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ARTÍCULO ORIGINAL

# Posible papel de la HmInx2 durante el desarrollo del sistema nervioso central de la sanguijuela (*Hirudo medicinalis*)

*Sánchez-González Alejandro, Perzabal-Corona Marisela, Portillo-Lopez Amelia*

Facultad de Ciencias, Universidad Autónoma de Baja California

Correspondencia: Km 103 carretera Tijuana-Ensenada, Baja California, C.P. 22860 México

E mail: AlejandroSG@uabc.edu.mx

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## Introducción

Existe un amplio número de Uniones de Hendidura (Uniones GAP; UGs) acopladas entre células del sistema nervioso central (SNC) durante la neurogénesis temprana en la sanguijuela medicinal (*Hirudo medicinalis*). Para analizar la función potencial de la expresión de proteínas específicas de la familia de las inexinas de la sanguijuela, se probaron dos técnicas que bloquean la formación de UGs en estados tempranos de desarrollo, apagar la expresión de inexinas utilizando RNA de interferencia (siRNA) intracelularmente, y bloquear de formación de UGs extracelularmente con péptidos miméticos. El trabajo que aquí se reporta está enfocado en la HmInx2, la cual se ha reportado se expresa exclusivamente en células gliales durante la gangliogénesis, en el desarrollo del SNC. El siRNA fue transferido en nanoparticulas de oro por medio de biobalística (pistola de genes), mientras que los péptidos miméticos fueron introducidos vía inyección extracelular. Los resultados iniciales sugieren que la HmInx2 participa en la interacciones, posiblemente adhesión, entre el macroglia (glia de empaquetamiento y neuropil) que da origen a la estructura ganglionar, debido a que las neuronas parecen estar desorganizadas y fuera de posición posteriores al contacto con siRNA. Los efectos obtenidos con los péptidos miméticos son similares pero más sutiles, indicando que la formación de UGs donde participa la HmInx2 es solamente afectada parcialmente por esta molécula extracelular. Estas observaciones sugieren que las UGs entre elementos celulares del CNS en desarrollo temprano tienen un papel en la formación y el mantenimiento de la integridad estructural. La adhesión mediada por UGs esta propuesta para jugar un importante papel en el desarrollo neural temprano en la sanguijuela medicinal, misma función que realiza en el sistema nervioso de mamíferos.

Palabras claves: Uniones GAP, inexinas, neurogénesis, gangliogénesis

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ORIGINAL ARTICLE

# Possible role of HmInx2 during development of the central nervous system of the medicinal leech (*Hirudo medicinalis*)

## Abstract

There is broad gap junctional (GJ) coupling among cells of the Central Nervous System (CNS) during early neurogenesis in the medicinal leech (*Hirudo medicinalis*). To assay potential roles of the expression of a specific GJ protein of the leech Innexin family, two techniques to block the formation of GJs at early stages of development were tested: (1) knock-down of expression by using RNA interference intracellularly, and (2) blocking assembly of GJs extracellularly with mimetic peptides. The work reported here focused on HmInx2, which has been reported to be expressed exclusively in glial cells, particularly during gangliogenesis as well as later stages of central nervous system (CNS) development. siRNA was delivered on gold particles by means of biolistics (gene gun), while mimetic peptides were delivered via extracellular microinjection. Initial results suggest that HmInx2 is involved in interactions, possibly adhesive, between the macroglia (packet and neuropil glia) that give rise to ganglionic structure, since neurons appear disorganized and displaced following siRNA intervention. The effects of mimetic peptide injection are similar but subtler, indicating that formation of HmInx2-based GJs is only partially affected by this extracellular reagent. These observations are consistent with the hypothesis that GJs between cellular elements of the CNS are necessary in early development for attaining and maintaining structural integrity. GJ-mediated adhesion is proposed to play an important role in early neural development in the medicinal leech, as it does in mammalian nervous systems.

Keywords: Gap Junctions, Innexins, neurogenesis, gangliogenesis.

## Introduction

Gap junctions (GJs) are intercellular channels formed by transmembrane proteins present in all metazoa. These channels allow intercellular ion exchange[1], and the passage of metabolites and other small molecules[2,3]. They are also fundamental for the propagation of electrical potentials in electrically coupled synaptic circuits and the coordination of cellular signaling among cells in various tissues, such as epithelia[4,5]. Three families of genes that encode GJ proteins have been characterized: connexins, pannexins and innexins. The first two are present in deuterostomes and the third in protostomes. All three families encode proteins with a similar topology, consisting of four transmembrane segments joining an intracellular loop to two extracellular loops and to amino and carboxy-terminals in the cytoplasm[6-8].

