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RESEARCH ARTICLE

HSP90 regulates larval settlement of the bryozoan *Bugula neritina* through the nitric oxide pathway

Xiao-Xue Yang[1,*], Yu Zhang[1,2,*], Yue-Him Wong[3] and Pei-Yuan Qian[1,‡]

ABSTRACT

The larvae of many sessile marine invertebrates go through a settlement process, during which planktonic larvae attach to a substrate and metamorphose into sessile juveniles. Larval attachment and metamorphosis (herein defined as 'settlement') are complex processes mediated by many signalling pathways. Nitric oxide (NO) signalling is one of the pathways that inhibits larval settlement in marine invertebrates across different phyla. NO is synthesized by NO synthase (NOS), which is a client of the molecular chaperone heat shock protein 90 (HSP90). In the present study, we provide evidence that NO, a gaseous messenger, regulates larval settlement of *Bugula neritina*. By using pharmacological bioassays and western blotting, we demonstrated that NO inhibits larval settlement of *B. neritina* and that NO signals occur mainly in the sensory organ of swimming larvae. The settlement rate of *B. neritina* larvae decreased after heat shock treatment. Inhibition of HSP90 induced larval settlement, and attenuated the inhibition of NO donors during larval settlement. In addition, the expression level of both HSP90 and NOS declined upon settlement. These results demonstrate that HSP90 regulates the larval settlement of *B. neritina* by interacting with the NO pathway.

KEY WORDS: Biofouling, Larval attachment, Larval metamorphosis, NO, Heat shock protein 90

INTRODUCTION

Many marine invertebrates have a biphasic life cycle, consisting of a pelagic larval stage and a sessile juvenile and adult stage (Bishop and Brandhorst, 2003). Attachment and metamorphosis (both processes are referred to, in the present paper, as 'settlement'), during which larvae become juveniles, play an important role in the life history of many marine invertebrates. It is generally believed that attachment is a reversible process, while metamorphosis is an irreversible process through which a larva transforms into a morphologically and physiologically distinct juvenile. Attachment and metamorphosis are temporally separated in larvae of some species of echinoderms (Strathmann, 1974) and molluscs (Bryan and Qian, 1998), while they can be tightly coupled in others, such as the bryozoans (Wendt, 2000). These processes are regulated by a variety of endogenous and exogenous factors such as temperature, chemical cues and signalling pathways (Jensen and Morse, 1990; Hentschel, 1999; Dahms et al., 2004; He et al., 2012).

Bugula neritina is a common fouling species that forms erect, branching colonies on various man-made and natural substrates (Mawatari, 1951). As with many other marine invertebrates, the life history of *B. neritina* consists of two stages, a planktonic swimming stage and a sessile juvenile/adult stage (Woollacott and Zimmer, 1971). The larvae released from *B. neritina* colonies can usually attach and metamorphose within 2 h (Jaeckle, 1994), while the swimming period lasts for 2–10 h (Woollacott and Zimmer, 1971). Attachment and metamorphosis are the two subsequent phases following the swimming stage, and they closely follow each other in bryozoan larvae (Wendt, 2000). *Bugula neritina* is a widespread invasive marine species in the waters of Hong Kong, and is of both economic and environmental concern (Fusetani, 2004; Mackie et al., 2006). Over the last 7 years, numerous studies have shed light on the molecular pathways triggering larval settlement of *B. neritina*. Through transcriptome and proteome profiling, the Wnt, MAPK signalling and nitric oxide (NO) pathways were identified in *B. neritina* (Wang et al., 2010; Wong et al., 2012). In addition, norepinephrine and dopamine have been reported to be involved in larval attachment and metamorphosis (Shimizu et al., 2000; Price et al., 2017). Although these discoveries have provided some insights into cellular signalling during *B. neritina* settlement, the underlying mechanisms remain ambiguous.

As a gaseous messenger molecule, NO actively participates in a wide range of physiological processes such as nerve transmission, apoptosis and vascular relaxation (Palmer et al., 1988; Dimmeler and Zeiher, 1997; Colasanti and Venturini, 1998). The role of NO during larval settlement has been intensively studied in marine invertebrates. Interestingly, NO can manipulate larval settlement of the barnacle *Amphibalanus amphitrite* by downstream cGMP signalling. Suppression of NOS and cGMP using chemical inhibitors increases the larval settlement rate significantly (Zhang et al., 2012). However, experimentation has shown that NO induces larval settlement in the ascidian *Herdmania momus* (Ueda and Degnan, 2013). At present, the crucial role of NO during larval settlement has been demonstrated in many phyla including Echinodermata, Mollusca, Crustacea and Urochordata (Bishop and Brandhorst, 2001; Hens et al., 2006; Comes et al., 2007; Zhang et al., 2012). In biological systems, NO is synthesized on demand by NO synthase (NOS) (Nathan and Xie, 1994) and one of the upstream regulators for NOS is heat shock protein 90 (HSP90). In mammalian cells, HSP90 acts as a molecular chaperone, facilitating the stabilization of signalling proteins that are conditionally activated or expressed (Neckers and Ivy, 2003). In addition, HSP90 binding to NOS enhances NO production by amplifying the enzymatic activity of NOS (García-Cardeña et al., 1998; Billecke et al., 2002). The association between HSP90 and NOS is thought to exist in a number of marine invertebrates. For example, several studies have focused on the role of NOS and HSP90 in larval

