University of Nevada, Reno

Genetic Analysis of *Drosophila* Stomatogastric Nervous System

A dissertation submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy in Cell and Molecular Biology

by

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Abstract

The enteric nervous system (ENS) is of critical importance to an organism, as it controls the function of the gastrointestinal system. The ENS is remarkably complex, and although sculpted early in development, has to maintain plasticity throughout adulthood in the face of changing food levels, intestine size and inflammatory insults. Compared to the central nervous system, the ENS is poorly understood, despite the impact of several medically significant conditions. Hirschsprung syndrome of colon and rectum (HSCR) is a multigenic congenital disease and occurs one in five thousand live births (0.02%). Affected children require corrective surgery due to a lack of neurons in the lower intestine. The main causative mutations for HSCR are in the Ret receptor tyrosine kinase, which responds to the Glial Cell Line-Derived Neurotrophic Factor (GDNF) ligand. Down syndrome patients suffer from a 100-fold increase in the incidence of HSCR (1-2%). Rather than loss-of-function mutations, HSCR in these patients is thought to be due to an extra copy of the Dscam gene (Down syndrome cell adhesion molecule). Loss of Ret signaling leads to an absence of neural precursors in the colon and rectum whereas Dscam is proposed to disrupt local axonal connections made by enteric neurons. The precise functions of both genes in ENS formation are still under debate.

The Drosophila stomatogastric nervous system (SNS) is the invertebrate equivalent of the ENS and with the fly's powerful genetics offers the opportunity to shed light on the molecular and cellular mechanisms functioning in ENS development. Ret gene expression is conserved in the migrating neural precursors and we have found Dscam protein is present in the axonal connectives. Although the adult SNS is well characterized in the fly, the embryonic SNS required tool development to allow genetic
In Chapter 1, I present the initial experimental design, which employs the genetically tractable model organism, *Drosophila*. The original goal of my thesis was to clarify the precise role of the Ret receptor in cell migration, survival and cell death. An additional goal was to determine whether the Ret and EGFR receptors cooperate during development by signaling through the same pathway (MAP kinase). A parallel objective was to elucidate the role of the Dscam receptor in normal ENS development and the increased prevalence of HSCR in Down syndrome. The rationale behind all these aims is outlined.

To develop the *Drosophila* embryonic SNS as a model system for the vertebrate ENS, I constructed and characterized tools for transgenic expression (Chapter 2). I cloned fragments of the Ret promoter to the GAL4 gene. Although expression was broader and weaker than hoped for, two lines RetP2A and P2B-GAL4 express in a large subset of SNS precursors and continue to be expressed throughout larval stages. I elaborate on a screen performed in collaboration with another graduate student, Logan Myers, which lead to the identification of the goosecoid (Gsc) promoter. The GscG-GAL4 driver is active during SNS development, but switches off at the end of embryogenesis. I then used these lines to demonstrate that the epidermal growth factor receptor (EGFR) plays a role during axon outgrowth as well as a previously documented role in precursor proliferation and migration. These results demonstrate that our genetic toolset may be employed for the manipulation of SNS precursors and functional analysis during larval stages.

In Chapter 3, I establish that Dscam1 receptor is expressed in the developing
stomatogastric nervous system. I present phenotypic analysis of Dscam1, Dscam1 frazzled and Dscam1 robo1 double mutants, showing that the subtle phenotypes observed in Dscam1 mutants alone are not enhanced nor suppressed by frazzled and/or robo mutations. Moreover, I aimed to model Down syndrome gut nervous system defects in order to elucidate the effect of Dscam trisomy on cell proliferation, migration and/or survival. To achieve this, I employed the tools described in Chapter 2, along with GFP tagged full length and dominant negative Dscam1 isoforms. Thorough phenotypic analysis of embryonic neuroanatomy revealed the SNS structure to be affected. An additional copy of Dscam1 causes overgrowth of the developing frontal nerve and motor neurons innervating the hindgut. We analyzed larval feeding behavior and, surprisingly, did not observe any obvious feeding problems. Consequently, we questioned whether the function of defecation was affected and developed an assay to evaluate possible defects. I found that Dscam1 overexpression in the SNS impairs the ability of larvae to clear food from their gut. We are currently trying to localize the origin of this defect.

Finally, in Appendix 1, I expand on the results obtained using a high-throughput larval locomotion assay. This sensitive assay was employed to assess for additional functional consequences of increased and altered neural connectivity. While driving dominant negative Dscam1 clearly increased overall kinesis of second instar larvae, an added copy of full length Dscam1 adversely affected locomotion. Interestingly, behavioral outputs were dramatically altered in the presence of a typical food odor, ethyl acetate.

In summary, genetic and functional analysis of Drosophila stomatogastric nervous system was facilitated by the new toolset described herein. These tools were used
to dissect the role of EGFR and Dscam signaling in SNS formation. The results provide a mechanistic insight into two complex, multigenic congenital diseases by analyzing the link between genes, nerves and behavior.
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Chapter 1

Developing a *Drosophila* Model of Hirschsprung Syndrome of Colon and Rectum

**Stomatogastric Nervous System**

The enteric nervous system (ENS) is a network of nerves that innervates the esophagus, stomach, and the small and large intestines. It operates in an autonomous fashion in regulating the gut functions of absorption, digestion and propelling the digested content through the gastrointestinal tract. In addition to functioning autonomously, the ENS can influence basic brain functions, which coupled with the surprising complexity has led the ENS to be termed the "second brain" (Michael Gershon). The importance of the ENS is emphasized by conditions such as Hirschsprung’s disease (HSCR), a congenital disease of complex genetic origin leading to a lack of neurons (aganglionosis) in the distal part of the gut. Without surgical correction, HSCR leads to secondary infections and can be fatal (NIH Genetics Home reference). Mutations in more than ten genes have been implicated in the pathogenesis of HSCR (Panza et al., 2012), and it is likely additional genetic loci are yet to be identified due to the genetic complexity of Hirschsprung’s. Down syndrome (DS) patients have a 100-fold greater incidence of HSCR compared to the general population of newborns (Korbel et al., 2009; Yin et al., 2012). However, the biological mechanisms associating HSCR and DS morbidity have not been studied. The *Dscam* gene was recently confirmed as an additional susceptibility locus for Hirschsprung’s in DS, providing a compelling rationale for us to test how Dscam could disrupt ENS formation (Korbel et al., 2009; Jannot et al., 2013).
The *Drosophila* stomatogastric nervous system (SNS) is the invertebrate equivalent of the vertebrate ENS (Spiess et al., 2008). This system is compact, relatively simple and easily analyzed. As part of my thesis I have pioneered the use of clearing agents to allow analysis without embryonic dissections. The SNS arises from three clusters of precursor cells, which migrate posteriorly early in development (Hartenstein, 1993). This process is remarkably similar to migration of the neural crest to give rise to the vertebrate enteric nervous system and conservation of gene expression strongly suggests a shared evolutionary origin (Hahn & Bishop, 2001). The well-established genetics of *Drosophila* suggests analysis of the SNS could lead to insights of the biological mechanisms underlying ENS formation and HSCR. The use of existing model systems in the mouse and chick are acknowledged (Schuchardt et al., 1995; O’Donnell & Puri, 2008), as well as the emergence of the zebrafish system (Shepherd & Eisen, 2011). Furthermore, a *Drosophila* model provides a genetically tractable system with single cell resolution to investigate multiple genetic loci involved in ENS developmental mechanisms and HSCR. Employing a fruit fly model also allows for testing of dose sensitive interactions, knocking out genes at different developmental stages to bypass earlier requirements and the use of the *GAL4/UAS* binary system for targeted gene expression in a temporal and spatial fashion (Brand & Perrimon, 1993).

**Molecules Involved in Migration of Neural Precursors**

Mutations in the *Ret* gene (Rearranged during transfection) are the most commonly implicated in Hirschsprung’s disease. Mice homozygous for *Ret* mutations have complete intestinal aganglionosis, while weaker loss-of function mutations in *Ret* lead to partial
colonic aganglionosis (Schuchardt, et al., 1994; de Graaff et al., 2001; Asai et al., 2006; Uesaka et al., 2007). Ret is a transmembrane receptor tyrosine kinase (RTK) expressed in ventral midline neurons, a subset of which are dopaminergic (Lundell & Hirsch, 1994), and in the developing ENS/SNS. Ret belongs to the dependence receptor family, which in the absence of ligand are cleaved by caspases inducing apoptosis (Goldschneider & Mehlen, 2010; Canibano et al., 2007). Ret can therefore both promote survival and trigger apoptosis (Uesaka & Enomoto, 2010). Ret requires the GFRα co-receptor to bind the glial cell derived neurotrophic factor (GDNF) ligand; binding promotes cell survival, proliferation and migration (Chalazonitis et al., 1998). Uesaka et al. report cell death in GDNF mutant mice, supporting the model that enteric precursors require trophic support, at least in part by GDNF, to migrate and populate the gut. The epidermal growth factor receptor (EGFR) is well known for promoting survival in many tissues, including the fly SNS (González-Gaitán & Jäckle, 2000). Using genetic tools I developed, I was able to demonstrate that EGFR signaling is not only required for early SNS development, but also during later stages of axon guidance (Hernández et al., 2015). I also generated stocks to investigate Ret-EGFR genetic interactions in the SNS, but was hampered by the lack of a strong loss-of-function Ret allele at the time. Prior to starting this work, I developed a model for ENS formation based on a novel biological mechanism. Although I was unable to test this model, I present an overview of it below as I believe it is still valid and could be tested by other members of the laboratory.
A Trophic Support Based Model of SNS Development

Vertebrate ENS formation is widely reported as migration induced by the Ret receptor tyrosine kinase signaling in neural precursors responding to chemoattractive ligands present in the developing intestine such as GFRa (Young et al., 2011; Chalazonitis et al., 2001; Chalazonitis et al., 2012). However, none of the ligands are present in the expected gradients and ablation experiments suggest that proliferation might be the underlying mechanism (Uesaka & Enomoto, 2010). EGFR signaling has been implicated in the mouse and in Drosophila ENS formation (Dumstrei et al., 1998; Hitch et al., 2012). A conservative goal was to test whether there was functional overlap between these systems as they share many downstream signaling components including the Ras/ERK pathway (Figure 1; Pandit et al., 1997; Crone et al., 2003; Croyle et al., 2008). In Drosophila, EGF is a survival factor for glia (Hidalgo et al., 2006). This gliatrophism includes the midline glia, specialized cells that reside at the center of the ventral nerve cord. In an intriguing experiment, Bergmann et al. showed that midline glia migrate to ensure access to axon derived survival factors and activating MAPK signaling can block migration. This argues that rather than directed migration, movement of these cells is simply to ensure trophic support. We speculated that the same mechanism might be operating in the developing SNS and ENS. First, EGFR signaling is also required and second, ~50% of SNS precursors die suggesting strong constraints on survival. The model also has the ability to explain discrepancies observed in the vertebrate ENS.
The fly model offers the opportunity to assess synergy of known pathways.

The hypothesis to be tested is a novel synthesis of current models that migration, and potentially proliferation, are stimulated by competition for trophic factors (Figure 1). The digestive nervous system appears to be the oldest nervous system as it is present in the simplest of animals, preceding the appearance of nerve cords. The principles of its development are very likely evolutionarily conserved. The ease of testing in the fly will allow a significant number of loci to be tested relative to what could be done in a vertebrate model.
Specific Aims

Hirschsprung Syndrome of Colon and Rectum (HSCR) is characterized by a lack of enteric neurons in the distal part of the gut. Despite occurring at a frequency of 1 in 5000 live births, the underlying biological mechanisms are unknown. Although key susceptibility loci have been identified, the inheritance patterns are complex and likely involve many unidentified genetic loci. HSCR is also associated with additional syndromes, notably Down syndrome, but the reason for this relationship is unexplained (as none of the known single gene mutations lie on chromosome 21). This proposal aims to test a novel biological mechanism underlying HSCR and in doing so will assess the potential of novel genetic loci contributing to the disease, including the Down syndrome cell adhesion molecule (DSCAM).

Mutations in the Ret receptor tyrosine kinase (RTK) critically contribute to half of HSCR cases. Drosophila has an excellent homologue (dRet) that is expressed in cells and tissues homologous to the vertebrate gene, including the migrating precursors of the enteric nervous system (ENS). Ret activates the MAPK signaling pathway, which is prominently linked to cell survival and proliferation. Almost 50% of fly ENS precursors die suggesting that ENS survival factors are limited. The epidermal growth factor receptor (EGFR) RTK has been shown to drive migration of CNS midline glia as they seek axons that provide EGF family ligands for trophic support. Increased MAPK signaling is sufficient to block glial migration. We hypothesize that ENS precursors also migrate in a search for trophic support. EGFR activates the same pathways as Ret and is also expressed in the ENS precursors. Our preliminary data reveals that dRet mutants have subtle embryonic phenotypes suggesting Ret signaling functionally overlaps with other
pathways (Figure 1). We have identified the wingless (wg) signaling pathway as likely involved in ENS survival as well. Our laboratory recently demonstrated that Dscam is required for neuronal survival. The latter two pathways would function in parallel to RTK signaling. We will test the hypothesis that dRet genetically interacts with the EGFR, and Dscam signaling pathways to promote normal ENS cell migration and survival. This work will determine if EGFR and Dscam are additional genetic loci implicated in ENS development and will provide biological insights into the mechanisms of Hirschsprung's in humans.

**Specific Aim 1:** Characterization of apoptosis and migration in *Drosophila Ret* mutants.

We will assess the role of apoptotic signaling in the developing ENS by transgenically increasing and decreasing cell death. We will also thoroughly analyze the phenotypes of dRet mutants with respect to cell death and cell number. We have developed transgenic tools that will allow us to manipulate and visualize the fly ENS at high resolution and will use the lines to drive expression of cell death inhibitors and activators in the ENS. We predict that ENS migration will be altered and that dRet mutants will display defects in the overall number of ENS neurons.

**Specific Aim 2:** Determine the functional overlap between EGFR and Ret RTK signaling in ENS development. EGFR drives cell migration in the CNS through trophic signaling. We will test the hypothesis that EGFR and dRet synergistically promote cell survival and migration in the developing ENS by activating the Ras/MAPK pathway. We will employ a variety of mutant combinations, transgenic RNAi and over-expression using our ENS
drivers to manipulate these signaling pathways. We predict that elevated survival signaling will block ENS migration. This work could identify a novel mechanism for ENS formation that would be relevant to vertebrates, as well as identifying EGFR as a novel locus contributing to Hirschsprung's Disease.

**Specific Aim 3:** Identify the mechanism by which Dscam contributes to Hirschsprung's phenotype. We hypothesize that increased Dscam levels will disrupt ENS formation and that synergy with the Ret locus will be observed. Compared to the general population, individuals with Down syndrome have a 100-fold increased risk of HSCR. The region of chromosome 21 responsible for DS-HSCR phenotype is large, but centered on the *Dscam* gene. Both Dscam and Ret receptors are expressed in the neural precursors of the gut nervous system. Preliminary results from our laboratory indicate that Dscam is a novel receptor protecting against cell death. We will identify the role of Dscam in ENS development using both loss and gain of function approaches alone, and in combination with *dRet* mutants. We predict that Dscam and Ret will cooperate to promote normal ENS formation.

**RESEARCH STRATEGY**

**Innovation**

*Drosophila* dRet activates the same signaling pathways as human Ret (Abrescia, 2005), and it is expressed in the ENS precursors prior to and during migration (Figure 2), additionally to several other tissues homologous to the human tissues that express the Ret RTK (Anders et al., 2001; Hahn & Bishop, 2001). dRet promoter fragments are also
expected to provide tools for studying dopaminergic and kidney cells (Malpighian tubules in the fly). These studies are innovative in that the fly enteric nervous system is poorly studied, although in other invertebrates the stomatogastric nervous system is a classic model for neural networks. The tools available for the study of the ENS are limited and for this reason I have been generating new transgene drivers based on the dRet promoter (Figure 3) as well as screening recently generated transgenic lines from HHMI: Janelia Farm (Figure 5). These tools will allow for visualization of the ENS at high resolution, and accurately map ENS development. More importantly, the tools will allow manipulation of genetic functions via transgenic RNAi and expression of transgenes at different stages of development, and in specific subsets of cells.

**Significance**

HSCR is a relatively common congenital disorder with two major challenges for the field: (i) identification of susceptibility loci to allow understanding of the complex genetics and ultimately predictive tests, and (ii) understanding of the mechanisms underlying the disease that may allow therapeutic design in the future. This proposal aims to develop the fly ENS as a system to contribute to both problems.

**Approach**

We will use classical genetics and transgenics to manipulate gene expression, survival and apoptotic processes in the fly ENS. We will employ a combination of novel and existing tools and mutant combinations to understand the mechanisms of ENS development with a focus on neural precursor migration. A key advantage is that reporter
genes and antibodies will allow us to locate and count the number of cells at given stages so that subtle alterations can be detected.

