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Cloning and Expression of Hydra Innexin 2, a Gap Junction Protein Required for Coordinated Contraction of the Body Column

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Cloning and Expression of Hydra Innexin 2, a Gap Junction Protein Required for Coordinated Contraction of the Body Column

by

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ABSTRACT OF THESIS

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In invertebrates gap junctions are formed by the innexin family of proteins. Remarkably, the genome of *Hydra magnipapillata* contains 17 innexin genes. This study focused on *Hydra* innexin-2 (h-Inx2) which is expressed in nerve cells and plays a role in contraction of the body column. The gene sequence of H-Inx2 was obtained from the National Center for Biotechnology Information (NCBI), the gene was synthesized externally and transferred to a vector suitable for expression in *Xenopus* oocytes (pcDNA3.1 CT-GFP TOPO). The TOPO CT-GFP vector includes a priming site for RNA polymerase which allows in *vitro* preparation of RNA. Another advantage is the optional GFP tag on the c-terminal tail, which could be useful for localization studies or protein purification. Also, the vector has been successfully used for expression of *Drosphila* innexins in oocytes. RNA encoding H-Inx-2 with the C-terminal GFP tag was transcribed *in vitro* and injected into *Xenopus* oocytes. The oocytes were then paired to allow formation of gap junctions, and the following day, coupling levels we assessed using electrophysiology. Injection of h-Inx2 RNA did not facilitate the formation of gap junctions. Positive controls included gap junctions formed by mouse Cx50, *Drosophila* innexin ShakBN16, and Hydra Inx3. *Hydra* innexins have not previously been expressed exogenously and to our knowledge this is the first attempt to express a GFP-tagged innexin in oocytes. Future work will involve insertion of a STOP codon to remove the GFP tag from H-Inx2 followed by studies of other *Hydra* innexins.

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Hydra is a genus of small, predatory fresh-water cnidarian with a small tubular body that is about 5-10 mm in length. In many species the body is secured to a non-living surface by an adhesive foot called the basal disc *(*Burnett and Dielh, 1964). An image of *Hydra vulgaris* is shown in **Figure 1**. *Hydra* is comprised of two main body layers, separated by a gel-like substance called mesoglea. Both cell layers are composed primarily of epithelial cells, the outer layer representing the outer epidermis and the inner layer representing a gastro-dermis. These layers are commonly referred to as the ectoderm and endoderm respectively and are apparent in the diagram in **Figure 2** (Chapman *et al.,* 2010). Epithelial cells of both layers communicate with one another through gap junctions.

Both ectodermal and endodermal epithelial cells contain contractile filaments and function in movement. They can therefore be further classified as myoepithelial cells. The contractile filaments are referred to as myofibrils, and are organized at 90° angles to one another, facilitating circular contractions of the body column via the endodermal layer and longitudinal contraction via the ectodermal layer (Campbell *et al.,* 1976). *Hydra* exhibits several types of movement. The animal is capable of moving slowly along the surface to which it is attached in a snail-like manner (while standing upright) via small contractions of the basal disk. It is also capable of rolling, flipping, somersaulting, and moving prey with its tentacles. When prey contacts a tentacle, the tentacle bends forcing the prey toward the mouth-like structure called the hypostome (Burnett and Diehl, 1964). Movements initiated by sensory cells in the tentacles are related to feeding behavior and are mediated by a nerve net. This basic nerve net connects sensory cells located in the body wall and tentacles with epitheliomuscular cells (Westfall *et al*., 1980). Nerve cells are also connected via gap junctions. While there are fewer gap junctions between nerve cells than between

epithelial cells, gap junctions are easily visible by electron microscope as shown in **Figure 3** (Takaku *et al*., 2014).

Gap junction channels of invertebrates are formed by a family of proteins known as the innexins (Phelan *et al*., 1998; Landesman *et al.,* 1999). The majority of knowledge about innexins is derived from studies of *Drosophila* and *C. elegans* where they were identified in the late 1990's. Innexins are proteins with four transmembrane domains, two extracellular loops and cytoplasmic amino and carboxyl termini (Skerrett and Williams, 2016). The innexin membrane topology is shown in **Figure 4**. Innexins are similar in structure to their vertebrate counterparts (connexins) and also to their vertebrate homologs, the pannexins which form membrane channels rather than gap junctions (Kelly *et al*., 2014). Although they have the same membrane topology there is little sequence similarity between connexins and innexins and they are believed to have evolved convergently (Alexoupolous *et al.,* 2004).

The *Hydra magnipapillata* genome encodes 17 different innexins as discovered by genome sequencing (Chapman *et al.,* 2010*)*. This is surprisingly high, especially in light of the fact that relatives such as the starlet sea anemone (N. vectensis) may not have any genes encoding innexins. The only innexin-like gene in *N. vectensis* appears to be more closely related to pannexins than innexins (Chapman *et al.,* 2010) and there do not appear to be any innexin-like genes in coral (*Acropora digitifera*, Shinzato *et al.,* 2011). In contrast there are eight innexin genes in the *Drosophila* genome and 25 in the *C. elegans* genome (Phelan and Starich, 2001).

Gap junctions are defined as dense arrays of channels connecting the intracellular environments within almost all animal tissues (Skerrett and Williams, 2016). Essentially, they facilitate the

communication between apposed cells by providing aqueous conduits. Both connexins and innexins can also form hemichannels which are half channels connecting the intercellular and extracellular environments. Structural studies indicate that gap junctions composed of connexins and innexins closely resemble each other. However, channels composed of innexins are hexadecameric (16 subunits/channel) while channels composed on connexins are comprised of 12 subunits (Oshima *et al.,* 2016). Innexin-based gap junction channels also tend to be larger in diameter than their connexin-based counterparts (Skerrett and Williams, 2016). **Figure 5** shows organization of connexin subunits into hemichannels and intercellular channels. Innexin channels are assembled in the same manner. A single subunit (top) oligomerizes with other subunits to form a transmembrane channel (aka hemichannel, bottom right). When two hemichannels dock together an intact gap junction channel is formed (bottom left). An innexin-based hemichannel involves eight innexin subunits and 16 subunits are required to form one gap junction channel (Oshima *et al.,* 2016).

In a study by Takaku *et al*. (2014) *in situ* hybridization was used to demonstrate the regions where H-Inx2 was expressed. The gene was found to be expressed in nerve cells in the peduncle region as well as in the base of newly forming organisms, and to a minimal extent at the mouth (Takaku *et al.,* 2014). The results of the *in situ* hybridization experiments are shown in **Figure 6A**. Unfortunately, the sequence of the *in situ* probe was not described. Further studies were completed with a H-Inx2 antibody directed against the first extracellular loop. Takaku *et al*. (2014) used the H-Inx2 antibody to demonstrate that the protein is localized to neurons of the peduncle region. As shown in **Figure 6B** staining was apparent in the peduncle region but not further up the body. Surprisingly, Takaku *et al.* (2014) found that treating live animals with antibody against H-Inx2

caused the loss of H-Inx2 gap junctions. The authors expected that applying antibody to live animals would result in binding and potentially block of gap junctions. However the H-Inx2 proteins appeared to have been internalized as H-Inx2-staining was not observed in these animals. Furthermore, the antibody-treated animals developed altered patterns of spontaneous contractions, contracting less frequently than untreated animals (**Figure 7A).** The anti-Inx2 animals maintained the ability to contract when touched with fine forceps as shown in **Figure 7B.** This suggests that the reduction in spontaneous contractions after treatment with Inx2 antibody is related to the initiation of contractions rather than a disruption of the neuromuscular system.

In summary, there is strong evidence that H-Inx2 is expressed in nerve cells of *Hydra,* specifically neurons in the peduncle region. These neurons likely play a role in regulating the rate of spontaneous contractions and gap junctions likely create a network of synchronously firing neurons that underlie spontaneous contractions. Understanding the properties of H-Inx2 junctions may help understand this pacemaker activity.

The goal of this project was to create a construct that would allow expression of H-Inx2 in *Xenopus* oocytes. The oocyte expression system has been used to characterize innexins of *c. elegans*, *Drosophila* and other invertebrates (Phelan *et al.,* 1998, Starich *et al.,* 2009). After developing a construct that facilitates expression of H-Inx2, the construct will be used to create RNA that can be injected into oocytes inducing the expression of innexin proteins. Pairing the oocytes together allows formation of gap junctions which can be studied using dual cell two-electrode voltage clamp. Hence the creation of an expression construct is a crucial step in furthering knowledge of H-inx2 structure, function and regulation.