In general, six of these membrane proteins interact to form a transmembrane pore, called a connexon, pannexon or innexon, respectively. Gap junctions are formed pairs of these hexamers on two apposed cells are selectively coupled through their extracellular loops[7,8].

Electrical synapses and the corresponding GJs have been described extensively in the H. medicinalis central nervous system (CNS) of both embryos and adults. Development of the CNS occurs from head to tail, and it takes around 12 days to be complete (E12). The nervous system of the medicinal leech is formed by 2 cerebroid ganglions joined by a peri-oesophageal collar to a sub-oesophageal nerve mass, innervating the oral sucker, and composed by coalescence of the ganglions of the first 5 metameres (segments). The ventral nervous system, located within the ventral sinus, consists of 19 pairs of ganglions, with one per metamere. It has 32 ganglia's or motor brains with connective nerves between them and lateral roots connected to the muscles.

During the development of the nervous system the electrical synapse connections are spatially and temporally regulated; peculiarly during the formation of circuits[8,9]. Hirudo nervous system, has proven to be an ideal model for innexin research. From 21 innexins present in the medicinal leech only 15 are expressed in the central and peripheral nervous system, neurons and glial cells[4,8,9].

Trying to mention the advances in terms of innexines knockdown, there are chemical mechanisms, siRNA, anti-peptide antibodies, antisense oligonucleotides or morpholines and mimetic peptides, in this study we use siRNA and mimetic peptides that have shown successful results of gene shutdown. Inhibition and gene "knock out" by siRNA has proved to be an important tool for the comprehensive study of central nervous system development biology. The siRNAs are molecules approximately 21 to 25 nucleotides long that are complementary to the mRNA target that will be inhibited, they are specific and highly effective. Connexin mimetic peptides are molecules that can inhibit gap junction[10]. Mimetic peptides are from 12-25 aminoacids long and they can interfere with innexins, iterrumping the channel formation. Mimetic peptides work on gap junctions albeit in a limited way. They do not affect existing gap junction channels but they do interfere with their formation. The Gap junctions mimetic peptides containing sequences of the extracellular loops of connexins inhibit the formation of new channels, but do not impair the function of existing ones because the peptides only inhibit the formation of new gap juntions but do not interfere with exiting cell-cell channels, also the turnover rate of gap junctions is important to be considered (Gerhard Dahl, 2007)[11]. We found that they have specificity action on innexins family of H. medicinalis leech. In this work we are focus on Innexins 1, 2, 3 and 6 of the medicinal leech.

From all the experiments of knocking down innexins, HmInx2 presents the more drastic effect on the H. medicinalis CNS development.

## HmInx2

HmInx2, it is known that its role in gap junctions is essential for epithelial morphogenesis in the embryos of Drosophila spp. It was evidenced that both the loss of function and the gain of it in mutants denote serious developmental effects due to cell death and failure of epithelial morphogenesis[12]. It has also been shown that in Xenopus sp. Oocyte pairs, injection of Dm-inx2 mRNA results in the formation of voltage-sensitive channels in 40% of cell pairs[13]. On the other hand, Stebbings and co-workers investigations on Dm-inx2 and Dm-inx3 genes show that they are expressed in overlapping domains throughout embryogenesis,

especially in the epidermal cells bordering each of the segments. It was shown that the ectopic expression of Dm-inx2 *in-vivo* has limited effects on the viability of Drosophila melanogaster.

In addition, Drosophila Inx2 mutants of innexin 2 show a feeding defect that arises from a failure of epithelial cells to migrate and invaginate during organogenesis and it is reported that the gene encoding for innexin 2 controls the development of the anterior part of the intestine in response to wingless signaling[14].

Inx2 within the CNS it is expressed in the macroglia, and also in the peripheral nervous system (PNS). Here we used specific siRNA and mimetics peptides with the gene gun and microinjection techniques respectively, with the objective of knowing the role of HmInx2 function in the H. medicinalis nervous system.

## Methodology

### Animal preparation

We used E6-E10 (6 to 10 days before born) *Hirudo medicinalis* embryos to study the CNS development. The cocoons were obtained at 24°C. Embryos were anaesthetized in a solution of 8% ethanol in sterile artificial pond water and placed ventral side up (Sylgard 184, Dow Corning, Midland, MI). Immediately before shooting particles or injection, the ventral surface of the embryos was uncovered by removing part of the solution. After treatment, the embryos were transferred to artificial pond water and kept in a darkness chamber at room temperature until processed for imaging.

