Putative Role of Innexins in Neuronal Arbor Formation

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

in

Biology

by

Ryan Gregory Natan

Committee in charge:
Professor Eduardo Macagno, Chair
Professor William Kristan
Professor Nicholas Spitzer

2009
The Thesis of Ryan Gregory Natan 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

Acknowledgements ............................................................................................................................... vii

Abstract of the Thesis ............................................................................................................................. viii

Introduction ........................................................................................................................................... 1

Experiments and Results ....................................................................................................................... 13

Conclusion ............................................................................................................................................ 28

Materials and Methods .......................................................................................................................... 31

Appendix .............................................................................................................................................. 50

Figures .................................................................................................................................................. 56

Tables ................................................................................................................................................... 72

References .............................................................................................................................................. 76
LIST OF FIGURES

Figure 1 – Leech anatomy.................................................................56
Figure 2 – AP morphology and ablation studies..............................57
Figure 3 – Coupling coefficient before and after 48h incubation in L15...59
Figure 4 – Inx1 RNAi and electrical coupling in Adult Retzius neurons...60
Figure 5 – AP pair coupling increases from E12 to E18.........................63
Figure 6 – Inx1 and Inx 6 RNAi treatments and AP neuron coupling.......66
Figure 7 – Inx1 and Inx 6 RNAi treatments and AP neuron morphology...69
Figure 8 – Time dependence of the luminance ratio in coupled neurons...70
Figure 9 – Exposure to alcohol and embryonic development...............71
LIST OF TABLES

Table 1 – Summary ‘p’ significance values in Figure 6A..................................72
Table 2 – AP pair coupling in Inx 1 and Inx 6 RNAi experiments......................72
Table 3 – Summary of coupling and action potential data (E12-E18)...............73
Table 4 – Inx1 RNAi in AP pairs experimental data ..........................................74
Table 5 – Inx6 RNAi in AP pairs experimental data...........................................75
ACKNOWLEDGMENTS:

First and foremost, I would like to express my utmost gratitude to my advisor, Dr. Eduarodo Macagno, for providing me the opportunity to pursue this research in his laboratory. As a mentor and teacher, he has guided me not only in this project but in my growth as a scientist. Secondly, I would like to express my appreciation for the continual instruction and assistance of Dr. Michael Baker, who helped make this thesis possible. I would also like to recognize fellow lab members Dr. Alejandro Sanches, Brandon Kandarian, Constante Firme III, and Duc Hoang, and Jasmine Sethi for their supporting work and encouragement. Finally, I would like to thank Krista Todd for her advice in this work and the Kristan Lab for their support.
ABSTRACT OF THE THESIS

Putative Role of Innexins in Neuronal Arbor Formation

by

Ryan Gregory Natan

Master of Science in Biology

University of California, San Diego, 2009

Professor Eduardo Macagno, Chair

Gap junctions have been implicated in a multitude of developmental phenomena, most recently gaining attention for their central role in neurodevelopment. Here, I explore an unusual function that gap junctions may serve based on investigations of the medicinal leech CNS. A pair of anterior pagoda (AP) neurons, found in 20 segmental ganglia, develops mirrored
morphology throughout embryogenesis. Each AP extends secondary neurites which interact with those of their contralateral and ipsilateral segmental homologs. During this 5 to 8 day period, functional gap junctions link these cells cytoplasmically as demonstrated through imaging and dye- and electrical coupling. Normally following this period of gap junction formation, these branches degenerate and recede establishing their adult AP morphology. However, if an AP is ablated, these branches can overtake the vacated territory and persist into adulthood. Indeed, subsequent ablation studies have established that AP homologs normally inhibit each others’ neurite growth. What is the molecular nature of homologous inhibition? I propose that innexin gap junctions mediate the inhibitory interaction. Invertebrate gap junctions are composed of hexameric hemichannels of the protein innexin and there are 14 innexin genes found within the leech genome. In this study, I have used single cell RNAi techniques for two innexins, Hm-inx1 and Hm-inx6, in order to functionally remove gap junctions in individual AP neurons. Gap junction knock-down experiments may disrupt AP homolog communication and prevent inhibition. Should this be the case, gap junctions have a novel developmental role; direct cell to cell mediation of an inhibitory neural growth signal.
**Introduction:**

**Gap Junctions: An Historical Perspective**

For over half a century, physiologists have been studying the structure and function of gap junctions. The first structural description of gap junctions was produced from electron microscopy on thinly sliced sections of the synapses of the giant motor neurons in crayfish. The structure was found to consist of curiously tightly associated membranes (1) that in fact join the membranes of adjacent cells (2). Around the same time, it was demonstrated that some cells could pass current directly to adjacent cells, even at subthreshold levels (3). For a time, this direct transfer of passive current remained a mysterious phenomenon without mechanism. As crayfish neurons were known to allow passive flow as well, Robertson suspected that these synaptic densities may be the site of direct current passage. Further study revealed a hexagonal array of proteins (2), an assembly of these gap junction proteins spanning the membrane. The findings suggested these sheets could provide for aqueous channels between cells. Finally, another group was able to demonstrate that these hexagonal sheets were able to pass small molecules between cells (4). Thus, the term ‘gap junction’ was coined to explicitly link the structure to the function of these intriguing specializations for cellular
communication. Since then, several more studies describing and linking the structure to function of gap junctions have arisen.

Much more is now known about gap junction structure. Early X-ray diffraction and electron microscopy on isolated gap junction proteins produced predictions about the geometry of its associations (5, 6). A model was proposed in which a gap junction hemichannel is formed as six subunits oligomerize to form a hexameric torus. More recent electron crystallography corroborated this model and established that these opposing hemichannels form a tight seal allowing the exchange of substances between cells without extracellular leakage (7). The smallest unit of a functional gap junction channel is a pair of hemichannels, one from each cell, apposed in the narrow intercellular gap between neighboring cell membranes (8). The structure these proteins remains controversial even as the gap junctional genes have been cloned sequenced and analyzed. So, what are these proteins?

The Molecular Basis of Gap Junctions

It wasn’t until 1986 that the first sequence encoding gap junction proteins was discovered (9, 10). This gene, encoding connexin32, has since been shown to be part of an enormous family of connexin genes. To date, 20 connexin genes have been found in mice and 21 in humans (11). Connexins
are expressed in most vertebrate tissues; expression has been found in all tissues except for skeletal muscle and some blood cells (12). However, connexins are restricted to vertebrates, hence whole genome scans prove their complete absence from invertebrates such as *C. elegans* and *D. melanogaster* (11).

Next, invertebrate connexin-like protein genes were isolated and characterized from *D. melanogaster* and *C. elegans* (13). Dubbed ‘innexins’, they have since been found in virtually every group of invertebrates (14, 15). Yet again, innexins compose a large gene family with many orthologs and pararologs; there are 25 innexins in *C. elegans* and 8 *D. melanogaster* (16). Though they form the gap junctions like connexins, innexins bear little sequence homology to connexins (13).

Most recently, the existence of an innexin-like gene family was discovered in vertebrates, three of which have identified members in humans and mice (17, 18). Given the name ‘pannexins’ for the families ubiquitous distribution in the animal kingdom, these gap junction proteins are likely part of the same superfamily as innexins and represent a vertebrate form of innexins (8,15). Pannexins or innexins are present in virtually every invertebrate and chordate genome investigated thus far (8). Although innexins pannexins are not related to connexins directly at the amino acid sequence level, they may have
possible common ancestors. Though evolutionarily distant, the three families of gap junction proteins share common structure and physiology.

It is evident that all gap junctions are composed of hexameric hemichannels of innexins, pannexins or connexins (8). Though primary structure is not identical, secondary gap junction protein structure is highly conserved. The monomers share the same topology, with four alpha-helical transmembrane domains connected by two extracellular loops and a single cytoplasmic loop while both N- and C-termini are intracellular (19). The proteins share other common features such as pore size, voltage-gating properties and sensitivity to the same classes of pharmacological agents (16, 20, 21). Clearly, these proteins are so similar that study of invertebrate innexins can enlighten our understanding of vertebrate gap junction proteins as well, especially pannexins.

**Gap junction in Neurons:**

From cellular genesis to apoptosis, gap junctions have been shown to play many functional roles in a variety of tissues (22, 23). In neurons, gap junctions form electrical synapses which mediate electrical coupling, the direct exchange of ions between neighboring cells (20, 24, 25). At the cellular level, electrical coupling has long been known to allow fast electrical cell to cell
communication, and propagation of action potentials among other unique electrical properties (8). More recently, Electrical synapses have gained attention for their role in assemblies of neurons (26). Electrical coupling has been demonstrated to be functionally important for electrical dynamics of neural networks (27).

As well as electrical coupling, electrical synapses are responsible for chemical coupling, allowing the exchange of small signaling molecules, second messengers and metabolites between neurons (20, 24, 25). The functional roles of neuronal chemical coupling are less clear but there are many interesting possibilities. For instance, accumulating evidence suggests that innexins, pannexins and connexins play crucial roles in normal development of the nervous system (28, 29, 30) and cellular exchange of messengers may be a possible mechanism of action.

**Gap junctions in Neuronal Development**

The first clue that gap junctions are central to neural development is that connexin gene expression is tightly regulated in neural tissues throughout development (31). If gap junctions play a role in neural development, connexin expression regulation would control where, when and what cells become coupled. Gap junctions can provide signaling cue specificity in for neural
development. Connexin genes appear to serve several other roles in development. Connexon gap junctions have been implicated in neuronal migration, differentiation, neurogenesis and formation of neural circuits (29, 32, 33, 34, 35). In addition, the developmental importance of gap junctions need not depend on cellular coupling; a growing myriad of connexin interacting proteins, including cytoskeletal elements, junctional proteins, and enzymes, gap junctions may serve these purposes (36). Gap junctions may act as signaling complexes that regulate cell function and transformation. As connexins are structurally and physiologically similar to innexins, it is not surprising that innexins play many of the same developmental roles in invertebrates.

Mutation of several innexin genes in *C. elegans* leads to developmental defects in the nervous system and behavior, including neurogenesis, muscle control and chemical sensitivity (37). Like connexins, Innexin proteins also interact with other proteins crucial developmental proteins (38). Innexins are necessary for the normal formation of some neural circuits. The development of left-right asymmetry between two neurons in *C. elegans* has been shown to be mediated by innexin gap junctions (39). Lastly, Innexins are implicated in the sculpting of axonal projections. Growth cones become electrically coupled to guidepost cells, and this coupling is necessary for the proper development of
that neural circuit (40).

