Neurotoxin localization to ectodermal gland cells uncovers an alternative mechanism of venom delivery in sea anemones

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Jellyfish, hydrams, corals and sea anemones (phylum Cnidaria) are known for their venomous stinging cells, nematocytes, used for prey and defence. Here we show, however, that the potent Type I neurotoxin of the sea anemone Nematostella vectensis, Nv1, is confined to ectodermal gland cells rather than nematocytes. We demonstrate massive Nv1 secretion upon encounter with a crustacean prey. Concomitant discharge of nematocytes probably pierces the prey, expediting toxin penetration. Toxic efficiency in sea water is further demonstrated by the rapid paralysis of fish or crustacean larvae upon application of recombinant Nv1 into their medium. Analysis of other anemone species reveals that in Anthopleura elegantissima, Type I neurotoxins also appear in gland cells, whereas in the common species Anemonia viridis, Type I toxins are localized to both nematocytes and ectodermal gland cells. The nematocyte-based and gland cell-based envenomation mechanisms may reflect substantial differences in the ecology and feeding habits of sea anemone species. Overall, the immunolocalization of neurotoxins to gland cells changes the common view in the literature that sea anemone neurotoxins are produced and delivered only by stinging nematocytes, and raises the possibility that this toxin-secretion mechanism is an ancestral evolutionary state of the venom delivery machinery in sea anemones.

Keywords: sea anemone; neurotoxin; nematocyst; venom

1. INTRODUCTION

The phylum Cnidaria consists of venomous carnivores such as hydrams, jellyfish, corals and sea anemones [1], of which some comprise risks to human health [2]. Despite unusual morphological and genetic diversity, all cnidarians bear specialized cells named nematocytes that contain miniature stinging devices, nematocysts, used for hunting and defence [3,4]. Nematocysts are highly complex intracellular proteinaceous structures and their release is considered to be among the fastest biological processes in nature [5]. It is widely assumed that the venom of cnidarians is produced in the nematocytes and is injected via the nematocysts upon encounter [1,3]. Direct proof for this assumption relies mostly on chromatographic fractionation of nematocysts from several species [6–8], and on immunolocalization of phospholipases and pore-forming toxins to the nematocysts of a single jellyfish and a single sea anemone species, respectively [9,10]. Intriguingly, the nematocyst-derived fractions were often less toxic than whole tentacle extracts, raising the possibility that toxins may also reside in non-nematocyst compartments [11,12]. Among the better studied cnidarian venom components are the potent sea anemone Type I neurotoxins. These are peptides of 46–51 amino acids that inhibit the inactivation of voltage-gated sodium channels, thus inducing paralysis of prey or predator (reviewed in [13,14]). The starlet sea anemone Nematostella vectensis of the northwest Atlantic serves as a model organism in the study of cnidarians owing to the easily inducible spawning, fast embryogenesis and its completely sequenced genome [15–17]. While studying the evolution and expression patterns of sea anemone neurotoxins, we found that N. vectensis produces a single Type I neurotoxin, Nv1, which is encoded by multiple genes [18]. Moreover, the Nv1 transcript in young life stages of N. vectensis is unspliced, introducing a pre-mature stop codon and raising the possibility that the neurotoxin is expressed solely in adult anemones [19]. This finding is surprising since nematocytes in N. vectensis appear as soon as 24 h after fertilization [20]. Therefore, we examined immunochromatically at which developmental stage the toxin can be detected. This analysis revealed an unexpected cellular localization of Nv1, raising the question as to its mechanism of delivery.

2. MATERIAL AND METHODS

(a) Animal culture

Nematostella vectensis polyps were raised in the laboratory and spawning was induced as previously described [15].

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Anemone viridis polyps were collected at Michmoret, Israel, and kept in artificial sea water at 28 °C in order to minimize the number of symbiotic zooxanthellae. Anthopleura elegantissima polyps were kindly provided by Dr D. Abed-Navandi (Haus des Meeres, Vienna, Austria). The polyps were fed twice a week with freshly hatched Artemia salina nauplii. Zebrafish larvae were kindly provided by Dr Y. Gothilf (Tel Aviv University) and were handled according to standard procedures [21].

(b) Antibody production and enzyme-linked immunosorbent assay (ELISA)

Nv1 was produced in recombinant form using a method previously described for the A. viridis toxin Av3 [22]. Toxin purity was assessed by mass spectrometry. Purified toxin was used to immunize New Zealand white rabbits (performed by Adar Biotech, Israel; the detailed immunization protocol is available upon request from the authors). Specificity of the antisera was assayed by ELISA according to a protocol detailed in electronic supplementary material, figure S1.

