Innexin gap junctions in nerve cells coordinate spontaneous contractile behavior in Hydra polyps

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Nerve cells and spontaneous coordinated behavior first appeared near the base of animal evolution in the common ancestor of cnidarians and bilaterians1–3. Experiments on the cnidarian Hydra have demonstrated that nerve cells are essential for this behavior, although nerve cells in Hydra are organized in a diffuse network and do not form ganglia. Here we show that the gap junction protein innexin-2 is expressed in a small group of nerve cells in the lower body column of Hydra and that an anti-innexin-2 antibody binds to gap junctions in the same region. Treatment of live animals with innexin-2 antibody eliminates gap junction staining and reduces spontaneous body column contractions. We conclude that a small subset of nerve cells, connected by gap junctions and capable of synchronous firing, act as a pacemaker to coordinate the contraction of the body column in the absence of ganglia.

**Results**

Innexin-2 is expressed in gap junctions in nerve cells in the peduncle of Hydra. In Hydra large gap junction plaques are present between ectodermal epithelial cells and between endodermal epithelial cells4–6 and these junctions have been shown to mediate dye coupling and electrical coupling9. Gap junctions are also formed between ectoderm and endoderm via thin cytoplasmic tubules connecting epithelial cells across the mesoglea9. Finally, gap junctions have been documented between nerve cells in Hydra, although the reported numbers were small11. We have screened EM thin sections for gap junctions between nerve cells and found examples in all parts of Hydra. Figure 1A shows a typical nerve-nerve gap junction in peduncle tissue, which will be the focus of this
Six innexin monomers form a hemi-channel in the cell forming two extracellular loops containing conserved cysteine residues which are membrane proteins with four transmembrane domains forming a protein with two extracellular loops and N- and C-terminal ends located intracellularly (Figure 2A). Four conserved cysteine residues are present in the first loop and 2 cysteines in the second loop.

A screen of innexus gene expression in Hydra by in situ hybridization showed that innexus-2 is expressed in a population of nerve cells in the lower peduncle of adult polyps (Figure 1C–E). Innexus-2 positive nerve cells were also present in the peduncle of late stage buds (Figure 1C, left side) but not in earlier stage buds (Figure 1C, right side). To localize innexus-2 protein to gap junctions in these cells, we prepared an antibody to the first extracellular domain of innexus-2. The antibody stained recombinant innexus-2 in western blots (Figure 2B) and also in Hydra tissue transfected with an innexus-2 gene (Figure 2C–F). To localize innexus-2 in tissue we carried out immunofluorescence staining on whole mounts of fixed Hydra. Figure 2 shows confocal images from different positions along the body column. Numerous small spots of innexus-2 staining were observed in the peduncle region but not further up the body column (Figure 21–K). The spots varied considerably in size and were frequently arranged in chains (Figure 2L) localized along nerve cell processes (Figure 2M and 3E). Individual innexus-2 spots were 300–500 nm in diameter and thus similar in size to the nerve-nerve gap junctions visualized in peduncle tissue by EM (Figure 1A). Quantitative scoring of innexus-2 gap junctions per peduncle led to values of 2000–3000 per peduncle. Since there are about 100 innexus-2 positive nerve cells in the peduncle (Figure 1D–E), we conclude that there are 20–30 gap junctions per nerve cell, i.e. the cells are well connected electrically and could fire synchronously.

To confirm that the innexus-2 spots were localized to nerve cells, we co-stained the animals with an anti-tyrosine-tubulin antibody, which has been shown previously to stain nerve cells in hydrozoans. The images in Figure 2M and 3D–E show that innexus-2 spots are closely associated with the tubulin-stained processes of nerve cells. We also carried out immunogold staining of EM thin sections. Figure 2H shows a patch of gold particles about 100 nm long representing an innexus-2 gap junction (compare to Figure 2G). Such patches were found in peduncle sections, but not in sections from the gastric region.

