Functional Analysis Of HLH-3, A bHLH Achaete/Scute Protein, In HSN Maturation

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THESIS

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Manuscript in Chapter VII (Appendix A)

Section 7.3, Figure 7.3

Ryan Doonan gathered or generated most of the strains used in the study, conceived and conducted the experiments reported in figures 1, 3, and 4, and obtained the data in Table 1. He also wrote the manuscript. Julia Hatzold and Barbara Conradt isolated the bc248 and bc277 deletion alleles and performed the RT PCR analysis reported in figure 2. Most of the work was conducted in Aixa Alfonso's laboratory. She provided the physical resources for the work and along with the other authors provided feedback on the manuscript. I generated the strain and obtained the data and images reported in figure 5. This portion of the study comprised the analysis of *punc-40::gfp* expression in the region of the vulva and the cells that innervate the vulval muscles, the HSNs and VCs

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List of Abbreviations

5HT	Serotonin
ACh	Acetylcholine
bHLH	basic helix loop helix
βME	beta-mercaptoethanol
BSA	bovine serum albumin
bp	base pairs
cDNA	complementary DNA
DIC	differential interference contrast
EST	expressed sequence tag
GFP	green fluorescent protein
HSN	hermaphrodite specific motorneuron
kb	kilobases
N2	*C. elegans* Bristol strain
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
Unc	Uncoordinated

Summary

hlh-3 belongs to the Achaete Scute family of proneural genes and encodes a protein with 59% identity to the Drosophila proneural protein Asense. *hlh-3 (tm1688)* mutant hermaphrodite animals are egg-laying defective and the serotonergic HSNs are not detected by 5HT staining. My goal with this work is to understand the fate of the undetectable HSNs and the regulatory mechanism for *hlh-3* expression. I show that: (1) the undetectable HSNs do not undergo inappropriate programmed cell death or change their fate to the sister cells. (2) The HSNs are less differentiated in the absence of *hlh-3* function and fail to reach the final maturation stage. (3) Many HSNs that do mature have axon pathfinding defects.

My analysis of expression of transcriptional and translational reporter gene fusions and fosmid constructs show *hlh-3* expression limited to the embryos with weak or no detectable expression in the HSN post hatching. This is remarkable as HSN differentiation defects are not seen in the earlier stages of development. The fosmid is able to rescue the serotonergic as well as the pathfinding defect in the HSNs in *hlh-3 (tm1688)* animals. Lineage analysis also shows the expression of *hlh-3* in the HSN lineage up until the mother of the HSNs.

hlh-3 (bc277) animals are not egg-laying defective but they have mature HSNs with pathfinding defects. These HSNs have ectopic branches that make synapses with the vulva muscles. The *hlh-3 (bc277)* deletion includes a putative exon with no known function to date. Reporter gene fusion has shown that the putative exon can be transcribed suggesting the existence of two potential protein isoforms having different functions. I propose the shorter abundant isoform, present in WT and *hlh-3 (bc277)*, is necessary for maturation (complete differentiation) and the longer less abundant isoform present only in WT is necessary for normal pathfinding.

Chapter I: Introduction

Chapter 1

INTRODUCTION

Neurogenesis is the process of generation of neurons from the neuronal stem cells. Neurons are generated from pluripotent embryonic stem cells (ESC) that have the capacity to give rise to all types of cells in the nervous system including glia and oligodendrocytes (Johnson & Glasgow, 2009). ESCs generate this wide array of neuronal populations (neurons, glia and oligodendrocytes) important for a functional nervous system from a pool of neuronal precursor cells (NPCs) that develop from ESCs (Temple, 2001). Neuronal differentiation requires exit from the cell cycle and involves the expression of genes that impart a neuronal fate in a spatially and temporally regulated manner (Hardwick, Ali, Azzarelli, & Philpott, 2015). Epigenetic repression of developmentally relevant genes leads to fate restriction by DNA methylation and chromatin modification (Hirabayashi & Gotoh, 2010). Studies have shown that the Polycomb and Trithorax group of proteins play an important role in maintaining the pluripotency of the ESCs by trimethylating nucleosomes of developmentally relevant genes such that they are maintained in a repressed or activated state (bivalent state) (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006; Lee et al., 2006). During progression through the neural lineage, histone modifications lead to resolution of the bivalent stage by activating the genes necessary for differentiation (Mikkelsen et al., 2007). In *C. elegans,* neuron generation is not clonally driven but lineage based (Hobert, 2005). About 84% neurons are derived from the AB blastomere which is generated from the cleavage of the zygote (Sulston et.al, 1983). In *C. elegans* epidermal-like cells further divide into a neuronal and hypodermal precursor cell. Sister cells (like HSNL/R) do not share the same mother cell in their final differentiation journey (discussed further in Chapter 5) (Frank et.al, 2003). This differentiation from undifferentiated stem cell to a progenitor cell is

controlled by a group of genes called proneural genes that sets up the stage for the final differentiation into neurons and glia.

1.1 Proneural genes in neurogenesis:

Terminal differentiation of NPCs to neurons is set up by the activation of the proneural bHLH (basic helix loop helix) transcription factors (Yoo & Crabtree, 2009). Proneural proteins control multiple aspects of differentiation and also work with homeodomain proteins to specify the subtypes of neuronal cells (Bertrand et.al, 2002; François Guillemot, 2007). A final step in neuronal differentiation is the expression of neuron-type specific characteristics that are guided by neuron-type specific genes, these genes may not necessarily be unique to every neuron but are expressed in a combination that is specific to an individual neuron (Hobert et.al, 2010). Neuron-type specific characteristics include the neurotransmitter phenotype, ion channels, cytoskeletal proteins, cell surface adhesion molecules etc. that are controlled by terminal selector genes (e.g. UNC-86 and MEC-3 in *C. elegans* touch receptor neurons) (Flames & Hobert, 2009, 2011; Xue, Tu, & Chalfie, 1993). Thus, the highly specialized and complex nervous system with its intricate connections develops as a result of the initial activity of the proneural genes that play an important role in the process of fate acquisition of the developing progenitor cells.

1.2 Proneural genes in Drosophila:

Proneural genes of the Ac/Sc type; Achaete, Scute, Lethal of scute and Asense were first discovered in Drosophila because of their ability to confer neural fate to ectodermal cells (Ghysen & Dambly-Chaudière, 1988; Skeath & Doe, 1996). Additional proneural genes belonging to the atonal family (ato, cato, amos and tap) were discovered later (Gautier et.al, 1997; Goulding, White, & Jarman, 2000; Goulding, zur Lage, & Jarman, 2000; Jarman, Grau,

Jan, & Jan, 1993). Loss of function (*lof*) mutations in Achaete, Scute, Lethal of scute and Asense, resulted in loss of sensory bristles (García-Bellido & de Celis, 2009; Rodríguez et.al, 1990). Thus, proneural genes are necessary for the generation of neuronal populations. They are also sufficient for the generation of neuronal populations because ectopic expression of proneural genes leads to the development of ectodermal cells into neurons (Bertrand et al., 2002; Kiefer et.al, 2005; Skeath & Doe, 1996). Proneural genes are expressed in an equivalence cluster of ectodermal cells and through Notch signaling silence the surrounding cells and specify their fate as epidermal cells thus letting only one (or a few) to develop as neuronal cells (Gibert & Simpson, 2003; Ware et.al, 2014). In addition to driving the generation of neuronal lineages, Ac/Sc genes also serve as neuronal subtype differentiation factors. They have been shown to reprogram neuronal lineages based on gain of function (*gof*) experiments (Bertrand et al., 2002; Diogo S. Castro et al., 2006). Depending on their time of expression they can specify the fate of a differentiating neuron. Thus proneural Ac/Sc genes can play a dual role depending on the context that they are expressed in. Early expression within a specific lineage determines the generation of that lineage and later expression leads to fate determination of a particular neuronal subtype (Diogo S. Castro et al., 2006; Powell & Jarman, 2008). The proneural proteins belong to class II bHLH proteins that hetero-dimerize with class I bHLH proteins to activate the transcription of downstream genes. The dimerization is mediated by the helix-loop-helix (HLH) domain whereas the basic region mediates DNA binding (Bertrand et al., 2002; Ma et.al, 1994). The class I bHLH protein in Drosophila is encoded by the gene *daughterless (da)* (Cabrera & Alonso, 1991). The basic region is important for subtype specification as swapping the basic region of *Achaete* for that of *atonal* leads to the development of chordotonal organs instead of sensory organs (Chien et.al, 1996).

1.3 Proneural genes in vertebrates:

In vertebrates there are only two Ac/Sc homologue genes, *Mash-1/Ascl-1* and *Mash-2/Ascl-2* but a variety of Atonal homologues belonging to distinct gene families: Neurogenins, Neurogenic differentiation genes and Olig genes (Bertrand et al., 2002). As in Drosophila, the vertebrate proneural proteins form heterodimers with the E proteins (vertebrate counterparts of the Daughterless proteins) (Massari & Murre, 2000; Murre et.al, 1989). *Mash-1* has been shown to play an important role in the generation of CNS and PNS neurons and *lof* mutations in Mash-1 resulted in loss of neurons in the central and autonomic nervous systems but *Mash-2* is not expressed in the nervous system (Bertrand et al., 2002; Casarosa et.al, 1999; F Guillemot & Joyner, 1993; F Guillemot, 1999; Kusakabe et al., 2002; Lo et.al, 1994).

1.4 Proneural genes in *C. elegans:*

There are three members of the Ac/Sc family in *C. elegans* (*hlh-3, hlh-6, hlh-14*) and a well characterized *atonal* ortholog gene *lin-32* (Doonan et.al, 2008; Ledent & Vervoort, 2001; Zhao & Emmons, 1995). Loss of *lin-32* leads to defects in the development of rays in the *C. elegans* male tail and peripheral sensory organs (Miller and Portman, 2011; Portman & Emmons, 2000; Zhao & Emmons, 1995). *hlh-14* seems to have a proneural role as loss of *hlh-14* results in a loss of neurons in the HSN/PHB lineage (Frank et al., 2003). *hlh-6* is not expressed in neurons but is required for expression of PGM-1 necessary for differentiation of the pharyngeal glands (Smit et.al, 2008). *hlh-3* is a homolog of the Drosophila *asense* and does not seem to have a classical proneural role (Doonan, 2006).

1.5 HLH-3 regulates the differentiation of HSNs:

Previous studies have shown widespread expression of *hlh-3* in the embryo (Doonan, 2006; Krause et al., 1997). HLH-3 could act by turning on a sequence of downstream effectors (such as *tph-1)* that are important for proper development and differentiation. Even though there is widespread expression of *hlh-3*, loss of function mutant animals are viable and are able to survive to adulthood (Doonan, 2006). Moreover, there seems to be no defect in the generation and function of most neurons and the only visible phenotype in the hermaphrodite is an egg-laying defect; the *lof* mutants retain almost twice as many eggs as wild type (Doonan et al., 2008). This phenotype can be attributed to the axon pathfinding defect in the hermaphrodite specific motor neurons (HSNs) that are the primary neurons in the egg-laying machinery along with the ventral cord type C motor neurons (VCs) and the vulva muscles. Pharmacological treatment with serotonin and imipramine suggest that the rest of the egg-laying machinery is normal and it is the serotonergic HSNs that are important for the defect in egg-laying. In *hlh-3 lof* mutants, the HSNs are born and migrate to their positions behind the vulva. However, 60% of them are deficient in the synthesis and accumulation of their neurotransmitter – serotonin and among those that can be detected using 5HT immunoreactivity 65% have axon pathfinding defects. Thus, formation of serotonin is not the only aspect of differentiation controlled by *hlh-3* as 65% of the axons are abnormal. Thus, the mutant HSNs are born and migrate to their appropriate positions but do not seem to acquire their final identity (i.e. make serotonin, reach their appropriate targets). Even though some of the mutant HSNs are detected by antibody staining, pharmacological assays with the serotonin reuptake inhibitor imipramine show that these HSNs are nonfunctional (Doonan et al., 2008).

hlh-3 (bc277), a deletion in the upstream genomic region that leaves the bHLH encoding region intact, shows a very contrasting phenotype. These animals have WT-like egg-laying but

the HSNs are abnormal in their pathfinding. They do mature, however, unlike the HSNs in *hlh-3 (tm1688);* their HSNs have collateral branches that seem to synapse on the vulva muscles. Thus, the missing genomic region seems to play a role in axon guidance as compared to differentiation. So, questions addressed in this project are, how does *hlh-3* regulate the differentiation of HSNs, and what are the molecular mechanisms by which this regulation is brought about?

1.6 Axon pathfinding is abnormal in *hlh-3* mutants:

Once neurons have established their identity, further development leads to the formation of the axon towards its target. In *C. elegans*, the HSNs are born in the tail and migrate towards their final position just posterior to the vulva before the embryos hatch. The process of axon development starts only around the third and fourth larval stages (L3-L4 stage) with the polarization of the cell body by distribution of UNC-40, the receptor, ventrally and in response to the secreted extracellular UNC-6/Netrin signal (Adler et.al, 2006).

HSNs in *hlh-3* mutant animals that acquire their serotonergic fate have abnormal pathfinding indicating that *hlh-3* function is involved in HSN axons finding their correct target. This is a novel function of a proneural gene of the bHLH family. Axon development involves neuronal polarization to form a site for axon formation, axon growth and elongation and branching and connections. All these processes are regulated (Lewis et.al, 2013; O'Donnell et.al, 2009). The polarization of UNC-40 to the ventral side of the HSN cell body determines the site of axon formation. This distribution of UNC-40 is regulated by UNC-6 and SAX-3. In worms, the vulval epithelial cells are a source of UNC-6/Netrin that is detected by the receptor, UNC-40 expressed in the developing HSN growth cone. This interaction triggers a downstream signaling

cascade that involves Rac GTPases and modulates the actin cytoskeleton thus leading to the development of the axon and axonal growth (Norris et.al, 2014; Quinn et.al, 2008, Adler et al., 2006; Tang & Wadsworth, 2014; Xu et.al, 2015).

Axons have to travel long distances through various environments to find their correct synaptic partner. The axons can make connections as they travel or at their final destination. In response to UNC-6/Netrin, the HSN axon first develops ventrally and then turns dorsally and finally carries on anteriorly towards the nerve ring. It makes 'en passant' synapses with the vulva muscles during its development (Desai et.al, 1988). These synapses are responsible for the stimulation of the vulva muscles that lead to opening of the vulva in the process of egg-laying. These connections are very important because the HSNs are the command neurons in the egg-laying circuitry and animals having HSN defects are egg-laying defective (Desai et al., 1988; M Zhang et al., 2008).

The adaptor protein SYG-1 on the developing axon recognizes its partner SYG-2 on the vulva muscles to form a stable synapse as the axon keeps on its anterior journey towards the nerve ring (Özkan et al., 2014; Shen & Bargmann, 2003a). Once the axons are on their normal trajectory, synapses are formed only at specific sites due to these molecules. The vulval epithelial cells serve as guidepost cells that express SYG-2. SYG-1 is the receptor on the developing growth cone that recognizes the guidepost molecule SYG-2 that leads to the formation of a stable synapse (Chia et.al, 2014; Özkan et al., 2014; Shen & Bargmann, 2003). Synaptic specificity is very important for the development of functional synapses as the axons encounter variety of targets during their growth. The interaction is very specific and helps in the stabilization of the synapses. Expression of *syg-2* is necessary for localization of SYG-1 and misexpression of *syg-2* leads to mislocalization of SYG-1 (Chia et al., 2014; Shen et.al, 2004). Thus, accurate axon

pathfinding is a result of multiple processes falling into place starting from the response to the guidance cue, reorganization of the actin cytoskeleton and the recognition of the guidepost cell by the receptor on the growth cone of the developing axon.

1.7 Current work:

Doonan (2006) showed that *hlh-3* belongs to the Ac/Sc family of proneural genes and is an orthologue of Drosophila *asense*. It is widely expressed in the developing embryo but the expression is restricted to fewer postembryonic cells including the HSNs and the VCs in the hermaphrodite. *hlh-3 (lof)* mutant hermaphrodites are Egl. The Egl phenotype is due to the differentiation and axon pathfinding defect in the HSNs and VCs. In order to characterize the molecular mechanisms regulating *hlh-3* expression I undertook a detailed characterization of the expression pattern using constructs of various sizes to elucidate the genomic regions necessary for *hlh-3* expression. My analysis confirms the extensive expression pattern of *hlh-3* in the early embryo but reveals little to undetectable expression in the postembryonic HSNs in the larva/adult nervous system.

One of the *hlh-3* mutant alleles isolated by Barbara Conradt's laboratory showed no Egl defect. This allele, *hlh-3 (bc277),* is a deletion in an upstream genomic region with the potential to encode an additional exon, an exon we have called the curated exon (1'), transcription of this exon is detectable by RT-PCR in the strain harboring the mutant allele *hlh-3 (bc248)* (Doonan et al., 2008). As one of the goals of my study was to characterize the genomic region necessary for *hlh-3* expression, I decided to characterize the allele further. I found that although this allele had fewer differentiation defects in the HSNs, they had more pathfinding defects. Even with the pathfinding defects, the animals were WT like for egg-laying because of the branches that they sprouted from the abnormal axons that made synapses on the vulva muscles. Although, *hlh-3*

(bc277) HSNs were functionally normal, they showed pathfinding defects suggesting a role for the deleted region in axon pathfinding.

Doonan 2006 also showed differentiation defects in HSNs in *hlh-3 (bc248) lof* mutants. *hlh-3 (bc248)* had a deletion in the bHLH coding region. My analysis with another bigger deletion allele, *hlh-3 (tm1688)* showed similar results. 60% of the HSNs were undetectable using various markers like *tph-1::gfp* and 5HT antibody staining. These HSNs were undifferentiated and were arrested in the lamellipodia stage (Chapter 5).

Thus, in this study I have characterized the embryonic expression pattern of *hlh-3* and using various alleles I have functionally analyzed the role of HLH-3 in HSN development. My findings suggest an isoform specific role for HLH-3 in axon development and a unique role for HLH-3A in axon pathfinding.

Chapter II: Materials and Methods

Chapter 2

MATERIALS AND METHODS:

2.1 *C. elegans* strains:

Nematodes were cultured and maintained using standard techniques (Brenner, 1974). Bristol strain was used as wild type and additional strains were generated and used for this study (Table 2.1). NGM plates seeded with OP50-1 (Johnson et al., 1988) were used for nematode growth and culture. In general, four liters of NGM was autoclaved for 45 minutes and cooled for 20 minutes at 55^{o}C in a water bath. Supplements were added to the cooled agar before being poured into plates. The strains were maintained at 22^{o}C unless otherwise noted. Double mutant and transgenic strains were made using standard genetic techniques.

2.2 5HT immunostaining:

Young adult animals were synchronized by letting the mothers lay eggs for two hours on a seeded NGM plate and letting the eggs hatch and grow at 22^{o}C for 48 hours. The plates were washed with M9 three times once the animals reached the late L4/young adult stage. The animals were fixed in 4% paraformaldehyde at 4^{o}C overnight (ON) (approx. 16 hrs). They were washed thrice with 0.5% Triton X-100/PBS (190 ml 1X PBS and 10 ml 10% Triton X-100). Immunostaining was done by incubating them in 5% Beta mercaptoethanol/0.5% Triton X-100 PBS in a 37^{o}C ON. This was followed by two washes of 1% Triton X-100/0.1M Tris, pH 7.4 and one wash of collagenase buffer (1 mM $CaCl_2$/1% Triton X-100/0.1% Tris, pH 7.4). The animals were further incubated with Collagenase type IV (2000u/ml; Sigma C 5138) for 20 min in the 37^{o}C shaker followed by three washes with 0.5% Triton X-100/PBS. The animals were then incubated in 1% BSA (Sigma A-9306)/0.5% Triton X-100/PBS for 3 hours on a rocker at room

Strain	**Genotype**
AL128	*hlh-3 (tm1688)II*
MD1252	*hlh-3 (bc248)II*
AL217	*hlh-3 (bc277)II*
AL132	*icIs132 (unc-40::gfp)*
AL133	*hlh-3 (bc248)II; icIs132*
CB271	*unc-40 (e271)I*
AL134	*hlh-3 (bc248)II; unc-40 (e271)I*
CB1416	*unc-86 (e1416)III*
MT2405	*ced-3 (n717); unc-26 (e205)IV*
BL5717	*inIs179 (ida-1::gfp)II; him-8 (e1489)IV*
AL152	*icIs159 (hlh-3::yfp,ttx-3::mcherry)*
AL165	*hlh-3 (bc277)II; inIs179*
AL166	*hlh-3 (tm1688)II; inIs179*
AL162	*icIs162 (hlh-3^{fosmid}::yfp; ttx-3::Mcherry)*
AL163	*hlh-3(tm1688)II; icIs162*
AL164	*hlh-3(bc277)II; icIs162*
RTD16	*icIs2[hlh-3^{prom}::gfp;rol-6 (su1006)]*
RTD19	*icIs 103[hlh-3^{genomic}::gfp; rol-6 (su1006)]V*
GR1366	*mgIs42 [tph-1::GFP + rol-6 (su1006)]*
AL219	*hlh-3 (bc277)II;mgIs42*
TV2412	*wyIs97 [punc-86::myrgfp; punc-86::MCherry::rab-3; podr-1::gfp]*
AL220	*hlh-3 (bc277)II; wyIs97*
AL221	*hlh-3 (tm1688)II; wyIs97*
MT1082	*egl-1 (n487)IV*
AL218	*hlh-3 (tm1688)II; icIs169[punc-86::hlh-3cDNA::gfp;rol-6 (su1006)]*

AL252	*hlh-3 (bc277)II;egl-1 (n487)IV*
AL253	*unc-86 (e1416)III, icIs162*
AL254	*unc-86 (e1416)III; icIs2*
AL255	*hlh-3 (tm1688)II;icIs2*
AL256	*hlh-3 (tm1688)II;icIs103*
AL257	*icIs257 (hlh-3$^{prom1st\ ex}$::gfp)*

Table I: List of *C. elegans* strains

temperature. Then they were incubated with 1:50 rabbit serotonin antibody (Sigma S 5545) ON at RT on a rocker and washed three times with 0.5% Triton X-100/PBS followed by an hour incubation with 0.1% BSA/0.5% Triton X-100/PBS. Secondary antibody staining was done with 1:200 Cy3 conjugated donkey anti rabbit IgG (Jackson) at 4^{o}C ON in a dark chamber by covering the Eppendorf tubes in aluminum foil. The animals were then washed twice with 0.5% Triton X-100/PBS followed by an hour's incubation in 0.1% BSA/0.5% Triton X-100/PBS. These animals were mounted on 2% agarose pads and imaged under Zeiss Axioplan confocal microscope.

2.3 PCR fusion:

The *hlh-3*$^{prom\text{-}intron}$*::gfp* and *hlh-3(bc277)*$^{prom\text{-}intron}$*::gfp* constructs were generated using the PCR fusion approach developed by the Hobert laboratory (Hobert, 2002). In this protocol, two primary PCR products, one the region of interest and another containing the coding sequence for GFP, are fused together with a set of nested primers such that the product is ready for injections without the need for purification. I amplified the genomic region of *hlh-3* from the WT animals using program HLHFUS (98°C for 30 sec; followed by 30 cycles of 98°C for 10 sec, 58°C for 30 sec, 72°C for 4 min 30 sec; followed by final extension of 72°C for 10 mins) on the MJ research PT-150 minicycler using primers UIC268/298. I further amplified GFP from pPD95.75 using the primers UIC 299/300a with the GFP program on the minicycler. In the next step, I used primers UIC 301a/302a to fuse the products together. The PCR products were checked by running on a gel and used for micro-injections to generate transformants (described below).

UIC 268: 5'CCAAGCTTCAAAGATCGGCAAGATGG3'

UIC 269: 5'AAGTCGACAGTTGAGGAGGTGGATGC3'

UIC 298:

5’AGTCGACCTGCAGGCATGCAAGCTTTGAATGTAACGGGCGGCTTCACGAA3’

UIC 299: 5’AGCTTGCATGCCTGCAGGTCGACT3’

UIC 300a: 5’AAGGGCCCGTACGGCCGACTAGTAGG3’

UIC 301a: 5’GGAAACAGTTATGTTTGGTATATTGGG3’

UIC 302a: 5’GCTTCAAAGATCGGCAAGATGGCAAG3’

2.4 Construction of expression clones:

2.4.1 Characterization of *hlh-3A::gfp* expression:

A 2.7 kb HinDIII/SalI PCR fragment was generated from *C. elegans* genomic DNA and subcloned into pPD95.77 to generate *hlh-3A::gfp* (pSR2), that was co-injected with pRF4 (Kramer, French, Park, & Johnson, 1990) at 10ng/ul to generate transgenic lines having extrachromosomal arrays. These lines were integrated with UV-TMP integration protocol (described below) to generate integrated lines that were outcrossed twice. The animals were mounted on 2% agar pads and imaged with a Zeiss Axioplan confocal microscope.

2.5 Analysis of the double heterozygote:

A heterozygote line was generated by crossing *hlh-3 (bc277)* and *hlh-3 (tm1688)*. Young *hlh-3 (bc277)* males were mated with L4 *hlh-3 (tm1688)* hermaphrodites. 20 F1 L4 hermaphrodites were picked on individual plates and allowed to self-fertilize. A heterozygous plate was selected by PCR of the F1 progeny. From the heterozygous plate, 30 F2 hermaphrodites were selected and allowed to lay eggs for 2 hours on an NGM plate seeded with OP50-1. Young adult F3 hermaphrodites were individually mounted on a 2% agarose pads and imaged under a fluorescence microscope to observe the axon pathfinding defect. The animal was

then recovered by flooding the slide with M9 and sliding the cover slip gently over. It was then allowed to grow on an NGM plate seeded with OP50-1 for 40 hrs post L4 and screened for the number of eggs retained (see unlaid egg assay) and a single worm PCR was subsequently carried out to determine the genotype of the animal.

2.6 Thrashing assay:

Hermaphrodites were allowed to lay eggs in an NGM plate for two hours and removed from the plate. The resulting eggs were allowed to hatch and develop at 22^{o} C for 3 days. Young adult hermaphrodites were picked individually in a drop of M9 in a watch glass. They were allowed to recover for a minute and their body bends were counted for another minute. The total number of body bends was averaged over the number of individuals.

2.7 Germline transformation of *C.elegans*:

2.7.1 Microinjections:

Young adult hermaphrodites were mounted on 2% agarose pads that were previously prepared. The pads were prepared by melting agarose (Invitrogen) in ddH_2O for two minutes. The hot solution was pipetted on a No.1 coverslip and sandwiched immediately by another coverslip such that a flat layer was formed. The coverslips with the agarose were cooled for 3 hours and subsequently pulled apart gently. The resulting pads were dried at 65^{o}C overnight. Two young adult hermaphrodites with one to two eggs were carefully selected and mounted on the agarose pads on a drop of mineral oil. The pad was subsequently mounted on a Nikon Diaphot light microscope equipped with a Narishige hydraulic micromanipulator. Microinjection needles were made using glass capillary tubes pulled with a Sutter instruments needle puller

(Model P-87) using program #6. These needles were pre-filled with a hand pulled micropipette using a personal mouth pipette. A picospritzer set at 3 millisecond duration at 40psi compressed nitrogen pressure was used for microinjection. Injected animals were recovered by submerging them in M9 solution for 3 minutes and picking them into individual seeded NGM plates.

