

UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

Evaluating innexins quantitatively and qualitatively in the developing leech : identified neurons in *Hirudo medicinalis* express unique subsets of the innexin genes / c Brandon Sarkis Kandarian

Permalink

<https://escholarship.org/uc/item/7zd2r5f8>

Author

Kandarian, Brandon Sarkis

Publication Date

2009

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Evaluating innexins quantitatively and qualitatively in the developing leech: identified neurons in *Hirudo medicinalis* express unique subsets of the innexin genes.

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Brandon Sarkis Kandarian

Committee in charge:

Professor Eduardo Macagno, Chair
Professor Immo Scheffler
Professor Gina Sosinsky

2009

The Thesis of Brandon Sarkis Kandarian is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2009

TABLE OF CONTENTS

Signature Page.....	iii
Table of Contents.....	iv
List of Figures.....	v
List of Tables.....	vi
Abstract.....	vii
Introduction.....	1
Results.....	4
Discussion.....	20
Methods.....	28
References.....	37

LIST OF FIGURES

Figure 1. After PCR was run with the primers shown in Table 1, the dissociation curves were verified by running the PCR product on a 1.5% gel.....	7
Figure 2. Relative quantification using either RS60 or ELF-1 as reference genes.....	14
Figure 3. PCR assays were performed to determine which innexins were present in leech Retzius, AP, and P ₁ neurons. Panel B shows a schematic diagram of the locations and identities of neurons on the ventral aspect of a single ganglion in <i>Hirudo medicinalis</i>	19
Figure 4. This diagram depicts both junctional and non-junctional innexins, used from Bao and Macagno 2006.....	27

LIST OF TABLES

Table 1. Primers were designed for Hm-inx1 through Hm-inx14, and for reference genes Hm-Rs60, Hm-Elf-1 and Hm-Act1.....	6
Table 2. Comparison of previous whole mount in situ hybridization results in the embryonic leech (Dykes et al. 2006) to qPCR results found in this study.....	17
Table 3. Contamination check with copy DNA (cDNA) and no template controls (NTC).....	30
Table 4. Quantitative assay with Hm-inxs 1 and 2 and three reference genes.....	32
Table 5. Assay run on E12 and adult to determine innexins present in the central nervous system (CNS).....	34
Table 6. Assay run to determine which innexins were expressed in single cells.....	36

ABSTRACT OF THE THESIS

Evaluating innexins quantitatively and qualitatively in the developing leech: identified neurons in *Hirudo medicinalis* express unique subsets of the innexin genes.

by

Brandon Sarkis Kandarian

University of California, San Diego, 2009

Master of Science in Biology

Professor Eduardo Macagno, Chair

Gap junctions are clusters of intercellular channels which enable direct cell-to cell cytoplasmic communication both electrically and chemically. Gap junctions play an important role in determining cell fate and function during development, underlining the functionality of their plasticity in developing systems. In the work presented here, *Hirudo medicinalis*, a European leech, was used to study gap junctional protein expression in both the whole embryonic and adult leech central nervous system (CNS) as well as in

single neurons. First, quantitative studies revealed that Hm-inx1, a neural specific innexin, and Hm-inx2, a glial specific innexin, are both expressed at relatively higher levels in the embryo than in the adult. Aside from suggesting greater possible coupling among cells in the early leech CNS, this up-regulation may explain how glial cells regulate synapse numbers in neurons early in leech development. Second among 12 previously characterized innexins and two novel innexins, Hm-inxs 13 and 14, four (Hm-inxs 4, 11, 12, and 13) were found to be expressed in the adult CNS but not in the young embryo, while one (Hm-inx8) was found to be expressed in the young embryo but not in the adult. Finally, three specific types of neurons, Retzius (R) serotonergic neurons, anterior pagoda (AP) motor neurons, and pressure (P₁) neurons, were found to express unique subsets of innexins. All three cells express Hm-inxs 1 and 6. In addition, R cells express Hm-inx2, AP cells express Hm-inx4, and P₁ cells express Hm-inx3 and Hm-inx11.

Introduction

Developing neurons, as well as many other embryonic cell types, are often connected to one another by gap junctions, intercellular channels that allow the direct transfer of electrical signals and small molecules between the cytoplasm of coupled cells (3). A gap junction is formed by either homotypic or heterotypic half junctions on adjacent cells. The half junctions are composed of six monomers from a multi-gene family of protein subunits. Connexins and pannexins are the gap junction subunits found in vertebrates, while innexins are the protein subunits present in invertebrates (23, 27).

Because of the similarity in morphology and function of invertebrate gap junction systems to vertebrate systems, much of our knowledge about the properties of neuronal communication has been discovered through invertebrate research. Leeches have been used extensively in neuronal studies because their large neurons allow for in-vivo analysis in both the embryo and the adult stages. Also, the shapes, positions, and functions of leech neurons are consistent among animals, making it possible to study how short-term and long-term changes come about at various levels, including synaptic connections between specific cells. The leech used in the research I will present here, *Hirudo medicinalis*, is particularly favorable for developmental studies; it has a fairly short generation time, simple cultivation, and a hardy embryo.

It has been reported that developing neurons in the vertebrate spinal cord, retina, and cortex are initially interconnected by gap junctions that are lost as they develop (15). In the leech, embryogenesis is a dynamic process involving several well-defined stages that ultimately result in the formation of the central nervous

system (CNS). Central synapses do not acquire their adult properties until after movements have begun (33).

In general, gap junctions link the earliest born non-neuronal cells in embryos and are essential for *C. elegans*, *Drosophila*, and mammalian embryogenesis (27). Recent studies of the expression of gap-junctional proteins in vertebrates indicate that neuronal coupling is exceptionally high in the first two postnatal weeks and declines thereafter in rodents (5). The work to be reported here includes an exploration of the dynamics of expression of gap junctional genes, or innexins, in the leech.

The way to evaluate the variability in innexin expression both in the embryo and adult is through CNS transcript quantification. Quantitative real-time PCR (qPCR) has become the method of choice for detection and quantification of mRNA (Bustin, 2000). Quantification can be relative or absolute. Relative quantification, used to determine changes in mRNA transcription levels relative to the same sequence in a calibrator sample, was used in order to compare expression pattern changes from the embryo to the adult. For accurate gene quantification analysis, normalization of qPCR data is essential to eliminate template variations between samples due to variations in initial sample amount, mRNA recovery, mRNA integrity, mRNA purity and reverse transcription efficiency, as well as pipetting errors (22, 25, 38). Normalization is most frequently achieved by use of internal controls, generally provided by measuring the expression of housekeeping genes whose expression levels are thought to be relatively invariant throughout the life of an animal. For *Hirudo medicinalis*, the housekeeping genes I employed in these experiments were Elongation Factor-1 (Hm-Elf1), 60S ribosomal

protein (Hm-Rs60), and Actin-1 (Hm-Rs60).

Here I report on the variability of gap junction protein expression in *Hirudo medicinalis* both in the embryo and the adult. Quantitative real time PCR measurements will be discussed first in embryonic stages, documenting the dynamics of innexin expression from birth to adulthood. Embryogenesis, from the time fertilized eggs are deposited in an egg case or cocoon to the emergence of juveniles from the egg case, takes approximately 28-30 days at room temperature (31). I first consider levels of expression of two known innexin genes during this time period as well as two post-embryonic stages relative to the adult leech. I then present the results of an initial study of the expression of innexins by single adult neurons, which suggest that different neurons can express unique subsets of the innexin genes, a property that could be important in defining their adult patterns of synaptic connections.

Results

Novel Innexins and Primer Design:

Twelve innexins (Hm-inxs 1 through 12) had previously been described in the adult (10) or embryonic leech (11). Innexins are known to be part of a multi-gene family, which lead to the initial investigation to describe these twelve innexin genes. A search for a more complete set of innexins expressed in the leech yielded two novel innexins, Hm-inxs 13 and 14 (Alejandro Sanchez, work unpublished) bringing the total number of known medicinal leech innexins to 14. Because the leech genome has not been sequenced, there may be more innexins that have not been identified yet. The leech innexins range in size from 381 aa (44.4 kDa; Hm-inx12) to 480 aa (55.8 kDa; Hm-inx6). Typically the sequences of the extracellular loops and transmembrane domains are well conserved, but the intracellular domains are quite variable (11). Please refer to (29), Figure 1 for a diagram of the four transmembrane domains of innexin channels.

Primers were designed for all innexins using primer-3 software (32). The purpose of the primers is to either quantitatively or qualitatively evaluate the 14 known innexins with qPCR using mRNA extracted from different parts of the leech. The melting temperatures were 60 +/- 0.5 C° and the expected product was kept approximately within 100-200 bps. The forward and reverse primers were screened in BLAST to ensure the sequences were specific for the gene of interest (see Methods).

Primer Characterization:

When primers are used in q-PCR, a melting point analysis is performed after the PCR product is formed, resulting in a dissociation curve. Each dissociation curve is specific for the gene of interest and is identified by measuring the peak melting temperature. A positive control run was performed in triplicate for each of the primers in Table 1. The resulting PCR products were then run on a 1.5% polyacrylamide gel (Figure 1) to verify that the dissociation curves were representative of the correct PCR products. The peaks of the dissociation curve were then used in all future experiments to verify if a gene of interest was present.

Table 1. Primers were designed for Hm-inx1 through Hm-inx14, and for reference genes Hm-Rs60, Hm-Elf-1 and Hm-Act1. The forward and reverse primers each are 20 bases long, and the expected product was kept within an approximate range of 100-200 bps. A PCR was run for all the primers using a positive control (full body cDNA). The dissociation curve of the PCR product was then verified by running the product on a 1.5% gel. The Hm-inx14 primers did not produce a product and were not used in the remaining experiments. The dissociation curves generated in these trials were used in subsequent trials to confirm the presence of mRNA from the innexins that were tested.

