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Functional characterization of an invertebrate gonadotropin-releasing hormone receptor in the Yesso scallop *Mizuhopecten yessoensis*

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Abstract
The neuropeptide control of bivalve reproduction with particular reference to gonadotropin-releasing hormone (invGnRH) is a frontier yet to be investigated. Bivalves are unique because they have two forms of the invGnRH peptide; however, there has been no functional characterization of the peptide–receptor pair. Therefore, the identification of a cognate receptor is a preliminary step toward exploring the biological roles of invGnRHs in bivalves. In this study, we functionally characterize an invGnRH receptor (invGnRHR) of a bivalve, the Yesso scallop *Mizuhopecten yessoensis*. In the receptor assay, HEK293 cells were transfected to transiently express the *M. yessoensis* invGnRHR (my-invGnRHR), which was found to be localized on the plasma membrane, confirming that my-invGnRHR, similar to other G-protein-coupled receptors, functions as a membrane receptor. Using both forms of invGnRH as ligands in a function-receptor assay, my-invGnRH11aa-NH_2 stimulated intracellular Ca^{2+} mobilization but not cyclic AMP production, whereas my-invGnRH12aa-OH did not induce increase in Ca^{2+} levels. Therefore, we concluded that my-invGnRHR is an endogenous receptor specific to my-invGnRH11aa-NH_2 which is hypothesized to be the mature peptide. To the best of our knowledge, this is the first study reporting the functional characterization of a bivalve invGnRHR.

Keywords : Mollusc, receptor assay, second messenger, AKH, CRZ, ACP, GnRH

1. Introduction

Transcriptome and genome surveys and subsequent sequence homologies and synteny analyses have been used to study a wide range of neuropeptide receptor genes, including those encoding gonadotropin-releasing hormone receptors (GnRHRs) in diverse invertebrates such as ascidians, amphioxuses, echinoderms, annelids, and molluscs (Hauser and Grimmelikhuijzen, 2014, Roch et al., 2014). Specifically, members of *Mollusca* (except for those of Cephalopoda) only possess the invertebrate GnRHR (invGnRHR) and adipokinetic hormone receptor (AKHR) among the related G-protein-coupled receptors (GPCRs) that constitute the GnRHR superfamily (Roch et al., 2014), including AKHR, corazonin receptor (CRZR), AKH/CRZ related peptide receptor (ACPR), and GnRHR.

Several bioassays have provided functional evidence regarding molluscan invGnRHRs that are stimulated by invGnRH peptides (Table 1). In Cephalopoda, the *Octopus* invGnRHR receives the invGnRH signal not only for muscle contraction in peripheral tissues (e.g., heart, oviduct, and radula retractor muscles) but also for the brain control of autonomic function, feeding, memory, and movement (Iwakoshi-Ukena et al., 2004, Iwakoshi et al., 2000, Kanda et al., 2006, Minakata et al., 2009). In Gastropoda, *Aplysia* invGnRH modulates muscular contraction for controlling the parapodia and foot and head movements (Sun et al., 2012, Tsai et al., 2010). However, invGnRH signaling and its physiological functions have yet to be fully investigated. In particular, only a few cognate receptors of invGnRHs have been identified at the functional level, and the elucidation of invGnRHs/receptor pairs is necessary to understand the biological roles of GnRHergic systems in molluscs.

In Bivalvia, invGnRH has so far been characterized in two marine shellfish, the Pacific oyster *Crassostrea gigas* (Bigot et al., 2012) and Yesso scallop *Mizuhopecten yessoensis* (Nagasawa et al., 2015b). These studies revealed that the following two forms of invGnRH peptides were present in the nerve ganglia: invGnRH12aa-OH and invGnRH11aa-NH_2 (Table 1). The *in vivo* administration of the oyster invGnRH11aa-NH_2 peptide was demonstrated to induce spawning in the Sydney rock oyster *Saccostrea glomerata* (In et al., 2016), although differences in bioreactivities of the two forms of the oyster invGnRHs were not assessed. In the Yesso scallop, the invGnRH11aa-NH_2 peptide was found to stimulate spermatogonial cell proliferation and masculinization (Nagasawa et al., 2015a, Nakamura et al., 2007, Treen et al., 2012).