2.7.2 Selection of transformants:

Transformants were selected from the F3 generation using the selectable markers in each case. These animals were followed for subsequent generations to check for stability of the array. Animals from stable lines, displaying the marker, were mounted for microscopy.

2.7.3 Integration of extrachromosomal arrays:

2.7.3.1 Gamma irradiation:

A single mosaic line was selected and around 25-30 animals were selected and irradiated together in an unseeded NGM plate at 1850 rads. Post irradiation the animals with selectable markers were transferred to individual plates to screen for stable 100% transmission of the trait over subsequent generations.

2.7.3.2 UV-TMP integration:

20 µl of 1 mg/ml TMP (Trimethyl Psoralen in DMSO, prepared by dissolving 1 mg TMP in 1 ml of DMSO at 37°C for 3 hours with continuous rocking) was added to 380 µl M9, resulting in a final concentration of 50 µg/ml. 40-50 animals from an array containing line were added to the TMP solution. After letting them sit for 15 mins at RT, they were transferred to an unseeded plate covered with aluminum foil. The foil was removed to expose the animals to 350

µJ(x100) long wave UV Stratalinker 1800 (Room 4082) for 2 min. After adding 200 µl of concentrated OP50-1, the plate was covered in foil and maintained at RT for 5 hours. Three array containing L4 worms were picked to ten seeded plates each as P_0 for a total of 30 worms. After two days, the adult P_0s were transferred to ten new plates. 100 F1s were picked from the original plates on 100 individual seeded plates from the original plates. After three days, two F2s were picked from each F1 plate on individual seeded plates for a total of 200 plates. These plates were scored for 100% GFP fluorescence using a GFP filter.

2.8 Unlaid egg assay:

Animal populations are synchronized by letting adult hermaphrodites lay eggs over two hours. The eggs are allowed to hatch and develop at 22^{o} C. At the midpoint of their reproductive cycle (40 hrs post L4) adult hermaphrodites are selected and subjected to alkaline sodium hypochlorite lysis. The solution is prepared with 7.5 ml ddH2O, 2 ml sodium hypochlorite, 0.5 ml 10N NaOH. Add a drop of alkaline sodium hypochlorite to a watch glass and pick an adult hermaphrodite to this drop. It takes about 1 minute for the animal to lyse and release its eggs that were counted under a dissecting scope.

2.9 Characterization of GFP/YFP tagged construct expression pattern:

Embryos were harvested from gravid well-fed hermaphrodites by alkaline sodium hypochlorite lysis and washed three times in ddH_2O. They were then fixed with 4% paraformaldehyde and rinsed three times with ddH_2O. These embryos were mounted on a Fisher glass slide (cat. no 12-550-14) on M9 and covered with a No.1 coverslip and observed at 10x and imaged at 40x/60x under the blue filter/green filter and on the Zeiss Axioplan confocal

microscope. Synchronized adult animals were mounted on a 2% agarose pad in 10mM levamisole by picking individually, covering with a No.1 coverslip and imaged as above.

Chapter III: hlh-3 is expressed in the embryonic HSNs

Chapter 3

hlh-3 IS EXPRESSED IN THE EMBRYONIC HSNs

3.1 Introduction:

C. elegans is a free-living soil nematode that is an extremely important model organism in genetic studies because of its simple life cycle, compact genome and the ability to produce a large number of offspring in a short period of time. The life cycle involves the embryonic phase, larval phases and adulthood. Embryogenesis is characterized by a **proliferation phase** that results in the formation of about 558 cells that are not fully differentiated from a fertilized oocyte, and an **organogenesis/morphogenesis phase** where most cells undergo terminal differentiation along with gastrulation, morphogenesis, organogenesis and elongation of the embryo to produce a small larva with fully differentiated tissues and organs (Notable exceptions are the P cells that undergo terminal differentiation in the post-embryonic stages) (Fig. 3.1) (Sulston J.E et.al, 1983; www.wormatlas.org, www.wormbook.org)

The cell lineage of the *C. elegans* is mostly invariant and the fates are specified either by a transcriptional cascade that triggers expression of specific genes unique to the cell type or inheritance of cytosolic factors. As a result of stereotypic cell divisions and the constraining egg shell, newly arising cells tend to be located in reproducible locations in the embryo; thus, developing cells have been mapped to the exact locations. The cells can be traced from their origins to their final locations. Using Nomarski optics, various cells including the hermaphrodite specific neurons (HSNs) have been studied and traced from their birth to their final location in the adult (Fig. 3.2) (Desai & Horvitz, 1989; Sulston et.al, 1983). Because of the ease of cellular identification, gene expression patterns can be easily studied in the developing embryo. Using various reporter tools like fluorescent markers, we can study the gene expression in the early

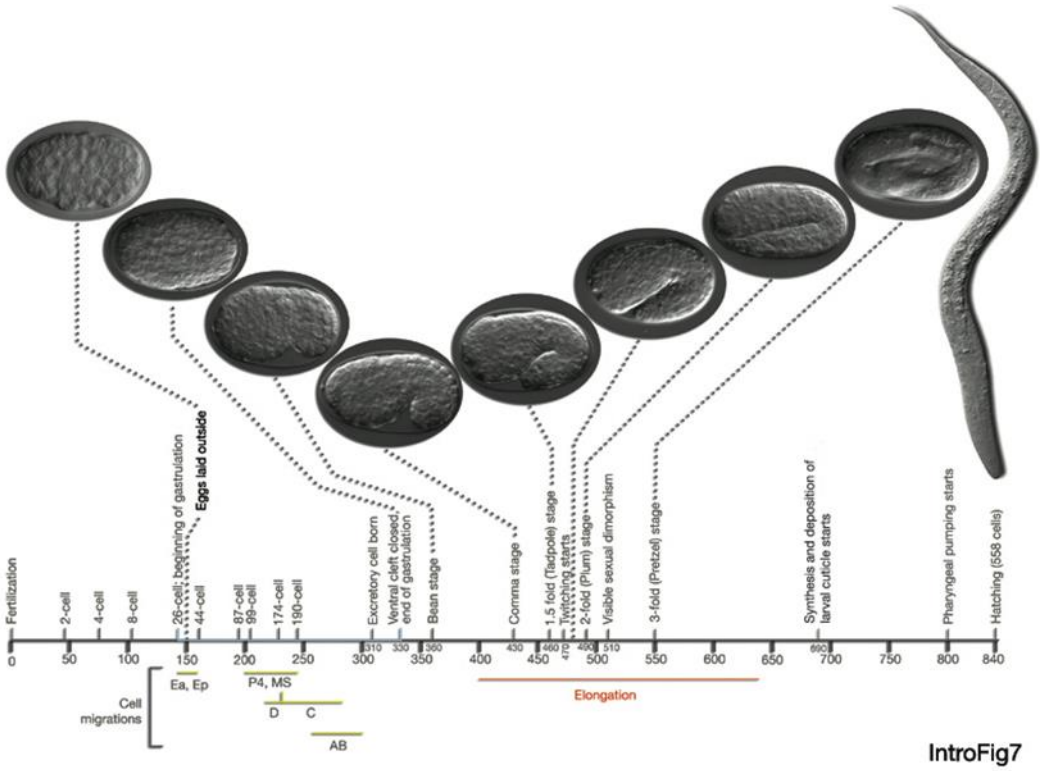


Fig 3.1. Diagram of embryonic development of *C. elegans* showing the timeline of various developmental stages with key milestones. Eggs are laid at the 30 cell stage and rest of the development is extra-uterine. Reprinted with permission from Wormatlas. Altun, Z.F. and Hall, D.H. (www.wormatlas.org)

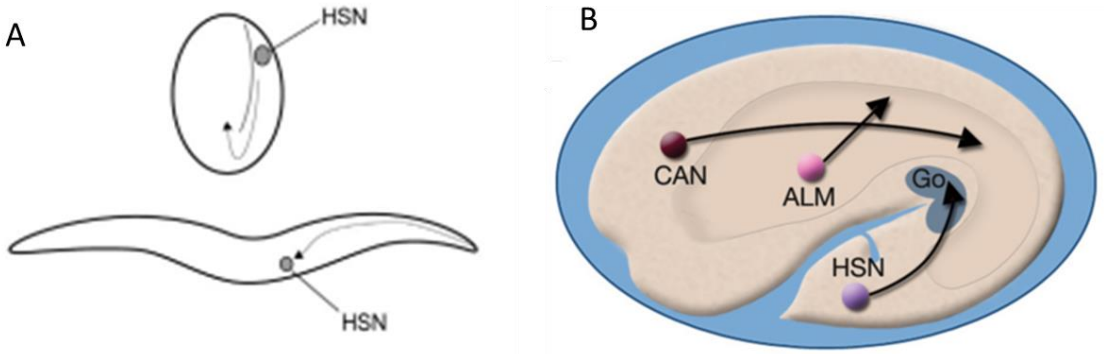


Figure 3.2. The HSNs are born in the tail and migrate to the midbody before the larva hatches. Migratory path of a developing HSN from the tail in the embryo (A and B) to the mid-body in the just hatched L1 (A)

[A has been taken from (Kennedy, Pham, & Grishok, 2013) and B Reprinted with permission from Wormatlas. Altun, Z.F. and Hall, D.H. (www.wormatlas.org)]

embryo. The development of optical tools and imaging software has provided an even higher level of detail. Studies performed by Waterston and Murray have been able to successfully characterize gene expression from the birth of a cell till the animal starts twitching at the one and a half fold stage (Bao et al., 2006; Murray et al., 2008).

The temporal and spatial expression pattern of genes of interest can give us insight into the functional significance of genes like transcription factors that can turn on a cascade of genes later on in the development of an animal but may get turned off early on, e.g. *egl-46* (Yu et.al, 2003). *hlh-3* is a gene in *C. elegans* encoding a helix loop helix transcription factor (HLH-3) whose homologs have been shown to play a proneural role in Drosophila and vertebrates (Aamodt & Aamodt, 2006; Doonan et.al, 2008; Powell & Jarman, 2008; Wilkinson et.al, 2013). *C elegans* HLH-3 is a homolog of Drosophila Asense that is required for maintaining the morphology of sensory bristles on the wing margin (Jarman et al., 1993). *hlh-3* has 2 exons encoding a 170 amino acid protein. *hlh-3* transcripts are transpliced to SL1/SL2 (Figure 3.3). A 93 nucleotide long upstream potential coding sequence has not been linked to any cDNA clones from the genome sequencing project. In addition RT PCR analysis has not identified a wild type transcript containing the sequences encoded by the genomic region we have designated as the curated 1st exon or exon 1' (Doonan et al., 2008; Krause et al., 1997). However, exon 1' can be theoretically transcribed and is in frame with the other two exons, it is detectable in RNA from the strain harboring the *hlh-3 (bc248)* mutant allele only. We propose this exon has a role to play in the function of *hlh-3* (discussed in Chapter 4, Section 4.2.4).

Helix loop helix transcription factors of Class I are ubiquitously expressed and interact with helix loop helix transcription factors of Class II, like HLH-3; together they activate gene transcription of downstream genes (Castro et.al, 2011). HLH-3 was discovered in a cDNA

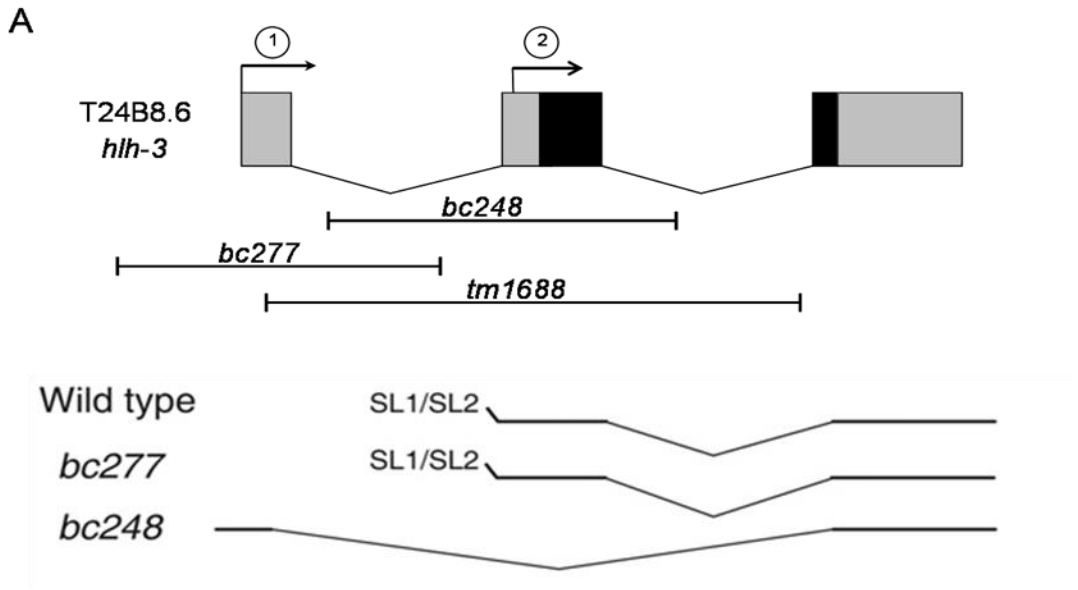


Figure 3.3. *hlh-3* gene structure. *hlh-3* has 2 transcription initiation start sites but only one transcript was detected by RT-PCR analysis of WT individuals. Individuals harboring *hlh-3 (tm1688)* have no detectable transcript(s) but *hlh-3 (bc277)* individuals contain a WT like transcript. (Image adapted from Doonan et al., 2008; Doonan, 2006).

expression library screen as a binding partner for HLH-2 (a Class I HLH protein). This interaction has been supported by co-expression studies of *hlh-3::gfp* and *hlh-2* reporters as well as protein interactions between the two gene products using Y2H binding assays (Grove et al., 2009; Krause et al., 1997; Vermeirssen et al., 2007).

hlh-3 appears to be expressed in many neuronal precursors. Previous characterization of *hlh-3* expression as monitored by a GFP translational reporter containing 3.3 kb of upstream sequences showed widespread and dynamic expression in the embryo and the persistence of *hlh-3* expression in the larval stages. In addition to some cells in the head and tail of hermaphrodites, the reporter was expressed in the P_na cells (neuroblasts) and its descendants in the first larval (L1) stage, and the HSNs and VCs at the third and fourth larval (L3 and L4) stages (Doonan 2006).

Given this expression pattern, it was expected that *hlh-3* function is required in the later stages of HSN differentiation because of its role in the differentiation of the HSNs (Doonan et al., 2008; Doonan, 2006). Here I have attempted to characterize the bonafide expression pattern of *hlh-3* using various constructs designed to characterize the function and relevance of various upstream DNA sequences in the promoter region.

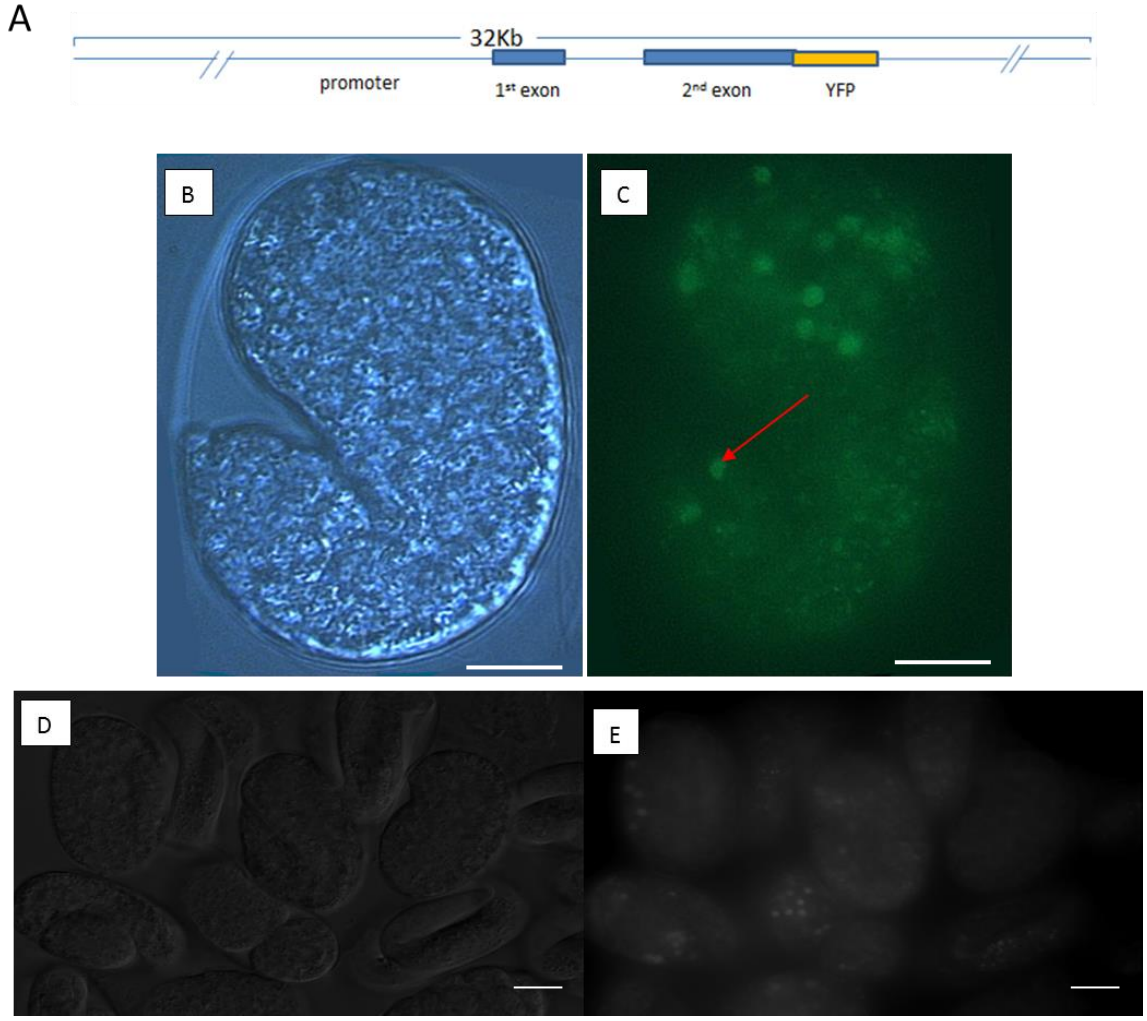


Figure 3.4. *hlh-3*^{fosmid}*::yfp* is expressed in the HSNs in the embryo (A) Diagram of the fosmid construct (~32kb). (B). DIC image of a one and half fold stage embryo. (C) Same embryo as in (B) under fluorescent light showing *hlh-3* expression in the HSN in the tail (red arrow) (D) DIC image of embryos at various stages of development. (E) Same image as in (D) under fluorescent light showing widespread expression of *hlh-3* in many embryonic cells. Scale bars represent 10µm.

3.2 Results:

3.2.1 *hlh-3* is robustly expressed in the embryonic HSNs and there is little to no detectable expression in the post embryonic stages:

Gene expression patterns are important in understanding gene function and in *C. elegans* various fluorescent reporters help in direct visualization of gene expression (Chalfie et.al, 1994). However, promoter gene fusion constructs commonly have a limitation in the length of upstream DNA sequence it can include and since it is known that regulatory regions can be located in various positions including introns or downstream regions of the gene these constructs could be missing important regulatory elements (Boulin et.al, 2006; Conradt & Horvitz, 1999). To address these concerns and develop a reporter that would include the gene coding sequences and much of the upstream and downstream regulatory regions, the Hobert laboratory developed a recombineering technique using the fosmid *C. elegans* library (Tursun et.al, 2009).

To address the bonafide expression pattern of the gene we characterized the expression pattern of WRM0625aA01, a *hlh-3::yfp* fosmid provided by Oliver Hobert's laboratory (Fig 3.4 A). This fosmid contains a 32Kb genomic fragment that encompasses the entire *hlh-3* coding region (T24B8.6) including potential upstream and downstream regulatory sequences as well as the coding sequences for T24B8.5 a protein coding gene 3,466 bases upstream of the predicted 5'end of *hlh-3*, and C07H4.1 and C07H4.2, protein coding genes about 2 and 6 kb, respectively downstream of the 3' end of *hlh-3*. The fosmid also has information for nine non-coding RNA genes upstream of the 5'end of *hlh-3* (T24B8.11, T24B8.13, T24B8.15, T24B8.16, T24B8.17, T24B8.18, T24B8.19, T24B8.20 and T24B8.23); and three non-coding RNA genes downstream of the *hlh-3* 3'end (T24B8.9, C07H4.3 and C07H4.4). T24B8.9 is about one kb downstream of

the 3'end of *hlh-3*. The yellow fluorescence protein (yfp) coding sequence is fused in frame before the stop codon in the 2nd exon (Tursun et al., 2009).

My analysis confirms widespread expression of the *hlh-3*^{fosmid}*::yfp* in the embryonic stages. Specifically, it shows expression in the HSNs in the one and half fold stage in the tail (Fig. 3.4B, C) in addition to other cells in the embryo which I have not characterized as they are numerous and beyond the scope of this study (Fig. 3.4 D, E). The cells that I have assigned as the HSNs are consistent in position with the HSNs that were analyzed by Nomarski optics by Sulston et al. 1983 doing the lineage analysis. Whether this *hlh-3*^{fosmid} expressing cell in the tail of the embryo is the newly born HSN can be tested in an *egl-1 (gof)* mutant background - the HSNs die via programmed cell death in an *egl-1 (gof)* mutant and *hlh-3* expression would not be detected in a *hlh-3*^{fosmid}::*egl-1 (gof)* transgenic strain.

Even though expression of *hlh-3*^{fosmid} is detectable in numerous embryonic cells, since the total loss of function phenotype in hermaphrodites is restricted to an egg-laying defect, then it is likely that other genes may have a redundant function in the additional cells. We do not believe the widespread expression in the embryo is artefactual because many other transgenic lines expressing reporters, including a second GFP tagged fosmid and different length promoter fusions (discussed further in 3.2.3, 3.2.4, 3.2.5) show similar patterns of expression.

In contrast to our published analysis (Doonan et al., 2008) there is little or no detectable expression of *hlh-3*^{fosmid} in the HSNs post-hatching, suggesting that *hlh-3* expression from the fosmid is being down-regulated in the HSNs once the animals hatch. This is surprising because *hlh-3* mutant hermaphrodites show normal development and have no overt abnormal phenotype other than the egg-laying defect that results from abnormal terminal differentiation of the HSNs that takes place at the L3-L4 stage or later (Doonan, 2006; Doonan et al., 2008; Chapter 5). Thus,

it appears that embryonic expression of *hlh-3* turns on a transcriptional cascade early on that is sufficient for events that take place in the later developmental stages. There is also a possibility that the fosmid construct bleaches due to exposure. However, this interpretation is unlikely because the GFP fosmid construct obtained from the mod-encode consortium also showed similar results (Nacke and Alfonso, unpublished).

3.2.2 Promoter fusion constructs show similar expression pattern as the longer fosmid constructs:

One of the initial goals of my project was to identify the cis-acting elements that are important for *hlh-3* expression. I intended to use multiple transcriptional reporter gene fusions to determine which genomic regions were important for *hlh-3* expression in *C. elegans.* To address that goal, I used a construct with the 3.3kb *hlh-3* promoter region with the 1st eight amino acids of the 1st exon fused in frame with GFP (Fig 3.5A). We have assigned as the promoter region that region of the genome on chromosome II that is upstream of the start codon of the 1st exon up to end of the previous gene (T24B8.5). Although this construct could be missing enhancer regions located much further upstream and not in the immediate promoter region, such an analysis provides a baseline of the gene expression pattern. Also, the possibility of missing out on enhancer elements can be eliminated by analyzing the fosmid lines (discussed in 3.2.1) that encompass vast areas of the genome. In order to analyze the *hlh-3* promoter region and delineate the regulatory regions required for expression of *hlh-3*, I analyzed *hlh-3*^{promoter}*::gfp* (3.3kb *hlh-3* promoter region fused with *gfp*).

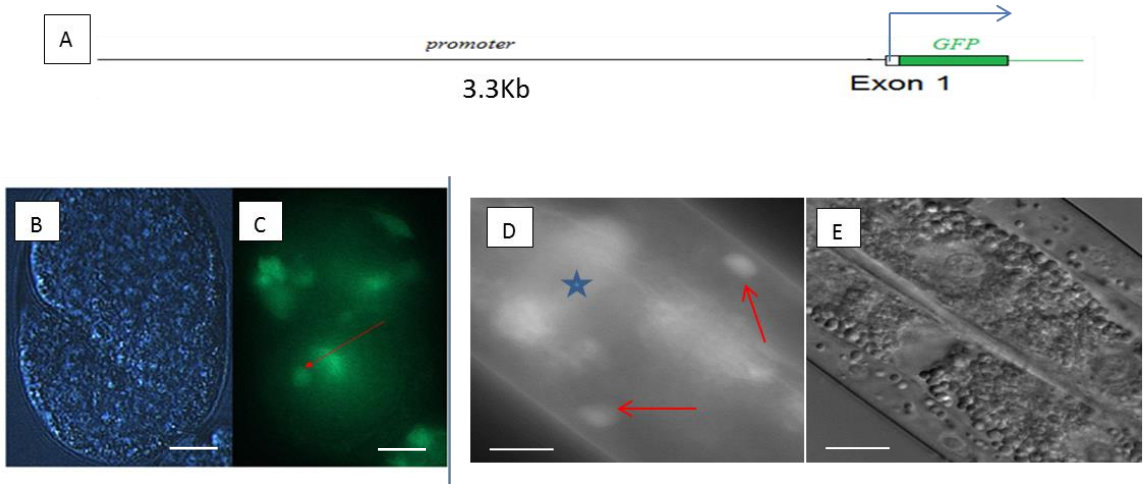


Figure 3.5. *hlh-3*^{promoter}*::gfp* is expressed in the embryo in the HSN. (A) Diagram of the construct (3.3kB). (B) DIC of the one and half fold embryo. (C) 1½-fold stage embryo under fluorescent light showing *hlh-3* expression in the HSN in the tail (red arrow). (D) L4 larva showing *hlh-3* expression in the HSNs (red arrows). Additional fluorescence represents autofluorescence. (E) DIC image of same animal in (D) (Ventral view with anterior to the left and vulva marked with a star). Scale bars represent 10µm.

hlh-3^{promoter}*::gfp* is expressed in the embryo, in a pattern similar to that of the *hlh-3*^{fosmid}. Expression can be seen in the HSNs in the tail of the embryo (Fig. 3.5 B, C). The only difference between the fosmid and this line is the persistence of faint expression in the adult HSNs in 33% of the animals (Fig. 3.5 D). The difference in expression between the fosmid and the promoter construct could be due to perdurance of GFP due to lack of regulatory elements that are present in the fosmid which prevent or result in low levels of expression beyond the embryonic stage.