Gene	Forward Primer (5'-3')	Tm	Reverse Primer (3'-5')	Tm	Product Size (bp)	Dissociation Curve
Hm-inx1	TCAGACAGCA CTCTCGGATG	60.14	CAGTAGAGGGA GCGGAACAG	60.01	160	87
Hm-inx2	CTGCAGAGAA CCGGGATAAG	60.07	AGACGAGGAGC AGGAACCTG	59.87	192	88.3
Hm-inx3	TGTCCACGAA GCAGTACGTC	59.9	CCCCTAAACAG AAGGCATGT	59.99	152	87.8
Hm-inx4	CAGACGGTCC CCTACTACCA	59.98	GCCTCTCCTTCT CCGATCTT	59.92	167	87
Hm-inx5	GCACCTGTCTC TACGGCTTC	60.02	GTGTCGTTGAC GGAGGATTT	59.97	130	84.7
Hm-inx6	TCAAGGACGA TTTCCAAAGG	60	CAACACGGAAA CATCACAGG	60	171	86.4
Hm-inx7	GCGACAAGCG TCACTGAATA	60.02	CTGCTTCAGAC GACAATGGA	59.98	121	86.4
Hm-inx8	AAGATATTTGC ACCCGAACG	59.98	ACGCTACGGTA ACCATCCAG	60.02	194	87.4
Hm-inx9	ACGGAATCGA AGTCATCAGG	59.97	CTGGCCAAAGA GGATACAGC	59.97	162	87
Hm-inx10	CCATTAAGACC GGTTGCACT	59.99	AGTGAGCCAA GACATTGGT	59.87	145	85.9
Hm-inx11	GCCAAGGTTT ATTTGGAAGA	60.05	CTTCGCTGCAC CTTTCTC	59.99	101	83.5
Hm-inx12	TCTCGATATTC GTCCCGTTC	60.04	AATGAAGACCA GCCACATCC	59.93	113	83.8
Hm-inx13	GGCAGTTTT AACAGCAGA	60.03	GTGCTTCCCGTC GTTATTGT	60	164	87
Hm-inx14	CCTACGCCACT GGCTACTTC	59.90	TTCCCGAAGAT CCGTATTG	59.89	219	N/A
Hm-Rs60	AGTGGAAGT TTGGATTGG	59.97	TTTGACAGCCT CTTCCTTGG	60.04	189	85
Hm-Elf1	CCAGTTGTTGA TTGCCACAC	60.01	ATCTCCAGCCTT CACATTGG	60.07	120	85
Hm-Act1	ACGTCGACATC AGGAAGGAC	60.12	GTATTTGCGCTC AGGAGGAG	59.98	155	89

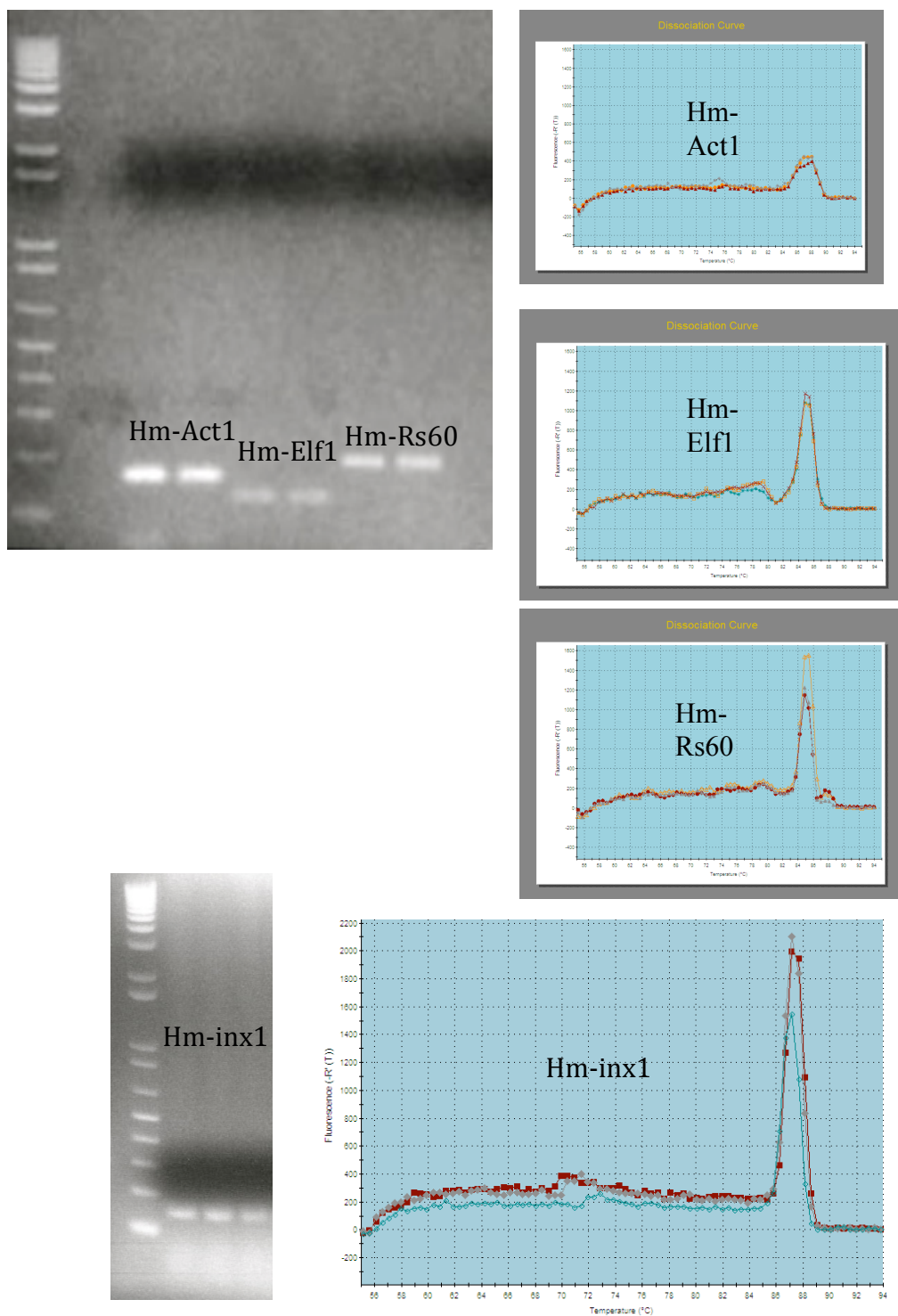


Figure 1. After PCR was run with the primers shown in Table 1, the dissociation curves (right) were verified by running the PCR product on a 1.5% gel (left).

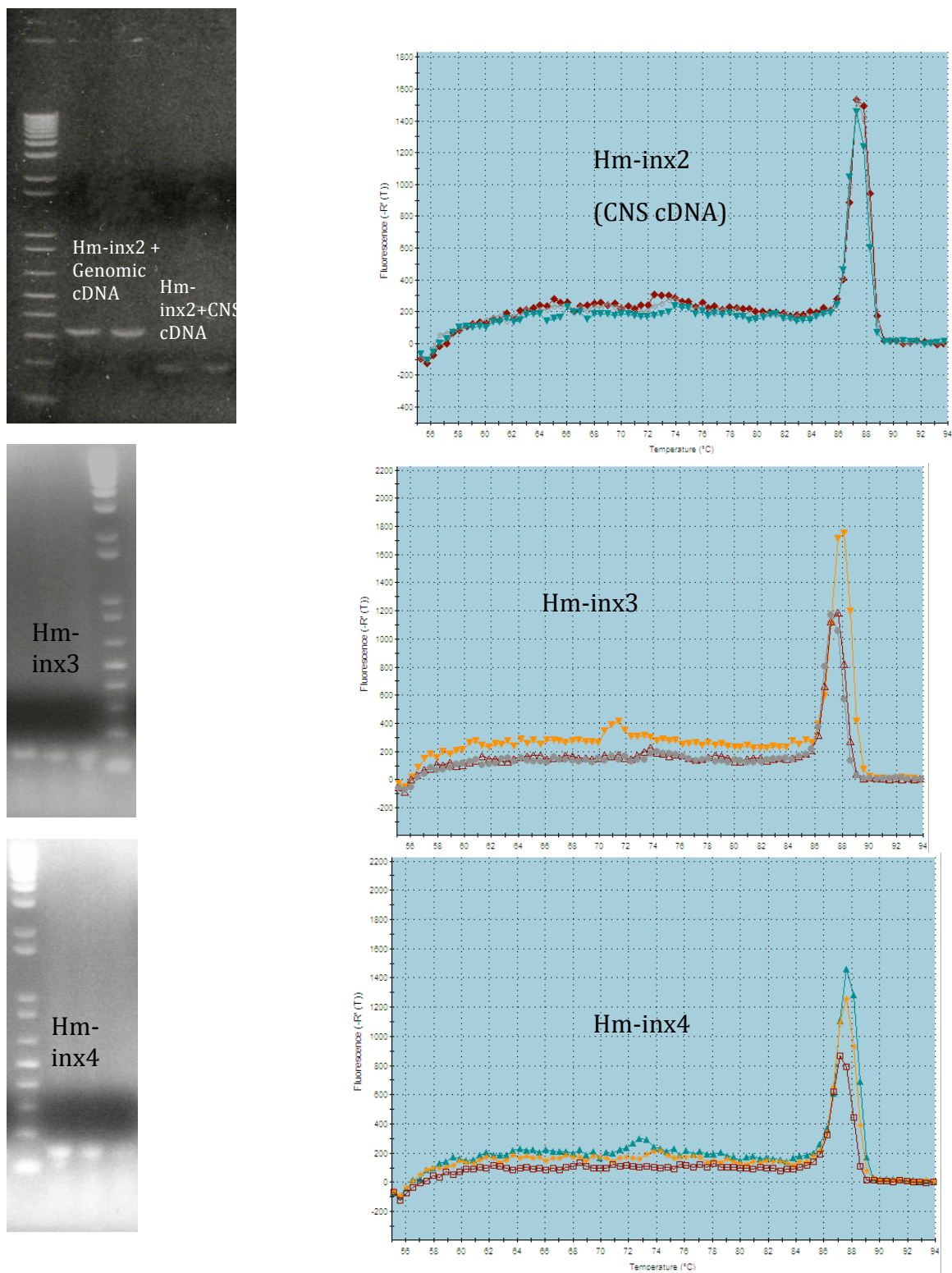


Figure 1. (continued) After PCR was run with the primers shown in Table 1, the dissociation curves (right) were verified by running the PCR product on a 1.5% gel (left).