These observations encouraged us to assess the ligand properties of the two forms of bivalve invGnRH peptides. To analyze ligand–receptor interactions, we recently cloned a putative candidate of the Yesso scallop invGnRHR, my-invGnRHR, using RNA-Seq of the ganglia (Nagasawa et al., 2017). The present study provides evidence for my-invGnRHR as a specific receptor of my-invGnRH11aa-NH_2.

2. Materials and methods

2.1. Plasmid construction and transfection

An open reading frame of my-invGnRHR (GenBank Acc. LC128621, *M. yessoensis GnRHR* mRNA for the complete coding sequence of GnRHR) was amplified using gene-specific primers with extensions to vector ends (Fw_my-GnRHR: AGCACAGTGGCGGCCAGAATGAATACTGATCATTCAA; Rv_my-GnRHR: TAGACTCGAGCGGCCATTGATCAACGGTGCAGGG). The amplified fragments were subcloned into the linearized mammalian expression vector pcDNA4/V5 (Thermo Fisher Scientific, Waltham, MA, USA) using an In-Fusion HD cloning kit (TaKaRa bio, Japan) according to the manufacturer's instructions. The expression vector was transiently transfected into a genetically engineered human embryonic kidney (HEK293MSR) cells using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Transfection efficiency (approximately 40–60%) was evaluated by an immunodetection method as described below.

2.2. Fluorescence immunocytochemistry

Immunocytochemistry was performed using confocal laser microscopy as previously described (Matsubara et al., 2017). Briefly, 1 μg of the expression vector was transfected into HEK293MSR cells in a glass-bottom dish (diameter, 35 mm). Immunoreactions were performed using V5 tag antibody (diluted to 1:300; Thermo Fisher Scientific) and anti-mouse IgG-Alexa488 antibody (diluted to 1:500; Thermo Fisher Scientific). Nuclei were stained with DNA fluorochrome 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) according to the manufacturer's instructions. The subsequent day, signal was visualized using the confocal laser microscope Fluoview FV1000 (Olympus, Tokyo, Japan).

2.3. Second messenger assays

Intracellular Ca^{2+} mobilization and cAMP production were assessed as previously described (Matsubara et al., 2017). Briefly, 2×10^6 HEK293MSR cells were cultured on a polystyrene culture dish (diameter, 100 mm). A day later, 10 μg of pcDNA-based my-invGnRHR expression vector, pcDNA-based ci-GnRHR1 expression vector (Sakai et al., 2010), or empty pc-DNA vector was transfected into HEK293MSR cells. After incubating for 24 h, 6×10^4 cells were plated in each of a 96-well plate for the following assay using the synthetic peptides: my-invGnRH11aa-NH_2, my-invGnRH12aa-OH, tunicate GnRH6, which was prepared by the custom order service of MBL Co. Ltd (Japan). Peptide purities were >95%. The real-time fluorescence assessment of Ca^{2+} mobilization was performed using the FLIPR Calcium 5 kit (Molecular Devices, Sunnyvale, CA, USA) with a FlexStation II Multi Mode microplate reader (Molecular Devices). The mean of relative fluorescent units (RFU) value (max–min) was calculated. Moreover, the end-point observation of cAMP production was observed using the CatchPoint Cyclic-AMP fluorescent assay kit (Molecular Devices) according to the manufacturer's instructions and measured with a FlexStation II Multi Mode microplate reader (Molecular Devices). Results are shown as means ± standard error of the mean of three independent experiments.

2.4. *Phylogenic analysis*

Bayesian phylogenic analysis was performed as described elsewhere (Nagasawa et al., 2017). In brief, all amino acid sequences were trimmed within the region TM1–TM7. The trimmed sequences were aligned using ClustalW (https://clustalw.ddbj.nig.ac.jp/) and were used for Bayesian inference (MrBayes v3.1.2, http://mrbayes.csit.fsu.edu/) using a mixed model of amino acid substitution (1,000,000 generations, sampling every 10th generation and burning the first 10,000 trees). Graphical representation of the phylogenic tree was obtained using FigTree v1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree/).