**Preliminary Studies and Results**

Bioinformatic analyses of the *dRet* promoter region in different *Drosophila* species revealed significant conservation in the first and third introns, suggesting the presence of enhancer elements that produce the highly specific pattern of Ret expression. Neural genes frequently utilize intronic enhancers, and such an element has been found in the human Ret gene and polymorphisms linked to HSCR susceptibility (Emison et al., 2005; Moore & Zaahl, 2012). To date, I have successfully cloned four enhancer fragments, two 600 bp, one 1.5 kb and one 3.9 kb into the pBPGUw, GAL4 expression vector using Gateway technology described by Pfeiffer et al., 2008 (Figure 3). This system allows for specific in vivo genomic targeting to the third chromosome using the PhiC31 integrase.

We outsource the labor-intensive task of transgenic line generation to a commercial company as is now standard in *Drosophila* research (Genetic Services, Inc.). Enhancers contained within the genomic fragments will drive expression of the GAL4 gene, a yeast transcriptional activator, and the patterns can be analyzed by crossing the transgenic lines to reporter genes containing GAL4 binding sites, UAS (upstream activation sequence) (Brand & Perrimon, 1993). We have identified alternative ENS-specific expressing genes by literature analysis. These include: fork head (fkh), expressed in anterior and posterior gut primordia, Goosecoid (Gsc), a transcription factor involved in ENS and foregut development, Munin, Putative homeodomain protein (Phdp) and Tupens (Goriely et al., 1996; Hahn & Jäckle, 1996; Pfeiffer et al., 2008). We have characterized *fkh-GAL4*
(Figure 5A), Munin-GAL4 (Figure 5B), Gsc-GAL4 fragments (Figure 5C), which have the potential to specifically drive expression in individual clusters of migrating cells or in their adjacent tissues (Figure 5A-C) (HHMI: Janelia Farm).

**Specific Aim 1:**

**Characterization of migration and apoptosis in *Drosophila* Ret mutants.**

**Rationale**

The *Drosophila* Ret gene, *dRet* is expressed in ENS precursors, which appear to be the functional equivalent of vertebrate neural crest cells, enteric neuron precursors. Homozygous *dRet* deficiencies display subtle changes to the ENS (Figure 4). Additional mutations in the *dRet* gene are being generated in the Kidd lab by imprecise excision of a P element transposon and will be employed after their characterization. Subtle ENS phenotypes were noted during initial characterization of these mutants. Detailed characterization of these mutants is imperative to dissecting the exact role of the *dRet* gene and *Drosophila* Ret receptor tyrosine kinase in ENS development.

**Approach**

An extensive array of available antibodies will aid in the identification of any phenotypic differences between the existing *dRet* mutants and wild type embryos. We will employ 22c10 (anti-Futsch) and 1D4 (anti-Fasciclin; FasII; Grenningloh et al., 1992; González-Gaitán & Jäckle, 1995) to examine the components of the ENS and their relative positioning with respect to the gut epithelium tissue, midline and brain. I am determining the optimal conditions to visualize Ret-expressing cells in the embryo employing a
readily available antibody against activated Ret phosphorylated at tyrosine residue 905 (Cell Signaling Technologies). Furthermore, we have recently developed collaboration with Dr. Irene Miguel-Aliaga from Imperial College London. Her research interest in gut signaling and metabolism suitably complements ours and we have obtained a dRet-specific antibody from her laboratory. We will quantify for decreased proliferation (of ENS precursors) in dRet mutants employing the anti-Fkh (fork head) and anti-Gcm (glial cells missing) antibodies (Jones et al., 1995). Differences in gene expression between wild type and dRet mutants will be analyzed by in situ hybridization (ISH). We will block apoptosis using a variety of GAL4 drivers in combination with the enhancer line UAS-p35, a viral apoptosis inhibitor (Duffy et al., 2002; Hay et al., 1994). We will employ readily available drivers, such as fkh- GAL4, Gsc-GAL4 (Goriely et al., 1996; Hahn & Jäckle, 1996; Pfeiffer et al., 2008), as well as the transgenic reporter constructs I have generated for dRet (Figure 3). We will ablate individual clusters by inducing UAS-reaper under control of fkh- or Gsc-GAL4 to analyze the manner in which the other two clusters respond (Figure 2) (White et al., 1996; Vernooy et al., 2002). Apoptotic cell death will be quantified via conventional techniques: TUNEL staining (terminal transferase dUTP nick end labeling) and anti-activated-caspase antibodies (Nezis et al., 2010; Gurtu et al., 1997). Cell death counts will be analyzed using an automated cell counter and the Volocity software available in our department.

**Expected outcome**

Based on our initial evaluations, the dRet mutants have minimal ENS phenotypes (Figure 4). Specifically, embryos homozygous for the dRet deficiencies will have subtle or no
significant disruption to the normal symmetry of the ENS. However, the amount of cell
death occurring in dRet mutants will be significantly increased. The quantity of dRet
mRNA and dRet-expressing cells should be significantly decreased, while motor neuron
and glial cell positioning in respect to the midline and brain will not be considerably
affected. The evidence obtained from these detailed characterizations will reinforce our
hypothesis that additional RTKs are highly involved in ENS development (Figure 1).

Potential problems and solutions
The dRet deficiency mutations are embryonic lethal when homozygous; therefore,
analysis of the ENS at postembryonic stages is unfeasible. However, embryonic
phenotypic examination, along with gene and protein expression analyses of dRet
deficient heterozygote larvae may serve for further characterization. Furthermore, we will
characterize cell positioning and quantify apoptosis in the additional dRet mutants being
developed employing the P element transposon. The Drosophila H99 mutant is deficient
of the apoptotic genes grim, hid, and reaper. Alternatively, we may take advantage of the
characteristic lack of developmental cell death in H99 mutants to investigate the
consequences of blocking cell death. We may also employ additional transgenic tools
available to drive and block cell death, (e.g. Drosophila caspase inhibitor, DIAP1) (Wang
et al., 1999).
Specific Aim 2:

Determine the functional overlap between EGFR and Ret RTK signaling in ENS development.

Rationale

EGFR amplification, along with Ret gain of function mutations and activating mutations in components of the MAPK pathways are key hallmarks of hereditary and sporadic thyroid cancer, and colorectal cancer (Antonelli, 2011; Antonelli, 2012; Guérin, 2012). Specifically, the ERK pathway is one of the most studied of the MAPK family members of signaling kinases and it regulates cell cycle progression and differentiation (Mouchel-Vielh, 2011), and phosphorylated ERK colocalizes with Ret. In vertebrates, ErbB2, a member of the EGFR/ErbB family of RTKs is required for postnatal maintenance of the ENS (Crone, 2003). The successful development of several Drosophila models for human brain cancers, such as glioma, strongly supports the notion of using the fruit fly as a model for HSCR (Read, 2005; Read, 2009). Our initial attempts to generate Ret-EGFR recombinants have produced heterozygous flies, which show circling and hyperactive behavior. This suggests that both genes are collaborating in a CNS midline function and therefore could also collaborate in the ENS. Moreover, our Ret-EGFR heterozygous flies display a substantial amount of cell death when combined with a balancer chromosome having an activated form of Ras1 expressed under control of the sevenless enhancer/promoter (Karim, 1996) (Figure 6C). Despite a wealth of evidence for interaction of these receptors in various cancers, the collaborative role of these two RTKs in normal ENS development has not been thoroughly assessed. The hypothesis to be tested is that EGFR and Ret synergistically promote cell survival and proliferation.
in the developing SNS by activating the Ras/ERK pathway.

**Approach**

The *Drosophila* epidermal growth factor receptor homolog, Torpedo or DER, has a complex biology and is expressed in two consecutive steps of early SNS development. EGFR and Ret are expressed in the same cells during SNS development and akin to Ret signaling, EGFR RTK signaling is involved in Malpighian tubule development (González-Gaitán & Jäckle, 2000). Like Ret, dRet is expressed in the migrating precursors of the ENS and our preliminary results suggest that Ret is an evolutionarily conserved central regulator of ENS formation. Using standard means, we are constructing mutant combinations between the EGFR and Ret deficiencies, as well as an EGFR loss of function (amorphic allele) with the Ret deficiency. Mutant embryos will be analyzed for phenotypic consequences, such as neuronal number, fate, position and connectivity utilizing a broad set of commercially available antibodies. These antibodies include anti-EGFR, anti-Ret, and anti-fork head. CNS and SNS neurons and axon subsets will be visualized using the 22c10 and 1D4 antibodies. There are fewer members of the MAP kinase family in *Drosophila* than in vertebrates, but the three main families ERK1/2, p38 and JNK are represented. Synergistic activation of the Ras/ERK pathway will be quantified by standard biochemical methods, such as immunoblotting and immunoprecipitation. Moreover, embryonic antibody labeling with anti-Ras, anti-Raf, anti-MEK, anti-ERK and anti-phosho-ERK (Sigma, CST) (Sackton et al., 2007) will aid in visualizing activation of the Ras/ERK pathway. At the molecular level, the extent of expression of the genes of interest will be analyzed by in situ hybridization.
**Expected Outcome**

We expect to observe synergistic phenotypes in dEGFR and dRet combination mutants. We predict that neuronal proliferation will be significantly decreased and cell positioning will be disrupted. The ideal outcome would be that the overlapping components of the two RTK signaling pathways would be synergistically affected. Activated signaling molecules of the Ras/ERK pathway will be significantly downregulated, modeling the disrupted kinase signaling in Hirschsprung’s.

**Potential Problems and Limitations**

The proposed genetic methodologies are standard in our laboratory (Kidd, et al., 1999; Bashaw, et al., 2000) and my experience analyzing EGFR and Ras/ERK expression and signaling under altering conditions in cancer models employing a variety of quantitative biochemical approaches (Rogers, 2010) will complement this expertise. The majority of the genes of interest are located on the second chromosome; constructing combination mutants will require recombination, which is only feasible given that a reasonable genetic distance separates the genes. The EGFR and Ret loci are significantly far apart which will increase the odds of recombination. In case the deletion of both RTKs has affects on earlier development, the transgenic tools we have developed and characterized will allow the use of tissue specific RNAi in combination with sensitized genetic combinations as an alternative approach. During our initial attempts to construct Ret-EGFR recombinants, we attempted to maintain the recombinants over a balancer chromosome with an allele of wingless. Subsequent analysis strongly suggests that this triple heterozygote mutation is
embryonic lethal. We repeated the experiments with a different balancer and successfully recovered the desired recombinants. Based on these observations, we will alternatively characterize the functional overlap between Ret, EGFR and wingless. We have a range of markers for the visceral mesoderm (anti-myosin), the endoderm (GATAe probe) and foregut (anti-Crumbs) to detect underlying developmental abnormalities that could lead to indirect effects on SNS formation. As a visualization alternative, the gut can be dissected out of embryos and larvae to allow closer examination. Biochemical techniques for protein expression analyses may be adopted as alternatives to embryonic antibody labeling.

**Specific Aim 3:**

**Identify the mechanism by which Dscam contributes to Hirschsprung's phenotype.**

**Rationale**

Increasingly strong genetic evidence implicates trisomy of the Dscam gene as responsible for the greatly increased incidence of HSCR in Down syndrome patients (Korbel, 2009; Jannot, 2013).

Dscam is expressed by the neural crest cells that give rise to the ENS (Yamakawa, 1998) and by enteric neurons in the mouse embryo (G. Mastick, pers. comm.). The most obvious mechanism would appear to be that Dscam disrupts the guidance of enteric precursors, and yet the known ligands for Dscam are not expressed in gradients. Dscam activates p21 activated kinase (PAK) (Schmucker, 2000; Li & Guan, 2004), a pathway that promotes cell survival via MAP kinases. **We will test the hypothesis that increased Dscam expression disrupts survival signaling in enteric precursors resulting in**
modified migration and survival patterns. Interactions with Ret mutants will also be tested.

Approach

We will crudely mimic trisomy for Dscam by using our identified transgenic lines to over-express Dscam in the developing SNS (Figure 5). We will utilize four different Dscam transgenes that are splicing variants and that have different signaling capacities (Lee, 2009). The effects of over-expression will be assayed using the 22c10 and 1D4 monoclonal antibodies that stain SNS neurons, and anti-Repo to visualize SNS glia. The transgenes themselves are tagged with GFP allowing further visualization either direct fluorescence or immunohistochemistry to determine cellular and subcellular localizations. We will express the transgenes in wild and mutant Ret backgrounds to detect potential synergies between Dscam and Ret in HSCR. Observed phenotypes will be analyzed for neuronal and axonal positioning, glial position and cell number allowing biological interpretation of the results. Immunoblotting and immunoprecipitation will be employed to further quantify protein expression and analyze protein-protein interactions.

Expected result

If our model is correct, increased Dscam signaling will disrupt MAPK signaling and reduce the need for neuronal precursors to migrate in search of survival cues. Alternatively migration may be minimally affected and axon guidance may be disrupted.

Potential Problems and Limitations
We have the genetic tools and reagents necessary for Dscam loss and gain of function experiments in our laboratory. Our laboratory has extensive experience constructing and working with Dscam double and triple combination mutants (Andrews, et al., 2008). Tissue specific RNAi may be employed as an alternative to obliterate dRet and/or Dscam expression, to circumvent requirements earlier in development. Further experiments may be pursued to dissect the role of PAK in signaling; this is a feasible alternative as the sponsor has done extensive p21 activated kinase screens.
Figure 1. Parallel signaling pathways hypothesized to cooperate in ENS development.
Figure 2. Expression of the *Drosophila Ret* gene in migrating SNS precursors.

Embryos stained to show Ret mRNA expression (blue) by in situ hybridization and counterstained with 22c10 (brown) to reveal the PNS and a subset of CNS axons. (A) Stage 14 embryo showing two SNS clusters expressing Ret mRNA (arrowheads) as well the fly equivalent of the kidneys, the Malphigian tubules (asterisk). (B) Side view of a same stage embryo showing the clusters (arrows) migrating along the digestive tract and previously undescribed Ret expression in the anterior portion of the midgut (asterisk). These figures illustrate the dynamic nature of Ret expression as the anterior cluster is clearly weaker in expression, and the third SNS cluster is no longer visible in either embryo.
Figure 3. Promoter analysis of the dRet gene.

The fruit fly has an obvious homologue of the human Ret gene, dRet. The indicated genomic regions will be used to drive expression of the GAL4 gene in transgenic animals with the objective of generating SNS specific reagents for the proposed aims. Six transgenic lines comprising dRet exon 1 and/or intron 1 have been generated and tested for SNS-specific expression patterns.
Figure 4. Disruption of the *Drosophila* stomatogastric nervous system.

B, C embryos stained with 1D4 monoclonal antibody to reveal the SNS and brain commissure. (A) Diagram from González-Gaitán and Jäckle, 1995 illustrating the topography of the SNS (red) at the end of embryogenesis. Two frontal ganglia (FG) lie underneath the brain lobes and are joined by the frontal commissure (fcm). From the center of the frontal commissure, the recurrent nerve (rn) projects backwards to the two sets of esophageal ganglia (EG1, EG2). (B) A wild type embryo with normal positioning of the frontal commissure (arrow), brain commissure (long arrow) and part of the esophageal ganglia (asterisk) (C) An embryo lacking Ret activity (homozygous *dRet* deficiency) showing a break in the recurrent nerve (arrowhead). Subtle disruptions to the normal symmetry of the SNS are seen in virtually all mutant embryos (three independent mutants examined, at least 10 embryos per mutant all crossed to the *dRet* deficiency). The embryo shown represents the severest phenotype seen. The asymmetry can affect neuron position as well suggesting dRet plays a role in both neuron migration and axon guidance. The frontal commissure (arrow) appears thinner than normal (compared to the slightly younger embryo in B) suggesting there may be less neurons. The esophageal ganglia appear clumped (asterisk).
Figure 5. Transgenic expression in the *Drosophila* SNS.

Embryos labeled with anti-beta-Galactosidase antibody, which detects expression of the UAS-taulacZ (A, B) and UAS-nuclear-lacZ (C) reporters. (A) A fragment of the forkhead promoter drives expression of tau-lacZ in three migrating clusters of SNS cells (arrowheads), which will give rise to the gut nervous system. (B) An older embryo exhibiting the migrating clusters labeled by a fragment of the Munin promoter as they approach their destinations (arrowheads). (C) A fragment of the Goosecoid promoter labels the cells underlying the SNS precursor clusters allowing manipulation of the migratory environment (arrow).
Figure 6. Ret and EGFR synergistically interact in a dosage sensitive manner.

Adult compound eyes carrying an activated Ras construct. (A) Heterozygous deficiency for Ret has no effect on the Ras phenotype. (B) Mild enhancement of the Ras phenotype by the presence of one copy of an EGFR deficiency is revealed by a rough appearance. (C) Synergistic interaction between the Ret and EGFR deficiencies (both heterozygous) leads to dramatically increased cell death as revealed by black necrotic tissue and unpigmented cells. (These proof of principle experiments will be repeated with both dRet and EGFR point mutations.)
Literature cited


Chapter 2
Genetic Tools for the Analysis of Drosophila Stomatogastric Nervous System Development

Preface


*These two authors contributed equally to this work.