### Preparation of siRNA-coated particles

The specific siRNA for each innexin, was prepared using the AM1620 Silencer siRNA construction kit from Invitrogen. S08d gold particles (Seashell Technology, LLC; average diameter 0.5 µm) were coated as described in the manufacturers protocol. Samples were prepared using 10 µg of siRNA per 1 mg of gold carrier particles, washed once with ethanol, sonicated briefly in bath sonicator and dried onto glass slides. This dry powder was charged in the gene gun.

### Gene gun charge of gold nanoparticles

About 10 mg of SO8 gold particles (Seashell Technology, LLC; average diameter 0.5 µm) were spread evenly onto a glass slide. After the solvent evaporated, the particles were suspended in 0.5 mL of distilled water and sonicated 5 min to prevent the formation of clusters. The sonicated solution was dried onto a clean glass slide. The siRNA was prepared using AM1620 silencer siRNA kit (ThermoFisher Sci, Invitrogen) and mixed with the gold particles. We used 30 ul of the shooting solution to introduce the siRNA in to the ganglia cells using a pneumatic nanogene gun15. Around 30 gold particles were counted inside the cell with a shooting range of 40 micrometers to select a good quality prep.

### Gene gun technique

Collect the dry powder with a razor knife and with a wax paper, Load the cartridge of the gun and shoot the desired target. We used 30 ul of siRNA-gold particles to load the gen gun. Generate a shot by clicking the trigger once for 0.1 s, causing the injection of a bolus of particles from the gene gun in to the embryo. The delivery region can have lateral dimensions of ~50-150 µm and extends over ~15 µm around the mean penetration depth, which is adjustable between 0 and 50 µm. This delivery has the advantage of being able to target a limited number of cells in a selected location and we standardize the technique do deliver aroun 30 gols nanparticles per shoot. After the particle delivery, place the embryos back into artificial pond water, preferably one embryo per well in a multi-well plate and wait for 2 days.

### Mimetic peptides preparation

Stock solutions for HmInx1-MimPep (seq. VTLCEFEIRQHSRMHNYIVQ); HmInx2-MimPep (seq. MGEDQAASDRFPRVTMCDFK); HmInx3-MimPep (seq. SNRELVRHQETRFPKV); HmInx6-MimPep (seq. GELVNFQLDQGRFPKVTMCD), 20 mM were prepared. For a working solution, we added 4 µl of our 100x stock solution along with 0.5 µl of fluorescent dye Alexa 568 and 0.5 µl of 10x Wenning's solution to account for the embryo's osmolality. Two micro-liter of our working solution was loaded into each electrode. On the leech embryo, a small, window-like incision was performed ventrally just above the eighth ganglion through a tungsten wire hook.

For this procedure to be accomplished, the embryo was fixed to a dissection plate on an 8% ethanol Wenning's solution to induced narcosis. Once the incision was made, the embryo was placed under a fluorescence microscope and, using a 40x immersion objective, a puncture was made in the ganglion through the filled electrode. Once inside the ganglion, several discharges of the peptide were performed at an increasing aperture range from 15 to 150 µm.

Success was verified through fluorescence filters. Even though dispersion was clearly observed throughout the ganglion, there was no sign of leakage into the sinus. Finally, the embryo was left to recover in a Wenning's solution-filled well with no ethanol.

### Mimetic peptides Micro-Injection

A stock solution of mimetic peptides (20 mM) targets transmembrane proteins expressing HmInx1, HmInx2 and HmInx6 were used. Then they were put into Wenning's solution 8% ethanol to anaesthetize. 2 µl of working solution (4 µl of the stock solution, 0.5 µl of Alexa 568 and 0.5 of 10X Wenning's solution) were injected into a ~6 to 10 (E6, E10) days embryos of *Hirudo medicinalis*, they have around 40-50% nervous system development. The micinyector were prepared with a mechanical puller. The embryos were fixed 24h and 48 h posterior to injection.

### In Situ Hybridization and Immunocytochemistry on whole-mount embryos

To assay for Innexin 1, 2, 3 and 6 (HmInx1, HmInx2, HmInx3 and HmInx6 respectively) mRNA, digoxigenin-labeled leech Innexin riboprobes were hybridized to whole leech embryos, using Nardelli-Haefliger and Shankland (1992) protocol. A full-length Innexin riboprobe was synthesized with digoxygenin labeled nucleotides. The template for in vitro transcription reaction was a full length HmInx cDNA.

### Immunohistochemistry

The embryos were dissected 24 and 48 hours after they were shooted or injected, the whole embryos were fixed in 4% paraformaldehyde phosphate buffered saline (PBS) and were left overnight at 4ºC, along with control embryos. The embryos were rinsed in PBX (PBS + 0.3% Triton X100). Then blocking solution was applied (BS; Goat serum + PBX) and left overnight at 4ºC. Next the primary antibody was applied (1:200 mouse anti-acetylated-tubulin in 100 µl BS). Afterwards, the embryos were covered by wipes soaked in PBX and left overnight at 4ºC. The embryos were rinsed again and the secondary antibody was applied (1:200 Alexa 568 conjugated with donkey anti-mouse in 100µl BS) and the embryos were left overnight at 4ºC.

