The Origins of Neurons and use of Glutamatergic Signaling in the Ctenophore: *Pleurobrachia bachei*

[1,4]JoshSwore, [1,3]Leonid Moroz, [1,2]Billie Swalla, [1,2]Andrea Kohn

Marin Genomics
Spring 2012

[1]Friday Harbor Laboratories
[2]University of Washington
[3]University of Florida
[4]Northwestern College, St. Paul

Contact Information
Josh Swore
Biology Department
Northwestern College
3003 Snelling
St. Paul, MN 55113

Introduction

Ctnenophores are a group of marine, jelly like, comb bearing metazoans. This phylum consists of an estimated 100-200 species (Mills CE, 2012). Ctenophores are similar to cnidarians in that they have a homologous mesoderm consisting of a jelly like substance called mesoglea. They differ in that they do not have venomous stinging nematocysts but instead have numerous sticky colloblasts lining the tentacles. They also differ in locomotion, cnidarians typically use a contracting pulse of the bell to propel themselves, whereas, ctenophores use 8 rows of ctene plates that paddle the animal through the water. It is believed that they have true striated muscle and a primitive central nervous system (CNS) consisting of the aboral organ and polar fields. The Aboral organ is composed of a balancing statocyst that is attached to all ctene rows by a series of balancers and ciliated furrows. Signaling occurs from the aboral organ to the ctene rows. Each row is capable of beating separately from any of the others, indicating a complex signaling. Extending from this CNS are two nerve nets. One nerve net is located in the ectoderm while the other extends through the mesodermal mesoglea. Genetic data and morphology are both used in comparing basal metazoans and determining evolutionary ancestors.

In this phylum, 2 species genomes have been sequenced, *Mnemiopsis leidyi* and *Pleurobrachia bachei.* Sequencing these species has been crucial in comparing and understanding the evolution of basal metazoans. Significant controversy has arisen in regards to which metazoan is most basal. With the completion of the sponge genome it was proposed that they were the most basal of metazoans (Srivastava et al. Nature. 2010). Through sequencing of *Pleurobrachia bachei* we will present evidence that suggests otherwise. We will propose that ctenophores are actually most basal, yet share high identity with other phyla.

The project has also led to major breakthroughs in understanding the physiology of the animal. Previous studies have shown that ctenophores rely completely on a complex ciliary locomotion for active predation (Mills, CE 2012). *Pleurobrachia bachei* uses 8 rows of fused ciliated plates and 2 tentacles to capture and consume its prey. Ctenophores are the largest animals that rely solely on cilia for locomotion. This is unique to the phylum and must require complex neuronal signaling to accommodate this action. Typically, we would assume that a variety of classical neurotransmitters would be used for these behaviors. Data found through sequencing has indicated that ctenophores have an expanded version of the glutamatergic signaling pathway.

The glutamatergic pathway is used for a wide variety of neuronal responses within the organism. Sequencing has shown that the *Pleurobrachia* has all necessary proteins to carry out this signaling pathway. It is critical for them to have transporting, catalyzing, reception and processing proteins. We take a look at one protein in each of these categories including the synaptic adhesion molecule *Neurexin*. The Glutamatergic signaling pathway aids in the depolarization of neuron to neuron. Neurons are are connected to one another by two special neuronal specific proteins called *Neurexin* and *Neuroglin. Neurexin* is located on the pre synaptic membrane, complementary to the postsynaptic *Neuroglin*. Glutamate is utilized by many different organelles in the cell and needs a way to cross these intracellular membranes. To do so requires the sure of glutamate transporters. Typically we see two types the Vesicular Glutamate Transporters (VGluTs) and the Sialins. Ctenophore transporters have a higher identity to Sialin. These sialins may be associated with allowing the glutamate to pass through the membrane of synaptic vesicles which will fuse with the plasma membrane upon a [Ca^{++}] spike releasing the transmitter into the synapse. This same influx in [Ca^{++}] causes conserved cytoplasmic C2 domains on *synaptotagmin* to bind to the Ca^{++} ions and catalyze the fusion of synaptic vesicles

with the plasma membrane. When the glutamate has entered the synaptic cleft it will bind with a glutamate receptor in the post synaptic membrane. Two types of glutamate receptors are present here, the *metabatropic* (mGluR) and *ionotropic* (iGluR)(Featherstone. *ACS Chemical Neuroscience.* 2009). The iGluR are known to be faster acting and are a focus in this paper due to the distinct expansion of receptors in *Pleurobrachia*. Upon reception the molecule must be processed. *Glutamate Decarboxylase* (GAD) is a degrader of glutamate and a synthesizer of GABA that actually may be used to process the neurotransmitter upon reception. GAD breaks down glutamate into GABA which is then used as an inhibition molecule. Backlog of GABA will suppress further transmission completing the glutamatergic signaling pathway (Schuske et al. *Trends in Neuroscience* 2004). Ctenophores sole use of this signaling pathway has both significant evolutionary and physiological implications. We would like to suggest through this data that Ctenophores had an independent evolution of neurons that solely use the glutamatergic signaling pathway to complement their unique active predation.

Methods

Cloning

Create primers of desired gene using greater than 50% GC nucleotide identity. Place in eppendorf tube 4ul PCR product, 1ul salt solution, 1ul TOPO vector, fill final volume to 6ul with MQ H2O. Add 2 ul of TOPO cloning reaction into vial of one shot bacteria. Incubate on ice 30 minutes. Heat shock for 30 seconds at 42C. place in 250 ul S.O.C. medium and shake at 37C for 1 hour. Spread 10 to 50ul on warmed agar plate and incubate O/N at 37C. add 20ul of SOC for even spreading. Harvest cells and place into testube containing SOC medium and place

Probe Design

Probe generation for *In situ* hybridization Moroz Lab 2011

Day1

1. **Put up culture** in 3 ml of LB broth with appropriate antibiotic for clone of interest and grow O/N
    a. Determine direction of insert relative to vector (TOPO)
        i. Want antisense for probe, sense can be the control
    b. Determine RNA polymerase: T3 (Cat # 11031163001, Roche)or T7 (Cat # 10881767001, Roche) if TOPO, T7 or Sp6 (Cat # 10810274001, Roche) if pGemT
    c. Determine restriction enzyme: Not1 for T3 and PmeI for T7
    d. Determine if restriction enzyme cuts insert

Day 2

2. **Do miniprep of culture**, elute with 50 ul of MQ H_2O (30 ul if low concentration)
    a. Run 1 ul on a 1 % agarose gel to check for concentration
3. **Linearized plasmid DNA** with Restriction enzymes, Not1or PmeI depending on direction

    x ul 1 ug plasmid DNA

    5.0 ul 10 x Buffer #4

    1.0 ul Not-HF (200units) (Cat # R3189, NEB) or PmeI (200units) (Cat # R0560S, NEB) enzyme both use Buffer4

    0.5 ul of 100x BSA

    x ul MQ H_2O

    50 ul total

4. Incubate at 37^0C for 1 Hour
    a. Run a 2.5 ul on a 1% agarose gel to see if lineraized, also run 2.5 ul of uncut plasmid to compare
5. Clean-up to remove enzyme and salts using MinElute PCR Purification Kit (Cat # 28004, Qiagen).
    a. Add 5 volumes (250 ul) of PB, mix well and place in column provided
    b. Centrifuge for 1 minute at 1000g then turn to maximum speed for 1 minute, discard flow-through
    c. Place column back in tube and add 750 ul of PE
    d. Centrifuge maximum speed for 1 minute, discard flow-through
    e. Then centrifuge maximum speed for an additional1 minute, to dry column
    f. Place column in a new 1.5 ml tube and add 10-14 ul of MQ H_20, depending on concentration

g. Incubate for 1-3 minute

h. Centrifuge for 1 minute at 1000g then turn to maximum speed for 1 minute.

i. Tube now contains linearized plasmid and template for transcription

6. Run 1 ul on a 1 % agarose gel to check for concentration (Can stop here)

Day 3

7. ***In vitro* transcription reaction (for Digoxigenin or Fluorescein)**

x ul 1 ug linearized plasmid DNA

2.0 ul 10 x Transcription buffer (In polymerase kit)

2.0 ul 10 x Dig RNA labeling mix (Cat #11277073910, Roche)