Nerve cells expressing innexus-2 gap junctions coordinate contraction of the body column. Hydra polyps in an undisturbed dish in the dark exhibit spontaneous behavior, contracting regularly 7–10 times per hour (see Figure 4A). Each contraction consists of a rapid series of strong contractions of the ectodermal epitheliomuscle cells oriented along the long axis of the polyp. This behavior has been termed a contraction burst and is accompanied by large electrical signals. Removal of nerve cells from Hydra tissue completely eliminated spontaneous contraction bursts in the body column. Such nerve-free polyps were nearly motionless for hours. Nevertheless, such polyps still responded to strong mechanical stimulation (pinching with forceps) with propagation of electrical signals and coordinated contraction of the body column. Thus contraction of the body column is regulated at two levels: (1) epitheliomuscle cells are connected by gap junctions and can propagate an electrical signal mediating contraction in response to exogenous stimuli and (2) spontaneous initiation of epitheliomuscle cell contraction is controlled by the nervous system, since it is absent in nerve-free animals.
To investigate the role of innexin-2-coupled nerve cells in initiating spontaneous contractions, we treated live animals with innexin-2 antibody in the presence of 0.05% DMSO to facilitate antibody access to tissue. Quite strikingly, after 3 days of treatment innexin-2 gap junctions could no longer be stained in the peduncle region of treated animals (Figure 3A–C). By comparison, DMSO treated control animals contained innexin-2 gap junctions (Figure 3D–E). While we had expected that the innexin-2 antibody would bind to and inhibit

Figure 2 | Immunofluorescent staining of innexin-2 in gap junctions in Hydra. (a) Schematic drawing of the predicted structure of hydra innexin showing four transmembrane domains and conserved cysteine residues in the extracellular (ec) loops. N- and C-terminus are intracellular (ic). The first extracellular loop of innexin-2 (aa48–134) was used for antibody production. (b) Purified recombinant GFP-tagged innexin-2 (aa48–134) from E.coli was detected by the innexin-2 antibody at the appropriate size (ca. 35 kD) in immunoblot. The antibody did not detect GFP. (c–f) Ectopic expression of GFP (used as a transfection marker) and untagged innexin-2 in hydra epithelial cells transfected with the particle gun. To visualize innexin-2 expression, animals were fixed and stained with innexin-2 antibody 48 hours post-transfection. Transfected GFP-expressing epithelial cells displayed a punctate innexin-2 pattern in immunofluorescence when co-transfected with innexin-2 (d). Control animals transfected with GFP only have no detectable innexin-2 signal (f). Scale bar: 10 μm. (g) and (h) Immunogold staining of innexin-2 gap junction in peduncle tissue. (g) TEM image of a typical gap junction (enlarged from Fig. 1A); (h) immunogold staining of innexin-2 gap junction in peduncle tissue. Scale bar: 100 nm. (i–k) Immunostaining of whole animals with innexin-2 antibody revealed innexin-2 positive green spots primarily in the peduncle region (k); more apical areas in the peduncle showed decreased amounts of antibody staining (i and j); the gastric region contained no innexin-2 positive green spots. (l) High magnification image of an innexin-2 positive cell in the peduncle region. The innexin-2 positive green spots were often clustered as strings along nerve processes. Nuclei stained with DAPI. Projections of confocal images covering a depth of 2–3 μm. Scale bar: 10 μm. (m) Co-immunostaining of hydra with innexin-2 antibody and tyrosine-tubulin antibody (Sigma) showed that innexin-2 staining in nerve cells was localized along nerve cell processes. The anti tyrosine-tubulin staining in nerve cell nuclei is regularly observed but appears to be an artifact (see also Figure 3). Nuclei stained with DAPI. Projections of confocal sections covering a depth of 2 μm. Scale bar: 10 μm.
gap junctions, it appears to eliminate the junctions altogether. This loss of gap junctions was also observed in EM sections of animals treated with innexin-2 antibody.

To analyze the behavior of antibody-treated animals, we made time-lapse videos of undisturbed animals in red light, which Hydra cannot detect (See Supplemental movie 1 and 2). Figure 4A shows that DMSO-treated control Hydra contracted roughly every 5 minutes during a one-hour interval. By comparison, polyps treated with innexin-2 antibody contracted less frequently, about once every 10–12 minutes (Figure 4B). Furthermore, the contraction bursts were markedly shorter than in control animals. These results suggest that nerve cells containing innexin-2 gap junctions are required to initiate and coordinate contraction bursts.

To confirm that the innexin-2 antibody did not affect the intrinsic ability of the body column to contract, we used a mechanical stimulation assay. When normal Hydra were pinched with a pair of forceps, the animals responded with rapid contraction of the body column (Figure 5A). Contraction was also detected when nerve-free animals were pinched (Figure 5B), indicating that nerve cells are not required to coordinate body column contraction. Animals treated with innexin-2 antibody also showed normal contractions after pinching (Figure 5C) indicating that treatment with innexin-2 antibody did not affect the ability of the body column to contract when mechanically stimulated. From these results we conclude that the inhibitory effect of antibody treatment on spontaneous contractile activity is due primarily to inhibition of the neuronal signal initiating contraction.