In another fruitful neurodevelopmental model, the leech *Hirudo Medicinalis*, recent research has demonstrated the importance of innexin gap junctions in the development of neural circuits and behavior. By injecting the small gap junction-crossing molecule, Neurobiotin, into single neurons during development, functional electrical connections are revealed (41). Experiments using this technique have shown that gap junctions are expressed in specific patterns of the leech CNS which precede those of chemical synapse connectivity (42). Gap junctions form premature but functional behavioral circuits before those same circuits are established in the adult form (43). In view of the fact that gap junctions prefigure the connectivity of multilayered neural networks, innexins must be able to form direct and specific connections between types of neurons. Innexin gap junctions clearly have an enormous repertoire of developmental functions, and there are certainly more roles yet to be discovered.

Accordingly, several observations in this lab lead us propose novel role; Innexin gap junctions mediate the normal development of neural circuits in the leech central nervous system (CNS) through contact inhibition.

**Gap Junctions in the Leech Central Nervous System**
The CNS of the leech, *Hirudo Medicinalis*, provides an excellent system in which to explore the role of innexins in neuronal development for several reasons. First, like other invertebrates, leeches lack connexins and pannexins which could complicate the study of innexin gap junctions in its CNS. Fourteen *H. medicinalis* innexin genes have already been cloned (inx1-14) of which the embryonic expression profile of inx1 through inx13 has been explored through in-situ hybridization (44; Kandarian B, MS Thesis, UCSD 2009; unpublished findings). In the CNS, for example, inx1 is predominantly expressed in all neurons while inx6 is expressed in a select few neurons and inx2 is expressed only in glia.

Unlike smaller invertebrate model systems (e.g. *Drosophila* and *Caenorhabditis*), the nature of the leech CNS makes single cell exploration of innexin function possible. The developing neurons are large enough to be seen with the aid of a compound light microscope. The model is amenable to single cell electrical recordings and substance delivery though sharp electrodes which are established experimental methods (45). Experimental manipulations are highly repeatable as each segment of the worm contains a stereotyped set of neurons. Between the head and tail ganglia of the leech CNS, there are 21 mid-body ganglia (MGs) (Fig 1A). Each ganglion is bilaterally symmetrical and contains roughly 400 neurons, of which many are uniquely recognizable from
ganglion to ganglion (Fig 1B) Neurons, like the Anterior Pagoda (AP) and Retzius (Rz), are highly stereotyped by location, morphology, physiology and biochemistry (45, 46). Gap junctional coupling and developmental patterns of these neurons has been extensively studied.

**Gap Junction Mediated Inhibitory Interaction**

The neurite development of the AP, a unipolar bilaterally paired neuron, is particularly intriguing (Fig 2A). This neuron, and its contralateral homolog, first extend branches toward the midline early in development. Once across, it turns posteriorly, bifurcates, and both branches exit the ganglion through the contralateral nerve roots. Around E9-10, several other exploratory branches extend from these primary neurites, most notably, branches that exit the ganglion through the anterior and posterior connectives, and branches that project back over the midline toward the ipsilateral nerve roots (47). The connective branches of ipsilateral segmental AP homologs intermingle closely for 5-8 days and form gap junctions (48). Presumably, functional gap junctions have formed between these neurites because during the entirety of contact, AP neurons are electrically and dye coupled (49). When this interaction is complete, around E15-18, secondary neurites degenerate and retract and by E25, the neuron has taken on its adult morphology (47). However some
exceptional treatments cause the AP to retain the secondary braches, permanently altering adult morphology.

A series of studies performed in this lab demonstrated that AP neurons mutually inhibit the continued extension and functional development of some of its homologs’ secondary neurites (Fig 2B). When an AP neuron is ablated before E14, the connective neurites of ipsilateral segmental AP homologs overtake the otherwise unoccupied territory by adulthood (47, 48, 49). Here, the ablation occurs while connective neurites are still in contact with each other. If instead an AP is ablated after E15, after connective branches have begun to retract, the contralateral homolog develops bilateral projections, again occupying the vacated territory (49). These experiments provide strong evidence that normal retraction and axonal inhibition is mediated though AP homolog axo-axonal contact. In other words, when APs were ablated, gap junctions were not present to provide inhibitory signals and axons were free to grow into vacated territory. Many questions remain: Do innexin gap junctions mediate the inhibitory signal? Does this represent a new mechanism for contact mediated repulsion? How might a mechanism for gap junction mediated inhibition work at the molecular level?

There are several possible mechanisms through which gap junctions may mediate this inhibitory interaction. Dscams are self recognizing cell
surface proteins that mediate self-avoidance and homophilic repulsion in drosophila and murine models (50). Already, innexins and connexins are known to interact with other regulating molecules (36, 37). Perhaps like Dscam, innexins act as a form of self- or homophilic-recognition activating an inhibitory cascade upon gap junction formation. Other possibilities invoke the unique coupling capabilities of gap junctions. Gap junctions may act as a sink for second messengers which regulate axon extension. Growth cone extension is dependent on high local concentrations of cAMP (51). As secondary neurites of AP homologs grow past each other, they form gap junctions. These channels could act as a sink into the neighboring axon lowering the local concentration of cAMP, thus halting the extension of the axon. Lastly, gap junction mediated inhibition may work though Hebbian learning principals, like spike timing dependent plasticity or long term depression. Calcium transients are a second messenger for LTD in some systems (52). The strong ionic fluxes of coincidental action potentials arriving at both sides of a gap junction simultaneously may, for instance, stimulate axon growth. But AP neurons have weak and desynchronized action potentials during this critical period. The small ionic fluxes and calcium transients may halt growth cone extension. Even if only one neuron produced action potentials, the small ionic currents or calcium transients may leak into the neighboring synapse as well.
There are many relevant and exciting opportunities to explore within the model of AP neuron development. In fact, an AP gap junction knock-down study (using inx1 RNAi) has demonstrated that AP to P cell electrical coupling prefigures the chemically connected adult circuit (K.L. Todd, unpublished findings). Therefore, innexins help sculpt the AP morphology, at least indirectly. Though several functional roles of gap junctions in developmental processes have been demonstrated, innexins have yet to be directly linked to inhibitory axo-axonal interaction. The experiments discussed here use single cell inx RNAi, electrical and Neurobiotin dye coupling in an effort to address this possibility.
Experiments and Results:

Electrical coupling coefficient remains stable during incubation:

Observation of physiological knock-down of gap junctions depends heavily on the turnover rate of innexins, which is not known. Gap junctions are renewed daily by quick connexin turnover on the order of hours (19). However, Innexin gap junction turnover, due to invertebrates’ significantly slower metabolism, may take days, meaning the detection of measureable declines in coupling may take days. MGs can be left in L15 for a number of days and remain alive and healthy. Studies have shown that injury to connective nerves can lead to rapid and dramatic changes in gene expression, which may include innexin expression (53). In order for RNAi to take effect, the neurons must be incubated for a period of time. In order to incubate them, they were prepared as described in methods, which involves cutting the roots and connectives, producing traumas to the neurites which may alter the neurons expression profiles. For these reasons it was necessary to test adult MG preparation and incubation methods for an effect on electrical coupling coefficient.

Four consecutive MGs were dissected from an adult leech and prepared as described in methods, without siRNA treatment. Electrical coupling coefficients were recorded from the Retzius pairs of each MBG with the 2
electrode setup. The coupling coefficient was calculated by ‘automated’ bridge balancing (see methods). The preparations were then incubated in L15 for 48h. After incubation the Rz pairs were assayed for their coupling coefficient again.

Recordings show that there is no significant change in coupling coefficient between contralateral Rz cells after 48hrs in incubation. Before incubation, the measured coupling coefficient was 0.39 ± 0.05mV. Just after incubation, 48h later, the coupling coefficient stood at 0.30 ± 0.04mV (Fig 3). A paired T-test on this data returns a value of 0.35 so there is no significant evidence that incubating the preparations changes coupling coefficient.

Since coupling coefficients remains stable through the 48h incubation period, I am confident that any changes in coupling in future experiments can be attributed to experimental treatments rather than necessary manipulations for the preparation.

**Inx1 RNAi treated Retzius pairs are less strongly electrically coupled:**

This experiment was performed to show that treating adult neurons with Inx1-targeting siRNA reduces the coupling coefficient of neurons that are normally coupled in the leech CNS. Retzius neurons are just one of many stereotyped neurons which are coupled during adulthood (45). Inx1 has been shown to be expressed throughout the CNS neurons while Inx 6 is expressed
in few specific cells (44; Kandarian B, MS Thesis, UCSD 2009; unpublished findings). Preliminary results from our lab have shown that adult Retzius neurons express Hm-inx1, 2, 6, and 9, but inx1 may be expressed most strongly. Assuming that inx1 monomers are the major component of gap junctions, we expect inx1 RNAi should greatly reduce gap junction function. A method for gap junction knock-down in the Retzius neuron would likely be generalizable to other coupled neurons of the leech CNS.

In this experiment, the coupling coefficient of Retzius pairs was recorded before and then 48h after one of the cells was injected with a single type of siRNA. Six siRNA sequences were designed, synthesized and delivered into these neurons. Four of these siRNAs target inx1 (siRNA 32, 49, A, B) one of which has previously been shown to be effective in inx1 RNAi experiments (siRNA B), and another which has been shown to be ineffective (siRNA A) (K.L. Todd, unpublished data). Two of these siRNAs were designed to target Inx6 (siRNA 13, 54). Each sequence targeted dissimilar parts of their respective genes, both in sequence and location (see Appendix), so cross inhibition of inx1 and inx6 expression is unlikely. I expect pairs treated with siRNAs 32, 49, and B (experimental) to show a decline in coupling coefficient while treatment with siRNAs 13, 54 and A should show no such effect:

Adult leech ganglia were prepared, treated and recorded from as
described in Methods. Consecutive MGs were prepared from three individual leeches. A combination of 15μM siRNA and 5% carboxyfluorescein in water was injected into one Retzius neuron from each MG. Each of the six siRNAs was injected into several preparations. After injection, each preparation was placed in L15 medium and allowed to incubate for two days. After 48h incubation, each Retzius pairs’ coupling coefficient was assessed again and dye filled (Alexa Dextran 488/neurobiotin).

As expected Retzius pairs treated with siRNA inx1 32, 49, and B show lower coupling coefficients than those treated with siRNA inx6 13, and 54 and inx1 A (Fig 4A). Although coupling coefficients before treatment were widely distributed between leeches, the coupling coefficient for control and experimental groups were similar within each leech.