(c) Immunohistochemistry and imaging

For whole-mount immunostaining, Nematostella and Anemonia samples were fixed for 1 h in 4 per cent paraformaldehyde in 0.1 per cent Tween-20 in phosphate buffered saline (PBS) (1.86 mM NaH₂PO₄, 8.4 mM Na₂HPO₄, 175 mM NaCl, pH 7.4). After fixation, the samples were washed in 0.1 per cent Tween-20 in PBS and stored in absolute methanol at −20 °C until use. After stepwise dehydration, the samples were blocked for 2 h with 20 per cent sheep serum (Sigma) and 1 per cent bovine serum albumin (BSA) (fraction V) in PBS containing 0.2 per cent Triton X-100. Primary antibodies were added at various dilutions for optimal results (electronic supplementary material, table S1). The samples were then washed five times with 0.2 per cent Triton X-100 in PBS, blocked again, and then incubated with secondary antibodies (electronic supplementary material, figure S1). After overnight incubation at 4 °C, the samples were washed five times with 0.2 per cent Triton X-100 in PBS, blocked again, and then incubated with secondary antibodies (electronic supplementary material, table S1). The samples were then washed five times with 0.2 per cent Triton X-100 in PBS and mounted on microscope slides in SlowFade (Invitrogen, USA) or VectaShield (Reactolab SA, Switzerland). Maceration was performed as described for Hydra [23]. The immunostaining procedure thereafter was similar to that of the whole-mount. The samples were visualized using a Zeiss LSM 510 META scanning confocal microscope (Carl Zeiss, Germany), a Leica TCS SP5 X scanning confocal microscope (Leica, Germany) or a Nikon Eclipse 80i fluorescent microscope.

(d) Protein extraction from tentacles

Tentacles of sea anemones starved for at least 4 days were removed and frozen at −80 °C. After 1 h, the tentacles were thawed on ice and cold non-denaturing extraction buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40 (NP-40), 2 mM ethylenediaminetetraacetic acid (EDTA)) with protease inhibitor cocktail (Roche, Germany) was added. The tentacles were incubated at 4 °C under slow rotation for 3 h and then centrifuged at 20 000g for 10 min. The supernatant was centrifuged through an Amicon Ultra centrifugal filter (Millipore, USA) with a 10 kDa cutoff according to the manufacturer’s instructions. The filtrate was then applied to an Amicon Ultra centrifugal filter (Millipore) with a 3 kDa cutoff and concentrated to a minimal volume. The buffer was then exchanged with 50 mM Tris HCl. Samples were kept at −20 °C until use.

(e) Mass spectrometry

Peptides were separated with an ultraHPLC Eksigent system (Axel Semrau G, bH and CoKG, Germany) using a C₁₈ reversed-phase column (150 mm, 100 μm internal dimensions, Chromolith, Merck, Germany) directly coupled to an Orbitrap XL mass spectrometer (Thermo Scientific, Germany). The peptides were eluted with a 40 min gradient of 0–60% acetonitrile. The Orbitrap was run in Fourier transform full scan positive mode with a resolution of 100 000. The m/z range was set from 600 to 1800 for expected three to five times charged peptide masses.

(f) Nv1 ectopic expression

Primers bearing PciI and AsclI (New England Biolabs, USA) restriction sites were used to amplify by PCR one of the Nv1 genes together with the 1.8 kb region upstream of the transcription start site. The PCR product was cloned at the corresponding restriction sites of a Nematostella expression vector bearing the mCherry reporter gene [24]. To ensure the expression of an Nv1-mCherry chimera, the TAA stop codon of Nv1 and the ATG translation start site of mCherry were removed. A (Gly-Gly-Ser)₂ linker between Nv1 and mCherry was introduced by the PCR primer in order to improve folding. Injections into fertilized Nematostella oocytes were performed as previously described [24].

3. RESULTS AND DISCUSSION

(a) The Nv1 neurotoxin of Nematostella vectensis is localized to non-nematocyte cells

In order to localize Nv1 expression in N. vectensis, we used the anti-Nv1 serum raised in rabbits. Whole-mount immunohistochemistry revealed no staining at early life stages such as the 3–6 day-old planula larvae (figure 1), which was in accordance with our previous observation at the RNA level [19]. In 9 day-old primary polyps, which settle and begin to hunt, a few cells containing Nv1-positive vesicles were observed in the four primary tentacles (figure 1), whereas numerous cells were stained in the tentacles of the adult polyp (figures 1 and 2a, and electronic supplementary material, movie S1). The staining did not co-localize with the numerous nematocytes easily observed by light microscopy owing to their dense nematocysts (figure 1c). Interestingly, some toxin appeared in clumps outside of the ectodermal layer (figure 2a and electronic supplementary material, movie S1).