Or

2.0 ul 10 x Fluorescein RNA labeling mix (Cat # 11685619910, Roche)

2.0 ul RNA polymerase, 20 Units/ul (T3, T7 or Sp6)

x ul MQ H_2O

20 ul total

8. Incubate at 37^{0}C for 2 hours

a. Check on 1 ul on a 1 % agarose gel for concentration

9. Add 2 ul of DNAase (2Units/ul) 15 min 37^{0}C (TURBO DNA-free™ Kit Cat # AM1907, Ambion)

10. Precipitate probe

30 ul LiCl Solution (7.5 M Lithium Chloride, 50 mM EDTA, pH 8.0, Cat # 9480, Ambion)

70 ul cold 100% Ethanol

11. Leave in -20^{0}C for > 2hours or O/N (longer better)

Day 4

12. Centrifuge maximum speed >13,000g for 15 min at 4^{0}C

a. Mark outside of tube so you know where pellet is located

b. Carefully remove liquid not disturbing pellet

13. Wash with 1 ml of cold 70% Ethanol

14. Centrifuge maximum speed >13,000g for 5 min at 4^{0}C

a. Carefully remove liquid not disturbing pellet and dry

15. Bring up probe in 10-14 ul MQH_2O depending on concentration on gel

a. Run 1 ul on a 1 % agarose gel for concentration and final check of amount to add for *in situ*

16. Check 2 ul on 2.0 Fluorometer with RNA assay kit (Cat # Q32852,Invitrogen life technologies) want to add 200-400 ng

In Situ Hybridization protocol

Pleurobrachia *In situ* hybridization – Modified 2011

Adapted from Derelle and Manuel 2007 and Moroz

Day1

Fix whole specimen in 4% paraformaldehyde in Filtered Sea Water (FSW) overnight (O/N) at 4^0C

Place no more than 10 animals in a 50 ml conical tube. To mix, hold on side and rotate gently.

.

Day2

Rinse 3 x for 10 min in PTW (PBST) at Room Temperature

To mix, hold on side and rotate gently. Dispose of all solutions in hazardous waste.

PTW (PBST) 50ul Tween 20 in 50mL 1 x PBS (1 x PBS is 5 ml of 10x PBS in 40 ml MQ H_2O)

Wash in 1:1 Methanol (MeOH)/PTW (to equilibrate to MeOH) 10 min at Room Temperature

1:1 MeOH/PTW (25 ml Methanol and 25 ml PTW)

Store in 100% MeOH at -20C for 2 hours up to a week

Place on side in freezer to allow animals to be separated

Day 3

Rehydrate specimen for 10 minutes in MeOH/PTW 3:1, 1:1, 1:3, 0:1 at Room Temperature

30mL/20mL/10mL MeOH fill to 40 ml with MQ H_2O

Wash in 1:1 solution of hybridization buffer (HB) and PTW for 15 minutes at Room Temperature

25ml PTW in 25 ml HB buffer

Incubate (prehybridize) in HB buffer for 1 hours at 60^0C

Hybridization buffer (HB) (50% formamide, 5mM EDTA, 5X SSC, 1X Denhardt solution (in 1.5 ml tubes at -20^0C) (0.02% ficoll, 0.02% polyvynilpirrolidon, 0.02% BSA), 0.1% Tween 20, 0.5 mg / ml

*yeast RNA (in–20^{0}C (Invitrogen) = (**for 50 ml**) 25 ml formamide, 0.5 ml 0.5M EDTA, 12.5 ml 20X SSC, 50 ul Tween20, 1 ml 50X Denhardt s-n, 25 mg yeast RNA)*

Incubate (hybridize) in HB with DIG-RNA probe O/N at 60^{0}C

Mix 1 ml of hybridization buffer (HB) with 2-10 ul(200-400 ng Qubit) of probe.Then remove prehybridization buffer from tube with animals and add mixed hybridization buffer. Very gently rock O/N.

Day 4

Wash in HB for 30 min at 60^{0}C

Remove old HB buffer and replace with 1 ml fresh HB in same well

Wash in 1:1 HB/PTW for 30 min at 60^{0}C

Remove old HB buffer and replace with 1 ml 1:1 HB/PTW in same well

Wash in PTW for 30 min at Room Temperature

Remove old 1:1 HB/PTW and replace with 1ml PTW in same well

Block in 10% Goat Serum (GS) for 60 min at Room Temperature

Remove old PTW and replace with 1ml 10% Goat Serum in same well

1mL GS (in 1.5 ml tubes at –20^{0}C) in 10mL PBT

Incubate in anti-DIG 1/2000 at 4^{0}C O/N

Remove 10% Goat Serum replace with 1 ml 1% GS + 1:2000 (0.5 ul / 1 ml 1% GS) of alkaline phosphatase - conjugated DIG-antibodies in same well

0.1mL GS (in 1.5 ml tubes at –20^{0}C) in 10mL PBT

1:2000 (0.5 ul alkaline phosphatase -conjugated DIG-Antibody/ 1 ml 1% GS)

Day 5

Wash 4 x 30 min in PBS Room Temperature

Prepare 24 well plates label wells with marker the names of probes.

Make detection buffer and aliquot 1mL into clean well for each sample. When ready to develop, add 20uL of NBT/BICP mix until dissolved. Should be yellow in color! **NOW** add samples. **Put on ICE and cover with tin foil.**

*Detection buffer (100 mM NaCl, 50mM MgCl2, 0.1% Tween 20, 1mM levamisole, 100mM Tris HCl = (**for 50 ml**) 1 ml of 5 M NaCl, 2.5ml of 1 M MgCl(10g/50mlH_2O), 0.012g levamisole, 50ul Tween 20, 5ml of 1M Tris-HCl pH=8; adjust PH to 9.5 with 10M NaOH, filter the resulting solution)*

Do not keep detection buffer longer than 2 weeks or if it become cloudy.

Watch for appropriate color development Stop in 4% paraformaldehyde in MeOH

Stop in PBS leave days with several changes at 4^0C

Can skip and get less crystals Have wells with PBS, transfer animals to fresh well with PBS to stop

Wash in 4% paraformaldehyde in MeOH 30 min Room Temperature

Timing of this step depends on the strength of the signal and the background. This time is for high signal with low background

Change solution couple times

5.4mL Formaldehyde in 44.6 ml Methanol

Wash 3x 10 min in Ethanol (EtOH) Room Temperature

Store in 100% EtOH

Mount

Add animals to Methylsalicylate, until they sink

Put animal onto microscope glass, clean, absorb methylsalicylate leftovers, add a drop of Permount, put on the cover slip

Fluorescent Label

Day 5

Wash 5 x 15 min washes with PBS Room Temperature

Prepare 24 well plates label wells with marker the names of probes.

Make detection buffer and aliquot 1mL into clean well for each sample Room Temperature

Developing solution *(*100mM Tris (PH 8.2-8.5), 0.2% Tween-20= *(**for 50 ml**) 100ul Tween 20, 5ml of 1M Tris-HCl pH=8; adjust PH to 8.2-8.5 with 10M NaOH, filter the resulting solution)*

Detection buffer *-Remove buffer and prepare developing solution using Vector Red Alkaline Phosphatase kit I(To 5 mls of developing solution add 2 drops of reagent 1, mix, 2 drops of reagent 2, mix and 2 drops of reagent 3 mix).*

Watch for appropriate color development. The vector red product is visible by eye (it can be used for non-fluorescent in situs) and in the Rhodamine, Cy3 channel The expression pattern will be brighter on the confocal than what you can see by eye.

Stop the reaction by washing 3x 5 min washes in PBS

I**nactivate** the Peroxidase by incubating in stop solution (10mM HCl, 0.2% Tween-20) for 10 min

Inactivation buffer (10mM HCl, 0.2% Tween-20) = ***(for 50 ml)*** *100ul Tween 2, 100 um of 5N HCl fill to 50 ml MQ H2O*

Wash 6x 20 min washes in PBS (~ 2 hours of washes)

Stop and mount in glycerol:PBS antifade

Double/Fluorescent Label

Follow protocol except what is mentioned below

Day2

For double label in situ prepare a second less bright probe with Fluorescein RNA labeling mix

Day3

Incubate (hybridize) in HB with DIG-RNA probe and **Fluorescein RNA probe** O/N at 60^0C

Day 4

Nothing changed

Day 5

Wash 5 x 15 min washes with PBS Room Temperature

Prepare 24 well plates label wells with marker the names of probes.