METHODS

1. Gene Analysis

Gene identification and sequence analysis focused on the *Hydra vulgaris* genome and was conducted to identify the sequence of h-Inx2. DNA sequence information was obtained from National Center for Biotechnology GeneBank. Sequences were analyzed initially using Biology Workbench (SDSC) and subsequently with SnapGene (GSL Biotech).

2. Gene Synthesis

The H-Inx2 sequence as determined from genome sequencing (Chapman *et al*., 2010) was synthesized by Genscript USA (Piscataway NY). This was necessary because the gene had not been cloned.

3. Transferring H-Inx-2 to a *Xenopus* Expression Vector

A vector known as pcDNA3.1 CT-GFP TOPO (Life Technologies, Grand Island NY) was identified as a candidate. The vector was obtained from Life Technologies and amplified for the purpose of cloning. Although only a small percentage of this TOPO vector was expected to be circular in its original form a transformation reaction was performed using One-Shot® TOP10 cells (Thermofisher Scientific, Grand Island NY). Colonies were formed on LB AGAR + AMP plates and these were selected to inoculate LB+AMP broth. Standard miniprep procedure (Qiagen

Germantown, MD) was carried out following overnight growth. Approximately 500 µg of pcDNA3.1 CT-GFP TOPO plasmid was sent to Genscript for subcloning of the H-Inx2 gene.

4. Amplification and Purification of Plasmid DNA

Once the TOPO GFP/H-Inx2 construct was received from Genscript (Piscataway NJ), plasmid DNA was amplified and purified. A small amount of purified plasmid was utilized to transform One-Shot® TOP10 cells (Thermofisher Scientific, Grand Island NY). The competent cells were plated on standard LB+AMP agar plates. A visual summary of the transformation and plating steps is shown in **Figure 8.** The following day colonies were individually transferred to LB+AMP media and incubated overnight in a shaking 37 °C incubator. After overnight growth a plasmid "miniprep" Qiagen SpinPrep Protocol (Qiagen Inc, Germantown MD, USA) was used to isolate plasmid DNA from small pelleted bacterial cells. To pellet the bacterial cells, about 1.5 ml of broth was transferred to a 1.5 ml microfuge tube and centrifuged at 13,000 rpm in a bench- top centrifuge for 10 minutes. All broth was removed by decanting and pipette. The standard protocol provided by Qiagen (Germantown, MD USA) was followed leading to lysis of bacterial cells, precipitation of unwanted DNA and protein, and finally, plasmid DNA which adhered to the miniprep spin column. Plasmid DNA was then eluted from the Qiaprep spin column with 50 µl of elution buffer..

5. Gel Analysis, Linearization and *In Vitro* Transcription

Prior to *in vitro* transcription the DNA was linearized downstream of the H-Inx2 gene and analyzed by gel electrophoresis. For gel electrophoresis, 5 µl of sample (1 µl plasmid DNA + 4 µl of loading dye) was used. A 1% agarose gel was cast incorporating a small amount of SYBR Safe gel stain

(ThermoFisher, Grand Island, NY). The DNA was then carefully transferred to the gel and run alongside a DNA ladder (Zipruler 2, Fermentas Inc, Waltham, MA). The gel ran for approximately 45 minutes at 75 volts. DNA bands were visualized with the SYBR platform and linearization was confirmed. The intensity was also compared to that of DNA bands in the ZipRuler marker in order to estimate DNA concentration in plasmid samples.

An *in vitro* transcription reaction was used to create RNA. The mMessage mMachine T7 protocol (Ambion/ThermoFisher, Grand Island, NY) was used with LiCl precipitation. RNA was then quantified by gel electrophoresis, using an RNA 250 standard (Ambion/ ThermoFisher, Grand Island, NY).

6. Expression in *Xenopus* Oocytes

RNA was diluted to 125 ng/µl prior to injection. Diluted RNA was injected (NanojectII microinjector, *Drummond Scientific, Broomall, PA)* into defolliculated *Xenopus* oocytes (Ecocyte Inc, Austin TX) with injection volumes ranging between 9.2 and 41.1 nl per oocyte. (total injected RNA varied between 1 ng/ oocyte and 5 ng/ oocyte). One day after injection, oocytes had their vitelline layers removed with fine forceps and were paired in shallow agar (Dahl *et al.,* 1987). Approximately 24 hours was allowed for the gap junctions to form between the cells after they were paired together. **Figure 9A** summarizes steps beginning with linearization of plasmid DNA to expression of protein in oocytes. Oocytes were maintained in modified Barth's (MB1) solution (88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 1 mM $MgCl_2$, 0.33 mM $Ca(NO_3)_2$, 20 mM HEPES, pH 7.4).

7. Voltage Clamp Analysis

The movement of ions between the injected *Xenopus* oocytes was assessed by way of a voltage clamp (*Axopatch200B, Molecular Devices, Sunnyvale CA*). **Figure 9B** shows a voltage clamp for a single oocyte, which is typically used to study ion channels. One electrode is referred to as the voltage electrode and the other as the current electrode. The tips of both electrodes were inserted just below the membrane surface. Electrodes are filled with a conductive electrolyte solution (in our case 100 mM KCl) which allows current to flow through to the voltage clamp amplifier. Through a rapid feedback mechanism the amplifier compares the actual voltage to a set value and injects current to maintain the defined command potential. The injected current is therefore a measure of the current flowing across the membrane at a specific clamped voltage. A dual-cell two-electrode voltage clamp works by the same mechanism, with both cells under individual control. One cell is maintained at a constant voltage while the voltage of the other cell is manipulated. In this way, the current required to clamp the first cell represents current flowing across the junction. The programs Clampex10 and Clampfit10 (Molecular Devices, Sunnyvale CA) were used for data acquisition and analysis. The program Origin8 (OriginLab, Northampton, MA) was used for graphing and statistical analysis.

.

RESULTS

The H-Inx2 Construct

The nucleotide sequence of *Hydra vulgaris* Inx2 was obtained from GenBank (NCBI) as show in **Figure 10** (top). The coding sequence (mRNA) is 1473 nucleotides in length and codes for a protein 490 amino acids in length. The amino acid sequence of H-Inx2 is also shown in **Figure 10** (bottom). Detailed information about splice variants is lacking but it appears that H-Inx2 includes one intron flanked by two coding sequences. The intron was removed in the published sequence and both exons were included in our construct expression construct. The sequence was sent to Genscript (Piscataway, NJ) and the company synthesized the gene in a pUC57 vector. The pUC57 vector lacked attributes for expression in *Xenopus* oocytes. The vector pcDNA3.1 CT-GFP TOPO (Life Technologies, Grand Island NY) was identified as an excellent candidate for expression because it induced high levels of gap junction protein expression in *Xenopu*s oocytes in previous experiments conducted in Dr. Skerrett's lab (J. Cutre, MA Thesis Buffalo State; Marks and Skerrett, 2014). It includes a priming site for RNA polymerase, a multiple cloning region, an optional C-terminal green fluorescent protein tag, and confers resistance to ampicillin. All of these characteristics make it ideal for harboring the gene of interest and expressing it in oocytes. The vector is shown in **Figure 11**. Genscript (Piscataway NJ) used a PCR-based method known as CloneEZ to transfer the gene from pUC57 to the new vector. This method involves amplification of the gene to be transferred using primers that incorporate a restriction site. The restriction site engineered by Genscript was a Mlu1 recognition site, the region of insertion was downstream of the T7 promoter and can be seen at the position labelled "PCR Insert" in **Figure 11**. Our goal was to include a STOP codon at the end of the H-Inx2 gene preventing C-terminal GFP fusion. Upon further examination and correspondence with

Genscript it was discovered that the H-Inx2 gene was directly linked to the GFP sequence. A 48 nucleotide linker region (including multiple cloning sites downstream of the PCR insert shown in **Figure 11**) is missing from the construct. Hence, RNA transcribed from the T7 promoter included a regular START codon for H-Inx2 and a STOP at the end of the GFP sequence.

One aspect of the vector that had made it amenable to our project was the presence of restriction sites downstream of the insert, for instance one that allowed linearization with Xba1 (our original goal was to include a STOP codon at the end of H-Inx2 and to bypass the GFP). However, due to the missing linker region in the construct, as well as the lack of a STOP codon the vector lacked the necessary Xba1 site for linearization. The construct passed quality controls at Genscript before going into use for this project (**Figure 12, Figure 13**) and although we checked the Genscript sequencing files they did not include information about the linker region. After several unsuccessful attempts to express H-Inx the entire vector was sequenced by Genscript and revealed the absence of a linker region between H-Inx2 and GFP. This suggests that there was a mistake in the QC file regarding the statement "flanking sequences of the cloning site are correct". Ultimately the construct used for preparation of RNA involved H-Inx2 directly linked to GFP.