While tentacle nematocytes in N. vectensis are thin and long like in other sea anemone species (electronic supplementary material, figure S2), the anti-Nv1-stained cells are thick, slightly round and filled with vesicles positive for the antibody (figures 1 and 2b), strongly suggesting that the Nv1-positive cells are gland cells. To validate these results, we co-stained Nematostella tentacles with anti-Nv1 and antibodies against the tubule component chondroitin and the capsule component Nv-Ncol 3, two
molecular components detectable at early developmental stages of nematocysts [25–27]. No co-localization was observed and each antibody stained distinct structures in the tentacle, precluding the possibility that the anti-Nv1-stained cells are developing nematocytes that contain vesicles (figure 2b,c). To verify the observations made on the whole-mounts, we immunostained tentacle macerates. As shown in figure 2d and electronic supplementary material, figure S3, the cells containing the Nv1 toxin indeed differ substantially from nematocytes in shape, and they lack nematocysts. Granule-rich gland cells that highly resemble the immunostained cells are common in the Nematostella tentacle ectoderm ([28]; electronic supplementary material, figure S2) and were also described in tentacles of other sea anemone species [29–31]. Although accessibility of nematocytes for antibody staining is highly increased upon maceration with acetic acid that leads to their discharge, not a single nematocyst stained with anti-Nv1 was observed in the macerated Nematostella tissues.

(b) Localization of Type I neurotoxins in other sea anemone species

Anemonia viridis (previously named Anemonia sulcata) is a common Mediterranean sea anemone species distantly related to N. vectensis [32]. It produces several Type I neurotoxins with moderate sequence similarity (approx. 50%) to Nv1 [14]. Nevertheless, the structure of Av2, the most abundant A. viridis neurotoxin, resembles that of Nv1 [19] and the anti-Nv1 antibody recognizes recombinant Av2 in ELISA (electronic supplementary material, figure S1).

Whole-mount anti-Nv1 staining of A. viridis tentacles revealed structures resembling the Nv1-containing gland cells described for N. vectensis (figures 3a and electronic supplementary material, figure S3). However, these cells were less abundant in A. viridis. In addition to the gland cells, numerous long threads were stained in the tentacle. Induced discharge of nematocysts by acetic acid substantially increased the number of the stained threads (figure 3b). Maceration of tentacles enabled a better examination of the gland cell structure (figure 3c) and also revealed that the stained threads are tubules of discharged nematocysts (figure 3d). Although the Nv1 antibody stained mostly the discharged nematocysts, several charged nematocysts were still accessible to the antibody (figure 3e), possibly owing to fractures in the capsule wall during maceration. Indeed, nematocytes in 10 μm thick tentacle cryosections were stained (figure 3f). The staining pattern in these stinging cells suggests that the toxin is associated with the tubule prior to discharge (figure 3e). The coiled tubule and the nematocyst structure indicate that the stained capsules are microbasic b-mastigophores, the nematocyte type previously identified in A. viridis tentacles (figure 3e) [33]. Toxin staining in these cells is restricted to the tubule and is not observed in the barbed shaft at the base of the tubule (figure 3d). This staining pattern supports the suggestion by Klug et al. [34]; that the venom is distributed along the tubule. It is important to note that various antibodies were reported to adhere non-specifically to capsules and threads (e.g. [35]), but in our experiments, the pre-immune serum and secondary antibodies did not stain nematocyst threads without anti-Nv1, indicating specific staining of Type I toxins (electronic supplementary material, figure S4).

The common pacific species A. elegantissima is much closer to Anemonia than to Nematostella based on morphological traits and molecular phylogeny (figure 4a) [32]. It produces several Type I neurotoxins with remarkable homology to Av2 (up to 96% identity and 98% similarity) [36]. We macerated A. elegantissima tentacles and stained them with the anti-Nv1 serum. Surprisingly, no stained nematocyst threads were observed, whereas clear staining was found in vesicle-containing cells, resembling the staining of the N. vectensis Type I toxin (figure 4b).