[1]Division of Life Science, The Hong Kong University of Science and Technology, Hong Kong SAR, People's Republic of China. [2]Guangdong Engineering Research Center for Marine Algal Biotechnology, College of Life Sciences and Oceanography, Shenzhen University, Shenzhen 518060, People's Republic of China. [3]Department of Biotechnology, Akita Prefectural University, Japan.
*These authors contributed equally to this work

‡Author for correspondence (boqianpy@ust.hk)

P.-Y.Q., 0000-0003-4074-9078

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settlement (Bishop et al., 2001; Bishop and Brandhorst, 2001; Ueda and Degnan, 2013). In the sea urchin *Lytechinus pictus*, inhibition of NOS or HSP90 increases the larval settlement rate (Bishop and Brandhorst, 2001). More recent research has shown that NO donor treatment increases HSP90 and NOS activity in the larvae of the ascidian *H. momus* (Ueda and Degnan, 2013). In the coral *Eunicea fusca*, thermal stress raises the expression of HSP90, and in turn enhances activity of expressed NOS. Remarkably, the HSP90 induced by heat stress is capable of co-immunoprecipitation with NOS (Ross, 2014). Collectively, these suggest that interaction between HSP90 and NOS exists in a variety of marine invertebrates.

In the present study, to investigate the regulatory role of NO, HSP90 and their interaction during the larval settlement of *B. neritina*, we used quantitative real-time polymerase chain reaction (PCR), fluorescence localization, *in situ* hybridization, western blotting and pharmacological treatments. Through these investigations, we characterized the expression pattern of HSP90 and the localization of NO in *B. neritina*. In particular, we performed pharmaceutical bioassays to assess the effect of a NO donor, a NO scavenger, a HSP90 inhibitor and co-incubation of these chemicals during larval settlement.

MATERIALS AND METHODS

Larval sample

Adult *B. neritina* (Linnaeus 1758) colonies were collected from floating buoys off a beach on Sharp Island, Hong Kong (22°21′21.9″N, 114°17′43.9″E) between December 2015 and March 2016. The adult colonies collected were used within 7 days of collection. Colonies were induced to spawn by exposing them to light, and swimming larvae were collected according to the methods of Woollacott and Zimmer (1971). At every targeted developmental stage, namely swimming larvae (SW), 2 h post-attachment (2 h), 4 h post-attachment (4 h) and 24 h post-attachment (24 h), approximately 1000 individuals of *B. neritina* were collected. Freshly released SW were collected using a 100 µm mesh sieve and transferred to 1.5 ml Eppendorf tubes. Different stages of attached individuals were collected according to the methods of Wang et al. (2010). Briefly, larvae were placed in polystyrene Petri dishes (Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA) containing 10 ml of autoclaved 0.2 µm filtered seawater (AFSW), and incubated in the dark to induce settlement. *Bugula neritina* larvae settle in the dark on any surface without requiring the presence of a chemical inducer. Any unattached larvae were removed after 30 min to avoid inconsistencies in their development. Newly attached individuals were collected after 2, 4 and 24 h of development in AFSW. Different stages of attached individuals were scraped from the Petri dishes after 2, 4 and 24 h of development, and transferred to 1.5 ml Eppendorf tubes. The larval samples collected from each stage were fixed in RNAlater solution (Ambion, Austin, TX, USA) for RNA extraction. The remaining larvae were frozen with liquid nitrogen for western blot analysis.

Gene cloning and sequence characterization of HSP90

Total RNA was extracted from each of the four developmental stages (SW, 2 h, 4 h and 24 h) using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. Using the method described by Zhang et al. (2013), cDNA was prepared from the total RNA. Based on the raw sequence of HSP90 acquired from a transcriptome database of *B. neritina*, nested PCR was performed using two designed pairs of primers (Table 1) and polymerase PrimeSTAR HS (Premix) (Takara Bio, Mountain View, CA, USA) to amplify HSP90. The conditions used were as follows: 98°C for 10 s, 60°C for 5 s, and 72°C for 60 s. The PCR products were then purified, and further confirmed by the Sanger sequencing method [BGI (Beijing Genomics Institute), Shenzhen, China].

Table 1. Primer used to amplify the HSP90 gene

Primer name	Primer sequence (5′ to 3′)
HSP90F1	GCTGCACAGGGTGGTGGTG
HSP90F2	CCAGGCTGAAATTGCTCAGTTG
HSP90R1	GTTTAATGTAGCAGGCGAG
HSP90R2	CATAAATGCAGCTGGCAGCAC

The sequences were compared with previously deposited sequences using the BLAST program at the National Center for Biotechnology Information (NCBI: http:// www.ncbi.nlm.nih.gov/blast). The HSP90 sequence of *B. neritina* was then aligned with other species using the Cluster W function of the BioEdit software, version 7.2.5 (Hall, 1999). The conserved domains were identified in the conserved domain database (CDD) on the NCBI website (Marchler-Bauer et al., 2015).