Abstract

The Drosophila stomatogastric nervous system (SNS) is a compact collection of neurons that arises from the migration of neural precursors. Here we describe genetic tools allowing functional analysis of the SNS during the migratory phase of development. We constructed GAL4 lines driven by fragments of the Ret promoter, which yielded expression in a subset of migrating neural SNS precursors and also included a distinct set of midgut associated cells. Screening of additional GAL4 lines driven by fragments of the Gfri/Munin, forkhead, twist and goosecoid (Gsc) promoters identified a Gsc fragment with expression from initial selection of SNS precursors until the end of embryogenesis. Inhibition of EGFR signaling using three identified lines disrupted the correct patterning of the frontal and recurrent nerves. To manipulate the environment traveled by SNS precursors, a fasII-GAL4 line with strong expression throughout the entire intestinal tract was identified. The transgenic lines described offer the ability to specifically manipulate
the migration of SNS precursors and will allow the modeling and in-depth analysis of neuronal migration in ENS disorders such as Hirschsprung’s disease.

**Introduction**

The invertebrate stomatogastric nervous system (SNS) has provided a wealth of information on the functioning of simple neural networks (Marder & Bucher, 2007). In *Drosophila*, all aspects of the adult gut including the enteric nervous system (ENS) have received intense attention in recent years (Lemaitre & Miguel-Aliaga, 2013). After initial characterization of the embryonic development of the SNS primarily by the Hartenstein and Jäckle groups (Dumstrei et al., 1998; Forjanic, Chen, Jackle, & Gonzalez Gaitan, 1997; Gonzalez-Gaitan & Jackle, 1995, 2000; Hartenstein, 1997; Hartenstein, Tepass, & Gruszynski-Defeo, 1994, 1996), the early SNS has received relatively little consideration. This is surprising as the SNS is a simple developmental system and likely to be of clinical relevance to vertebrate ENS disorders.

The SNS begins as three epithelial pouches in the primitive mouth (stomatogastric) that delaminate and migrate along the developing foregut as coherent clusters (referred to as invaginating SNS precursors or iSNSPs; reviewed in (Hartenstein, 1997)). An additional group of cells (dSNSPs) delaminate in front of the iSNSPs (Hartenstein et al., 1994). The SNS anlage is located within the roof epithelium of the stomodeum, the primitive mouth of the embryo. Within the anlage, three single cells, called tip cells (tSNSPs), are selected by the action of the proneural (*achate-scute*), neurogenic (*Notch*) and wingless (*wg*) genes (Gonzalez-Gaitan & Jackle, 1995). The tip cells secrete an Epidermal Growth Factor (EGF), Spitz, which induces EGF receptor (EGFR) signaling in the surrounding
cells, inducing them to delaminate from the epithelium and form migratory vesicles (Dumstrei et al., 1998; Gonzalez-Gaitan & Jackle, 2000). These three clusters of cells migrate along the foregut and then start to produce daughter cells that separate and migrate both anteriorly and posteriorly to form discrete ganglia (Hartenstein et al., 1994). In anterior to posterior order, the ganglia are: the frontal ganglion which lies on top of the pharynx anterior to the brain commissure, two sets of esophageal ganglia which lie alongside the esophagus, and the proventricular ganglion which innervates the crop-like proventriculus that forms at the junction of the foregut and midgut (Spiess, Schoofs, & Heinzel, 2008). Cells from each iSNSP cluster contribute to each of the ganglia, whereas the dSNSPs contribute only to the frontal ganglion (Hartenstein et al., 1994).

The fly SNS has strong parallels with the vertebrate neural crest as epithelial cells delaminate and migrate to their final destinations. In vertebrates, the RET receptor tyrosine kinase has a critical role in the migration of enteric neuron precursors and mutations are a key cause of Hirschsprung’s disease in which the colon and rectum have severely decreased innervation (Ibanez, 2013; Lake & Heuckeroth, 2013; McKeown, Stamp, Hao, & Young, 2013). Intriguingly the fly Ret gene is expressed in the migrating SNS precursors suggesting there may be a shared evolutionary origin (Hahn & Bishop, 2001). Drosophila Ret mutants affect dendrite growth but have not yet been examined for SNS defects (Soba et al., 2015). We wished to generate transgenic reagents specific to the developing SNS as many developmental genes affect multiple stages and tissues during development, which can hinder phenotypic analysis. Some of the reagents may allow functional assays of feeding and peristalsis to be conducted in larvae. We constructed fragments of the Ret promoter to the GAL4 gene and also screened additional GAL4
lines. Three specific GAL4 lines, Gsc-GAL4, Gfrla-GAL4 and RetP-GAL4, were identified that allow the manipulation of SNS precursors and these will be made available to the research community.

Materials and Methods

Molecular Biology
A 527 base pair fragment upstream of the Ret transcription start site was amplified with Phusion high fidelity DNA polymerase from genomic DNA derived from an Exelixis isogenic stock (Thibault et al., 2004) with CCAGGTAAACCCTTTTATCG (forward) and CCGCGGAAATACTTTTTGG (reverse) primers (written from 5’ to 3’), cloned into pCR8/GW/TOPO (Life Technologies Inc.) and subcloned into the StuI and EcoRI sites of pPTGAL (Addgene; (Sharma, Cheung, Larsen, & Eberl, 2002)). P-element injections were performed by Genetic Services, Inc. (Sudbury, MA) and one transformant was recovered. The same fragment was cloned into pENTR/D-TOPO (Life Technologies Inc.) and subcloned into pBPGUw (Addgene; (Pfeiffer et al., 2008) using LR Clonase II (Life Technologies Inc.). Two additional fragments were amplified (Figure 2) and cloned the same way using the forward primer above and GTATGACTGCTAATTATT (reverse), and GTCGTATGTTATTAGCAT and CGGATATTAGACCACGAAC primers. Sequencing of constructs was performed by the Nevada Genomics Center. Injection using phiC31 integrase into the attP2 landing site (Bloomington #25710 nos-phiC31-int.NLS, attP2) was performed by Rainbow Transgenics (Camarillo, CA) and Genetic Services Inc. Six additional transformants were recovered.
Immunohistochemistry

Antibody staining was performed as described in (Patel, 1994), and in situ hybridizations per (Kopczynski, Davis, & Goodman, 1996). Ret probe was generated by transcription of a 3 kilobase genomic fragment cloned in pBluescript. We generally use 70% glycerol in PBS or 0.1M Tris pH 8.0 as clearing agents. However, the lipid rich midgut can be hard to resolve with microscopy, so we tested several clearing protocols including ClearT (Kuwajima et al., 2013). We found that the best results were obtained with either Focus Clear and Rapid Clear (Cedarlane; (Liu & Chiang, 2003)); both reagents were also useful for imaging late stage 17 embryos.

Drosophila Genetics

Janelia Farm GAL4 lines were all obtained through the Bloomington Drosophila Stock Center (BDSC). Gsc-GAL4 lines are: 46772, 48376, 46773, 48377, 40381, 40382, and 40383. Gfrl-GAL4 lines are: 47237, 47238, 47239, 47275, 40663, 40664, 40665, 40666 and 40667. fkh-GAL4 lines are: 47326, 48746, 48764, and 48795. twi-GAL4 lines are: 46150, 48725, 48729, and 48760. Please note some of these lines are no longer available but we are happy to supply them on request. The stock number for fasII-GAL4 is 46123, UAS-EGFR RNAi on III is 36770 and UAS-EGFR-DN on II and III 5364. UAS-nuclear-lacZ and UAS-CD8-GFP were obtained from the BDSC. UAS-tau-lacZ was obtained from M. Fujioka. Several of the GAL4 lines are no longer available from Bloomington and we are more than willing to supply them upon request. The w^{1118} stock was the most reliable wild type stock as other reference stocks do not consistently display the wild type neuroanatomy described in previous publications.
Statistics

For each genotype, stage 17 embryos were collected at random and scored for the presence, absence or thinning of the frontal nerve, and for defasciculation defects in the recurrent nerve. At least ten embryos were collected for each genotype. The 95% confidence interval and the Fisher exact test with two tails for the phenotypes was calculated using the GraphPad website ([www.graphpad.com/quickcalc](http://www.graphpad.com/quickcalc)). Statistical significance was assessed using the Bonferroni correction.

Results

**Ret expression in the developing SNS**

Expression of the *Ret* gene has been thoroughly documented in the *Drosophila* embryo (Hahn & Bishop, 2001). We confirmed expression in the migrating SNS precursors (Figure 1A-D, G-I). *Ret* expression is dynamic, with expression reduced in SNS cells that have completed migration (Figure 1E-F). We also noted expression in the anterior midgut, which is present throughout the midgut by the end of embryogenesis (Figure 1E-F). Gut expression is robust but appears weaker than expression in other tissues. We additionally noted expression in a paired set of CNS neurons at the level of the subesophageal ganglia (Figure 1H) along with an ordered row of midline cells in the ventral nerve cord.

**Generation of Ret-GAL4 lines**

Traditional pan-neural promoters do not express during SNS precursor migration and previously identified promoter elements either have broad or highly limited expression
(Gonzalez-Gaitan & Jackle, 2000; Hartenstein et al., 1994). We chose to place fragments of the Ret promoter in front of the GAL4 gene with the goal of generating more SNS specific reagents. Ret is distinguished by a short promoter region upstream of the transcriptional start and three large introns (Figure 2A). We cloned the promoter region into the pPTGAL vector (Sharma et al., 2002) and generated transformants using P element transposase (RetP1-GAL4). We also placed the promoter into the pBPGUw vector (RetP2-GAL4) (Pfeiffer et al., 2008), as well as the promoter fused to the first intron (RetPI-GAL4), and the second half of the first intron (RetI-GAL4; Figure 2A); transformants were generated using the PhiC31 integrase. Transformant recovery proved especially difficult for all transgenes and even identical constructs integrated into the same site yielded differences in expression (Figure 9S; Table 1), suggesting there may be negative selective pressure towards the Ret control regions when fused to GAL4. The promoter constructs yielded broad expression particularly in the epithelial lining of the midgut (Figure 2B and 7S). Expression occurs after migration of the endodermal cells (Hartenstein et al., 1994) and often appears continuous, but then becomes restricted to a large number of discrete cells, sometimes in linear arrangements (Figure 2C, D). Midgut expression is most pronounced in the RetP1-GAL4 construct. Expression was also seen in the brain (Figure 2E), in what may be a single lineage for either the mushroom bodies or a more basal lateral cluster (Sprecher, Reichert, & Hartenstein, 2007). The pBPGUw insertions (RetP2A,B-GAL4) displayed expression in a subset of the migrating SNS precursors as defined by Ret expression (Figure 2F-G and 7S). Additional isolated cells throughout the head region express Ret as has been seen for Ret mRNA (Hahn & Bishop, 2001), including a subset of cells projecting through the subesophageal commissure.
Expression of *Ret-P2* persists to the end of embryogenesis and was found to label a subset of cells in the frontal and esophageal ganglia (Figure 2H). Strong expression of reporters was observed in SNS cells at the proventriculus and additional labeled cells further along the gut (Figure 2I, 2K, 2L). Finally expression was also observed in larval midgut and body wall neurons (Figure 2L’). Additionally, we cloned the entire first intron into pBPGUw yielding strong expression in the midgut and hindgut, but not in the SNS (Figure 9S; Table 1). The second half of the first intron produced expression in gut related tissues but not the SNS (Figure 9S; Table 1). The *RetP2* transgene appeared the most useful for SNS manipulation even though expression was only observed in a subset of cells, because expression persists into larval stages primarily in the proventricular ganglion (Figure 2L).
**Identification of Additional SNS Specific GAL4 Lines**

The limited SNS expression and additional expression of the *Ret* promoter fragments prompted us to look for additional reagents. We examined the Janelia Farm Fly Light GAL4 lines (Jenett et al., 2012; Pfeiffer et al., 2008) for driver fragments derived from genes with known SNS expression. *Ret* has an evolutionarily conserved co-receptor known as Gfrl or Munin in flies (Kallijarvi et al., 2012), and we tested nine *Gfrl* lines for SNS expression (Table 1). One line (#47237) had highly specific SNS expression from initial delamination of the SNS precursors until the end of embryogenesis (Figure 3A,B). This line (which we will refer to as *Gfrl-GAL4*) also displays brain lobe expression strongly resembling that of the RetP1 construct. Two additional lines (#47238, #47239) had broader expression in the esophagus and likely the SNS too (Figure 3C-F). #47239 also has brain lobe expression. A fourth line expresses in cells at the leading edge of dorsal closure (Figure 3G,H). These lines have expression that appears significantly more restricted to the SNS than the Ret lines, with *Gfrla-GAL4* having the greatest potential for SNS manipulation. We also tested fragments of the *forkhead* (*fkh*), *Goosecoid* (*Gsc*) and *twist* (*twi*) genes as their expression has been reported in the SNS (Gonzalez-Gaitan & Jackle, 1995; Hahn & Bishop, 2001; Hahn & Jackle, 1996; Hammonds et al., 2013). Two *Gsc* lines were of interest, #40382 with esophageal and likely SNS expression (Figure 3I,J) and #40383 with strong specific SNS expression (see below). Three *fkh* lines had midgut and hindgut expression (Figure 3K-P), with #47326 having brain lobe expression like Ret-P1, #48764 having very broad expression and #48795 strongly resembling Ret-P1 in the overall expression pattern. One *twi* line #46150 has potential expression in a very small subset of the SNS. However *Gsc* line
#40383 stood out for its striking SNS specificity and duration of expression so we chose to characterize it further.

**Maternal Effect of the attP2 Integration Site Chromosome**

The Fly Light lines are integrated into a third chromosome site, attP2, using the phiC31 site-specific integrase system (Groth, Fish, Nusse, & Calos, 2004). We noticed that several GAL4 lines, but especially GscG-GAL4 produced shorter embryos. This effect was regardless of reporter used and was only observed when the GscG-GAL4 was the mother. The embryos themselves appear completely normal when assessed with 22c10 or 1D4 staining, just compressed along the anterior-posterior axis. A large number of embryos fail to hatch and the lines were quite difficult to maintain as homozygotes. Balancing in combination with a dominant male sterile mutation helped significantly. For all crosses, we used the GAL4 line as the male parent.

**Characterization of a Gsc-GAL4 line**

A transposable element, SNS1-GAL4, inserted in the Goosecoid (Gsc) gene had been previously used to drive early SNS expression (Gonzalez-Gaitan & Jackle, 2000), which led us to test Gsc promoter fragments. Gsc #40383 expresses in all three SNS clusters from the start of SNS delamination until the end of embryogenesis (we will refer to this line as GscG-GAL4). This element contains parts of exon coding regions from the first and second exons of Gsc (Figure 4A). Early stage 11 expression of GscG-GAL4 may be restricted to the three delaminating clusters but rapidly broadens to include cells in the underlying esophagus (Figure 4B,C). Expression in stage 16/17 is subsequently
restricted to the migrating SNS precursors as well as the cluster of brain cells seen in RetP1-GAL4 and the Gfrl-GAL4 lines (Figure 2E, 3B,F, L). Additional cells are seen in the head region as seen for Ret mRNA. At the end of embryogenesis the line has strong expression in the frontal nerve, frontal commissure, recurrent nerve, and proventricular ganglion (Figure 4E-G). This line has the most complete expression in the anterior elements of the SNS, while RetP1-GAL4 appears to express in more proventricular cells and for longer.

Manipulation of SNS Migration using Gfrl-GAL4 and RetP2-GAL4

To test the utility of the identified GAL4 lines we chose to manipulate EGFR function. As noted above EGFR plays an important role in delamination of the SNS precursors and this early phenotype would preclude later phenotypes from being observed. We used RetP2A- and RetP2B-GAL4 to drive an EGFR dominant negative construct (Buff, Carmena, Gisselbrecht, Jimenez, & Michelson, 1998; Ni et al., 2009). In each case we observed frequent loss or reduction of the frontal nerve and disruption of the recurrent nerve (Figure 5B, B', B'', C, C', E, F, G). We observed the same phenotype with Gfrla-GAL4 driving EGFR RNAi (Figure 5D, D', D''). Obtaining the same phenotype with three different GAL4 lines and two different constructs validates the lines identified. Similar results were obtained with GscG-GAL4 and both dominant negative and RNAi transgenes. The observed phenotypes indicate that EGFR signaling is likely required for axon growth/guidance of the frontal and recurrent nerves. Based on the appearance of the esophageal ganglia (Figure B'', C'') cell number is not disrupted by EGFR inhibition. We could not accurately determine whether cells in the frontal ganglia are lost as anti-fasII
staining does not reveal the cell bodies of the frontal ganglia in later stages (when the frontal nerve has developed). Most of the recurrent nerve axons project from the esophageal ganglia and project anteriorly, but a few appear to originate in the frontal ganglia and project posteriorly (Spiess et al., 2008). The recurrent nerve phenotype appears to arise from esophageal ganglion axons projecting anteriorly and defasciculating from the nerve (Figure 5D’’). The axons can either change direction after passing under the brain commissure, or may fail to join the recurrent nerve in the first place projecting around the commissural surface of the brain lobe. Confocal microscopy could not further distinguish the origins of the phenotype (Figure 10S). Similar results were observed with Gsc-GAL4 and both EGFR RNAi and the dominant negative transgene (Figure 5F,G, 11S). With our current level of analysis, we cannot rule out alternative explanations including non-cell autonomous effects on brain neurons although we think this unlikely.