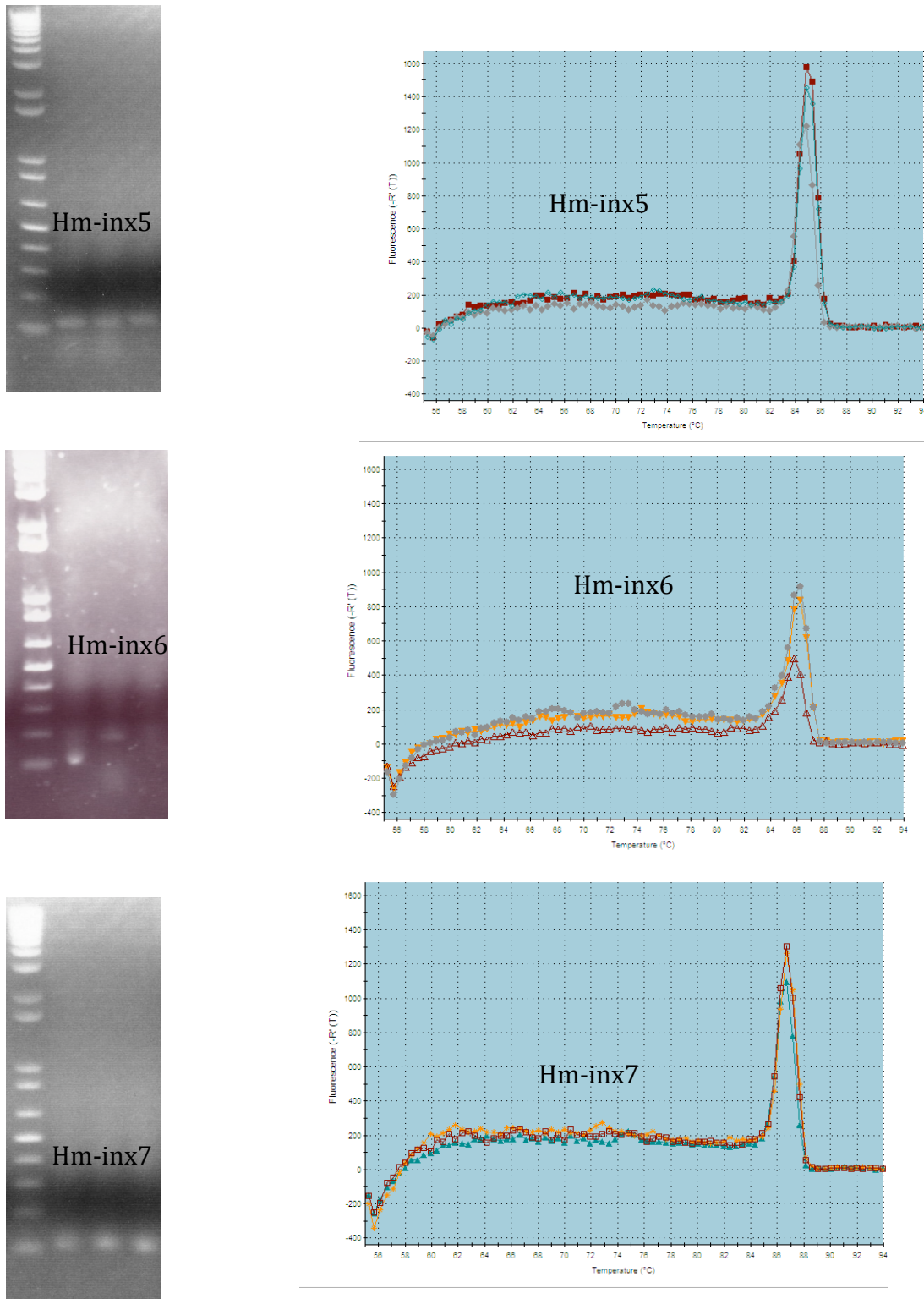


Figure 1. (continued) After PCR was run with the primers shown in Table 1, the dissociation curves (right) were verified by running the PCR product on a 1.5% gel (left).

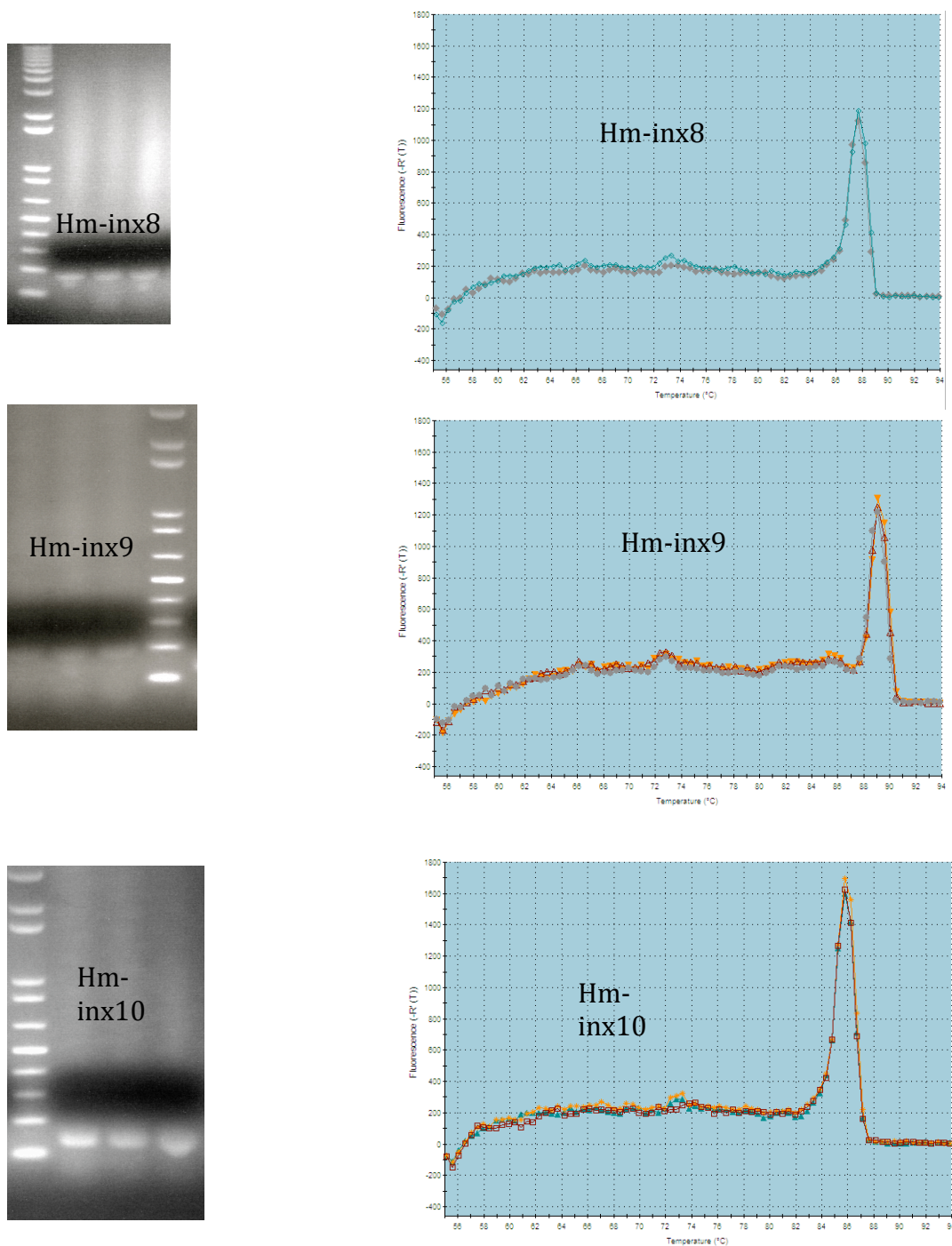


Figure 1. (continued) After PCR was run with the primers shown in Table 1, the dissociation curves (right) were verified by running the PCR product on a 1.5% gel (left).