Localization of NO production in *B. neritina* larvae

Nitric oxide production and location in the swimming larvae were visualized using the NO-specific fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) (Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's protocol. DAF-FM DA is a pH-insensitive fluorescent probe that emits increased fluorescence when reacting with active NO intermediates formed during the autoxidation of NO (Kojima et al., 1999). Approximately 600 newly released larvae were divided equally into three groups as follows: control, untreated +DAF-FM DA, and treated+DAF-FM DA. One group of larvae served as a negative control, the second group was kept in AFSW for 1 h (untreated group), while the third group was treated with 5 µmol l^{-1} of geldanamycin (a HSP90 inhibitor) for 1 h (treated group). The treated and untreated groups of larvae were incubated with 5 µmol l^{-1} of DAF-FM DA in the dark at room temperature for 30 min. After this, larvae were washed twice with AFSW to remove excess DAF-FM DA and then incubated in AFSW for an additional 30 min to allow complete de-esterification of the intracellular diacetates. DAF-FM DA fluorescence images of four different developmental stages were acquired using a fluorescent microscope (Olympus BX51), with 460–490 nm excitation, 505 nm dichroic beam splitter and 510 nm bandpass emission filter set. The larvae not incubated with DAF-FM DA were used as the negative control.

Protein extraction and western blot analysis

To study the expression profile of HSP90 and NO during larval settlement, the total protein of each developmental stage before and after settlement (swimming larval stage, 2 h, 4 h and 24 h after attachment juvenile stages) was extracted using a protein extraction buffer (8 mol l^{-1} urea, 40 mmol l^{-1} dithiothreitol and commercial protease and phosphatase inhibitor cocktails; Roche, Indianapolis, IN, USA; pH 7.4). To investigate the effect of the HSP inhibitor on NOS expression, the total protein from swimming larvae treated with 1.25, 2.5 and 5 µmol l^{-1} geldanamycin was also extracted using the above buffer. The protein samples were then homogenized for 5 s, at an amplitude of 20% and a pulse of 1 s on, 1 s off, five times (QSonica Q125 sonicator, Newtown, CT, USA), followed by 15,000 ***g*** centrifugation for 20 min at 4°C to collect the supernatants. The protein concentration was measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). A total of

120 µg of each extracted protein sample was subjected to a 4–20% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Genscript, Jiangsu, China), and then transferred onto polyvinylidene difluoride (PVDF) membranes (0.22 µm pore size; Millipore, Billerica, MA, USA). The membranes were blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h, and then incubated with either rabbit anti-HSP90 (no. 4877, Cell Signaling Technology, Danvers, MA, USA) or goat anti-NOS antibodies (ab1376, Abcam, Hong Kong) in 3% bovine serum albumin (BSA) in TBST solution (1:3000 dilution) at 4°C overnight. The membranes were then washed with TBST for 15 min, three times, to remove excess antibodies, and later incubated with goat anti-rabbit or donkey anti-goat HRP-linked secondary antibodies [Boster (Wuhan, China) 1:5000 dilution; Abcam 1:5000 dilution] at room temperature for 2 h. The membranes were further washed with TBST at room temperature for 15 min, four times. Images were acquired using a Western Bright peroxide chemiluminescent detection reagent (Advansta, Menlo Park, CA, USA), and recorded with a ChemiDoc Touch Imaging System (Bio-Rad). β-Actin expression (Boster) was used as a loading control.

Settlement bioassay

To further examine the involvement of HSP90 and NO in the settlement of *B. neritina* larvae, the HSP90 inhibitor geldanamycin (Blagosklonny et al., 2001; Miyata, 2005), the NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolineoxyl-1-oxyl-3-oxide (carboxy-PTIO) were used to perform larval settlement bioassays. As SNAP and geldanamycin are soluble in dimethyl sulfoxide (DMSO), but only weakly soluble in water, they were prepared as 250 mmol l^{-1} and 6.25 mmol l^{-1} stock solutions in DMSO, respectively. Carboxy-PTIO was prepared as a 200 mmol l^{-1} stock solution in Milli-Q deionized water. Dilution series of SNAP (100, 200, 400 and 800 µmol l^{-1}), geldanamycin (0.625, 1.25, 2.5 and 5 µmol l^{-1}) and carboxy-PTIO (5, 10, 20 and 40 µmol l^{-1}) were prepared in AFSW for the bioassays. The potential interaction between HSP90 and NOS was investigated by carrying out a co-incubation assay using 800 µmol l^{-1} SNAP and a dilution series of geldanamycin (1.25, 2.5 and 5 µmol l^{-1}).

The highest concentrations of DMSO used in geldanamycin, SNAP and geldanamycin+SNAP co-treatments were 0.08, 0.32 and 0.4%, respectively. Therefore, larvae treated with 0.4% DMSO, which was the highest concentration of DMSO used in these experiments and did not affect larval settlement (data not shown), were chosen as the negative controls. For the carboxy-PTIO experiment, AFSW was used as control solution. The larval settlement bioassays were performed using 24-well polystyrene plates (Fisherbrand). Approximately 20 larvae were collected immediately upon release, and placed in each well containing 2 ml of AFSW with the concentrations of chemicals described above. All of the control and treatment groups were kept at room temperature in the dark. In the inductive (carboxy-PTIO or geldanamycin) assays, percentages of settlement were scored 2 h post-treatment. In both the inhibitive (SNAP) and rescue (SNAP+geldanamycin) assays, percentages of settlement were scored 4 h post-treatment. Settled and unsettled larvae were distinguished based on the absence or presence of a ciliated surface, which could be easily observed under a light microscope. All experiments were repeated four times.

Statistical analysis

Statistical analysis was performed with SPSS 24. Simultaneous multiple comparisons of treatment means with a control were performed using one-way ANOVA, followed by Tukey's honest significant difference (HSD) *post hoc* test. A homogeneity of variance test was performed to ensure that variability between treatments was low.