We note that similar recurrent nerve phenotypes were observed for the misshapen/Ste20/l(3)6683 kinase that regulates MAPK signaling (Forjanic et al., 1997).

The Gsc expression pattern suggests there could be a cell type boundary at or near the defasciculation point at the pharynx-esophageal junction (Hahn & Jackle, 1996), perhaps indicating that secreted cues change at this position and that there could be choice point for growing axons.

### A GAL4 Line for Gut Specific Expression

We anticipate wanting to be able to manipulate the esophagus and gut tissue over which the SNS precursors migrate and differentiate. We identified a GAL4 line with a fragment of the fasII promoter (#46123) that expresses strongly from the earlier stages of gut
formation until the point at which SNS precursors stop migrating (Figure 6). The line is striking as it expresses simultaneously in the foregut, midgut and hindgut. As the gut is derived from different cell populations and germ layers (Tepass & Hartenstein, 1994), GAL4 lines typically express in a subsection of the gut rather than the entire tissue (Ward et al., 2002). The fasII line identified expresses in the intestinal epithelium as opposed to the visceral muscle that surrounds the gut. Over-expression of the EGFR ligand Spitz led to extremely disrupted embryos as would be expected from high expression of a potent growth factor. Additional over-expression experiments employing activated $d\text{Ret}^{\text{MEN2B}}$ produced subtle SNS phenotypes and in some instances, ectopic neural growth (Figure 12D).

**Discussion**

**Lines identified in this study**

Our analysis has identified three GAL4 lines that have embryonic specific SNS expression with limited expression in other tissues. The $\text{RetP2-GAL4}$ line expresses in a subset of SNS cells with strong persistent expression in the proventricular ganglion in the first instar larva. The $\text{Gfrl-GAL4}$ line expresses in most SNS cells until the end of embryogenesis. The $\text{Gsc-GAL4}$ line expresses in likely all SNS cells, and based on reporter gene expression is the strongest driver we identified in the embryo, although SNS expression does not persist into the larvae. All three lines have utility in SNS manipulation as they produce EGFR phenotypes that occur after EGFR’s role in the specification of SNS precursor clusters. As SNS delamination was unaffected by any of the GAL4 lines used, these lines may not be used to manipulate the earliest stages of SNS
migration. The strength of reporter genes suggests the GAL4 lines may have relatively low expression. We are currently building stocks to amplify or permanently switch on expression to assess whether the lines will be useful in larval analysis.

**Relationships between identified gene fragments**

Striking similarities are observed between the expression patterns of the promoter fragments analyzed. RetP1-GAL4, RetP2A-GAL4, RetP2B-GAL4, Gfrl-GAL4 (2 different lines), fkh-GAL4 and Gsc-GAL4 all express in cells near the back of the brain lobes, in the migrating SNS precursors and/or the underlying esophageal cells, suggesting that these genes may be functionally linked. It has been suggested that Ret and Gfrl did not function as a cis receptor-coreceptor pair before the emergence of GDNF family ligands in vertebrate lineages (Kallijarvi et al., 2012). The shared expression patterns of Ret and Gfrl regulatory elements suggest a functional relationship exists in flies and likely other invertebrates. We note that Ret and Gfrl share expression both in the SNS and in the malphigian tubules (Hahn & Bishop, 2001; Kallijarvi et al., 2012), the fly homologue of the vertebrate kidney where Ret also plays a role (Pachnis, Mankoo, & Costantini, 1993; Schuchardt, D'Agati, Larsson-Blomberg, Costantini, & Pachnis, 1994). We are currently generating Ret mutants in the fly to establish the precise role it plays in SNS formation.

The Ret gene is one of the key markers for neural crest cells migrating into the gut (Sauka-Spengler & Bronner-Fraser, 2008) and plays a key role in enteric nervous system formation (McKeown et al., 2013). Neural crest cells acquire their identities through the expression of neural crest specifier genes such as the Snail, FoxD3 and SoxE genes. The fly Sox10 ortholog is not expressed in the SNS and appears to have been co-opted into
neural crest development during the course of evolution through altered expression patterns (Cossais et al., 2010). It is therefore interesting to note that although the fly FoxD3 homologue is not expressed in the SNS (Hacker, Grossniklaus, Gehring, & Jackle, 1992), the related *fkh* gene is required for SNS formation (De Velasco, Shen, Go, & Hartenstein, 2004). Similarly, *Gsc* homologues have roles in neural crest development (Gottlieb, Hanes, Golden, Oakey, & Budarf, 1998; Parry et al., 2013). Identifying regulators of SNS development in the fly and their functional relationships has the potential to shed light on vertebrate neural crest formation. Other insects display a similar migration of enteric precursors (Copenhaver & Taghert, 1990, 1991; Knipp & Bicker, 2009), suggesting that SNS precursor migration is an ancient developmental program. It will be interesting to see whether there is an evolutionarily conserved regulatory network driving *Ret* expression. Such information will likely be useful in unraveling the genetic complexity of Hirschsprung’s disease in humans.

**Future directions**

Identification of three independent SNS specific drivers with different expression characteristics provides an opportunity to investigate the development of the fly SNS. Relatively few SNS developmental components have been identified and fewer have been characterized to date, particularly in the later stages of embryonic development. Reporter genes can be used as markers to dissect phenotypes in greater detail. The ability to complement loss of function data with gain of function data is an important tool in analyzing function, as is the ability to rescue phenotypes. Some of the lines such as *Ret-P2* may be useful in larval feeding assays that can provide comprehensive functional
Hirschsprung’s disease occurs one hundred times more frequently in Down syndrome patients and overexpression of the Dscam gene is the leading candidate gene (Jannot et al., 2013). The SNS drivers will facilitate modeling of Hirschsprung's disease in a simple organism.
<table>
<thead>
<tr>
<th>Gal 4 Driver Line</th>
<th>Expression Time</th>
<th>Expression Place</th>
<th>Primary Expression Feature</th>
<th>Embryonic SNS expression (stage)</th>
<th>Larval Expression (mCD8 GFP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gsc A 46772</td>
<td>embryo 11–17</td>
<td>Surrounding brain lobes and weak midline (15–17)</td>
<td>Exterior brain lobes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gsc B 48376</td>
<td>embryo 11–17</td>
<td>Broad brain lobe expression (11–17); anterior sensory neurons (11–17); lining of esophagus (13–16)</td>
<td>Full brain lobes</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Gsc C 46773</td>
<td>embryo 13–17</td>
<td>Weak CNS expression (11–17)</td>
<td>Weak CNS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gsc D 48377</td>
<td>embryo 12–17</td>
<td>Strong CNS expression (12–17)</td>
<td>Complete CNS</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Gsc E 40381</td>
<td>embryo 11–17</td>
<td>Weak CNS expression (12–17)</td>
<td>Weak CNS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gsc F 40382</td>
<td>embryo 12–16</td>
<td>Esophagus and foregut/esophageal ganglion (12–16); mild outer brain lobe (12–16); small hindgut segment (12–16)</td>
<td>SNS</td>
<td>st 12–16</td>
<td>Esophagus (pharyngeal muscles?); hindgut</td>
</tr>
<tr>
<td>Gsc G 40383</td>
<td>embryo 11–17</td>
<td>Pre-migrating SNS (11); early esophagus and foregut (11–13); proventriculus/foregut (16/17); frontal ganglion and FNJ (17); posterior brain lobe cluster (15–17)</td>
<td>Foregut/SNS</td>
<td>st 11–17</td>
<td>-</td>
</tr>
<tr>
<td>Mun α 47237</td>
<td>embryo 10–17</td>
<td>Brain lobes (12–17); SNS precursors (10/11); anterior end of midline (12–16)</td>
<td>Very early SNS</td>
<td>st 11–14</td>
<td>Anterior midgut cell bodies; hindgut</td>
</tr>
<tr>
<td>Mun β 47238</td>
<td>embryo 11–17</td>
<td>Anterior tip of esophagus (13–15); esophageal ganglion (13); brain lobes (15–17); large posterior brain lobe cluster (16–17); anterior receptor cell clusters (11–17)</td>
<td>Mid-stages SNS marker</td>
<td>st 11–13</td>
<td>Anterior midgut cell bodies</td>
</tr>
<tr>
<td>Mun γ 47239</td>
<td>embryo 11–17</td>
<td>Lining developing esophagus (12–16); optic lobe precursors (11–12); weak brain lobe (15–17); receptor cells in anterior end of embryo (16–17)</td>
<td>Early SNS/anterior sensory neurons</td>
<td>st 11–15</td>
<td>-</td>
</tr>
<tr>
<td>Mun δ 47275</td>
<td>embryo 11, 15–17</td>
<td>SNS precursors (11); anterior sensory receptors (16–17)</td>
<td>Mild anterior sensory neurons</td>
<td>st 11</td>
<td>-</td>
</tr>
<tr>
<td>Mun ε 40663</td>
<td>embryo 13–17</td>
<td>Dorsal closure (13–17)</td>
<td>Dorsal vessel</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mun Z 40664</td>
<td>embryo 11–17</td>
<td>Midline precursors (13–17); presumptive foregut/hindgut (8–11); lining of developing esophagus (13–15); developing brain lobes (13–17)</td>
<td>Developing CNS</td>
<td>st 13</td>
<td>Similar expression as Mun I</td>
</tr>
<tr>
<td>Mun H 40665</td>
<td>embryo 13–17</td>
<td>Broad expression in small punctate 13–17</td>
<td>Nonspecific</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mun Ω 40666</td>
<td>embryo 11–17</td>
<td>Pre-migrating SNS and early esophagus (11); sponadric and nonspecific esophageal tissue (16–17); receptor cells in anterior end of embryo (13–17)</td>
<td>Anterior dorsal sensory neurons</td>
<td>st 11</td>
<td>None observed</td>
</tr>
<tr>
<td>Mun I 40667</td>
<td>embryo 11–17</td>
<td>Presumptive hindgut (11); spread along developing esophagus (13–17); weak midline glia expression (13–17); brain lobes (14–17)</td>
<td>Early foregut/hindgut; midline glia</td>
<td>st 15–17</td>
<td>Posterior to PV; anterior midgut cell bodies; midbrain?; IMR?</td>
</tr>
<tr>
<td>Fkh 1 47326</td>
<td>embryo 11–17</td>
<td>Hindgut lining (11–17); light expression in whole midgut (11–17)</td>
<td>Hindgut lining</td>
<td>-</td>
<td>Hindgut</td>
</tr>
<tr>
<td>Fkh 2 48746</td>
<td>embryo 11–17</td>
<td>Weak CNS expression (11–17)</td>
<td>Non specific</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fkh 3 48764</td>
<td>embryo 9–17</td>
<td>Foregut/hindgut (9–12); complete Intestinal tract (13–17); Malpighian tubules (13–16); mild CNS expression (13–17)</td>
<td>CNS/Intestinal Anatomy outline</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fkh 4 48795</td>
<td>embryo 13–17</td>
<td>Similar to Fkh 3; intestinal tract expression (11–17); Malpighian tubules (13–16); mild CNS expression (13–17)</td>
<td>Intestinal anatomy outline</td>
<td>st 11–15</td>
<td>-</td>
</tr>
<tr>
<td>Twi 1 46150</td>
<td>embryo 13–17</td>
<td>SNS cluster (13); esophagial/pharyngeal muscles, dorsal side of esophagus/EGF (15–17)</td>
<td>Esophagial/pharyngeal clusters</td>
<td>st 13–17</td>
<td>Anterior sensory neurons</td>
</tr>
<tr>
<td>Twi 2 48725</td>
<td>embryo 13–17</td>
<td>Mild CNS expression (13–17)</td>
<td>Weak CNS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Twi 3 48729</td>
<td>embryo 11–17</td>
<td>Developing anterior Sensory receptors (11–17); mid CNS (13–17);</td>
<td>Anterior sensory neurons</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Twi 4 48780</td>
<td>embryo 11–17</td>
<td>Developing CNS (11–17)</td>
<td>CNS</td>
<td>st 13,17</td>
<td>-</td>
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</tbody>
</table>

Table 1. Summary of GAL4 line embryonic expression patterns.
<table>
<thead>
<tr>
<th>Gal 4 Driver Line</th>
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<th>Expression Place</th>
<th>Primary Expression Feature</th>
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<th>Larval Expression (mCD8 GFP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RefP1 (Hema#1 in pPTGAL)</td>
<td>embryo 12–17</td>
<td>Distinct expression in cells of the dorsal fold (14); putative adult midgut precursors and other endoderm/endoderm adjacent cells (14–17); specific subset of brain cells (15–16); periventricular ganglion, dorsal pharyngeal muscles, Malpighian tubules (17)</td>
<td>CNS; SNS; foregut/midgut/hindgut; brain lobes</td>
<td>st 13–17</td>
<td>Brain; posterior to PV; anterior midgut cell bodies and hindgut</td>
</tr>
<tr>
<td>RefP2A (Hema#2 in pBPGLw)</td>
<td>embryo 12–17</td>
<td>Pre-migrating SNS clusters (11–12); migrating SNS clusters (13–16); specific subset of brain cells, putative adult midgut precursors and other endoderm/endoderm adjacent cells and Malpighian tubules (15–17)</td>
<td>CNS; SNS subset; foregut/midgut/hindgut; brain lobes</td>
<td>st 12–17</td>
<td>SNS; posterior to PV; anterior midgut cell bodies</td>
</tr>
<tr>
<td>RefP2B (Hema#3 pBPGLu)</td>
<td>embryo 12–17</td>
<td>Distinct expression in cells in the esophageal clusters (13–14); esophageal and periventricular ganglion, specific subset of brain cells, putative adult midgut precursors and other endoderm/endoderm adjacent cells (15–17)</td>
<td>CNS; SNS subset; foregut/midgut/hindgut; brain lobes</td>
<td>st 12–17</td>
<td>SNS; posterior to PV; anterior midgut cell bodies</td>
</tr>
<tr>
<td>RefPIA (Hema#4 pBPGLu)</td>
<td>embryo 13–17</td>
<td>Tracheal/peripheral (ventral) expression (13–14); midgut lining (endoderm/endoderm adjacent cells) (14–17 and later)</td>
<td>PNS (ventral); midgut</td>
<td>-</td>
<td>Midgut and hindgut</td>
</tr>
<tr>
<td>RefPIB (Hema#5 pBPGLw)</td>
<td>embryo 13–17</td>
<td>Proventriculus (13–14); minimal midgut/hindgut lining (endoderm/endoderm adjacent cells) (15–17); cephalopharyngeal ganglia/pharyngeal muscles (17)</td>
<td>Proventriculus; midgut; hindgut lining</td>
<td>st 13–14 (Proventriculus)</td>
<td>-</td>
</tr>
<tr>
<td>RefPIC (Hema#6 pBPGLw)</td>
<td>embryo 12–17</td>
<td>(Anterior) midgut lining (endoderm/endoderm adjacent cells) (14–17); hindgut lining (16–17)</td>
<td>Proventriculus</td>
<td>-</td>
<td>Midgut and hindgut</td>
</tr>
<tr>
<td>RefPA (Hema#7 pBPGLu)</td>
<td>embryo 12–17</td>
<td>CNS, broad PNS expression, trachea (12–16); proventriculus (16–17)</td>
<td>CNS/PNS; proventriculus</td>
<td>st 16–17 (Proventriculus)</td>
<td>-</td>
</tr>
<tr>
<td>RefPB (Hema#8 pBPGLw)</td>
<td>embryo 11–17</td>
<td>Developing CNS (11); distinct expression in cells in the esophageus (12–17); proventriculus (16–17); (anterior) midgut lining (endoderm/endoderm adjacent cells) (15–17)</td>
<td>Proventriculus; anterior midgut</td>
<td>st 12–17 (Proventriculus)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1. Summary of GAL4 line embryonic expression patterns (continued).
Figure 1. Ret expression in the developing SNS.
Figure 1. *Ret* expression in the developing SNS.