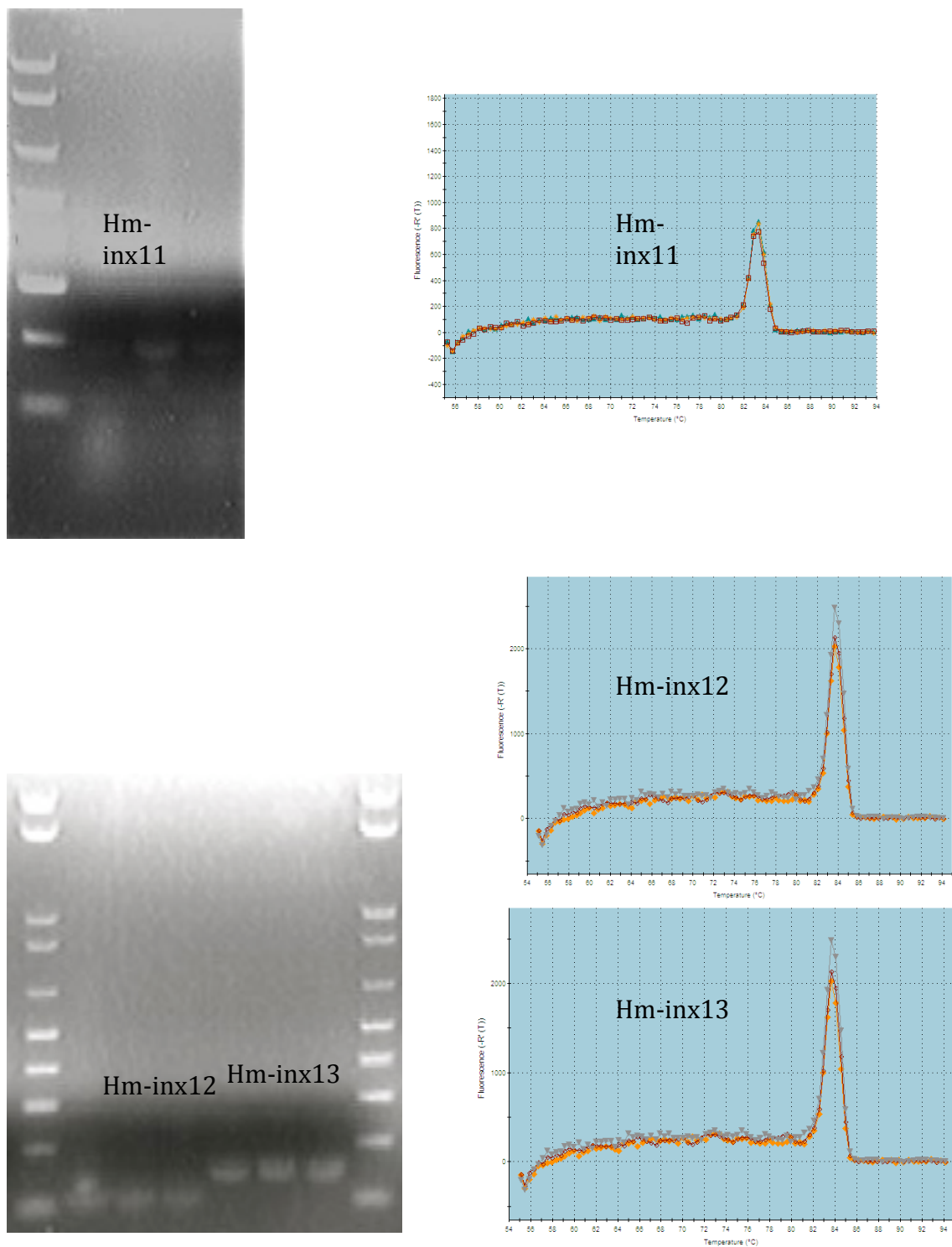


Figure 1. (continued) After PCR was run with the primers shown in Table 1, the dissociation curves (right) were verified by running the PCR product on a 1.5% gel (left).

Measuring the Dynamic Expression of Innexins 1 and 2:

During the embryonic stage of the leech, the organism undergoes enormous morphological and physiological changes, including CNS formation, initiation of spontaneous movements, and the generation of neuronal circuits underlying complex behaviors such as swimming. To explore what happens to gap junctional communication during the embryonic stages of the leech, mRNA levels were measured for two innexins that have been shown to be present in the leech both in early embryonic stages and in the adult (10). More specifically relevant to my project, Hm-inx1 is expressed in neuronal cells and Hm-inx2 is expressed in glial cells. Two different reference genes were used, and for both the results showed similar trends in the growing leech. At embryonic stages E12 and E22, I determined that both innexins express statistically significant higher levels of mRNA relative to the adult levels, while at the postembryonic stages PE2 and PE12, neither of the innexins expressed mRNA levels significantly different from those observed in the adult (Figure 2).

The values from the different stages were normalized with respect to the reference genes Hm-Rs60 and Hm-Elf-1 and compared to the adult values by using the $\Delta\Delta C_t$ equation provided by Stratagene with their q-PCR kit. Normalizing the data for embryonic and post-embryonic stages relative to the adult measurements, a value greater than 1 indicates up-regulation and a value less than 1 signals down-regulation compared to the adult. As shown in Figure 2, the measurements using both reference genes yield consistent results. Hm-inxs 1 and 2 were upregulated more than two fold at the E12 stage when using Hm-Rs60 as the reference gene (Figure 2B), and more than 1.5 fold when using Hm-Elf-1 as the reference gene (Figure 2C). At the E22 stage, Hm-inxs 1 and 2

remained upregulated more than 1.5 fold using RS60 as the reference gene, and upregulated 1.2 to 1.4-fold when using Elf-1. These differences were shown to be statistically significant by means of a paired T-test (Figure 2A and 2C).

By contrast, in the PE2 or PE12 stages neither of the innexins showed statistically significant differences in expression relative to the adult, using either of the reference genes (Figure 2A and 2C). Thus, Hm-inx1 and Hm-inx2 are expressed at higher levels during embryogenesis, particularly at earlier stages, but the adult levels seem to be attained quickly post-embryonically. The replicability of both reference genes underlines their potential to be used as reference genes for future quantitative assays of gene expression with *Hirudo medicinalis*.

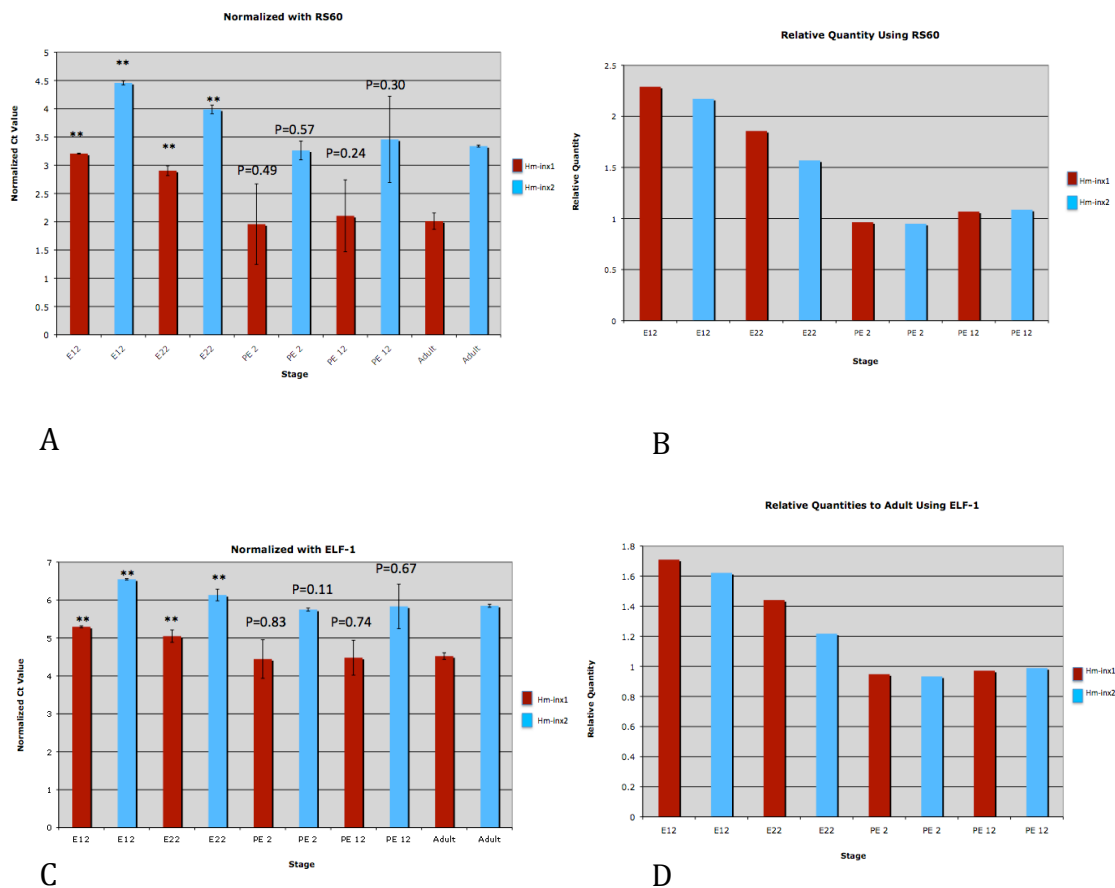


Figure 2. In panels A (Rs60 reference gene) and C (Elf-1 reference gene), each stage was compared to the adult mRNA levels using a paired T-test. Hm-inxs 1 and 2 levels were significantly different at E12 and E22 compared to adult levels, with P-values less than 0.001 (shown with **). Both innexin levels were not significantly different in the PE2 or PE12 stage. In panels B (Rs60 reference gene) and C (Elf-1 reference gene), the $\Delta\Delta C_t$ Method was used to determine whether the normalized genes at each stage were upregulated or downregulated relative to the adult: $2^{-(\Delta\Delta C_t)}$, where $\Delta\Delta C_t = (C_{t_{GOI}} - C_{t_{norm}})_{adult} - (C_{t_{GOI}} - C_{t_{norm}})_{unknown}$.

Characterizing Innexins Present in the Embryonic and Adult CNS

Previous *in situ* hybridization (ISH) assays determined the expression patterns of innexins in different stages of the embryonic leech (11). Specifically, the data was used to determine whether an innexin was expressed in the CNS of the leech, among other parts of the animal (Table 2). Results from ISH were limited to stages up to about E12 because of reagent penetration in the embryonic whole mounts. This left a large gap of hypothetical plasticity as the leech continues to develop approximately 18 more days before it has reached its juvenile stage.

qPCR was found to be an alternative method that could be used to continue the characterization of neuronal expression patterns in later stages of leech development and in the adult. Although the specific location of the innexin expression within the nervous system cannot be determined using this approach, the expression level of an innexin in the CNS or in the rest of the animal can be measured with great accuracy. mRNA for these assays was purified from ganglia in the leech, converted to cDNA, and then run with all primers shown in Table 1. cDNA made from the full body of E12 leeches was used as a positive control and run for each primer. No-template controls were run in duplicate for each primer to ensure the primers weren't contaminated. A contamination check (see Methods) was run on each set of cDNA to ensure there was no genomic cDNA and that only cDNA from the CNS was amplified. The dissociation curves for the PCR products were used to identify whether mRNA for an innexin was present. Assays were first run on multiple ganglia in E12 leeches. The PCR results matched expression patterns found with previous ISH observations (11), verifying q-PCR as a simplified alternative to *in situ* hybridization.