RESULTS

Sequence characterization of HSP90

The amino acid sequence of *B. neritina* HSP90 was aligned with HSP90 from *Paracyclopina nana* (ADV59561.1), *Mytilus coruscus* (ALL27016.1), *Lissorhoptrus oryzophilus* (AHE77376.1), *Pomacea canaliculata* (AIZ03410.1) and *Saccharomyces cerevisiae* (P02829.1). Based on *S. cerevisiae* HSP90 domain information from Hainzl et al. (2009), the HSP90 from *B. neritina* contained characteristic conserved ATP-binding domains, a protein interaction domain, a flexibly charged linker region, the middle domain and a dimerization region. It had 80% positional identity with the sequence from *P. nana* (Fig. 1). The charged linker region contributed to the major difference in the amino acid sequences.

NO localization pattern in *B. neritina* larvae

A DAF-FM DA NO-specific probe was used to detect the spatial localization of endogenous NO. This sensitive probe is cell permeable, and is a non-fluorescent reagent that combines with NO to form a fluorescent benzotriazole (Kojima et al., 1999). At the swimming stage, NO was confined to the ciliated corona region (locomotory organ), which covers most of the lateral surface of the larva. From an aboral view, concentrations of NO occurred at the pyriform complex and the neural plate within the apical disc of the larva, which are, respectively, an important sensory organ and the larval nervous system (Fig. 2A).

After treatment with a HSP90 inhibitor (5 µmol l^{-1} geldanamycin), the signal at the pyriform complex and neural plate is clearly weakened (Fig. 2B,C), suggesting that geldanamycin treatment can suppress NO production.

Characterization of HSP90 expression

The expression levels of HSP90 and NOS in *B. neritina* larvae at different stages were analysed by western blot. Both HSP90 and NOS were strongly expressed during the swimming larval stage (Fig. 3A,B). In contrast, lower expression levels were detected at 2, 4 and 24 h post-attachment stages. For HSP90 expression, the lowest level was detected at the 4 h post-attachment stage, but the expression level increased at the 24 h post-attachment stage. Furthermore, the expression level of NOS in swimming larvae dropped significantly in response to all geldanamycin treatments (Fig. 3C).

The involvement of HSP90 and NO in the regulation of larval settlement

Geldanamycin is a potent and specific inhibitor of HSP90 (Blagosklonny et al., 2001; Miyata, 2005). As shown in Fig. 4A, larvae incubated with 1.25, 2.5 and 5 µmol l^{-1} of geldanamycin showed significantly higher settlement rates than the control group after 2 h ($P<0.001$).

However, the settlement of swimming larvae was significantly inhibited by treatment with SNAP, a widely used NO donor, at 200, 400 and 800 µmol l^{-1} ($P<0.001$). As shown in Fig. 5, at all tested concentrations (5, 10, 20 and 40 µmol l^{-1}), carboxy-PTIO induced significant numbers of larvae to settle ($P<0.05$).

To investigate the interaction between HSP90 and NO, swimming larvae were co-incubated with 800 µmol l^{-1} SNAP and serial dilutions of geldanamycin (1.25, 2.5 and 5 µmol l^{-1}). The

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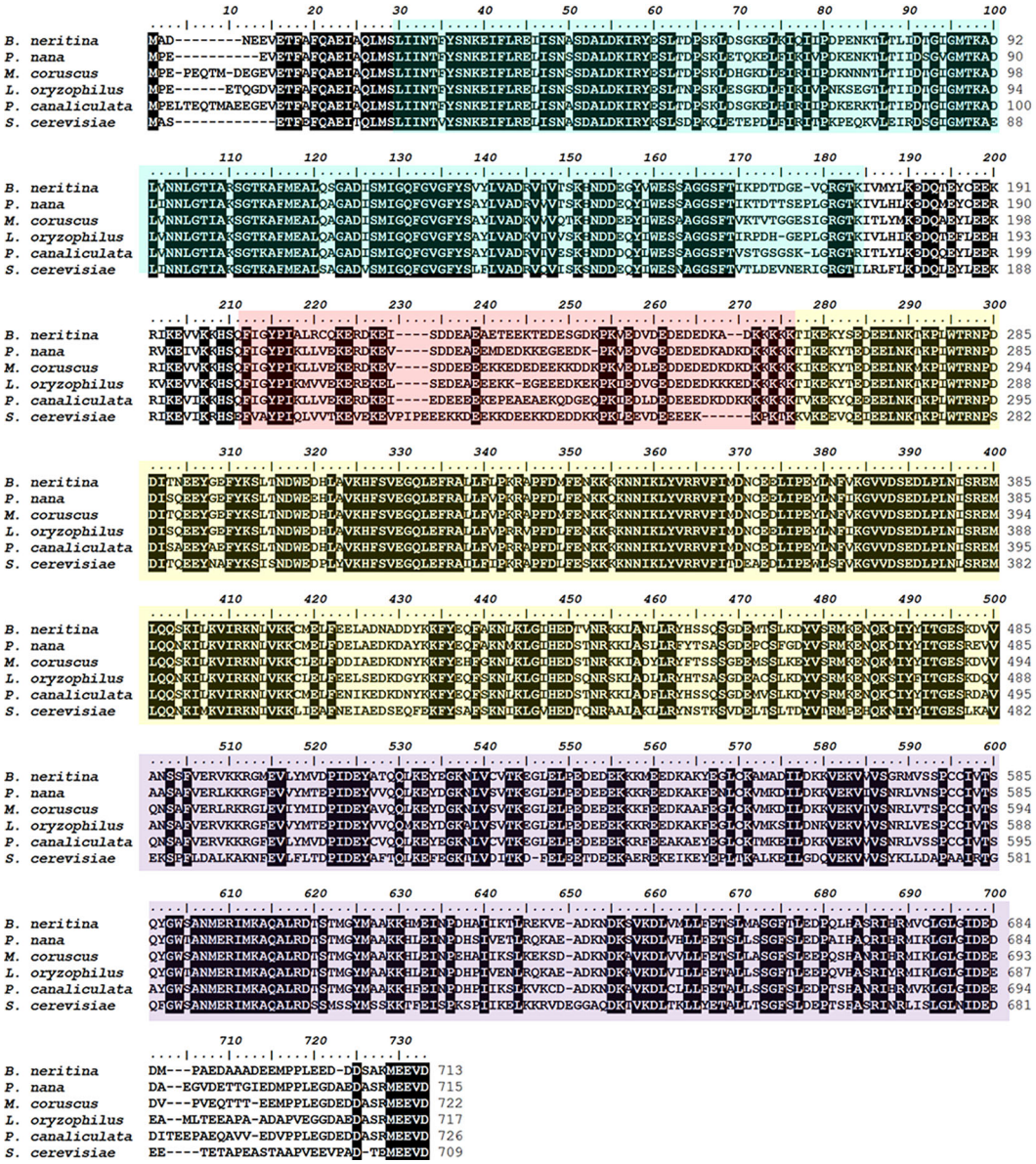