***In vitro* Transcription**

The construct was received as lyophilized powder and in order to create RNA for injection into oocytes the plasmid was reconstituted and amplified. Gel analysis was used at each step to confirm quality and/or quality of the products. As can be seen in **Figure 14** five samples of plasmid DNA were obtained, all of similar quality and concentration. Two were selected for further use and treated

with Xba1. Due to accidental deletion of the linker region the Xba1 site was not present, suggesting that the linearization was not achieved. It is possible to synthesize RNA without linearization, however the yield of transcripts is reduced because the polymerase creates long transcripts which are only partially useful.

Both *in vitro* transcription reactions yielded RNA in the microgram per microliter range which was determined by running the RNA alongside an RNA250 control (250 ng/µl) on the gel (**Figure 14**). The RNA 250 allows estimation of RNA concentration based on the intensity of the band representing 250 ng or RNA. In Figure 14 the RNA samples were much brighter and more diffuse than the RNA 250 sample suggesting concentrations in the microgram range. Samples were diluted prior to injection, with a target concentration of 125 ng/µl. Diluted RNA was reassessed next to the RNA 250 and further diluted if necessary.

Oocyte Expression

RNA was diluted to a final concentration of about 125 ng/µl and was injected into *Xenopus* oocytes that had been defolliculated and pre-injected with antisense oligonucleotide directed against *Xenopus* Cx38, the endogenous connexin that occasionally forms gap junctions. The volume injected varied between 9 nl and 41nl allowing expression to be tested for injection quantities ranging from 1 ng/oocyte to 5 ng/oocyte. Positive control oocytes were injected with RNA encoding gap junction proteins known to form functional channels in oocytes including Cx50 (gift from Leah Volk), ShakBL (gift from Xiang Li) and H-Inx3. All oocytes were pre-injected with an anti- XeCx38 morpholino oligonucleotide (Genetools Inc, Philamel OR). Oligo-injected oocytes also served as the negative control.

After injection and overnight pairing the dual cell two electrode voltage clamp technique was used to assess coupling. **Figure 15** shows summarized junctional conductance measurements for three experiments. In the first experiment oocytes injected with H-Inx2 (41 nl of RNA at 125 ng/µl) died after pairing. In the second experiment a lower volume of RNA was injected in one group (Inx 2 - 9 nl) to address the potential issue of RNA toxicity. This failed to induce coupling however the oocytes were healthy. In this experiment, injections of 41 nl did not appear to compromise the health of the oocytes however only one of five pairs was coupled and this pair showed poor responses under the voltage clamp. Inx2 also failed to induce coupling in the third experiment when Cx50 positive controls demonstrated an average of 12 µS and responded well under voltage clamp. **Figure 16** shows examples of currents recorded from paired oocytes during voltage clamp experiments. Negative control (anti-sense negative control; Inx2/antisense; TOP) did not couple as evident by the lack of induced current in the lower trace. In this experiment crrrents were recorded from a continuously clamped oocyte while its partner was pulsed in 100 mV increments to ± 100 mV. Positive control (Cx50; positive control; MIDDLE) displayed junctional currents that decayed in a time- and voltage-sensitive manner. In this experiment currents were recorded from a continuously clamped oocyte while its partner was pulsed in 10 mV increments to induce voltage across the junction of ± 100 mV. The experimental group (h-Inx2; BOTTOM) did not couple as evident by the lack of induced current in the lower trace. In this experiment currents were recorded from a continuously clamped oocyte while its partner was pulsed in 100 mV increments to ± 100 mV.

Figure 17 demonstrates the successful expression of Hydra Inx3 in *Xenopus* oocytes. currents were recorded from oocytes injected with 41 nl of 125 ng/µl of H-Inx3. This experiment served as a positive control for Hydra innexins. The gene was synthesized by Genscript (Piscataway, NJ)

in pUC57, transferred to pSP64T a vector that includes *Xenopus B*-globin regions for enhanced expression in oocytes. RNA was prepared as for H-Inx2. Oocytes were paired and incubated overnight. The results were as follows: H-Inx3/H-Inx3 = 0 μS, 3.5 μS, 2 μS; H-Inx3/ Oligo = 0 μS, 0 μS, 0 μS, 0 μS.

Lastly, to ensure we were working with a H-Inx2 sequence that produced a full-length innexin protein with correct membrane topology a hydropathy analysis was performed. The membrane topology prediction allowed us to confirm that the protein contained four transmembrane domains, cytoplasmic amino and carboxy termini as well as two extracellular loops. Hydropathy analysis was carried out using the PHOBIUS interface (Stockholm Bioinformatics Center, **http://phobius.sbc.su.se//**) which is designed to visualize membrane protein topology and signal peptides. Results from this can be seen in **Figure 18** and reveal all the standard topological components typical of an innexin.

DISCUSSION

It is probable that H-Inx2 did not express well in oocytes due to the accidental inclusion of the C-terminal GFP tag combined with the lack of linker region in the construct. There are several effects that this error could have caused. For example, the linkage of the GFP at the C-terminus has the potential to restrict trafficking or disrupt function. In addition, the absence of the 16 amino acid (48 nucleotide) linker region between H-Inx2 and GFP would be expected to reduce flexibility in the C-terminus. The absence of the linker region also meant that there was no Xba1 restriction site for linearization. This likely lead to excessively long RNA transcripts that reduced the efficiency of in vitro transcription and in vivo translation in the oocytes. We were able to produce a good RNA yield in the in vitro transcription reactions but dilution of the RNA was based on the expectation that transcript was of similar length to that of the RNA 250 (about 1 Kb). A construct is being developed that includes a stop codon at the end of the h-Inx2 sequence and function will be assessed in future studies.

Several other challenges should be considered when expressing new innexins including issues related to expressed sequences (splicing) and glycosylation with the biggest challenge likely related to understanding the way genes are spliced. At the current time, our sequence information comes from whole genome sequencing and computational analysis (Chapman *et al.,* 2010) with some addition information from whole animal studies such as the *in situ* hybridization and antibody studies of Takaku *et al*. (2014). In the case of *Hydra* Inx-2, the splicing options seem limited since computational analysis predicts only one intron which was removed from the predicted sequence shown in **Figure 10.** However many of the innexins have splice variants that perform different functions in cells. For instance *c. elegans* UNC-7 gene encodes three isoforms

(UNC7-L, UNC7-S and UNC7-SR). UNC7-S and UNC7-SR are widely expressed and seem to be functionally equivalent, while the role of UNC7-L remains unknown (Starich *et al.,* 2009). Similarly three innexins with different expression patterns and properties are formed from the ShakB locus in *Drosophila*. These include ShakingB(Neural+16) and ShakingB(Lethal) which readily form gap junction channels and also couple heterotypically to form rectifying junctions (Phelan *et al*., 2008; Marks and Skerrett, 2014). However a third variant ShakingB(Neural) does not seem to form functional channels (Phelan *et al*., 2008). Moving forward we would like to use RNA sequencing to better understand splicing of innexins in *Hydra*. It is also expected that further analysis by other labs at the genetic, cellular, and molecular levels will reveal more information about potential splice variants and their physiological roles.

All innexins include glycosylation sites within the extracellular domains and glycosylation could prevent the formation of gap junctions. We did not anticipate this to be an issue with *Hydra* innexins because it has not been the case for innexins from *Drosophila* or *C. elegans* which express reliably in oocytes (Phelan *et al.,* 1998; Landesman *et al*., 1999; Starich et al., 2009; DePriest *et al.,* 2009). However in future it might be possible to treat oocytes with a nucleoside antibiotic called tunicamycin. As well as directly inhibiting gap junction formation, glycosylation is known to regulate trafficking (eg. pannexin proteins - Boassa *et al.,* 2008; Park *et al*., 2015). It is possible to regulate glycosylation of membrane proteins in oocytes by treating with tunicamycin (Xu *et al.,* 2008) making this an easy experiment for future analysis of H-Inx2.

Another possibility for future work could involve the use of an antibody to detect H-Inx2 protein after RNA is translated in *Xenopus* oocytes. This would confirm if protein was being produced

but not forming channels. Gap junction proteins expressed in oocytes are amenable to Western blot (Skerrett *et al*., 2001) and it represents a relatively easy way to confirm expression. Surface labelling is also suitable for oocytes and could help to confirm both expression and trafficking to the cell membrane. It may be possible to obtain some of the antibody used by Takaku *et al.* (2014) or to use the c-terminal GFP tag.