Next day the embryos were rinsed and they were mounted on glass slide with a drop of 80% glycerol/ PBS and a coverslip to observe under the confocal microscope (Olympus FV1000) at 568 nm.

## Results

Particles coated with HmInx2 siRNA knock down HmIn2 expression in target ganglia. To demonstrate that siRNA delivered on gold particles knocks down the expression of this innexin in a target embryonic ganglion, we use *in situ* hybridization (ISH) to assay expression of Inx2 in embryos at various times following the particle delivery to an intact anesthetized embryo. Delivery was carried out in embryos at stages E6 to E10, and the hybridization with the digoxigenin-labeled probe was performed post-fixation after 24, 36hr or 48hr-intervals.

HmInx2 has been shown to be expressed strongly by the macroglia of the CNS, specifically the 2 neuropil glia and 6 packet glia, as well as the macroglia that reside in the connective nerves (*ref.; figure 2A*). Knock-down was generally very efficient, with the target ganglia showing no evidence of HmInx2 mRNA being present by 24hrs, while adjacent ganglia showed standard patterns of expression (*figure 2B*).

We detected no evidence of a decrease in the expression of HmInx2 when any of the siRNAs delivered corresponded to one of the other leech innexins (see below). Also apparent in *figure 2B* is that the gold particles are confined to the target ganglion and a small region surrounding it, which was achieved by placing a metal baffle with a small opening over the ganglion of interest (see Methods). Most preparations showed a diffuse distribution of these particles, ranging from 5-10 to over 50-60. Occasionally, the gold particles were found to have remained stuck together, and physical damage (a hole) was clearly visible in the target region. Those preparations were discarded.

*Figure 1.* Pneumatic gene gun. On loan from Prof. Alex Groisman (UCSD)

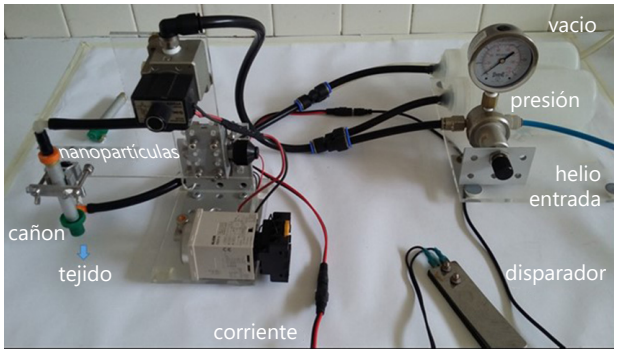
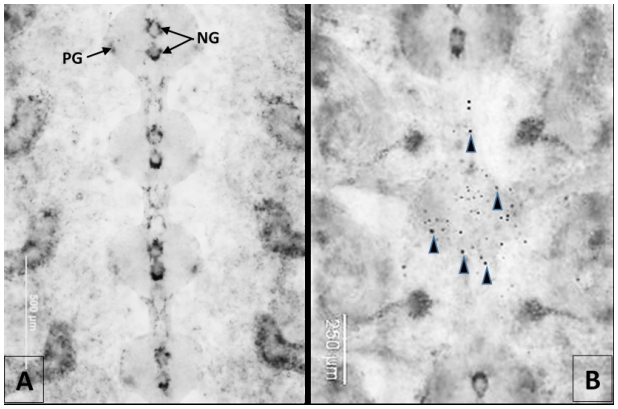



*Figure 2.* In-situ hybridization (ISH) with a digoxigenin-labeled HmInx2 probe. (**A**) Normal preparation, showing a ventral region of an E8 embryo comprising four body segments. The neuropil glia (NG) and packet glia (PG) in the four segmental ganglia show ISH label in their perinuclear regions. (**B**) Ventral region of an experimental E8 embryo, 24hr after ballistic delivery of HmInx2 siRNA. The image includes parts of SG10 and SG12, and SG11 containing ~30 gold nanoparticles (arrowheads) coated with HmInx2 siRNA within its boundaries and several more in the surrounding tissues. In comparison with the adjacent ganglia (arrows), the siRNA delivered to SG11 appears to have knocked down completely the detectable expression of HmInx2.