In addition, mRNA from single adult *Hirudo medicinalis* ganglia was extracted and run in a qPCR assay with all the primers in Table 1. Results show that there are differences in CNS expression patterns in the embryonic and adult leech. For example, Hm-inx4, Hm-inx11, Hm-inx12, and Hm-inx13 began being expressed in the CNS at some point after the E12 stage. Expression of Hm-inx8 in the CNS was turned off some time after the E12 stage. These findings demonstrate that the set of hemichannels expressed in the CNS continues to change throughout embryonic development.

Table 2. Comparison of previous whole mount in situ hybridization results in the embryonic leech (11) to qPCR results found in this study. Primers for the same 12 innexins were used with the addition of Hm-inx13, a novel leech innexin. cDNA was made from three tail, middle, or head ganglia in E12 leeches and one tail, middle, or head ganglia in the adult leech. Two animals were used for each stage. Each innexin primer was run in triplicate with CNS cDNA, the negative control run in duplicate, and positive control (full body or FB cDNA with the gene of interest) run in a single trial. The PCR product was verified using the dissociation curves listed in Table 1. Results differing from the previous in situ hybridization study have been boxed.

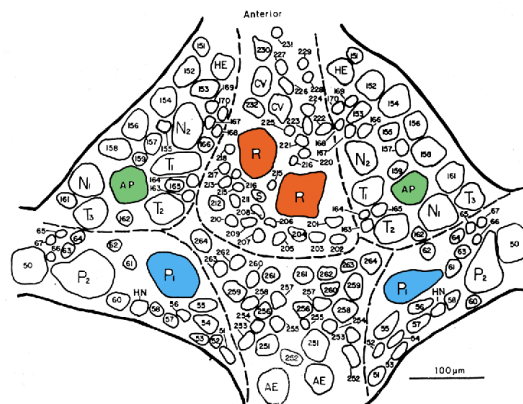
Gene	Present in the E12 CNS (In Situ Results, Dykes et al. 2006)	Present in the E12 CNS	Present in the Adult CNS
Hm-inx1	Y	Y	Y
Hm-inx2	Y	Y	Y
Hm-inx3	Y	Y	Y
Hm-inx4	N	N	Y
Hm-inx5	N	N	N
Hm-inx6	Y	Y	Y
Hm-inx7	N	N	N
Hm-inx8	Y	Y	N
Hm-inx9	Y	Y	Y
Hm-inx10	N	N	N
Hm-inx11	N/A	N	Y
Hm-inx12	N/A	N	Y
Hm-inx13	N/A	N	Y
Hm-Rs60	N/A	Y	Y
Hm-Elf1	N/A	Y	Y
Hm-Act1	N/A	Y	Y

Characterizing Innexins Present in Single Neurons:

Adult neurons were screened with all innexin primers that tested positive in the adult CNS to determine if neurons expressed all CNS innexins or a select subset. mRNA was purified from Retzius, AP, and P₁ cells, and cDNA made. Three cells were taken for each type of neuron from three different animals and run with each gene of interest in triplicate. cDNA made from the full body of E12 leeches was used as a positive control and run for each primer. No-template controls were run in duplicate for each primer to ensure the primers weren't contaminated. A contamination check (see Methods) was run on each set of cDNAs to ensure there was no genomic cDNA and that only cDNA from the single cell was amplified. The dissociation curves for the PCR products were used to identify whether mRNA for an innexin was present. The results (Figure 3) showed that each cell type had a unique innexin expression pattern. All three neurons were producing mRNA for Hm-*inx1*. Retzius cells were also making mRNA for Hm-*inx2*. By comparison, AP cells were also making mRNA for Hm-*inx4*. P₁ cells were also making mRNA for Hm-*inx3* and Hm-*inx11*. All of the tested neurons expressed Hm-Rs60 and Hm-Elf-1, showing the potential to perform quantitative studies on single neurons in future experiments.

Gene	Innexins Present in Adult CNS	Retzius Cell	AP Cell	P Cell
Hm- <i>inx1</i>	Y	Y	Y	Y
Hm- <i>inx2</i>	Y	Y	N	N
Hm- <i>inx3</i>	Y	N	N	Y
Hm- <i>inx4</i>	Y	N	Y	N
Hm- <i>inx6</i>	Y	Y	Y	Y
Hm- <i>inx9</i>	Y	N	N	N
Hm- <i>inx11</i>	Y	N	N	Y
Hm- <i>inx12</i>	Y	N	N	N
Hm- <i>inx13</i>	Y	N	N	N
Hm-Rs60	Y	Y	Y	Y
Hm- <i>Elf1</i>	Y	Y	Y	Y
Hm- <i>Act1</i>	Y	Y	Y	Y

A



B

Figure 3. PCR assays were performed to determine which innexins were present in leech Retzius, AP, and P₁ neurons. The PCR product was verified using the dissociation curves listed in Table 1. Panel B shows a schematic diagram of the locations and identities of neurons on the ventral aspect of a single ganglion in *Hirudo medicinalis*. Those with color fills were screened in this experiment. The diagram was altered from (21).

Discussion

Measuring the Expression of Innexins 1 and 2 in Different Life Stages:

In addition to their role in neural circuits, gap junctions also play a role in embryonic development by allowing low molecular weight signaling molecules to pass between cells. In the leech, for example, gap junctions were hypothesized to mediate the inhibition of projections in a central neuron during development (37). At early stages in development, many cells are typically electrically coupled, dye coupled, or both. These patterns of coupling between cells typically change as the animals develop (4).

Embryonic days 6 to 12 is the period during which the main features of the central nervous system are established. By E12 the embryo has a CNS containing differentiated neurons as well as circulatory and excretory systems. Thus the up-regulation of mRNA for Hm-inxs 1 and 2 in E12 and E22 leeches reflects the increased presence of gap junctions in the developing embryo. Increased gap junction formation in the early stages of leeches might be a reflection of the need to have more permissive cell-cell connections to facilitate development. Many of these connections are likely lost after the embryonic stage, highlighted by the return of Hm-inxs1 and 2 mRNA levels to adult levels in PE2 and PE12.

In another study in the leech, electrical coupling between developing neurons and glial cells, which was observed in leech embryos near 45% ED, disappeared when the neurons started growing their secondary central branches, eventually forming synapses (20). It has been found that glial cells play an important role in the control of synapse number, which may explain their early coupling with neuronal cells (36). The mechanism

for how these cells become coupled is unknown, however. This study, in which the amounts of Hm-inxs 1 and 2 were measured during development may offer an explanation. Hm-inx1 has been found in neuronal cells but not glial cells and Hm-inx2 is expressed in glial cells but not in neuronal cells, but both can form heterotypic gap junctions with each other. The fact that Hm-inxs 1 and 2 were both upregulated at the E12 and E22 stages of the leech and downregulated thereafter may explain the mechanism of this coupling observed by Marin-Burgin and others.

Also, gap junctions formed between the transient projections of the developing AP neurons mediates the exchange of signals that permit homologues to recognize each other and inhibit further outgrowth (37). However the identity of the transiently expressed innexin is unknown in this case. It is known that most if not all neuronal cells in the leech CNS express Hm-inx1 and that this innexin forms homotypic gap junctions (1). Thus the upregulation of Hm-inx1 during the early embryonic stages may help direct the growth of neurons in the CNS.

Quantitative real-time PCR is the most sensitive method to quantify transcripts, provided the gene transcription patterns are normalized to a reference gene evaluated in parallel. This experiment began with three reference genes, Hm-Rs60, Hm-Elf1, and Hm-Act1. After trials with E12, E22, and adult tissues, it became clear that Hm-Act1 was not an effective reference gene (data not shown) and it was left out of data analysis and the remaining trials. But the other two reference genes produced replicable results in both embryos and adults. In addition, mRNA for these reference genes was found in the single neurons that were investigated. Therefore both reference genes may be invaluable to future quantitative trials with *Hirudo medicinalis*.

Characterizing Two Novel Innexins:

Partial sequences for novel innexins 13 and 14 have been determined. However, the primers for Hm-inx14 did not work, but those for Hm-inx13 proved fruitful. It was found that Hm-inx13 was not expressed in the CNS at the E12 stage, but it was found to be expressed in the adult stage. Although the specific neurons that express this innexin are unknown, it is possible that Hm-inx13 plays a developmental role in the later stages of the leech. The isolation and characterization of this novel gene should form the basis for further study of the functional events that occur during development and neuronal communication in *Hirudo medicinalis*. Moreover, new primers and perhaps a new region of the transcript need to be explored in order to obtain expression patterns for the Hm-inx14 gene.

Characterizing Innexins Present in the Embryonic and Adult CNS:

In situ hybridization experiments had been performed to determine the location of innexin expression in *Hirudo medicinalis* up to E12, but could not be used reliably after that age to map innexin expression in embryonic whole mounts (11). By showing that my qPCR results agree with the in situ hybridization results at the same stage, qPCR has been confirmed as a valid alternative to in situ hybridization in characterizing the expression of innexins in the leech at the tissue level. My results indicate that Hm-inxs 4, 11, 12, and 13 begin to be expressed in the CNS at some point after the E12 stage (or approximately 51% ED). Previous in situ hybridization results found Hm-inx4 to be expressed in the reproductive and excretory system very early in the embryo. In situ hybridization results

for Hm-inxs11 and -12 were inconclusive, and were not performed for Hm-inx13 as it was not identified at the time (11). The innexins which were turned on after E12 may play a part in the later stages of development.

Since gap junction proteins are encoded by multi-gene families, the complexity of these families allows the assembly of a large number of gap junction channels, each containing a unique combination of subunits and hence potentially unique properties with respect to, for example, permeability, gating, and cell-cell recognition. Thus each innexin may help to form very specific gap junctions with other cells or non-junctional hemichannels, specific for components needed during development.