Fig. 1. Multiple sequence alignment of shock protein 90 (HSP90) from *Bugula neritina* and other species. The amino acid sequence of *B. neritina* HSP90 was aligned with homologues from *Paracyclopina nana* (ADV59561.1), *Mytilus coruscus* (ALL27016.1), *Lissorhoptrus oryzophilus* (AHE77376.1), *Pomacea canaliculata* (AIZ03410.1) and *Saccharomyces cerevisiae* (P02829.1). Black blocks illustrate 100% identical residues. The ATPase domain (green), charged linking region (red), middle domain (yellow) and the dimer region (violet) are highlighted by colour blocks.

co-incubation assay showed that geldanamycin had a very strong rescue effect at all concentrations tested (Fig. 6). Even at the lowest concentration (1.25 µmol l^{-1}), geldanamycin attenuated the settlement inhibition effect of SNAP and increased settlement to 30.7±10.6% in comparison with 11.1±4.0% in the SNAP treatment group (P<0.05). At higher concentrations (2.5 and 5 µmol l^{-1}), geldanamycin restored larval settlement to approximately 71% (P<0.01) in comparison with the SNAP-treated group.

DISCUSSION

HSP90 binds to NOS and consequently triggers NO production by mammalian cells (Billecke et al., 2002, 2004; Peng et al., 2012). In marine invertebrates, HSP90 may affect larval settlement by interacting with NOS (Bishop and Brandhorst, 2001; Bishop et al., 2001; Ueda and Degnan, 2013), but this interaction has not been studied widely. Using BLAST analysis, the amino acid sequence of HSP90 from *B. neritina* was found to be conserved. Like HSP90 in other species, it possesses ATP-binding and protein interaction domains, a flexibly charged linker middle domain, and a dimerization domain, as previously demonstrated by Csermely et al. (1998). The HSP90 amino acid sequence from *B. neritina* shares 80% of its positional identity with *P. nana* HSP90 protein. The band from the western blot result coincides with the molecular mass (90 kDa) of HSP90.

Bugula neritina larvae stained with the NO-specific probe DAF-FM DA show strong signals at the neural plate and the pyriform complex regions. Weak signals were also observed at the corona. In swimming larvae, the neural plate within the apical disc extends nerves that reach beneath the pyriform complex (Woollacott and Zimmer, 1971), indicating a connection between these important sensory organs. Strong NO signals in the sensory organ suggest that NO plays a crucial role in signal transduction in *B. neritina* larvae.