Make detection buffer and aliquot 1mL into clean well for each sample Room Temperature

Developing solution *(*100mM Tris (PH 8.2-8.5), 0.2% Tween-20= ***(for 50 ml)*** *100ul Tween 20, 5ml of 1M Tris-HCl pH=8; adjust PH to 8.2-8.5 with 10M NaOH, filter the resulting solution)*

Detection buffer *-Remove buffer and prepare developing solution using Vector Red Alkaline Phosphatase kit I(To 5 mls of developing solution add 2 drops of reagent 1, mix, 2 drops of reagent 2, mix and 2 drops of reagent 3 mix).*

Watch for appropriate color development. The vector red product is visible by eye (it can be used for non-fluorescent in situs) and in the Rhodamine, Cy3 channel The expression pattern will be brighter on the confocal than what you can see by eye.

Stop the reaction by washing 3x 5 min washes in PBT

I**nactivate** the Peroxidase by incubating in stop solution (10mM HCl, 0.2% Tween-20) for 10 min

Inactivation buffer (10mM HCl, 0.2% Tween-20) = ***(for 50 ml)*** *100ul Tween 20, 100 ul of 5N HCl fill to 50 ml MQ H2O*

Wash 6x 20 min washes in PBS (~ 2 hours of washes)

Incubate in Anti-Fluorescein-POD 1/2000 at 4^0C O/N

Remove 10% Goat Serum replace with 1 ml 1% GS + 1:2000 (0.5 ul / 1 ml 1% GS) of alkaline phosphatase - conjugated DIG-antibodies in same well

0.1mL GS (in 1.5 ml tubes at –20^{0}C) in 10mL PBT

1:2000 (0.5 Anti-Fluorescein-POD / 1 ml 1% GS)

Day 6

Wash the POD antibody by extensive washing all day ~12x 30 min washes in PBS

Make Detection buffer

In 250µl amplification buffer, supplied in the Tyramide plus kit, dilute the Tyramide-Green 1:50

Develop incubate with diluted Tyr-Green for 3-5 min, 1 hour and 2 hour

Wash 4x 15 min washes PBT

Wash overnight in PBS at 4^{0}C

Day 7

Wash 8x 15 min. Basically wash for 2-3 hours in the morning (* Extensive washing on days 4 and 5 is essential to reduce background from the Tyramide kit)

Mount in Vecta Shield and image.

Phylogenetic Analysis

The Phylogenetic anaysis was performed on the Phylogeny.fr platform. Sequences aligned with MUSCLE (v3.7) configured for highest accuracy. Ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b) Reconstruct tree using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT).Selected WAG substitution model assuming an estimated proportion of invariant sites (of 0.060) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. Estimated gamma shape parameter directly from the data (gamma=1.929). Reliability for internal branch was assessed using the

aLRT test (SH-Like).Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3).

Results/Discussion

Glutamate Receptors in *Pleurobrachia bachei*

Ctenophores have coded both metabotropic and Ionotropic GluR's (iGluR) within their genome. Due to the complex locomotion of the animal I focused on the faster acting iGluR's. It was found in the genome that they have coded 14 different variants. Of these iGluRs there are the NMDA, AMPA and KAinate subfamilies. *Pleurobrachia* shows that its iGluR are unique from any of these subfamilies in that they share highest identity to one another. To compensate for this they have a distinguished expansion of their own GluR's. It is typical in all species to find multiple genes for GluRs. For example, Human and Mouse both have 15 *iGluRs*. When we compare *Pleurobrachia* to other basal metazoans however, we see that this expansion is unusual. *Trichoplax* has 11 *iGluRs*, *Nematostella* has 4 *iGluRs* and sponge has 0 *iGluRs*. This suggests an independent evolution of neurons. Further phylogenetic analysis shows that that these genes cluster together and are significantly divergent from other basal metazoan *GluRs*. Figure 3 shows us that *Pleurobrachia's GluR's* share highest identity with one another but have closer identity to plants than other organisms. This is probably due to a long branch attraction rather than close relationship. Exon arrangement of these genes is as dramatic as the expansion of genes themselves. Exons in the genes can range from 2, the smallest known exon organization, to 20, more exons than human providing further evidence that they have had an independent evolution separate from other metazoans. This unusual, dramatic expansion of receptors with little identity to other metazoans indicates that the neurons of *Pleurobrachia*

have undergone an independent evolution early on while conserving many ancient characteristics.

When we look at this from a physiological standpoint, we assume that these receptors are being expressed and localized in different areas of the organism and being used to signal different functions. *In Situ* hybridizations and semi quantitative PCR allowed us to view these expression patterns. The results were as expected. We found that each receptor had individualized localization. The polar fields for example expressed numerous GluR's, however they were expressed in different regions (Figure 4). This would indicate that as our brains have various regional functions so too does *Pleurobrachia.* The semi quantitative PCR showed us vividly different transcription patterns in the mouth and aboral organ. As seen in figure 5 the mouth shows high concentration of GluR 3, whereas, the aboral organ has a higher concentration of GluR 7. Each of the receptors has its own exclusive transcription pattern indicating individual functionality of the iGluR's in *Pleurobrachia.*

Sialin

Glutamate is used by multiple organelles in the cell and, therefore, needs a way to cross intracellular membranes. Transporters are the membrane bound proteins that allow for this transmission. In the molecular world of biology two different types of transporters exist, the Vesicular Glutamate Transporters and the Sialins. These molecules differ slightly in location, function, and domain organization. The *Pleurobrachia* genome has 8 different glutamate transporter genes. When gene alignments with various types of transporters were performed we found that they had highest identity to *sialin* transporter genes. We then compared them to other basal metazoans and found that they had an expansion of these genes (Figure 1) within *Pleurobrachia*. This expansion led to the question: Is there a correlation of variable expression

between the receptors and the transporters. Comparative semiquatitative PCR led to our conclusion that there was no correlation (Figure 15). Three different transporters are expressed evenly though out the body plan of *Pleurobrachia.* In situ hybridazations showed that there was similar expression to iGluR's in the ctenes of the comb rows (Figure 2). We have not seen any expression in the polar fields but further experiments with other *sialin* probes have potential to reveal otherwise. The phylogenetic analysis then revealed that *Sialin* grouped by themselves in two distinct clades. They were also most basal to other phyla (Figure 1). These distinct clades represent two separate evolutionary expansions. This expansion combined with evidence of expanded iGluR's and differential expression of iGluR's suggests the hypothesis that an independent evolution of neurons occurred in *Pleurobrachia*. In regards to the phylogenetic gene tree we do conclude that it is possible that there is a second possible rooting. If this were the case there would be only a single expansion of these transporters. None the less Pleurobrachia would still be most basal among other organisms.

Synaptotagmin in *Pluerobrachia bachei*

Pleurobrachia has predicted 1 *Synaptotagmin* (SYT) gene. This membrane trafficking protein is located in the presynaptic membrane of neurons. It is conserved by its two repeated C2 domains (Figure 8). Most of these molecules are associated with the regulated release of neurotransmitters. Through alignment and phylogenetic analysis, the SYT gene in *Pleurobrachia* has shown highest identity to the SYT1 gene in other organisms. This gene is required specifically for the fast acting release of neurotransmitters across the synapse. SYT is a perfect complement to the fast acting iGluR expressed though out the organism.

SYT possesses two C2 domains, C2a and C2b, and an N terminus trans membrane domain (figure 8). It is typically seen that both C2 domains will bind with multiple Ca^{++} ions in

order to function, however this is not always the case. On occasion the C2b Domain will bind with a PIP3 or PIP2 in absence of Ca^{++}. As the [Ca^{++}] concentration increases within the cell, the SYT molecule will bind to Ca^{++} and induce exocytosis of the neurotransmitter by catalyzing fusion of the synaptic vesicle with the plasma membrane (Craxton *BMC Genomics* 2010). Without the presence of SYT, fusion of these neurotransmitter vesicles is slower and over all signaling occurs takes a longer period of time. Presence of both the catalyzing SYT and fast acting iGluRs are crucial the complex ciliary motion of the organism. Without both present, ctenophores would be critically hindered in signaling and reaction.