Although some innexins were turned on after the E12 age, my observations show that one innexin was turned off. Expression of Hm-inx8 in the CNS was turned off at some time after the E12 stage. Between E9 and E10, ISH showed Hm-inx8 to have highly restricted expression in two adjacent cells in the CNS, which are predicted to be motor neurons (11). It is possible that Hm-inx8 formed transient electrical connections needed for the development of the nervous system but not for the developed system, causing them to be down-regulated and ultimately absent by the adult stage. It is known that motor neurons typically form many electrical connections with other motor neurons to allow neurons to generate behavior very early, even before chemical synaptic transmission is established in the CNS (19). These electrical connections may play an important role in guiding the later formation of chemical synapses. In many organisms, neurons form transient electrical synapses near the times of chemical synapse formation (24, 26), suggesting that transient electrical synapses may be necessary for the formation of permanent chemical synapses (12, 35). Thus Hm-inx8 may help certain motor neurons

establish temporary electrical connections early in leech development, which helps to guide the formation of chemical synapses later in development.

The leech has been found to develop many novel behaviors after the E12 stage (~51% ED) such as circumferential indentation (indentation around the entire stimulated segment) at 52.5% ED, avoidance (the body bends away from the stimulus) at 56.5% ED, local bending at 59% ED, elongation at 63.5% ED, and crawling at 81.5% ED (Reynolds et al. 1998). The gap junctions that were turned on or off after E12 may have a role in these developmental events. But although the determination of a difference in the innexins expressed early in the embryo from the adult is a step forward, more tests must be done to determine the exact role of gap junctions in leech development. Finally, the determination of the innexins present in the adult CNS allowed the following single cell characterizations to be performed more efficiently. Thus this data alone may be useful for future experiments involving gap junctions in the adult CNS.

Characterizing Innexins Present in Single Neurons:

Hm-inx1 can form homotypic gap junctions (e.g. inx1-inx1) with each other. As a consequence of this characteristic of this innexin, the results showing that R serotonergic neurons, AP motor neurons, and P₁ sensory neurons each express Hm-inx1 demonstrates that they all have the potential to form gap junctions with each other. In addition, all three cells express Hm-inx6, Retzius cells express Hm-inx2, and P-cells express Hm-inx3. This is interesting because Hm-inxs 1, 2, 3, and 6 have been shown to form non-junctional hemichannels (1). The non-junctional innexons permit the exchange between the cytoplasm and extracellular space of molecules in the size range of second messengers,

seen in Figure 4. Although all gap junction channels and hemichannels close upon reduction of cytoplasmic pH, the sensitivity of the channels formed by the various gap junction proteins varies by almost one pH unit. The fact that each innexin has a different sensitivity to cytoplasmic pH may mean that each has a unique role for the cell. Thus it is possible that each of these innexins which can form non-junctional hemichannels may have a specific role during development in the leech, such as allowing certain ions or second messengers into the neuron.

Also, I found that P₁ cells express Hm-inx11 and AP cells express Hm-inx4. Both of these innexins are not expressed prior to the E12 stage (~51% ED). A previous study has shown that AP and P₁ cells are electrically coupled in the adult stage but not in the 65% ED stage (20). Based on this time frame, the expression of Hm-inx4 or Hm-inx11 after the E12 stage may facilitate the formation of an electrical synapse between these two neurons. Also, some of the P₁ electrical synapses to interneurons present at 65% ED were replaced by excitatory chemical synapses (20). The expression of Hm-inx11 after the E12 stage may have a role in these synapse changes.

During embryogenesis, motor neurons such as AP cells extend anterior and posterior projections that become coupled to a segmental homologue before being retracted. The function of this coupling is to ensure complete innervation. One proposed hypothesis for how this happens is that these motor neurons transiently express a unique innexin which confers specificity (37). My data showing that innexins are turned on and off during development give additional weight to this hypothesis.

My observation that Hm-inx2 is expressed in an adult leech neuron (the Retzius

cell), although it is not expressed by any neurons at E12, was unexpected. This innexin has been previously thought to be specific to glial cells. However, the idea that only glial cells express Hm-inx2 is based only on embryonic studies, and were inconclusive in adults (10). Further studies should be conducted to verify that Hm-inx2 is not specific to glial cells in adult leeches. Hypothetically, if Hm-inx2 is in fact expressed in R cells some time after the E12 stage, this innexin may play a part in the hypothesized physiological role of R cells in development. The elevation of serotonin by Retzius cells when stimulated by tactile mechanosensory neurons increases the responsiveness of leeches to swim-inducing sensory input (14). The swimming response in the leech is not seen until much later in the embryonic development. Based on this time frame, Hm-inx2 may play a role in the activation of the swimming response in the leech.

Overall the results found in single cells allow for hypothetical developmental roles to be postulated. It is a novel concept that each neuron type expresses a unique set of innexins. The previous data showing which innexins were expressed in the adult CNS paired with the protocol developed for single cell characterization will facilitate future efforts to map the expression patterns of different neurons in the CNS. A more complete map of the expression patterns of neurons may elucidate why the cells have only select gap junction proteins. In addition, each single cell was found to express Hm-Rs60 and Hm-Elf1, revealing the possibility of quantifying the different innexins present in single cells. Ultimately the results from this study have added to the understanding of CNS development in the leech. The mechanisms behind leech CNS development are important to understand because it may help understanding how the leech can repair its damaged neurons. This in turn may one day help us to devise ways to fix spinal cord injuries or

brain damage in humans.

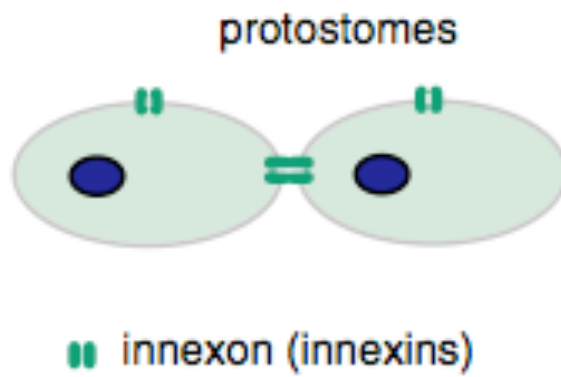


Figure 4. This diagram depicts both junctional and non-junctional innexins, used from (1).

Methods

Measuring the Expression of Innexins 1 and 2 in Different Life Stages:

Animals and Staging

Hirudo medicinalis embryos were obtained from an in-house breeding colony. The embryos were raised in artificial pond water after removing them from egg cases, generally no later than E9. The embryos were raised at 20-22°C and given the designation E for embryo or PE for postembryonic and a number to indicate the days after birth. To ensure there was no developmental variation based on ambient temperature, the ages of each stage were matched with percent development (31). The leeches were staged at E12, E22, PE2, PE12, and the adult stage.

Dissection

Each of the different stages required different techniques for the dissection of ganglia. Generally, animals were anesthetized in 8% ethanol in artificial pond water. Tools were cleaned with RNase prior to each dissection. For all embryonic leeches, the cryptolarval membrane was opened dorsally, and the yolk removed. Embryos were transferred to Wenning's solution (40 mM malic acid, 4 mM KCl, 10 mM succinic acid, 10 mM Tris-HCl, 1.8 mM CaCl₂, pH 7.4) containing 8% ethanol and pinned out flat. All experiments were performed using the same six ganglia from the mid-body of the animal (segments 7-12) in all trials in order to minimize any variability in age due to the anterior-posterior developmental gradient in the leech. Their location was determined using the sex organs as a marker. All tissues surrounding the ganglia and connectives

were then removed. Roots of all ganglia were cut first, then connectives of the 7th and 12th ganglia were cut. The tools were cleaned again using RNase to prevent contamination from non-CNS material. The ganglia were then placed in Trizol. The E22 dissections were similar to E12 dissections, with the exception that the yolk sac was removed by a jet of the medium from a pipette streaming across the embryo, while a fine metal wire was used to remove fat and muscle attached to the ganglia. PE2, PE12, and adult leeches were anesthetized in Ringer solution (in mM: 115 NaCl, 1.8 CaCl₂, 4 KCl, and 10 Tris maleate, pH 7.4) containing 8% ethanol. The leech was pinned in the head and tail, dorsal side up and then opened using a razor. The preparations were then pinned open and the segments 7-12 ganglia were cleared of overlying tissues. Roots of all ganglia were cut first, then connectives of the 7th and 12th ganglia were cut. The tools were cleaned again using RNase and the 6 ganglia were then placed in Trizol.

RNA extraction

RNA was extracted from the tissues using the Trizol reagent from Invitrogen. The ganglia were placed in 250 μ L of Trizol, spun down, instantly frozen with dry ice, crushed, and then an additional 250 μ L of the same reagent was added. This process was repeated until there was 1ml of Trizol with the cells. After the cells were homogenized, the total RNA was isolated according to the Trizol protocol. The optional step of adding 5 μ L of glycogen was performed to enhance RNA yield. The poly A RNA yield and purity was determined by measuring the absorbance at 260 and 280 nm.

Making cDNA and checking for contamination

The total RNA was then reverse transcribed into cDNA using the cells-to-cDNA II kit (Ambion) following their protocol. The cDNA was then checked for contamination with extraneous DNA using PCR. The negative control was Hm-inx10, a non-CNS innexin. Thus no amplification of Hm-inx10 with the CNS cDNA indicated that the cDNA was only from the CNS and also that there was no genomic contamination. The check consisted of the negative control: Hm-inx10 primer with and without cDNA, each run in triplicate. The positive control was Hm-inx1, a CNS innexin, run with and without cDNA, each run in triplicate (Table 3).

Table 3. Contamination check with copy DNA (cDNA) and no template controls (NTC).

Sample	1	2	3	4	5	6
Negative Control	Hm-inx10 + cDNA	Hm-inx10 + cDNA	Hm-inx10 + cDNA	Hm-inx10 NTC	Hm-inx10 NTC	Hm-inx10 NTC
Positive Control	Hm-inx1 + cDNA	Hm-inx1 + cDNA	Hm-inx1 + cDNA	Hm-inx1 NTC	Hm-inx1 NTC	Hm-inx1 NTC

Quantitative PCR

Primers were designed for both the reference genes and the innexins using the Primer-3 program. Hm-inx1 and 2 were selected because of their expression in the neuron and glia, respectively (11). Three reference genes were selected because of their theoretical ubiquitous expression throughout the leech in their different stages of life: Hm-60S ribosomal protein, Hm-actin-1, and Hm-elongation factor 1.