Overall this study represents a first attempt in expressing Hydra innexins in *Xenopus* oocytes. As far as we know *Hydra* innexins have not been expressed in oocytes or in any exogenous expression system. We made significant progress in identifying an efficient (2-4 weeks) and affordable (less than $500) method of gene synthesis with options for subcloning into any expression vector (Clone EZ, Genscript USA, Piscataway, NJ). Although our goal was to investigate the properties of H-Inx2 because of its interesting localization and potential role in pacemaker activity, we were able to demonstrate the expression of another *Hydra* innexin (Inx3). Further attempts to express H-Inx2 will involve insertion of a STOP codon via site-directed mutagenesis. This will uncouple the innexin from the GFP linked at the C-terminus and potentially induce expression. In addition, we plan to continue investigating methods for expression of H-Inx2 and other *Hydra* innexins in oocytes. The *Xenopus* oocyte expression system will allow characterization of *Hydra* innexin interactions, properties and regulation.

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FIGURES

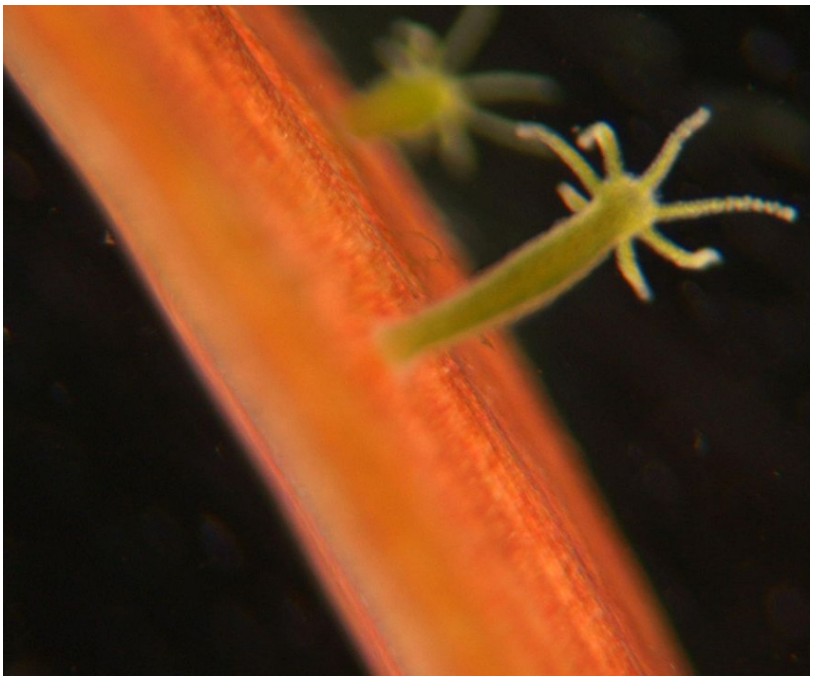

Figure 1. *Hydr*a attached to a substrate. Clearly visible are the tentacles, elongated body and basal disc by which the animal is attached to the substrate. The basal region is also known as the peduncle (*image from Wikipedia.com*).

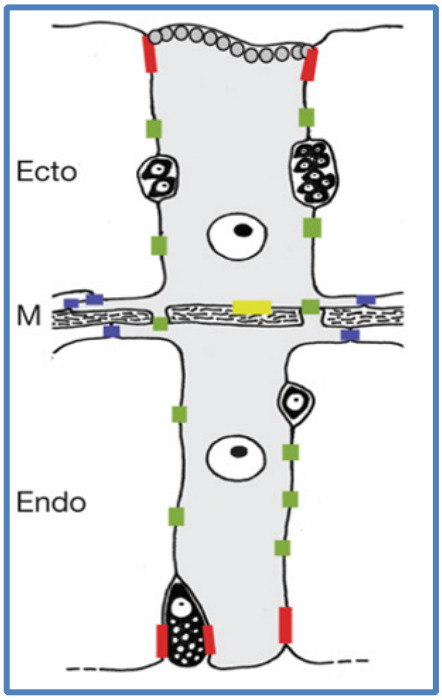

Figure 2. Illustration of cell junctions typically found between endodermal and ectodermal epithelial cells in *Hydra. GREEN = Gap junction; RED = Septate junction; YELLOW = Hemidesmosomelike cellation of variou; BLUE = spot desmosomes.* Gap junctions also exist between nerve cells which are not shown in this image. (Image from Chapman *et al.,* 2010)

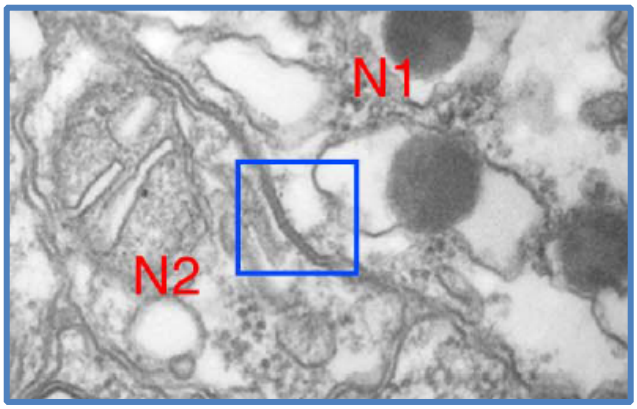

Figure 3. A gap junction between adjacent nerve cells (N1, N2) in *Hydra* is visible with electron microscopy. The gap junction is outlined by the blue box in the thin section EM from the peduncle region. Similar gap junctions are found in all body regions and range from 200–500 nm in length. Chemical synapses were also apparent between nerve cells but are not shown here (*from Takaku et al., 2014).*

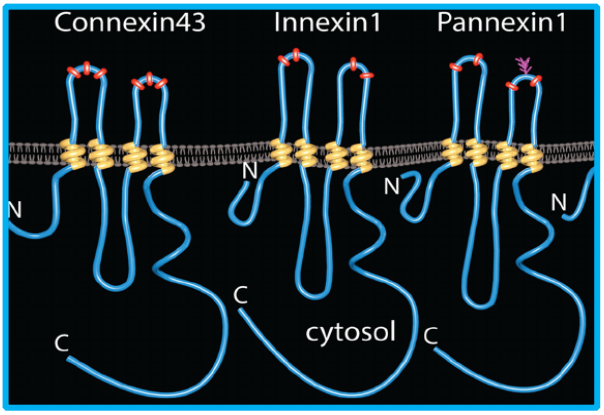

Figure 4. Membrane topology of a typical innexin protein (middle) with four transmembrane domains, cytoplasmic amino and carboxyl termini. Also shown are a vertebrate gap junction protein Cx43 and a vertebrate pannexin protein that is homologous to innexins but forms transmembrane channels (from Kelly *et al*., 2014).

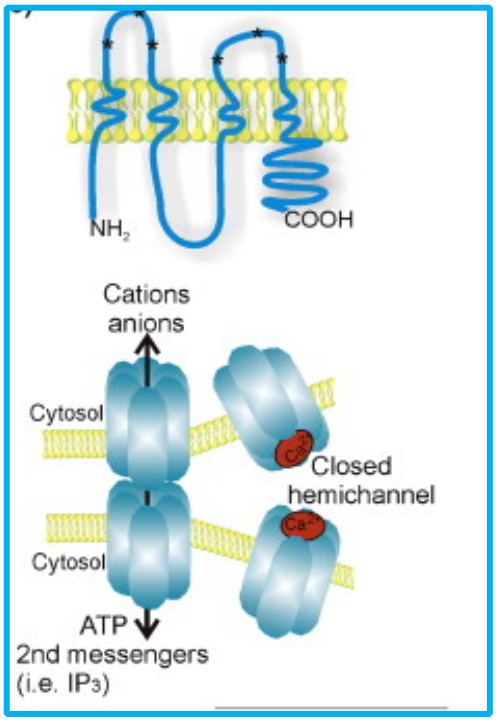

Figure 5. Innexins assemble in a similar way to connexins to form gap junctions except that eight innexin subunits oligomerize to form one hemichannel and 16 subnits are required to form one gap junction channel *(*Oshima *et al., 2016).*Gap junction plaques are composed of hundreds or thousands of intercellular channels and facilitate the transfer of ions and small molecules between cells. (from MacVicar and Thompson, 2010)