There are many more synaptotagmin genes ranging from *SYT1* to *SYT54* with variants seen in almost all genes. More than one of these genes is present in most organisms. Humans, for example have 17 different *SYT* genes and only 8 of which are calcium activated. Other basal metazoans such as *Trichoplax* and *Nematostella* possess two *SYTs* that are calcium activated. Similar to *Pleurobrachia* in lineage, *Amphimedon* retains one *SYT* gene (figure 7). In figure 10 you can see that each of these *SYT* genes in *Pleurobrachia* and *Amphimedon* cluster together. This is exactly what we would expect of two basal metazoan genes. We can therefor assume *SYT* is a conserved gene further indicating the basal lineage of ctenophores.

Neurexin

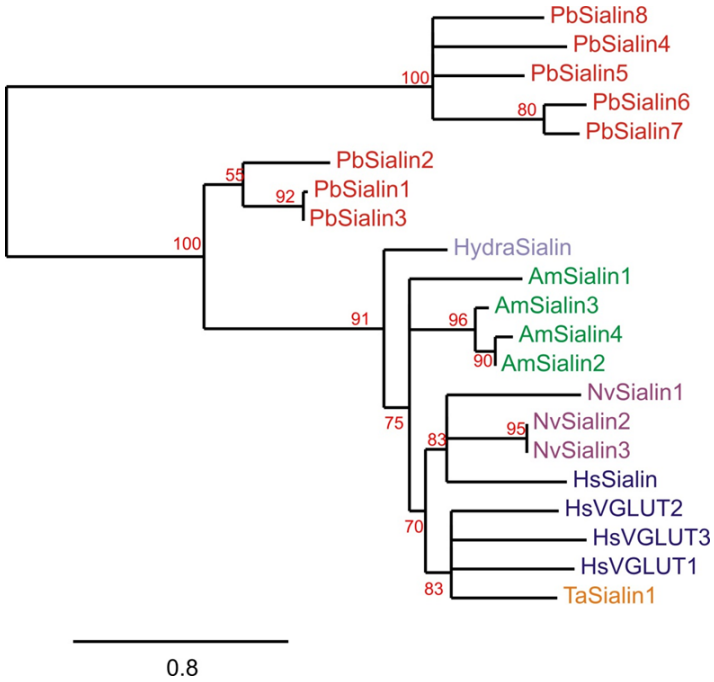
Neurexin is necessary in the glutamatergic signaling pathway in the sense that it keeps neurons attached to one another. This synaptic junction molecule brings two neurons close enough to allow a transfer of neurotransmitters from one neuron to the next. Until now it has always been seen with its complementary post synaptic partner *Neuroligin.* Sequencing has shown that *Neurexin* is alone in the genome of *Pleurobrachia.* The gene itself is conserved by four LamG domains, two EGF domains and a single transmembrane domain (figure 12). This

gene is actually much shorter when we compare it to other organisms. *Pleurobrachia* seems to be missing two LamG domain and one EGF. For instance Aplysia has six LamG domains and three EGFs. This seems to be the standard among other animals as well (figure 11). Further analysis also shows that *PbNeurexin* stands alone in the tree (Figure 11). It is again the outgroup but is well supported by bootstrap values. The deletion of multiple domains when paralleled to other organisms could be an indication of a loss, however we believe a gene duplication may have occurred after *Pleurobrachia* had diverged from the ancestor. The gene is still highly conserved due to the multiple repeated domains. How this shorter *neurexin* correlates to the absence of *neuroligin* is unclear. We do believe that this shorter *neurexin* has potential to help explain this absence though.

Glutamic Acid Decarboxylase

Glutamate Decarboxylase is a molecule typically used to synthesize gamma-aminobutyric acid (GABA) in the Cnidarian *Nematostella.* (http://www.sciencedirect.com/science/article/pii/S1744117X09000628). GABA is then used as an inhibitory neurotransmitter by suppressing pre and post synaptic transmission (http://www.ncbi.nlm.nih.gov/pubmed/9777643). To act as a signaling molecule the *Pleurobrachia* genome would be required to have GABA receptor genes. This, however, is not the case. Upon degradation, the glutamatergic pathway is complete. This would appear to suggest that GABA is being used in another way. It is possible that it could be as simple as regulating the glutamate concentration and nothing further. To determine why *pleurobrachia* shows GAD but no GABA receptors we performed a phylogenetic analysis, however we were unable to obtain a well-supported tree. The only thing we were left to do were perform in situ hybridizations to confirm its expression in the animal. We were successful in this and found

expression in multiple organs. GAD is expressed heavily in the mouth, aboral organ, polar fields and what appear to be specific elongated cells (figures 13 & 14). It is hypothesized that these elongated cells are neurons, but further analysis is needed to confirm this. To do this we may combine immunohistochemistry with *in situ*'s hybridizations to show staining of neuron with acetylated tubulin and expression of GAD. More genomic data is needed to complete a phylogenetic analysis of this gene. We can hypothesize however that this gene will not stray from common data retrieved from other genes in that it is most basal among other organisms.



Species	Sialins
Homo sapiens	1
Nematostella vectensis	3
Trichoplax adhaerens	1
Amphimedon queenslandica	4
Pleurobrachia bachei	**8**

Figure 1.
Pleurobraichas 8 sialins represent a dramatic expansion among other phyla. Phylogentics reveal two distinct clades among other phyla representing a dual expansion of transporters. A different rooting is possible on the node separating Pleuorobrachia from other phyla. If this is the case Pleurobrachia remains most basal but only a single expansion occurs.

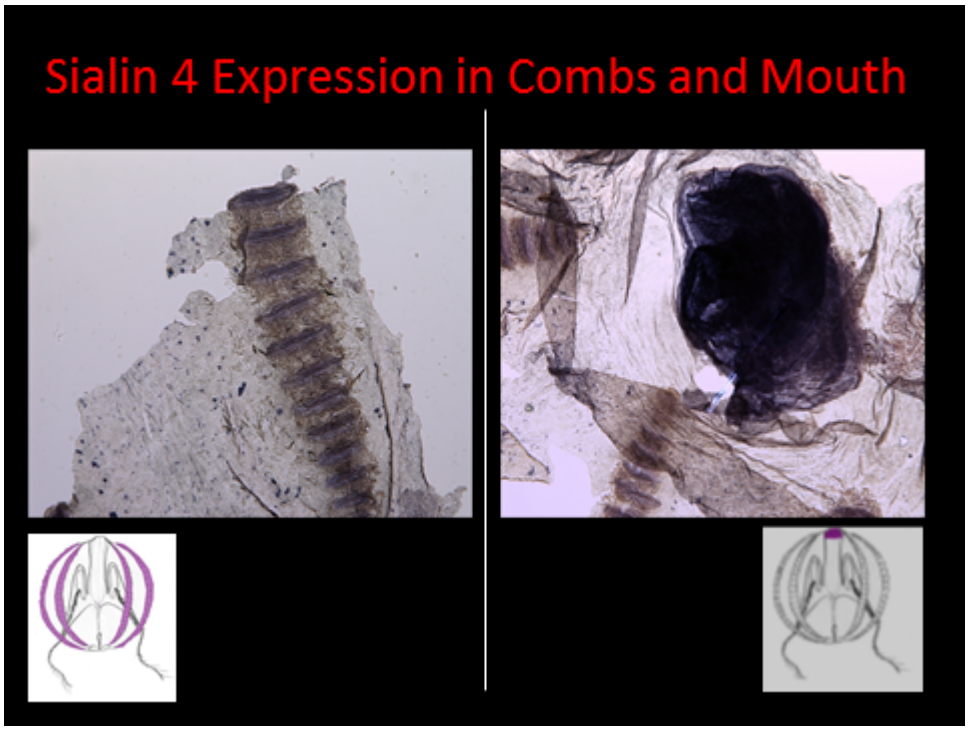


Figure 2.

Expression of *Sialin* 4 in ctenes of comb row. There is also heavy expression in the mouth.