**Biolistic knock-down of HmInx2 at early embryonic stages strongly affects ganglionic morphology.** *Figure 3* shows two examples of the effects of HmInx2 siRNA on the gross morphology of experimental ganglia after 48 hrs. The experimental preparations were in early stages of embryonic development, E8-9, when the posterior half of the germinal plate is still undergoing gangliogenesis. After an interval of 48 hrs, the embryos were fixed and stained for morphological analysis. Compared to adjacent ganglia (*figure 3A, 3C*), the affected ganglia appear smaller and often asymmetric, with a fuzzy perimeter indicative of a loss of the ganglionic connective tissue sheath and possibly some of the macroglia. We did observe a rough proportionality between the severities of the changes and the distributions and numbers of gold particles we counted as embedded in a ganglion (*table 1*). The extent of the effect was also proportional to time after particle delivery, with 36-hr and 48-hr intervals showing more pronounced structural abnormalities than preparations fixed after 12-hr or 24-hr.

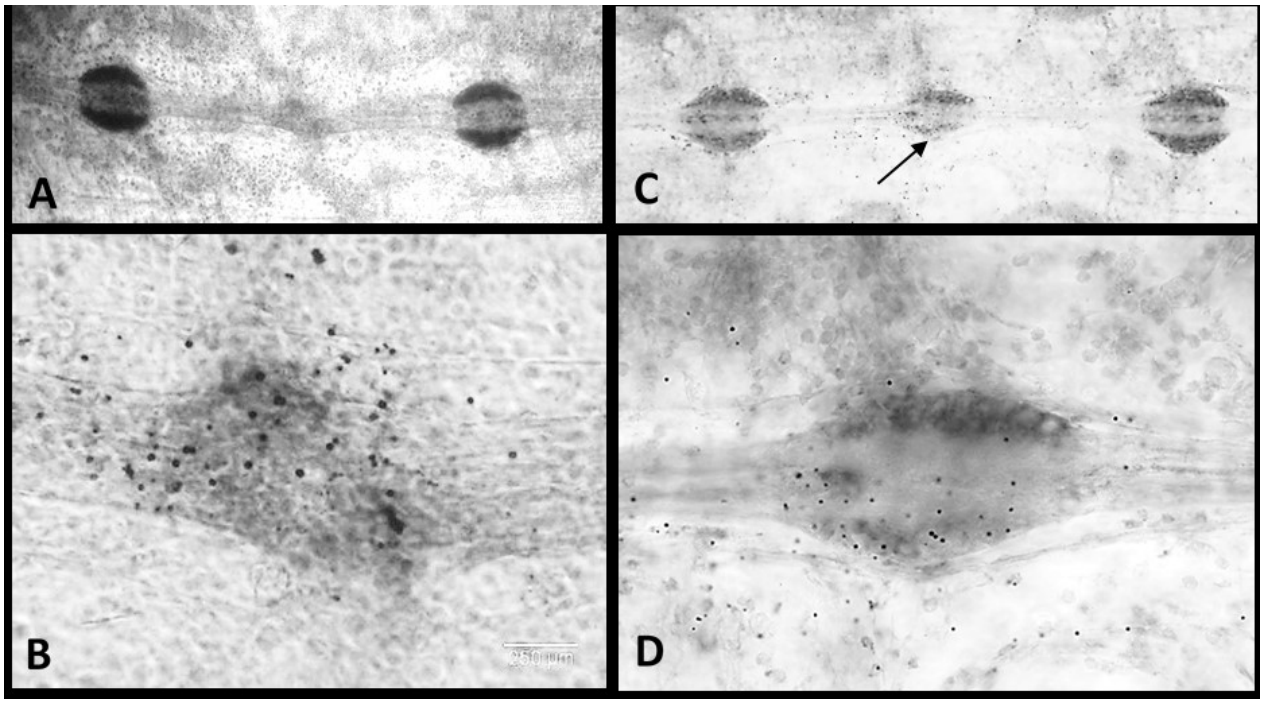



*Figure 3.* (**A** and **C**) Examples of the effects, after 48 Hrs, of ~30 gold nanoparticles coated with anti HmInx2 siRNA delivered to SG-13 (arrow) of an E9 embryo (**A**) and SG-15 (arrow) of an E10 embryo (**C**). Adjacent ganglia appear to be normal, indicating that the delivery of the HmInx2 siRNA is strongly localized to the vicinity of the gold particles. (**B** and **D**) Magnified images of the experimental ganglia in panels above, which shows more clearly the presence and distribution of gold particles.