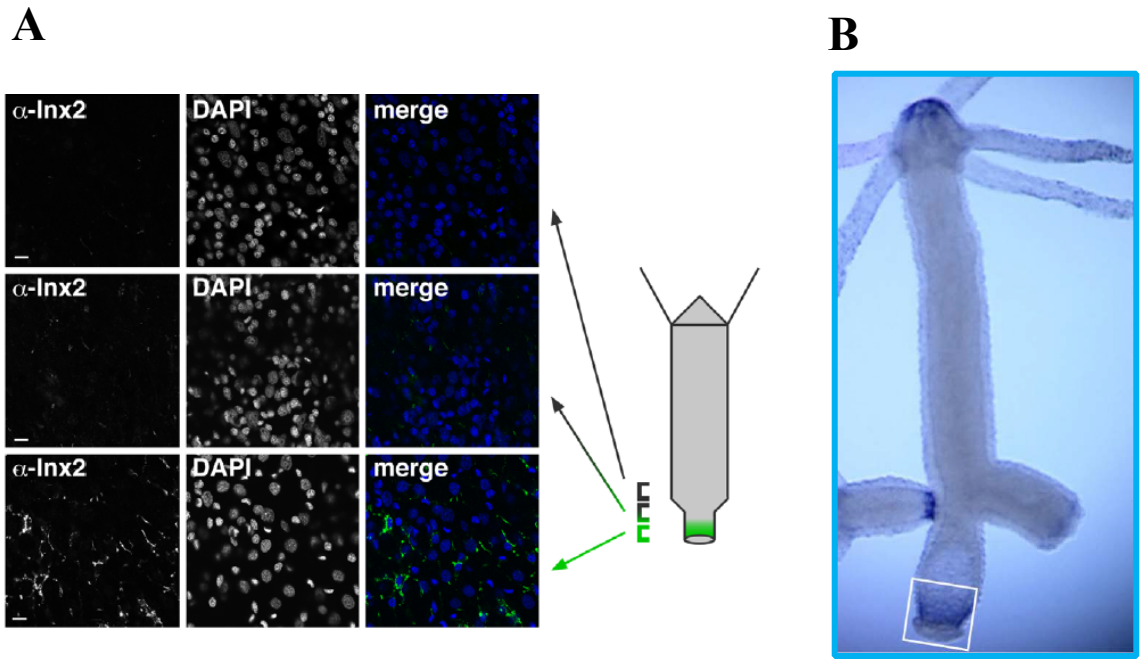

Figure 6. **(A)** Whole mount confocal images of *Hydra* tissue taken from different regions of the peduncle as indicated by the cartoon body shape on the left. Tissue was fixed and exposed to green fluorescently labelled antibody against Inx2 (first column, greyscale). Tissue was also stained with DAPI to indicate nuclei (middle column. greyscale). Images were then merged to show the presence of Inx2 relative to nuclei (third column, color). **(B)** Image of *Hydra magnipapillata* after *in situ hybridization* for Inx2. Staining was apparent in the peduncle (white square) as well as forming organism (bottom left) and, to a lesser extent, the mouth region and tentacles (*from Takaku et al., 2014*)

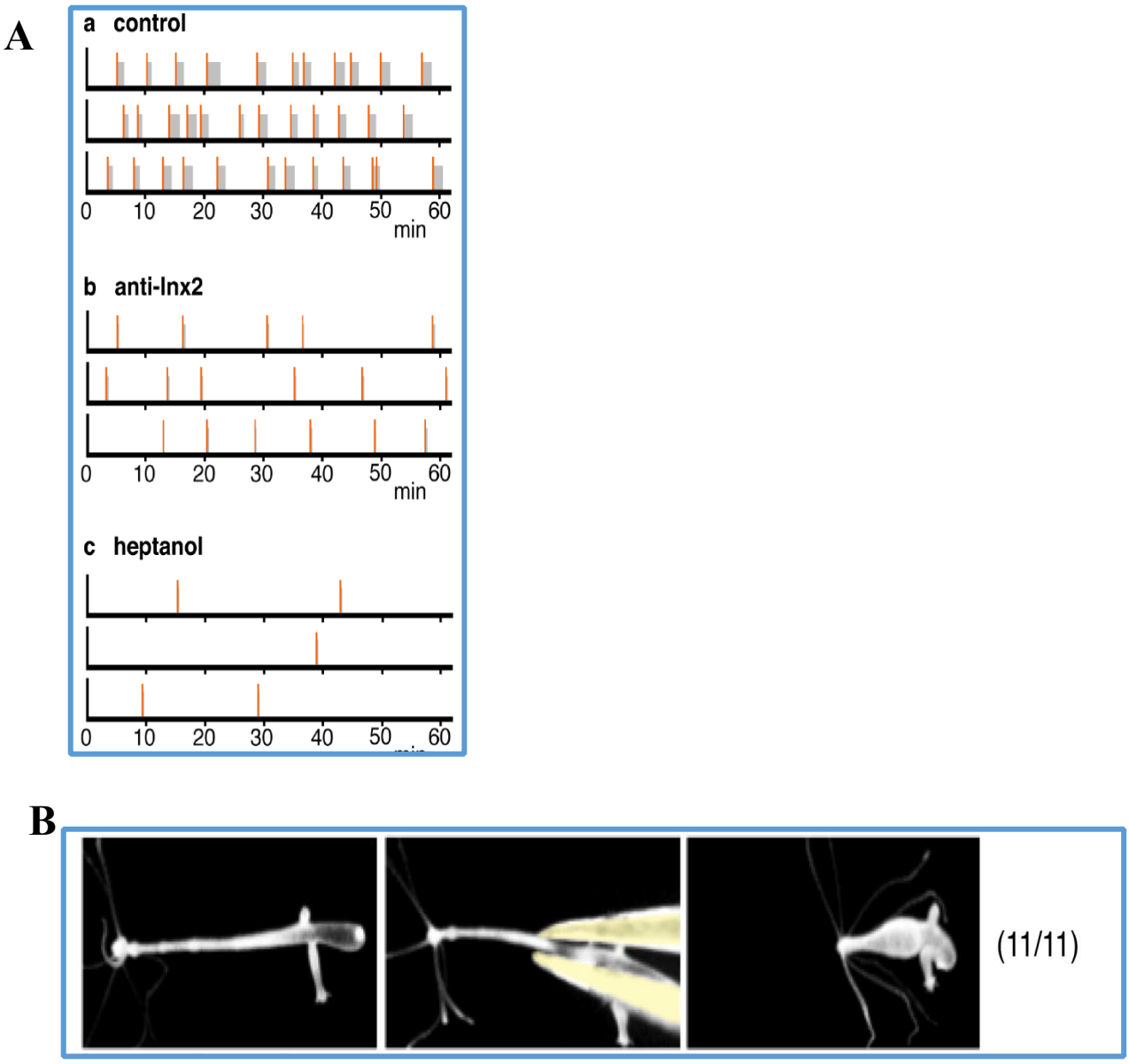

Figure 7. (A) Reduction in the number of spontaneous contractions after treatment with Anti-Inx2 antibody. Contractions are shown as function of time in minutes for *Hydra* in control, anti-Inx2, and heptanol-treated groups with three animals included in each graoup. Anti-Inx2 andtibody reduces the rate of spontaneous contraction. Treatment with heptanol, a gap junction blocker further reduces contractions. **(B)** *Hydra* treated with antibody directed against Inx2 contract in response to mechanical stimulation (middle image, pinched with forceps) indicating that the nerve net is attached and gap junctions continue to function. The number on the right indicates that 11 of 11 animals contracted in this treatment group (from Takaku *et al.,* 2014).

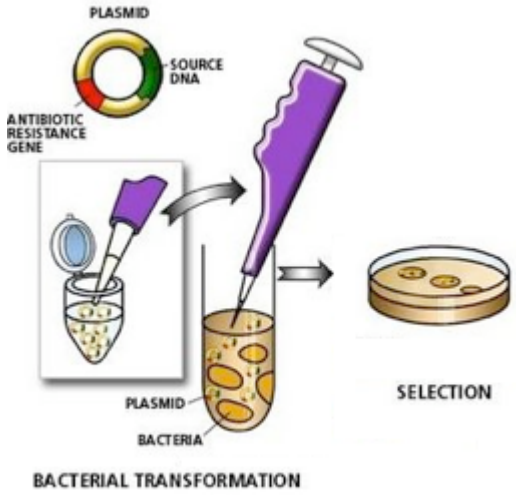

Figure 8. Illustration of transformation reaction. These steps were carried out at several times during the project, for instance after receiving plasmid DNA containing the H-Inx2 gene. Selection of colonies was followed by overnight growth in LB+AMP media and plasmid isolation (*modified from young-pearse-lab.bwh.harvard*).