The qPCR reaction was set up as follows: 10µl 2X SYBR Green Master Mix

(Biopioneer, San Diego, CA), 8 μ l deionized H₂O, 1 μ l 2.0 μ M forward and reverse primer, 1 μ l cDNA template. Experiments were performed using the Mx3000 Quantitative PCR system (Stratagene). An amplification-based threshold and adaptive baseline were selected as algorithms. Thermal cycling conditions were: 10 minutes at 95 °C followed by 40 cycles of 20 s at 95 °C, 20 s at 59 °C and 30 s at 72 °C. A second set of thermal cycling was run to get the dissociation curve: 1 minute at 95 °C to denature the PCR product, ramp down to 55 °C. For the dissociation curve, the temperature was ramped up from 55 °C to 95 °C at 0.2°C/sec and the fluorescence data was collected continuously on the 55-95°C ramp. Three individual leeches were used at each of the five different stages: E12, E22, PE2, PE12, and adult. Each trial was conducted with primers for Hm-inx1 and 2 as the genes of interest. Hm-Rs60, Hm-Act1, and Hm-Elf1 were used as reference genes. No template controls were used for each primer to ensure there was no contamination. Each of the primers were run in triplicate in each trial (Table 4).

Table 4. Quantitative assay with Hm-inxs 1 and 2 and three reference genes.

Sample	1	2	3	4	5	6
Gene of Interest	Hm-inx1 + cDNA	Hm-inx1 + cDNA	Hm-inx1 + cDNA	Hm-inx1 NTC	Hm-inx1 NTC	Hm-inx1 NTC
Gene of Interest	Hm-inx2 + cDNA	Hm-inx2 + cDNA	Hm-inx2 + cDNA	Hm-inx2 NTC	Hm-inx2 NTC	Hm-inx2 NTC
Reference Gene	Hm-Rts60 + cDNA	Hm-Rts60 + cDNA	Hm-Rts60 + cDNA	Hm-Rts60 NTC	Hm-Rts60 NTC	Hm-Rts60 NTC
Reference Gene	Hm-Elf1 + cDNA	Hm-Elf1 + cDNA	Hm-Elf1 + cDNA	Hm-Elf1 NTC	Hm-Elf1 NTC	Hm-Elf1 NTC
Reference Gene	Hm-Act1 + cDNA	Hm-Act1 + cDNA	Hm-Act1 + cDNA	Hm-Act1 NTC	Hm-Act1 NTC	Hm-Act1 NTC

PCR product dissociation curves, previously verified on a 10% gel, were used to confirm the threshold cycle (Ct) levels were measurements of correct PCR products. The Ct is the point in qPCR where the fluorescence from the transcript gives signal over the background and is in the linear portion of the amplified curve. The CT values were then used to calculate the expression level of the genes of interest for E12, E22, PE2, and PE12 relative to the adult. This was done using the $\Delta\Delta\text{Ct}$ Method: $2^{-(\Delta\Delta\text{Ct})}$, where $\Delta\Delta\text{Ct} = (\text{Ct}_{\text{GOI}} - \text{Ct}_{\text{norm}})_{\text{adult}} - (\text{Ct}_{\text{GOI}} - \text{Ct}_{\text{norm}})_{\text{unknown}}$. $\Delta\Delta\text{Ct}$ values greater than 1 signaled upregulation in the organism and values less than 1 signaled down regulation. Incremental stages of the leech were tested until the $\Delta\Delta\text{Ct}$ value approached 1.

Characterizing Innexins Present in the Embryonic and Adult CNS:

RNA Extraction and cDNA Synthesis

Adult leeches and E12 leeches were dissected (see earlier dissection section). Single ganglia were cleaned and placed in 25 μ l of a master lysis solution of 0.7 μ l β -ME combined with 100 μ l of Lysis Buffer (Absolutely RNA Nanoprep Kit from Stratagene). This was frozen with dry ice, crushed, and then 25 μ l of the master lysis solution was added. This procedure was repeated until the ganglia were homogenized in 100 μ l of the master lysis solution. RNA was then extracted according to their protocol. 5ug of RNA were then made into cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was then checked for contamination using PCR. The check consisted of the negative control: Hm-ix10 with and without cDNA, each run in triplicate. The positive control was Hm-inx1 with and without cDNA, each run in triplicate.

Primer Design

Primers were designed for the 10 additional innexins expressed in the leech (11). In addition, primers were designed for novel innexins Hm-inx13 and Hm-inx14. The primers were designed using the Primer-3 program. All primer sequences were run through BLAST to ensure the primers were specific for the innexin of interest.

PCR mRNA Detection

Assays were then run with primers for all 14 innexins (Table 5). 10 μ l 2X SYBR

Green Master Mix (Biopioneer, San Diego, CA), 8µl deionized H₂O, 1µl 1.5µM forward and reverse primer, 1 µl cDNA template. The cDNA was from three tail, middle, or head ganglia in the E12 and one tail, middle, or head ganglia in the adult leech. Each innexin primer with CNS cDNA was run in triplicate, the negative control run in duplicate, and positive control (full body or FB cDNA with the gene of interest) run in a single trial. Three animals at the adult stage and E12 stage were used. PCR product dissociation curves were used to confirm the identity of the PCR products.

Table 5. Assay run on E12 and Adult to determine innexins present in the CNS

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hm- inx1 + cDNA	Hm- inx1 + cDNA	Hm- inx1 + cDNA	Hm- inx1 NTC	Hm- inx1 NTC	Hm- inx1+ FB cDNA	Hm- inx2+ cDNA	Hm- inx2+ cDNA	Hm- inx2+ cDNA	Hm- inx2 NTC	Hm- inx2 NTC	Hm- inx2 + FB cDNA
B	Hm- inx3 + cDNA	Hm- inx3 + cDNA	Hm- inx3 + cDNA	Hm- inx3 NTC	Hm- inx3 NTC	Hm- inx3 + FB cDNA	Hm- inx4 + cDNA	Hm- inx4 + cDNA	Hm- inx4 + cDNA	Hm- inx4 NTC	Hm- inx4 NTC	Hm- inx4 + FB cDNA
C	Hm- inx5+ cDNA	Hm- inx5+ cDNA	Hm- inx5+ cDNA	Hm- inx5 NTC	Hm- inx5 NTC	Hm- inx5 + FB cDNA	Hm- inx6 + cDNA	Hm- inx6 + cDNA	Hm- inx6 + cDNA	Hm- inx6 NTC	Hm- inx6 NTC	Hm- inx6 + FB cDNA
D	Hm- inx7 + cDNA	Hm- inx7 + cDNA	Hm- inx7 + cDNA	Hm- inx7 NTC	Hm- inx7 NTC	Hm- inx7 + FB cDNA	Hm- inx8+ cDNA	Hm- inx8+ cDNA	Hm- inx8+ cDNA	Hm- inx8 NTC	Hm- inx8 NTC	Hm- inx8 + FB
E	Hm- inx9 + cDNA	Hm- inx9 + cDNA	Hm- inx9 + cDNA	Hm- inx9 NTC	Hm- inx9 NTC	Hm- inx9 + FB cDNA	Hm- inx10 + cDNA	Hm- inx10 + cDNA	Hm- inx10 + cDNA	Hm- inx10 NTC	Hm- inx10 NTC	Hm- inx10 +FB cDNA
F	Hm- inx11 + cDNA	Hm- inx11 + cDNA	Hm- inx11 + cDNA	Hm- inx11 NTC	Hm- inx11 NTC	Hm- inx11 + FB cDNA	Hm- inx12 + cDNA	Hm- inx12 + cDNA	Hm- inx12 + cDNA	Hm- inx12 NTC	Hm- inx12 NTC	Hm- inx12 + FB cDNA
G	Hm- inx13 + cDNA	Hm- inx13 + cDNA	Hm- inx13 + cDNA	Hm- inx13 NTC	Hm- inx13 NTC	Hm- inx13 + FB cDNA	Hm- inx14 + cDNA	Hm- inx14 + cDNA	Hm- inx14 + cDNA	Hm- inx14 NTC	Hm- inx14 NTC	Hm- inx14 + FB cDNA

Characterizing Innexins Present in Single Neurons:

Single Cell Extraction and cDNA Synthesis

All tools were first washed with RNase. An adult leech was anesthetized in Ringer

solution containing 8% ethanol. Leeches were pinned in the head and tail dorsal side up and then opened using a razor. They were pinned open and a single ganglion was cleaned. The roots of the ganglia were cut first and then connectives. The ganglia (with roots) was placed in a sylgard dish with Ringer's containing 8% ethanol and pinned down. Then using fine forceps in one hand and fine scissors in the other, the ganglionic sheath was cut. Once the ganglion was desheathed, the cell was held with fine forceps and cut at the base with fine scissors. Retzius cells were identified by their position and size (the largest cells in the ganglia). The AP and P₁ cells were visualized by injecting them with 5% fast green in 0.2M KCl beforehand. The cell was then placed away from the ganglia in the sylgard dish. A customized thick capillary glass pipette (1 mm OD, 0.5 mm ID) was made using a Sutter Instrument micropipette puller with the following specifications: heating=835, pull=60, velocity=80, time=250. The tip was then broken to increase the diameter until it was large enough to pick up the cell. The pipette was then inserted into a rubber tube fitted with an adapter and the cell was sucked up. The cell was then placed in the cap of an eppendorf tube containing 10 uL of a master lysis solution of 0.7 µl β-ME combined with 100 µl of lysis buffer. The buffer was then sucked in and out of the pipette three times to ensure the cell didn't stick inside the micropipette. The cap was put back onto the eppendorf tube (already containing 90 uL of lysis buffer) and centrifuged to ensure the cell was brought down with the lysis buffer. RNA was then extracted using the Absolutely RNA Nanoprep Kit using their protocol (Stratagene). 5ug of RNA were then made into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). The cDNA was then checked for contamination using PCR. The check consisted of the negative control, Hm-ix10 with and without cDNA, each run in

triplicate. The positive control was Hm-ix1 with and without cDNA, each run in triplicate.