3.2.3 The *hlh-3 (bc277)* promoter construct is expressed in the HSNs, similar to the fosmid and promoter fusion:

hlh-3 (bc277) is a 664bp deletion in the upstream promoter region of *hlh-3*. It encompasses a curated exon that has no ESTs or mRNA detected with RT PCR under wild type conditions (Doonan et al., 2008), but can be theoretically transcribed and is in frame with the other two exons. I show in the next Chapter that this exon has a potential role in the function of *hlh-3* (discussed in Chapter 4, Section 4.2.4). Since we already had a deletion construct in the upstream promoter region, my goal was to analyze this genomic region to see if I could assign a role in the expression or function of *hlh-3*.

I generated a reporter fusion using the promoter region of *hlh-3 (bc277)* that lacked the 664 bp fragment upstream and fused *gfp* coding sequences in frame with the intron such that the construct included the 1st exon, the intron and 8 amino acids of the 2nd exon in frame with *gfp*, called *hlh-3*^{promoterbc277}*::gfp* (Fig 3.6a). If the deleted upstream genomic region has a role to play in *hlh-3* expression, then this construct should not be expressed anywhere. My observations do not support this assertion. I detect *hlh-3*^{promoterbc277}*::gfp* expression in the HSNs and other cells in

the embryo (Fig. 3.6 b, c); and there is no expression post hatching. This pattern is similar to the other constructs, *hlh-3*^{fosmid}*::yfp, hlh-3*^{promoter}*::gfp* and *hlh-3*^{promoterintron}*::gfp* (Fig. 3.7) suggesting that the deleted region is not necessary for the spatial and temporal regulation of *hlh-3* expression in the HSNs.

Thus, the analysis of the expression of multiple constructs supports the conclusion that *hlh-3* is expressed in the embryo, notably in the HSNs and is either turned off or is below the detection limit in the post embryonic stages. This is surprising as the HSNs do not mature until the L3/L4 stage suggesting a potential early role of HLH-3 in the HSNs.

3.2.4 The HSNs from individuals harboring *hlh-3 (tm1688)* and *hlh-3 (bc277)* alleles have variable terminal differentiation defects

To further characterize the function of the *hlh-3* gene we analyzed the consequence to HSN terminal differentiation of two deletion alleles, *hlh-3 (tm1688)* and *hlh-3 (bc277)*. The *hlh-3 (tm1688)* mutant allele (WormBase release WS187; kindly provided by S. Mitani) represents the largest deletion (1242 bp) in the gene, spanning exon I and eliminating 65% of the bHLH coding region (Fig.7) (Doonan et al., 2008). Hermaphrodites harboring the *hlh-3 (tm1688)* allele are Egl (Doonan et al., 2008; Table 3.1).

The inability to lay eggs normally is not surprising given that only 28% of the *hlh-3 (tm1688)* HSNs are 5HT immunoreactive and among those that have 5HT immunoreactivity 72% have abnormal pathfinding (Table 3.1). The degree of the absence of detectable 5HT and the axon pathfinding defects are identical to that of *hlh-3 (bc248)* HSNs (Doonan et al., 2008).

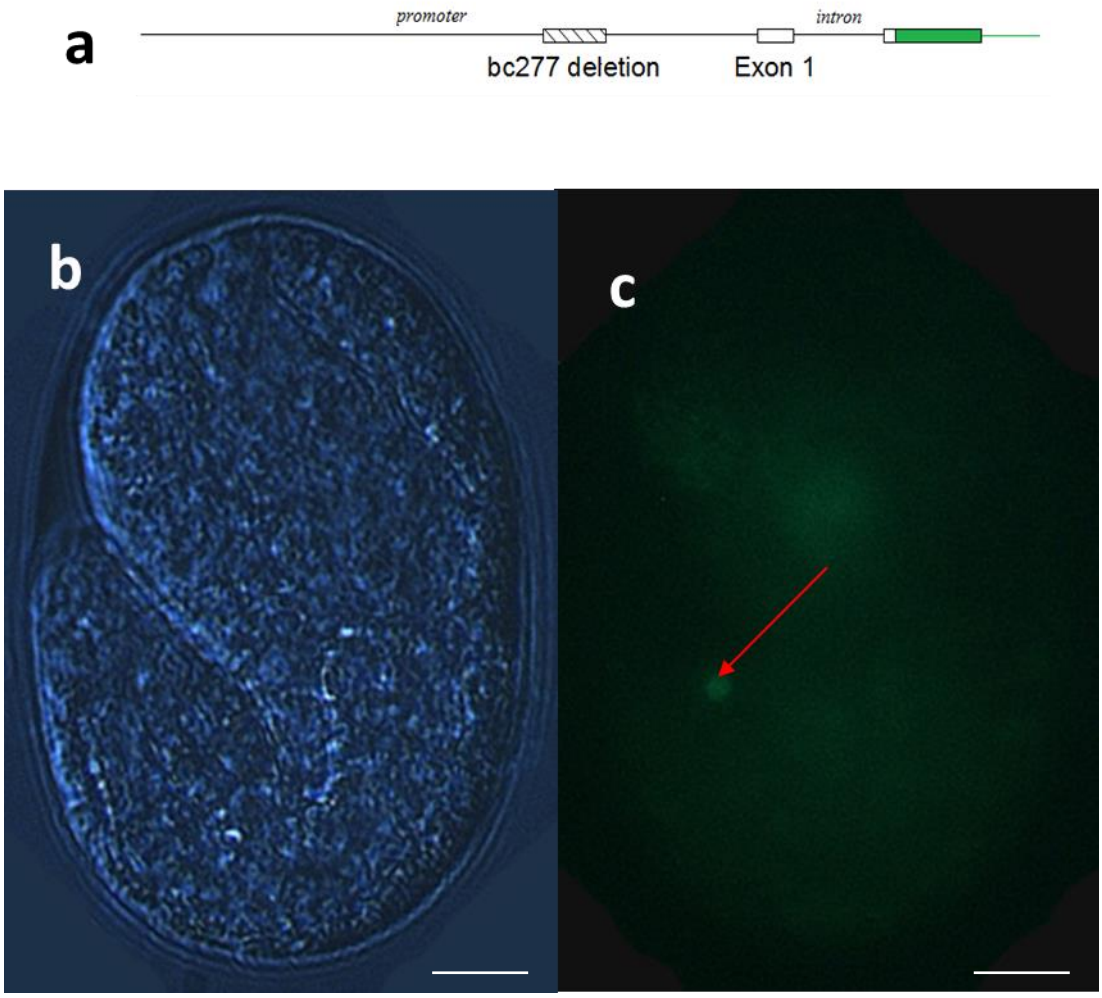


Fig. 3.6. *hlh-3*^{promoterbc277}*::gfp* is also expressed in the tail, likely in the HSNs. (a) Diagram of the construct (3.9kb) (hatched box represents deleted sequence). (b) DIC image of a 1½-fold stage embryo. (c) Same embryo as in (b) showing *hlh-3* expression in the tail (red arrow). Scale bars represent 10µm.

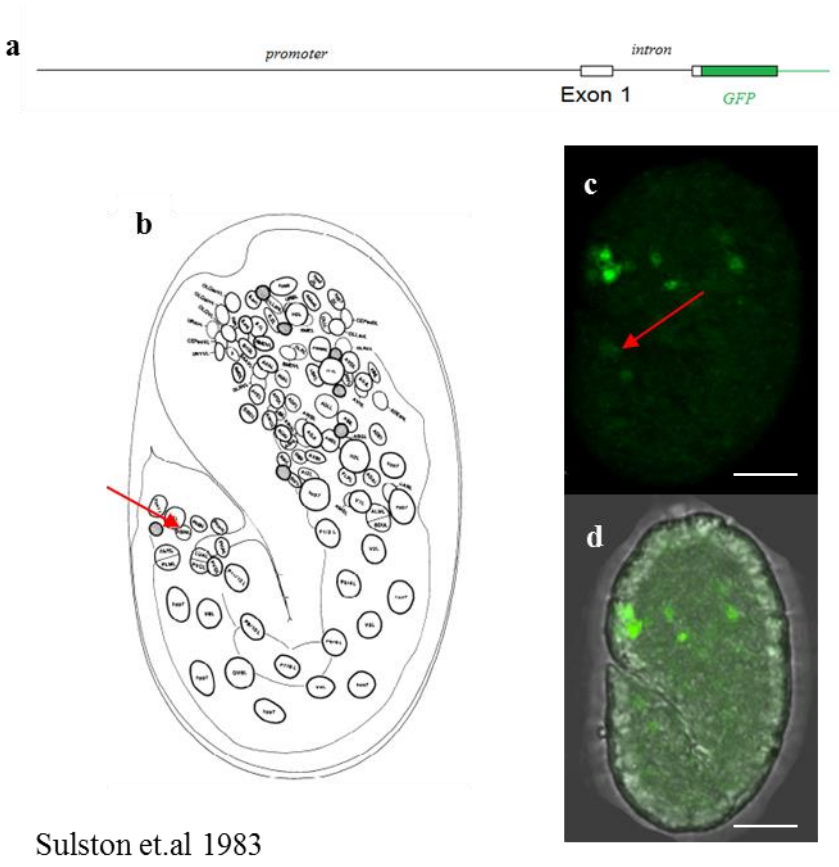


Figure 3.7. *hlh-3*^{promoterintron}*::gfp* is expressed in the HSNs in the embryo. (a) Diagram of the construct (4.5kb). b) Diagram of the embryo at one-and-a-half-fold stage. (c) 1½-fold stage embryo under fluorescent light showing *hlh-3* expression in the HSN in the tail (red arrow). (d) Same embryo as in (c) under transmitted light (Sulston et.al., 1983). Scale bars represent 10µm.

In contrast, hlh-3 (bc277) hermaphrodites are not Egl (Doonan et al., 2008; Table 3.1). As mentioned in section 3.2.3 the hlh-3 (bc277) deletion maps to 160 bp upstream of the presumptive *hlh-3* open reading frame and removes 664 bp (Doonan et al., 2008) (Figure 3.3). In our initial characterization of this allele we suggested that *hlh-3 (bc277)* represented a mutation in a cis-acting regulatory region affecting spatial or temporal expression of the gene but unlikely to affect HSN differentiation. As shown in the previous section, the missing sequences are not necessary for turning on expression of the gene. However, to determine whether *hlh-3 (bc277)* HSNs differentiate normally; I performed immunocytochemical detection for 5HT and characterized the location and pathfinding of the stereotypic axon. I find that 90% of the *hlh-3 (bc277)* HSNs accumulate 5HT (stained positive for 5HT) at the L4 larval stage, but 70% have pathfinding defects (Table 3.1). The pathfinding defect was primarily consisting of the HSN axon traveling anteriorly and not ventrally as it normally does. Even though *hlh-3 (bc277)* HSNs have pathfinding defects, 83% of individuals also have branches emanating from the abnormal axon that seem to make synapses on the vulva muscles (discussed in Chapter 4, section 4.2.2).

3.2.5 The fosmid rescues the HSN defects:

As discussed in section 3.2.1, the *hlh-3* fosmid contains a 32Kb piece of genomic DNA, it is expressed in a number of cells in the *C. elegans* embryo, and its expression in the HSNs disappeared as the animals hatched. This pattern of expression was different from our previous and published observations where *hlh-3* expression persisted in the cells involved in the egg-laying circuit. In order to answer the question whether the fosmid was a functional construct, we used it to determine if it would rescue the *hlh-3* mutant phenotype. Hermaphrodites harboring the loss of function *hlh-3 (tm1688)* allele have defects in axon pathfinding and serotonergic

	Phenotype	5HT+ HSNs		5HT- HSNs
		Normal ventral process	Abnormal process	
N2 (wt) (n=65)	+	92%	6%	2%
***hlh-3(bc277)*(n=58)**	+	20%	70%	10%
***hlh-3(tm1688)*(n=52)**	Egl	8%	20%	72%

Table II: *hlh-3 (bc277)* hermaphrodites are not Egl but their HSNs have a significant problem in axon pathfinding. Egl is defined as laying of eggs more slowly leading to bloating typically leading to more than two rows of eggs and accumulation of late stage eggs in the uterus. Non-Egl *hlh-3 (bc277)* animals have 70% of HSNs with abnormal pathfinding. Individual HSNs were detected via immune reactivity to 5HT. An abnormal process is one that does not follow the stereotypic ventral, then anterior trajectory. Percent of HSNs with normal and abnormal processes are calculated from the HSNs that are detectable.

differentiation. They are also stalled in their maturation (Chapter 5, section 5.2.4). Expression of the *hlh-3*^{fosmid} was able to rescue all these defects (Fig. 3.8, Table 3.2) suggesting that endogenous gene expression is primarily, if not restricted to the embryo primarily if not exclusive in the HSNs and that *hlh-3* function in the embryo is necessary for proper axon guidance, neurotransmitter expression and maturation of HSNs. This observation is again consistent with *hlh-3* setting up a cascade of events that manifest or drive events much later in development.

3.2.6 Lineage analysis shows *hlh-3* is expressed in the mother of the HSNs:

Lineage analysis involves multidimensional analysis of *C. elegans* embryos, to understand the transcriptional profile of embryonic cells, in order to study development of cells in molecular detail (Murray et al., 2008). It involves using reporter gene-*histone gfp/Mcherry* fusions to detect the cells and follow the lineage in a developing embryo. Gene expression pattern of any given cell is important for learning the interacting pathways functioning in those cells.

The transparent *C. elegans* embryo with a fixed number of somatic cells and a fixed lineage relationship makes it a great model system for using this tool. It provides great spatiotemporal resolution and reduces the need for anatomical expertise as this process can be automated using various standardized algorithms such as Starrynite and Acetree that allow for accurate detection and follow up of cells from the 4D movies generated from the time lapse imaging (Bao et al., 2006). This overcomes the drawbacks in older methods like *in-situ* hybridization, where spatiotemporal data could not be accurately obtained. Using fluorescent reporters also allows the study to happen in real time and provides an advantage over differential

	5HT^{+} HSNs	Normal pathfinding
hlh-3 (tm1688)	65%	60%
hlh-3 (tm1688); hlh-3^{fosmid}::yfp	83%	96%

Table III. 5HT immunostaining of *hlh-3 (tm1688)* (n=30) and *hlh-3 (tm1688); hlh-3^{fosmid}::yfp* (n=48). *hlh-3 (tm1688); hlh-3^{fosmid}::yfp* shows increased 5HT^{+} HSNs that have normal pathfinding compared to *hlh-3 (tm1688)* alone.

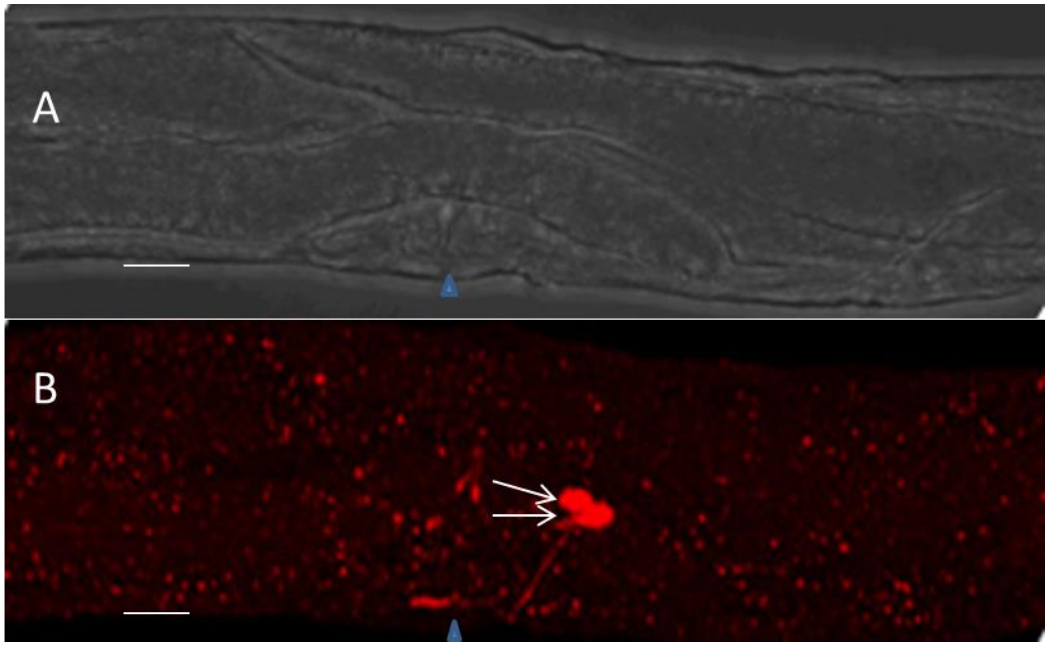


Figure 3.8. *hlh-3*^{fosmid}*::yfp* rescues *hlh-3 (tm1688).* The pathfinding defect of HSNs (white arrows in (B) in *hlh-3 (tm1688)* animals is rescued by expression of the fosmid. (A) DIC image of a L4 hermaphrodite with anterior to the left and ventral side at the bottom. (B) Same hermaphrodite as in (A) stained with anti-5HT antibody. The blue Δ indicates the position of the vulva, the white arrows point to the cell bodies of the HSNs. Scale bars represent 10µm.

interference contrast imaging by providing a good contrast with the dark cytoplasm and labelling the mitotic figures that assist in assigning the daughter cells to their appropriate mothers (Bao et al., 2006; Murray et al., 2008; Araya et al., 2014). We have looked at various reporter gene fusions as well as two fosmid lines to elucidate the expression pattern of *hlh-3* in the embryonic stage. However, I have relied on the anatomical location and developmental timing of the embryo for my analysis. We used the automated lineage analysis in collaboration with Dr. Murray, to independently validate our findings that suggested the expression of *hlh-3* is restricted to the embryonic HSNs. This analysis using *icIs159 (hlh-3::yfp,ttx-3::mcherry)* revealed that *hlh-3* is indeed expressed in the grandmother and mother of the HSNs (Fig.3.9).The drawback of this analysis is we cannot see expression in the HSNs themselves as it is only possible to trace the lineage only up to the 350 cell stage because the embryo starts twitching after that and the final stage of embryonic division is not traced. However, the fact that *hlh-3* is expressed in the HSN lineage strongly supports our conclusion that it has a role to play in the development of the HSNs.

3.2.7 Antibodies generated against HLH-3 peptides shows non-specific staining:

Transcriptional or translational fusion constructs using GFP or other markers are an excellent way of analyzing gene expression. However, integrated transgenic reporter lines do not always recapitulate endogenous gene expression due to various reasons like a high copy number, abnormal localization due to the presence of a tag etc. (Mello and Fire 1995). Immunohistochemistry is the tool of excellence to look at endogenous gene expression using antibodies that bind to the protein of interest (Vermeirssen et al., 2007). In collaboration with BioSynthesis Inc. polyclonal antibodies were generated against two unique peptides

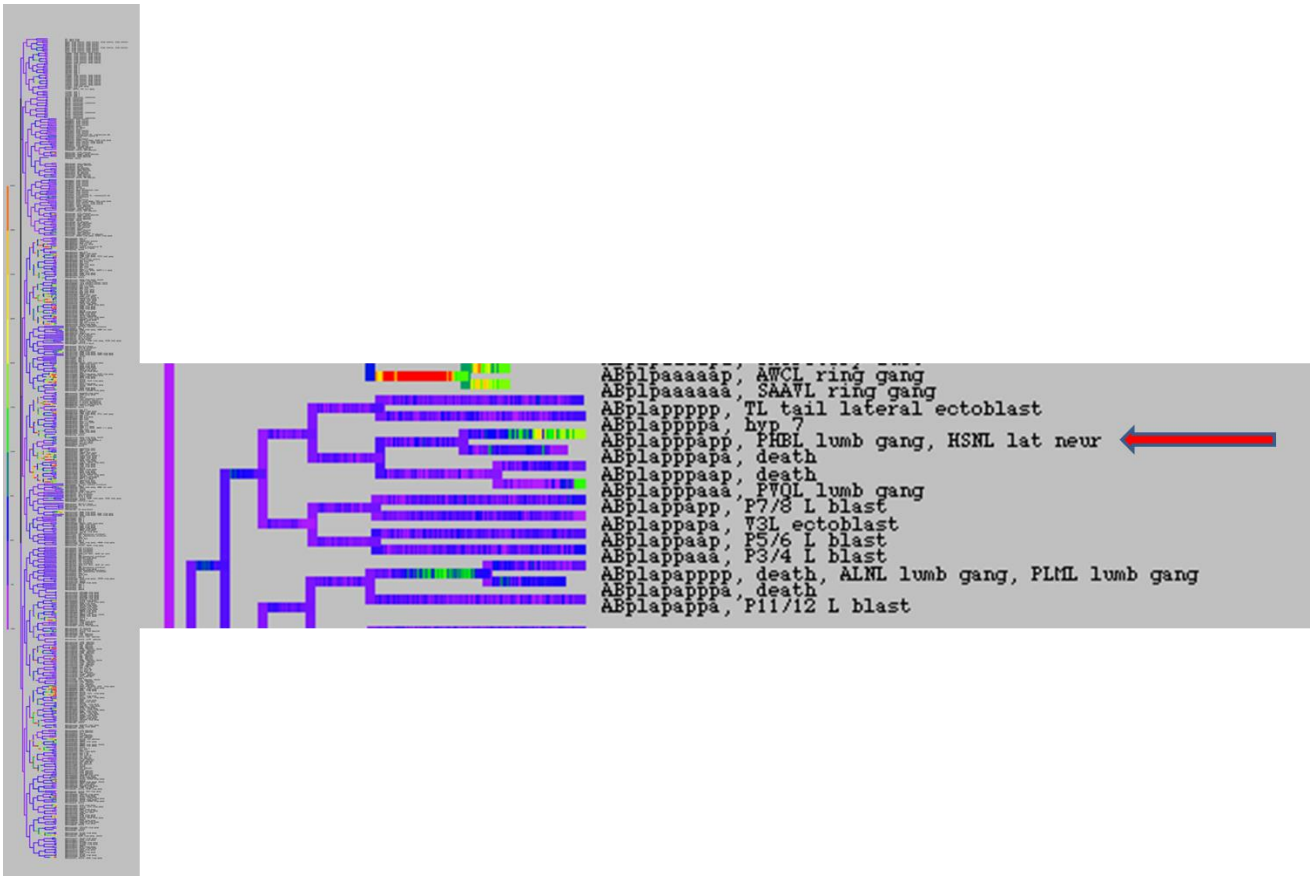


Figure 3.9. *hlh-3::gfp* is expressed in the mother of HSNs. Lineage analysis using the reporter *icIs159 (hlh-3::yfp,ttx-3::mcherry)* reveals the expression of *hlh-3* in the mother of HSNs/PHBs. Further imaging is not possible due to twitching of the embryo. (Image courtesy of Dr. Murray, personal communication)

(1.C- SSASTSGDHHSFYSHYRNF (152-170); 2.C-STPSTSTKIPSSSKSSVTKQTKQ (7-26)) in HLH-3. These peptides were highly specific to the *C. elegans* HLH-3 polypeptide. Unfortunately, neither elicited a specific staining pattern. Immunocytochemical staining resulted in non-specific body wall muscle staining. There was no detectable staining in the neurons at any of the larval stages. This result could possibly be because there is no *hlh-3* expression in larval stages. The staining of muscle is not specific for HLH-3 because staining of the *hlh-3 (tm1688)* animals, which should make no polypeptide, is identical to the staining of wild type animals. To determine whether there was some non-specific immunoreactivity in the sera, I incubated *hlh-3 (tm1688)* animals with the serum to get rid of the non-specific antibodies (that might be causing non-specific binding in the WT animals) and used the supernatant on the wild type animals. None of the protocols detected any neuronal staining. Since staining with the pre-immune serum showed no immunoreactivity, then the muscle staining that we see is because of something in the immune serum. It is very surprising that antibody against such a specific epitope should give a muscle staining.

Since the robust expression is only detected in embryos with the fosmid constructs, I attempted to use the freeze cracking method of embryo staining to characterize *hlh-3* expression in the early embryo. However, I wasn't successfully able to stain the embryos and could not detect any antibody in any of the cells. This can be looked at more diligently as I did not pursue this after two failed attempts since there was non-specific staining in the adults. Wormbook Chapter on immunohistochemistry describes various protocols for antibody staining that can be used for analysis with the antibody (Duerr, 2006). There is no evidence of any role or expression of *hlh-3* in the muscle cells, more importantly detection of muscle immunoreactivity

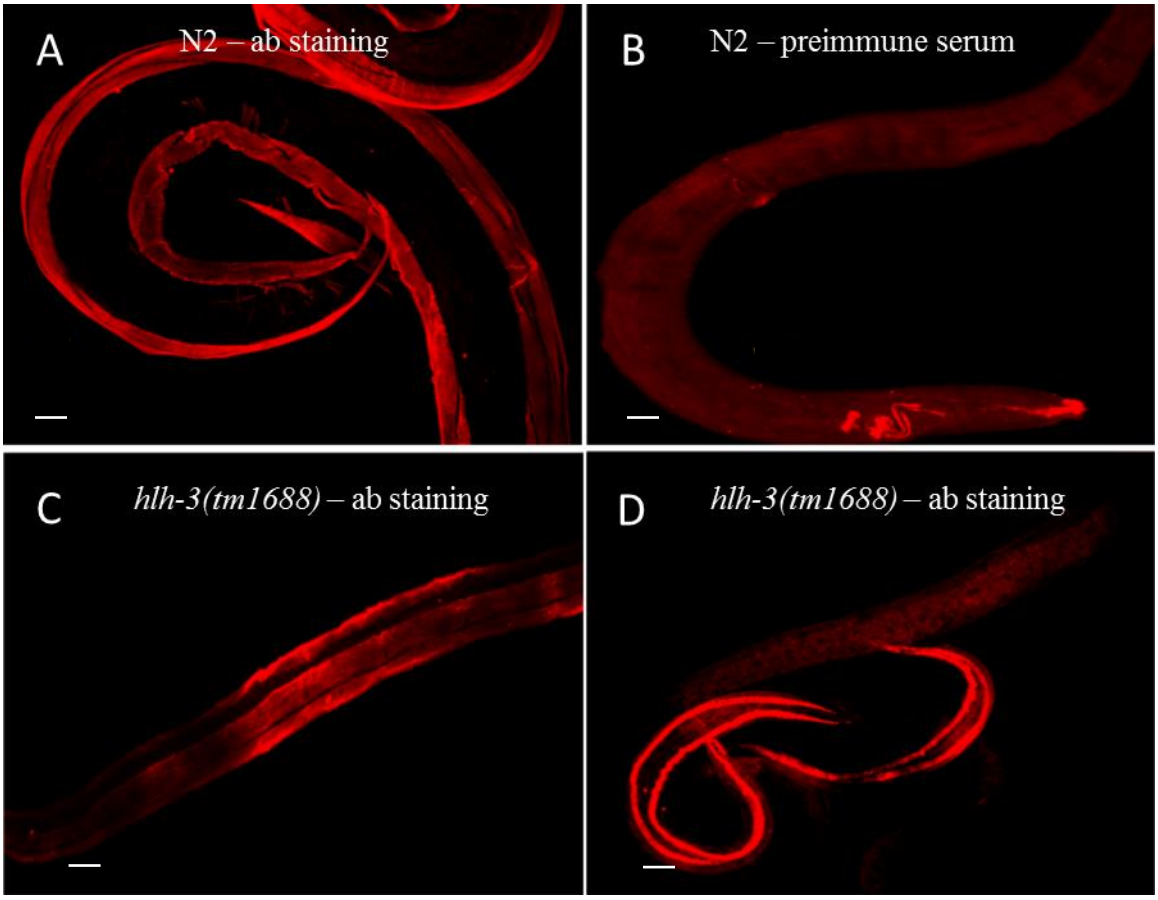


Fig 3.10. Immunostaining with antibodies against HLH-3 peptides label body wall muscle in wild type and *hlh-3 (tm1688)* animals. (A) WT L4 animal with HLH-3 antibody showing body wall muscle staining. (B) No specific staining is seen in WT animals with pre-immune serum. (C) and (D) *hlh-3 (tm1688)* animals show similar pattern of staining as WT in L4 (C) and younger larvae (D). Scale bars represent 10µm.

is unrelated to whether there is *hlh-3* around. Thus, we do not currently have a tool to look at endogenous protein expression.