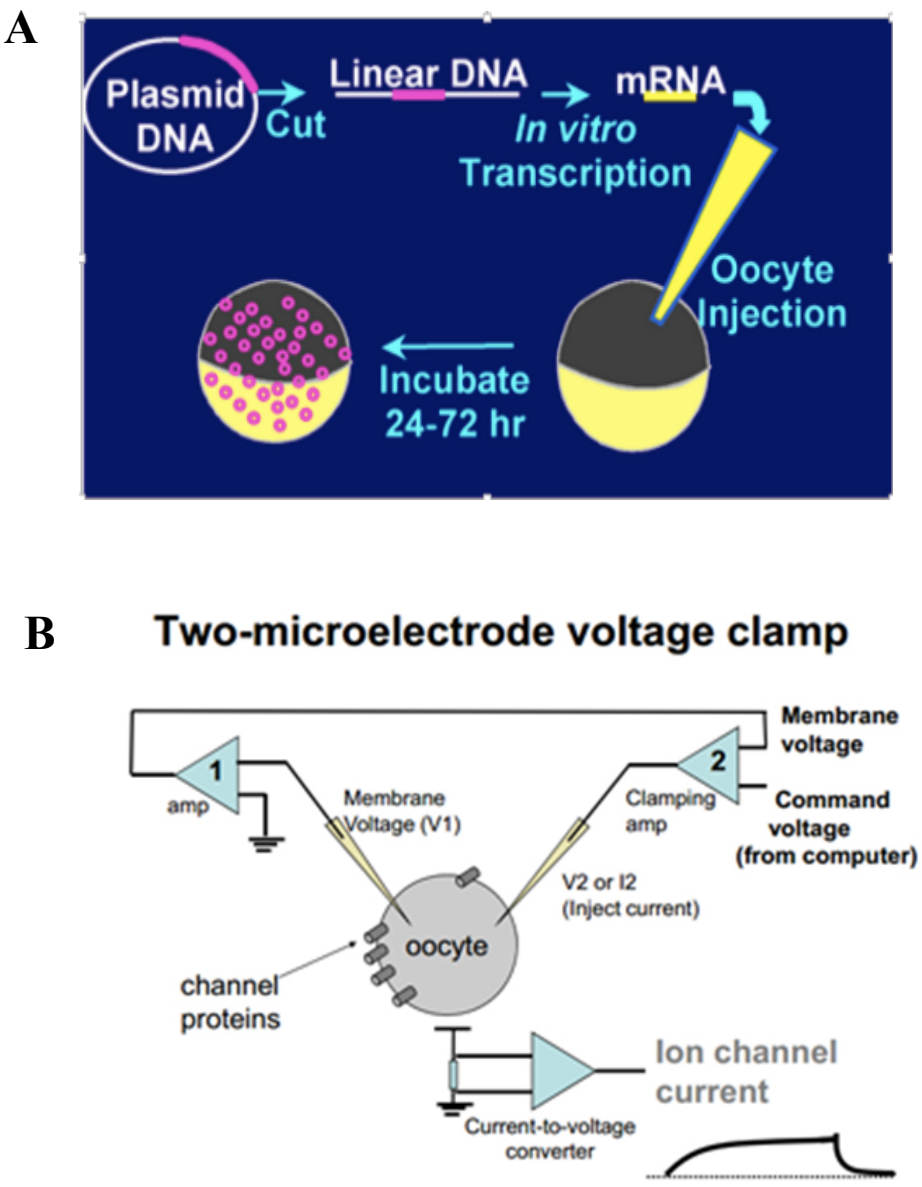

Figure 9. **(A)** Summary of procedure leading to the expression of membrane protein in oocytes. Plasmid DNA is linearized, RNA synthesized and injected with a fine glass needle. After incubation, proteins are made within the oocyte cytosol, trafficked through the intermembrane system and inserted in the plasma membrane. (image from kasturisem2biochem.wordpress.com) **(B)** Example of two-electrode voltage clamp of a single *Xenopus* oocyte. Membrane current is a measure of the current required to clamp the cell at a specified voltage (command voltage). The voltage clamp amplifier works as a comparator, assessing membrane voltage (V1) and injecting current to maintain it at a set potential. For gap junction analysis oocytes are paired and current is recorded from a continuously clamped oocyte with voltage is changed in the other (*from Wikipedia.org).*

PREDICTED: Hydra vulgaris innexin inx2-like (LOC100209836), mRNA NCBI Reference Sequence: XM_002166895.3

>XM_002166895.3:239-1399 PREDICTED: Hydra vulgaris innexin inx2-like (LOC100209836), mRNA

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Figure 10. Sequence of the Inx2 gene from *Hydra vulgaris*. TOP: Predicted nucleotide sequence based on computational analysis and genome sequencing. Predicted introns have been removed. The sequence was obtained from Genbank NCBI and represents one of many *Hydra* gene sequences available after the *Hydra* genome (Chapman *et al*., 2010) was sequenced. BOTTOM: amino acid sequence of the h-Inx2 protein

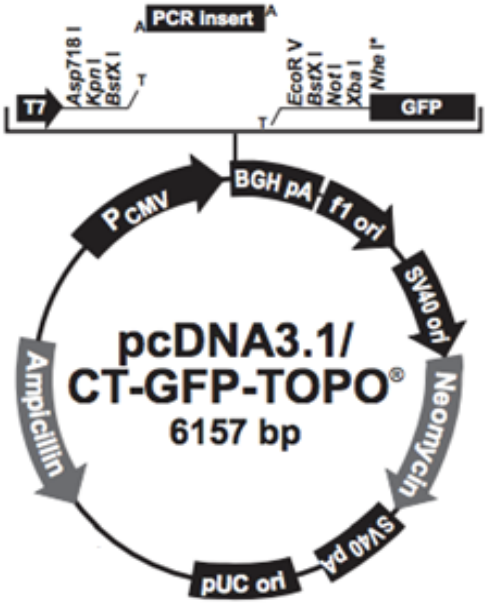

Figure 11. Vector used for expression of *Hydra* Inx-2 gene. The gene was synthesized in pUC57 vector and subcloned by Genscript (Piscataway, NJ) with insertion at the site marked "PCR Insert". This vector is available for purchase from Life Technologies (Grand Island, NY). It is known to work well for expression of gap junction proteins in oocytes, allows for optional GFP-tagging, and is suitable for selection and amplification as it includes Ampicillin resistance genes. The T7 site allows for *in vitro* synthesis of RNA. (Image from: Life Technologies, Grand Island, NY)

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Certificate of Analysis

Project ID: U0806CG060-2

Construct Information:

Gene Name: Hydra Inx2_pcDNA3.1/CT-GFP-TOPO

Clone ID: K41025 **Gene Length:** 1179 bp

Cloning Vector: pcDNA3.1/CT-GFP-TOPO **Cloning Strategy:** CloneEZ

QC Items	Specifications	Results	
Sequencing Alignment	Sequencing results are consistent with the targeted insert sequence.	Pass	Consistent
Vector Sequence	The flanking sequences of the cloning site are correct.	Pass	Correct Shown in the SQD file
Restriction Digests	The size of inserted fragment is correct and free of unexpected bands suggesting contamination.	Pass	Correct Shown in attachment 1
DNA Quality	Miniprep: 4 μg OD260/280=1.8~2.0 Free of contamination	Pass	⩾ 4 μg OD260/280=1.80 Pure
Quality grade	Research Grade	Pass	Research Grade
Appearance	Clear and free of foreign particles.	Pass	Clear Free of foreign particles
Additional Test		N/A	

Figure 12. The new construct Hydra Inx2 pcDNA3.1/CT-GFP-TOPO was created by Genscript USA (Piscataway, NJ) and passed quality control tests for gene sequence, vector sequence and restriction analysis. Approximately 4 μg of lyophilized plasmid was received by mail.