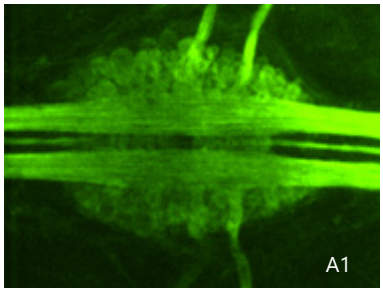
*Table 1.* In this table we show the effects of the gold nanoparticles present in the ganglia. All the experiments were performed in the same ganglionary segment with an N > 10 individuals per experiment. Ganglia dissolved means that there were just the connectors with no ganglia shape.

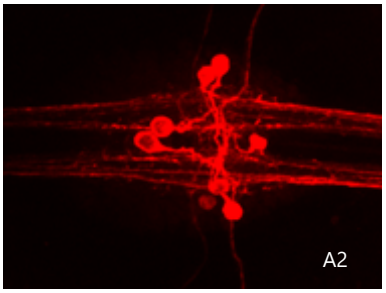
| PARTICLE COATING | # PARTICLES | DAMAGE | CHARACTERISTIC |
|---|---|---|---|
| ONLY GOLD | 25-30 | - | NO EFFECT |
| | 40-50 | x | DEFORMED |
| HEMERITRINE | 15-30 | - | NO EFFECT |
| | 40-50 | | REDUCED SIZE |
| HmInx1 | 1-5 | - | NO EFFECT |
| | 10-15 | - | NO EFFECT |
| | 20-25 | - | NO EFFECT |
| | 30-35 | - | NO EFFECT |
| HmInx2 | 5-10 | - | NO EFFECT |
| | 10-20 | xxx | Ganglia disintegrated |
| | 25-30 | xxx | Ganglia disintegrated |
| | 35-40 | xxx | Ganglia disintegrated |
| HmInx3 | 1-5 | - | NO EFFECT |
| | 15-30 | - | NO EFFECT |
| | 35-40 | x | SMALL DENT |
| HmInx6 | 5-10 | - | NO EFFECT |
| | 15-20 | - | NO EFFECT |
| | 25-30 | - | NO EFFECT |

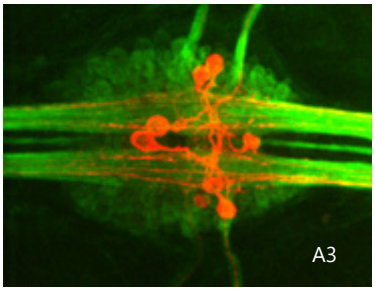
To gain more detailed information about the nature of the abnormal morphology of the experimental ganglia, particularly differential effects on the cellular components (neurons and glia, sheath), we used two different methods to stain ganglia in which innexin expression had been reduced by biolistic delivery of HmInx2 siRNA. To observe the overall effects on neurites and ganglionic boundaries, we used an anti-tubulin antibody which stains fiber tracts, cell soma perimeter and often the ganglionic sheath (*figure 4A1*); to examine possible loss or displacement of identified neurons, we used a serotonin antibody that labels 4 pairs of neurons in most ganglia (*figure 4A2, 4A3*; see Methods). A nuclear stain, Hoechst, was often included in the staining to observe general distributions of cells in tissues.

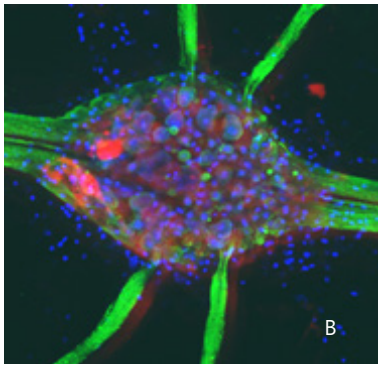
Several examples shown in (*figure 4B, 4C* and *4D)* illustrate the changes we observed, from imperceptible to major deletion of cells and absence of the target ganglion macroglia. In some cases, the ganglion appears to have disintegrated, though some of the labeled cells are still present, as are the connective nerves and some roots (*figure 4D* By contrast, delivery of gold particles carrying siRNAs for other leech innexins fail to cause detectable structural changes (*figure E1, E2 and E3*), as further discussed in the next section, suggesting that it is the lack of HmInx2 that leads to the morphological changes.