3.3 Discussion:

3.3.1 *hlh-3* is expressed in the embryonic HSNs and is necessary for HSN differentiation and axon pathfinding:

hlh-3 belongs to the Achaete-Scute family of genes with a proneural role but this *C. elegans* gene does not have a classical proneural role even though there is widespread expression in the developing *C. elegans* embryo. The protein encoded by *hlh-3* was first discovered in a cDNA expression library screen as a binding partner for HLH-2, and was seen to be co-expressed with its binding partner HLH-2 (Krause et al., 1997). Genes of the As/Sc family in vertebrates and Drosophila show similar widespread expression patterns in the CNS and PNS and their loss leads to a loss of the sensory organs in Drosophila and change in fate from neurons to glia in vertebrates. However, not all members of the As/Sc family show neuronal loss and some like the Drosophila Asense, that is the ortholog of the *C. elegans hlh-3*, is known to play a role later in neural differentiation (Jarman et al., 1993). The widespread expression of *hlh-3* in the embryonic stages suggests multiple functions involved in various systems, possibly a necessary requirement for the survival of the animal. However, not only does the mutant hermaphrodites mature and are viable but their only overt phenotypic anomaly is in the egg-laying system.

This is not surprising as *hlh-3* is expressed in the HSNs in the embryo. Previous work from our laboratory (Doonan, 2006) has provided evidence for the expression of *hlh-3* in various

neuronal lineages. Here we focused on the role of *hlh-3* in the HSNs. Using various reporter and fosmid constructs and lineage tracking we have shown that *hlh-3* is expressed in the embryonic HSNs. Loss of *hlh-3* results in abnormal HSN differentiation such that there are maturation defects (Chapter 5) and axon pathfinding defects in the mature HSNs. The fosmid can rescue these defects in the null *hlh-3 (tm1688)* mutant, suggesting that *hlh-3* is necessary for the normal HSN function.

3.3.2 There is little to no detectable post embryonic *hlh-3* expression in the HSNs:

It is surprising that there is no detectable expression in the late larval stages (L3 and L4) when the HSNs become functionally mature. This is surprising, because unlike other proneural genes that result in a loss of neuronal populations, the role of *hlh*-3 in the hermaphrodite seems to be restricted to the developing HSNs and their differentiation is not initiated until the late L3, early L4 stage (Adler et.al, 2006). The exact role of proneural genes in regulating neurogenesis, that is whether they regulate other transcription factors or effector genes, was unclear until evidence in studies of Ascl-1 revealed a role of As/Sc family genes in the regulation of different transcription factors (Castro et al., 2011). Overall my studies suggest that a transcriptional cascade is turned on early in the development of the animal by *hlh-3* which then gets turned off once the said cascade is set in motion. The Drosophila ortholog Asense has a similar role in that it is expressed early but its function is required later in Drosophila sensory bristle differentiation (Jarman et al., 1993). Other *C. elegans* transcription factors like *egl-46* have been known to function in this manner where they are expressed in the embryonic stages but functionally needed in the later stages (Yu et al., 2003). The expression of *hlh-3*^{promoter}*::gfp* in 33% of the

HSNs of the transgenic line could be because of perdurance of GFP after the initial expression in some of the animals. Another possibility is that the level of expression of the endogenous gene is low and hard to detect after the eggs hatch. The absence of a reliable antibody to assess expression pattern further compounds the problem. An antibody raised to unique residues of the *hlh-3* coding region failed to give specific staining. Although the serum stained body wall muscle, since *hlh-3* mutant animals are not impaired in locomotion or thrashing then the observation is meaningless.

3.3.3 The deleted sequence in the allele *bc277* has no role in regulating *hlh-3* expression but may play a functional role in later differentiation events:

hlh-3^{prombc277}*::gfp* drives a pattern of expression in the HSNs that is similar to the fosmid and full length promoter constructs. The *hlh-3*^{prombc277}genomic fragment contains a 664 bp deletion that includes the curated exon (1') that has no ESTs or mRNAs detected by RT PCR but is in frame, can be theoretically transcribed and thus encode a longer protein. The expression pattern of this deletion construct revealed no potential role for the missing sequences in regulation of *hlh-3* expression. However, the *hlh-3 (bc277)* mutant hermaphrodites show a phenotypic defect that is different from the null mutant *hlh-3 (tm1688)* (Table 3.1). The *hlh-3 (tm1688)* hermaphrodites have differentiation defects as seen by the abnormal neurotransmitter phenotype. The *hlh-3 (bc277)* animals have highly differentiated serotonergic HSNs that are pathfinding defective. The abnormal axon pathfinding defects in the differentiated *hlh-3 (bc277)* HSN axons suggest a role for the curated exon in the pathfinding of the HSNs (discussed in

Chapter 4). It also suggests that there is no enhancer or regulatory activity in the 664 bp deleted fragment that regulates *hlh-3* expression in the HSNs.

3.4 Future Directions:

3.4.1 Further characterization of *hlh-3* expression

3.4.1.1 Lineage analysis:

Our current analysis using lineage tracing techniques has been able to give us limited insights as it is only possible to image the embryo until the one and half fold stage after which the animal starts to twitch making imaging difficult. Using this method, *hlh-3* expression has only been followed up to the mother of the HSNs. In collaboration with Dr. Daniel Colon-Ramos at Yale, using an iSpim microscope, should let us image the embryos until they hatch and would allow us to delineate the timeline of *hlh-3* expression in the HSNs.

3.4.1.2 Antibody staining:

Immunohistochemistry using monoclonal antibodies raised against peptides in HLH-3 did not give a specific pattern of staining. My analysis has revealed that *hlh-3* is expressed in the embryo and there is little or no detectable expression in the HSNs after hatching. To further corroborate this observation, anti-GFP antibodies could be used to stain the *hlh-3*^{fosmid}*::yfp* and *hlh-3*^{fosmid}*::gfp* strains. There is a possibility that the fosmid construct expression is very weak to be detectable in larvae post hatching under current conditions. Anti-GFP antibodies can detect the gfp and yfp encoding region of the construct and will be able to stain HSNs in larvae and adults to provide information on post embryonic expression of *hlh-3*.

3.4.1.3 Western Blotting:

The monoclonal antibodies generated against the HLH-3 peptides did not show specificity on whole worms, but they could be tested by Western blotting and used to detect the presence of HLH-3 in whole embryo extracts. They could be tested in an embryo preparation first to determine if they detect HLH-3 expression and then in larval and adult enriched extracts. If the antibody is able to bind to HLH-3 in a Western blot, this can be a great tool for comparison between embryonic and adult protein expression. The fosmid is not detectable in an adult HSN, but if HLH-3 is detected in a Western in an adult preparation, then this shows that the levels of expression are too low to be detected by fluorescence.

Chapter IV: Characterization of the hlh-3 (bc277) phenotype

Chapter 4

CHARACTERIZATION OF THE *hlh-3 (bc277)* PHENOTYPE

4.1 Introduction:

hlh-3 (bc277) mutant hermaphrodites are not Egl (Doonan et al., 2008) but do have HSN pathfinding defects (described in Chapter 3). The *hlh-3 (bc277)* deletion maps to 160 bp upstream of the presumptive *hlh-3* open reading frame and removes 664 bp (Doonan et al., 2008). In our initial characterization of this allele we suggested that *hlh-3 (bc277)* represented a mutation in a regulatory cis-acting region affecting spatial or temporal expression of the gene but not overtly affecting HSN differentiation. To determine whether *hlh-3 (bc277)* HSNs differentiate normally, we performed immunocytochemical detection for 5HT and characterized the location and pathfinding of the stereotypic axon. As shown in Chapter 3 (Table II) we find that 90% of the *hlh-3 (bc277)* HSNs accumulate 5HT (stained positive for 5HT) at the L4 larval stage, but 70% have pathfinding defects. Given that *hlh-3 (bc277)* hermaphrodites are not Egl, I decided to characterize the phenotype in detail. My goal was to answer the overall question, why are *hlh-3 (bc277)* animals normal in egg-laying even though their HSNs have axon pathfinding defects?

4.2 Results:

4.2.1 HSNs in *hlh-3 (bc277)* are required for normal egg-laying:

Normal egg-laying involves a subset of neurons, at least two neurotransmitters, neuropeptides and muscles acting together to bring about a single egg-laying event. The HSNs have been shown to be the command neurons for normal egg-laying (Schafer, 2005; M Zhang et

al., 2008). The mechanism by which the HSNs bring about an egg-laying event involves the stimulation of the vm2 vulva muscle by the neurotransmitter serotonin acting on G protein coupled receptors (GPCRs) and *en passant* synapses that are made by the anteriorly travelling axon (Bastiani et.al, 2003; Desai et al., 1988; Hobson et al., 2006; Xiao et al., 2006). In *hlh-3 (bc277)* animals 70% of the HSNs had pathfinding defects, and zero egg-laying deficient animals (Chapter 3, Table II). To answer the question, whether HSNs are required in *hlh-3 (bc277)* hermaphrodites for normal egg-laying, we made a doubly mutant strain containing the *hlh-3 (bc277)* allele and the gain of function (*gof*) *egl-1 (n1084)* allele. *egl-1* encodes an apoptotic protein with a BH3 (Bcl2 homology region 3) domain and inhibits the product of *ced-9*, the anti-apoptotic Bcl-2 homolog in worms, in the programmed cell death pathway (Conradt & Horvitz, 1998).

egl-1 (n487) is a gain of function mutation in which the HSNs inappropriately undergo apoptosis in a hermaphrodite (Conradt & Horvitz, 1999). Under normal developmental conditions the *egl-1* gene is suppressed in a WT hermaphrodite. The *gof* mutation results in an active *egl-1* gene that leads to inappropriate HSN apoptosis in the hermaphrodites and makes the animals Egl. *hlh-3 (bc277); egl-1 (n487)* animals are Egl to the same extent as *egl-1(gof)* animals are, suggesting that loss of HSNs makes the *hlh-3* (*bc277)* animals deficient in egg-laying (Table IV). This result strongly suggests that the HSNs in *hlh-3 (bc277)* animals, even though they are not fully normal, are required for normal egg-laying.

	Egl animals(%)
N2 (0/27)	0
hlh-3(bc277) (0/27)	0
egl-1(n487) (24/25)	96
hlh-3(bc277); egl-1(n487) (24/27)	89

Table IV: The HSNs in *hlh-3 (bc277)* are necessary for normal egg-laying, even though they do not make normal processes. *hlh-3 (bc277); egl-1 (n487)* hermaphrodites are Egl (egg-laying defective).
Egl assay: Adult hermaphrodites 30 hours past L4 from a synchronized population were bleached and the eggs in each were counted. Count >25 was considered as Egl.

4.2.2 Abnormal axons of *hlh-3 (bc277)* HSNs develop collateral branches that seem to synapse on the vulva muscles:

The HSNs are the command neurons for egg-laying and loss of HSNs makes the animals egg-laying defective (Desai et al., 1988; Schafer, 2005; Singhvi et.al, 2008). However, *hlh-3 (bc277)* animals are still able to lay eggs normally. To address the discrepancy between the behavior, normal egg-laying, and the differentiation defect, abnormal axonal processes, we used the *tph-1::gfp* transgene driven by the *unc-86* promoter to detect the processes of the HSNs. *tph-1* encodes tryptophan hydroxylase, a key enzyme in the serotonin biosynthetic pathway and *unc-86* encodes a POU homeodomain protein, both are expressed in the HSNs (Finney & Ruvkun, 1990; Sze et.al, 2000). We find that the axons of the *hlh-3 (bc277)* HSNs extend collateral branches near the vulva region from the main axon shaft even though the axons have abnormal trajectories; moreover, the abnormal branches seem to make synapses on the vulva muscles (Figs. 4.1, 4.2). These branches extend from the main axonal process and could be equivalent to the ones that develop from the WT axon near the vulva region (Chia et al., 2014).

4.2.3 *hlh-3(bc277)* mutant animals have fewer *rab-3::Mcherry* positive vesicles localized at the synapse:

We used RAB-3 as a synaptic vesicle marker to study the location of synapses in an *hlh-3* mutant background. RAB-3 is a small GTP binding protein that is associated with synaptic vesicles and regulates synaptic transmission (Nonet et al., 1997; Sieburth et al., 2005). The localization of RAB-3::Mcherry (driven by the *unc-86* promoter - *wyIs97 [punc-86::myrgfp; punc-86::MCherry::rab-3; podr- 1::gfp])* to synapses indicates a stable synapse

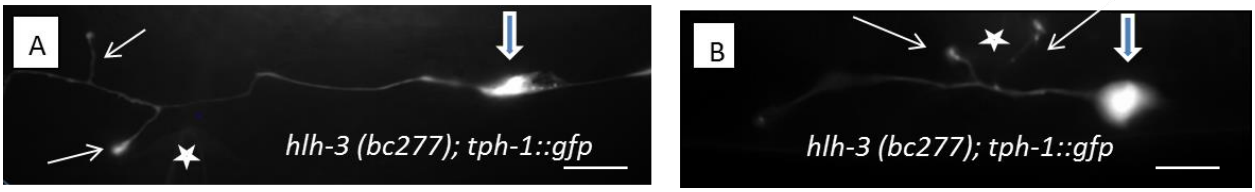


Fig 4.1. *hlh-3 (bc277)* hermaphrodites have HSNs with branches in the vicinity of the vulva muscles with the potential to make synapses. Representative images of *hlh-3 (bc277);tph-1::gfp* HSN axons with abnormal pathfinding and detectable varicosities at the end of the branches. Both panels show lateral view of L4 hermaphrodites with anterior to the left, posterior to the right, ventral side down and dorsal side up. A and B show anteriorly directed *hlh-3 (bc277)* HSN axons that develop collateral branches (white arrows) near the vulva (star). The HSN cell body is indicated with a block arrow. Scale bars represent 10µm.

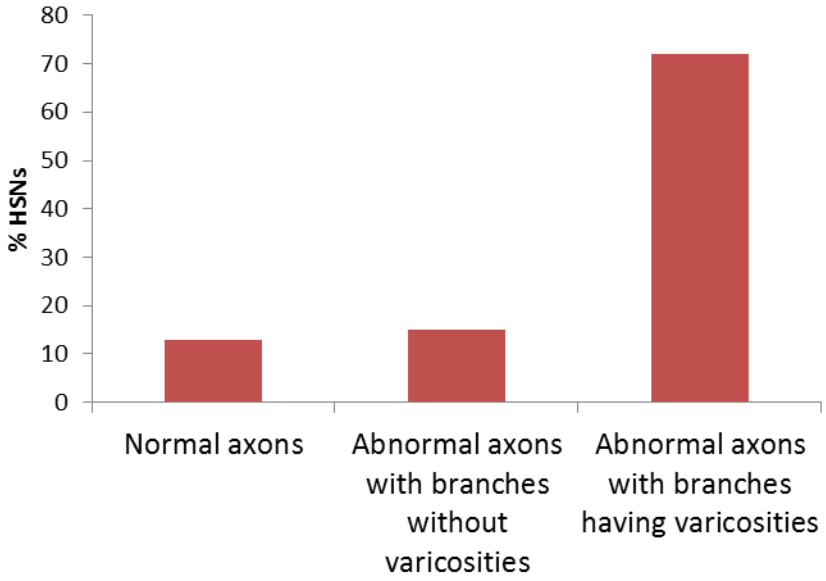


Figure 4.2. *hlh-3 (bc277)* hermaphrodites have HSNs with branches in the vicinity of the vulva muscles with the potential to make synapses. Quantification of *hlh-3 (bc277);tph-1::gfp* animals displaying normal HSNs with axons having a ventral process, abnormal HSN axons with detectable or undetectable varicosities **(**n=52).

(Fischer von Mollard et al., 1990; Sieburth et al., 2005). I analyzed the localization of this marker to the HSN synapses in *hlh-3 (bc277)* and *hlh-3 (tm1688)* mutant backgrounds. I had expected to see similar number of vesicles as WT, localized to the synapses in an *hlh-3 (bc277)* mutant background since I hypothesized that *hlh-3 (bc277)* animals are not Egl because of the stable synapses made by the collateral branches. However, the numbers of vesicles located in an *hlh-3 (bc277)* background were far fewer (only about 50%) as compared to WT indicating that these HSNs do not form stable synapses in all cases (Figs. 4.3, 4.4).

4.2.4 *hlh-3* potentially has multiple isoforms that encode distinct functions:

In Doonan et.al., 2008 we argued that the *hlh-3* WT transcript must consist of two exons encompassing the bHLH coding domain but devoid of a curated first exon because there is no evidence (through RT-PCR or in expressed sequence tags) for a transcript with three exons, and the deletion in *hlh-3 (bc277)* did not result in an egg-laying defect (Doonan et al., 2008). However, the fact that *hlh-3 (bc277)* HSNs are abnormal prompted me to re-examine the role of the curated putative first exon further.

Since what we call exon 1' is transcribed and detected in the hermaphrodites harboring the *bc248* allele (Doonan et al., 2008), there is a possibility that *hlh-3* encodes two isoforms, one of them is made in abundant quantity and can be detected by RT PCR (2 exons) and the other is not very abundant and difficult to detect (3 exons). The hypothetical longer transcript, *hlh-3A* has three exons (1', 1 and 2), and is presumably missing in *hlh-3 (bc277*) animals whereas *hlh-3B*

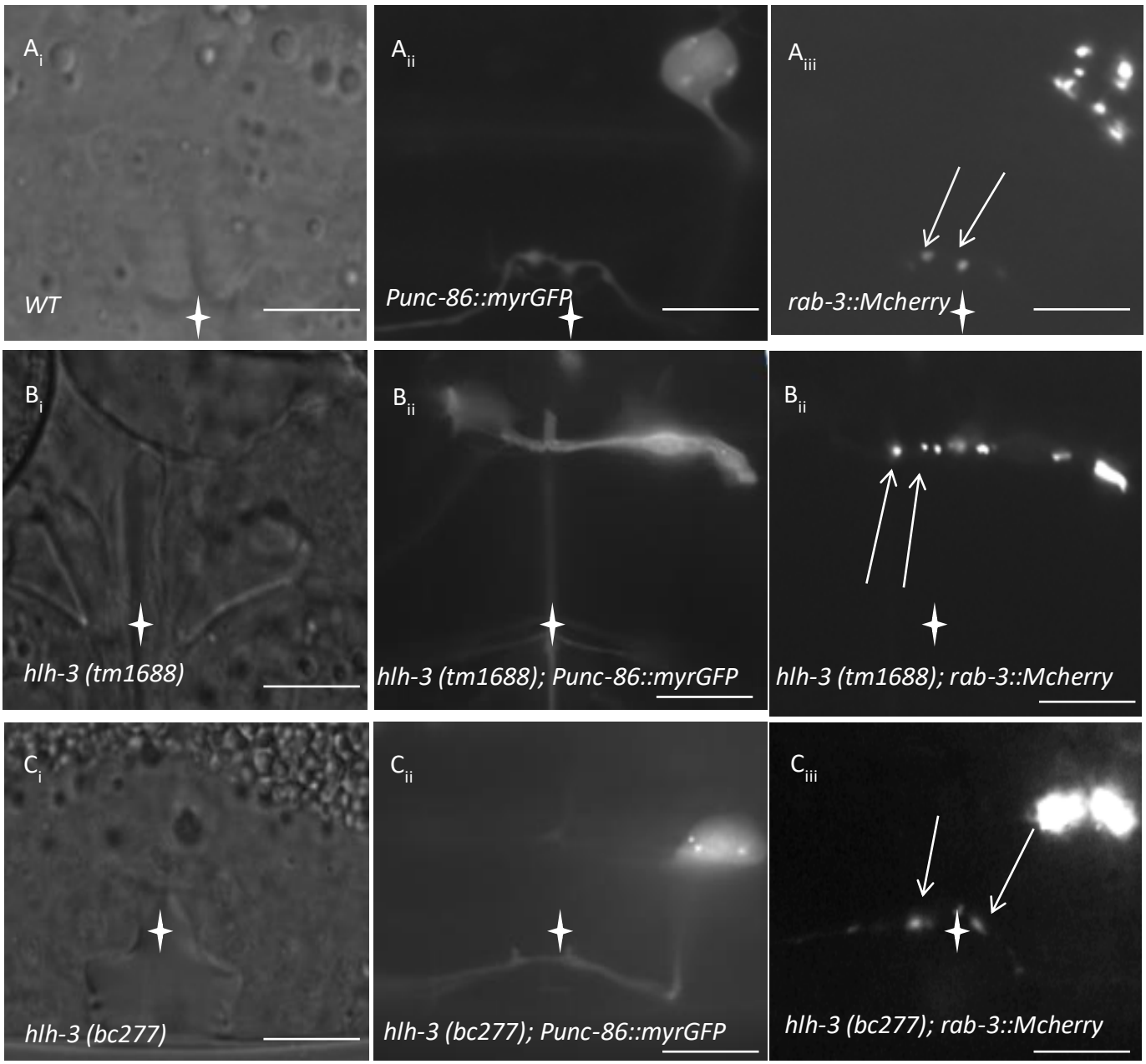


Figure 4.3. The HSNs of *hlh-3 (bc277)* hermaphrodites have fewer Rab-3::Mcherry positive vesicles but they are at normal locations. Rows A, B and C show representative N2 (WT), *hlh-3 (tm1688)* and *hlh-3 (bc277)* individuals expressing *wyIs97 [punc-86::myrgfp; punc-86::MCherry::rab-3; podr- 1::gfp]* imaged with DIC optics (i), or fluorescence microscopy in the region of the developing (Ai and Ci) or fully developed (Bi) vulva. All panels show lateral view of L4 hermaphrodites with anterior to the left, posterior to the right, ventral side down and dorsal side up. Each strain is expressing *Punc-86::myrgfp* (ii) and *rab-3::Mcherry* (iii). Images of the same animal were obtained with a green filter and a red filter. Arrows represent *rab-3::Mcherry* puncta. Puncta in Aiii and Ciii are at presumptive synapses. The *hlh-3 (tm1688)* individual (Bi) is older than those in Ai and Ci but has an immature HSN. ✦ indicates the position of the vulva. Scale bars represent 10µm.

is shorter, contains exons 1 and 2, and is expressed in WT and *hlh-3 (bc277)* animals. To determine whether the first exon (1') is transcribed, we fused a GFP reporter to the 2.7kb upstream promoter region from the transcription start site of the curated exon (*hlh-3A^{promoter}::gfp)* (Fig. 4.5 A). *hlh-3A^{promoter}::gfp* is widely expressed in the embryo including the HSNs but the expression is turned off post embryonically (Figure 4.5 B-E). This pattern of expression is similar to that of the *hlh-3^{fosmid}::yfp* expression pattern described in Chapter 3 (section 3.2.1, Figure 3.4). Thus *hlh-3A^{promoter}::gfp* shows a spatial and temporal expression pattern similar to the *hlh-3* fosmid, and it is transcribed and by extension could be translated. The fact that exon 1' can be transcribed suggests the possibility of a 2nd protein isoform, from a longer transcript which I named as isoform A, HLH-3A. The protein encoded by the shorter transcript with 2 exons readily detected in RT PCR was named HLH-3B. Analysis of expression patterns (discussed in Chapter 3) of a GFP reporter fused to a 3.3 kb upstream region to the 1st exon (*hlh-3^{promoter}::gfp)*, the fosmid (*hlh-3^{fosmid}::yfp)* and the 2.7kb reporter construct (*hlh-3^{bc277promoter}::gfp)* showed similar pattern to *hlh-3A^{promoter}::gfp*. These similarities in expression patterns show that transcription can be initiated and include the anomalous exon (1'). The only difference between the expression of the tagged fosmid and the shorter transcriptional GFP reporters is that in the latter case there is detectable GFP expression in other cells post embryonically but not the HSNs. This prolonged expression could reflect the presence of additional regulatory elements in the fosmid and missing in the shorter transcriptional reporters. However, based on the varying phenotypes in *hlh-3 (tm1688)* and *hlh-3 (bc277)*, I propose that the two isoforms have different roles in HSN differentiation. The genomic sequences in *hlh-3 (bc277)* can only direct expression of HLH-3B as exon 1' is deleted. Since *hlh-3 (bc277)* hermaphrodites have HSNs with abnormal

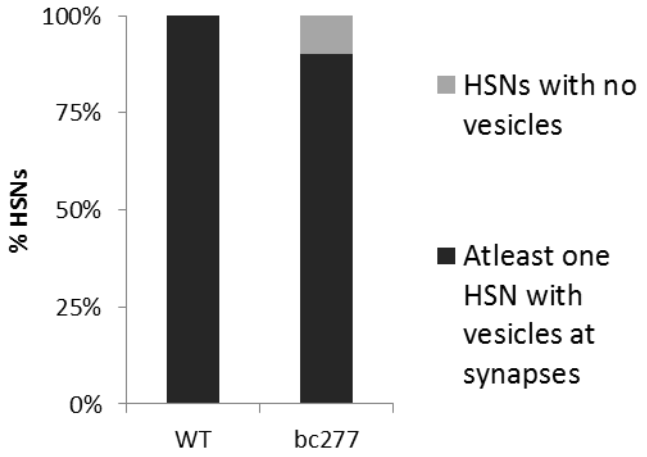


Figure. 4.4. The HSNs of *hlh-3 (bc277)* hermaphrodites have fewer RAB-3::Mcherry positive vesicles but they are at normal locations. Quantification of the number of HSNs in each strain with at least one HSN with a RAB-3::Mcherry positive vesicles at a branch (*wyIs97 [punc-86::myrgfp; punc-86::MCherry::rab-3; podr- 1::gfp])*WT n= 52, *hlh-3 (bc277)* n=48.