When control and experimental siRNAs are grouped together the results are more clear (Fig 4B). The control group consists of samples where an Rz neuron was injected with an siRNA that were intended to have no effect and included inx6 13 and 54 and inx1 A treated samples. The experimental group consists of samples where the siRNA injected should be reasonably expected to knock down inx1 expression, including inx1 32, 49 and B. Before injection, control and experimental groups had nearly indistinguishable coupling coefficients of 0.34 ± 0.175 and 0.373 ± 0.135, respectively. Two days after
treatment with siRNA each groups coupling coefficient were significantly different; 0.450 ± 0.0862 and 0.346 ± 0.015, respectively with a P-value 0.041. In other words, Retzius pairs treated with inx1 RNAi have significantly lower coupling coefficients than controls. Comparing the difference in Cc before and after siRNA treatment reveals a more dramatic effect. Coupling in the experimental group declined by -0.02667 ± 0.125831 while Cc in the control group increased 0.156667 ± 0.090185, p = 0.008 (Fig 4C). While coupling in each group before and after treatment is not significant different, these results show that the change in coupling is significantly different between the inx1 RNAi and control groups.

It is interesting that there is an increase in coupling in the control Rz pairs which is not observed in the experimental group. This contradicts results of the previous experiment which showed no change in coupling during incubation. In that experiment, the preparations were treated identically apart from the micro-injection of siRNAs. Perhaps the increase in coupling can be attributed to siRNA micro-injection generally, which might be a traumatic procedure. It is possible that if micro-injection of siRNA causes brief over expression of inx1, the coupling coefficient could increase in these pairs. Coupling coefficient would remain virtually unchanged in pairs injected with effective inx1 siRNAs, since RNAi will prevent an increase in inx1 expression.
Inx1 RNAi may be acting as a somewhat of a rescue treatment, preventing the increased expression of inx1 which would otherwise lead to a high Cc phenotype seen in controls. Nevertheless, it looks as though injection with effective siRNA Inx1 prevents this increase in coupling coefficient. Thus single cell injection of these siRNAs can be regarded as an effective treatment for the knock-down of new inx1 expression, affecting the function of gap junctions.

There was no significant difference between the dye coupling observed in control and experimental groups (data not included). Though the inx1 knock-down may be complete, there was measurable electrical coupling in each pair suggesting that functional gap junctions still exist. Though these small differences in coupling may affect the dynamics of dye coupling, current methods of dye coupling measurement are not sensitive enough to detect such differences.

Though there is incomplete elimination of gap junctions between adult Retzius pairs, inx1 RNAi may lead to complete elimination of gap junctions in embryonic neurons. Adult leech metabolism may be too slow for observable changes too occur but the embryonic metabolism is much faster, and developing neurons must express high levels of innexins in order to form developing gap junctions.
Changes in AP coupling during development:

In order to characterize the development of homologous coupling during embryonic development, various properties of AP neurons were measured through a window of development. Not only does this give us a greater understanding of AP neuron development, but also informs us on when we should start RNAi. Observations have shown that RNAi in leeches has a limited window of effectiveness, on the order of days, in which its effects are measurable, which has been shown in other model systems (54; K.L. Todd, unpublished observations). So, it is necessary to treat neurons with RNAi when those cells are becoming coupled in order to prolong the period of development in which the cells remain uncoupled. Clearly, we would want to inject siRNA to knock-down inx1 around the time when AP neurons become electrically coupled, thus preventing this coupling from occurring. Since specific neurons, like the Rz and AP, become reliably distinguishable only around E11-12 (50-53%ED), this is the stage at which probing begins.

Ten leeches from a single cocoon were used in these measurements for embryos aged E12 (51%ED), E14 (58%ED) and E16 (64%ED). Four leeches from a second cocoon were used for measurements at age E18 (70%ED). Results show that AP pair coupling increases from ages E12 to E18. I observed that 0%, 33%, 71%, and 100% of AP pairs were coupled at E12,
E14, E16, and E18, respectively (n = 6, 9, 7, 4) (Fig 5A) (Table 3). Similarly, there is a significant increase in average coupling strength every 4 days (p = <0.01) as well as significant increases during the 2 days between (p = <0.05) E12 to E14 and E16 to E18 (Fig 5B, Table 1). The average coupling strength from E12 to 18 was 0.07, 0.30, 0.58, and 1.82mV, respectively. The concurrence of an increase in coupled pairs and increased coupling strength is not surprising as coupling is defined by coupling strength, but these measurements do demonstrate that gap junction connectivity increases from virtually no pairs to all pairs in this six day window. It also worth noting that by E12 AP axons already cross the midline and AP neurites overlap extensively. So, why do these neurons only become coupled days later? Does the upregulation of inx1 in these neurons coincide with coupling? Also, this period of coupling precedes the time when supernumerary secondary neurites degenerate. Is coupling responsible for this recession?

I also observed that the initiation of the production of action potentials coincides with increases in coupling. Following the above trend, the number of AP neurons producing action potentials increases through these stages. At stage E12 through E18, 8%, 39%, 57% and 100% of AP neurons tested were able to produce action potentials (n = 12, 18, 14, 8) (Fig 5C). Proportions of AP producing neurons and coupled pairs are highly similar at each stage. In
addition, for pairs between E12 and E16, AP pairs that produce action potentials have much higher coupling coefficients than those that lacked action potentials \((p= 0.02)\). Where action potential producing APs had an average coupling coefficient of \(0.49 \pm 0.40\text{mV/nA}\), neurons without action potentials had an average coupling strength of \(0.09 \pm 0.13\text{mV/nA} \) \((n=11, 10, p=0.001)\) (Fig 5D). Taken together, these observations suggest a relationship between maturation, the development of electrical coupling and the initiation of action potential production. This leads us to ask very interesting questions about the nature of the relationship; is one developmental phenomenon dependent on the other? Does coupling activate action potential production, or vice-versa?

A commonly used method to establish the health of neurons is to survey the presence of action potentials. These measurements suggest that, during development, AP neurons lack the ability to produce action potentials until they are coupled. If RNAi for inx1 successfully prevents the formation of gap junctions or otherwise reduces coupling, it is possible that these cells will not produce action potentials. They may physiologically resemble uncoupled AP neurons of early development. Thus, the presence or lack of action potentials may not be a good indicator of the health of neurons in future experiments.

**Treatment with Inx1 siRNA eliminates electrical coupling in developing**
AP neurons.

Next, I performed a series of experiments to test the hypothesis; that gap-junctional communication is an essential component in the normal development of neurons like the APs. Using inx1 RNAi, knocking-down inx1 expression in embryonic neurons should prevent the formation of functional gap junctions, thus preventing and/or delaying direct cell to cell communication. The embryonic neurons are allowed to continue development during a phase when the adult morphological patterns are established. I would expect to see that neurons treated with inx1 RNAi should not only lack electrical coupling but also might have altered branching patterns.

I chose to treat AP neurons with RNAi at E15 because the results of the previous experiment show that this is a stage at which AP pairs still lack full electrical coupling. Beginning RNAi during this period should delay coupling at least through E18, the time at which all AP pairs are normally coupled. Furthermore, previous work in this lab has shown that ablation of an AP neuron after E15 leads its contralateral homolog to extend projections through its ipsilateral nerve roots by adulthood (49). This bilateral projection phenotype might be easily detected within a few days of RNAi treatment, if gap junctions indeed fulfill the hypothesized developmental role.

Two separate experiments were carried out. In the first experiment, an
AP neuron of each embryo from two cocoons was treated with inx1 siRNA B at E15 as described in methods. In the second experiment, RNAi treatment consisted of inx6 siRNA 13. Inx1 siRNA sequence B was used as the experimental treatment because previous results from adult RNAi suggest that this sequence is highly effective. In addition, this sequence has been used previously by others in the field for similar purposes (K.L. Todd, unpublished experiments). The inx6 siRNA sequence is intended as a control for inx1 treatment, but, recent observations suggest that adult AP neurons express inx6, at least at levels (Kandarian B, MS Thesis, UCSD 2009; unpublished findings). It is possible that inx6 RNAi could have unexpected results. AP pairs from ganglia adjacent to the treated ganglion were also measured as untreated controls.

After treatment with inx1 siRNA, AP pairs were uncoupled at E18. While 100% of untreated controls were coupled (n=4), only 14% of inx1 RNAi treated pairs were coupled (n=7). The one treated pair that was coupled showed an unusually high coupling strength (3.82mV/nA) was statistically eliminated as an outlier in further analysis (data summarized in Table 4). The coupling strength was significantly lower among treated pairs as compared to untreated pairs, 0.18 ± 0.14mV/nA and 1.81 ± 0.73 mV/nA, respectively (p = >0.01) (Fig 6A). In fact, the coupling strength of the treated group resembles
that of background noise, suggesting complete knock down of inx1 expression and lack of gap junction connectivity. There were no significant differences between the groups in resting membrane potential, input resistance or morphology as detected by dye fill, suggesting that treated neurons were otherwise healthy. All AP neurons of untreated pairs produced action potentials, while only 50% of those of treated pairs produced action potentials. However, as previously discussed, the lack of action potentials is likely to be a sign of uncoupling rather than cell damage. This experiment suggests that inx1 RNAi treatment was extremely successful in keeping AP homologs uncoupled for up to three days. Though all measures of cellular health suggest that treated APs were not damaged, the controls used here are unsatisfactory, as the RNAi treatment procedure itself may be responsible for the observed uncoupling through unexpected changes in the neurons. To consider this possibility, another experiment was carried out in the same manner treating AP neurons with inx6 siRNA instead as a control (data summarized in Table 5).

Contrary to inx1 RNAi, inx 6 treated AP pairs and controls had no detectable significant differences. These two groups were similar in many respects. For the treated and untreated groups, the resting membrane potentials and input resistance were \(-22.48 \pm 10.14\) mV and \(-27.62 \pm 12.18\) mV, and \(158.65 \pm 55.85\) M\(\Omega\) and \(159.54 \pm 75.15\) M\(\Omega\), respectively. Morphological
assessment revealed no obvious difference between the two groups. Most importantly, the treated and untreated groups had no significant difference in coupling strengths which were $0.19 \pm 0.26\text{mV/nA}$ and $0.27 \pm 0.31\text{mV/nA}$, respectively. These results would suggest that inx6 treatment caused no detectable effects on electrical coupling. However, a caveat needs to be considered; unlike the previous experiment, the untreated AP pairs in this experiment were mostly uncoupled. Only 29% of untreated and 33% of treated pairs were coupled (Table 2). While both groups are similar in their fraction of coupled pairs, this lack of coupling in controls of the inx6 RNAi experiment is problematic.