*Figure 4.* (A1, A2, A3) Control ganglion, stained with anti-tubulin (A1) and anti-serotonin (A2). Both stains are shown together in (A3). (A, B, C) Hm-Inx2 anti sense siRNA shoot effect tubulin green, serotonin red and Hoechst blue staining. (E1, E2, E3) Ganglion shot with gold particles coated with HmInx1 siRNA show no detectable morphological changes. Images of SG13 in an E10 embryo. No effect on ganglion or neuron morphology is seen with anti-tubulin stain (left) and anti-serotonin stain (center), nor when it is imaged with transmitted light (right) in order to visualize the gold nanoparticles inside and surrounding the ganglion. Scale bars = 200µm




A2


A3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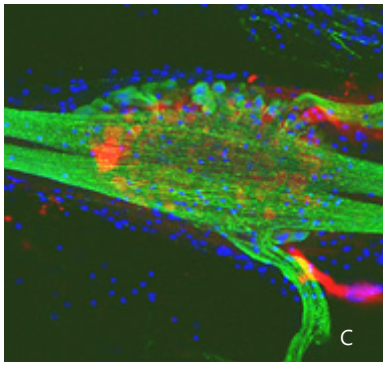
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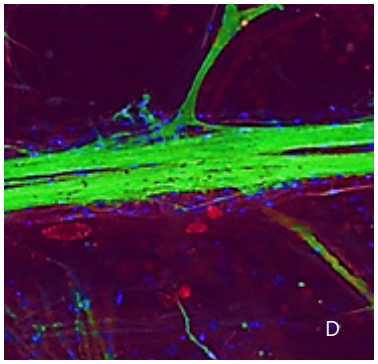
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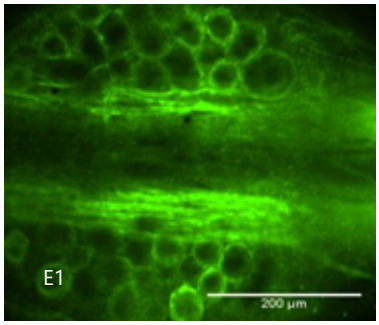
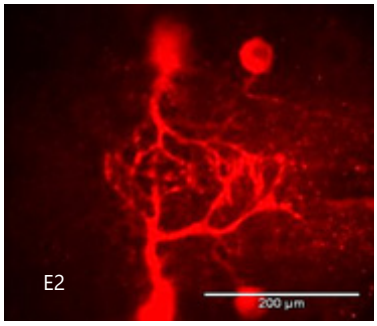
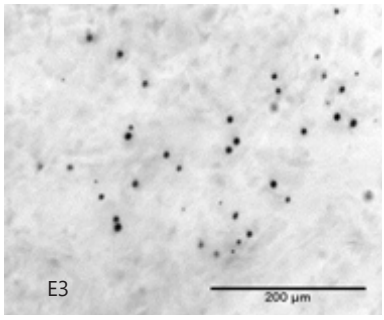

E1
200 µm


E2
200 µm


E3
200 µm

**Normal or near-normal ganglionic morphology following biolistics with siRNAs for HmInx1, HmInx3 and HmInx6.** The specificity of the effects of HmInx2 knock down was assessed by coating gold particles with siRNAs to three other innexins that are known to be expressed in embryonic leech CNS: HmInx1, which is expressed by all neurons, HmInx3 which is expressed at low levels by both neurons and glia, and HmInx6, which is expressed strongly by only 3 neurons in each ganglion, and possibly weakly by a few others. An example for HmInx1 siRNA is shown in (*figure 4E1-3*).

**Extracellular injection of HmInx2 mimetic peptides yields minor effects on ganglionic morphology** We observed that the shutdown of HmInx2 expression using anti sense siRNA, have a direct effect on the formation of the structural packing glial during the development of E12 or younger embryos nervous system (between E6-E10) . The effect using mimetic peptides was more subtle, only 2 out of 10 cases, presented drastic effects on the morphology, we suggest that this was caused because the mimetic peptides micro injection had an extracellular effect without introducing inside the neurons. Still the low number of cases, we obtain an effect that this observation supports the hypothesis where it is suggested that the role of HmInx2 plays an important role in the formation and structural maintenance of the ganglia in the nervous system of the leech. When using the mimetic peptides, in embryos of the same stage of development, a similar behavior was observed, but less efficient since only in some cases we had absence of the packing glial.

*Figure 5.* Comparison of effects of siRNA injection vs mimetic peptide injection. (A) Control ganglion with tubulin stained capsule clearly visible; (B) Ganglion from a preparation injected with HmInx2 siRNA. Dextran-fluorescein (green spot) was included as tracer during the injection. The capsule and packet glia are clearly absent. (C) Ganglion from a preparation injected with HmInx2 mimetic peptide. The ganglion's capsule, and possibly some macroglia, are missing.