3. Results and discussion

First, we examined whether my-invGnRHR (Nagasawa et al., 2017) was expressed and translocated to the plasma membrane of HEK293 cells. The V5-tagged my-invGnRHR candidate was transiently transfected into HEK293 cells. Immunocytochemical fluorescence confocal microscopic detection of the V5 tag confirmed that my-invGnRHR was localized to the cell surface of HEK293MSR cells (Fig. 1A–C), similar to that observed for ascidian GnRHR ci-GnRHR1 (Fig. 1D–F) (Sakai et al., 2010) as well as for the GPCRs of other marine invertebrates, including sea cucumber (Wang et al., 2017) and oyster (Jia et al., 2018). These results suggest that HEK293 is capable of analyzing ligand–receptor interactions of molluscan invGnRHRs.

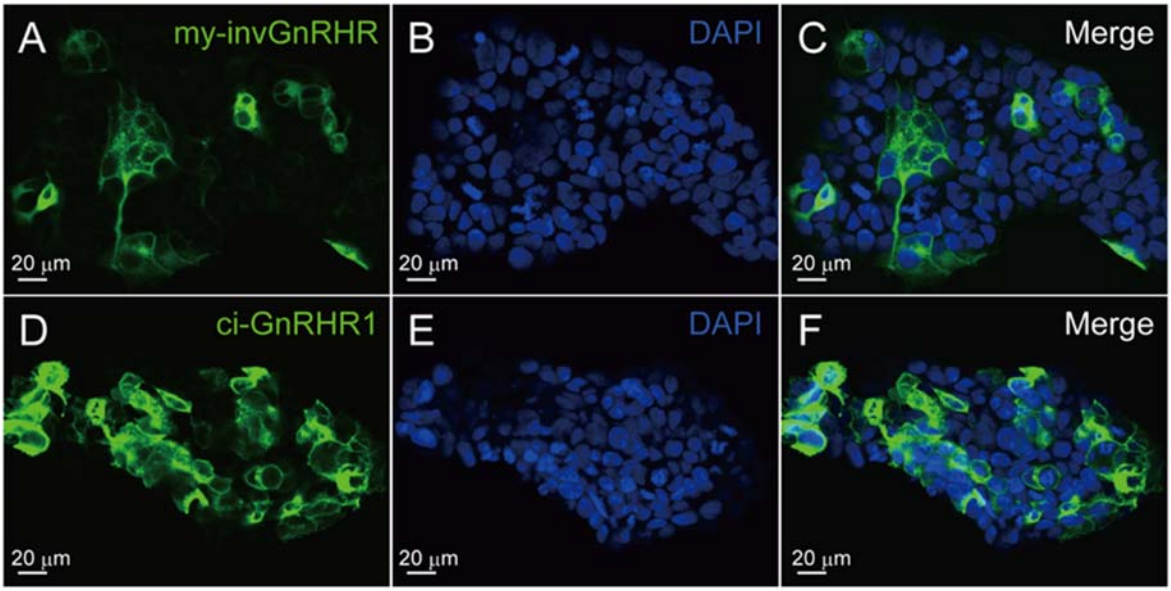

Fig. 1. Confocal microscopic observation of immunostained transfectants of my-invGnRHR.
A–C) Transfectants expressing the Yesso scallop my-invGnRHR. D–F) Transfectants expressing the ascidian ci-GnRHR1. A, D) Expressed receptors in HEK293MSR cells were immunostained by V5 tag antibody conjugated with Alexa 488 (green). B, E) Nuclei were stained with DNA fluorochrome 4′,6-diamidino-2-phenylindole (DAPI, blue). C, F) Merged views of the transfectants. Bar = 20 μm.