Searching for a reason behind this difference, I compared inx1 RNAi results with those of inx6 RNAi and found only a small significant difference in membrane resistance ($p=0.5$) (Fig 6B). Though significant, these values were within range of each other, and within the limits of normal healthy neurons’ input resistance. Still this difference of input resistance may be symptomatic of other problems. Other measures, like resting membrane potential revealing nothing extraordinary (Fig 6C). Lastly, morphological assessment of each group reveals no major differences between all groups (Fig 7). In sum, all pairs included in this analysis appear to be healthy by all measures. So why are these AP in the inx6 experiment pairs mostly uncoupled?
Though these experiments are well designed and tightly controlled, there are possible issues that arise when these experiments were carried out at different times. Perhaps differences in the duration of the injection procedure, and thus EtOH exposure time between the inx1 and inx6 experiments might have damaged and/or uncoupled the AP pairs in the latter. Since these embryos came from different cocoons, slight age difference may be to blame, although any differences should be less than 2%ED or a fraction of a day’s development. Again, inx6 RNAi may have unexpected and unintended consequences. Clearly, this experiment should be repeated, along with repetitions with other controls.

The effects of inx1 RNAi on developing AP neuron morphology are inconclusive. If gap junctions indeed have an inhibitory role in neurite development in AP neurons, I might expect to see supernumerary branching remaining in the treated neuron, its contralateral homolog, or both. Methods which were intended to reveal the morphology of AP neurons were not always sufficient to expose the extent of the neurite arbor. And due to the low numbers of samples involved and high noise in these images, few conclusions can be drawn from data obtained thus far.

Unfortunately, these experiments fall short of definitively showing that inx1 RNAi can electrically uncouple developing neurons, or that homologous
coupling is necessary for normal CNS development. The data is far too noisy to
and procedures need some improvement. But, taken together, the results are
very encouraging and suggest that repeating these experiments with several
improvements would indeed show that inx1 siRNA completely knocks-down
gap junction formation specifically in these neurons causing changes to adult
AP morphology.
Conclusion:

Though not entirely conclusive, my research has shown that single cell inx1 RNAi treatment is a valuable method for knocking down inx1 expression, effectively preventing the formation of functional gap junctions thus eliminating any electrical coupling between that neuron and others.

In order to address the ambiguities in these results, I am planning future experiments with the necessary experimental repetition, methodological improvements, and appropriate controls. To begin with, simple repetition of previous experiments to increase the sample number to the point of statistical significance could resolve some ambiguity in the data, lower the prominence of noise and reveal previously unseen trends. Nonetheless, these experiments will not be undertaken without some methodological changes. Control and experimental RNAi treatments should be performed at the same time and on embryos from the same cocoon to avoid procedural differences that might arise. Also, filling treated AP neurons with LY dye alone rather than dye combinations, as well as superior imaging equipment may improve the quality of images and lead to more valuable morphological data. Future experiments planned involve the use of ‘scrambled sequence’ siRNAs and ‘sham’ injections as controls. Thus far, Inx6 RNAi was used as a control for inx1 RNAi because inx6 is unlikely to play an integral role in gap junction formation and coupling, due to its
low or nonexistent expression believed in embryonic AP neurons (Kandarian B, MS Thesis, UCSD 2009; unpublished findings). However, other controls may reduce the uncertainties of this experiment as inx6 siRNA has potentially unforeseen effects. In a scrambled siRNA, is a fully functional siRNA in which the nucleotide sequence has been randomly rearranged. Since it cannot bind to and inhibit the target mRNA, this siRNA is designed to be entirely ineffective and a good negative control for the presence of siRNA in single cells. Sham treatments involve impaling and injecting the AP neuron with dye, but without any siRNA. This control would reveal any coupling or morphological changes due to a potentially damaging injection procedure.

Still, the current experiment may be insufficient to show morphological dependence on gap junctions. In the leech, siRNA's effectiveness diminishes in the days following treatment (K.L. Todd, personal communication) and the development of mature neurite arbors takes weeks from beginning to end (49). In AP ablation studies, contralateral homologs indeed sprouted and maintain novel projections to the ipsilateral nerve roots, but they were only observed seven days after ablation (49). Perhaps the current RNAi technique works over too short a time for a detectable phenotype to develop. We are addressing this problem by developing another technique for single cell RNAi. Based on a RNAi 'short hairpin' RNA expression system, a plasmid would be injected into
embryonic CNS nuclei which would constitutively express shRNA thereafter (55, 56). An embryo treated in this manner with inx1 RNAi could be allowed to develop for much longer periods of time, even well into maturity, allowing for the development of atypical phenotypes.

The possible role of gap junctions as channels for direct cell to cell communication in guiding development may only be elucidate when better techniques are developed. If successful, the experiments discussed here and techniques in development open many other interesting avenues of research in leech neurodevelopment.
Materials and Methods:

Staging:

Embryos were staged using two systems which have been previously described (41, 47, 57). Both use external morphological features to place animals on a scale from 0-100% embryonic development (ED) or embryonic day E0 to E30. In these schemes, fertilized eggs are deposited into a cocoon at 0% ED or E0. The last observable morphological feature to appear during embryogenesis marks 100% ED. Embryogenesis is considered to end with hatching which occurs at E30. Staging between morphologically identifiable stages was accomplished by interpolating between the preceding and subsequent morphological markers, counting 24h as 3% (Reynolds et al. 1998) or 1 embryonic day. Prior to experimental manipulation, leech embryos were maintained at 25°C. Once removed from the cocoon, leech embryos were kept at 25°C in *Helobdella* medium (48μM CaCl, 34μM MgCl) and the medium changed every 24h. Leeches were kept at 25°C, 22°C (room temperature) or 16°C for four days or less during experimental incubation periods. Incubation temperature can affect the speed of development, but incubation periods were determined to be too short to cause differences of more than 1% ED or a fraction of an embryonic day. Leeches from 48% (E10) to 65% (E16), as well as adult leeches, were used in these experiments.
All experiments were performed in mid-body ganglia MG7 through MG13, minimizing any variability in staging due to anterior-posterior developmental gradient in the leech. The developmental difference between the anterior-most MG7 and posterior-most MG13 used in these experiments should be no greater than 1%ED or a fraction of 1 embryonic day (42).

**Adult Ganglion Preparation:**

Before dissection, adult leeches were immobilized on ice for 10min. The leech was then placed on a chilled wax surface and pinned through the head and tail and stretched longitudinally. Several midbody segments of the animal were then opened along the dorsal midline with a razor blade. The cut usually opened the gut, which was washed thoroughly of food blood with chilled leech Ringer’s. Several more pins were then placed along the cut edge wall to stretch the body wall. Before proceeding with further dissection, the leech was washed and bathed in 10% EtOH in Leech Ringer’s and remained in this solution thenceforth in order to reduce muscular contractions. Using small iridectomy scissors and forceps, the gut tissue was cut longitudinally and pulled away in order to access the ventrally-located nerve cord in the ventral blood sinus. A series of specific cuts removed the blood sinus from an individual ganglion and its connectives, while sinus tissue was maintained attached to the
roots for pinning. To excise individual ganglia, both connectives were cut midway between adjacent ganglia, followed by cutting the roots with attached sinus tissue away from the body wall. The ganglion was then transferred to a 30mm Sylgard-coated Petri dish and submerged in normal leech Ringer’s. Both connectives and both pairs of roots were stretched gently and pinned to the Sylgard with 1mil tungsten wire pins.

**Embryo Preparation for siRNA Injection:**

After removal from the cocoon, embryos were rinsed every 24h and maintained in *Helobdella* medium with 6.6mg/l Terramycin (Pfizer) at 25°C or room temperature in a dark environment until experimental manipulations began. A single embryo was transferred with the polished end of a cut-off Pasteur pipet to a 30mm Sylgard-coated Petri dish which had a small groove cut into the Sylgard to locate and hold the embryo. The embryo was submerged in normal Wenning’s saline (4mM KCl, 1.8mM CaCl$_2$, 40mM malic acid, 10mM succinate, 20mM NaCl, 10mM TrisCl, pH 7.4). Using blunted forceps, the embryo was maneuvered into the groove in such a way that the ventral surface faced up and the CNS was visible. Minuten pins bent into the shape of a staple, or thin strips of latex were placed over the embryo and secured to the Sylgard. Two or three staples or strips were used to secure the
anterior and posterior portion of the embryo, allowing stability of access to some of the midbody ganglia. Using an etched tungsten point, a small portion of body wall covering a single ganglion was opened allowing access to that ganglion. Finally, the normal Wenning’s was replaced with 8% EtOH in Wenning’s in order to prevent muscular contractions during intracellular injection.

**SiRNA Design, Synthesis and Preparation:**

Two siRNAs, (sequence A and B for Inx1) were obtained as a gift from the laboratory of William Kristan. All other siRNAs used were designed according to guidelines provided by Ambion. SiRNAs were synthesized using the Silencer siRNA construction kit (Ambion) as directed. Each batch of siRNA was tested for purity and quality. In each experiment, the siRNA was diluted to 15μM in the final solution to be injected directly into cells.

**SiRNA Injection:**

The rig used for single cell siRNA injection in has a microelectrode amplifier (Neuroprobe model 1600, A-M systems, inc) (Model 5, Getting), a Picospritzer (Picospritzer II, General Valve corporation), a stimulator (S88, Grass), a micromanipulator (MO-103, Narshige) and a compound microscope
(Eclipse E600FN, Nikon) equipped with epifluorescence optics 10X, 20X, 40X and 60X water-immersion objectives. The adult and embryonic preparations were placed on the microscope stage with a grounding electrode submerged in the pool of Wenning’s.

For embryos, glass electrodes (Thin wall, A-M systems, inc) were pulled (P-87, Sutter Instruments CO.) and filled by capillary action with a combination of siRNA (10-20μM) and 2.5% Alexa Dextran 488 (10kDa) or 5-10% Lucifer Yellow in milliQ water. For adults, thick walled glass electrodes were pulled, filled with siRNA (15μM) and 5% Carboxyfluorescien in water, and beveled on a micro-pipette beveller (BV-10, Sutter Instruments CO.) to 80-100MΩ. These electrodes were then back filled with 0.5M K acetate and mounted on the electrode holder of the micromanipulator. AP and Rz neurons were identified by location and the size of the cell.