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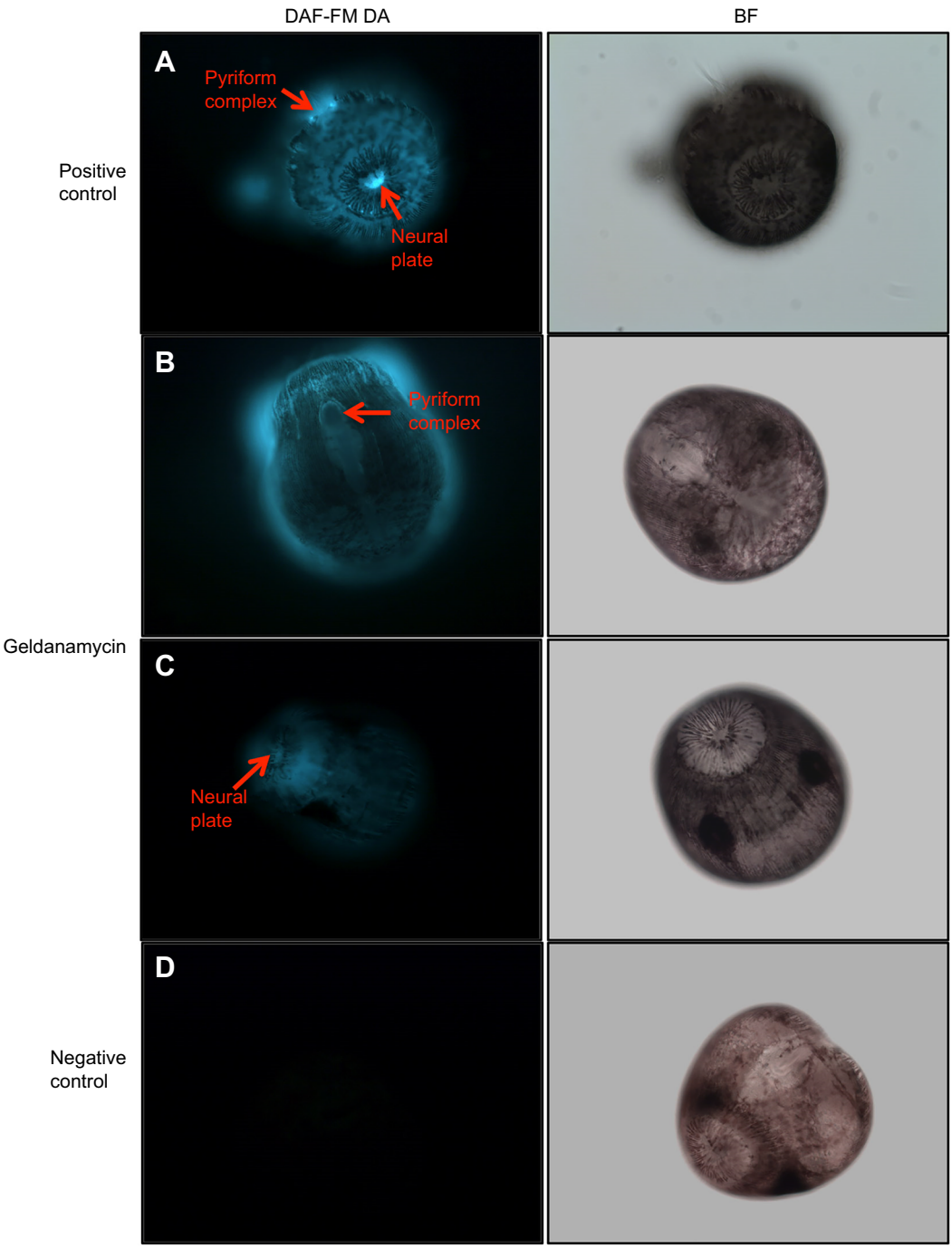

Fig. 2. Detection of endogenous nitric oxide in *B. neritina* larvae. (A) In the positive control, the larvae were stained with the DAF-FM DA probe and strong NO signals were observed at the neural plate and pyriform complex. (B,C) In larvae treated with the HSP90 inhibitor geldanamycin, the signals of NO at the pyriform complex and the neural plate were weakened. (D) No clear signals were detected in the negative control larvae. DAF-FM DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; BF, bright field.

Previous experimental evidence illustrates the involvement of NO in sensory functions in various organisms including the locust, lobster, snail and tunicate (Scholz et al., 1998; Ott et al., 2001; Thavaradhara and Leise, 2001; Leise et al., 2004; Comes et al., 2007). After geldanamycin treatment, the signal in the pyriform complex and neural plate was reduced, implying a decline in NO abundance in these regions of the organs. Shimizu et al. (2000) detected biogenic amines in larval nervous systems of *B. neritina*, in regions such as the apical plate, nerve cord and equatorial nerve ring. The nervous system of swimming larvae may perceive stimulants from the substratum through sensory organs. Thus the hypothesis that reduced NO abundance in the pyriform complex, the major sensory organ and the neural plate of swimming larvae may induce settlement, is consistent with the bioassay results.

The protein expression levels of both HSP90 and NOS declined during larval settlement of *B. neritina*. One reason for the down-regulation of NOS after settlement might be that NO is localized to the neural plate, and NOS is mainly responsible for producing NO in the neural system (Kriegsfeld et al., 1999). However, the larval nervous system of bryozoans is sacrificed after settlement, as the

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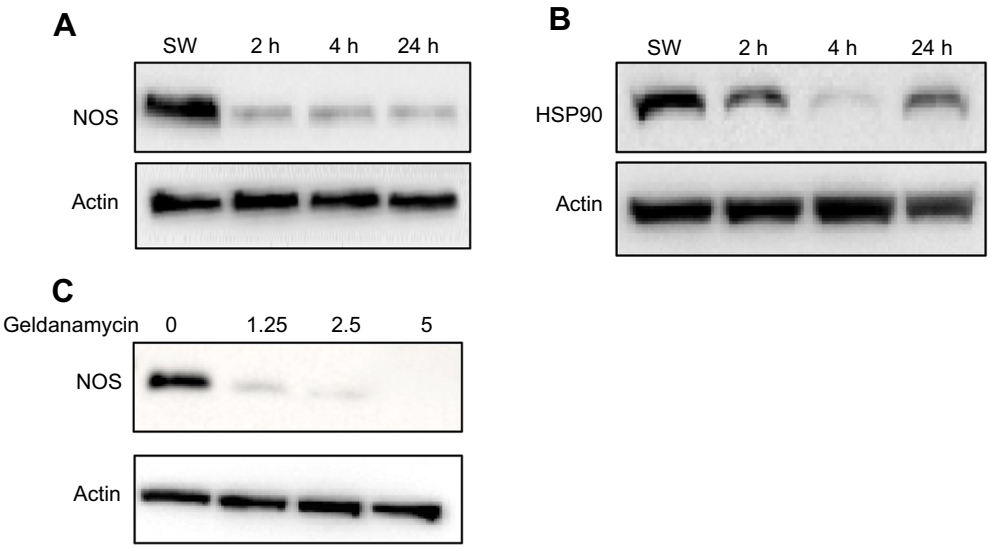