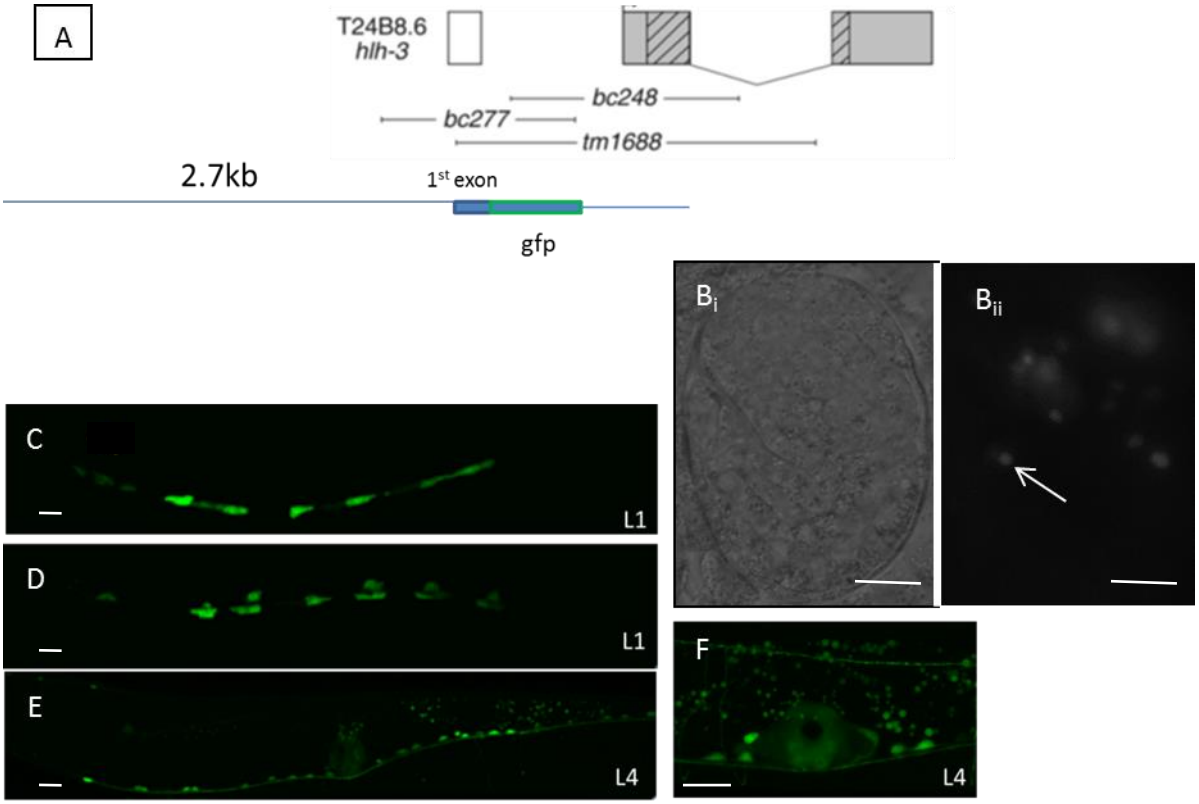


Figure 4.5. The first curated exon [1'] (deleted in *bc277)* can be transcribed. (A). Diagram of a translational fusion construct (*hlh-3A*^{prom}*::gfp)* that includes the region upstream from the 1st curated exon with the 1st eight amino acids fused to the GFP coding sequence. (B) This fusion construct is expressed in the embryonic HSNs (B_i - DIC image, B_{ii} - fluorescent image of the same embryo as B_i). Expression is also detected in the P cells at the L1 stage (C and D) and perdures in some P cell descendants through the L4 stage (E and F). Expression is not detectable in the postembryonic HSNs. Panels C-F show a lateral view of hermaphrodites with anterior to the left, posterior to the right, ventral side down and dorsal side up. Scale bars represent 10µm.

pathfinding but are mature (i.e. they make serotonin and most reach their target) then isoform HLH-3B must be sufficient for promoting HSN maturation. The genomic sequences in allele *hlh-3 (tm1688)* cannot generate transcription of either isoform as the deletion encompasses both exon 1'or exon 1. Since the absence of both isoforms results [as in *hlh-3(tm1688)*] HSNs with abnormal pathfinding and maturation defects (discussed in Chapter 5) then I propose that HLH-3A is necessary for normal pathfinding.

One way to test whether the *hlh-3A* transcript exists in wild type embryos is by quantitative PCR. I have shown the expression of the curated axon (1') in the presumptive HSNs is similar to the other constructs. RT PCR analysis of *hlh-3 (bc248)* derived RNA can detect the transcription of exon 1' (Fig. 3.3), possibly because in the absence of the genomic sequences for the abundant *hlh-3B* mRNA, the primers are able to bind to the low abundance mRNA containing the curated exon. Quantitative PCR should help in the detection of all *hlh-3* gene expression products in the WT (if they exist) and different mutant backgrounds. Our working hypothesis is that the *hlh-3A* transcript is made in very low amounts as compared to *hlh-3B*, hence undetected in WT and *hlh-3 (bc277)* animals. Yet, it is functional when HLH-3B is absent in *hlh-3 (bc277)* animals but cannot fully fix the HSN differentiation defects.

4.2.5 HLH-3B rescues HSN defects:

To address whether HLH-3B is involved in HSN maturation, I performed HSN-specific rescue by expressing the *hlh-3B* cDNA using the *unc-86* promoter. Although *unc-86* is expressed in other cells in the embryo, the HSNs are the only cells in the egg-laying circuit that express *unc-86* (Finney & Ruvkun, 1990b). Surprisingly the *hlh-3B* cDNA was able to rescue the maturation phenotype (discussed in Chapter 5) as well as the pathfinding phenotype (Figure 4.6,

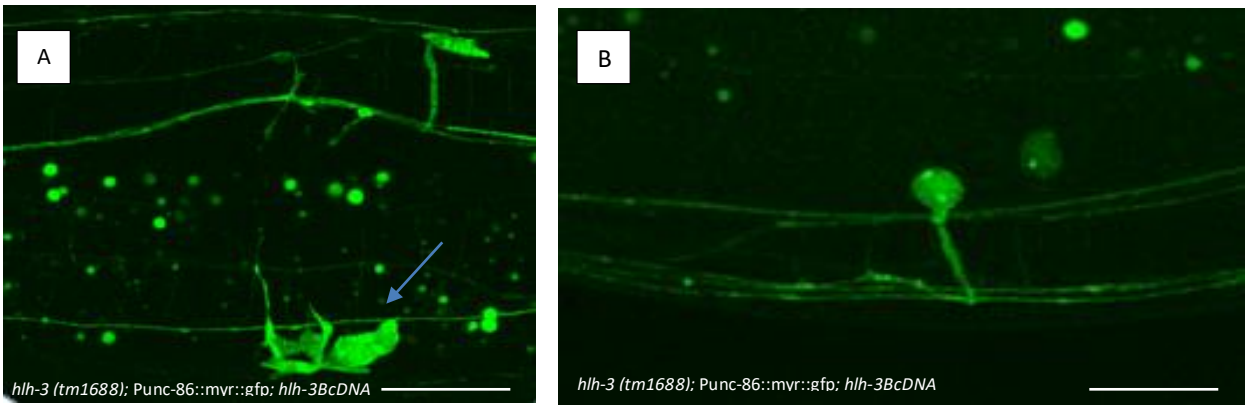


Figure 4.6. Expression of HLH-3B rescues maturation defect of HSNs in *hlh-3 (tm1688)*. Representative images of mutant *hlh-3* hermaphrodites expressing the myrystoylated GFP and the *hlh-3B* cDNA construct (A) Ventral view of an *hlh-3 (tm1688)* rescued hermaphrodite with one normal and an abnormal HSN (arrow). (B) Shows a lateral view of another rescued hermaphrodite with two normal HSNs (the 2nd HSN is out of focus). Scale bars represent 10µm.

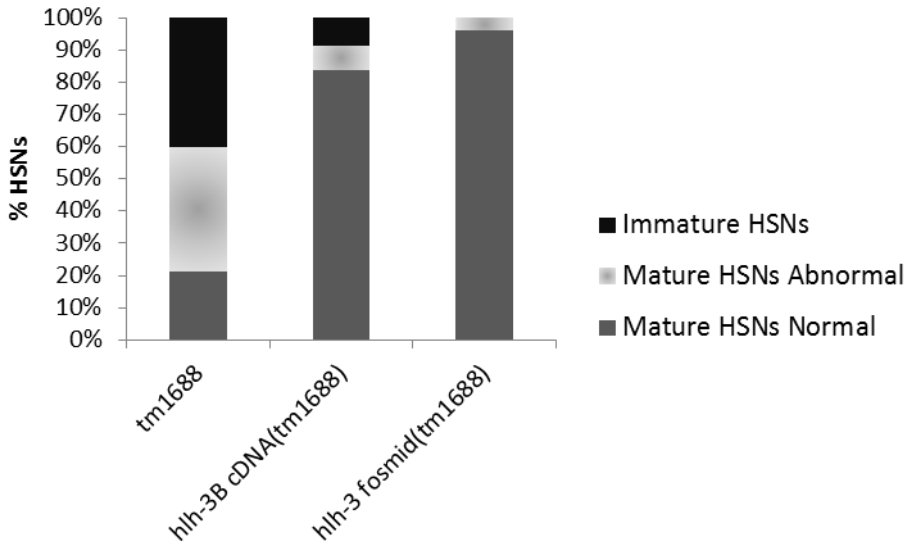


Figure 4.7. Expression of HLH-3B rescues maturation defect of HSNs in *hlh-3 (tm1688)*. Quantification of HSN maturation rescue by expression of *hlh-3BcDNA* or *hlh-3* fosmid (WRM0625aA01). *tm1688 n=80, hlh-3cDNA (tm1688) n=75, hlh-3 fosmid (tm1688) n=58.*

4.7). This finding was surprising because I had hypothesized HLH-3A to be necessary for normal HSN axon pathfinding.

A possible explanation for this observation is that this may be a result of high copy number in the transgenic line expressing *punc-86::hlh-3B*. It is also possible that HLH-3A is made in very small amounts with HLH-3B being the dominant isoform. We propose that HLH-3A cannot fix the differentiation defects by itself (as is the case in *hlh-3 (bc277)* animals) but when HLH-3B is overexpressed it can make HLH-3A redundant. Quantitative RT-PCR (discussed above) should be able to help us differentiate between these possibilities.

4.2.6 *hlh-3 (bc277)* is not a hypomorphic allele:

hlh-3 (bc277) animals are not Egl but 65% of *hlh-3 (tm1688)* animals are. Likewise, *hlh-3 (bc277) / hlh-3 (+)* and *hlh-3 (tm1688) / hlh-3 (+)* heterozygous animals are not Egl, suggesting that one wild type copy of *hlh-3* is sufficient to maintain normal egg-laying in these animals (Doonan et.al, 2008). To address the question whether *hlh-3 (bc277)* has some residual function we created a trans-heterozygote between *hlh-3 (bc277) / hlh-3 (tm1688)* to analyze the egg-laying defect. We hypothesized that *hlh-3 (bc277)* makes HLH-3B, which is sufficient for maturation of HSNs and egg-laying and HLH-3B is enough for normal egg-laying. As shown in Figure 4.8, the trans-heterozygote between *hlh-3 (bc277) / hlh-3 (tm1688)* retains more eggs than *hlh-3 (bc277)* homozygotes but fewer than *hlh-3 (tm1688)* homozygotes.

Although the statistical analysis revealed that the number of retained eggs in a trans-heterozygote is not significantly different from that of either homozygous mutant alone, the trans-heterozygote is somewhere in between. This suggests that the trans-heterozygote is not better or worse than either homozygous mutant alone. Thus, having a single dose of HLH-3B in *hlh-3 (bc277) / hlh-3 (tm1688)* is enough to make the animals better at laying eggs and look

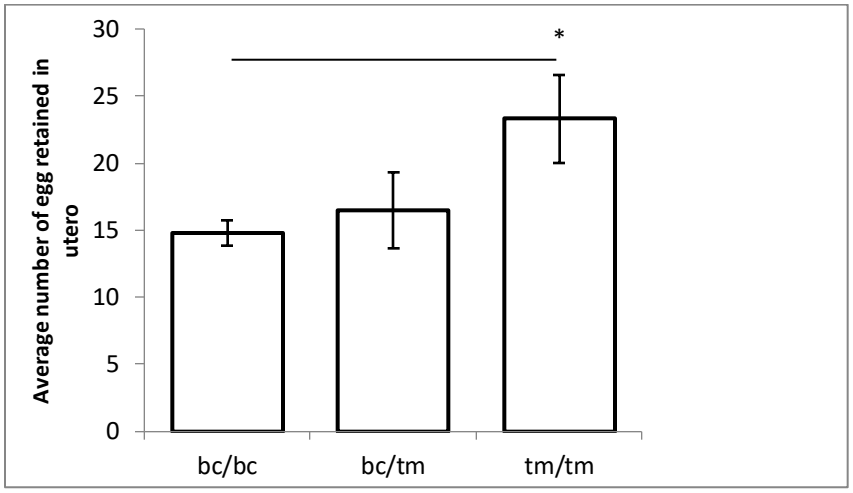


Figure 4.8. The trans-heterozygote does not rescue the *hlh-3 (tm1688)* egg-laying defect. The number of eggs retained by the trans-heterozygote (*bc277/tm1688*) is not significantly different from *hlh-3 (tm1688/tm1688)*

more like *hlh-3 (bc277)* hermaphrodites. Moreover, *hlh-3 (bc277)* homozygotes retained significantly fewer numbers of eggs than *hlh-3 (tm1688)* homozygotes (Doonan et al., 2008). This suggests that having one copy of *hlh-3B* is enough to provide egg-laying like that of having two wild type copies [as is the case in *hlh-3(bc277)*]. However, it does not completely rescue *hlh-3 (tm1688)* that has no HLH-3B as the number of eggs retained in the trans-heterozygote is not significantly different from homozygous *hlh-3 (tm1688)*.

4.3 Discussion:

My findings reveal that the *hlh-3* gene has the potential to encode more than one functional entity. Hermaphrodites harboring the deletion allele *hlh-3 (bc277),* a deletion that maps to 160 bp upstream of the presumptive *hlh-3* open reading frame and removes 664 bp, have partially differentiated HSNs. My analysis shows that *hlh-3 (bc277)* HSNs have a well-developed cell body and an axon but a majority of their axons have abnormal pathfinding. The difference between the wild type and *hlh-3 (bc277)* alleles is the 664bp genomic region that also includes an exon with an ORF that can potentially be transcribed. RT PCR analysis did not detect a transcript and no ESTs have detected this exon either. Nevertheless, this genomic region has the potential to serve as an enhancer or encode a portion of a novel isoform that can fulfill a different role than the established shorter isoform or potentiate its role. The shorter isoform is made and detectable in *hlh-3 (bc277)* individuals (Doonan et al., 2008), hence they are able to mature like wild type HSNs; yet the presence of the shorter isoform is not sufficient to provide all function. It is not uncommon for genes to encode multiple isoforms with unique functions (Lo et al., 2008; Mohamed & Chin-Sang, 2011; Stavoe et.al, 2012).

Although we have no direct evidence that this exon is normally transcribed and part of a longer transcript, the fact that these hermaphrodites lay eggs comparably to wild type but have

HSNs that are not fully normal suggests that the transcript detected in *hlh-3 (bc277)* is not sufficient to provide all function. Here we show that a construct with the regulatory sequences upstream of exon 1' and the coding sequences of the first few amino acids can be transcribed in transgenic animals. Moreover, this construct shows a temporal expression pattern similar to the fosmid. The fact that it is transcribed, strongly suggests it could be translated. Since exon 1' can be transcribed and the *hlh-3 (bc277)* HSNs are not fully normal we suggest that it is likely a 2nd protein isoform exists, encoded from a longer transcript, which we named isoform HLH-3A. The shorter transcript with 2 exons readily detected in RT PCR was named HLH-3B.

hlh-3 (bc277) hermaphrodites show HSNs with pathfinding defects. This observation did not make sense when considering that the hermaphrodites had normal egg-laying. The HSNs are the command neurons in the egg-laying pathway (Desai & Horvitz, 1989). Multiple lines of evidence have shown that although the HSNs are the major neurons and serotonin is the major neurotransmitter for egg-laying, there are other neurons like the VCs or the uterine uv1 cells that may lead to normal egg-laying (Bany et.al, 2003; Waggoner et.al, 1998; Mi Zhang et.al, 2010b). This may be the reason why, in spite of abnormal synapses and fewer synaptic vesicles, the *hlh-3 (bc277)* animals can still lay eggs normally.

My analysis of the *tph-1::gfp* transgene (*mgIs42 [tph-1::GFP + rol-6 (su1006)]*) in the *hlh-3 (bc277)* animals, support the conclusion that there are branches emerging from the main axon and they appear to make synapses on the vulva muscles. I know that the development of these branches in *hlh-3 (bc277)* animals requires the UNC-40/UNC-6 guidance mechanism suggesting that they responded to the same signals that led to normal axon development (Appendix). One possible reason for these ectopic branches could be the timing at which these axons develop. In *unc-86* mutant animals, there is a well-developed anteriorly projecting axon at

the L1 stage (Olsson-Carter & Slack, 2011). I considered that perhaps *hlh-3 (bc277)* animals had a precocious axon, when there is no guidance molecule UNC-6/Netrin to direct its growth, or perhaps the receptor UNC-40 was not properly localized, either scenario could lead to development of the abnormal branches that we see in *hlh-3 (bc277)* animals. However, when compared to wild type animals, we do not detect developmental timing defects in *hlh-3 (bc277)* animals (Appendix, section 7.6). The RAB-3::Mcherry vesicle marker (*wyIs97 [punc-86::myrgfp; punc-86::MCherry::rab-3; podr- 1::gfp])* allows us to see vesicles at the synapses (varicosities) and we detect at least one HSN, if not both, even when they are abnormal in pathfinding, to form synapses (varicosities). The ability of *hlh-3 (bc277)* HSNs to form synapses even though the pathfinding is not normal is likely to explain why the animals have normal egg-laying. In the absence of HSNs, *hlh-3 (bc277)* hermaphrodites become Egl. The *lof* deletion mutants, *hlh-3 (bc248)* and *hlh-3 (tm1688)* do not have these branches and are Egl, suggesting that these branches and the HSNs are necessary in *hlh-3 (bc277)* animals for normal egg-laying.

4.4 Future directions:

4.4.1 Rescue of the *hlh-3* mutant phenotypes with HLH-3A isoform:

According to my analysis, HLH-3A is the isoform that is not made in either *hlh-3 (bc277)* or *hlh-3 (tm1688)* mutant animals and plays a role in axon pathfinding of HSNs whereas HLH-3B is made *hlh-3 (bc277)* animals. I have shown that *hlh-3A* can be transcribed and translated. We have generated an integrated strain with *hlh-3AcDNA::gfp* driven by the *unc-86* promoter (Nacke and Alfonso., unpublished). This strain can be crossed into the *hlh-3 (bc277)* mutant background for rescuing the axon pathfinding phenotype. Phenotypic rescue by HLH-3A would provide further evidence of its function and help explain the *hlh-3 (bc277)* phenotype.

This strain can also be crossed into the *hlh-3 (tm1688)* mutant background. Although 65% HSNs do not mature in these animals, 60% of the mature HSNs have pathfinding defects. It will be interesting to see if HLH-3A rescues the pathfinding defect of the already matured HSNs further providing insight whether the two isoforms act in conjunction or independently.

4.4.2 Rescue of axon pathfinding defects by *hlh-3BcDNA* in *hlh-3 (bc277):*

HLH-3B was able to rescue the maturation phenotype of the *hlh-3 (tm1688)* mutant animals. However, it also rescued the pathfinding phenotype in these animals. This is surprising but could be because of overexpression of HLH-3B due to the strong *unc-86* promoter driving the *hlh-3BcDNA* leading to compensation for the lack of HLH-3A. In order to test this, the construct driving *hlh-3BcDNA* can be crossed into *hlh-3 (bc277)* animals. If overexpression leads to compensation, then axon pathfinding of *hlh-3 (bc277)* animals should be rescued.

Chapter V: Characterization of the fate and maturation defects of the HSNs in the absence of hlh-3 function

Chapter 5

CHARACTERIZATION OF THE FATE AND MATURATION DEFECTS OF THE HSNs IN THE ABSENCE OF *hlh-3* FUNCTION

5.1 Introduction:

Neuronal birth and differentiation are important for a functional nervous system. Neurons and glial cells develop from a set of neural progenitor cells. The highly specialized and complex nervous system with its intricate connections develops due to the activity of the proneural genes that impart a final fate to the developing progenitor cells. Various proneural genes play a role in the differentiation of the post-mitotic neurons. Proneural genes belonging to the basic helix loop helix family (bHLH) play an important role in the process of fate acquisition (Kiefer, 2005; Wilkinson et.al, 2013b)

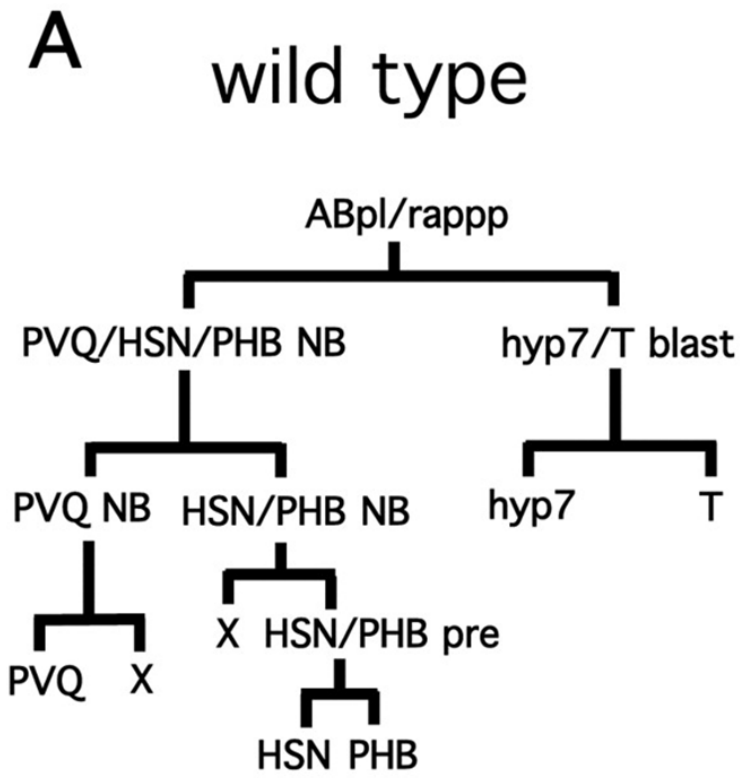
Proneural genes are necessary and sufficient for the generation of neuronal populations and ectopic expression of proneural genes leads to susceptible cells developing into neurons. In addition to their role in the generation of neuronal lineages, Ac/Sc genes also serve as neuronal subtype differentiation factors. They have been shown to reprogram neuronal lineages based on gain of function (*gof*) experiments (Castro et al., 2006; Skeath & Doe, 1996). Depending on their time of expression they can specify the fate of a differentiating neuron. Thus, proneural Ac/Sc genes can play a dual role depending on the context that they are expressed in. Early expression within a specific lineage determines the generation of that lineage and later expression leads to fate determination of a particular neuronal subtype. Thus they seem to serve a dual purpose, that of lineage determination and terminal differentiation (Powell & Jarman, 2008).

We have reported previously that total loss of *hlh-3* function results in hermaphrodites with defects in the terminal differentiation of the hermaphrodite specific neurons (HSNs) and as

a consequence are egg-laying defective (Egl) (Doonan et.al, 2008). The HSNs have been shown to be important for normal egg-laying in *C. elegans*. They form part of the circuit, along with the VCs and vulva muscles that regulate egg-laying (Schafer, 2005). *hlh-3* is a homolog of *Mash-1,* a gene that is important for the generation of neurons in the autonomic nervous system in vertebrates. However, unlike *Mash-1, hlh-3* does not seem to be necessary early in the lineage as the HSNs are born and migrate to the proper location behind the vulva. Interestingly *hlh-3* behaves more like its Drosophila homolog *Asense*, a gene that is expressed later in the neural precursor cells and is necessary for terminal differentiation. (Doonan et al., 2008; Jarman et.al, 1993). In the absence of *hlh-3* function the HSNs are not detectable by the usual late differentiation HSN markers; that is, they do not make serotonin and have pathfinding defects. Thus *hlh-3* seems to be involved in late stage differentiation. When we started this project, we did not know what happened to the HSNs that were not detectable. Thus, we set out to determine whether they were present and if so at what stage.

5.1.1 Development and migration of HSNs:

The HSNs are born in the tail of both XX and XO embryos. They develop from the PVQ/HSN/PHB neuroblast, which undergoes further divisions to give rise to the PVQ, the HSN and the PHB (Fig. 5.1). In XO embryos HSNs undergo programmed cell death shortly after their birth (Conradt & Horvitz, 1998). In XX embryos they migrate to their normal position in the mid-body near the vulva before hatching. The cell bodies then send their axons ventrally and



Figue 5.1. HSNs develop from the PVQ/HSN/PHB neuroblast through a series of divisions.

(Taken from C. Andrew Frank et al. Development 2003; 130:6507-6518)

anteriorly to synapse on the vulval muscles and finally to receive synaptic input on the nerve ring (Desai et al., 1988; Shen & Bargmann, 2003b). *hlh-3 (bc248)* mutant animals show normal generation of the HSNs and migration to their proper position (Doonan et al., 2008)

5.1.2 Late differentiation defects:

The features of late differentiation of the HSNs include 5HT immunoreactivity, axonal pathfinding and synapse formation. The HSNs are serotonergic and the axons follow a typical path making synapses on the vulval muscles *en route* to the nerve ring (Desai et al., 1988). *hlh-3 (lof)* mutant animals have defects in their HSNs. As mentioned in Chapter 3, *hlh-3* mutant hermaphrodites had 65% HSNs that were not detectable by anti 5HT staining and over 60% of the detectable HSNs show abnormal pathfinding. Analysis of the HSNs using a *tph-1::gfp* transgene showed similar results as 5HT staining (Doonan et al., 2008) (Chapter 4, section 4.2.2). Since the *tph-1* reporter is detectable in other serotonergic cells besides the HSNs in the absence of *hlh-3* function, then the absence of detectable serotonin in the HSNs appears to be a problem that goes beyond regulation of the biosynthetic pathway. Likewise, the problem in pathfinding is beyond the presence of the pathfinding receptor and its ligand. HSN axons have a stereotypical pathfinding in which the axon first develops ventrally and then turns anteriorly, making 'en passant' synapses with the vulva muscles before continuing anteriorly to the nerve ring. In contrast, *hlh-3* mutants have various defects – some axons don't have a ventral process, some axons had a posterior process and some axons were seen wandering (Doonan et al., 2008).