Once the electrode tip dimpled the surface of the neuron, the cell membrane was penetrated simply by micromanipulation alone, tapping the side of the microscope, or electrode vibration. In adults, successful Rz impalement could be confirmed by a -10 to -50mV voltage drop and presence of action potentials. Successful impalement of embryonic neurons could sometimes be confirmed by a drop in membrane voltage or observation of action potentials, but neurons at this early stage of development (E10-E15)
lacked action potentials or an observable membrane voltage. Therefore successful impalement could be confirmed by the observation of fluorescent dye diffusing evenly within the cell body after a pressure or electrical pulse delivered dye into the cell. Similarly, AP neurons were identified morphologically during injection. A few brief (4-20msec) pressure pulses were delivered to the neuron, expelling siRNA and dye from the electrode tip into the cell. The dye was allowed to diffuse for a minute or more, and then the ganglion was observed under fluorescence. If the branch pattern did not resemble AP morphology, the electrode was carefully withdrawn and used to impale another candidate cell. If the branch pattern observed revealed the stereotypical and dramatic morphology of the primary neurite of the AP neuron, pressure injection was continued.

The duration of the pressure pulses were increased incrementally until dye could be observed leaving the electrode tip. When electrode tip became blocked, weak (-/+1nA) and short (500ms) current pulses were used unblock the electrode tip. Electrodes that did not become unblocked within a few pulses were simply removed and replaced. A series of the smallest (shortest) possible pressure pulses were used to fill the cell with siRNA and dye. Pressure injection continued for maximum of 5 min, until secondary neurites were visible, the neurons voltage deteriorated or dye filled vesicles could be seen leaving
the cell. The neuron was then imaged with a digital camera (Sensicam QE, Cooke) for fluorescence measurement. The electrode tip was gently withdrawn from the cell, and the whole prep was removed from the microscope.

For embryo preparations, the 8% EtOH Wenning’s was immediately replaced with chilled normal Helobdella medium. Embryos were exposed to 8% EtOH for no longer than 15 min. Staples and latex strips were removed, and the embryo was transferred to a new small Petri dish with fresh Helobdella medium. During incubation (2-4d), embryos were kept in individual dishes, rinsed every 24h, and maintained in Terramycin treated Helobdella medium at 25°C or room temperature in a dark environment until the next experimental manipulations.

For adults, the preparation was rinsed with fresh chilled Leech Ringer’s and placed in L15 medium. The ganglia were incubated in a sealed Petri dish in L15 at 16°C for one to three days.

In order to estimate the relative amount of siRNA material injected into each neuron, each neuron was imaged after injection as described above. Using image processing software (Image-Pro Plus 4.1, Media Cybernetics), the injected neurons cell body was encircled and the mean pixel intensity (I_i) calculated. In the same image, the mean pixel intensity of a background area of the same size (I_b) was calculated. If the ratio of I_i/I_b was less than 3, the sample preparation was not included in analysis.
**Estimating Quantities of siRNA Delivery:**

After intracellular siRNA injection was complete, each injected cell was fluorescence imaged. Images were acquired with the digital camera and paired software (Image-Pro Plus 4.1, Media Cybernetics). Each image is comparable since parameters for imaging were never changed. For every image, siRNA was always delivered to the cell in a solution with 2% Alexa Dextran 488 (10kDa), imaging software acquisition settings were kept the same, and room and microscope lighting was controlled.

Using features of the imaging software, the mean pixel intensity of the injected cell’s soma and a sample of background were measured. The two areas, cell and background, were encircled by hand and were roughly equal in size. Any somas which exhibit a pixel intensity less than x3 that of background was rejected from analysis. The relative intensities of cell somas can be used as a relative comparison of the amount of siRNA injected into those cells.

A rough estimate of the absolute amount of siRNA material calculated. The amount of siRNA delivered to the Rz was estimated by the number and duration of pressure pulses during injection. Each 5msec pressure pulse was roughly calculated to be 0.52 to 4.2pl.
Dissection of Embryos for Physiological Recording and Dye Fills for Imaging:

Embryos were partially immobilized in ice cold Helobdella medium. The embryo was opened by longitudinally tearing the cryptolarval membrane, or the dorsal fusion line in older embryos, freeing the cocoon’s cryptolarval fluid. The majority of cryptolarval fluid and the gut tissue were removed with forceps. Cryptolarval fluid of older embryos (>E13) is more viscous and the convoluted gut tissue due to gut pouch formation make its removal more difficult. In these embryos a stream of saline was necessary to wash out the remaining cryptolarval fluid and careful tearing of gut tissue was necessary for gut removal. The embryo was then anesthetized by transfer to a Sylgard (Dow-Corning) coated Petri dish and submerged in 8% EtOH in sterile Wenning’s. The embryo was pinned onto the Sylgard, ventral side up, with one pin through each the head and tail sucker. Once the embryo was stretched longitudinally and positioned in the middle of the dish, several more pins were placed, through either cryptolarval membrane or distal embryonic body wall tissue, in order to stretch the tissues surrounding the MBGs in all directions. Stretching the tissue prevents the MBGs from moving due to persistent muscle contractions. To expose the CNS, a longitudinal incision was made in the ventral body wall over 3-10 adjacent MBG using a pick made of etched tungsten wire. The pick was
also used to remove the transparent blood sinus surrounding each MBG. Finally, any remaining obstructing gut tissue was removed with the pick, and a stream of saline was applied to remove any remaining cryptolarval fluid or debris.

**Electrophysiology, Adult Ganglia:**

The electrophysiology rig for adult MBGs consisted of a dissecting microscope (type), three micromanipulators (Leitz), three microelectrode amplifiers (Neuroprobe 1600, A-M systems, inc) (5A, Getting instruments, Inc.), a stimulator (S88, Grass), and a digitizer (Digidata1322A, Axon Instruments). Sharp recording electrodes were made from thin walled capillary tubes on an electrode puller (P-87, Sutter Instruments CO.) and filled with 4M potassium acetate. Each electrode’s resistance was tested and those with resistance between 10 to 25MΩ were used. Depending on the specific experiment, recording and measurements of electrical coupling were performed with two or three electrodes. Ganglia were prepared as stated above, and rinsed and submerged in leech Ringer’s before staging.

With two electrodes, each Rz in a single ganglion was impaled by one electrode. Successful impalement could be verified by presence of action potentials, a negative membrane voltage, and visual confirmation. After five
minutes to ensure stability of recording, 500msec pulses of -1.0nA current were passed into one Rz, separated by at least 10sec. Electrical signals were simultaneously recorded from both electrodes. Pulses were repeated until six clear recordings were obtained (no action potentials preceding the pulse). These pulses were repeated, injecting current into the other cell. With the two electrode configuration, coupling strength ($\Delta mV/nA$) could be recorded directly or coupling coefficient could be calculated by eliminating current injection artifacts.

With three electrodes, one Rz was impaled with two electrodes and another electrode impaled the other Rz. In this configuration, an electrical pulse could be delivered to one Rz while the resulting voltage changes of both Rz’s could be recorded simultaneously with minimal artifact. Rz’s were impaled and confirmed as above. A series of 500msec current pulses (-0.5, -1.0, -2.0, -3.0, -4.0, -5.0nA) were delivered, each pulse separated by at least 10sec. This series was repeated twice more, after a 5min resting period. In this three electrode configuration, the Rz pairs coupling coefficient could be directly calculated ($\Delta mV_{Cell B}/\Delta mV_{Cell A}$) without the need to eliminated current injection artifacts.

**Electrophysiology, Embryonic Ganglia:**
The rig used for embryo electrophysiology has two microelectrode amplifiers (Neuroprobe 1600, A-M systems, Inc), a stimulator (S88, Grass), two micromanipulators (MO-103, Narshige) and a compound microscope (Eclipse E600FN, Nikon) equipped with epifluorescence optics and a 60X water-immersion objective. Electrical recordings were digitized (Digidata1322A, Axon Instruments). Sharp recording electrodes were made from thin walled capillary tubes (A-M systems) on an electrode puller (P-87, Sutter Instruments CO.) and filled with 0.5M potassium acetate. Each electrode had a resistance between 50 and 90MΩ.

Due to fragility of embryonic neurons and small working space under the compound microscope, only a two electrode configuration was used to record coupling strength. Likewise, neural fragility necessitated shorter initial and inter-trial resting periods (1min). Otherwise, coupling strength recordings were carried out similarly to those in adults. A series of negative current pulses (500ms) of increasing strength (-0.5, -1.0, -2.0, -3.0, -4.0, -5.0nA) were delivered to one cell, with 10sec intervals. Only negative current was used to avoid action potentials. This series was repeated, injecting the other cell. Pulses were repeated until 12 clear recordings were obtained (one trial). A minimum of one trial or maximum of three trials was carried out on every AP pair, before the neurons began to deteriorate.
Calculating coupling coefficient and coupling strength:

All electrophysiological recordings were digitized at 20,000Hz and stored on a computer. All data acquisition and processing was performed by digitizer paired software (Axoscope 10.0, Molecular Devices Corporation). Each pulse delivered was recorded as a 1.5sec ‘sweep’, which included 500msec preceding the pulse, 500msec of current delivery, and 500msec after the pulse. Each sweep includes simultaneous recordings of the voltage and current recorded by each probe and the trigger which marks the beginning of the current pulses. Selected sweeps were omitted from analysis for the following reasons; if there were action potentials obscuring the membrane voltage before or during the current pulses, too much noise, sudden atypical voltage changes or any other suspect phenomena.

For convention, the electrode passing artificial current into a cell is referred to as the ‘active electrode’. The electrode passively recording electrical signals is referred to as the ‘passive electrode’. In the two electrode configuration, the passive electrode records from Cell B. In the three electrode configuration, there are two passive electrodes, one recording from Cell B and another recording from Cell A. In both configurations, the active electrode passes current into and records from Cell A.
The change in membrane voltage of any cell due to the current pulse ($\Delta V_m$) was defined by the following equation:

$$\Delta V_m = V_{m_0} - V_{m_1}$$

Where $V_{m_0}$ is the cell's membrane voltage during current injection and $V_{m_1}$ is the initial membrane voltage just prior to current injection ($V_{m_1}$). $V_{m_1}$ was calculated as the average voltage at every time point for the 100msec immediately preceding the current pulse. $V_{m_0}$ was calculated as average voltage at every time point for the last 100msec of the current pulse. If a cell's response to a single current strength was recorded multiple times during one trial, the calculated $\Delta V_m$ for each sweep was averaged. Otherwise, $\Delta V_m$ was based on one successful recording.

In experiments involving two and three electrode recordings, the coupling coefficient ($C_c$) was calculated by the following equation:

$$C_c = \frac{\Delta V_{m_B}}{\Delta V_{m_A}}$$

Where $\Delta V_{m_A}$ represents the change in membrane voltage of Cell A and $\Delta V_{m_B}$ represents the change in membrane voltage of Cell B. $C_c$ should always be a number between 0 and 1 as $\Delta V_{m_B}$ should always be smaller, absolutely, than $\Delta V_{m_A}$. When many $C_c$'s were calculated for the same pair of cells using different pulse strengths, all $C_c$ at each pulse strength were averaged.