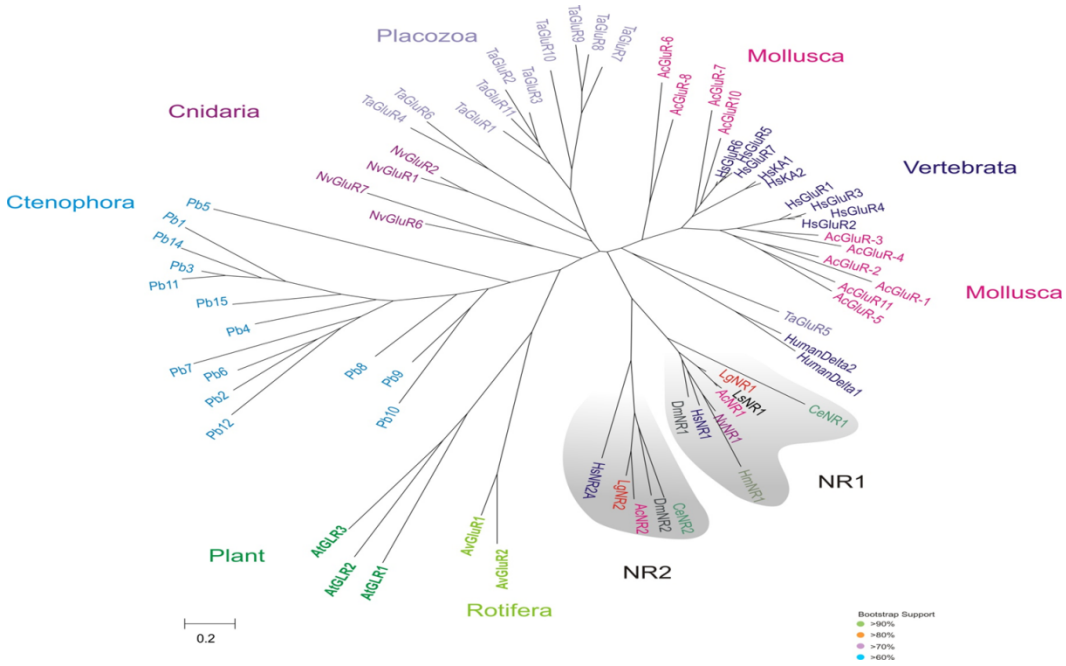


Figure 3.

Pleurobrachia represents a distinct clade among other phyla. The have highest identity to one another. Apparent closest relation is plant, however, this is probably due to long branch attraction and not due to relation.

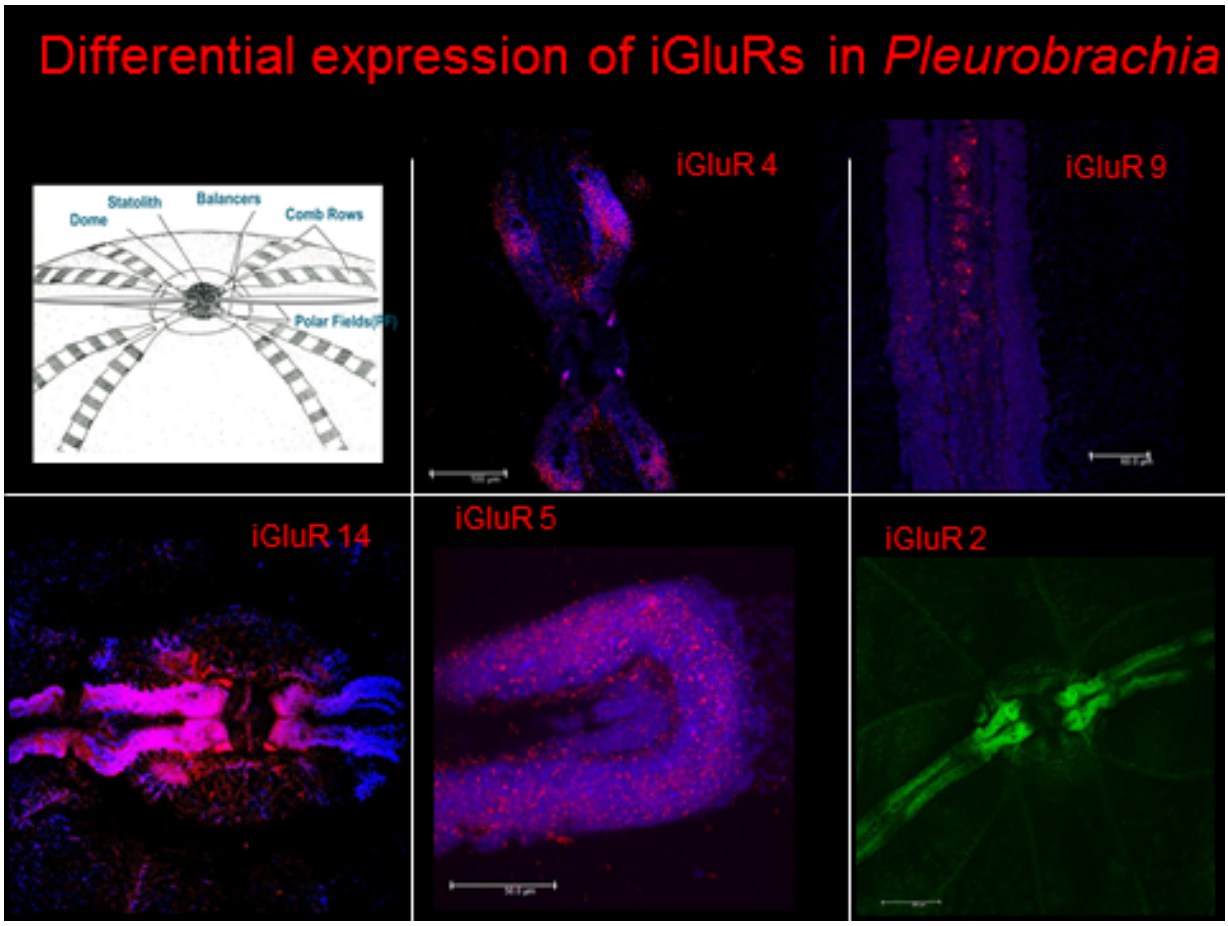


Figure 4.

The Glutamate receptors have differential expression in Pleurobrachia. iGluR 4 expresses in the balancers and polar fields. iGluR9 expresses down the center of the polar fields. iGluR expresses in the medial polar fields, balancers and statolith dome. iGluR 5 expresses laterally in the polar fields. Finally iGluR 2 expresses throughout the entire animal: polar fields, ciliated furrows, balancers; photo of comb rows and mouth expression of iGluR2 not shown.

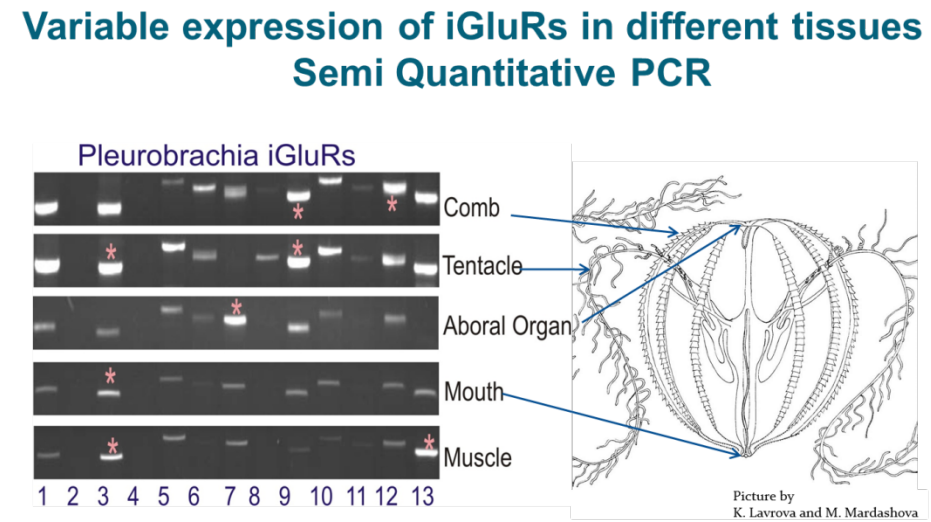


Figure 5. Semi quantitative PCR

Variable expression of iGluR's is seen throughout the animal. A) Combs express iGluRs 9 and 12 in highest concentrations. B) Tentacle expression of iGluRs 3 and 9 are highest. C) Aboral expression of iGluR 7 is highest. D) Oral expression of iGluR 3 is highest. E) Muscle expression of iGluR 3 is highest.