The above experiments show that nerve-free animals contract when mechanically stimulated by pinching. To confirm the role of gap junctions between epithelial cells in this contractile behavior we conducted further experiments using heptanol to inhibit gap junction communication. In the presence of 0.06% heptanol, contraction of normal animals stimulated by pinching was completely inhibited (Figure 5D). Contraction of nerve-free animals stimulated by pinching was also completely inhibited (Figure 5E). From these results we conclude that signaling between epithelial cells via gap junctions is required for contraction of the body column. In agreement with this conclusion, heptanol-treated animals also showed fewer spontaneous contractions and shorter contraction bursts than untreated control animals (Figure 4C).

**Discussion**

The present experiments have identified a population of nerve cells in the peduncle of Hydra that are linked by innexin-2 gap junctions. Furthermore, treatment of Hydra with an anti-innexin-2 antibody reduced spontaneous contractions of the body column but did not affect the intrinsic ability to contract when mechanically stimulated.
Cells are coupled via gap junctions and thus can fire in synchrony. Unable to identify with certainty the cellular basis for the activity. They referred to this activity as a pacemaker but were controlled periodic contractions of the body column, so-called contraction bursts. If this conclusion is correct, then removal of peduncle tissue from Hydra should remove the pacemaker and inhibit periodic contraction of the body column. Experiments of Shimizu and Fujisawa support this conclusion. They found that the frequency of contraction bursts was significantly reduced when peduncle tissue was excised. More than 50 years ago, electrophysiological experiments carried out by Passano and McCullough demonstrated rhythmic potentials (RPs) originating in the peduncle of Hydra and showed that they controlled periodic contractions of the body column, so-called contraction bursts. They referred to this activity as a pacemaker but were unable to identify with certainty the cellular basis for the activity. Based on results reported here, innexin-2-positive nerve cells in the peduncle of Hydra are the source of the pacemaker activity. These cells are coupled via gap junctions and thus can fire in synchrony. Hence they can signal ectodermal epitheliomuscle cells (the effector cells) around the periphery of the body column and thereby induce synchronous contraction of the body column.

Spontaneous rhythmic behavior is a characteristic feature of the earliest evolving animal phyla. Cnidarian medusae in particular show rhythmic swimming movements while polyps exhibit periodic contractile movements. In spite of this superficial similarity, the organization of the nervous system and the control of behavior are fundamentally different between different classes within the phylum Cnidaria. Hydrozoans have nerve cells with well-developed gap junctions and there is clear evidence of electrically coupled cohorts of nerve cells, which control behavior. By comparison, anthozoans and scyphozoans lack gap junctions and there is no evidence for electrical coupling between nerve cells. This difference in the occurrence of gap junctions is also reflected in the occurrence of innexin genes, which encode the proteins that make up gap junctions. The innexin gene family is highly expanded in both Hydra and Clytia, the two hydrozoans for which we have extensive EST and genomic databases. By comparison, the sequenced genomes of anthozoans lack clear evidence of innexin genes. Although the genome of the sea anemone Nematostella does contain a single innexin-like sequence, the encoded protein is more closely related to mammalian pannexins, which form hemi-channels and not gap junctions, than to innexins. No innexin related sequences could be identified in the genome of the coral Acropora digitifera. Thus, the striking expansion of the innexin gene family in hydrozoans and the extensive formation of gap junctions between different cell types, including nerve cells, appear to be unique to this group of cnidarians and may have enabled development of behavioral activities, which were advantageous for a wide variety of environmental niches.

**Methods**

**Strains and culture.** A standard wild-type strain of Hydra magnipapillata (strain 105) was used in the present study. Hydra were cultured at 18 ± 1 °C as described by Sugiyama and Fujisawa. They were fed on newly hatched brine shrimp nauplii six times a week. Experimental animals were starved for 24 hours prior to use and not fed during the experiment.

**Whole-mount in situ hybridization.** In situ hybridization was carried out as described by Grens et al. The concentration of the riboprobe used for hybridization varied from 50 to 200 ng/ml.