In a two electrode configuration, there is no passive electrode
recording from Cell A. The active electrode records a significant artifact due to the current pulse injection which must be eliminated (bridge balancing) in order to calculate the actual $\Delta V_m$ in Cell A. Time limitations precluded the use of bridge balancing operations during recording, so bridge balancing methods were implemented on recorded data. The voltage drop due to current pulse recorded by the active electrode consists of two components; a fast component due to electrode capacitance, and a slow component due to cellular capacitance. The fast component, or bridge, was balanced using one of two methods, depending on the experiment. In one method, manual balancing, $V_{m1}$ was defined as the membrane voltage of Cell A at one time point; when the membrane voltage of Cell B first began to drop after the beginning of the current pulse. This voltage drop was detected visually and occurred 2-3msec after the beginning of the pulse. This was generally enough time to entirely eliminate the fast component of rising phase leaving the majority of the slow component of the rising phase revealing the membrane voltage change as calculated above. The other method, automatic balancing, the fast component was isolated by recording a voltage change outside of any cell and then subtracted from the intracellular recording. During each experiment and after intracellular recordings were completed, a -1.0nA 500msec test pulse was delivered through the active electrode into solution and the resultant voltage
signal was recorded. This voltage change was then subtracted from the signal recorded while delivering a current pulse to Cell A, thus eliminating the fast component of the voltage change recorded in Cell A due to electrode capacitance, leaving only the slow cellular component. The electrode’s test curve was matched (visually) to the cells curve and subtracted.

In two electrode configurations where bridge balancing was found to be insufficient, coupling strength (Cs) was calculated rather than Cc. Calculating the Cs rather than Cc eliminates the need for a third electrode or unreliable bridge balancing on the active electrode. Cs was calculated with the following equation:

\[ Cs = \frac{\Delta V_{mB}}{I} \]

Where \( \Delta V_{mB} \) (mV) is the change in membrane voltage in the Cell B due to I (nA), the strength of current injected into the Cell A. The Cs’s calculated from each current injection strength (-0.5 to -5nA) were plotted (mV v nA) and a linear regression was fitted to the data. The average Cs for each cell pair is defined as the slope of the trend line. Current pulses were delivered to both cells in each pair, so the Cs calculated from both ‘sides’ were averaged. Then average Cs’s from repeated trials in the same pair were averaged.

In all recordings and calculations, only pulses of negative current were used because even small positive current pulses caused action potentials,
which complicated calculations. Therefore, all quantification on coupling is based upon negative current pulses.

AP pairs were said to be coupled if their average coupling strength exceeded background coupling values. Small coupling strength values observed between uncoupled neuron pairs can be attributed to 'background coupling', or electrode to electrode interference while coupling strengths were recorded. Background coupling was measured by leaving one electrode in a cell and pulling the other out of the cell, but leaving it submerged in solution. Current was passed through each electrode. The resulting voltage changes were used to calculate the background coupling strength as described above. Background coupling strength was 0.21 ± 0.19mV, so neural pairs that exceeded a coupling strength of 40mV were considered coupled.

**Dye Fills:**

Neurobiotin fills were performed nearly identically to previous methods (41, 42). Embryos were prepared as described above. Individual neurons were impaled with sharp electrodes. Dye fills were performed on a stage of a compound microscope equipped with epifluorescence optics. A combination of 2% (weight/volume) Neurobiotin with 2.5% Alexa Dextran Fluor 488 (10kDa), or 50mM Caryboxyfluoreci, or Lucifer Yellow or alone in milliQ water was
injected into cells. Dye was passed from the tip of a sharp microelectrode into the impaled cell with 500msec positive current pulses at 1Hz. Under continuous observation during dye injection, the identity of the cell was confirmed by its projection morphology. In adult ganglia, +2.0nA current pulses delivered dye for 30min. In embryonic ganglia, +0.5-2.0nA current pulses delivered dye for a minimum of 10 min and stopped before the cell began to deteriorate. After gently removing the microelectrode, the dye was allowed to diffuse for 5 min to 3 h at room temperature. The tissue was then fixed in 4% paraformaldehyde in PBS overnight at 4°C. The tissue was rinsed and washed four times for 30 min each with 0.5% Triton X-100 in PBS (PBX), followed by incubation with DyLight 549-conjugated streptavidin 1.7μg/ml (Jackson Immuno Research, West Grove, PA) in 0.2% PBX for 1 to 3 h. The tissue was then rinsed and washed six times for 15 min each with 0.2% PBX. The tissue was then cleared and mounted on a glass slide with 80% glycerol.

**Imaging:**

Each neuron injected with fluorescent dye was imaged on a confocal microscope (TCS-SP2, Leika), equipped with epifluorescence optics, and 10X, 20X, 40X and 63X objective lenses. Images were acquired with the accompanying software (Leika).
**Luminance Ratio Measurement:**

Luminance ratio measurement was methodologically similar to siRNA injection measurement. Using the same measurement software above mean pixel intensity was measured for the 568 component. Three areas were encircled; the injected cell, the coupled cell and another adjacent cell as a control. These measurements were made on only the 568 component of the image, in order to reveal neurobiotin coupling. Since background cells are nearly invisible in this component, 488 and/or transmitted light were used to help locate and encircle somas.
Appendix:

Temporal measurement of dye diffusion:

Neurons that form gap junctions are often dye coupled, meaning traceable molecules can pass from one cell through the synapse into the other. Measuring differences in dye coupling provides further evidence of gap junction functionality. In order to measure dye coupling, an amount of dye is delivered to the neuron and a certain amount of time given to allow the dye to diffuse from the soma to neurites through gap junctions and finally into the somas of coupled neurons. The following experiment was performed to elucidate the temporal dynamics of dye coupling and develop the dye coupling protocol to have a set diffusion time.

Six consecutive MGs from a single adult leech were prepared as described in methods. In each ganglion, one of the two Rz neurons was filled with a dye combination (2% neurobiotin, 5% carboxyfluorescein in water) by iontophoresis (5nA, 500msec, 1Hz) for 5min. After injection, the dye was allowed to diffuse for a 5, 20, 30, 40, and 60 min (10min sample omitted due to cell damage and dye leakage). Immediately after each preparation’s designated diffusion time, the ganglia were fixed in 4% paraformaldehyde and later stained with Strep-Cy3. Preparations were photo imaged for Cy3 as described in methods. Luminance ratios were measured as described in
methods, where the average pixel value of the non-injected cell divided by the average pixel value of the injected cell.

As expected, the measured luminance ratio increased with longer diffusion times (Fig 8). A 5m diffusion produced a luminance ratio of only 0.20 while a 60m diffusion time produced a luminance ratio of 0.68, and the overall trend of dye diffusion shows a clear time dependence. The nature though, of this dependence (linear, logarithmic) is not made completely clear. The standard error was significantly large. We suspect (and advised by Antonia Marin-Burgin) that the luminance ratios would be too variant, from preparation to preparation, to reveal subtle changes in dye coupling. Dye coupling would have to be nearly completely eliminated for significant differences in luminance ratio to be observed. These measurements were not pursued further.

**Acute exposure to ethanol measurably delays embryonic development**

In these experiments, it is crucial to be able to predict the age of an embryo given the specific amount of time since its age was last assessed. This should be reliable so that MG development is aligned with the rest of the embryos. Noticing that treated embryos were seemingly delayed development, I suspected that anesthetization in 8% EtOH for even short periods of time may be the cause developmental delay. To test this possibility, I performed the
following experiment:

20 embryos from one cocoon were staged and found to be 51% (E11). The embryos were split into control and experimental groups. The experimental group was exposed to 8% EtOH in Helobdella for 15 min and then placed back in regular Helobdella, while the control group was simply transferred to a fresh Petri dish in Helobdella. The next day (E12), 24 h later, the embryos’ age were assessed again. At E12 the experimental and control groups were 53.9 ± 1.4% and 55.3 ± 1.3%. The difference between developmental stages between the two groups at E12 was small but significant. It is quite possible that damage caused by ethanol exposure obscured cues used for staging, limiting the effectiveness of the percentage ED staging system. In fact, 2 of the 10 exposed embryos died within 24 h of exposure. 7 days after EtOH exposure, the embryos’ ages were assessed again. The experimental and control groups were 74.7 ± 2.1%ED and 75.8 ± 1.2%ED, respectively, which is not significantly different (Fig 9). While differences in development after one day were significant, a difference of 1% ED, or a fraction of a day, is unlikely to affect the outcome of these experiments.
**siRNA Target Sequences:** Target sequence is highlighted in the HM-innexin gene sequence listed below.