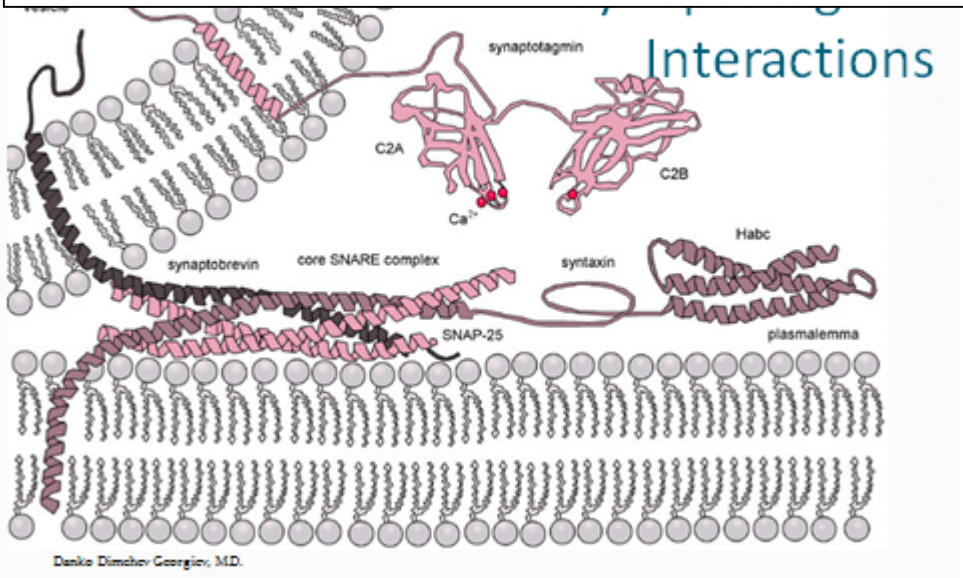
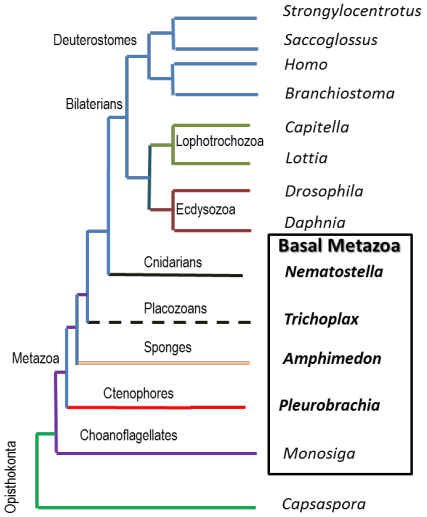


Figure 6.

Synaptotagmin's two C2 domains are activated by Ca^{++} causing an interaction with the snare complex. This interaction allows *synaptotagmin* to guide the synaptic vesicle to the plasma membrane. The synaptic vesicle will then fuse with the plasma membrane releasing the neurotransmitter.

Synaptotagmin in the Animal Kingdom



Synaptotagmin		
Species	Calcium Active	Total
Strongylocentrotus	4	15
Saccoglossus	3	7
Homosapiens	8	17
Brachiostoma	3	14
Capitella	3	9
Lottia	3	12
Drosophila	2	6
Daphnia	2	9
Nematostella	2	23
Trichoplax	2	5
Amphimedon	1	1
Pleurobrachia	1	1
Monosiga	0	0
Capsaspora	0	0

Figure 7. Synaptotagmin in the Animal Kindom

Only one *Pleurobrachia SYT* gene is conserved. As the animal becomes more derived more genes appear. More basal animals have fewer genes and in turn is more conserved to the ancestor.

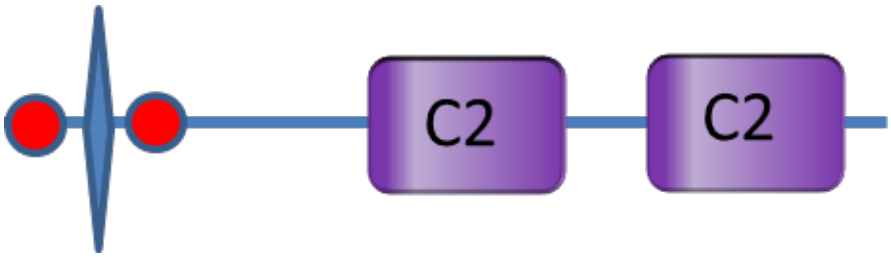


Figure8. Genomic Organization of SYT

Synaptotagmin in Pleurobrachia has two Ca^{++} binding C2 domains and a single transmembrane domain.

Pleurobrachia bachei Synaptotagmin

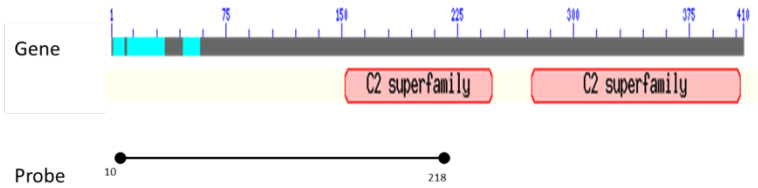


Figure 9. *In Situ* probe on the *Synaptotagmin* Gene.

SYT is a 410 amino acid protein with two conserved C2 domain. Our in situ probe covered a region starting at aa10 to aa218. This crossed over the first

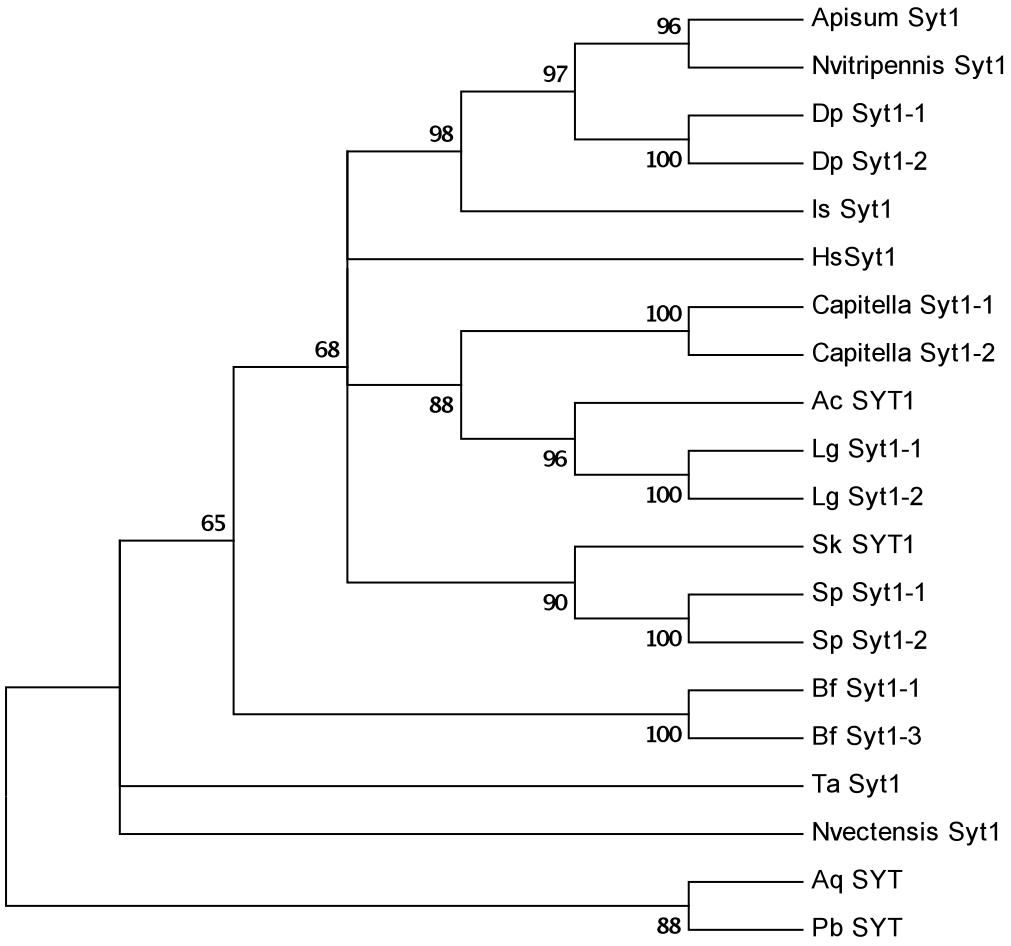


Figure 10. Phylogentic Analysis of SYT

Every species with multiple variants of SYT cluster together this indicates a slight change in the gene after a divergence of ancestor. Well supported bootstrap out grouping of *Pleurobrachia* and *Amphimedon* shows that these are the most basal metazoans. With only one SYT gene in each we can assume there is high conservation to the ancestor.