PCR mRNA Detection

Assays were then run on primers for all innexins present in the adult CNS, determined in the earlier experiments (Table 6). The assays contained 10 μ l 2X SYBR Green Master Mix (Biopioneer, San Diego, CA), 8 μ l deionized H₂O, 1 μ l 1.5 μ M forward and reverse primer, 1 μ l cDNA template. Innexins present in Retzius cells, AP cells, and P cells was determined. PCR was run on cDNA from three of each type of cell from three different animals. PCR product dissociation curves were used to confirm the identity of the PCR products.

Table 6. Assay run to determine which innexins were expressed in single cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hm- <i>inx2</i> + Retzius cDNA	Hm- <i>inx2</i> + Retzius cDNA	Hm- <i>inx2</i> + Retzius cDNA	Hm- <i>inx2</i> NTC	Hm- <i>inx2</i> NTC	Hm- <i>inx2</i> FB	Hm- <i>inx3</i> + Retzius cDNA	Hm- <i>inx3</i> + Retzius cDNA	Hm- <i>inx3</i> + Retzius cDNA	Hm- <i>inx3</i> NTC	Hm- <i>inx3</i> NTC	Hm- <i>inx3</i> FB
B	Hm- <i>inx4</i> + Retzius cDNA	Hm- <i>inx4</i> + Retzius cDNA	Hm- <i>inx4</i> + Retzius cDNA	Hm- <i>inx4</i> NTC	Hm- <i>inx4</i> NTC	Hm- <i>inx4</i> FB	Hm- <i>inx6</i> + Retzius cDNA	Hm- <i>inx6</i> + Retzius cDNA	Hm- <i>inx6</i> + Retzius cDNA	Hm- <i>inx6</i> NTC	Hm- <i>inx6</i> NTC	Hm- <i>inx6</i> FB
C	Hm- <i>inx9</i> + Retzius cDNA	Hm- <i>inx9</i> + Retzius cDNA	Hm- <i>inx9</i> + Retzius cDNA	Hm- <i>inx9</i> NTC	Hm- <i>inx9</i> NTC	Hm- <i>inx9</i> FB	Hm- <i>inx11</i> + Retzius cDNA	Hm- <i>inx11</i> + Retzius cDNA	Hm- <i>inx11</i> + Retzius cDNA	Hm- <i>inx11</i> NTC	Hm- <i>inx11</i> NTC	Hm- <i>inx11</i> FB
D	Hm- <i>inx12</i> + Retzius cDNA	Hm- <i>inx12</i> + Retzius cDNA	Hm- <i>inx12</i> + Retzius cDNA	Hm- <i>inx12</i> NTC	Hm- <i>inx12</i> NTC	Hm- <i>inx12</i> FB	Hm- <i>inx13</i> + Retzius cDNA	Hm- <i>inx13</i> + Retzius cDNA	Hm- <i>inx13</i> + Retzius cDNA	Hm- <i>inx13</i> NTC	Hm- <i>inx13</i> NTC	Hm- <i>inx13</i> FB

References

1. Bao, L., Samuels, S., Macagno, E., Muller, K., Dahl, G. (2007). Innexins form two types of channels. *FEBS Lett* vol. 581 (29) pp. 5703-8.
2. Baptista, C.A., and Macagno E.M. (1988). Modulation of the pattern of axonal projections of a leech motor neuron by ablation or transplantation of its target. *Neuron* vol. 1 (10) pp. 949-62.
3. Bennett, M.V., and Zukin, R.S. (2004). Electrical coupling and neuronal synchronization in the mammalian brain. *Neuron* 41, 495–511.
4. Bossinger, O., Schierenberg, E. (1992) Cell-cell communication in the embryo of *Caenorhabditis elegans*. *Dev Biol* 15 1:40 1-409.
5. Bruzzone, R., Dermietzel, R. (2006) Structure and function of gap junctions in the developing brain.
6. Bustin, S.A., (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 25, 169–193.
7. Bustin, S.A., (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* 29, 2339.
8. Carretta, M. (1988). The Retzius cells in the leech: a review of their properties and synaptic connections. *Comp Biochem Physiol A Comp Physiol* vol. 91 (3) pp. 405-13.
9. Curtin, K.D., Zhang, Z., and Wyman, R.J. (2002). Gap junction proteins expressed during development are required for adult neural function in the *Drosophila* optic lamina. *J. Neurosci.* 22, 7088–7096.
10. Dykes, I.M., Macagno, E. (2004). Molecular basis of gap junctional communication in the CNS of the leech *Hirudo medicinalis*. *J Neurosci* vol. 24 (4) pp. 886-94.
11. Dykes, I.M. and Macagno, E.M. (2006). Molecular characterization and embryonic expression of innexins in the leech *Hirudo medicinalis*. *Dev Genes Evol* vol. 216 (4) pp. 185-97.
12. Fischbach, G.D. (1972). Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Dev Biol* 28:407– 429.
13. French K.A., Chang J., Reynolds S., Gonzalez R., Kristan W.B. III. (2005). Development of swimming in the medicinal leech, the gradual acquisition of a behavior. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* vol. 191 (9) pp. 813-21.

14. Friesen, W.O., and Kristan, W.B. (2007). Leech locomotion: swimming, crawling, and decisions. *Curr Opin Neurobiol* vol. 17 (6) pp. 704-11.
15. Kandler, K., and Katz, L.C. (1995). Neuronal coupling and uncoupling in the developing nervous system. *Curr. Opin. Neurobiol.* 5, 98–105.
16. Kumar, N.M., Gilula, N.B. (1996) The gap junction communication channel. *Cell* 84:381- 388.
17. Levin, M., and Mercola, M. (1999). Gap junction-mediated transfer of left-right patterning signals in the early chick blastoderm is upstream of Shh asymmetry in the node. *Development* 126, 4703–4714.
18. Macagno, E.M. (1980). Number and distribution of neurons in leech segmental ganglia. *J Comp Neurol* vol. 190 (2) pp. 283-302.
19. Marin-Burgin, A., Eisenhart, F.J., Baca, M.S., Kristan, W.B. Jr, French, K.A. (2005). Sequential development of electrical and chemical synaptic connections generates a specific behavioral circuit in the leech. *J Neurosci* 25:2478–2489.
20. Marin-Burgin, A., Eisenhart, F., Kristan, W., French, K. (2006). Embryonic electrical connections appear to pre-figure a behavioral circuit in the leech CNS. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* vol. 192 (2) pp. 123-33.
21. Muller K.J., Nicholls, J.G., Stent, G.S. (1981). *Neurobiology of the leech*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
22. Nolan, T., Hands, R.E., Bustin, S.A., (2006). Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* 1, 1559–1582.
23. Panchin, Y.V., (2005). Evolution of gap junction proteins—the pannexin alternative. *J. Exp. Biol.* 208, 1415–1419.
24. Peinado, A., Yuste, R., Katz, L.C. (1993a) Gap junctional communication and the development of local circuits in neocortex. *Cereb Cortex* 3:488 – 498.
25. Peirson, S.N., Butler, J.N., Foster, R.G., (2003). Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res.* 31, e73.
26. Penn, A.A., Wong, R.O.L., Shatz, C.J. (1994). Neuronal coupling in the developing mammalian retina. *J Neurosci* 14:3805–3815.

27. Phelan, P., (2005). Innexins: members of an evolutionarily conserved family of gap-junction proteins. *Biochim. Biophys. Acta* 1711, 225–245.
28. Phelan, P., Starich, T.A., (2001) Innexins get into the gap. *Bioessays* 23:388-396.
29. Phelan, P., Bacon, J.P., Davies, J.A., Stebbings, L.A., Todman, M.G., Avery, L., Baines, R.A., Barnes, T.M., Ford, C., Hekimi, S., Lee, R., Shaw, J.E., Starich, T.A., Curtin, K.D., Sun, Y.A., Wyman, R.J. (1998). Innexins: a family of invertebrate gap junction proteins. *Trends Genet* 14:348-349.
30. Reynolds S.A., French, K.A., Baader, A., Kristan, W.B., (1998). Development of spontaneous and evoked behaviors in the medicinal leech. *J Comp Neurol* vol. 402 (2) pp. 168-80.
31. Reynolds, S.A., French, K.A., Baader, A., Kristan, W.B. (1998). Staging of middle and late embryonic development in the medicinal leech, *Hirudo medicinalis*. *J Comp Neurol* vol. 402 (2) pp. 155-67.
32. Rozen S., Skaletsky H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386.
33. Shankland, M. (1995). Formation and specification of neurons during the development of the leech central nervous system. *J Neurobiol* vol. 27 (3) pp. 294-309.
34. Starich, T., Sheehan, M., Jadrlich, J., Shaw, J., (2001). Innexins in *C. elegans*. *Cell Adhes Commun* 8:311–314.
35. Szabo, T.M., Faber, D.S., Zoran M.J. (2004). Transient electrical coupling delays the onset of chemical neurotransmission at developing synapses. *J Neurosci* 24:112–120.
36. Ullian, E.M., Sapperstein, S.K., Christopherson, K.S., Barres B.A., (2001). Control of synapse number by glia. *Science* 291:657–661.
37. Wolszon, L.R., Gao, W.Q., Passani, M.B., Macagno, E.R. (1994). Growth cone “collapse” in vivo: are inhibitory interactions mediated by gap junctions? *J Neurosci* 14:999 –1010.
38. Wong, M.L., Medrano, J.F., (2005). Real-time PCR form RNA quantitation. *Biotechniques* 39, 7585.
39. Yen M.R., and Saier M.H. Jr., (2007). Gap junctional proteins of animals: the innexin/pannexin superfamily. *Prog Biophys Mol Biol* vol. 94 (1-2) pp. 5-14.