**HM-Innexin 1:** Target sequence 49: AATGAATGCTATGGAGTGATG

**HM-Innexin 1:** Target sequence 32: AAGCTCTTCATCTTTTGCTTGG

**HM-Innexin 1:** Target sequence A: AAGAGATCCGGAGTCAATATG*

**HM-Innexin 1:** Target sequence B: AAATCAGACAGCACTCTCGGA*

**HM-Innexin 6:** Target sequence 13: AATGAATTATCCTCGATCAT

**M-Innexin 6:** Target sequence 13: AATTCTGATCAGACTCTTTAAA

*Gift from K.L. Todd

**Hm-Innexin Gene Sequences:**

Hm – Innexin 1 – Nucleotide sequence of *Hirudo Medicinalis* Innexin 1 gene

ACTTGTTCCTGGCCTGGTCTTCTCGTAGGGAGCAAAATGGGATCAGACTGTTT
AAGTCCGTGGTCTCAGGTATAAGGGAGATAATGCTCCGTATGGAGAATGACTACT
CGTCGACAGACTGAGTGCAGATACGGTCTGGTGTCATCCTCATCTGCTTTTG
GCTTGCCTCGTACGAGACGCACGTCTGTGCAGGACAGCTACTTGGCTG
GTGCCGGCTCGATTTCGCTCAGGACAGCGAGATCAGACTACAGGATGCTGTC
TGCTGTCGGCAGCAACACTTCTTTGCTCGGCCATGAGACGAGCTGAGG
CAGGACCACCTCTCCATCCACACTAACATCCATGATCAGCTATTACCACAA
TGGATAACCCTACTATCCATCCACTACGACTACTTCCAGGCCCTACTGTGCGTGTTCCATG
CCTACTGTGAGGGTTTGCTAACGAGGATCCGGAGTCAATATG*GCGGCC
ATCATGGAGCCTGCCGGGTGTCCGCTCAGGCCCAGGCCACTACGCTGGAGATCA
GGGAGAAGGCCATCAAGTTACGCTCAACAGAGATGAGTCAGTTGTT
GGCACAGCAGGAGTACAGGACCGGCTGCTGGTCAGGATCAAAACACGT
Hm – Innexin 6 - Nucleotide sequence of Hirudo Medicinalis Innexin 6 gene
CGTCCAGTGCGTCCTCCCAATCAACCTTTTCAACGAGAAAGTGTTTCTCA
TGCTCTGGGTTTGCTAGTTCTACTAATTCTGATCAGACTTTAAAAATCTAT
TCTCGTGGAAGCTCCTGTGATGTTTCGCTGTTCGAGGCTAGTTACATCAAA
CAACGCCTCAGACTGTTACCAGTTCCGAGAGGATCGACAGCATCCA
CAATGAAATTCTTTGACATCCTACATGGCAGTGATGGAGTCTTGACGCCTC
AAACTGTTAGCATCAAATTCGATGAAAGAGGGAGCAGCACGGTTGCTG
ACGGACTCTTGAGGACTACAGCACACAGTCTGACAGGTCAGGAAC
TTGGAAGGGAAGAAAGTGCAAGGTCATACAGAAGTAAGAAGAAAAA
ACCCAGACCAAGTGCTTTGCTAGGAAAGCAGTCAGAAAGAGGAATCAGAG
GGAAACGCCCACAAGGCACGACACAATTGCAATGCAACGAGGTCTAGAA
GGGGAGGTTATTTCTTGGACTAGTAACTCACAACGCGCAACAGGAG
CAGCAAACAAAGCTTTGCTAAAACCACATACGAAATAGTAAACTCCAAG
ACTGGAAACAAAAATACATAGAATGTTTCTTAAAAAAAACAAAAAAAAA
Figures:

A) Chain of ganglia of the leech CNS (45).

B) Atlas of leech neurons of the adult segmental ganglia (45). AP (yellow) and Rz (blue) neurons are highly stereotyped neurons in segmental ganglia, able to be identified by size and location.

Figure 1 – Leech anatomy
Figure 2 – AP morphology and ablation studies

A) AP neurons at E18. A fluorescence z-series image of showing the morphologies the Left and Right AP neurons. The left AP was filled with cy3 conjugated neurobiotin (red) and the right AP was filled with Lucifer yellow (green). The mirror symmetry is apparent. In each AP the primary neurite extends across the midline, turns posteriorly, bifurcates and sends projections out of the ganglion through the contralateral nerve roots. Extending from the primary neurite of the Left AP, secondary neurites are visible in this image. At this age, they have already begun to degenerate and retract.

B) Schematic drawings of AP morphology in three consecutive ganglia under normal and experimental conditions (49).

Left) From E10-E14, AP cells extend four axons, two of which grow into the connective nerves that join adjacent ganglia. These longitudinal projections overlap with those of ipsilateral, segmental AP homologs (indicated with solid circles) for 5-8 d. (Successive AP homologs are indicated with alternating solid and dotted lines to distinguish the axons of adjacent neurons). The other two projections exit the CNS via two contralateral nerve roots. On the opposite side of the ganglion lie the mirror-image contralateral homologs (indicated with open circles) but their projections are not illustrated, for simplicity.

Center Left) In the adult, AP cells possess only the contralateral projections, as the longitudinal axons are retracted during development. Retraction begins at E1 5-E18 and is nearly complete by the end of embryogenesis (E30).

Center right, Adult AP morphologies after ablating one AP (marked with an x) between E10 and E14). The ipsilateral homolog takes over territory vacated by the killed cell by adulthood (47).

Right) Deletion of an AP at E15 or later causes the contralateral homolog to generate bilateral projections by adulthood.
Figure 3 – Coupling coefficient before and after 48h incubation in L15. Error bars indicate standard error of the mean. The apparent difference in the coupling coefficient of Retzius pairs before and after incubation is not statistically significant (paired t-test, p=0.35).
Figure 4 – Inx1 RNAi and electrical coupling in Adult Retzius neurons.

A) Data from each one of the siRNAs tested is plotted separately. siRNA treatments were carried out in pairs on ganglia from the same leech, so the color of the line represents the experiment which the data was gathered. Closed circles represent ‘effective’ siRNAs (experimental) while closed circles represent ‘ineffective’ siRNAs (controls). In all three experiments, Rz pairs treated inx1 RNAi have lower average coupling coefficients after 48h incubation than those of control treatments.

B) Summary of the above chart. Control treatments are grouped and represented by the red line. Experimental treatments are grouped and represent by the blue line. There is a significant difference in Retzius pair coupling coefficients after treatment with inx1 RNAi (experimental) relative to controls.

C) Change in coupling coefficient before and after injection. Control and experimental groups are grouped as above. There is a significant difference in the change in coupling coefficient between control and experimental groups.
A) Coupling Coefficient Before and After RNAi Treatment

B) Coupling Coefficients are significantly different after Inx 1 siRNA treatment
Figure 4 – Inx1 RNAi leads to decreased electrical coupling in Adult Retzius neurons, Continued
**Figure 5** – AP pair coupling increases from E12 to E18.

A) The proportion of AP pairs that are electrically coupled increases from E12 to E18.

B) A similar increase in coupling strength occurs from E12 to E18. P values for significances are summarized in Table 1.

C) The proportion of neurons producing action potentials increases through these developmental stages.

D) AP neurons at E12, E14 and E16 show a trend suggesting that the ability to produce action potentials may be related to electrical coupling. When grouped together, the difference between coupling strength between action potential producing APs and those that lack action potentials is significantly different (p=0.001)
A) The Proportion of Electrically Coupled AP Pairs at E12, E14, E16, and E18 (n = 6, 9, 7, 4)

B) Electrical coupling in AP pairs at E12-18 (n = 12, 18, 14, 8)
Figure 5 – AP pair coupling increases from E12 to E18, Continued.
Figure 6 – Inx1 and Inx 6 RNAi treatments and AP neuron coupling.

A) Inx1 RNAi treatment eliminates electrical coupling (p=0.01).

B) There is a small but significant difference in input resistance between treated and untreated AP neurons of the inx1 experiment and those of the inx6 experiment.

C) There are no significant differences in the resting membrane potentials of either group of either experiment.
A) Coupling strength of AP neurons at E18

B) Rm of Control and Experimental Groups in inx1 and inx6 RNAi Experiments
Figure 6 – Inx1 and Inx 6 RNAi treatments and AP neuron coupling, Continued.
Figure 7 – Inx1 and Inx 6 RNAi treatments and AP neuron morphology. Lucifer Yellow fills of treated and untreated AP neurons from inx1 and inx6 RNAi experiments. In each, The primary and secondary neurites are clearly visible. These images show that the APs are morphologically healthy. B, C, and D show a streaky imaging artifact near the soma. A, B, C, and D correspond to representative examples of groups inx1 control, inx1 experimental, inx6 control and inx6 experimental, respectively. The scale bar represents 50μm.
Figure 8 – Time dependence of the luminance ratio in coupled neurons. Coupled Rz neurons allow the passage of neurobiotin tracer at a predictable rate on the order of minutes.
Figure 9 – Exposure to alcohol and embryonic development. A 15min exposure to 8% EtOH at E11 delays embryonic development.
**Tables:**

**Table 1** – Summary ‘p’ significance values in Figure 6A.

<table>
<thead>
<tr>
<th>ED v ED</th>
<th>18</th>
<th>16</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.0056</td>
<td>0.0101</td>
<td>0.0475</td>
</tr>
<tr>
<td>14</td>
<td>0.0064</td>
<td>0.0922</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.0102</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2** – AP pair coupling in Inx 1 and Inx 6 RNAi experiments. Dashes mean that no measurements were made on that condition.

<table>
<thead>
<tr>
<th>preparation</th>
<th>inx 1</th>
<th>control</th>
<th>experimental</th>
<th>inx 6</th>
<th>control</th>
<th>experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>yes</td>
<td>no</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>no</td>
<td>yes</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>yes</td>
<td>no</td>
<td>-</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 3 – Summary of coupling and action potential data (E12-E18).

<table>
<thead>
<tr>
<th>E Day</th>
<th>Prep</th>
<th>MBG</th>
<th>Coupling Strength (mV/nA), electrode</th>
<th>Coupled</th>
<th>Action P.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Right</td>
<td>Left</td>
<td>Average</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>1</td>
<td>0.0305</td>
<td>0.0523</td>
<td>0.0414</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.0318</td>
<td>0.0003</td>
<td>0.01605</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>-0.01</td>
<td>0.0294</td>
<td>0.0097</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td></td>
<td>0.0595</td>
<td>0.0637</td>
<td>0.0616</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.6226</td>
<td>0.0312</td>
<td>0.3269</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>-0.1073</td>
<td>0.0019</td>
<td>-0.0527</td>
</tr>
<tr>
<td>average</td>
<td></td>
<td></td>
<td>0.06715833</td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>sd</td>
<td></td>
<td></td>
<td>0.13299599</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>1</td>
<td>0.0413</td>
<td>0.23</td>
<td>0.13565</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.13</td>
<td>-0.1129</td>
<td>0.00855</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.073</td>
<td>0.1492</td>
<td>0.1111</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td></td>
<td>0.4003</td>
<td>0.4665</td>
<td>0.4334</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.0501</td>
<td>0.0534</td>
<td>0.05175</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.1125</td>
<td>0.0901</td>
<td>0.1013</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td></td>
<td>0.1197</td>
<td>0.1472</td>
<td>0.13345</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.0197</td>
<td>1.4377</td>
<td>0.7287</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>1.944</td>
<td>0.0212</td>
<td>0.9826</td>
</tr>
<tr>
<td>average</td>
<td></td>
<td></td>
<td>0.2985</td>
<td></td>
<td>33%</td>
</tr>
<tr>
<td>sd</td>
<td></td>
<td></td>
<td>0.34350903</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>1</td>
<td>0.2707</td>
<td>0.4697</td>
<td>0.3702</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.531</td>
<td>0.1674</td>
<td>0.3492</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.1027</td>
<td>0.1194</td>
<td>0.11105</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td></td>
<td>1.6517</td>
<td>1.264</td>
<td>1.45785</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.2952</td>
<td>0.6909</td>
<td>0.49305</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.2952</td>
<td>0.6909</td>
<td>0.49305</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td></td>
<td>0.9636</td>
<td>0.6611</td>
<td>0.81235</td>
</tr>
<tr>
<td>average</td>
<td></td>
<td></td>
<td>0.58382143</td>
<td></td>
<td>57%</td>
</tr>
<tr>
<td>sd</td>
<td></td>
<td></td>
<td>0.43909458</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>1</td>
<td>1.72</td>
<td>0.77</td>
<td>0.99387279</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>4.18</td>
<td>0.12</td>
<td>2.47156753</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>2.7</td>
<td>2.08</td>
<td>2.3916687</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>2.09</td>
<td>0.74</td>
<td>1.42</td>
</tr>
<tr>
<td>average</td>
<td></td>
<td></td>
<td>1.81927725</td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td>sd</td>
<td></td>
<td></td>
<td>0.72888715</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4 – Inx1 RNAi in AP pairs experimental data.