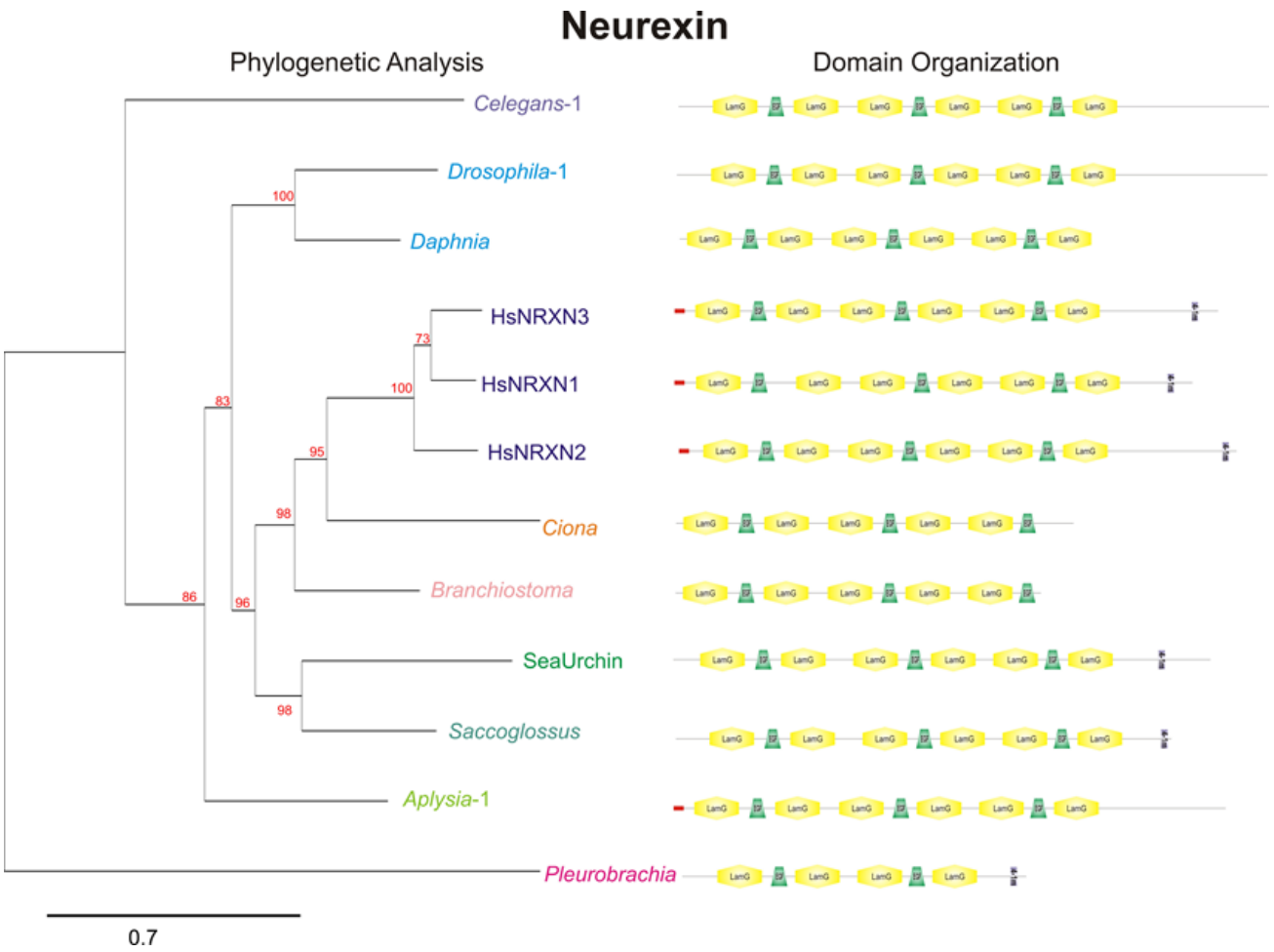


Figure 11. Phylogenetic Analysis and Genomic Comparison of Neurexin

Well supported bootstraps show that *Pleurobrachia neurexin* is most basal. Genomic comparison reveals that it is missing two Lamg domains and an EGF domain and is the shortest *neurexin* gene known It is still the out group and most basal organism indicating basal lineage.

Figure 12. Genomic Organization of *Neurexin* in *Pleurobrachia*

Pleurobrachia contains four LamG domains, Two EGF domains and one trans membrane domain. This is the shortest known *neuroligin* gene.

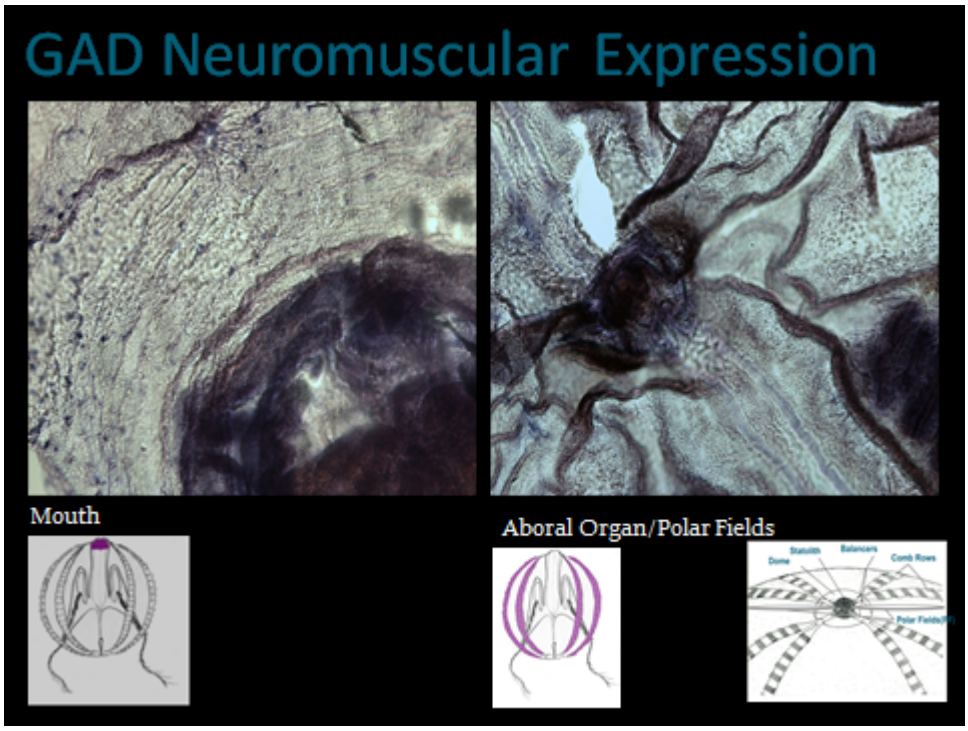


Figure 13.

Expression of GAD in the mouth, Aboral Organ, Comb Rows and Polar Fields. This confirms GAD is in the genome and being expressed in the animal.