To evaluate the activation of my-invGnRHR by two forms of my-invGnRH (Nagasawa et al., 2015b), we assessed the mobilization of intracellular Ca^{2+} and the production of cAMP following the stimulation of my-invGnRHR transfectants by each form of my-invGnRH. The Ca^{2+} mobilization assay (Fig. 2A) demonstrated that my-invGnRH11aa-NH_2 specifically induced the elevation of intracellular Ca^{2+} ($EC_{50} = 4.8 \times 10^{-9}$ M) in a concentration-dependent manner, while my-invGnRH12aa-OH did not upregulate Ca^{2+} concentration. In contrast, the cAMP production assay (Fig. 2B) demonstrated that neither my-invGnRH11aa-NH_2 nor my-invGnRH12aa-OH exhibited considerable increase in cAMP production in cells expressing my-invGnRHR, whereas a considerable stimulation of cAMP production was observed in the ascidian ci-GnRHR1 and the tunicate GnRH6 (tGnRH6) pair used as a positive control ($EC_{50} = 1.6 \times 10^{-10}$ M) as reported in a previous study (Adams et al., 2003, Sakai et al., 2010). Table 2 summarizes the second messengers of invGnRHRs in molluscs. Our results are consistent with the fact that molluscan invGnRHRs, similar to vertebrate GnRHRs (Chang and Pemberton, 2018), utilize an inositol triphosphate (IP_3)/Ca^{2+} second messenger cascade rather than cAMP cascade. In the members of Protostomia, AKHR and invGnRHR are thought to have originated via gene duplication (Sakai et al., 2017), whereas both the receptors are conserved in the members of Mollusca (Roch et al., 2014). Intriguingly, the activated *Aplysia* AKHR (ap-AKHR1-A and -B) results in the elevation of intracellular Ca^{2+} levels (Kavanaugh et al., 2016) similar to the activated oyster AKHR (Li et al., 2016). All members of the AKH/CRZ/ACP/GnRH receptor superfamily may have originated from an ancestral receptor and have been shown to induce the elevation of intracellular Ca^{2+} levels (Sakai et al., 2017). Moreover, in a deuterostome, the activation of a starfish GnRHR and CRZR was found to elevate intracellular Ca^{2+} levels (Tian et al., 2016). Collectively, the present study supports the general conservation of the

IP_3/Ca^{2+} second messenger cascade in the GnRH receptor superfamily.