*Drosophila* embryos with an in situ hybridization for the *Ret* gene (dark blue) and antibody staining with the 22c10 antibody (brown) to reveal the SNS, the PNS and elements of the CNS. A, C, E, G, H and I are dorsal views, B, D and F are lateral views. (A, B) Stage 13 embryo with expression in the migrating SNS clusters (arrows). Limited expression can also be seen in a discrete set of cells of the anterior midgut (arrowhead) and in the CNS midline at the bottom of panel B (CNS). (C, D) Stage 15 embryo in which the esophagus has started to loop. The three SNS clusters are immediately adjacent to one another within the loop and all express *Ret* (arrow). Additional *Ret* staining occurs in the developing frontal ganglion (FG). Faint expression can be seen in the anterior midgut (arrowheads), the ventral midline and PNS cells towards the anterior of the embryo. (E, F) Stage 17 embryo with *Ret* expression in some cells of the esophageal ganglion (EG) and proventricular ganglion (PG). Significant *Ret* expression is observed in the midgut (MG) and the CNS midline (CNS). (G) Expression of *Ret* in the three migrating SNS clusters in a stage 13 embryo. (H, H’) Two different focal planes of late stage 13 embryo. *Ret* is expressed in the SNS clusters which are clustered in the looping esophagus (compare to D), and in CNS cells that project through the subesophageal ganglion (arrows). (I, I’) Stage 14 embryo with diminishing *Ret* expression. Some axons of the subesophageal ganglion (SEG) are labeled by 22c10.
Figure 2. Expression of Ret-GAL4 transgenes.
Figure 2. Expression of Ret-GAL4 transgenes.

Embryos and larvae with Ret-GAL4 lines driving expression of either nuclear lacZ (nuc-lacZ) or tau-lacZ reporters (both brown) with select counterstaining with an in situ hybridization probe for Ret (blue). (A) Diagram of the Drosophila Ret gene showing the location of fragments used to construct GAL4 lines. (B) Expression pattern of RetP1-GAL4 driving tau-lacZ showing broad expression throughout the inner lining of the gut. (C) RetP1-GAL4 driving expression of nuc-lacZ showing expression in discrete gut cells. (D) RetP1-GAL4 driving expression of tau-lacZ with individual cells in the midgut sometimes aligning into linear arrays (arrows). (E) RetP1-GAL4 and tau-lacZ with expression in brain neurons (arrowheads), elements of the CNS and PNS at the anterior of the embryo (left), malphigian tubules (mp) and hindgut. (F) Dorsal view of an embryo with RetP2-GAL4 driving nuc-lacZ in a subset of migrating SNS precursors (arrows and blue stain) and the gut. (F’) Lateral view of the same embryo as F with SNS precursors and gut staining visible. (G) An independently recovered line of RetP2 showing similar staining as F. (H, H’) Stage 17 embryo showing persistence of RetP2 expression in the neurons of the frontal ganglion (FG) and brain neurons (arrows). (I) First instar larvae with RetP2 and tau-lacZ displaying prominent staining in the proventricular ganglion (arrow). (J) Late stage 17 embryo showing overlap of RetP2, nuc-lacZ and Ret mRNA expression in the proventricular ganglion (arrow). (K) First instar larva with RetP2-GAL4 and tau-lacZ expression on the proventricular ganglion (arrow). (L,L’) Expression of UAS-CD8-GFP under control of RetP2A-GAL4 in a second instar larva showing expression (arrows) in cells adjacent to and downstream of the proventriculus (pv).
Figure 3. Expression of *Gfrl*, *Gsc* and *Fkh* GAL4 lines.

Expression of *nuc-lacZ* or *tau-lacZ* (brown) by selected Janelia Farm GAL4 lines. The driver, reporter and embryo stage are noted on the figure panel. Please also refer to Table 1. (A) *Gfrl* fragment driving expression in the roof of the stomatodeum in presumptive SNS precursor clusters and a few additional cells. (B) *Gfrl* fragment with expression in the frontal ganglion (FG) and brain lobe clusters (arrows). (C, D) Dorsal and lateral views of a *Gfrl* fragment driving expression in esophageal and SNS cells (arrow), cells presumed to be the subesophageal ganglion and additional cells. (E) Esophageal, SNS and brain lobe expression of a *Gfrl* fragment. (F) Esophageal, SNS and brain lobe expression with additional CNS and PNS cells. (G, H) Expression in cells of the leading edge during dorsal closure. (I, J) Expression of a *Gsc-GAL4* line in foregut, esophageal, SNS and brain cells. (K, L) Expression of a *Fkh-GAL4* line in the midgut, hindgut, brain lobe cells (arrows) and additional cells near the anterior of the embryo (left). (M, N) Expression of a *Fkh-GAL4* line throughout the gut and CNS in a late stage embryo. (O, P) *Fkh-GAL4* expression in the esophagus, SNS, brain lobes and gut cells in a pattern that resembles *RetP1*. (Q) *Twi-GAL4* expression in a subset of SNS cells. (R) *Twi-GAL4* expression in the pharynx and additional unidentified cells that likely include parts of the SNS and PNS.
Figure 4. Characterization of the GscG-GAL4 line.

Developmental series of embryos expressing tau-lacZ (left hand columns) or nuc-lacZ (right hand columns) under control of the GscG-GAL4 line. (A) Schematic of the position of the promoter fragment of GscG-GAL4 inside the Gsc gene containing parts of exon 1 and 2. (B) Nuclear lacZ reporter displaying expression of GscG-GAL4 in the initial SNS precursor clusters in the stomatodeum (stage 11, see also panel D), expanding to include most esophageal cells (stage 13), and resolving into SNS, brain lobe, subesophageal ganglion and PNS specific staining in later stages (15-17; see also panels E-G). (C) Tau-lacZ reporter in a similar developmental series to panel B. All SNS cells are labeled by the end of embryogenesis including the frontal ganglion, esophageal ganglia and proventriculus. (D-E) Higher magnification views showing the initial three delaminating SNS clusters at stage 11 and the entire frontal ganglion and nerve at stage 17 (FG). The nuclear staining shows the brain lobe clusters (arrows) as well as the proventriculus (PV).
Figure 5. SNS manipulation using the *RetP2-GAL4* and *Gfrl-GAL4* lines.
Figure 5. SNS manipulation using the *RetP2-GAL4* and *Gfrl-GAL4* lines.

Anti-fasciclin II staining (monoclonal antibody 1D4) revealing the mature embryonic SNS. We use 1D4 as it reliably stains the frontal nerve (fn) whereas 22c10 frequently fails to stain this nerve in wild type. (A) Dorsal view of a stage 17 wild type embryo with elements of the SNS labeled. The recurrent nerve (rn, arrow) runs from the esophageal ganglion (eg) along the esophagus underneath the supraesophageal commissure (sec, also known as the brain commissure) to the frontal ganglion (fg). The frontal nerve (fn, arrowhead) projects anteriorly from the frontal ganglion (fg). (A') Lateral view of the same embryo. The frontal connective (fc) which links the frontal ganglion to the brain, and the esophageal nerve (en) which links the esophageal ganglia to the ventricular ganglion (vg) can be seen. The corpora cardiaca (cc) neuroendocrine organ is visible close to the dorsal surface. (A'') Close-up of the frontal ganglion (arrow), recurrent nerve (arrowhead) and the esophageal ganglia (EG). A slight expansion of the recurrent nerve (arrowhead) can be seen, marking a location where defasciculated axons are often seen in transgenic manipulations. (B) *RetP2A-GAL4* driving a dominant negative *EGFR* transgene. The frontal nerve (arrowhead) is absent and the recurrent nerve (arrow) exhibits a defasciculated axon or axons (asterisk). (B') Lateral view of the embryo in B. The frontal nerve is clearly missing (arrowheads). (B'') Close-up of the same embryo. The recurrent nerve displays a kink and expansion underneath the supraesophageal commissure (arrowhead). The frontal ganglion (arrow) is also visible. The number of cells in the esophageal ganglia appear comparable to wild type. (C) *RetP2B-GAL4* driving the dominant negative EGFR transgene. The frontal nerve is absent (arrowhead) and a defasciculated axon is crossing the recurrent nerve (asterisk). The latter axon may originate at the esophageal ganglia, but we have been unable to conclusively determine this for any examples studied. (C') Lateral view of the same embryo showing the absence of the frontal nerve (arrowheads) and axon defasciculation from the recurrent nerve (arrow). From this angle, at least one defasciculated axon appears to originate from the recurrent nerve itself. (D) *GfrlA-GAL4* driving transgenic RNAi for *EGFR*. The frontal nerve is absent (arrowhead) and the recurrent nerve (arrow) is defasciculated (asterisk). (D') Lateral view of the same embryo showing the absence of the frontal nerve (arrowheads) and a slight swelling of the recurrent nerve (arrow) at the point that defasciculation occurs. (D'') Higher magnification view showing a defasciculated axon growing alongside the recurrent nerve from the esophageal ganglia to the point of defasciculation. (E) Late stage 17 embryo with the *EGFR* dominant negative transgene driven by *RetP2B-GAL4* showing that the number of cells within the esophageal ganglia appears unaffected by *EGFR* inhibition. (F, G) Quantification of frontal nerve defects (reduced or absent; F) or recurrent nerve (defasciculation; G) defects in the genotypes examined. The error bars represent the 95% confidence interval. Statistical significance (*) relative to the wild type control (*w*1118) was assessed using the Fisher exact test with two tails and the Bonferroni correction.
Figure 6. A *fasII-GAL4* line drives in all parts of the developing gut.

Lateral views of embryos with a nuclear lacZ reporter (brown) driven by *fasII-GAL4* (#46123). (A) Stage 11 embryo in which the anterior (amg) and posterior (pmg) midgut are stained. (B) Stage 13 embryo displaying continuous staining throughout the gut tissue, including the foregut (fg), midgut (mg) and hindgut (hg). (C, C’) Early stage 15 embryos in which the esophagus (es) is starting to loop. By this stage the visceral mesoderm that forms the visceral muscles surrounding the gut has migrated to the epithelial lining, but we see no evidence that the *fasII* line expresses in this tissue. (D) Late stage 15 embryo with persistent staining throughout the gut. (E) Early stage 16 embryo in which the proventriculus (pv) has started to form. Expression is widespread and continues to stage 17/early larval stages but at weaker levels.
Figure 7 (Supplementary). Characterization of RetP2A-GAL4 expression in the developing hindgut.

Embryos with RetP2A-GAL4 line driving either nuclear lacZ (nuc-lacZ) or tau-lacZ reporters (both brown) with select counterstaining with an in situ hybridization probe for Ret (blue). (A, A’, B) Expression pattern of RetP2A driving expression of nuc-lacZ showing expression in the periphery, brain lobes and developing hindgut (arrowheads). (C) Late stage 15 embryo showing overlap of RetP2A, nuc-lacZ (arrowhead) and Ret mRNA expression in developing hindgut (arrow). (D, D’) Stage 15 embryo with RetP2A driving nuc-lacZ in a subset of cells in the hindgut (arrowheads) and overlapping expression with Ret mRNA (arrow and blue stain). (E) Stage 16 embryo showing persistence of RetP2A expression in the hindgut (arrowheads). (E’) Lateral view of the same embryo as E with ventral nerve cord (VNC) and hindgut staining visible (arrowheads). (F) Lateral view of stage 16 embryo showing RetP2A expression in posterior peripheral neuron(s) (arrowheads). (G) Lateral view of stage 16 embryo showing RetP2A expression in what appears as the (PDF-positive) efferent neurons in the posterior-most portion of the VNC (arrowheads). (H, H’) Two different focal planes of late stage 16 embryo showing persistence of RetP2A expression in the posterior-most neurons hypothesized to innervate the hindgut (arrowheads).
Figure 8 (Supplementary). The RetP2A-GAL4 line drives in posteriorly projecting (CNS) neurons.

Expression of tau-lacZ (brown) by RetP2A-GAL4 line. (A) Ventral view of early stage 16 embryo displaying specific expression in CNS or CNS neighboring neurons (arrowheads) and axons (arrows). (B) Early stage 17 embryo showing persistent expression of RetP2A in (CNS) neurons (arrowheads). (C, D) Lateral view of early stage 17 embryo showing RetP2A expression in CNS-specific cell bodies (arrowheads) and axons projecting posteriorly (arrow). (E, F) Additional focal planes revealing further expression in peripheral neurons in the posterior side of the embryo.
Figure 9 (Supplementary). Expression of Ret-GAL4 transgenes (continued).
Figure 9 (Supplementary). Expression of Ret-GAL4 transgenes (continued).

Embryos with Ret-GAL4 lines driving expression of either nuclear lacZ (nuc-lacZ) or tau0lacZ reporters (both brown) with select counterstain with an in situ hybridization probe for Ret (blue). (A) Expression pattern of RetPIA-GAL4 driving nuc-lacZ showing broad tracheal/peripheral expression. (B) Lateral view of stage 15 embryo showing midgut lining (arrows in A and B). (C) Early stage 15 embryo showing proventricular expression (arrow) of tau-lacZ driven by RetPIB-GAL4. (D) Late stage 15 embryo showing tau-lacZ expression in posterior midgut lining (arrows). (E, F) RetPIC-GAL4 drives expression along the midget lining (arrows). (G, H) RetIA-GAL4 is broadly expressed in developing trachea (arrows in G) and in CNS-specific neurons at stage 16 (arrows in H). (I, J) RetIB-GAL4 drives expression in the (anterior) midgut lining at late stage 14 (arrows in I) and in distinct esophageal cells at stage 16 (arrows in J).
Figure 10 (Supplementary). Confocal analysis of EGFR inhibition in the SNS.

Anti-fasciclin II staining (monoclonal antibody 1D4) revealing the mature SNS in lateral views. (A) Wild type (w1118) embryo showing the frontal nerve (fn), frontal ganglion (fg), recurrent nerve (rn), supraesophageal or brain commissure (sec), corpora cardiaca (cc), frontal connective (fc), esophageal ganglia (EG), esophageal nerve (en), ventricular ganglion (vg) and ventral nerve cord (vnc). (B) RetP2A-GAL4 driving the EGFR dominant negative transgene. The frontal nerve is absent (arrowheads) and the recurrent nerve is possibly less tightly bundled (arrow). (C) RetP2B-GAL4 driving the EGFR dominant negative transgene. The frontal nerve is absent (arrowheads) whereas the recurrent nerve looks normal.
Figure 11 (Supplementary). SNS manipulation using the *GscG-GAL4* line.

Anti-fasciclin II staining (monoclonal antibody 1D4) revealing the mature SNS in ventral views. (A) Stage 17 wild type embryo with elements of the SNS labeled. The recurrent nerve (rn, arrow) runs from the esophageal ganglion (EG) along the esophagus underneath the brain commissure (bc) to the esophageal ganglion (EG). (B) *GscG-GAL4* driving transgenic RNAi for *EGFR*. The recurrent nerve (arrow) is clearly disrupted. (C) *GscG-GAL4* driving an EGFR dominant negative transgene. The frontal nerve is missing (arrowhead) and the recurrent nerve is disrupted.
Figure 12 (Supplementary). Overexpression of activated dRet causes SNS phenotypes.

Anti-futsch staining (monoclonal antibody 22c10) revealing the mature SNS, stage 17. Dorsal (A) and lateral view (A’) of stage 17 wild type embryo with major SNS landmarks labeled (brown); frontal nerve (fn), frontal ganglia (fg), recurrent nerve (rn), supraesophageal commissure (sec) and esophageal ganglia (eg). (B) RetP2A-GAL4 driving activated dRet\textsuperscript{MEN2B}. An overgrowth of esophageal ganglia was detected and the recurrent nerve is clearly disrupted, as is the overall symmetry of the SNS topography (B’). (C) Driving the same dRet transgene with a different SNS-specific driver, GscG-GAL4, produced similar (eg) overgrowth phenotypes (arrowhead in C’). (D, D’) fasII-GAL4 driving activated dRet transgene. The overgrowth phenotype is exacerbated; the frontal and supraesophageal commissures are thickened. (D”’) Dorsal and lateral (D’’’) views of the same embryo showing abnormal/ectopic neuronal/axonal growth along the hindgut (hg, arrowhead).
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Literature cited


Chapter 3

*Dscam1* overexpression impairs the function of the gut nervous system in *Drosophila*

*Manuscript in preparation.*

**Abstract**

Down syndrome (DS) patients have a 100-fold increase in the risk of Hirschsprung syndrome of colon and rectum (HSCR), a lack of enteric neurons in the colon. The leading DS candidate gene is trisomy of the Down syndrome cell adhesion molecule (Dscam). We detected Dscam1 protein expression in the *Drosophila* enteric/stomatogastric nervous system (SNS) suggesting that the fly could be used to examine *Dscam1* function. *Dscam1* mutants have subtle defects in SNS anatomy that are not increased by removing the fra or robo1 receptors, suggesting that Dscam1 is not responding to its known ligands. Overexpression of *Dscam1* reduced the length of the frontal nerve but also resulted in an expansion of the anterior tip of the nerve. In contrast, expression of a dominant negative *Dscam1* (*Dscam1*-ΔC) frequently led to a complete absence of the frontal nerve. *Dscam1*-ΔC expression also resulted in branching of the hindgut nerve. Larval feeding and mortality were unchanged by SNS overexpression of *Dscam1* or *Dscam1*-ΔC. However, continuous SNS overexpression of *Dscam1* in larvae reduced the efficiency of food clearance from the larval gut. Larval locomotion is influenced by feeding state and we found that the average speed of larvae with *Dscam1* SNS expression was reduced, whereas overexpression of *Dscam1*-ΔC significantly increased the speed. In humans, HSCR results from disrupted neuronal migration but we
did not find any evidence for mis-positioned neurons. Our work demonstrates that overexpression of Dscam can perturb gut function in a model system and suggests that disruption of neuronal connectivity could be a secondary mechanism acting in HSCR.