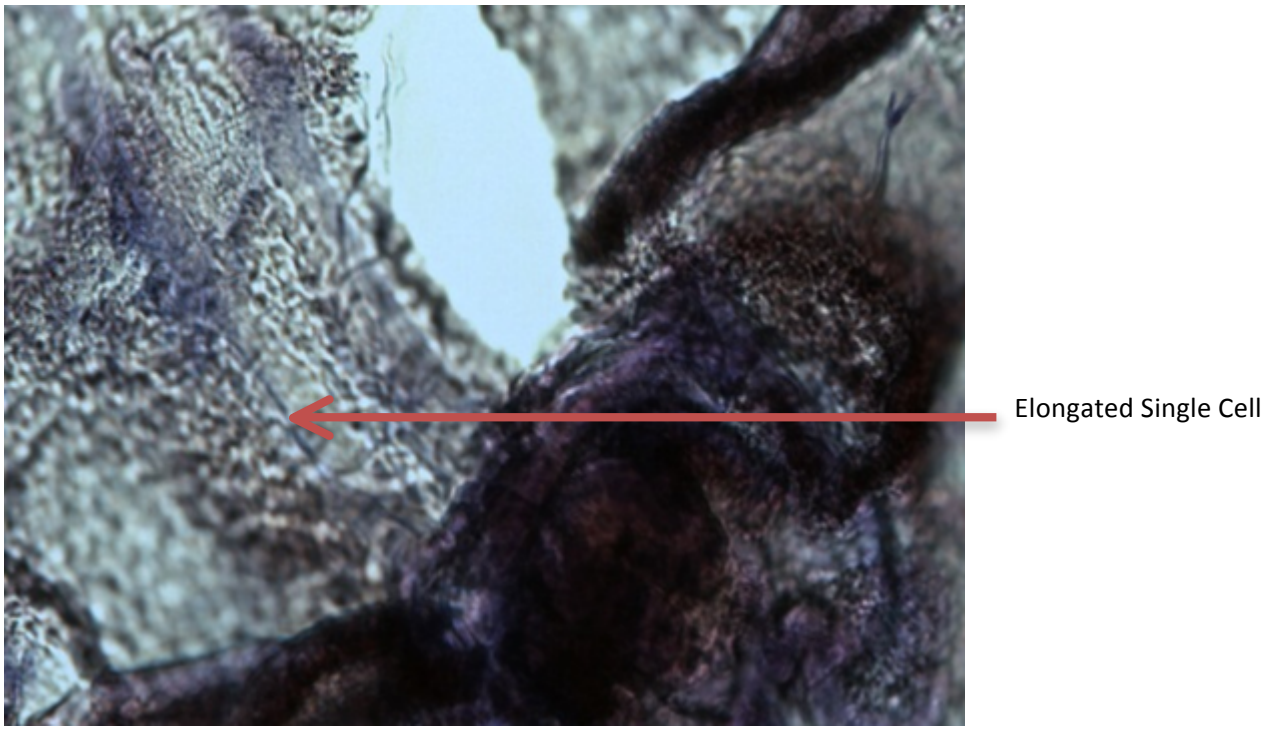


Figure 14.

Magnified expression of the Aboral Organ and Polar Fields. We can also see expression in the polar fields of an elongated single cell.

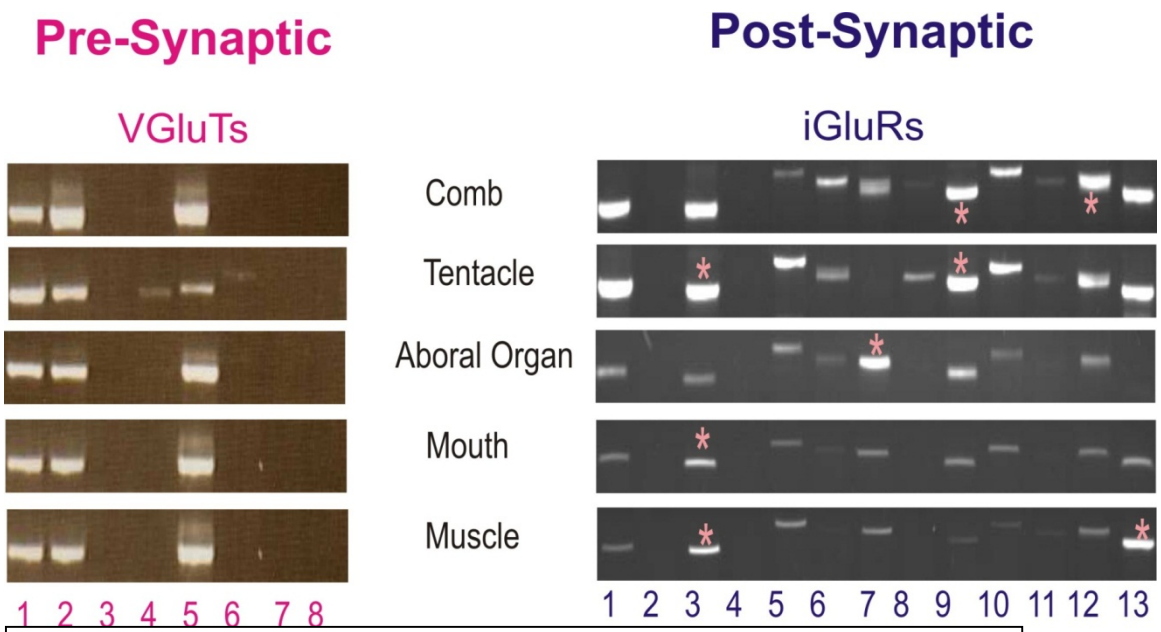


Figure 15.

Siallins 1,2,4 expressed equally throughout body of *Pleurobrachia.* Variable expression of *iGluR's.* this indicates no correlation of expression between *Sialins* and *IGluRs*

Acknowledgements

I first want to thank all of the professors here, Dr. Leonid Moroz, Dr. Billie Swalla, and Dr. Andrea Kohn for their sleepless nights and willingness to help me complete my project. Andrea especially for making sure I didn't ever quit on myself. Kevin Kocot for teaching my how to do bioinformatics and phylogenetics and realizing this is not the path in biology I should pursue. Yelena Bobkova at The Whitney Labs in Florida made all of the probes and stayed late many times so that we could have our materials the next day. Thank you to the other students for their help with everything and making this apprenticeship much more enjoyable. The TA's were helpful in preparing solutions and making sure we had what we needed for our experiments. I also want to thank my advisor at Northwestern College for encouraging me to do this apprenticeship and helping me find the funding to do it. My professors at Northwestern were on board as well and allowed me to finish classes thousands of miles away so I could par take in this wonderful opportunity. Finally, I want to thank Friday Harbor Laboratories for providing the facilities to do the research needed to complete this project.

References

1. Anctil, M. Chemical transmission in the sea anemone Nematostella vectensis: A genomic perspective.*Comp Biochem Physiol Part D Genomics Proteomics* **4**, 268-289 (2009).

2. Benquet, P., Gee, C.E. & Gerber, U. Two distinct signaling pathways upregulate NMDA receptor responses via two distinct metabotropic glutamate receptor subtypes. *J Neurosci* **22**, 9679-9686 (2002).

3. Craxton, M. A manual collection of Syt, Esyt, Rph3a, Rph3al, Doc2, and Dblc2 genes from 46 metazoan genomes--an open access resource for neuroscience and evolutionary biology. *BMC Genomics* **11**, 37 (2010).

4. Featherstone, D.E. Intercellular Glutamate Signaling in the Nervous Systemand Beyond. *ACS Chemical Neuroscience*, 9 (2010).

5. Dean, C.*, et al.* Neurexin mediates the assembly of presynaptic terminals. *Nat Neurosci* **6**, 708-716 (2003).

6. Schuske, K., Beg, A.A. & Jorgensen, E.M. The GABA nervous system in C. elegans. *Trends Neurosci***27**, 407-414 (2004).

7. Sreedharan, S*., et al.* Glutamate, aspartate and nucleotide transporters in the SLC17 family form four main phylogenetic clusters: evolution and tissue expression. *BMC Genomics* **11**, 17 (2010).

8. Srivastava, M*., et al.* The Trichoplax genome and the nature of placozoans. *Nature* **454**, 955-960 (2008).

9. Srivastava, M*., et al.* The Amphimedon queenslandica genome and the evolution of animal complexity.*Nature* **466**, 720-726 (2010).

10. Ziskind-Conhaim, L. Physiological functions of GABA-induced depolarizations in the developing rat spinal cord. *Perspect Dev Neurobiol* **5**, 279-287 (1998).