(c) Further verification of toxin localization to gland cells
The localization of Type I toxins to ectodermal gland cells rather than nematocytes was also analysed using fractionation and mass spectrometry. The tentacle protein was extracted without disruption of nematocyst capsules,
and following size-selection was analysed by mass spectrometry. Mass to charge ratios (m/z) identical or similar to those of the known Type I toxins of N. vectensis, A. viridis and A. elegantissima were detected in each of the corresponding samples (figure 4 and electronic supplementary material, figure S5). To further verify the specificity of the anti-Nv1 antibody staining, we performed a transgenesis assay. A measure of 1.8 kb of the sequence upstream of the Nv1 transcription start site was used to drive the expression of the Nv1::mCherry fusion (electronic supplementary material, figure S6). Injection of this construct into fertilized N. vectensis eggs [24] resulted in transient expression of mCherry in random patches 24 h after fertilization, which did not resemble the native expression pattern of Nv1 (electronic supplementary material, figure S6). A similar phenomenon was also observed for sequences upstream of several other Nematostella genes and may result from the absence of other regulatory elements (U. Technau 2011, unpublished results). Nevertheless, these embryos were fixed and double-stained with anti-mCherry and anti-Nv1 antibodies (electronic supplementary material, figure S6). Since the earliest endogenous expression of Nv1 protein is observed in the primary polyp (figure 1), it is clear that all Nv1 protein at the gastrula stage can only have transgenic origin. The signals of anti-Nv1 and

Figure 2. Nv1 is accumulated in gland cells of N. vectensis and is released to the water during preying. Nv1 antibody and an Alexa Fluor-conjugated secondary antibody were used to localize Nv1 (appear in red). Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue). (a) Nv1 is localized to thick gland cells inside the tentacle and to clumped structures at the tentacle surface (indicated by arrowheads). (b) Double immunostaining of Nv1 (red) and chondroitin (green; a proteoglycan involved in nematocyst tubule formation) shows that these molecules are also localized to different cells. Cells with Nv1-positive vesicles are designated by arrowheads. (c) Double immunostaining of Nv1 (red) and Nv-Ncol 3 (green; a minicollagen component of the nematocyst capsule) clearly shows that these peptides are localized to different cells. (d) An Nv1-containing gland cell in N. vectensis tentacle macerate. The vesicular structures suggest toxin accumulation in vesicles. (e,f) Nomarski and fluorescent images localize Nv1 to the surface of interaction between N. vectensis tentacles (shown as Nv) and Artemia salina (shown as As) during feeding. Scale bars: (a) 100 μm; (b) 25 μm; (c) 10 μm; (d) 20 μm and (e,f) 50 μm.
anti-mCherry clearly co-localized in the transgenic embryos and supplied sound evidence for the specificity of the Nv1 antibody for the toxin in the immunolocalization experiments (electronic supplementary material, figure S6).

**d) Delivery of sea anemone neurotoxins**

To gain further insight on neurotoxin delivery in *N. vectensis*, tentacles were fixed during and after predation of *A. salina* nauplii. The tentacles that *a priori* were charged with toxin were nearly depleted of stained gland cells upon contact with the crustacean, and in some preparations, the surface of the tentacle was rich in stained large granules located outside of the cellular layer (electronic supplementary material, figure S7). The intense staining observed at the tentacle surface and in the narrow space between the tentacle and the prey suggested toxin secretion during predation (figure 2e,f; electronic supplementary material, movie S2 and figure S7).

Figure 3. *Anemonia viridis* Type I neurotoxins are localized to both gland cells and nematocytes. Nv1 antibody and an Alexa Fluor 568-conjugated secondary antibody were used to localize Nv1 homologues in *A. viridis* (appear in red). Nuclei were stained with DAPI (blue). (a) In whole-mount tentacles, anti-Nv1 staining is observed in thick gland cells. (b) After treatment with acetic acid, numerous thread-like structures are stained in whole-mount tentacles. (c) Maceration of *A. viridis* tentacles reveals the structure of the gland cells (indicated by an arrowhead) and suggests that the neurotoxin is packed in vesicles (a non-merged image is provided as an inset). (d) Maceration reveals that the thread-like stained structures are tubules of *A. viridis* nematocysts (shown as Tu). Unlike the tubule, the capsule and the barbed shaft (indicated as Ca and Sh, respectively) are not stained. (e) The Nv1 immunostaining in charged nematocytes indicates that the tubule is coiled, and that the neurotoxin is probably packed on or inside the tubule prior to discharge (a non-merged image is provided as inset). (f) Cryosectioning of the tentacle enables anti-Nv1 immunostaining of a charged nematocyte (indicated by an arrowhead) without maceration. Scale bars: (a,b,d,f) 20 μm; (c) 50 μm and (e) 10 μm.