Fig. 3. Characterization of NOS and HSP90 expression at different stages, and NOS expression at several concentrations of geldanamycin in *B. neritina* larvae. Geldanamycin was used at the following concentrations: 1.25, 2.5 and 5 μmol l^{-1}. (A,B) Expression level was highest at the swimming (SW) stage for both NOS and HSP90, and was reduced upon larval settlement, except that HSP90 expression increased at the 24 h stage compared with the 4 h stage. (C) After all treatments with HSP90 inhibitor geldanamycin, the expression level of NOS was reduced in swimming larvae. Expression of actin was stable at all stages and in all experimental treatments.

juvenile nervous system develops (Wanninger, 2008). Another possibility is that high NOS activity and NO levels are essential to maintain the larval stage of *B. neritina.* This is also supported by evidence from the mollusc *Ilyanassa obsoleta*, which also shows a constant NOS expression level throughout the larval stage, but exhibits decreased expression during settlement (Hens et al., 2006). Moreover, the apical ganglion, which is part of the neural system in *I. obsoleta*, displays NOS-like immunoreactivity. As one of the functions of NO is preventing cells from going through apoptosis, Leise et al. (2004) found that the low level of expression of NOS triggered apical ganglion loss during metamorphosis, suggesting the necessity of reduced NOS expression to initiate nervous system breakdown. In terms of HSP90, the decreased expression level after attachment in comparison with swimming larvae is consistent with previous studies in the polychaete *Capitella*, which also shows the highest expression level of HSP90 in competent pelagic larvae, and a decreased expression level upon settlement (Chandramouli et al., 2011). Like *B. neritina*, this species has a biphasic life cycle, during which it undergoes metamorphosis into a benthic juvenile. Chandramouli et al. (2011) argued that non-feeding larvae might experience the destruction of the larval structures as well as constant substratum exploration, and that these factors could lead to oxidative stress, causing higher expression levels of HSP90 in the larval stage. This elevated HSP90 expression may act as a coping mechanism for non-feeding larvae under stressful conditions. After treatment with the HSP90 inhibitor geldanamycin, the percentage of larval settlement of *B. neritina* increased significantly in comparison with the control (Fig. 4). Furthermore, the expression level of NOS decreased in swimming larvae treated with geldanamycin (Fig. 3). It was previously reported that the degradation of HSP90-regulated proteins tended to proceed more rapidly in cells treated with geldanamycin (Pratt, 1998). Bender et al. (1999) also demonstrated that the NOS expression level of mammalian cells decreased significantly when the cells were treated with geldanamycin. Therefore, it is reasonable to suggest that geldanamycin might exert effects on *B. neritina* larvae by accelerating the breakdown of NOS, further reducing NO production, and consequently promoting larval settlement. Another interesting finding is that the lowest expression level of HSP90 was detected 4 h after the post-attachment stage, while the expression level was increased at the 24 h post-attachment stage. This suggests that HSP90 begins to be expressed after metamorphosis as juvenile structures mature. These results are in agreement with a previous study, which indicated that NADPH-diaphorase, an enzyme that histochemically co-localizes with NOS in neurons, shows a similar trend of activity change in several types of ganglia during metamorphosis of the gastropod mollusc *I. obsoleta* (Lin and Leise, 1996).

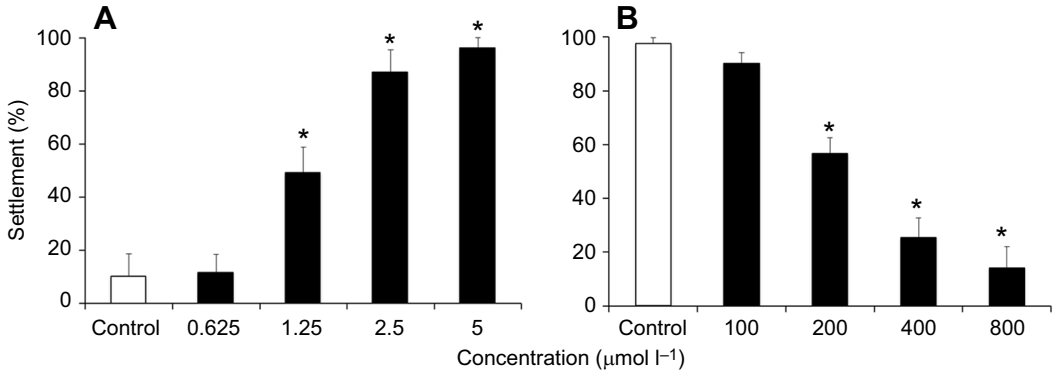

Fig. 4. Bioassays using the HSP90 inhibitor and the NO donor. DMSO (0.4%) in autoclaved filtered seawater was the control condition. Data presented are percentages of larvae settled 2 h after geldanamycin treatment and 4 h after SNAP treatment. Each bar represents the mean±s.d. (*N*=4). (A) Geldanamycin (HSP inhibitor) strongly induces larval settlement at high concentrations (1.25, 2.5 and 5 μmol l^{-1}). (B) Larval settlement was inhibited by SNAP (NO donor) when the concentrations were high (200, 400 and 800 μmol l^{-1}). *Significantly different from the corresponding control (P<0.05).