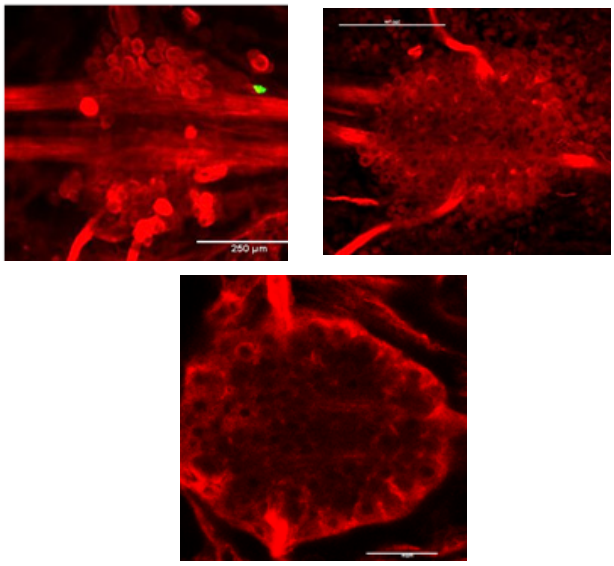
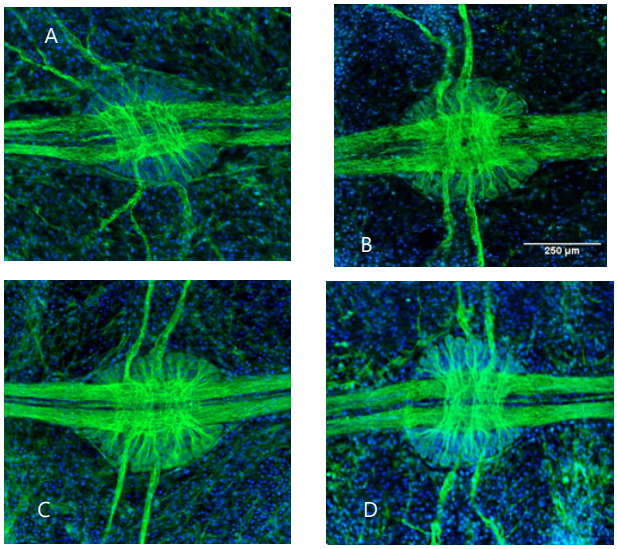



*Figure 6.* Images showing a selected number of collapsed confocal planes from preparations injected at stage E6 with mimetic peptides for (A) HmInx1, (B) HmInx3, (C) HmInx6, and (D) No peptide control. No detectable effects were observed after 36 hrs. The injections were made into the blood sinus proximal to segment 12. Tubulin Stain. Scale bar in B applies to all panels



## Discussion

Possible there is no equivalent cell type similar to package glial in Humans, but they are analogous to oligodendrocytes. Oligodendrocyte, is a type of neuroglia found in the central nervous system of invertebrates and vertebrates that functions to produce myelin, an insulating sheath on the axons of nerve fibers. Still they are not responsible to produce myelin in leeches, but we can say that they isolate and group neurons and give structure to the brain.

The neurons from the control ganglion seem compacted into a defined structure while the neurons from the shooted/injected embryo, seem dispersed, and the structure that surrounds them, the capsule, seems absent. Apparently, by comparing the images obtained and the images obtained before with gold nanparticles using siRNA, the capsule on both doesn't seem to be formed; causing the neurons desegregate away and dispersed around the body of the ganglion.

It can be inferred that INX-2 plays a role on the formation of the ganglion body by analyzing the results obtained from both experiments, the microbalistics using siRNA gold nanoparticles and the injection with mimetic peptides techniques.

Studying GJs is important for understanding neuron communication. The medicinal leech offers us an excellent biological model for the in-vivo experimentation of neurons, innexins in electrical synapsis and neurotransmitters in chemical synapsis.
Also we need to explore and understand better when both type of synapsis are working coupled together.

HmInx2 is particularly important for the functioning of the leech central nervous system, since it is expressed in the glial cells of young and adult leeches. Moreover, it is known that the interruption of its expression leads to consequences in the development and formation of lymph nodes, which causes a negative impact on both the development of the nervous system of the organism and an impact at a morphophysiological level. HmInx2 participates in the formation of the glial packing compartment, which gives shape and structure to the grouping of neurons that form the ganglion or motor brain in the leech.

## Conclussion

The lack of profound studies on molecules and mechanisms involved in the nervous system development has given us a slow progress in terms of treatments to combat the vulnerability of the nervous system against diseases and injuries such as cerebral ischemia, neurodegenerative diseases, Alzheimer's or Parkinson's disease and infections by bacteria or viruses that can infect the brain or spine, not to mention the exponential increase in tumor cases. It is very important to study this system to know it, understand it and be able to manipulate it in favor of humanity and the health of people. It is important that students, researchers and pharmaceutical industries make a commitment to develop these areas of knowledge.

Our experiments with HmInx2 knock-down has shown that the Inx2 key function is directly related to the formation and maintenance of the package glial that keeps all neuron compacted in the ganglia.

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