Introduction

Down syndrome (DS) is characterized by a wide range of symptoms including variable levels of intellectual disability, congenital heart defects and stereotypical facial features (Antonarakis and Epstein, 2006). DS patients additionally can have gastrointestinal abnormalities including Hischsprung disease of the colon and rectum (HSCR) (Arnold et al., 2009). In HSCR, neural precursors derived from the neural crest either fail to migrate to the distal regions of the gut or have survival defects leading to a lack of innervation (McKeown et al., 2013). Trisomy for the Dscam gene is the leading candidate for the increased occurrence of HSCR in DS (Jannot et al., 2013; Korbel et al., 2009). Dscam is a large single pass transmembrane protein with extensive roles in neural connectivity and other developmental processes (Montesinos, 2014; Schmucker and Chen, 2009). Dscam is expressed in the migrating neural crest cells that give rise to the enteric nervous system (ENS) (Barlow et al., 2002; Yamakawa et al., 1998). Upon arriving at the correct rostro-caudal position, ENS neural precursors migrate orthogonally to form ganglia and it has been proposed that an extra copy of Dscam disrupts this secondary migration to produce HSCR (Jannot et al., 2013).

In *Drosophila*, the *Dscam1* gene is characterized by a multitude of alternatively spliced isoforms that mediate homophilic cell adhesion, but this extensive diversity is not conserved outside of insects or in paralogous genes within species. Dscam1 can act as a
receptor for Netrin and Slit ligands (Andrews et al., 2008; Dascenco et al., 2015). Both cell adhesion and receptor functions are evolutionarily conserved (Agarwala et al., 2000; Liu et al., 2009; Ly et al., 2008; Yamagata and Sanes, 2008), although the function of ligand binding is unclear in vertebrates (Palmesino et al., 2012). In flies, over-expression of \textit{Dscam1} increases axonal arbor size (Kim et al., 2013; Sterne et al., 2015), produces synaptic targeting errors (Cvetkovska et al., 2013; Kim et al., 2013), alters dendritic patterning (Zhu et al., 2006) and induces ectopic midline crossing of CNS axons (Andrews et al., 2008). Disruption of \textit{Dscam1} function frequently leads to axon stalling (Chen et al., 2006; He et al., 2014), suggesting that \textit{Dscam1} normally acts to promote axon growth and expansion. Functional studies reveal that Dscam overexpression can have severe consequences for heart development and sensory perception (Cvetkovska et al., 2013; Grossman et al., 2011).

The principal gene underlying HSCR in humans is Ret, which is inherited as an autosomal dominant mutation subject to modification by other loci (Wallace and Anderson, 2011). In the \textit{Drosophila} equivalent of the vertebrate ENS, the stomatogastric nervous system (SNS), \textit{dRet} is expressed in the migrating neural precursors that give rise to the SNS (Hahn and Bishop, 2001). This pattern of expression matches that of the Ret vertebrate gene (Pachnis et al., 1993). There are additional evolutionary homologies between the ENS and SNS. The neural crest placode is marked by Six1/2 expression even in primitive chordates (Abitua et al., 2015), and the fly paralogue, \textit{sine oculis}, is required for SNS development (De Velasco et al., 2004). An additional set of motor neurons that innervate the hindgut, are responsible for defecation and are identified by \textit{Pigment-dispersing factor (Pdf)} expression (Zhang et al., 2014). PDF is functionally related to the
vertebrate vasoactive intestinal polypeptide (VIP) and orexin/hypocretion (Crocker and Sehgal, 2010; Vosko et al., 2007), and both genes can be expressed in the same ENS neurons (Naslund et al., 2002).

*Dscam1* is expressed in the embryonic SNS suggesting an evolutionarily conserved role. Based on prior over-expression studies (Cvetkovska et al., 2013; Sterne et al., 2015), we predicted that *Dscam1* over-expression in the SNS would lead to disruption of precursor cell migration and/or neural connectivity. We found that cell migration appears unaffected although subtle changes in cell number were evident. Axon connectivity defects were evident in the embryonic frontal nerve (innervates the muscles of the pharynx) and the hindgut nerves, with weaker effects in other parts of the SNS. Genetic manipulations of *Dscam1* levels in the SNS led to impaired abilities to clear food from the gut as well as effects on larval locomotion. We believe that Dscam is subtly increasing axonal or synaptic connections.

**Results**

*Dscam1* is expressed in the Stomatogastric Nervous System

To assess whether the fly SNS is a suitable model system for modeling Dscam enteric function, we stained *Drosophila* embryos with anti-Dscam1 antibodies (Figure 1). Two independent antibodies (gifts of the Schmucker and Zipursky laboratories) labeled the frontal connective and nerve and to a lesser extent the recurrent nerve. The majority of this staining was axonal rather than in cell bodies. There appeared to be very low levels of *Dscam1* protein in migrating neural precursors prior to axon outgrowth, but neither antibody was strong enough to detect expression in the migrating neural precursors due to
a low level of background staining. The staining pattern suggests that in flies Dscam1 is playing a role in axonal connectivity rather than precursor migration.

**Phenotypic Analysis of Dscam1 in the Stomatogastric Nervous System**

Having established that Dscam1 is expressed in the SNS, we examined Dscam1 mutants for alterations to SNS patterning using anti-Fas2 that reliably stains the frontal nerve (1D4; Figure 2). Dscam1 mutants often displayed a reduced frontal nerve but very few other defects compared to wild type. In the CNS, Dscam1 has a mild phenotype that is greatly enhanced by removal of the Netrin receptor frazzled (Andrews et al., 2008), but no enhancement was seen in the SNS. Dscam1 can bind Slit (Dascenco et al., 2015), and can form a complex with Robo1 (M. Alavi et al., submitted), therefore we examined Dscam1 robo1 double mutants and again saw no enhancement of the Dscam1 phenotype (Figure 2C,D). Dscam1, Dscam1 fra and Dscam1 robo1 mutations are embryonic lethal, which limited our analysis to embryonic neuroanatomy. These results suggest that either Dscam1 is playing a minor role in SNS patterning, functionally overlaps with other receptors or may be responding to an unidentified ligand.

**Expression of Dscam1 transgenes in the developing SNS**

To model trisomy for Dscam in Down Syndrome, we chose to overexpress full-length Dscam1 in the SNS. We used two independent isoforms of Dscam1 in the developing SNS and expressed them using the Gsc and Ret promoters (Hernandez et al., 2015). Both promoters express in the developing SNS, with Gsc-GAL4 shutting off expression at the end of embryogenesis. Ret-P2A and -P2B are independent isolates of identical transgenes
with slightly different expression patterns. In parallel, we over-expressed a $Dscam1$ construct that lacks the cytoplasmic domain (Zhu et al., 2006). We have found that this construct ($Dscam1DC$) is quite disruptive to CNS formation suggesting that it can act as a dominant negative by inhibiting other receptors (M. Alavi et al., submitted). Expression of full-length $Dscam1$ most obviously altered morphology and distribution of Fas2 in the frontal nerve (Figure 3B,C). Fas2 staining frequently did not extend the full length of the dorsal pharyngeal muscles. However, the tip of the frontal nerve was often expanded laterally, with axons following the junctions between muscles. The latter phenotype resembles the expansion of presynaptic arbors observed in C4da neurons when Dscam1 is overexpressed (Kim et al., 2013; Sterne et al., 2015). Expression of $Dscam1DC$ frequently led to absence of the frontal nerve and also caused defasciculation of the recurrent nerve (Figure 3D-F). We noted that anti-Fas2 also stains the hindgut neurons that control defecation (Zhang et al., 2014), and found that the drivers used express in these neurons. Expression of $Dscam1$ led to expansions at the tip of the hindgut nerves and increased numbers of side branches at the start of the axon trajectory. $Dscam1DC$ often induced splitting of the axon into two distinct branches (Figure 7D). We confirmed these results with the PDF-GAL4. Overall, expression of Dscam1 appeared to act positively on axons increasing the area innervated, whereas $Dscam1DC$ either inhibited growth or promoted branching.

**Increased $Dscam1$ expression impairs food clearance but not feeding**

To examine the consequences of manipulating SNS $Dscam1$ activity, first instar larvae were allowed to eat yeast paste mixed with carmine red dye to examine feeding behavior
(Zinke et al., 1999). No apparent differences in the amount of food ingested or the distribution within the larvae were observed (Figure 4A-G). Larval development proceeded normally through the larval stages without any increases in mortality. As the hindgut motor neurons were affected by Dscam1 manipulation, we assayed larval defecation by feeding red yeast until they reached second instar, washing them and transferring them to uncolored food for two hours. Wild type larvae (w^{1118}) had no traces of red food internally but larvae expressing Dscam1 under control of Ret promoter fragments still had traces of food inside (Figure 4A'-D'). Expression under control of Gsc-GAL4, which switches off expression at the end of embryogenesis had only a very minor effect on food clearance. This argues that continuous expression of Dscam1 is required to reduce food clearance. Expression of Dscam1DC under control of the Ret-P2A promoter reduced food clearance, but the other two drivers had no effect.

**Larval locomotion is affected by SNS Dscam1 manipulations**

Larval locomotor behavior is influenced by feeding state, with starved larvae displaying increased locomotion (Godoy-Herrera and Mora, 1987; Berni et al., 2012). In adult flies, foregut neurons are proposed to relay information from stretch receptors to the CNS, influencing feeding behavior (Al Anzi et al., 2010). We examined whether a failure to defecate influences locomotor behavior by recording the distance travelled and the average speed of larvae over-expressing full-length Dscam1. These larvae showed a small but statistically significant decrease in average speed compared to control larvae when the Ret-P2A and -P2B drivers were used (Figure 5B and Appendix 1). GscG-GAL4 did not display the same effect suggesting that persistent SNS expression of Dscam1-FL...
is required for this effect \((GscG-GAL4\) appears to turn off at the end of embryogenesis). Expression of \(Dscam1-DC\) produced an increase in speed when expressed by the \(RetP2A,B\) drivers (Figure 5B). The distance travelled was significantly changed for \(RetP2A::Dscam1\) and for both \(Ret-P2\) drivers expressing \(Dscam1-DC\) (Figure 5A), while the number of runs per tracking session (bout number) was unaffected in most genotypes (see Appendix 1). We note that the average speed of the larvae is correlated with ability to clear food from the gut. Both \(Ret-P2\) drivers expressing \(Dscam1-FL\) show significantly reduced speed and food clearance, whereas \(GscG\) does not (Figure 4H). The correlation is less clear with \(Dscam1-DC\) expression as food clearance levels are close to wild type (except \(RetP2A\)) whereas average speed is much greater in these genotypes. These results indicate that over-expression of \(Dscam1-FL\) and \(Dscam1-DC\) have opposite phenotypic effects, which we suspected based on the embryonic SNS neuroanatomy.

**PAK expression reproduces Dscam1 phenotypes**

In \(Drosophila\), \(Dscam1\) in axons signals via PAK (p21 activated kinase) (Schmucker et al., 2000). We expressed PAK (PAK-GPF) and an activated PAK isoform (myr-PAK) in the SNS to determine whether the same phenotypes were observed. Overexpression of wild type PAK reproduced the phenotypes observed when \(Dscam1-FL\) was over-expressed, specifically an expansion of the tip of the frontal nerve coupled with reduction of Fas2 staining in the frontal nerve itself (Figure 6B). Over-expression of activated PAK mimicked expression of \(Dscam1-DC\) causing the frontal nerve to be absent or highly reduced (Figure 6C). In the hindgut, PAK caused the hindgut motor neurons to grow much farther than usual, sometimes along the entire length of the hindgut as opposed to
approximately halfway in wild type (Figure 8B). We have observed this phenotype at a much lower frequency in Dscam1 gain-of-function embryos. Activated PAK expression induced the major branching characteristic of Dscam1-DC expression. These results indicate that Dscam1 may be signaling through PAK in the SNS. If Dscam1 was purely permissive for axon growth, then activated PAK should have the same effect as PAK over-expression. However, the similarity between activated PAK and Dscam1-DC strongly suggests that PAK activation all over the growth cone is as detrimental to axon growth as inhibition of Dscam1 signaling. It seems likely that Dscam1 is responding to localized cues in both the frontal nerve and the hindgut motor neurons.

**Discussion**

In this study we establish that over-expression of full-length Dscam1 has correlated effects on SNS neuroanatomy, food clearance and locomotor activity. Driving Dscam1 expression continuously throughout larval development leads to behavioral phenotypes (Ret-P2A and 2B drivers), notably a failure to clear food as efficiently as wild type. Expression solely in the embryo alters neuroanatomy, yet differentially affects larval behavior (GscG driver; Appendix 1). Based on prior work and our observations, Dscam1 over-expression may be increasing local connections between SNS neurons and the gut musculature or other neurons, impairing digestive function.

**Origins of the food clearance defect**

We observed expansions of the frontal and hindgut nerves when Dscam1 or PAK was over-expressed, consistent with previous observations of axonal arbors and synaptic
targeting. The expansions could be due to general promotion of axonal growth or errors in synaptic targeting. A failure to form correct synaptic connections or the formation of too many connections would clearly disrupt function of the SNS. We began this work assuming that disruptions to the SNS would impair feeding ability and were surprised to see no change. The larval tracking data provided a clear indication to us that our manipulations were having an effect and that \textit{Dscam1} and \textit{Dscam1-DC} expression was phenotypically opposed. This led us to perform the food-clearing assay. We also were surprised that the hindgut neurons were so strongly affected, as our initial survey of the promoters used did not indicate strong expression in these neurons. GFP marker analysis revealed that there is continuous expression by the \textit{Ret} promoters, leading us to quantify hindgut motor neuron defects. At this stage, we have three models for the food clearance defect. The \textit{Drosophila} SNS does not innervate the midgut, stopping shortly after the esophagus ends. The SNS may be responsible for initiating peristaltic contractions at the junction of the proventriculus and the midgut. The discovery of a set of enteroendocrine cells that are sufficient for larval gut motility argues against this model (LaJeunesse et al., 2010). A second model is that feedback from the midgut to the brain might require functional SNS connections (Al-Anzi et al., 2010). This mechanism seems more likely to regulate meal size and food search strategies rather than clearance of food that is already ingested. We currently favor the model that we have impaired the anal sphincter circuit. Disruption of signaling in the hindgut neurons has been shown to reduce the defecation rate and this would reduce food clearance (Zhang et al., 2014). We are currently testing this hypothesis by determining whether \textit{Dscam1} expression in the hindgut motor neurons alone is sufficient to induce the food clearance defects.
**Implications for the human ENS**

Based on prior studies we expected \textit{Dscam1} overexpression to increase the size of axons or the number of connections made. DS patients do frequently lack ENS neurons as is seen for HSCR in non-DS patients. However, Jannot et al. suggested that trisomy for Dscam could disrupt local connections that form after neural precursors have finished migration. Mouse studies have found that ENS neurons can be present in animals with impaired gut function, and this may be due to incorrect specification of neuron types (Musser et al., 2015). Consistent with this, innervation defects have been detected in human patients (Warren et al., 2015). A key next step is to determine which vertebrate enteric neurons are Dscam positive. If distant evolutionary relationships hold, our work predicts that neurons positive for VIP and/or orexin/hypocretin will express Dscam and be affected in DS patients.

**Methods**

\textbf{Drosophila Genetics}

The \textit{Ret-P2A} and \textit{-P2B} lines and the Janelia Farm lines \textit{GscG-GAL4} (#40383) and \textit{fasII-GAL4} (#46123) were described in (Hernandez et al., 2015). Additional stocks, \textit{PDF-GAL4} (#6899), \textit{UAS-PAK-GFP} (#52299) and \textit{UAS-PAKmyr} (#8804) were obtained from Bloomington \textit{Drosophila} Stock Center (BDSC). Full-length \textit{UAS-Dscam1-GFP} lines were obtained from P. Shen and Y. Pu, and are described in (Wang et al., 2004). Dscam lacking the cytoplasmic domain (\textit{UAS-Dscam1-ΔC}) was obtained from L. Luo (Zhu et al., 2006). \textit{UAS-nuclear-lacZ} and \textit{UAS-CD8-GFP} were obtained from BDSC. The most
reliable stock for neuroanatomy was $w^{118}$ as it displays the wild type SNS neuroanatomy described in previous publications.