<table>
<thead>
<tr>
<th>Exp</th>
<th>Condition</th>
<th>Prep</th>
<th>Probe</th>
<th>Vrp</th>
<th>Rm</th>
<th>Average</th>
<th>S.D.</th>
<th>Cs Avrg of Cs Avrg</th>
<th>Cs&gt;</th>
<th>S.D.</th>
<th>0.35</th>
<th>Aps</th>
<th>Luminance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inx1</td>
<td>Untreated</td>
<td>2</td>
<td>L</td>
<td>-19.63</td>
<td>167.83</td>
<td>1.72</td>
<td>0.77</td>
<td>0.99</td>
<td>yes</td>
<td>yes</td>
<td>100%</td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td></td>
<td>-31.36</td>
<td>144.58</td>
<td>0.27</td>
<td>0.17</td>
<td></td>
<td>yes</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>L</td>
<td>-41.56</td>
<td>186.67</td>
<td>4.18</td>
<td>4.32</td>
<td>2.47</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td></td>
<td>-25.93</td>
<td>296.57</td>
<td>0.76</td>
<td>0.12</td>
<td></td>
<td>yes</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>L</td>
<td>-13.07</td>
<td>220.46</td>
<td>2.70</td>
<td>1.50</td>
<td>2.39</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td></td>
<td>-58.45</td>
<td>398.05</td>
<td>2.08</td>
<td>3.15</td>
<td></td>
<td>yes</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>L</td>
<td>-37.95</td>
<td>286.56</td>
<td>2.09</td>
<td>0.97</td>
<td>1.42</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td></td>
<td>-16.22</td>
<td>561.76</td>
<td>0.74</td>
<td>0.34</td>
<td></td>
<td>yes</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td>2</td>
<td>L</td>
<td>-39.85</td>
<td>150.81</td>
<td>0.39</td>
<td>0.24</td>
<td>0.32</td>
<td>no</td>
<td>yes</td>
<td>300</td>
<td></td>
<td>192</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td></td>
<td>-17.43</td>
<td>143.65</td>
<td>0.24</td>
<td>0.06</td>
<td></td>
<td>yes</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>L</td>
<td>-56.20</td>
<td>336.84</td>
<td>5.22</td>
<td>5.31</td>
<td>3.82</td>
<td>yes</td>
<td>yes</td>
<td>477</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td></td>
<td>-23.34</td>
<td>382.66</td>
<td>2.42</td>
<td>0.74</td>
<td></td>
<td>yes</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>L</td>
<td>-20.87</td>
<td>325.43</td>
<td>0.17</td>
<td>0.30</td>
<td>0.07</td>
<td>no</td>
<td>no</td>
<td>192</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td></td>
<td>-18.89</td>
<td>355.83</td>
<td>-0.02</td>
<td>0.08</td>
<td></td>
<td>yes</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>L</td>
<td>-13.82</td>
<td>378.30</td>
<td>0.20</td>
<td>0.22</td>
<td>0.35</td>
<td>no</td>
<td>no</td>
<td>210</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td></td>
<td>-17.74</td>
<td>795.63</td>
<td>0.50</td>
<td>2.72</td>
<td></td>
<td>no</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>L</td>
<td>-15.18</td>
<td>280.83</td>
<td>0.09</td>
<td>0.11</td>
<td>0.05</td>
<td>no</td>
<td>no</td>
<td>457</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td></td>
<td>-11.00</td>
<td>416.83</td>
<td>0.01</td>
<td>0.04</td>
<td></td>
<td>no</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>L</td>
<td>-10.51</td>
<td>263.37</td>
<td>0.29</td>
<td>0.10</td>
<td>0.25</td>
<td>no</td>
<td>yes</td>
<td>1770</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td></td>
<td>-11.48</td>
<td>354.22</td>
<td>0.21</td>
<td>0.14</td>
<td></td>
<td>yes</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>L</td>
<td>-16.30</td>
<td>323.91</td>
<td>0.03</td>
<td>0.15</td>
<td>0.07</td>
<td>no</td>
<td>no</td>
<td>2052</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td></td>
<td>-21.53</td>
<td>354.95</td>
<td>0.11</td>
<td>0.06</td>
<td></td>
<td>no</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The table shows the experimental data for Inx1 RNAi in AP pairs. The columns represent the experimental condition, preparation, probe, Vrp, Rm, average Vrp and Rm, standard deviation, average of the ratio of Vrp to Rm, presence of Cs, siRNA injection, and luminance. The data is organized into two groups: Untreated and Treated. The table includes the average and standard deviation of the measurements for each group.


**Table 5 – Inx6 RNAi in AP pairs experimental data.**

<table>
<thead>
<tr>
<th>Exp</th>
<th>Condition</th>
<th>Prep</th>
<th>Probe</th>
<th>Vrp</th>
<th>Rm</th>
<th>Average</th>
<th>S.D.</th>
<th>Avrg Cs Averg</th>
<th>Cs&gt;</th>
<th>0.35</th>
<th>Aps</th>
<th>Luminance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inx6</td>
<td>Untreated</td>
<td>2 L</td>
<td>-20.02</td>
<td>113.10</td>
<td>0.00</td>
<td>0.04</td>
<td>0.00</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-16.03</td>
<td>104.15</td>
<td>0.00</td>
<td>0.04</td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 L</td>
<td>-19.04</td>
<td>89.62</td>
<td>0.10</td>
<td>0.20</td>
<td>0.18</td>
<td>no</td>
<td>yes</td>
<td>1223</td>
<td>1223</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-15.75</td>
<td>168.08</td>
<td>0.27</td>
<td>0.47</td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 L</td>
<td>-13.21</td>
<td>142.90</td>
<td>0.09</td>
<td>0.16</td>
<td>-0.02</td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-16.86</td>
<td>204.88</td>
<td>-0.14</td>
<td>0.23</td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 L</td>
<td>-45.35</td>
<td>139.16</td>
<td>0.40</td>
<td>0.13</td>
<td>0.46</td>
<td>yes</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-37.60</td>
<td>119.95</td>
<td>0.53</td>
<td>0.07</td>
<td></td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 L</td>
<td>-34.55</td>
<td>159.42</td>
<td>0.63</td>
<td>0.23</td>
<td>0.63</td>
<td>yes</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-25.24</td>
<td>204.28</td>
<td>0.64</td>
<td>0.31</td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 L</td>
<td>-13.06</td>
<td>154.19</td>
<td>0.03</td>
<td>0.16</td>
<td>0.04</td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-27.07</td>
<td>221.09</td>
<td>0.04</td>
<td>0.66</td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 L</td>
<td>-15.46</td>
<td>108.10</td>
<td>0.07</td>
<td>0.12</td>
<td>0.05</td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-15.41</td>
<td>292.17</td>
<td>0.02</td>
<td>0.22</td>
<td></td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>-22.48</td>
<td>158.65</td>
<td>0.19</td>
<td>0.19</td>
<td>0.19</td>
<td>29%</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD</td>
<td>10.14</td>
<td>55.85</td>
<td>0.26</td>
<td>0.26</td>
<td></td>
<td>33%</td>
<td></td>
<td></td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td>2 L</td>
<td>-32.99</td>
<td>93.56</td>
<td>0.07</td>
<td>0.01</td>
<td>0.07</td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>561</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-29.75</td>
<td>180.43</td>
<td>0.07</td>
<td>0.36</td>
<td></td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>1223</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 L</td>
<td>-32.96</td>
<td>101.04</td>
<td>-0.02</td>
<td>0.10</td>
<td>0.11</td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>2130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-14.33</td>
<td>161.84</td>
<td>0.24</td>
<td>0.25</td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
<td>yes</td>
<td>561</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 L</td>
<td>-10.38</td>
<td>125.01</td>
<td>0.06</td>
<td>0.13</td>
<td>0.05</td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>1344</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-20.36</td>
<td>344.85</td>
<td>0.05</td>
<td>0.11</td>
<td></td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>580</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 L</td>
<td>-25.88</td>
<td>165.73</td>
<td>0.75</td>
<td>0.26</td>
<td>0.70</td>
<td>yes</td>
<td></td>
<td></td>
<td>yes</td>
<td>561</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-40.47</td>
<td>255.01</td>
<td>0.66</td>
<td>0.29</td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
<td>yes</td>
<td>580</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 L</td>
<td>-9.03</td>
<td>128.52</td>
<td>0.80</td>
<td>0.29</td>
<td>0.64</td>
<td>yes</td>
<td></td>
<td></td>
<td>yes</td>
<td>1344</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-33.49</td>
<td>164.66</td>
<td>0.48</td>
<td>0.08</td>
<td></td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>1344</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 L</td>
<td>-49.32</td>
<td>82.80</td>
<td>0.07</td>
<td>0.10</td>
<td>0.07</td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>1344</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>-32.48</td>
<td>111.08</td>
<td>0.07</td>
<td>0.51</td>
<td></td>
<td>no</td>
<td></td>
<td></td>
<td>yes</td>
<td>1344</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>-27.62</td>
<td>159.54</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>33%</td>
<td></td>
<td></td>
<td>yes</td>
<td>1344</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S.D.</td>
<td>12.18</td>
<td>75.15</td>
<td>0.31</td>
<td>0.31</td>
<td></td>
<td>33%</td>
<td></td>
<td></td>
<td>yes</td>
<td>1344</td>
</tr>
</tbody>
</table>
References:


2. Robertson JD (1963) The Occurrence of a Subunit Pattern in the Unit Membranes of Club Endings in Mauthner Cell Synapses in Goldfish Brains. Journal of Cell Biology, 19, 201-221


Integral Membrane Proteins. The Journal of Membrane Biology. 217, 21-33


42. Marin-Burgin A, Eisenhart FJ, Kristan WB Jr, French KA (2006) Embryonic Electrical Connections Appear to Pre-Figure a Behavioral Circuit in the Leech CNS. Journal of Comparative Physiology, 192(2), 123-133


52. Graupner M, Brunel N (2007) STDP in a Bistable Synapse Model Based on CaMKII and Associated Signaling Pathways. PLoS Computational Biology, 3(11), e221