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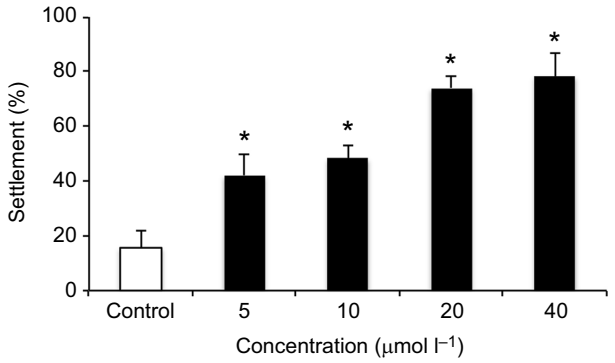

Fig. 5. Inductive effects of a NO scavenger on larval settlement of *B. neritina* larvae. Autoclaved filtered seawater was the control. Data presented are percentages of larvae settled 2 h after treatment. Each bar represents the mean±s.d. Larval settlement was induced by carboxy-PTIO at all concentrations tested. *Significantly different from the corresponding control (P<0.05).

NO inhibits larval settlement in various marine invertebrates (Bishop et al., 2001; Leise et al., 2001; Bishop and Brandhorst, 2003; Comes et al., 2007; Zhang et al., 2012). Our settlement bioassay with the NO donor SNAP revealed that NO inhibits larval settlement of *B. neritina* in a similar manner. In particular, the rate of spontaneous larval settlement significantly decreased in a concentration-dependent manner, after larvae were incubated with SNAP. This result is consistent with previous studies on other marine invertebrates, including Echinodermata, Mollusca, Crustacea and Urochordata (Bishop and Brandhorst, 2001; Hens et al., 2006; Comes et al., 2007; Zhang et al., 2012). Considering the interaction between HSP90 and NOS in mammalian cells (García-Cardeña et al., 1998), we speculated that HSP90 is engaged in the larval settlement process by its interaction with NOS. Also, HSP90 inhibitors can inactivate, destabilize and eventually degrade HSP90 client proteins (Neckers and Ivy, 2003). In this study, treatment with higher concentrations of the HSP90 inhibitor geldanamycin significantly promoted larval settlement. In addition, geldanamycin attenuated the inhibitory effect of SNAP under co-incubation conditions. We assume that the application of geldanamycin inhibited HSP90 synthesis and may have subsequently suppressed NOS activity or affected either the stability or degradation rate of NOS, thus reducing NO production. This reaction would affect the overall endogenous NO levels in larvae and diminish the inhibitory effect of SNAP on larval settlement. In the context of these results, this HSP90 inhibitor probably facilitates larval settlement in *B. neritina* by reducing NO production.

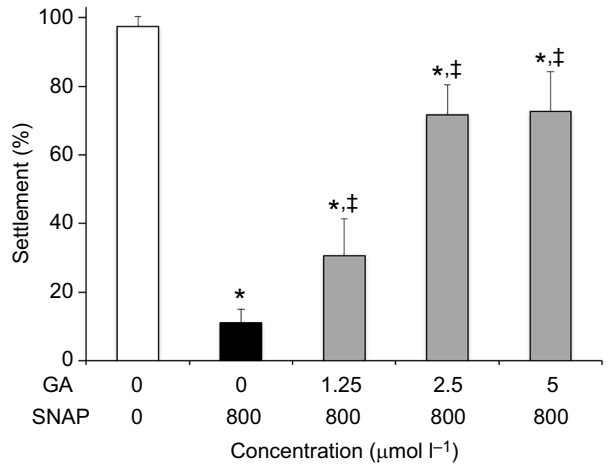

Fig. 6. Co-treatment using a NO donor and the HSP90 inhibitor on *B. neritina* larvae. DMSO (0.4%) in autoclaved filtered seawater was the control. Data presented are percentages of larvae settled 4 h after treatment. Each bar represents the mean±s.d. Larval settlement was significantly inhibited under SNAP treatment (800 μmol l^{-1}). All concentrations of geldanamycin (GA) tested (1.25, 2.5 and 5 μmol l^{-1}) attenuated the inhibitory effect of SNAP and increased larval settlement. *Significantly different from the corresponding control; ‡significantly different from SNAP treatment (P<0.05).

Overall, we revealed that HSP90 along with NO helps to maintain the larval state by repressing larval settlement of *B. neritina*. The application of a HSP90 inhibitor resulted in decreased NOS expression levels, as well as repression of NO production. Furthermore, the localization of NO in sensory organs of the larvae supports our conclusion that NO participates in the metamorphic signalling process. In summary, HSP90 mediates the larval settlement process of *B. neritina* by interacting with the NO pathway.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.Z., P.-Y.Q.; Methodology: X.-X.Y., Y.Z., Y.-H.W., P.-Y.Q.; Software: Y.-H.W.; Validation: X.-X.Y.; Formal analysis: X.-X.Y., Y.Z., Y.-H.W., P.-Y.Q.; Investigation: X.-X.Y., Y.Z., P.-Y.Q.; Resources: P.-Y.Q.; Data curation: Y.-H.W., P.-Y.Q.; Writing - original draft: X.-X.Y.; Writing - review & editing: Y.Z., Y.-H.W., P.-Y.Q.; Visualization: X.-X.Y.; Supervision: P.-Y.Q.; Funding acquisition: Y.Z., P.-Y.Q.

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