**Transmission electron microscopy.** Animals were fixed for 12 hours in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4), followed by three washes of 10 minutes each in 0.1 M cacodylate buffer (pH 7.4) containing 4% sucrose. The animals were then postfixed for 60 minutes in ice-cold 1% OsO4 in the same buffer and washed three times for 10 minutes each in ice-cold distilled water. Dehydration through a graded series of ethanol solutions was followed by embedding in an Epon-Araldite mixture. Ultra-thin sections (approximately 70 nm) were made, and stained with 2% uranyl acetate followed by 0.4% lead citrate for 5 minutes each. Samples were observed using a JEOL transmission electron microscope (JEM-1010, 80 kV). For immuno-electron microscopic observations, the post-embedding method of Hwang et al was used.

**Antibody generation.** The putative first extracellular loop (amino acids 48–134) of innexin-2 was cloned into the unique NotI and SalI sites of an Escherichia coli overexpression vector, pET28a (Novagen, Madison, WI). After verifying the insertion by sequencing, the plasmid was transformed into Escherichia coli BL21 (DE3). Overexpression of recombinant innexin-2 was induced with IPTG (isopropyl-1-thio-
3-D-galactoside) and the soluble protein was purified on Ni-NTA agarose (Invitrogen, Carlsbad, CA). Eluted fractions containing recombinant innexin-2 peptide were then subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A single band of 15 kD was isolated from the gel and delivered to OPERON Biotechnologies, K.K. (Tokyo, Japan) to raise a polyclonal antibody in rabbit. The polyclonal antibody was affinity-purified by protein A-Sepharose.

**Immunofluorescence staining.** Hydra polyps were relaxed with 2% urethane, fixed with 2% paraformaldehyde in hydra medium for 1 hour and then permeabilised in 0.5% Triton X-100/PBS for 15 minutes. Blocking was performed in 0.1% Triton X-100 and 1% BSA in PBS for 16 hours. Rabbit anti- innexin-2 antibody (see above) and mouse monoclonal anti-Tyrosine-Tubulin antibody (T9028, Sigma) were diluted 1:200 in blocking solution. Alexa488 anti-rabbit and Cy3-coupled anti-mouse antibodies were used as secondary antibodies. Antibody incubations were performed at room temperature for 1 hour.

Confocal images were acquired with a Leica (Leica Microsystems, Wetzlar, Germany) TCS SP confocal laser-scanning microscope as described previously.

**Behavior experiments.** The dynamic movements controlled by the peduncle in hydra were examined under a light microscope (Nikon OPTIPHOT). The observation was recorded with a Hi-band 8-mm formatted video recorder (SONY, EVO-9500A) through a CCD TV camera, which accepted visible and infrared light (Hitachi, KV-26). Experimental animals were placed in a plastic dish containing 10 ml of culture solution and the entire procedure was carried out at 18 ± 1 °C. Tentacles were cut off 3 hours before experiments to reduce the movement of animals around the dish. The animals were incubated for 3 days with a 300-fold dilution of the antibody in hydra culture medium containing 0.05% DMSO. Control animals were also treated with 0.05% DMSO for 3 days. These solutions were changed every 12 hours. In some
experiments, animals were treated with heptanol (0.06%; Wako) and, after incubation for 1 hour, analyzed for contractility activity.

**Plasmids.** Full-length innexin-2 was amplified from Hydra cDNA by PCR and subcloned in the E.coli expression vector by using the NheI and Smal restriction sites. Site-directed mutagenesis was used to introduce two adjacent stop-codons in between the innexin-2 open reading frame and the GFP sequence. This resulted in expression of untagged full-length innexin-2 after transfection into Hydra cells.

**Transfection of Hydra cells.** Hydra cells were transfected with a particle gun as described elsewhere.

**Figure 5** Contractile behavior induced by mechanical stimulation. (a–e) Contraction of the body column was induced by pinching with forceps. Control hydra (a), nerve-free hydra (b), innexin-2 antibody treated hydra (c), heptanol treated normal hydra (d) and heptanol treated nerve-free hydra (e). The number of animals responding with body column contraction is shown on the right.
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Author contributions
Y.T., A.W., A.B., C.N.D. and T.G. planned the experiments. Y.T., J.S.H., A.W. and H.S. performed the experiments. Y.T., A.W., C.N.D. and T.G. wrote the paper.

Additional information
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CORRIGENDUM: Innexin gap junctions in nerve cells coordinate spontaneous contractile behavior in Hydra polyps

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The Supplementary Movies that accompany this study were omitted from the original version of this Article.