**Immunohistochemistry**

Antibody staining was performed as described in Patel, 1994 and Hernandez et al., 2015. Anti-Dscam antibodies were generous gifts from D. Schmucker and S.L. Zipursky. Late stage 17 embryos were cleared in 70% glycerol in PBS and subsequently in RapiClear (Sunjin Labs; (Liu and Chiang, 2003)). For all genotypes 10+ embryos were randomly selected and analyzed.

**Feeding and Defecation Assays**

Feeding assays were performed as previous described in Zinke et al., 1999 and Melcher et al., 2005. Defecation assays were modified from Zhang et al., 2014. Adult flies were allowed to lay eggs in grape agar plates with yeast paste containing Carmine red, 0.02g per 10g of yeast paste (Sigma-Aldrich; St. Louis, MO, USA). Embryos were allowed to develop for 24 hours and counted to quantify mortality. For defecation assays, second instar larvae were imaged to document feeding state (time 0), then placed in a separate plate with regular yeast paste and monitored in 15 minute intervals. After two hours (time 2 hours), larvae were reimaged to assess gut clearance. Larval drug treatments were carried out as described in Sterne et al., 2005. Nilotinib dissolved in DMSO and added to yeast paste at a concentration of 380 µM (Abcam; Cambridge, MS, USA). For all genotypes, n > 20 second instar larvae.
Locomotion Assay

Larval tracking assays were executed as described elsewhere (Mathew et al., 2013). Larvae were collected from grape agar plates and gently washed in distilled water. Using a paintbrush, 22 to 26 larvae were aligned along the y-axis of a petri plate (22x22 cm) atop a thin layer of 1.5% agarose. The plate was covered to contain animals. Kinesis was recorded at 130 frames per minute, for five minutes with a CCD camera inside a dark chamber. Locomotion data was analyzed using MATLAB® software. For all genotypes, n > 60 second instar larvae.

Statistics

Neuroanatomy

For each genotype, stage 17 embryos were collected at random and scored for presence (wild type), absence/thinning or overgrowth/ectopic branching of the frontal nerve, altered frontal ganglion number, defasciculation defects in the recurrent nerve, altered esophageal ganglia number, proventricular ganglion and increased branching or bifurcation of the hind gut motor neurons. The 95% confidence interval and the Fisher exact test with two tails for the phenotypes was calculated using the GraphPad website (www.graphpad.com/quickcals). Statistical significance was assessed using the Bonferroni correction.

Locomotion assay

Statistical analysis was performed with Statistica® software using a Tukey HSD within a one-way ANOVA as this is the most conservative standard analysis.
Acknowledgements: We thank L. Luo, P. Shen and Y. Pu for *Drosophila* stocks, D. Schmucker and S.L. Zipursky for antibodies. L. Myers, H. Perera, K. Sweeney, M. Contreras and members of the Kidd and Mathew laboratories for technical assistance. KH was supported by the Michael (Mick) J. M. Hitchcock, Ph.D. Graduate Student Research Fund.
Figure 1. Dscam1 is expressed in the embryonic SNS.
Figure 1. Dscam1 is expressed in the embryonic SNS.

Anti-Fasciclin2 staining (monoclonal antibody 1D4) revealing the mature embryonic SNS. (A) 1D4 dependably labels the frontal nerve (fn) which projects anteriorly from the frontal ganglion (fg). The recurrent nerve (rn, arrow) runs from the esophageal ganglion (eg) along the esophagus underneath the supraesophageal commissure (sec, better known as the brain commissure) to the frontal ganglion (fg). Also labeled are the olfactory lobe (ol) and the frontal connective (fc). The frontal connective links the frontal ganglion to the brain. (B) Stage 16 embryo stained with Dscam1 antibody similarly labeling the frontal nerve, the frontal commissure and brain commissure (sec) connecting to the brain (br). (C) Dissected stage 17 embryo labeled with Dscam antibody followed by Ni stain (black) to enhance visualization showing Dscam expression the central nervous system, ventral nerve cord (vnc), axons connecting to the brain lobes (br), brain commissure (sec), frontal commissure and recurrent nerve.
Figure 2. *Dscam1, Dscam1 fra, Dscam1 robo1* mutants lack strong SNS phenotypes.
Figure 2. *Dscam1, Dscam1 fra, Dscam1 robo1* mutants lack strong SNS phenotypes.

(A) Anti-Fasciclin2 staining revealing the mature SNS of *w^{1118}* embryos. (B) Embryos lacking *Dscam1* display thinned frontal nerve (fn), slightly irregular frontal ganglia (fg), recurrent nerve (rn) and brain commissure (supraesophageal commissure, sec) (B’)

Lateral view of the same embryo; the frontal nerve is clearly thinner/absent. (C) Dorsal view of *Dscam1 fra* combination mutant with frontal ganglia and brain commissure defects. (C’) Lateral view of the same embryo with subtle frontal nerve phenotype and slightly irregular esophageal ganglion (eg). (D) Embryo lacking *Dscam1 robo1*. The overall SNS appears asymmetric, although the majority of the anatomical features appear wild type. (E) Lateral view of another *Dscam1 robo1* embryo showing variation in phenotype. The frontal nerve is absent and the recurrent nerve appears slightly abnormal.
Figure 3. *Dscam1* overexpression causes SNS defects.
Figure 3. *Dscam1* overexpression causes SNS defects.

Stage 17 embryos stained with monoclonal 1D4 to visualize motor neurons comprising the characteristic anatomical landmarks of the mature SNS. (A) Dorsal view of stage 17 wild type embryo illustrating stereotypical/symmetrical SNS structures. (A’) Lateral view of the same embryo clearly showing the intact frontal nerve (fn), recurrent nerve (rn), brain commissure (sec), corpora cardiaca (cc) and esophageal ganglia (eg). (B) Embryos carrying one copy of *ReP2A-GAL4* and one copy of *UAS-Dscam1-FL* transgene showing the frontal nerve extending anteriorly and ectopically expanding/innervating the pharyngeal muscles (arrows). (B’) Lateral view of the same embryo showing additional detail of the frontal nerve thickening/expansion phenotype. (C) Embryo carrying one copy of the embryonic driver *GscG* and one copy of the *UAS-Dscam1-FL* transgene showing the same ectopic expansion of the frontal nerve (arrows). (C’) Lateral view of the same embryo. (D) Close-up of lateral view of stage 17 embryo carrying one copy of the *RetP2A* driver and one copy of the *UAS-Dscam1ΔC* transgene. The recurrent nerve displays strong defasciculation and separation (arrows). The frontal nerve is drastically thinned. (E) Embryo of the same genotype as D exemplifying additional recurrent nerve defasciculation and appreciable asymmetry in reference to the brain commissure (sec) (arrow). (F) Close-up of lateral view of embryo carrying *RetP2B-GAL4* and *UAS-Dscam1-ΔC* transgene. The axons are defasciculated (arrow) and separated. Esophageal ganglia are also abnormal (arrow).
Figure 4. *Dscam1* overexpression affects larval defecation.
Figure 4. *Dscam1* overexpression affects larval defecation.

Second instar larvae showing no apparent feeding defects. (A-G) Second instar larvae with guts filled with colored food at time 0. (A’) The same larvae two hours after eating regular food. 100% of control larvae (*w^{1118}*)) had complete gut clearance after eating regular food (H). (B’) Larva carrying one copy of the *RetP2A-GAL4* driver and one copy of the *UAS-Dscam1-FL* transgene. Larvae failed to fully evacuate/clear their guts (time: 2 hours, arrow). Similar results were caused by *RetP2B*, but not by the *GscG* driver (C-D’). Overexpression of *Dscam1-ΔC* with the *RetP2A* driver impaired gut evacuation in more than half of the larvae (E, E’). Overexpression with *RetP2B* and *GscG* drivers caused a minor affect on gut clearance (F-G’). Arrowheads indicate external red food located on surface of the animals.
Figure 5. *Dscam1* overexpression affects larval locomotion.
**Figure 5. Dscam1 overexpression affects larval locomotion.**

(A) *Dscam1-FL* overexpression with the *RetP2A* driver decreased average run length (#p=0.00132, Tukey HSD within a one-way ANOVA). Continuous *Dscam1-ΔC* overexpression with *RetP2A* and -P2B drivers resulted in a significant increase of average run length (*p=0.000036 and p=0.000010). (B) *Dscam1-FL* overexpression with SNS-specific drivers, *RetP2A* and -P2B, significantly decreased average run speed compared to the outcross *UAS-Dscam1-FL* control (#p=0.000010). However, embryonic overexpression with *GscG-GAL4* significantly increased average run speed (**p=0.00010). *Dscam1-ΔC* overexpression had an opposite effect as all three drivers significantly increased average run speed compared to the *w^{1118}* control (*p=0.00010). *Dscam1-ΔC* overexpression with *RetP2A,B* drivers caused a significant increase in average run speed (**p=0.00001). For all genotypes, n > 60 second instar larvae.
Figure 6. *PAK* misexpression phenotypes.
Figure 6. *PAK* misexpression phenotypes.

To distinguish between proliferative and guidance effects, we overexpressed p21 activated kinase, *PAK* transgenes. (A) Dorsal view of stage 17 wild type embryo stained with 1D4 monoclonal antibody to reveal the mature SNS. (A’) Lateral view of the same embryo. (B) *RetP2B-GAL4* driving *UAS-PAK-GFP* transgene. The frontal nerve (fn) has ectopically expanded and innervated the pharyngeal muscles (arrows). (A’) Lateral view of the same embryo revealing the overgrown frontal nerve. (C) *RetP2B* driving *UAS-myristoylatedPAK*. From a dorsal view, the frontal nerve appears absent. (C’) Lateral view of the same embryo reveals the frontal nerve to be thinned, while the recurrent nerve is abnormally defasciculated.
Figure 7. Phenotypes of Dscam1 transgenes in the embryonic hindgut motor neurons.
Figure 7. Phenotypes of \textit{Dscam1} transgenes in the embryonic hindgut motor neurons.

Anti-Fasciclin2 staining (monoclonal antibody 1D4) to reveal hindgut innervating motor neurons. (A) Lateral view of a stage 17 wild type embryo with elements of the ventral nerve cord (vnc) labeled. The hindgut nerve (hgn) consists of axons which extend from the posterior-most pair of axon bundles in the nerve cord and innervate the hindgut (hg). (B) \textit{RetP2B-GAL4} driving a copy of \textit{UAS-Dscam1-FL} transgene. The hindgut nerve appears overgrown and defasciculated (arrowheads). (C, D) Two embryos with \textit{Dscam1-ΔC} overexpression by \textit{RetP2A-GAL4} to show distinct hindgut nerve guidance (C) and bifurcation/strong defasciculation (D) phenotypes (arrowheads). (E) Similarly, driving \textit{Dscam1-ΔC} overexpression with embryonic driver \textit{GscG-GAL4} resulted in hindgut nerve abnormalities.
Figure 8. Phenotypes of PAK transgenes in the embryonic hindgut motor neurons.
Figure 8. Phenotypes of PAK transgenes in the embryonic hindgut motor neurons.

(A) Anti-Fasciclin2 staining to reveal the hindgut innervating motor neurons of stage 17, wild type embryo (arrowheads). (B) RetP2A-GAL4 driving PAK-GFP transgene. The hindgut nerve (hgn) appears overgrown along the hindgut (hg) (arrowheads). (C) Overexpressing myristoylated PAK with RetP2B caused ectopic branching and defasciculation (arrowheads).


Chapter 4
Conclusion

The enteric nervous system is of considerable importance to the organism and controlling the gut cavity may have been one of the earliest functions for the nervous system Ghysen 2003. Ever since the Drosophila embryonic stomatogastric nervous system was described and characterized (Nässel et al., 1993; Hartenstein et al., 1992, 1994 & 1996; González-Gaitán & Jäckle, 1996) it has received relatively little attention. An anatomical and function description of the hindgut innervation of Drosophila was only recently described (Zhang et al., 2014). More importantly, the biological links between the anteriorly located SNS and posterior neural circuitry have yet to be established and well defined. My work reveals that the hindgut circuitry may be as important as the SNS in regulating passage of food through the gut with neural alterations and feedback mechanisms impacting organismal behavior.

The complexity involved in nervous system development requires investigation from various perspectives: genetic, anatomical and functional. Previous research in vertebrate models revealed the genetic complexity of ENS congenital pathologies, as although both mice and humans homozygous for Ret mutations have complete intestinal aganglionosis, human heterozygotes have HSCR whereas heterozygous mice do not (Shimotake et al., 2001). Warren et al. state that the high incidence of Hirschsprung disease (HSCR) phenotype (constipation) in Down Syndrome (DS) may have different underlying pathobiology, as well as different genetic basis compared to non-syndromic Hirschsprung (Warren et al. 2015). I hypothesize that the functional phenotype
(constipation) is due to abnormal synaptic targeting, overgrowth/ectopic neural branching instead of a lack of innervation – as previously proposed.

Through our efforts to investigate enteric nervous system development, I produced novel transgenic flies to manipulate normal stomatogastric nervous system formation and function (Chapter 2). Our investigations initiated with phenotypic analysis of neuroanatomy defects of the embryonic SNS. In some instances, we found specific mutations caused subtle perturbations to embryonic SNS architecture, despite dramatic disruption to the CNS. These observations suggest that other cell surface molecules function in SNS development, or that there is functional redundancy amongst known molecules. Although receptor tyrosine kinases play important roles in vertebrate CNS development, they seem to play more minor roles in fly CNS development. It will be important to characterize the individual roles of RTKs such as Ret and EGFR and their interactions with axon guidance receptors such as Dscam, Robo and Frazzled in SNS development.

In analyzing the role of the Dscam in SNS development we found Dscam receptor expression in axons of embryonic SNS, rather than the migrating cells bodies (vertebrate Dscam is strongly expressed in migrating neural crest cells – Chapter 3). Localizing Dscam protein expression to axons, matches predictions that Dscam overexpression in DS might disrupt local connectivity rather than neural precursor migration. Both phenotypes have been observed in human patients. Further expression characterization with additional tools is necessitated. We acknowledge the need for additional characterization of loss of function phenotypes as even in the CNS, Dscam mutants have mild defects revealed primarily by markers that label subsets of axons and cell bodies,
such as anti-Connectin (Andrews et al., 2008). Careful analysis of SNS structures and hindgut motor neuron precursors employing additional Dscam-specific antibodies, additional antibody labels, and mRNA probes (in situ hybridization; ISH) is merited. Functional analysis of Dscam loss of function mutants is needed and can be achieved by generating allelic combinations that allow larval behavior to be analyzed.

Subsequently, we set forth to mimic Dscam trisomy in gut specific neurons and determined that elevated Dscam levels cause ectopic neuronal growth of the frontal nerve into pharyngeal musculature. Surprisingly, SNS neural defects did not affect effective feeding functionality, but resulted instead in decreased gut clearance efficiency. As a result, evaluation of the hindgut innervating motor neurons revealed further abnormalities, overgrowth and excessive branching. Furthermore, we characterized additional larval behavioral aberrations and found the Dscam overexpression to be detrimental to locomotion (Appendix 1). We hypothesize that the observed decrease in overall larval kinesis is due to inefficient gut clearance. Interestingly, when presented with a common food odor, ethyl acetate, the slow-moving larvae change their behavior significantly and have increased run length and speed. This behavior may be an indication of affected feeding status, perhaps increased hunger and foraging behavior resulting in Lévy flight like locomotion (Troncoso et al., 1987; Koon et al., 2011; Berni et al., 2012).

Additional functional studies are warranted in order to understand the nature of the functional phenotypes we observed. Measurements of peristalsis and defecation rates will allow the functional defects to be localized. We began this work expecting SNS manipulation to alter feeding and peristalsis. Instead we observed a food clearance defect
that seems likely due to defects in hindgut circuitry. It is also possible that both phenotypes are present. Dscam has a well known role in synaptic targeting and it will be important to analyze synaptic distributions at the proventriculus and hindgut to understand the overgrowth and behavioral phenotypes. General examination of larval neuroanatomy will be just as important to determine if the embryonic defects are maintained as the larvae rapidly increase in size. In summary, this dissertation describes the use of a simple model organism to model a complex human condition as well as to increase our understanding of how the larval enteric nervous system is formed and functions. Our results are in accordance with previous observations that Dscam overexpression seems to promote axonal and synaptic growth, but suggest these increases are actually detrimental to function.

