of L-DOPA induces the production of L-DOPA, DA and NE by upregulating synthesising enzymes, potentially by interacting with DA receptors (after conversion to DA), until sufficient NE has been produced to interact with the adrenergic pathway. When exogenous EPI is present, however, adrenergic receptors are activated and production of NE or EPI is not required, thus enzymes are not upregulated. Interestingly, EPI might not be naturally synthesised by the Pacific oyster given that no PNMT homolog could be identified in the oyster genome. In fact, EPI biosynthesis might have been lost in *C. gigas,* as to our knowledge, detection of EPI has not been reported for this species. Whether this is species-specific or also occurs in other bivalve species is difficult to predict. A PNMT homolog has not been reported for any bivalve species or identified in the available database at NCBI, but while EPI was undetectable in scallop larvae (Cann-Moisan et al., 2002), concentrations of EPI have been reported in adult scallop species (Chen et al., 2008; Zhang et al., 2014; Zhou et al., 2011b; Pani and Croll, 1995). The upregulating effect of ifenprodil on catecholamine synthesising enzymes could be a result of its potential antagonistic effect on adrenergic receptors, which prevents the suspension of catecholamine synthesis by blocking the activating effect of NE/EPI on adrenergic receptors. Synthesised NE/EPI might activate the adrenergic pathway and reduce the expression for *CgTH*, *CgDDC2* and *CgDBH*, but not until the effect of ifenprodil has worn off.

Catecholamine biosynthesis including the associated enzymes is not well studied in invertebrate species and might differ from the vertebrate equivalents, particularly due to (i) the closely related biosynthesis pathways of the monoamines, tyramine and OA, (ii) expansion of homologous enzymes as seen for molluscs and (iii) differences in additional enzymatic functions. For example, THs in invertebrates seems to not only be involved in pathways leading to catecholamine synthesis (as proposed for vertebrates), but also assumed to be involved in pigmentation, immune responses and other developmental processes unrelated to the catecholamine pathway (Friggi-Grelin et al., 2003; Mapanao and Cheng, 2016; Liu et al., 2010; Gorman et al., 2007). The expansion of DDC homologs observed here in the Pacific oyster, and potentially in other molluscan species, might hint at additional regulatory functions for the different homologs during different developmental stages. Interestingly, based on our phylogenetic analysis the DDC homolog identified in *C. farreri*, it appears that CfDDC is not a direct homolog to vertebrate DDCs as previously reported (Zhou et al., 2012), but instead is a homolog to *Drosophila* TDCs, which are involved in tyramine synthesis rather than DA conversion (Cole et al., 2005; Blumenthal, 2009). The high expression of the *CfDDC* gene in veliger and pediveliger larvae might suggest the involvement of tyramine in late larval development and potentially during metamorphosis. Further functional analysis on CgDDC3 and CfDDC are required to verify their enzymatic involvement in either tyramine or DA synthesis, given that TDC homologs have not been investigated in molluscs. In depth analysis of the DβH/TβH groups is also needed given that synthesis pathways of NE and OA have not been unambiguously identified in invertebrate protostomes. The adrenergic pathways seem to have been lost in many insects and nematodes, but co-exist with octopaminergic pathways in molluscs and other invertebrate lineages (Bauknecht and Jékely, 2017; Gallo et al., 2016). Potential function for CgDβH is difficult to predict based on closely related homologs with vertebrates as DβHs mainly catalyses NE synthesis, but might also be suggested as the enzyme for synthesis of OA functioning as trace amine (Pryor et al., 2016). Tyramine-β-hydroxylases in insects produce OA (Monastirioti et al., 1996) and the AvTβH homolog in the terrestrial slug *Limax* co-localised with OA-receptor containing cells rather than with TH containing cells in the central nervous system (Matsuo et al., 2016). However, the annelid PdDβH was classified as adrenergic marker in annelid neurons (Randel et al., 2013). Hence, it cannot be excluded that CgDβH catalyses the synthesis of OA rather than NE, particularly as octopaminergic next to adrenergic pathways are potentially involved in bivalve metamorphosis (Joyce and Vogeler, 2018; Ji et al., 2016). Additional NE and OA catalysing enzymes might be provided by the expansion of the DβH group, which seems to apply to molluscan species with several DβH-like and DβH/TβH-like members per species. Although no information is available on which amines these enzymes might catalyse, studies in *C. farreri* (Zhou et al., 2011a, 2012) and *S. constricta* (Li et al., 2020), suggest an involvement of CfDβH and ScDβH in larval development and immune-response in relation to catecholamine regulation. Both enzymes classified as members of the DH-like group in our phylogenetic analysis. Both studies, however, have not discussed the possibility of these enzymes catalysing OA synthesis.

5. Conclusions

The cloning of three NMDA receptor subunits in *C. gigas* and localisation of *CgNR1* gene expression in larval organs linked to metamorphosis-induction, as well as the positive effect of NMDA receptor antagonists on metamorphosis, in particular MK-801, further strengthens our existing theory that NMDA receptors are negatively regulating metamorphosis by increasing intracellular calcium concentration for metamorphosis-related downstream signalling. Hence blockage or inactivation of NMDA receptors induces metamorphosis. Based on our results, however, NMDA receptors are unlikely to operate via a regulation of enzyme expression involved in catecholamine synthesis. Other putative downstream responses need to be investigated such as effects of NMDA receptors on catecholamine release. Further work should also be conducted to assess the aforementioned connection between NMDA receptors and nitric oxide production (Vogeler et al., 2018), to shed light on how the NMDA receptor pathway regulates metamorphosis in the Pacific oyster, and potentially other bivalves. The current research also provides additional evidence for the involvement of catecholamines during the onset of oyster metamorphosis. However, it also emphasizes the need for more in-depth research on enzymes involved in catecholamine, as well as monoamine synthesis, given that molluscan-specific expansions of these enzymes makes it difficult to compare to existing models such as octopaminergic pathways in insects or adrenergic pathways in vertebrates. Regulation of metamorphosis in bivalve species involves a complex cross-talk between various regulatory pathways. Further information on novel, less-explored pathways, such as the NMDA receptor pathway, as well as on established pathways are required to complete the picture.

Authors contribution

SV, AJ, SC, XL contributed to experimental design and conceptualization. Interpretation of data and manuscript preparation were conducted by all authors. All laboratory studies and experiments were completed by SV with assistance of JHI for the *in-situ* hybridisation.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2020.10.008.

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