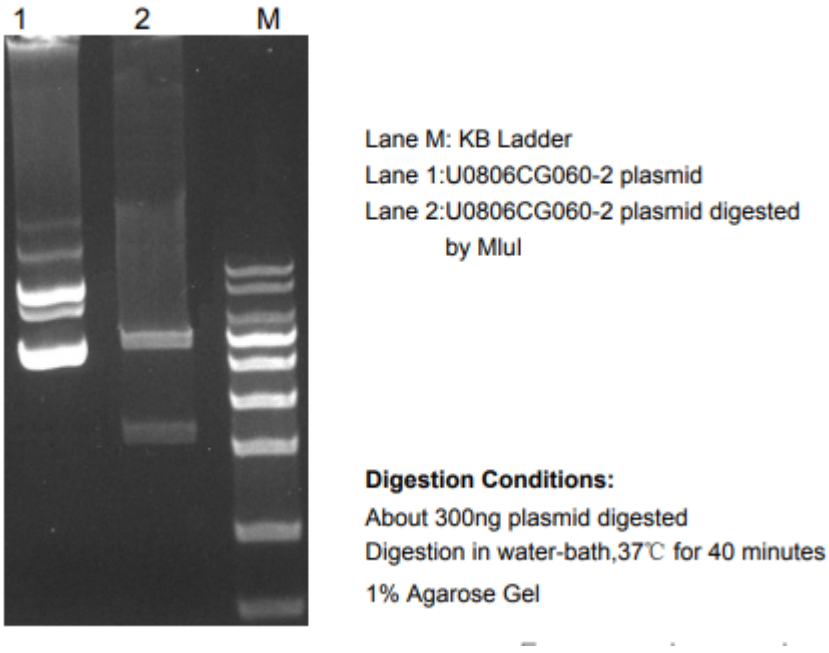
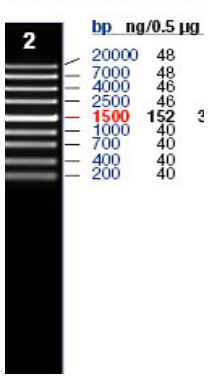


Figure 13. Analysis and quality control test of Hydra Inx2 pcDNA3.1/CT-GFP-TOPO by Genscript USA (Piscataway, NJ). Digestion with the restriction enzyme Mlu1 yielded two bands, one band representing the plasmid after release of the insert (approximately 5500 bp) and one band representing the H-Inx2 gene (about 2000 bp)



Plasmid DNA DNA Yield RNA

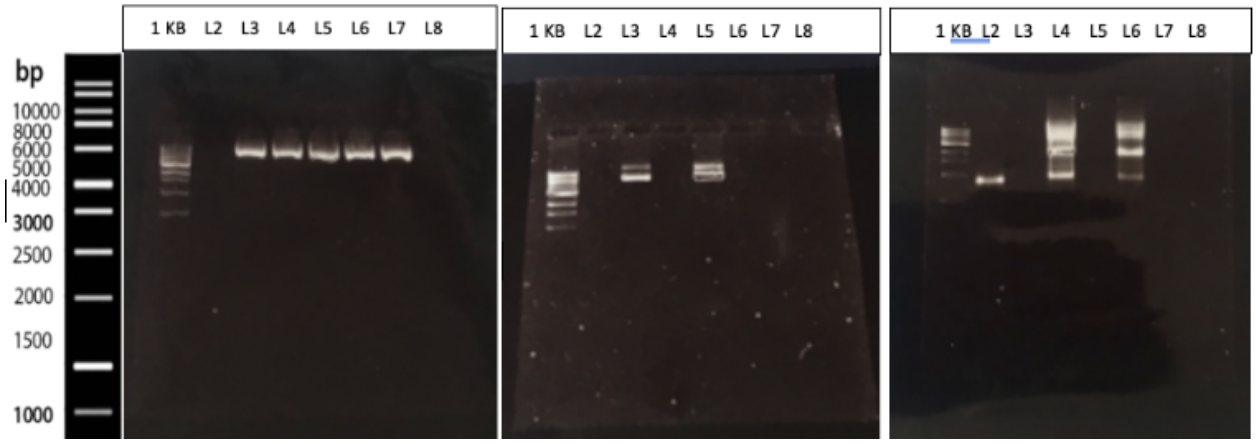


Figure 14. Gel analysis of plasmid DNA **(left**), DNA yield after linearization techniques were implemented **(middle**) and RNA yield (**right**). All are 1% agarose gels stained with SYBR Safe and viewed on the SYBR Safe platform. **Left:** Plasmid DNA was isolated from five colonies grown overnight after transformation of coli with the h-Inx2 construct. **Middle:** For two of the plasmid preparations, plasmid DNA was linearized using Xba1 in preparation for transcription. **Right:** RNA was created using the mMessage mMachine T7 protocol/LiCl precipitation method. RNA preparations from both linearized samples yielded concentrated RNA. Inset: ZipRuler2 DNA Ladder.

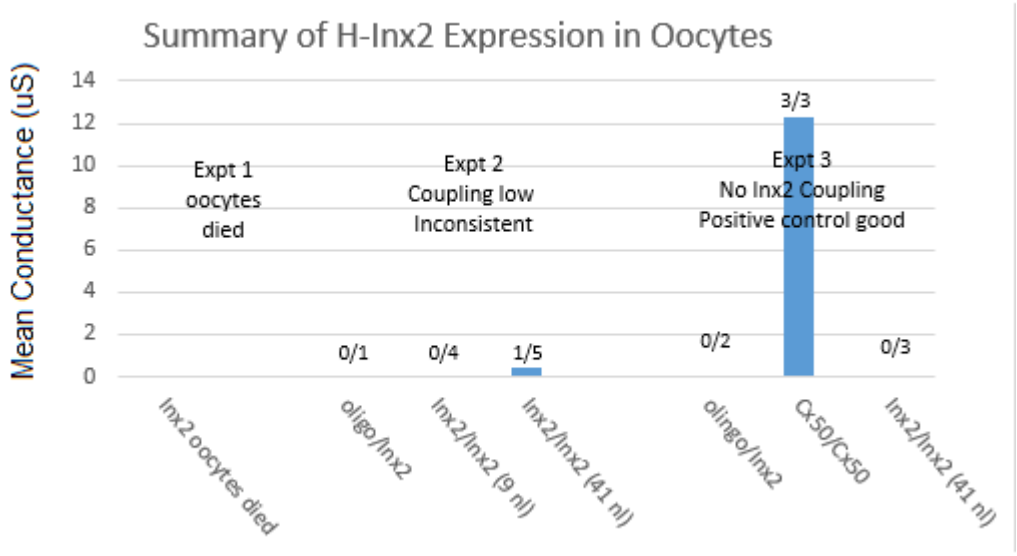


Figure 15. Summary of three experiments involving expression of H-Inx2 in *Xenopus* oocytes. In the first experiment oocytes injected with H-Inx2 (41 nl of RNA at 125 ng/µl) died after pairing. In the second experiment a lower volume of RNA was injected in one group (Inx 2 - 9 nl) and failed to induce coupling. In the 41 nl group only one of five pairs was coupled and this pair showed poor responses under the voltage clamp. Inx2 also failed to induce coupling in the third experiment when Cx50 positive controls demonstrated an average of 12 µS and responded well under voltage clamp.