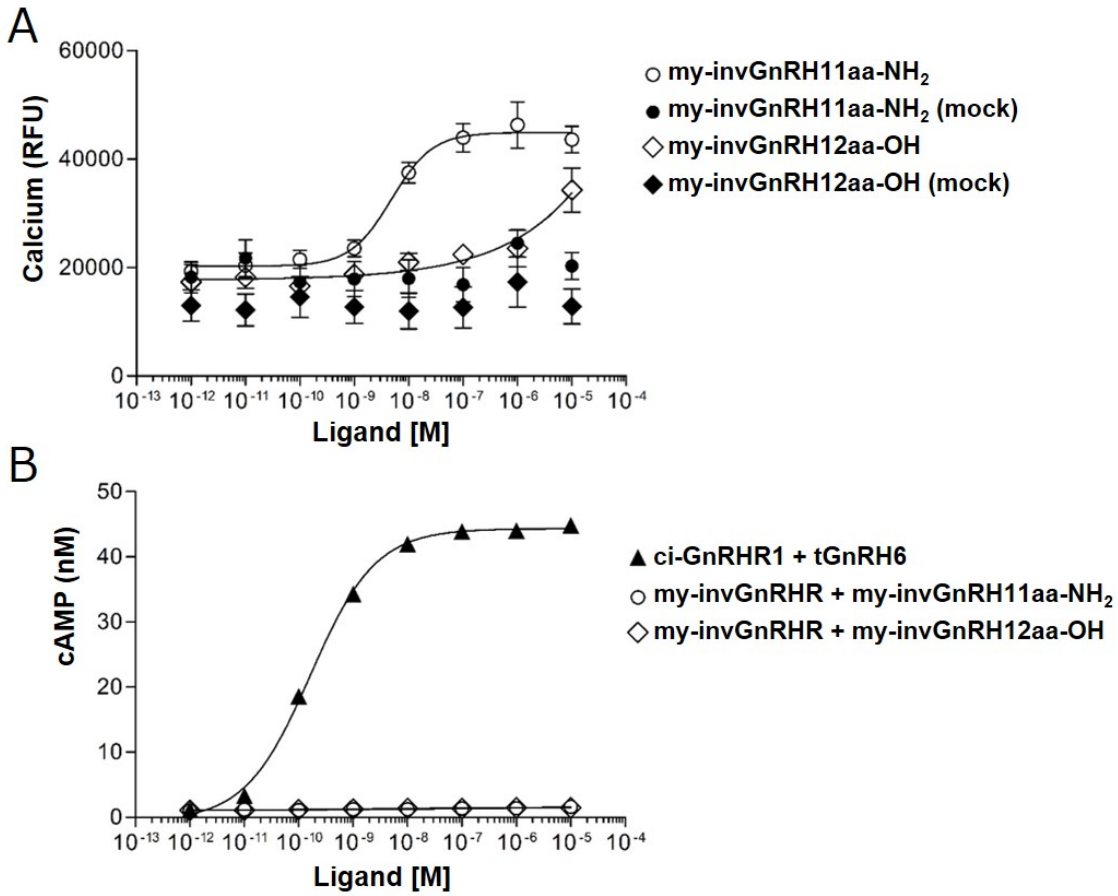

Fig. 2. Functional receptor assay of my-invGnRHR for assessing second messenger production
Dose-response curves of each my-invGnRH peptide in my-invGnRHR-expressing HEK293MSR cells. Ca^{2+} mobilization (A) and cAMP production (B) in HEK293MSR cells were measured following stimulation with my-invGnRH11aa-NH_2 or my-invGnRH12aa-OH. A) my-invGnRH11aa-NH_2 induced the elevation of intracellular Ca^{2+} ($EC_{50} = 4.8 \times 10^{-9}$ M), whereas my-invGnRH12aa-OH did not upregulate Ca^{2+} concentration. Empty vector was transfected as the negative control (mock). Data points are means ± standard error of mean of 3 three independent transfections. B) Neither my-invGnRH11aa-NH_2 nor my-invGnRH12aa-OH induced increase in cAMP production. A previously defined combination of invGnRHR–ligand pair i.e., ci-GnRHR1 and tGnRH6 pair (Sakai et al., 2010) was used as the positive control, which showed increase of cAMP production. Data points are means ± standard error of mean of 3 three independent transfections except for the positive control, which was obtained from two independent transfections.

Although induction of the elevation of intracellular Ca^{2+} levels is conserved by invGnRHRs in a wide range of species, some invGnRHRs have acquired species-unique features. Intriguingly, the bivalves have two forms of invGnRHs, which have been identified in both the Pacific oyster (Bigot et al., 2012) and the Yesso scallop (Nagasawa et al., 2015b). In the Yesso scallop, tissue localization and quantities of the two forms of invGnRH peptides were individually analyzed (Nagasawa et al., 2015b): In the visceral ganglion (VG) neurons, my-invGnRH11aa-NH_2 and my-invGnRH12aa-OH are colocalized in the same neuronal population; my-invGnRH12aa-OH (a precursor of my-invGnRH11aa-NH_2) signals were specifically observed in parts of the neuronal cell body, whereas my-invGnRH11aa-NH_2 (the mature peptide) signals were found in the axon hillocks and elongating fibers, inferring that the final amination process is going on. In contrast, in the cerebral and visceral ganglion (CPG) neurons, these two forms of my-invGnRHs were found in different neuronal populations. One type simply possessed my-invGnRH11aa-NH_2, suggesting that the precursor could be immediately

processed into the mature form. The other type possessed my-invGnRH12aa-OH without my-invGnRH11aa-NH_2, which may be the result of less amidation activity. This differential distribution of the two types of my-invGnRH neuronal populations may provide a clue to understand my-invGnRH function and suggest different neuronal controls of invGnRH under VG or CPG in bivalves. However, further study is needed for careful assessments of neuronal projection of two my-invGnRH neurons to find their target organs.

Phylogenic reconstruction visualized that the GnRHR superfamily was separated from oxytocin receptor (OXTR)/vasopressin receptor (VPR) and annetocin receptor (ANR)/cephalotocin receptor (CTR) families. In particular, my-GnRHR clustered with other mollusc/annelid invGnRHR sequences (Fig. 3A). The cluster of invGnRHR mollusk/annelid was closest to the CRZR arthropod cluster. In the present study, we unraveled the molecular characteristics of a bivalve invGnRHR. We found that my-invGnRHR is a specific receptor for my-invGnRH11aa-NH_2 but not for my-invGnRH12aa-OH and that my-invGnRHR exclusively induces intracellular Ca^{2+} mobilization as a second messenger without inducing cAMP production. To the best of our knowledge, this is the first report of the functional characterization of an invGnRHR in bivalves (Fig. 3B). Ligand selectivity of my-invGnRHR, combined with the differential distribution of the two my-invGnRHs (Nagasawa et al., 2015a, Nagasawa et al., 2015b), led to a hypothesis that my-invGnRH11aa-NH_2 is the mature form of the precursor my-invGnRH12aa-OH based on the known principals of post-transcriptional modification in neuropeptide processing (Fricker, 2005). Therefore, the identification of the receptor specific to my-invGnRH12aa-OH may be necessary to understand the species-specific regulation of invGnRHs in bivalves.