**Envenomation mechanisms in sea anemones**

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To examine the possibility that the toxin released into the medium affects the prey, we exposed intact *Artemia* nauplii to recombinant neurotoxin (0.5 mg ml\(^{-1}\) Nv1 or Av2). Notably, impaired swimming, paralysis and death were observed within several hours, while 5 mg ml\(^{-1}\) BSA had no effect on control animals (electronic supplementary material, movie S3). Intoxication of zebrafish (*D. rerio*) larvae by Av2 was much faster than *Artemia* nauplii, and 10 min after exposure to 0.5 mg ml\(^{-1}\) toxin, all fish were either paralysed or exhibited strong convulsions and impaired swimming (electronic supplementary material, movie S4). Identical doses of Nv1 caused tail twitching accompanied by impaired swimming after 20 min and complete paralysis within 45 min. By contrast, fish exposed to 5 mg ml\(^{-1}\) BSA (control) were active during the entire experiment and did not exhibit any of the symptoms induced by the toxins. Moreover, application of the weakly active mutant toxin Av2\(^{L5A}\) slightly affected the fish only after several hours [37]. Thus, Type I neurotoxins can penetrate into prey even without nematocysts and reach their sodium channel targets. However, the fast paralysis of prey within seconds of interaction with the tentacle, compared with the slower paralysis observed in our experiments using recombinant toxins, suggests that piercing by the numerous discharging nematocysts expedites toxin delivery during capture of prey.

In the light of their phylogeny and living habitat, the differences in toxin localization and mechanism of venom delivery between *Nematostella* and *Anthopleura* to *Anemonia* despite contrasting phylogeny (figure 4a) suggest that the ecology of these species might have played a major role in the development of the venom apparatus. It is likely that nematocytes loaded with toxins are more efficient in envenomation compared with the gland cell secretion mechanism. Both *N. vectensis* and *A. elegantissima* feed on a limited variety of small animals [38–40], whereas *A. viridis* exhibits a more active feeding behaviour and preys on a wider variety, including larger animals [41]. Moreover, while *N. vectensis* and *A. elegantissima* avoid other species by contraction and *Nematostella* can burrow in the substrate, *A. viridis* is unable to contract and is exposed in its rocky environment and attacked by nudibranch molluscs [42]. The different ecologies of *N. vectensis* and *A. viridis* are also reflected by their different neurotoxin content, with a much greater arsenal in *Anemonia* [14,18]. Previous studies have already shown that diet and ecology probably affected the evolution of the venom arsenal and delivery devices in reptiles [43,44]. Since the complex venom production and delivery mechanisms require considerable energetic resources, but are crucial for survival, ecological shifts might have imposed a strong selective pressure on these systems to either evolve or atrophy. Whether the production of neurotoxins in gland cells of *A. viridis* is an additional mechanism of venom delivery or a remainder of an ancestral delivery system is still unknown. In a previous study, chromatographic analysis of *Stichodactyla gigantea* fractions suggested that a major fraction of the
neurotoxins, including a Type I toxin are located in an unknown tentacle compartment other than the nematocyte [45]. Thus, it is possible that neurotoxin-producing gland cells are common in most actinian species. Moreover, neurotoxins have been isolated from tentacles of cubozoan and scyphozoan cnidarians and although their cellular origin was unclear, they were not derived from nematocytes as was shown chromatographically [11,12,46]. This may suggest that an alternative venom delivery system also exists in non-actinian cnidarians. Yet, such an assumption requires the study of toxin localization in other cnidarian classes, including non-actinian anthozoans.

It has been shown that both gland cells and nematocytes in Hydrozoa are derived from interstitial stem cells (for review see Bode [47]). Although the common cellular origin of gland cells and nematocytes has not been shown in Anthoza, the fact that Golgi vesicles are involved in both secretion from gland cells and also in nematocyte morphogenesis raises a possibility for a common evolutionary ancestry of these two seemingly distinct venom-containing cells. Secretion of neurotoxins by gland cells is a slower delivery mechanism in comparison with nematocyst discharge and therefore it is hard to imagine that cnidarians would give up on the fast mechanism in favour of a slow delivery mechanism. On the other hand, secretion by gland cells may allow for delivery of larger amounts of the toxin and a recovery of the toxin-synthesizing cell, whereas nematocytes are single-use cells. Thus, whether venom-secreting ectodermal gland cells are an adaptation to the environment and the food regime in some anemones, or whether they actually represent the ancestral venom-secreting cell type, remains to be shown by the investigation of more anthozoan species.

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