It is evident that *hlh-3* does not play a role in the initial differentiation of the HSNs but is needed at the later stages (Doonan et al., 2008, current work). I wanted to better understand the

role of *hlh-3* in HSN differentiation. The absence of detectable HSNs in *hlh-3* mutants raised the possibility of apoptotic cell death or fate transformation. Alternately, it is also possible that differentiation was halted due to developmental arrest. My analysis has shown that the latter is the case. The HSNs are arrested in the lamellopodia stage during development and I have called this stage as the maturation phase. The HSNs that are absent do not mature past the lamellopodia stage and thus do not form the necessary connections and final differentiation features.

5.2 Results:

5.2.1 *hlh-3* mutant HSNs do not undergo programmed cell death:

Programmed cell death is a genetic mechanism by which organisms maintain the appropriate cell number by activating a group of proteases called caspases that trigger a cascade of events leading the death of a cell (Elmore, 2007). *C. elegans* development is an ideal model system to study this phenomenon as 131 cells undergo programmed cell death at various points, which are invariant during development. There is also sex specific cell death as the HSNs are born in both sexes but die in the males (Conradt & Horvitz, 1999; Metzstein et.al, 1998). The absence of detectable HSNs in *hlh-3 (bc248)* mutant hermaphrodites could be a result of HSNs inappropriate apoptosis in XX embryos. *ced-3* encodes a caspase and is one of the cell death defective gene that regulates apoptosis in *C. elegans.* It's protease activity is essential to promote cell death (Xue et.al, 1996; Yuan et.al, 1993). Absence of *ced-3* function leads to the survival of cells that normally die in the animal (Liu & Hengartner, 1999).

To test whether undetectable HSNs undergo programmed cell death, I made a double mutant between *ced-3 (n717)* and *hlh-3 (bc248)* mutant animals. If the HSNs underwent apoptosis in *hlh-3 (bc248)* hermaphrodites then more HSNs would have been detected by

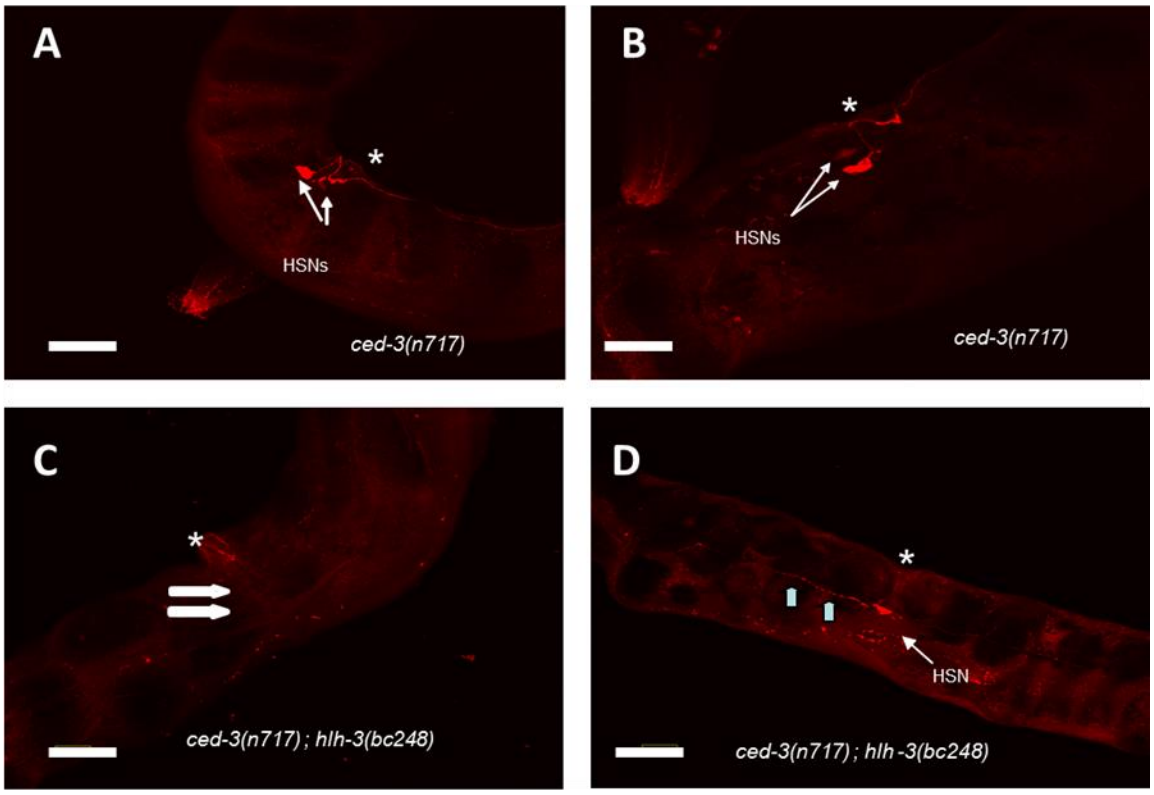


Figure 5.2. Loss of function mutations in *hlh-3* do not result in inappropriate cell death of the HSNs. Representative 5HT immunoreactive HSNs in the *ced-3 (n717)* worms (A, B, n=31) and *ced-3 (n717); hlh-3 (bc248)* double mutant (C, D, n=44). (A, B) Two 5HT immunoreactive HSNs in a *ced-3(n717)* worm show normal pathfinding and resemble those in wild type worms (data not shown). (C) No 5HT immunoreactive HSNs are detectable (block arrows) in this doubly mutant individual. (D) Only one 5HT immunoreactive HSN, with abnormal pathfinding (block arrows), is detected in this individual. These patterns (C, D) resemble *hlh-3 (bc248)* mutants alone (data not shown). Asterisk indicates the vulval region. Panels A, B, C are lateral views with anterior to the right. Panel D is ventral view. Scale bars represent 10µm.

5HT staining in the double mutant. My analysis shows that is not the case; 67% HSNs were absent in *hlh-3 (bc248); ced-3 (n717)* double mutant as compared to 65% in *hlh-3 (bc248)* alone (Fig. 5.2). This indicates that the HSNs in *hlh-3 (bc248)* animals do not die. Two other possible reasons for non-detection are: they either differentiate into another cell type or stall in their differentiation program.

5.2.2 Absence of 5HT immunoreactivity in the HSNs of *hlh-3* mutant animals does not represent cell fate change:

All cells develop from a zygote via mitosis; hence the differences in gene specificities in individual cells develop over time. These gene regulatory networks specify the final fate of cells. Loss of these specification factors lead to change in fate of the cell to another cell type (Maduro, 2010). Fate transformation to sister cells in bHLH mutant animals is not uncommon in *C. elegans* as *hlh-14* animals transform the fate of PVQ/HSN/PHB neuroblast to its sister cell, the hyp7/T blast (Frank et al., 2003)(Fig. 5.3). I tested the possibility that the HSNs not detected by anti 5HT staining may have undergone a fate transformation to their sister cells, the PHBs. To study fate transformation I used the *srb-6::gfp* reporter transgene that is expressed in PHBs (Troemel, Chou, Dwyer, Colbert, & Bargmann, 1995) and introduced it in the *hlh-3 (bc248)* mutant background. Any fate transformation should have led to detection of increased number of cells with *srb-6::gfp*. However, no additional cells were detected in the mutant background as compared to wild type indicating that there is no fate transformation of the HSNs to PHBs in *hlh-3* animals (Fig. 5.4)

DiI is a dye that stains phasmid neurons such as the PHBs. It is a fluorescein dye that is taken up by the neurons through their exposed sensory cilia and is transported retrogradely such

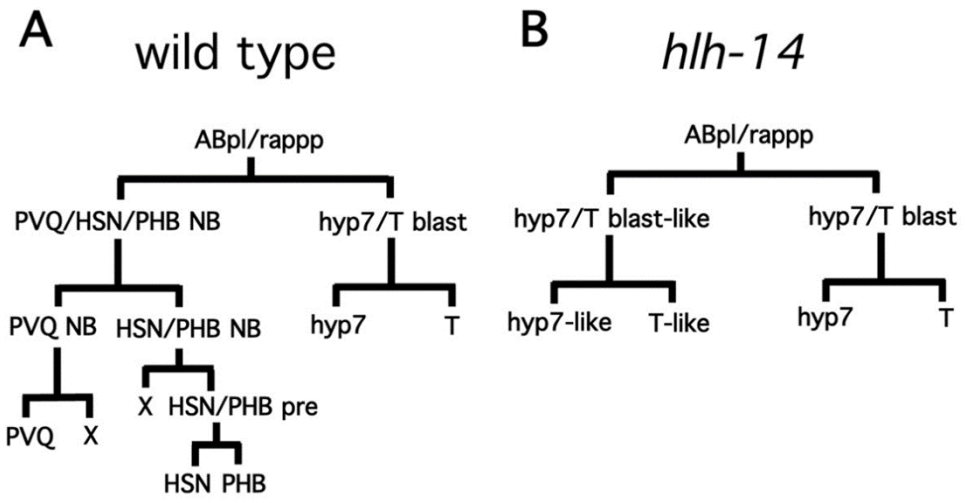


Figure 5.3. In *hlh-14* mutants (B) PVQ/HSN/PHB neuroblast undergoes fate transformation to its sister cell (hyp7/T blast)
(Adapted from C. Andrew Frank et al. Development 2003; 130: 6507-6518)

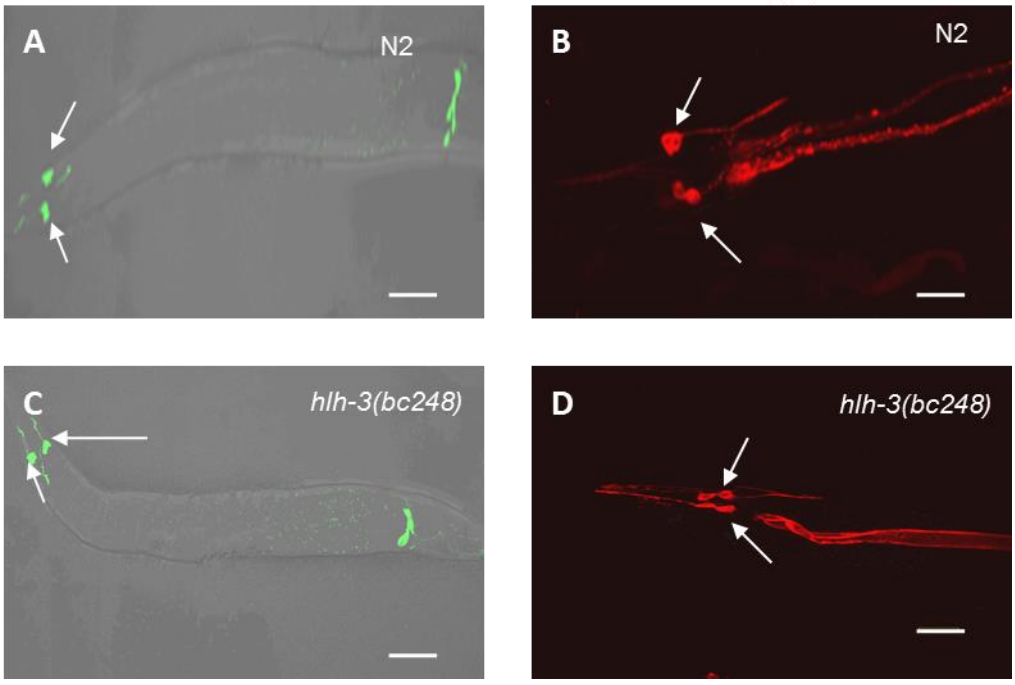


Figure 5.4. HSNs do not undergo fate change to their sisters in *hlh-3* mutant animals
Two phasmid neurons (arrows) in the tail express *srb-6::gfp*, (*gmIs12 [srb-6::gfp]*) in WT (A) as well as *hlh-3 (bc248)* (C) animals by. No extra cells are detected in the absence of *hlh-3* function. Similar results were seen with DiI staining (B, D). Head is to the right and tail is to the left. Scale bars represent 10µm.

that it stains the entire neuron (Hedgecock et.al, 1985; Perkins et.al, 1986). No additional cells were detected by DiI staining in *hlh-3* mutant animals (Figure 5.4 B and D).

5.2.3 Pioneer neuron, PVQ, appears normal in *hlh-3(bc248)* mutants:

In *C. elegans,* the PVQ pioneer neurons guide the HSNs along their anterior paths in the ventral nerve cord to their final destination in the nerve ring (Troemel et al., 1995). Since *hlh-3* was expressed in the PVQs (Doonan, 2006), I decided to test whether the pathfinding defects of the HSNs are due to the abnormal pathfinding of the PVQs. We crossed the *sra-6::gfp* reporter transgene expressed in the PVQs in a *hlh-3* mutant background and traced the path of PVQ axons (Frank et al., 2003). These neurons showed no pathfinding defects indicating no abnormality in the guidance of the pioneer neuron (Fig 5.5).

5.2.4 Total absence of *hlh-3* function results in HSNs with maturation defects at the L4 stage

Proneural bHLH proteins of the Ac/Sc family are responsible for the specification and differentiation of neuronal populations in vertebrates and Drosophila (F Guillemot, 1999; Kiefer, 2005). However, in *C. elegans* the functions of these proteins is more restricted to specific lineages instead of for example all motor neurons. For example, *hlh-14* mutants show the role of this gene in the lineage that gives rise to three neurons, including the HSNs (Frank et al., 2003). Likewise, although *hlh-3* expression in hermaphrodites is widespread and detected in numerous cells in the embryo, the neuronal defects seem to be restricted to the egg-laying

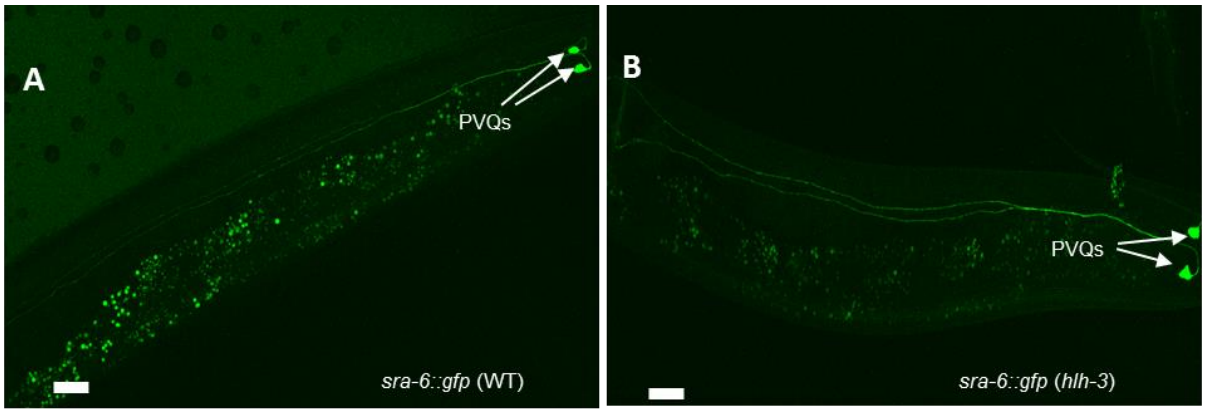


Figure 5.5. Differentiation of the PVQ pioneer neurons is not affected in hlh-3 mutant hermaphrodites. Representative images of the expression of *sra-6::GFP* *(kyIs39[sra-6::GFP + lin-15(+)])*in the WT(n=25) and *hlh-3 (bc248)* mutant background (n=31). The *sra-6::GFP* array is expressed in the pioneer neuron PVQ located in the tail (Troemel et.al., 1995). No difference was observed in the expression pattern in WT or *hlh-3 (bc248)* mutants. Ventral oblique view with anterior to the left. Scale bars represent 10µm.

machinery neurons, HSNs and VCs (Doonan, 2006, Chapter 1). In *hlh-3 lof* mutants the HSNs are generated but 60% of them are not detected by terminal differentiation markers.

To address the question, what is the fate of the HSNs that are not detectable by the various terminal differentiation markers, we utilized a strain containing a myristoylated GFP fused in frame with the *unc-86* promoter (wyIs97 [*Punc-86::myrgfp; Punc-86::mCherry::rab-3; Podr- 1::gfp*], an integrated array) kindly provided by Kang Shen) (Chia et al., 2014). This construct is expressed throughout the life of the animals and allows us to see the HSNs in their entirety, the only cells in the vulva region that express *unc-86.* We find that the HSNs in *hlh-3 (tm1688)* are stalled in their maturation (Fig. 5.6 B). Differentiation of HSNs involves multiple aspects (discussed in the Introduction). The HSNs are born in the tail of the embryo and migrate to the midbody before they hatch. Around the L3 stage axonal development starts by development of multiple lamellopodia and filopodia out of which one process stabilizes to give rise to the mature axon (Adler et al., 2006). Since HSN development is a multi-step process, differentiation defects could involve one or many of the aspects referenced above. In this work I define maturation as the formation of a stable axonal process from the various lamellopodia and filopodia. Moreover, I define HSN maturation as a sub-category of axonal pathfinding because although two alleles show differentiation defects in axonal pathfinding *hlh-3 (tm1688)* is different from *hlh-3 (bc277)* in some aspects of maturation. That is, at equivalent developmental stages (L4), *hlh-3 (tm1688)* HSNs are delayed in the formation of a stabilized axonal process and only have lamellopodia whereas the HSNs in WT and *hlh-3 (bc277)* hermaphrodites have progressed in their maturation and have formed a stereotypical axonal process. The new finding is that 67% of *hlh-3 (tm1688)* HSNs are immature; this observation is consistent with the

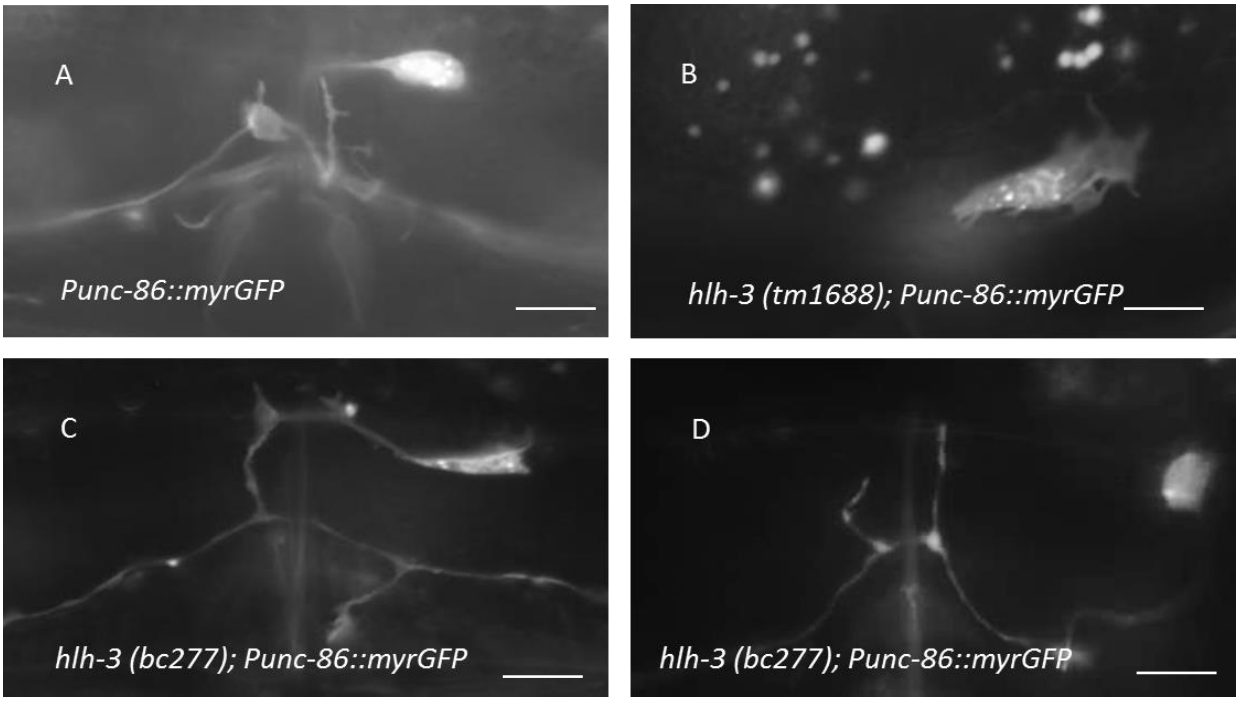


Figure 5.6. *hlh-3 (bc277)* HSNs are more mature than the HSNs in *hlh-3 (tm1688)* hermaphrodites but they are not entirely normal. All panels show lateral view of L4 hermaphrodites with anterior to the left, posterior to the right, ventral side down and dorsal side up. HSNs are detected with cell bodies and processes. All strains harbor wyIs97 [Punc-86::myrgfp; Punc-86::mCherry::rab-3; Podr- 1::gfp], an integrated array where the *unc-86* promoter drives expression of myristyolated GFP (A) Representative image of WT HSN showing stereotypic, normal pathfinding n=60 (a well-developed HSN axon traveling ventrally first, a short dorsal growth and then anterior growth towards the nerve ring). (B) Representative image of *hlh-3 (tm1688)* HSN arrested in its development n = 80 (lamellopodia and filopodia are detectable but no mature ventral process is present). (C and D) Representative image of *hlh-3 (bc277)* hermaphrodites where HSNs have a well-developed axon [compared to *hlh-3 (tm1688)*] that can be abnormal (C) or normal (D) in its pathfinding (n = 56). Scale bars represent 10µm.

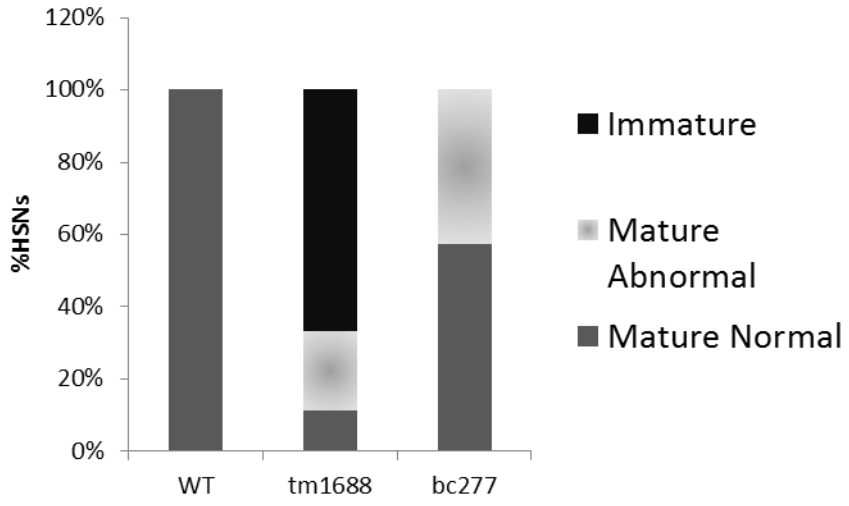


Figure 5.7. *hlh-3 (bc277)* HSNs are more mature than the HSNs in *hlh-3 (tm1688)* hermaphrodites but they are not entirely normal Quantification of detectable HSN processes in the indicated strains harboring wyIs97 [*Punc-86::myrgfp; Punc-86::mCherry::rab-3; Podr-1::gfp*]. "Immature" refers to cells with only lamellopodia and filopodia.
WT n=60; *tm1688* n=80; *bc277* n=56

expectation that an immature HSN does not express *tph-1* and thus cannot accumulate 5HT (Doonan et al., 2008). My finding that *hlh-3 (tm1688)* HSNs are immature also confirms that*tm1688* like *bc248* represent null alleles and the phenotypes displayed are not fully penetrant. About 65% of *bc248* (the second largest deletion in the gene) HSNs do not accumulate 5HT or express a *tph-1::gfp* transgene (Doonan et al., 2008). In contrast *hlh-3 (bc277)* HSNs mature at the same rate as the HSNs in WT animals. Even though *hlh-3 (bc277)* HSNs resemble wild type HSNs in their maturation 43% of the animals have an abnormal axon. This suggests that maturation of an HSN does not guarantee normal axon formation and the two processes can be regulated independently (Fig. 5.6 C, D, 5.7).

5.2.5 *hlh-3* mutant animals have VC differentiation defects

In the egg-laying circuitry, VCs play a supporting role to the HSNs that are the command neurons (Mi Zhang et al., 2010b). The VCs innervate the vm2 vulva muscles as well as the HSNs. However, they have a stimulatory role on the vulva muscles but inhibit the HSNs. When the HSNs stimulate the vm2 muscles, they also stimulate the VCs which in turn inhibit the HSNs. Thus they serve to regulate the frequency of egg-laying (Mi Zhang et al., 2008). The VCs on their own are able to stimulate the vm2 muscles to lay eggs to some extent (Mi Zhang et al., 2010b). Thus, mutants in which the HSNs are absent are not 100% Egl (Schafer, 2005; Waggoner et al., 1998; M Zhang et al., 2008; Mi Zhang et.al, 2010a)

Shorter *hlh-3* constructs are expressed in the P cells (ectodermal like cells and the VC precursors) (Doonan et al., 2008). Although we haven't focused on the VC defects, we do have preliminary data to suggest that VCs are not fully differentiated in *hlh-3* mutants. IDA-1 is a

protein tyrosine phosphatase- like receptor involved in the neuropeptidergic control of egg-laying that is expressed in the VCs and HSNs. To characterize the VC defects, we used the *ida-1::gfp* transgene *(inIs179 (ida-1::gfp)II; him-8 (e1489)IV)* in the *hlh-3 (tm1688)* and *hlh-3 (bc277)* mutant background (Zahn et.al, 2001).

Although, VC4 and VC5 are equally affected in *hlh-3 (tm1688)*, that is they lack *ida-1::gfp* expression to the same extent, VC5 is differentiated (ida-1 positive) in *hlh-3 (bc277)*, possibly contributing to the normal egg-laying in these animals. In addition, the VC processes appear to be abnormal (Table 5.1, Fig. 5.8). Since the focus of my work is the HSNs I have not quantified them. However, using a VC specific marker, it will be helpful to look at the processes in the future.