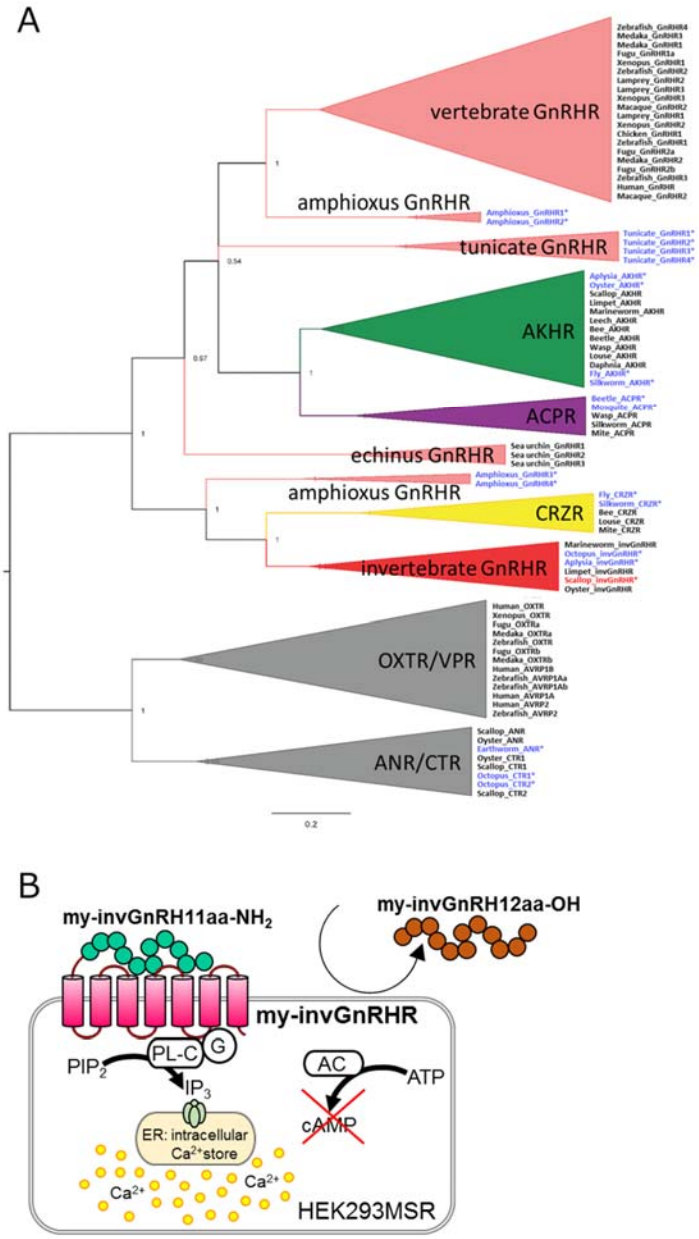

Fig. 3. Schematic representation of possible invGnRH signaling via my-invGnRHR

A) Condensed Bayesian phylogenic tree for the GnRHR superfamily, oxytocin receptor/vasopressin receptor, and annetocin receptor/cephalotocin receptor families. my-GnRHR (red) stands in the cluster of invertebrate GnRHR. For the receptors of non-vertebrates, asterisks (blue) indicate the authentic receptors evaluated by a functional assay with the ligand (refer in Table S1). Numbers at the nodes indicate Bayesian posterior probability. The scale bar indicates amino acid substitutions per site. GenBank accession numbers for sequences are listed in Table S1. ACPR, AKH/CRZ related peptide receptor; AKHR, adipokinetic hormone receptor; CRZR, corazonin receptor; GnRHR, gonadotropin-releasing hormone receptor; invGnRHR, protostome GnRHR; OXTR, oxytocin receptor, VPR, vasopressin receptor; ANR, annetocin receptor; CTR, cephalotocin receptor. B) my-invGnRH11aa-NH_2 stimulated the Yesso scallop my-invGnRHR to activate inositol triphosphate (IP_3) generation followed by intracellular Ca^{2+} mobilization, while cAMP production was not modulated. In contrast, my-invGnRH12aa-OH had no effect on second messenger production. CC, cerebral commissure; CG; cerebral ganglia; CVC, cerebro-visceral connective; FN, foot nerve; PG, pedal ganglia; PPN, posterior pallial nerve.

4. Conclusions

In conclusion, this study functionally identified the invGnRH and receptor pair in the Yesso scallop *M. yessoensis* using receptor assay. This study provides new insights into our understanding of the invGnRHR function in bivalves.

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Table 1. Details of molluscan invGnRH peptides subjected to mass spectrometry and/or bioassay in previous studies

Class	Species	Peptide sequence	Terminal modification	Length (aa)	Mass spectrometry identification	Bioassay