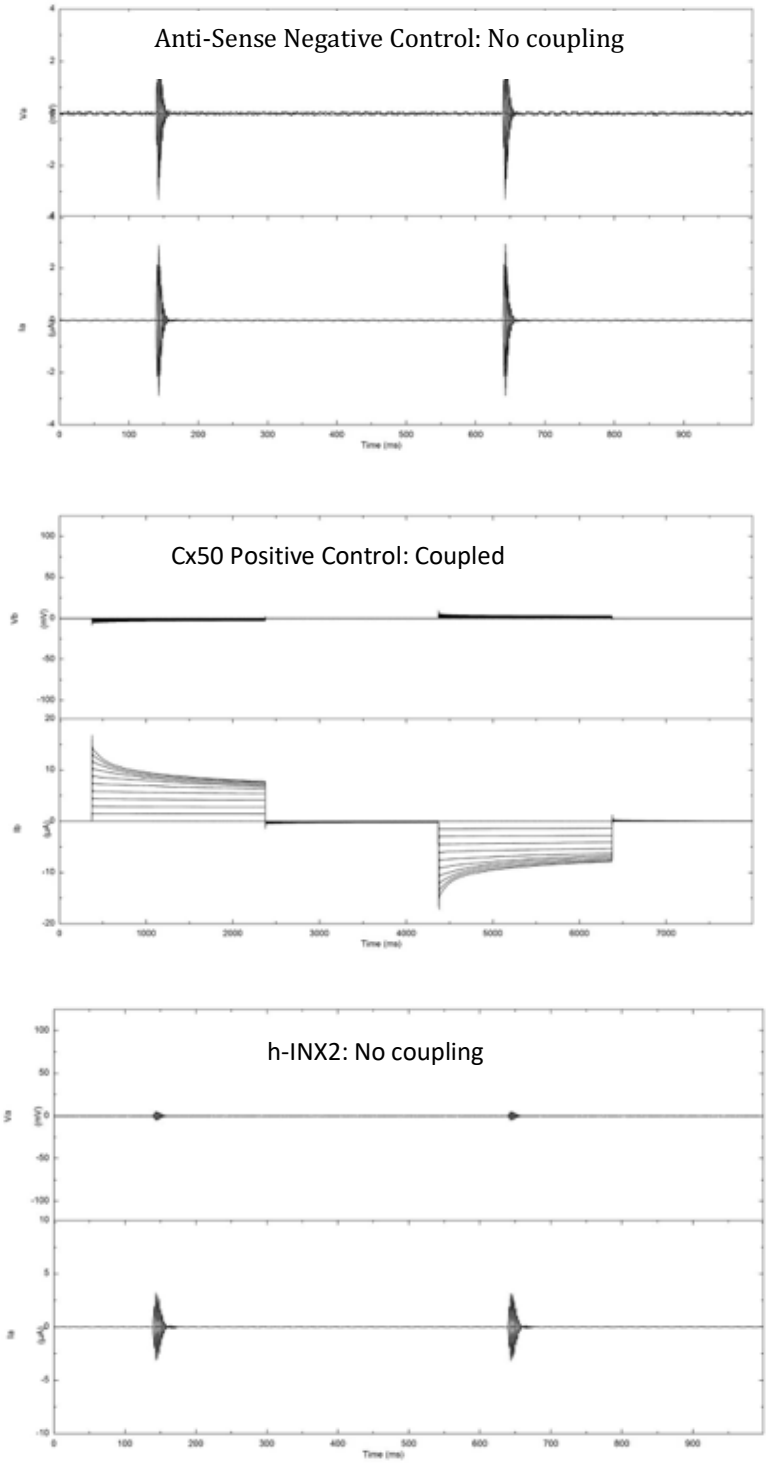


Figure 16. Currents recorded from paired oocytes using the dual cell two-electrode voltage clamp. TOP: Negative control anti-XeCx38 oocytes did not couple as evident by the lack of induced current in the lower trace. Currents were recorded from a continuously clamped oocyte while its partner was pulsed in 100 mV increments to ± 100 mV. MIDDLE: Positive control Cx50-injected oocytes displayed junctional currents that decayed in a time- and voltage-sensitive manner. Currents were recorded from a continuously clamped oocyte while its partner was pulsed in 10 mV increments to induce voltage across the junction of ± 100 mV. BOTTOM: Experimental h-Inx2 injected oocytes did not couple as evident by the lack of induced current in the lower trace. Currents were recorded from a continuously clamped oocyte while its partner was pulsed in 100 mV increments to ± 100 mV

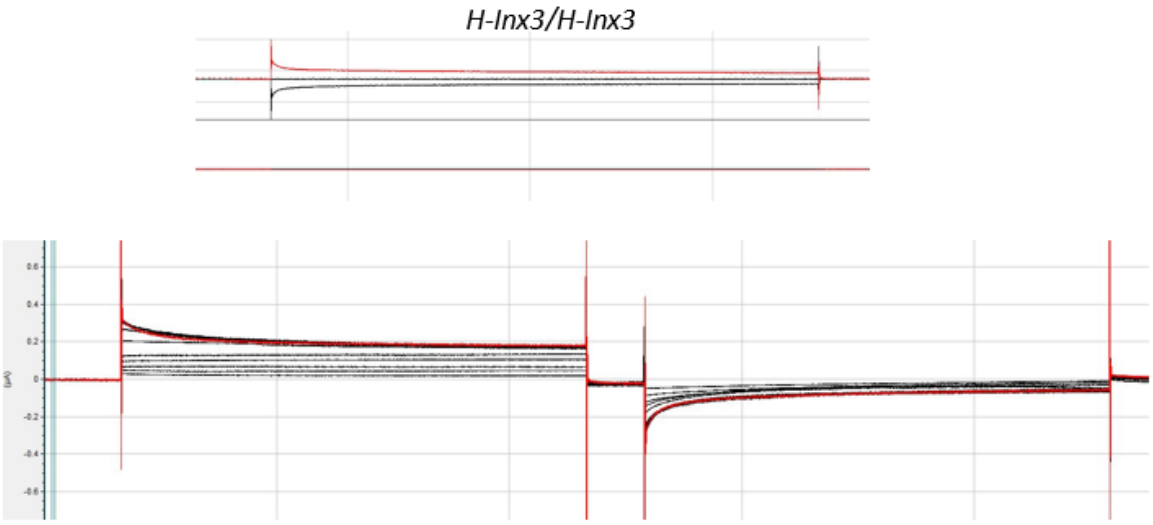


Figure 17. Example traces demonstrating expression of H-Inx3 in *Xenopus* oocytes. These currents were recorded from oocytes injected with 41 nl of 125 ng/µl of H-Inx3. This experiment served as a positive control for *Hydra* innexins. **TOP:** Currents were recorded from a continuously clamped oocyte while its partner was pulsed ± 100 mV in 200 ms short pulses. **BOTTOM:** Currents were recorded from a continuously clamped oocyte while its partner was pulsed in 10 mV increments to induce voltage across the junction of ± 100 mV. Each voltage pulse is 2 seconds long.

```
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FT   TRANSMEM     27     48
FT   TOPO_DOM     49    129       NON CYTOPLASMIC.
FT   TRANSMEM    130    152
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FT   TRANSMEM    196    217
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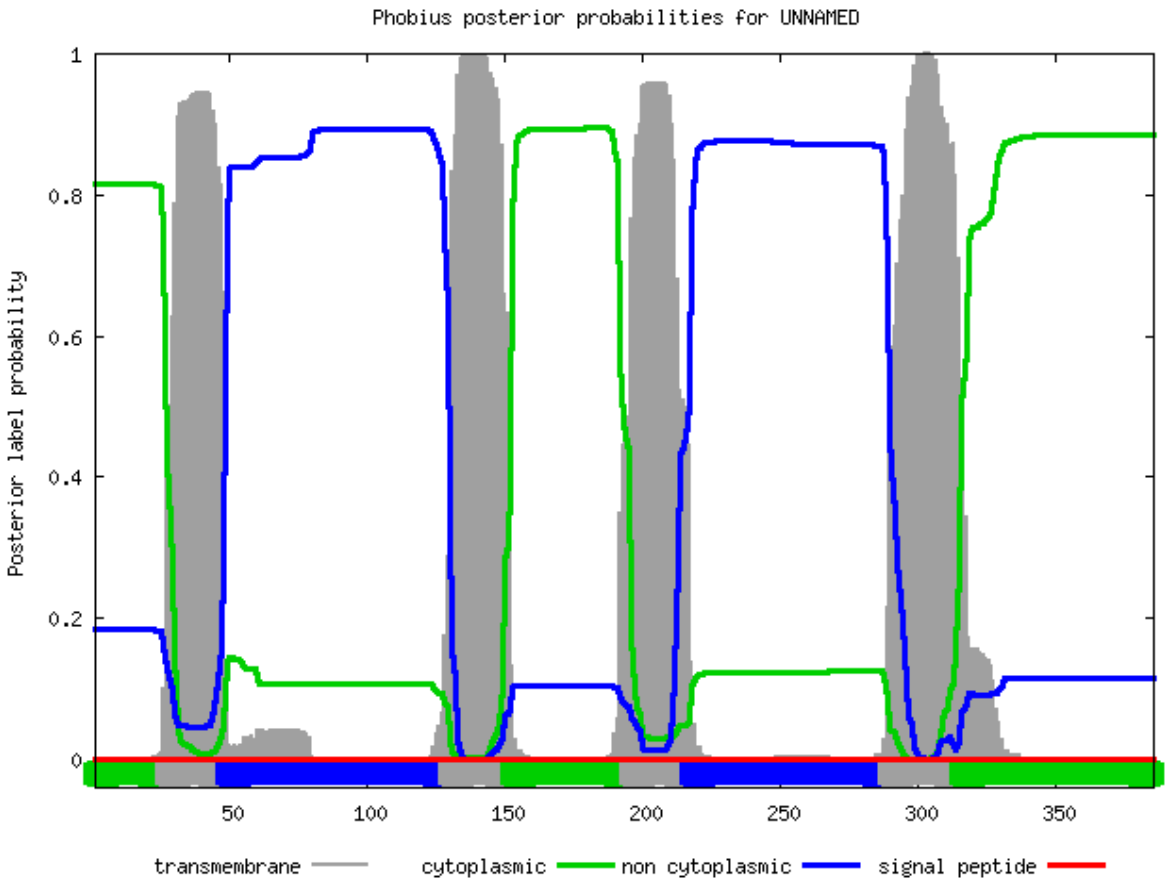

Figure 18. Membrane topology prediction with graphics by Phobius (Stockholm Bioinformatics Centre) with cytoplasmic amino terminus (amino acids 1 through 26), four transmembrane domains and a cytoplasmic carboxyl terminus (amino acids 316 through 386). Other topology prediction programs yielded similar results. This is topologically characteristic of a functional innexin.