5.3 Discussion:

The HSNs in *hlh-3 (tm1688)* mutant animals were not detectable 65% of the time when monitoring *tph-1::gfp* expression or performing 5HT antibody staining (Doonan et al., 2008). When a cell differentiates, its subtype identity is determined by various markers like the neurotransmitter it produces, the synaptic connections it makes. These are features that differentiate its identity from other cells. When a cell isn't identified by the usual markers of differentiation, it could be because the cell has undergone lysis due to misexpression of the apoptotic pathway or changes in regulatory pathways have led to a fate change. *hlh-3* has been shown to be required in the programmed cell death pathway of another neuronal lineage, the NSM lineage. In *hlh-3 (bc248)* mutants the NSM sister cells can survive the apoptotic pathway

	VC4		VC5	
ida-1::gfp	22/22	100%	22/22	100%
bc277;ida-1::gfp	10/22	45%	21/22	95%
tm1688;ida-1::gfp	8/23	35%	11/23	48%

Table V: VC4 and VC5 have differentiation defects in *hlh-3* mutants. In *hlh-3 (tm1688) (lof)* expressing *ida-1::gfp (inIs179 (ida-1::gfp)II; him-8 (e1489)IV)* both VC4 and VC5 differentiation is abnormal whereas in *hlh-3 (bc277)* differentiation of VC4 is affected.

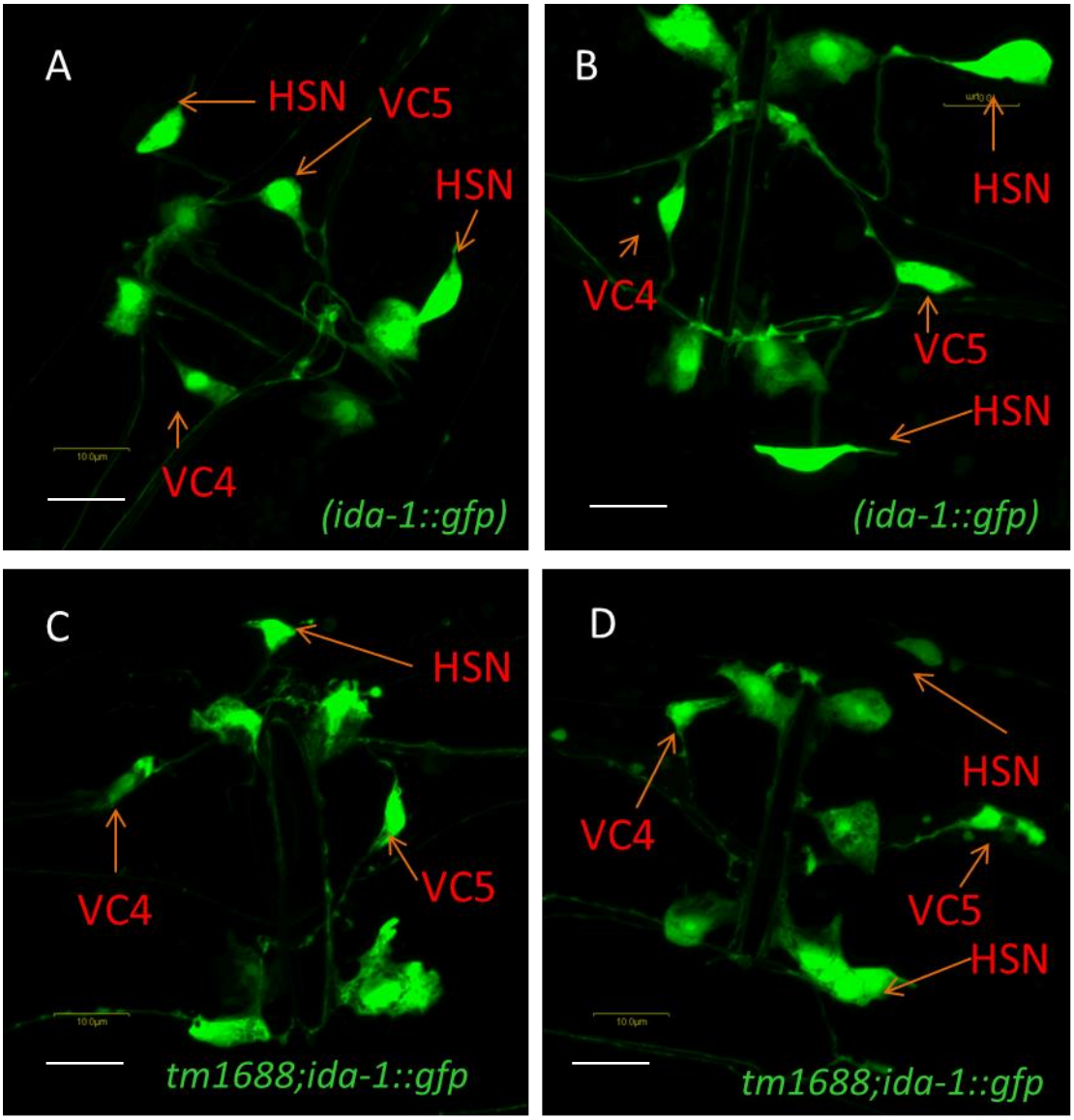


Figure 5.8. VC4 and VC5 are abnormal in *hlh-3 (tm1688)* animals. Panels A and B show wild type animals expressing *ida-1::gfp (inIs179 (ida-1::gfp)II; him-8 (e1489)IV)*in the HSNs and the VC4 and VC5. Panels C and D show *hlh-3 (tm1688)* animals with VC differentiation defects. In (C) VC5 is normal whereas VC4 is abnormally positioned with a missing process. In (D) VC4 is abnormal in position and VC5 is missing a process. Scale bars represent 10µm.

and take on the characteristic of its sister cells, the NSMs (Thellmann, 2003). One of the Ac/Sc genes in *C. elegans, hlh-14,* acts in the HSN lineage and is necessary for fate determination of the PVQ/HSN/PHB neuroblast (NB). This NB undergoes multiple asymmetric divisions to result in a PVQ, HSN and a PHB neuron. *LOF hlh-14(gm34)* mutants show a transformation in cell fate such that the mother of the NB divides into two epidermal (hypodermal) blast cells instead of a hypodermal blast cell and the NB (Frank et al., 2003). In *hlh-3* mutants neither do the HSNs undergo programmed cell death, nor are they transformed into its sister, the PHBs.

5.3.1 HSNs in hlh-3 (*lof*) mutants are immature:

My finding is that these HSNs are arrested in a late stage of differentiation that prevents a mature axon from developing. I have called this a maturation defect. It is known that the development of the axon requires an interaction between the UNC-6/UNC-40 signaling pathway where the ligand UNC-6 interacts with the receptor UNC-40 to initiate axon formation and drive appropriate axon pathfinding. UNC-6/Netrin secreted by the vulval epithelial cells polarizes UNC-40/DCC in the HSNs to the ventral side of the cell body. It is due to this polarization that a mature axon develops from multiple lamellopodia (Adler et al., 2006; Norris & Lundquist, 2011; Yong Yang et.al, 2014). One mechanism by which *hlh-3* could regulate HSN axon maturation is by directly regulating *unc-40* expression as the *unc-40* promoter region has canonical E boxes that bHLH transcription factors like HLH-3 could bind to (Grove et al., 2009). However, we have shown that *hlh-3* does not regulate *unc-40* directly as in the absence of *hlh-3, unc-40* is still expressed in 60% of the HSNs (Doonan et al., 2008). It is also known that in the absence of *unc-40* function HSNs mature normally, although they have abnormal pathfinding (Alexander et al., 2009). Thus HLH-3 may not directly regulate *unc-40* but it may have a role in

the polarization of UNC-40/DCC, the receptor. Loss of *hlh-3* may cause uniform distribution of UNC-40 such that there is an immature HSN or an abnormal axon formation.

Differentiation of the HSNs involves multiple processes – from being born in the tail to migrating to its normal position posterior to the vulva to developing structurally, to expressing neurotransmitter, to making the proper synapses. The HSNs in *hlh-3 (tm1688)* mutants are able to complete the initial steps of differentiation. But in about 65% of the cases they do not form a mature process that can make appropriate synapses and make serotonin. Although, *tph-1* is not expressed in about 65% of the HSNs, it is not because *hlh-3* regulates *tph-1* directly. If differentiation is a sequential process, when one step gets halted then all subsequent steps will be abnormal. Thus not making serotonin or expressing the synaptic markers could be a direct result of the HSNs not maturing into their final state. *hlh-3 (tm1688)* animals have HSNs that get arrested in the lamellopodia stage and do not form a mature axon. The HSNs that develop a process are not all normal either with about 65% having pathfinding defects. This suggests that maturation itself is not enough for proper axon development and even the axons that do end up maturing may need finer regulation to develop normally. This is further corroborated by *hlh-3 (bc277)* animals in which HSNs do mature but 90% of the axons are abnormal. Thus maturation and axon pathfinding can be uncoupled, one not being a prerequisite for the latter. The two isoforms discussed in Chapter 2 may have different roles to play in the two processes. Thus, it appears that *hlh-3* regulates other developmental processes in the HSNs via a pathway independent of *unc-40* activation or downstream from *unc-40*.

Axon development takes place due to actin remodeling of the cytoskeleton, initiated by activation of the Rac pathway by UNC-6/UNC-40 signaling. Multiple parallel Rac pathways are activated that redundantly drive axon development, no one pathway has significant pathfinding

and outgrowth defects (Alexander et al., 2010; Gitai et.al, 2003; Lundquist et.al, 2001; Quinn et al., 2008; Struckhoff, 2003). Since the HSNs have abnormal pathfinding, *hlh-3* could be acting downstream on one of the signaling pathways to regulate axon outgrowth. One of the actin remodeling proteins that we tested is encoded by *unc-115*, as mutations in the gene lead to axon defects and it is in a list of HLH-3 targets derived from bioinformatics (Grove et al., 2009; Yieyie Yang & Lundquist, 2005). Our analysis shows that *hlh-3* does not regulate *unc-115* (A. Alfonso, unpublished). However, there are many more targets like Arp2/3 complex, UNC-73, MIG-10 that need careful evaluation (Alexander et al., 2009; Chang et al., 2006; Norris et.al, 2009).

hlh-3 belongs to a bHLH family of genes that are proneural; wherein mutations in bHLH family members result in a loss of neuronal populations. We show in this work that the role of *hlh-3* is not classically proneural as evidenced by the fact that HSNs are born and migrate to their normal locations in hermaphrodites in mutants. However, the phenotype is not completely penetrant and only 40% develop a serotonergic identity and 35% of these HSNs have normal axon development. *hlh-3* ortholog Asense in Drosophila is expressed later in the external sense organs and results in abnormalities in sensory bristles rather than a loss of cells (Brand et.al, 1993; Domínguez & Campuzano, 1993). Other *C. elegans* Ac/Sc family members like *hlh-14* and *lin-32* do play a role in lineage determination and loss of these proteins results in loss of neurons, it is not clear why *hlh-3* has a more limited role in subtype specification in the hermaphrodites although it is expressed very early on. One possible explanation could be other bHLHs may be playing a redundant role in fate specification. bHLH proteins recognize an E box motif consisting of the nucleotides CANNTG in the promoters of their target genes (Murre et al., 1989). Using protein binding microarrays, the Walhout laboratory has determined the *in vitro*

DNA binding specificities of bHLH dimers and combined that with bHLH-DNA and computational modeling to infer the interaction between a bHLH monomer and an E box half site (CAN) that was validated with mutational analysis (De Masi et al., 2011; Grove et al., 2009). *hlh-14* and *hlh-3* share the CAG half site and have at least two E box binding sites in common with each other. Thus, *hlh-14* may be playing a redundant role in *hlh-3* mutants by turning on transcription of target genes in the PVQ/HSN/PHB lineage.

5.4 Future directions:

5.4.1 Characterization of HSN maturation in *hlh-3 (tm1688)* adults:

The current analysis on the maturation phenotype was performed on L4 animals. It is possible that there may be a delay in maturation rather than a developmental arrest. To distinguish between these two possibilities, the *hlh-3 (tm1688)::myrgfp* animals can be imaged at 40 hours post L4 to look for developmental changes.

5.4.2 Characterization of VC abnormalities:

VCs are an important part of the egg-laying circuit that make synapses with the HSNs and the vulva muscles. They are also abnormal in *hlh-3* mutants. Since the focus of this study was on the HSNs, I did not characterize the VCs in detail. Preliminary analysis revealed differentiation and axon pathfinding defects, similar to the HSNs. It will be important to compare and contrast the abnormalities with the HSNs and analyze the defects in the two mutants in relation to the HSNs. Many reporters are in our hands such as the *myr::gfp, ida-1::gfp* or the more VC specific *lin-11::gfp* can be used.

Chapter VI: Conclusions

Chapter 6

CONCLUSIONS

6.1 *hlh-3* is expressed embryonically but its functions are needed at the L3-L4 stage or later

I characterized *hlh-3* expression using various constructs (promoter, genomic, fosmid) and observed that it is expressed in the HSNs in the tail in the one-and-a-half-fold stage embryo. Lineage analysis helped corroborate these findings where *hlh-3* expression was detected in the mother of the HSNs. This shows that *hlh-3* is expressed early on in development. In hermaphrodites, *hlh-3* is expressed in many other cells, other than the HSNs but the *lof* mutants, *hlh-3 (tm1688)* and *hlh-3 (bc248),* show no other overt developmental anomalies besides the fact that they are egg-laying defective in the adult stage. The only detectable phenotype is the abnormal differentiation of the HSNs and VCs in the egg-laying circuit. HSNs are born in the embryo but are not functional until L3-L4 stages when they undergo terminal differentiation by developing an axon, making the appropriate synapses and developing the serotonergic phenotype. Thus, *hlh-3* is an atypical proneural gene that has a late differentiation role in the neurons.

6.2 *hlh-3* encodes two isoforms that have different functions in the differentiation of the HSNs

hlh-3 gene has three exons, which we call 1', 1 and 2. We propose that Exon 1' is only present in a low abundance transcript. Transcripts with exons 1 and 2 were readily detectable in WT RNA by RT-PCR analysis and considered as the only functional exons. Since *hlh-3 (bc277)* has a deletion of the region encoding exon 1' but contains the information for exon 1 and 2 it has a detectable transcript as in WT animals and results in animals that are normal in their egg-

laying, these observations were considered as further evidence of the existence of a single transcript. However, *hlh-3 (bc277)* animals had 5HT positive HSNs that had axon pathfinding defects. Also, I provide evidence that exon 1' can be transcribed and translated. Thus, we propose that *hlh-3 (bc277)* individuals are missing a function, provided by isoform HLH-3A, which is required for axon pathfinding. Further we propose that isoform HLH-3B is required for HSN maturation since *hlh-3 (tm1688)* animals that lack both isoforms have maturation and pathfinding defects.

6.3 *hlh-3* is not a classical proneural gene but is required for sub-type specification

Mutations in classic proneural genes in vertebrates and Drosophila lead to a loss in neuronal populations in which they are functional. HSNs in *hlh-3 lof* mutants are born and migrate to their normal positions. They do not differentiate and are not detectable by usual terminal differentiation markers. There can be many reasons why this could be happening, the cells may be undergoing programmed cell death, or changing their fate to that of their sister cells. In these mutants, however, the neurons are stalled in their development and do not get past the lamellopodia stage. This is novel and not reported in any other bHLH proneural gene. *hlh-3* may regulate axon pathfinding by regulating the Rac GTPases that function downstream in the UNC-40/UNC-6 signaling pathway and it will be worth testing some of the probable candidates by genetic techniques.

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Appendix A

Chapter 7

APPENDIX A

7.1 UNC-86 does not regulate *hlh-3* expression:

The expression patterns of both *unc-86* (Finney & Ruvkun, 1990, Sze, Zhang, Li, & Ruvkun, 2002) and *hlh-3* (Doonan et al., 2008), as well as the completely penetrant non-serotonergic phenotype of *unc-86* vs. the partially penetrant non-serotonergic phenotype of *hlh-3* suggests that *unc-86* might regulate the expression of *hlh-3*. Together this also suggests that *unc-86* is upstream of *hlh-3* and regulates the expression of *hlh-3* as it has a more severe phenotype. One of the goals of this study was to characterize the interaction between *unc-86* and *hlh-3* in greater detail by using various genetic tools and microscopy.

I wanted to test whether UNC-86 regulates *hlh-3* expression. There are UNC-86 binding sites on the *hlh-3* promoter and intron. To address this question I used a strain where a genomic fragment 3.3kb upstream of the *hlh-3* coding region was fused in frame with GFP (*hlh-3^{genomic}::gfp*). Using standard genetic techniques I crossed *hlh-3^{genomic}::gfp* in an *unc-86 (e1416)* null mutant background. My initial results suggested that UNC-86 regulates *hlh-3* as 75% of the HSNs did not express *hlh-3 ^{genomic}*::*gfp* in these neurons in *unc-86 (e1416)* mutants.

However, to further analyze the regulatory mechanisms by which UNC-86 regulates *hlh-3* and to test which upstream and downstream regions are important for regulation, I introduced other *hlh-3* constructs into the *unc-86 (e1416)* null mutant background. These were the *hlh-3^{promoter}::gfp* and the *hlh-3^{fosmid}::yfp* constructs. The *hlh-3^{promoter}::gfp* construct is smaller than the *hlh-3^{genomic}::gfp* construct in that it only has the 3.3kb upstream promoter region with the 1st eight amino acids of exon 1 in frame with the GFP coding sequences. The *hlh-3^{fosmid}::yfp*

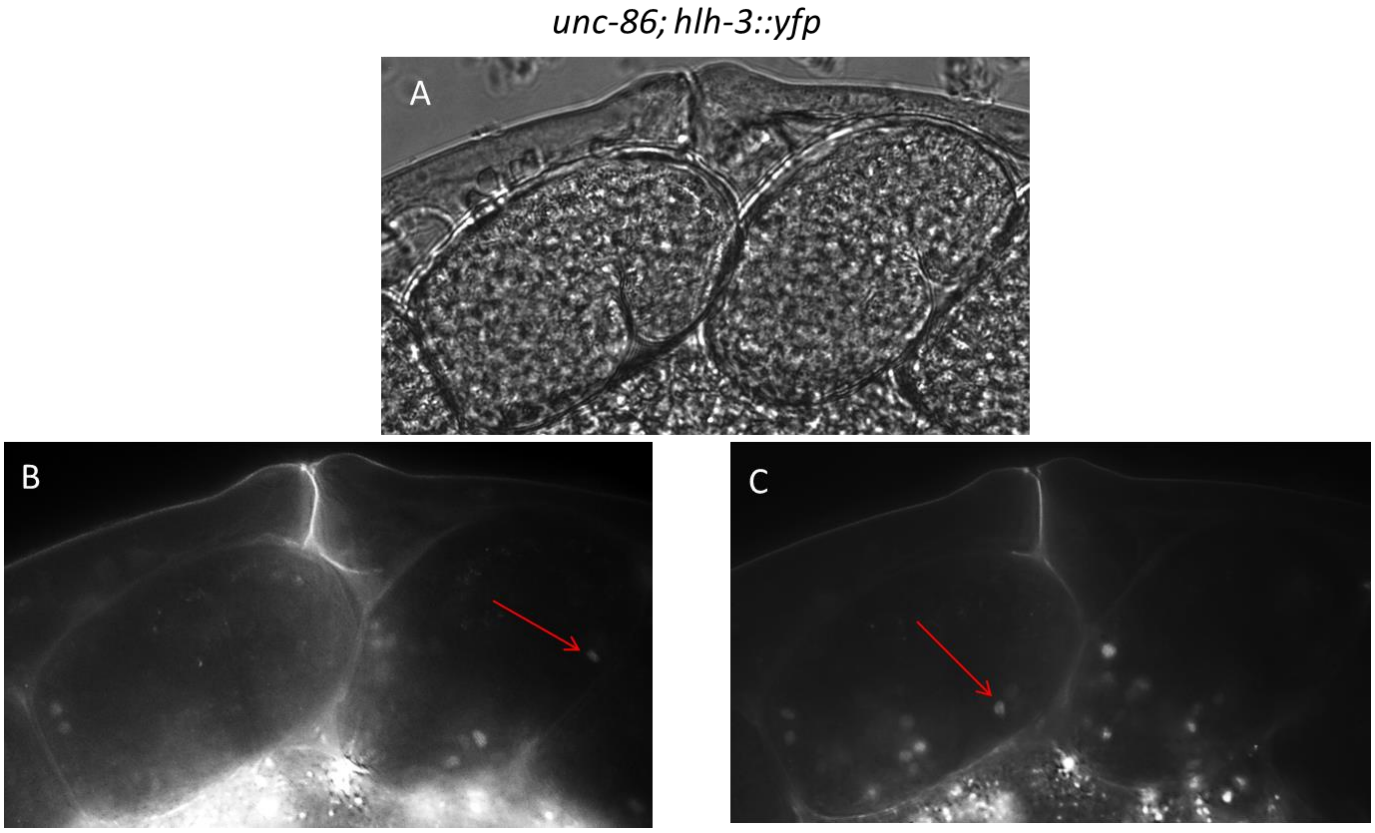

Figure 7.1. UNC-86 is not necessary for initiation of *hlh-3* expression in embryos.

The *hlh-3*^{fosmid}*::yfp* is expressed in the embryos (arrows, in B and C) in an *unc-86 (e1416)* mutant background (B and C are fluorescent images of the same animal as shown in A)

contains a 32Kb genomic fragment that encompasses the entire *hlh-3* coding region including upstream and downstream regulatory sequences (Fig. 3.2). The *yfp* is fused in frame before the stop codon in the 2nd exon (Tursun et.al, 2009). If UNC-86 regulates *hlh-3* expression, then expression of *hlh-3* will be turned off in an *unc-86 (e1416)* mutant background.

I found that, the *hlh-3*^{promoter}*::gfp* and the *hlh-3*^{fosmid}*::yfp* constructs I tested were expressed in an *unc-86* null mutant background (Fig. 7.1). In both instances *hlh-3* expression is seen in the HSNs in the embryos in the one and half fold stage in *unc-86* mutant animals. It is possible that *unc-86* may be regulating *hlh-3* expression in other cells but I have not carefully analyzed this possibility. This finding suggests that *unc-86* does not regulate *hlh-3* in the HSNs. The discrepancy between both sets of observations is puzzling and has not been resolved.

7.2 *hlh-3* does not regulate its own expression:

Transcription factors have been known to regulate their own expression. There are examples where they are required for maintenance of their expression either by themselves or by an interaction with another transcription factor (Gibert & Simpson, 2003). Various genes from diverse gene families are known to autoregulate their expression in a positive or a negative feedback loop (Karp & Greenwald, 2003; Milton & Okkema, 2015; Teng et.al, 2004; Turner et.al, 2014). HLH-3 binds to E boxes (CANNTG) to initiate the transcription of downstream genes, with a high specificity for CACCTG (Grove et al., 2009; Thellmann, 2003). The *hlh-3* promoter also has E boxes (CANNTG) in its sequence. Thus, I hypothesized that HLH-3 autoregulates its own expression.

To test this, I crossed the *hlh-3*^{promoter}*::gfp* strain in an *hlh-3 (tm1688)* mutant background using standard genetic techniques. I find that there was no loss of *gfp* expression in the mutant

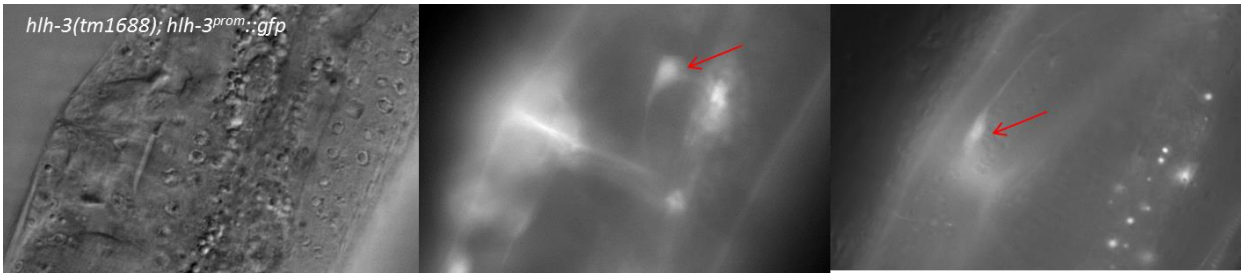

Figure 7.2. HLH-3 does not regulate its own expression. *hlh-3^{promoter}::gfp* is expressed in *hlh-3 (tm1688)* animals (arrows showing GFP+ HSNs in *hlh-3 (tm1688)* L4 animals).

background as compared to WT (Fig.7.2). This observation strongly suggests that there is no autoregulatory loop by which HLH-3 maintains its own expression.

7.3 *hlh-3* mutant animals have some HSNs that do not express *unc-40*:

HSN axons find their target by using the Netrin signaling pathway. UNC-6/Netrin is a signaling molecule that is secreted by the vulval epithelial cells (C. C. Quinn et al., 2006). UNC-40/DCC is a receptor that belongs to the immunoglobulin superfamily. It responds to the attractive netrin cue and directs the growth cone of the cell where it is expressed towards the source of netrin (Adler et.al, 2006; Xu et.al, 2009). UNC-6 regulates the polarization of UNC-40 in the HSNs in the L2 – L3 stage which leads to the ventral guidance of the developing axon. In the absence of such polarization, the axon develops abnormally and has a posterior or an anterior projection without a ventral projection (Adler et al., 2006; Z. Xu et al., 2009). Activation of the UNC-40 receptor activates a signaling cascade that regulates axonal growth by remodeling the actin cytoskeleton. *unc-40* is expressed in the HSNs and mutations in *unc-40* show an abnormal pathfinding phenotype similar to *hlh-3* mutants. A preliminary search of the *unc-40* promoter region shows that it has E boxes to which HLH-3 has the potential to bind to. I hypothesized that *hlh-3* regulates the expression of *unc-40.*

To test this I crossed *unc-40::gfp* (a rescuing translational construct from the Culotti laboratory (*evIs103[unc-40p::unc-40 minigene::gfp])* into the *hlh-3* mutant background using standard genetics techniques.