Cepharlopoda	*Octopus vulgaris*	pQNYHFSNGWHPG-amide	Amidated	12	Iwakoshi et al. 2002	Iwakoshi-Ukena et al. 2004
Gastropoda	*Aplysia californica*	pQNYHFSNGWYA-amide	Amidated	11	–	Tsai et al. 2010
Bivalvia	*Crassostrea gigas*	pQNYHFSNGWQP-amide	Amidated	11	Bigot et al. 2012	In et al. 2016
		pQNYHFSNGWQPG	Unmodified	12	Bigot et al. 2012	–
	Mizuhopecten yessoensis	pQNFHYSNGWQP-amide	Amidated	11	Nagasawa et al. 2015b	Nagasawa et al. 2015a
		pQNFHYSNGWQPG	Unmodified	12	Nagasawa et al. 2015b	–

Table 2. Functional receptor assays of invGnRHR in molluscs

Phylum	Class	Species	English name	Receptors	Ligands [a]	Binding assay [b]	Ca^{2+} (P) [c]	Ca^{2+} (B) [d]	cAMP [e]	IP_1 [f]	Expression system	Reference
Mollusca	Cephalopoda	*Octopus vulgaris*	Octopus	oct-GnRHR	oct-GnRH	-	Yes	-	-	-	*Xenopus* oocytes	Kanda et al. 2006
					c-GnRH-I, II	-	No	-	-	-		
					dm-AKH-I, II	-	No	-	-	-		
					dm-CRZ	-	No	-	-	-		
	Gastropoda	*Aplysia californica*	Sea hare	ap-GnRHR-L	ap-GnRH-amide	Yes	-	-	No	Yes	S2 cells	Kavanaugh et al. 2016
				ap-GnRHR-S	ap-GnRH-amide	No	-	-	No	No		
				ap-GnRHR-L	ap-AKH-amide	No	-	-	-	-		
				ap-GnRHR-S	ap-AKH-amide	No	-	-	-	-		
	Bivalvia	*Mizuhopecten yessoensis*	Yesso scallop	my-invGnRHR	my-invGnRH11aa-amide	-	-	Yes	No	-	HEK293MSR cells	This study
					my-invGnRH12aa-OH	-	-	No	No	-		

a) Abbreviations of species providing ligands: ap: *Aplysia californica*, c: *Gallus gallus* (*chicken*), cg: *Crassostrea gigas*, dm: *Drosophila melanogaster*, my: *Mizuhopecten yessoensis*, and oct: *Octopus vulgaris*

b) Binding assay: Radioreceptor assay

c) Ca^{2+} (P): Patch clamp for the measurement of membrane Cl^- currents coupled to the inositol phosphate/Ca^{2+} cascade

d) Ca^{2+} (B): Bioluminescence measurement of intracellular Ca^{2+}

e) cAMP: Immunoquantification of cyclic AMP (cAMP) using ELISA

f) IP_1: Immunoquantification of inositol monophosphate (IP_1) as a metabolite of IP_3 using ELISA