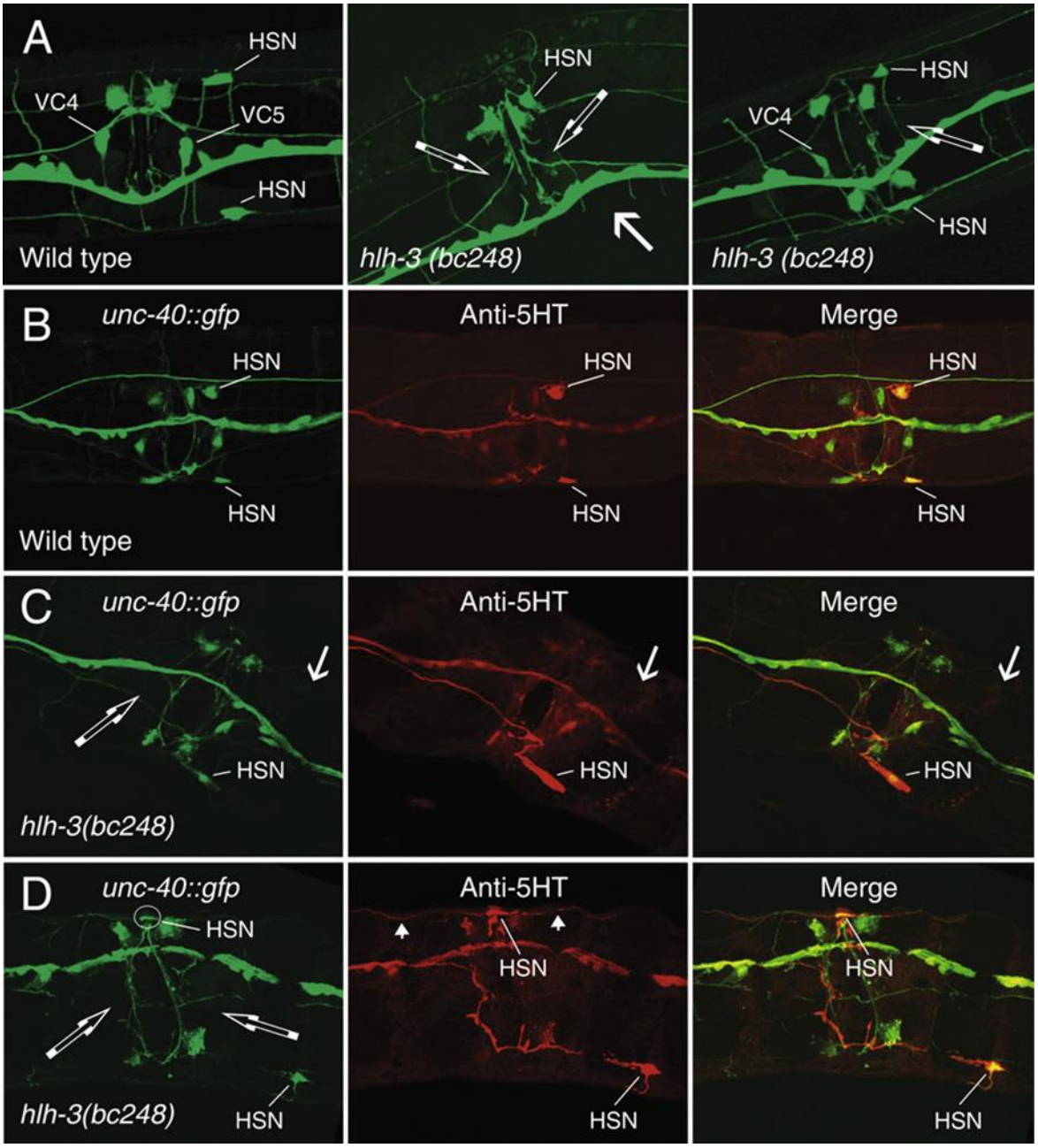

Figure 7.3. *hlh-3* mutant animals lack UNC-40 expression in some HSNs and VCs.
(A) *hlh-3 (bc248)* mutants showing no *unc-40::gfp* in HSNs(solid arrow) and VCs (open arrow) (B) WT animals showing *unc-40::gfp* expression and anti-5HT antibody staining in HSNs and VCs (C) *hlh-3 (bc248)* animal showing one normal HSN (D) *hlh-3 (bc248)* animal showing two abnormal HSNs and missing VCs.

(Adapted from Doonan et.al, 2008)

I observed that *unc-40* expression is absent in 25% of the HSNs in an *hlh-3 (bc248) lof* mutant (Fig. 7.3). However, I also noticed that *unc-40* expression was not detectable every time that 5HT immunoreactivity was absent. These results support the conclusion that HSNs lacking 5HT and UNC-40::GFP are cells that failed to commit to HSN differentiation. I also see that 60% of the HSNs that do show *unc-40* expression have abnormal axons. Since this construct, *unc-40::gfp* is a functional fusion (Culotti, personal communication), it shows that having a functional *unc-40* is not enough to have normal HSN axons and HLH-3 must have a regulatory role elsewhere and outside of the UNC-40 mediated pathway.

7.4 *unc-86* and *hlh-3* act in different pathways to regulate axon pathfinding in the HSNs

unc-86 encodes a POU homeodomain transcription factor that has a role in various aspects of HSN differentiation. Loss of *unc-86* gives rise to the various defects like loss of 5HT and axon pathfinding (Finney & Ruvkun, 1990a; Ro, Ro, Donhauser, & Baumeister, 2000). This makes the animals egg-laying defective (Desai & Horvitz, 1989; Schafer, 2005). Loss of *hlh-3* gives rise to similar defects although they are not as severe as *unc-86 (lof)* defects. Based on reporter gene analysis, we know that *hlh-3* does not regulate *unc-86* (Doonan, 2006) and the work reported in this manuscript shows that *unc-86* does not regulate *hlh-3*. However, based on their similar defects, it is possible that they act in parallel pathways and the double mutant may be more severe than either of the single mutants.

We analyzed the HSN axons in *unc-86* and *hlh-3* single mutants and the double mutant using the functional *unc-40::gfp* reporter transgene. The axon pathfinding defects in the *unc-86; hlh-3* double mutant is similar to the *unc-86* mutant alone. In an *unc-86* mutant, 95% of HSNs

are abnormal as compared to 60% in an *hlh-3* mutant. However, in an *unc-86; hlh-3* double mutant, we find 95% HSNs with abnormal pathfinding. This suggests that either *unc-86* is upstream of *hlh-3* or it is a dominant partner in regulating axon pathfinding in HSNs. The latter seems to be the case, as we know that *unc-86* does not regulate *hlh-3* (current work).

We also performed a thrashing assay to look at the number of body bends that each animal makes when suspended in water to measure the level of uncoordination (Table 7.1). Previous work has shown that *hlh-3* mutants are not uncoordinated (Doonan, 2006), and our current analysis supports it (Mean body bends/min – N2 = 84, *hlh-3* = 74.4*). unc-86* mutants are severely UNC (Mean body bends/min = 23.91). Surprisingly the double mutant is more UNC than the single mutant alone (Mean body bends/min = 7.91). This observation is statistically significant using a Tukeys pairwise analysis. This suggests that although *hlh-3* mutants do not display an overt defect in locomotion, they appear to have some deficit that is exacerbated when *unc-86* function is also missing. Perhaps this defect is related to the fact that *hlh-3* is expressed in the postembryonic ventral cord motor neurons (Doonan et al., 2008).

7.5 *tph-1* expression is unchanged in *hlh-3 (bc277)* animals:

TPH-1 is the enzyme needed for catalyzing the conversion of tryptophan to serotonin. This is the rate limiting step in the serotonin (5HT) production pathway. Serotonin is the primary neurotransmitter released by the HSNs to stimulate the vulva muscles. As the HSNs are abnormal in *hlh-3 (bc277)* animals, but the animals do not show Egl defects, I hypothesized that

Thrashing assay:

N2 , hlh-3, unc-86;unc-40::gfp and unc-86;hlh-3;unc-40::gfp

	Mean body bends/min
unc-40::gfp	84
unc-40::gfp(hlh-3)	74.4
unc-40::gfp (unc-86)	23.91
unc-40::gfp(unc-86;hlh-3)	7.91

Using ANOVA, F=100.61, there is significant variation among the different strains

Using Tukey's HSD test: (for pairwise analysis)

Difference between means:

	unc-40::gfp (unc-86)	*unc-40::gfp (unc-86;hlh-3)*	*unc-40::gfp*	*unc-40::gfp(hlh-3)*
unc-40::gfp (unc-86)	-	16	60	50
unc-40::gfp(unc-86;hlh-3)	-	-	76	66
unc-40::gfp	-	-	-	10
unc-40::gfp(hlh-3)	-	-	-	-

The difference is significant among every individual strain except between N2 and hlh-3.
Notably, the double mutant is more uncoordinated than unc-86 alone.

Table 7.1. *unc-86 (e1416); hlh-3 (tm1688)* doubly mutant individuals are more uncoordinated than the *unc-86* mutant alone

the HSNs serve as a humoral source of serotonin and in *hlh-3 (bc277)* individuals this is enough to stimulate the vulva muscles to contract and lay eggs normally. I compared the *tph-1::gfp* expression in a WT and a *hlh-3 (bc277)* background by standardizing it against fluorescent beads. In the two sets of animals there was no significant difference between the intensities in the expression level (Fig. 7.4). This suggests that the TPH-1 levels in *hlh-3 (bc277)* are similar to WT and given that programmed cell death of the HSNs in an *hlh-3 (bc277)* mutant background makes the animals Egl (this manuscript), it is likely that the HSNs in the mutant animal serve as sources of serotonin for normal egg-laying.

7.6 HSNs in *hlh-3 (bc277)* animals do not show precocious development:

Axon development is a well-timed process, with lamellopodia and filopodia developing at developmental time points when their growth is most conducive to the animal (Chia et.al, 2014; Peckol et.al, 1999). HSNs are born in the tail during the embryonic stage and migrate to the midbody before the animal hatches (Desai & Horvitz, 1989). The wild type HSN starts axon development only during the late L3 stage when the receptor UNC-40 on the developing growth cone locates the ligand UNC-6 secreted by the vulval cells (Adler et al., 2006; Gitai et.al, 2003; Yang et.al, 2014). In an *unc-86* mutant background, the HSN axon development starts early in the L1 stage and in the absence of the ligand UNC-6 the axon develops anteriorly without a ventral outgrowth (Olsson-carter & Slack, 2010, 2011). The abnormal axons in *hlh-3 (bc277)* mutant background have similar anterior growth without the ventral component. Hence I hypothesized that these mutants had a similar precocious development of the axons before UNC-6 became available to guide the axons in the ventral direction.

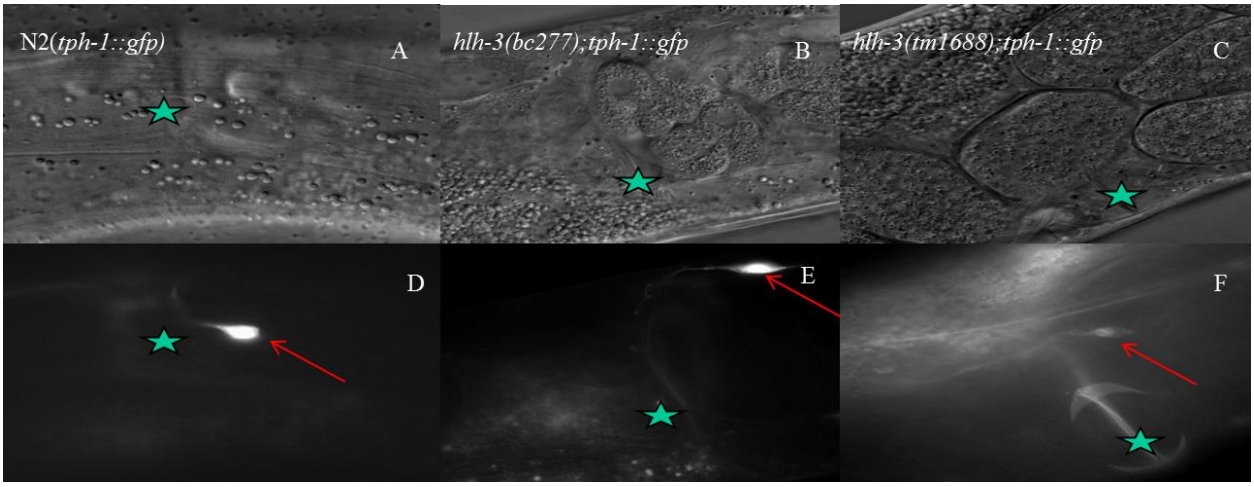

Figure 7.4: The expression of *tph-1::gfp* in *hlh-3 (bc277)* (B&E) animals is similar to WT (A&D). The intensity of expression was comparable to WT. *hlh-3 (tm1688)* (C&F) which is a null mutation showed a reduced intensity of expression as compared to WT. * indicates the position of the vulva.

To test this, I generated transgenic lines that expressed *ida-1::gfp* in a *hlh-3 (bc277)* mutant background and imaged the expression of *ida-1* in the HSNs at various developmental stages. IDA-1 is a protein tyrosine phosphatase receptor, expressed in peptidergic neurons including the VCs and HSNs (Zahn et.al, 2001). The axons in *hlh-3 (bc277)* hermaphrodites develop similar to WT HSN axons and there is no precocious development (Fig. 7.5). These axons are abnormal, however, I do know that the *hlh-3 (bc277)* animals are normal for egg-laying due to the branches that sprout from the abnormal axon and make synapses on the vulva muscles. HLH-3A, the longer isoform missing in *hlh-3 (bc277)* may be responsible for pathfinding and the absence of this isoform possibly gives rise to pathfinding defects.

7.7 *unc-40* and *hlh-3* act in the same pathway:

UNC-40 is the receptor that responds to the ligand UNC-6 during HSN axon pathfinding and is expressed in the HSNs. *unc-40 (e271)* individuals have HSN axon pathfinding defects similar to those shown by *hlh-3 (bc248)* individuals. However, *hlh-3* does not regulate the expression of *unc-40* (this study). I hypothesized that *hlh-3* and *unc-40* act in different pathways to regulate HSN axon pathfinding. Since the *unc-40 (e271)* and *hlh-3 (bc248)* animals are not 100% Egl, if the hypothesis is true then the double mutant should be more Egl than any of the single mutants alone. However, the double mutant is not any worse than the single mutants. This suggests that the two act in same pathway and *hlh-3* may be regulating some other candidate gene in the *unc-40* pathway and may not have direct effects on *unc40* itself. Several candidate genes have been detected based on bioinformatics analysis that could be possible targets of *hlh-3* (Grove et al., 2009).

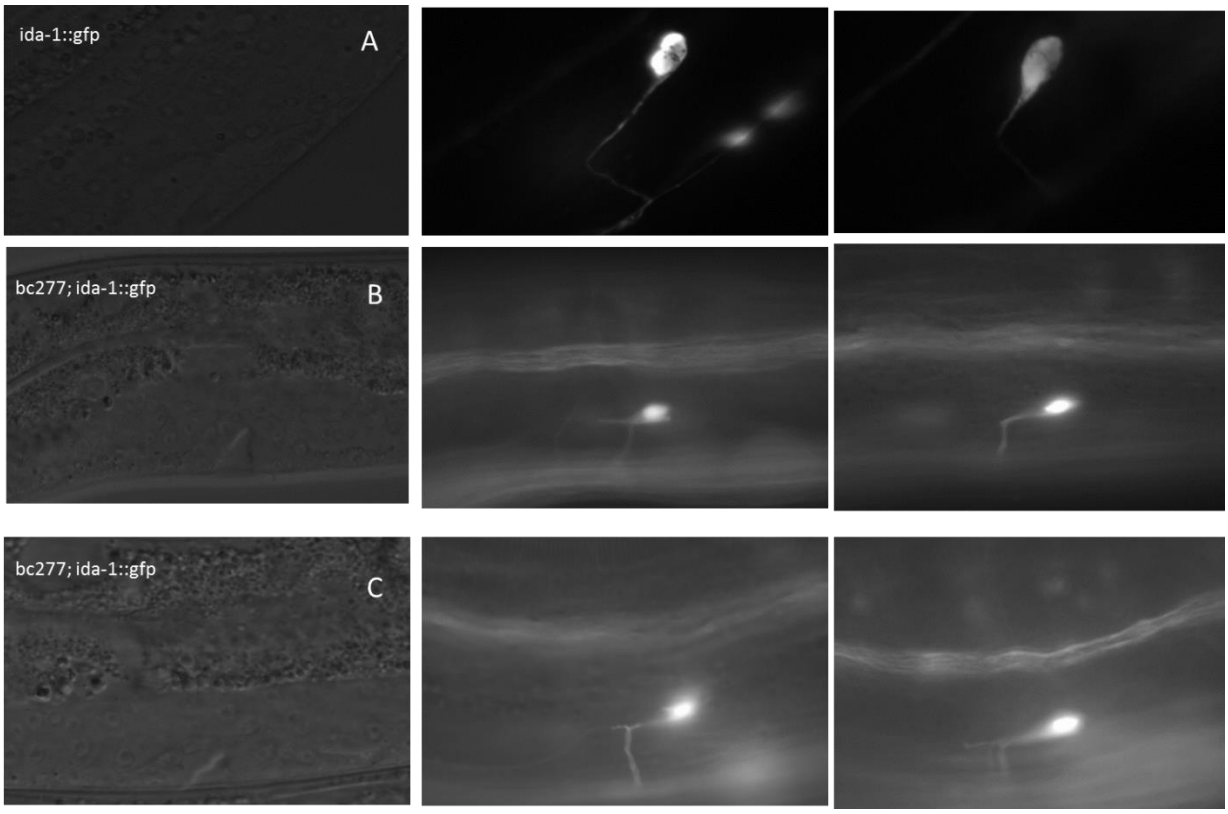

Figure 7.5: HSNs in *hlh-3 (bc277)* animals do not show precocious development. Row A shows a WT L4 animal with normal well developed axon. Rows B and C show L4 *hlh-3 (bc277)* animals with well-developed HSNs.

7.8 *syg-1, syg-2* and *tph-1* are not regulated by *hlh-3:*

syg-1 and *syg-2* are important guidepost cells that are needed in generating HSN synapses with the vulva muscles and the VCs (Shen et.al, 2004). Synaptic specificity is important for forming functional connections and the interaction between these two molecules ensures that the synapses are formed at the right places even though the growing HSN axon comes in contact with multiple cells and neurons (Miller, 2007; Shen et al., 2004). *syg-1* is expressed on the developing HSN growth cone whereas *syg-2* is expressed on the guidepost vulval epithelial cells. They play a role in synaptic specification rather than axon pathfinding. *syg-1* mutant animals have a defect in the clustering of the synaptic vesicles at the HSN synapse but do not have an egg-laying defect (Shen & Bargmann, 2003b). The role of these cell adhesion molecules in the developing growth cone pursued us to look at the interaction between *hlh-3* and these molecules as it was also one of the targets of *hlh-3* based on yeast 2 hybrid assays (Vermeirssen et al., 2007) and the promoter of *syg-1* had E boxes to which bHLH proteins bind to. However, *hlh-3* does not regulate *syg-1* or *syg-2*. The guidance molecules probably play a role in synaptic function and have no role in the development of the axon.

7.9 SEM-4 and ZAG-1 do not regulate *hlh-3* expression:

ZAG-1 is a Zn finger homeodomain protein. In *zag-1* mutants (*lof)*, the HSNs are born, migrate to midbody, and express *unc-86* but not *tph-1*. The HSN axons detected with *tph-1::gfp* had branching problems at the vulva. *zag-1* animals have pathfinding defects and nuclear morphology defects (Clark & Chiu, 2003). SEM-4 is also a Zn finger homeodomain protein required for the differentiation of the HSNs. In *sem-4* mutants, most HSNs fail to accumulate serotonin and to acquire their distinctive nuclear morphology. They also show an abnormal pattern of axonal outgrowth (Basson & Horvitz, 1996; Toker, Teng, Ferreira, & Emmons, 2003).

The range of defects seen in *sem-4* and *zag-1* are similar to the ones seen in *hlh-3* mutant animals but much more penetrant and much more severe. Hence, I hypothesized that these transcription factors regulate *hlh-3* expression.

If SEM-4 and ZAG-1 regulate *hlh-3* expression, then *hlh-3*^{genomic}*::gfp* (*hlh-3* promoter with *hlh-3* genomic fragment fused in frame with GFP) will not show gfp expression in the HSNs in *sem-4* and *zag-1* mutants. I crossed *hlh-3*^{genomic}*::gfp* in both the mutant backgrounds using standard genetic techniques. I assayed for the presence of gfp expression in the HSNs and observed that *hlh*-3 expression was unaffected in either *sem-4* or *zag-1* mutant backgrounds. My results indicate that *sem-4* and *zag-1* do not regulate *hlh-3* expression.

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CHAPTER 8

APPENDIX B

LICENSES AND PERMISSIONS

Saleel Raut <saleelraut@gmail.com>

Permission to use images from wormatlas

5 messages

Saleel Raut <saleelraut@gmail.com> Tue, Dec 6, 2016 at 5:25 PM
To: david.hall@einstein.yu.edu, zeynep.altun@einstein.yu.edu, laura.herndon@einstein.yu.edu

Dear all,

I would like to request permission to include two figures from www.wormatlas.org,
in my PhD dissertation/thesis for the purpose of illustrating a concept.
Specifically, I would like to use Intro figure 7, Chapter 1 General
Introduction from the Hermaphrodite Anatomy section
authored by Z. F. Altun & D. H. Hall.
And, Neuro Fig 4B from the Hermaphrodite Handbook from Nervous system General description.
If allowed to use It, I would fully credit the authors and source. Could you let me know if this is permissible or whom I
should contact for permission to use it?
Sincerely,
Saleel Raut.
Department of Biological Sciences,
University of Illinois at Chicago,
840 W Taylor St, Rm3067SEL
Chicago, IL 60607.

David Hall <david.hall@einstein.yu.edu> Wed, Dec 7, 2016 at 7:55 AM
To: Saleel Raut <saleelraut@gmail.com>

Hi Saleel,

Yes, I hereby give you permission to show those figures in your thesis, with the stipulation that you credit us as
the source in the Figure legends, and add a reference to WormAtlas in your overall reference list.
Suggestions for how to write a reference to WormAtlas are given here:
http://www.wormatlas.org/howtocite.htm
Best regards,
David Hall
Albert Einstein College of Medicine
Bronx, NY

From: Saleel Raut <saleelraut@gmail.com>
Date: Tuesday, December 6, 2016 at 6:25 PM
To: David Hall <david.hall@einstein.yu.edu>, Zeynep Altun-Gultekin
<zeynep.altun@einstein.yu.edu>, Laura A
Herndon <laura.herndon@einstein.yu.edu>
Subject: Permission to use images from wormatlas
[Quoted text hidden]
Zeynep AltunGultekin

<zeynep.altun@einstein.yu.edu> Wed, Dec 7, 2016 at 9:15 AM
To: Saleel Raut <saleelraut@gmail.com>, David Hall <david.hall@einstein.yu.edu>, Laura A Herndon
<laura.herndon@einstein.yu.edu>
Dear Saleel;
You can use the images with the acknowledgement:
Reprinted with permission from Wormatlas. Altun, Z.F. and Hall, D.H. (www.wormatlas.org)www.wormatlas.org
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Zeynep

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VITA

Name: Saleel Raut

Education:

University Of Illinois at Chicago (UIC)
Ph.D Biological Sciences (2015) GPA 3.6/4.0

Georgia State University, Atlanta GA
M.S Biology (2005) GPA 4.0/4.0

Topiwala National Medical College (T.N.M.C), India
Bachelor of Medicine Bachelor of Surgery (M.B.B.S) (2002)

Research Experience:

Research Assistant with Dr. Aixa Alfonso
Conducted research funded by an NSF grant in the field of Neurobiology using the nematode *C. elegans* as a model system elucidating the role of a transcription factor HLH-3,in the differentiation of the hermaphrodite specific motor neurons (HSNs) using genetic techniques, immunohistochemistry, confocal microscopy and cloning techniques.

Teaching Experience:

Teaching Assistant at UIC (2008-2011, 2014)
Courses taught - **BIOS 221 Genetics Laboratory**. Directed laboratory sections in Genetics for Sophomores and Juniors. Graded exams, quizzes and lab reports. (Average enrollment = 24)
BIOS 220 and BIOS 222 Discussion sections for Genetics and Cell Biology Lecture courses
Conducted discussion section that involved troubleshooting problems in Genetics and Cell biology.

Adjunct Faculty at Westwood College (Loop Campus)(2005-2006)
Instructed undergraduates in Biology, Earth Science and Physical Science
Prepared, administered and graded exams and term papers

SKIT fellow for the High School Transformation Project (2006-2008)
Worked as a SKIT fellow under the IDS (Instructional Delivery Systems) program with 3 Chicago Public Schools over the course of 2 years. Helped the teachers with the implementation of the IDS Biology curriculum. Interacted with my mentor teachers and the IDS team for lesson planning. Helped with lab set up and conduction by providing the students with an extra resource in the classroom to answer questions and guide them in the right direction.

'AVID' Tutor for Chicago Public Schools (2005-2006)
Tutored underprivileged High School students in Math and Science to get them ready for college for the Chicago Public Schools under the AVID (Advancement Via Individual Determination) Program. (Average enrollment=150 students in 6 classes/day)

Teaching Assistant at Georgia State University (2002-2005)
Courses taught: **BIOL 1107 – Principles of Biology** and **BIOL 1110 – Anatomy and Physiology**
Conducted laboratory sections for freshmen, sophomores and juniors using instruction and demonstration. Graded and conducted exams and quizzes. (Average enrolment = 24)

Publications:

HLH-3 is a C. elegans Achaete/Scute protein required for differentiation of the hermaphrodite-specific motor neurons
Ryan Doonan, Julia Hatzold, Saleel Raut, Barbara Conradt, Aixa Alfonso

HLH-3 has a unique role in maturation and axon pathfinding in the hermaphrodite-specific motor neurons
Saleel Raut and Aixa Alfonso (In progress)

Awards:

Departmental award for Teaching Excellence for BIOS 221 – Genetics laboratory at UIC (2010, 2011)
Departmental travel award:
C. elegans topics meeting, University of Wisconsin at Madison (2008, 2010, 2014)
International *C. elegans* meeting, University of California at Los Angeles (UCLA) (2011)
Society for Neuroscience meeting, New Orleans, LA (2012)
College of Liberal Arts and Sciences travel award:
Society for Neuroscience meeting, New Orleans, LA (2012)
Graduate College travel award:
C. elegans topics meeting, University of Wisconsin at Madison (2008, 2010)
International *C. elegans* meeting, University of California at Los Angeles (UCLA) (2011)
Society for Neuroscience meeting, New Orleans, LA (2012)
Graduate student council travel award:
C. elegans topics meeting, University of Wisconsin at Madison (2008, 2010)
Society for Neuroscience meeting, New Orleans, LA (2012)

Volunteer Experience:

Board member with **AID-Chicago** (Association for India's Development), a 401c(3) non-profit organization working on development projects in India (2008-2014)
Served as '**Community Outreach Coordinator'** on the Indian Graduate Student Association (IGSA) board at UIC (2011-2012)
Volunteered at the **NATO conference** (2012) in Chicago in the hospitality department, with **World Sport Chicago** for various sporting events in Chicago like the USA Volleyball Junior Beach tour (2008), AIBA World boxing championship (2007), **Radio DJ with 89.3 FM** (WNUR) as a host for a Radio Show – Lotusbeat, featuring South Asian Music