**Future Directions**

Further development of the fly model: I proposed specific experiments above related to larval gut function that may be required for publication of a manuscript. However, many further avenues of work present themselves. Additional anatomical markers such as GAL4 lines expressing in SNS and hindgut subsets, combined with confocal microscopy will allow closer examination and quantification of the phenotypes. Analysis can be performed during the larval stages where the SNS structures are correspondingly larger. Dscam1 signals through multiple pathways, including the non-receptor kinases Abl and PAK, either through direct physical interaction or through adaptor proteins such as Dock. Examining the loss of function phenotypes for these cytoplasmic effectors should be quite informative and warrant detailed characterization. Reagents are available for
assaying Abl gain of function phenotypes. The small molecule Bcr-Abl-specific inhibitor, Nilotinib, has been reported to counteract Dscam overexpression and led to us testing it in the food clearance assay. Preliminary results show a beneficial effect on food clearance in the Dscam overexpressing larvae, but an adverse effect on wild type animals. This may reflect a normal role for Abl in SNS/hindgut nerve development, which we would like to uncover by analysis of mutant phenotypes. Alternatively, PAK has been linked to Dscam repeatedly and may be the more relevant molecule to inhibit. Again, drug studies combined with analysis of loss of function mutations will guide our understanding. The conservation of signaling pathways from flies to humans suggests that this work will be relevant to Down Syndrome patients.

Both PAK and Abl are strongly linked to both cystoskeletal rearrangements and cell proliferation and survival. My original hypothesis that searching for trophic support might drive SNS formation may still be valid. Our laboratory has shown that Dscam mutants lead to increased cell death in combination with frazzled. Cell death occurs at a remarkably high rate in the SNS and the ventral nerve cord segments from which the hindgut neurons emerge. Mutational analysis of Dscam manipulation suggested increased cell numbers in the SNS and I would like to follow up on this to directly test if Dscam is blocking cell death in the SNS.

**Translational experiments:** two well-characterized mouse models of Down Syndrome are available, Ts65Dn and Ts1Cje and although they have been widely used to analyze CNS function, the ENS has not been examined (Cefalu et al., 1998; Faizi et al., 2011). Establishing the specific cell types and developmental stages at which Dscam is
expressed in the ENS would be a critical first step. Examination of neuroanatomy in the DS models would quickly follow. These studies could be combined with observations on passage of food through the gut, frequency of stool expulsion and stool content.

Intriguingly, the ENS has been found to mimic the CNS defects seen in disorders such as Parkinson's disease and such assays have been carried out (Taylor, Green & Miller, 2010). The expertise also exists at the University of Nevada, Reno in the smooth muscle groups of Kent Sanders and colleagues. Detailed neuroanatomical and functional studies would follow, but perhaps most excitingly, positive results would allow for the testing of PAK and Abl inhibitors in these mice. The single most important initial experiment would be to establish whether the mouse models of DS have constipation or other gut motility defects. In the absence of a phenotype (and different threshold levels for gene activity between mice and humans are often observed), then direct observation of drug effects on enteric neurons and behavior could allow progression towards the clinic.
**Specific Aims:**

1. Determine if elevated Dscam protein levels affect cell death in the developing SNS and hindgut innervating motor neurons. The approach will be to employ readily available genetic tools (employing Drosophila and mouse models) to quantify basal cell death levels in wild type, loss of function and gain of function mutants. The hypothesis to be tested is that amplified Dscam levels result in increased cell survival and in turn, ectopic neural growth.

2. Identify the role of Dscam in synaptic targeting and formation. This will be done by thoroughly evaluating synaptic boutons and neuro-muscular junctions of the proventricular ganglion and hindgut motor neurons with their respective targets. The question to be answered is whether augmented Dscam levels cause flawed wiring and result in defective gut functionality.
Appendix 1

*Dscam1* overexpression affects larval locomotion

**Introduction**

Here I provide the extensive analysis of locomotion phenotypes of all the transgenic flies discussed in Chapter 3. This line of research may be considered as tangential; nonetheless, it illustrates the complex behaviors orchestrated by interconnected neural networks. As noted from previous studies in *Drosophila*, elevated Dscam protein levels alter dendritic patterning; induce ectopic midline crossing of nerve cord axons and increase axonal arbor size (Zhu et al., 2006; Andrews et al., 2008; Kim et al., 2013; Sterne et al., 2015). Furthermore, Dscam overexpression leads to errors in synaptic targeting consequently causing functional defects in sensory perception (Cvetkovska et al., 2013). Likewise, we found that SNS-targeted *Dscam1* overexpression caused increased and abnormal neural development. Based on these observations, we decided to employ a sensitive, high-throughput assay to assess for further functional consequences (see Chapter 3).

**Methods**

*Drosophila Genetics*

See Chapter 3. The *w*¹¹¹⁸ line was used as a wild type control. Additional controls: GAL4 lines were crossed to *UAS-mCD8-GFP* to verify expression (patterns) and to discard possible effects caused by driver transgenes. *UAS* lines were outcrossed to *w*¹¹¹⁸ to
circumvent possible effects caused by the varying genetic backgrounds and to examine for and discard the possibility of leaky expression.

**Larval Locomotion**

See Chapter 3. The paradigm described in Mathew et al., 2013 was employed to assess for locomotion phenotypes. 22 to 26 fed, second instar larvae were placed along the central y-axis of a 22 cm by 22 cm plate containing a thin layer of 1.5% agarose. Clear covers were used to seal the arena and to contain larvae. Kinesis was recorded with a CCD camera for five minutes inside a dark chamber. Odorant: 50 µL of ethyl acetate was diluted $10^{-2}$ in paraffin oil (vol:vol).

**Statistics**

For each genotype 60+ second instar larvae were selected at random and subjected to locomotion analysis. Locomotion videos were analyzed using MATLAB ® (MathWorks ®). Parameters examined: average number of tracks per run (number of bouts), run length, run speed and track length divided by displacement. Statistical analyses were performed employing the Tukey’s honest significance test in the Statistica® software.

**Results and Discussion**

First, we wished to confirm that variations in behavioral parameters were not due to the transgenic driver lines employed: *RetP-GAL4, GscG-GAL4, fasII-GAL4* and *PDF-GAL4* (Figures 2-5). However, we found the *PDF-GAL4::UAS-GFP* control to have significantly increased run length (Figure 3). Notably, a single copy of either *Dscam1-FL* or *Dscam1-*
ΔC significantly increased run speed in a wild type genetic background (outcross to w^{118}, Figure 4). As a result, we included the UAS-Dscam outcrosses as additional and necessary controls in subsequent evaluations.

In Chapter 3, I employed three distinct SNS-specific drivers to specifically target Dscam1 overexpression: RetP2A-GAL4, RetP2B-GAL4 and GscG-GAL4. These lines are thoroughly described in Chapter 2. Although the three lines share embryonic expression patterns, we found each to have a distinct affect on locomotion (Figures 7-10). The distinction is evident in analyzing average run speed. While continuous Dscam1-FL overexpression with RetP2A,B drivers significantly decreased larval run speed. This may be correlated with failure to defecate (within a normal rhythmic manner – see Chapter 3 Figure 4H and Discussion). Expression under control of Gsc-GAL4, which switches off expression at the end of embryogenesis, caused a substantial increase in the same parameter (Figure 8). This suggests that persistent SNS expression of Dscam1-FL is detrimental to overall motility (see Chapter 3). Additionally, we summarized alterations to behavioral parameters employing principle component analysis (PCA; Figure 10). This particular analysis was performed in four-dimensional space incorporating average number of bouts, run length, run speed and track length divided by displacement. Interestingly, overexpression of the Dscam1 transgenes using the hindgut motor neuron-specific driver, PDF-GAL4, resulted in contrasting behavioral outputs (Figures 7-10). These results suggest that SNS-neurons (anterior) and the hindgut innervating motor neurons (posterior) operate through distinct feedback mechanisms (see Chapter 3 Discussion).
Schmucker et al found that, in axons, the Dscam receptor signals through the p21 activated kinase, PAK. We found locomotion to be affected upon expressing PAK (PAK-GFP) and activated PAK (myristoylated). Particularly, an added copy of PAK-GFP decreased average run length and speed, whereas activated PAK caused significant increases in the same parameters (Figures 12 and 13). Principle component analysis of behavioral responses altered by PAK overexpression further illustrates the discrepancies between the two RetP drivers. More importantly, it is obvious that the two PAK transgenes cause distinct affects, as do Dscam1-FL in contrast with Dscam1-ΔC (Figure 15).

Lastly, we questioned if Dscam1 overexpression affected larval response to a food odor. We used an odor known to attract wild type larvae, ethyl acetate. To our surprise, driving Dscam1-FL with RetP2B-GAL4 resulted in significantly augmented run length and speed (Figures 18 and 19). Driving with RetP2A did not have similar results; in fact, RetP2A::Dscam1-FL larvae behaved alike wild type in the presence of the attractive odor. Furthermore, the hyperactivity previously observed in larvae having Dscam1-ΔC overexpression, was not amplified in the presence of ethyl acetate. I speculate that the increased activity observed in RetP2B::Dscam1-FL larvae is due to abnormal (increased) neural connectivity (Chapter 3, Figures 3 and 7B).

**Future Directions**

Much of the published work pertaining to the Pigment-dispersing factor (PDF) is in the circadian rhythm field. Thorough and detailed embryonic and larval neuroanatomical analysis of the hindgut innervating motor neurons would further clarify if Dscam
overexpression causes overgrowth and abnormal branching/arborization phenotypes. This can be achieved employing available genetic tools, *HGN1-GAL4, PDF-GAL4*, etc.

Likewise, possible hindgut innervation phenotypes should be fully analyzed in *Dscam1* and combination mutants, as my characterizations of these mutants were preliminary and limited to the SNS (anterior).

Elevated Dscam protein levels could cause a number of perturbations to the hindgut innervating motor neurons that have yet to be identified and studied. I hypothesize that three copies of the *Dscam* gene (Dscam X3) cause ectopic axonal growth, arborization/branching, flawed synaptic targeting and, consequently, abnormal hindgut neural circuit function. Moreover, analysis of peripheral neurons and glia would shed light on additional perturbations in the developing embryo and larvae.
Table 1. Summary of \textit{GAL4}/\textit{UAS} transgenic lines employed.

<table>
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<th>Controls:</th>
<th>\textit{Dscam1} Gain of function:</th>
<th>\textit{PAK} Gain of function:</th>
<th>Response to Ethyl acetate</th>
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<td>\textit{w}^1118</td>
<td>RetP2A-gal4 :: UAS-Dscam1-FL</td>
<td>RetP2A-gal4 :: UAS PAK-GFP</td>
<td>\textit{w}^1118</td>
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<tr>
<td>RetP2A-gal4 :: UAS \textit{mCD8-GFP}</td>
<td>RetP2B-gal4 :: UAS-Dscam1-FL</td>
<td>RetP2B-gal4 :: UAS PAK-GFP</td>
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<td>GscG-gal4 :: UAS-Dscam1-FL</td>
<td>RetP2A-gal4 :: UAS myrPAK</td>
<td>RetP2A-gal4 :: UAS-Dscam1-FL</td>
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<td>fasII-gal4 :: UAS-Dscam1-FL</td>
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<td>PDF-gal4 :: UAS-Dscam1-\textit{ΔC}</td>
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Figure 1. Overexpressing \textit{Dscam1} transgenes affects larval locomotion.

(A) Locomotion tracking set up. Larvae are placed on a large Petri plate containing a thin layer of 1.5% agarose. CCD camera captures kinesis information at 130 frames per minute. (B) Representative locomotion trajectories for wild type control (\textit{w}^{1118}) and \textit{RetP2A} driving \textit{Dscam1-FL} (C) or \textit{Dscam1-AC} overexpression (D).
For all genotypes, n > 60 second instar larvae. None of the transgenes, neither GAL4 nor UAS-Dscam1, caused an affect on average bout number compared to the \( w_{118} \) control.
Figure 3. **GAL4** transgenes do not affect larval run length.

Average run length of **GAL4** drivers crossed to a benign reporter, *UAS-mCD8-GFP*. No significant difference was detected between the three SNS-specific drivers: *RetP2A,-2B* and *GscG-GAL4*. GFP expression in the hindgut motor neurons, PDF, caused a significant increase in average run length (p=0.0158, Tukey HSD within a one-way ANOVA for all p values). Note: as single copy of *Dscam1* transgene (*Dscam1-FL* and *Dscam1-ΔC* outcross to *w*1118) did not affect average run length compared to the *w*1118 control. For all genotypes, n > 60 second instar larvae.
Figure 4. *GAL4* transgenes do not affect larval run speed.

Average run speed of *GAL4* drivers crossed to a benign reporter, *UAS-mCD8-GFP*. No significant difference was detected between *fasII*-*, PDF-GAL4* and the three SNS-specific drivers. Dscam transgenes outcrossed to the *w^{118}* had an increased average run speed (p=0.000055 and p=0.000033 respectively, Tukey HSD within a one-way ANOVA). For all genotypes, n > 60 second instar larvae.
Figure 5. *GAL4* transgenes do not affect track length over displacement.

None of the transgenes, neither *GAL4* nor *UAS-Dscam1*, significantly affected average track length divided by displacement compared to the w^{1118} control. For all genotypes, n > 60 second instar larvae.
**Figure 6. Overexpressing Dscam1 transgenes mildly affect bout number.**

For all genotypes, n > 60 second instar larvae. Average bout number of larvae with specifically targeted Dscam1-FL or Dscam1-ΔC overexpression. Driving Dscam1-FL in the hindgut innervating motor neurons, PDF, caused a significant decrease in bout number (p=0.017597, Tukey HSD within a one-way ANOVA). In contrast, Dscam1-ΔC overexpression in the same motor neurons caused a significant increase (*p=0.028592) compared to the w1118 and Dscam1-ΔC outcross controls. Driving Dscam1-ΔC with the SNS-specific driver, RetP2B, caused a significant decrease (#p=0.044120).
Figure 7. *Dscam1* overexpression affects larval run length.

Average run length of larvae with specifically targeted *Dscam1* transgene overexpression. Interestingly, *Dscam1*-FL overexpression decreased average run length only when driven by *RetP2A* compared to the outcross control (*p=0.004538, Tukey HSD within a one-way ANOVA). An inverse effect was caused in driving *Dscam1*-FL with *fasII* and in the PDF motor neurons (*p=0.000020 and *p=0.000020 respectively). *Dscam1*-ΔC overexpression with *RetP2A* and *RetP2B* resulted in significant augmentation of average run length (*p=0.000138 and *p=0.000020 respectively). For all genotypes, n > 60 second instar larvae.
Figure 8. *Dscam1* transgene overexpression disrupts average run speed.

Average run speed of larvae with specifically targeted *Dscam1* transgene overexpression. Both outcross controls had significantly increased average run speed (*p=0.000214 and p=0.000029, Tukey HSD within a one-way ANOVA). However, compared to outcross controls, *Dscam1-FL* overexpression with *RetP2A* and *RetP2B* significantly decreased average run speed (#p=0.000020 and ###p=0.000020 respectively). In contrast, driving the same *Dscam1* transgene with the embryonic SNS-specific *GscG* driver resulted in a significant increase compared to both controls (* and ###p=0.000020). *Dscam1-ΔC* overexpression caused opposite effects: significant increase with *RetP2A* and -2B drivers (###p=0.00020 for both) and substantial decrease with PDF-GAL4 driver (###p=0.000020). For all genotypes, n > 60 second instar larvae.
Figure 9. Dscam1 overexpression has no affect on exploratory behavior.

For all genotypes, n > 60 second instar larvae. Average track length divided by displacing (exploratory behavior or wandering) was unaffected in all transgenic larvae.
Figure 10. PCA of various behavioral parameters.
Figure 10. PCA of various behavioral parameters.
Figure 10. PCA of various behavioral parameters.

Principle component analysis (PCA) measures Euclidean distance between differing behaviors for desired genotypes in a multi-dimensional space (in this instance, four-dimensional space). Behaviors analyzed: number of runs per track (number of bouts), run length, run speed and distance/displacement of track. Genotypes: 1) w^{118}, 2) Dscam1-FL outcross, 3) RetP2A::Dscam1-FL, 4) RetP2B::Dscam1-FL, 5) GscG::Dscam1-FL, 6) fasII::Dscam1-FL, 7) PDF::Dscam1-FL, 8) Dscam1-ΔC outcross, 9) RetP2A::Dscam1-ΔC, 10) RetP2B::Dscam1-ΔC, 11) GscG::Dscam1-ΔC, 12) fasII::Dscam1-ΔC and 13) PDF::Dscam1-ΔC
Figure 11. Bout quantity is unaffected by PAK transgene overexpression.

Average number of bouts for transgenic larvae. Driving myristoylated PAK with RetP2A caused a significant decrease in number of bouts compared to overexpressing an extra copy of PAK-GFP (p=0.011470, Tukey HSD within a one-way ANOVA). For all genotypes, n > 60 second instar larvae.
Figure 12. Average run length is augmented by myristoylated PAK.

Average run length was increased by driving myristoylated PAK with SNS-specific drivers, \textit{RetP2A} and -2B (*p=0.000017 and *p=0.021255 respectively). For all genotypes, \(n > 60\) second instar larvae.
Figure 13. *PAK* transgene overexpression affects run speed.

Overexpressing an additional copy of GFP tagged PAK with *RetP2A* resulted in a significant decrease in average run speed (#p=0.001806, Tukey HSD within a one-way ANOVA). Conversely, overexpression of myristoylated PAK caused an increase in run speed (*p=0.000017) with both *RetP* drivers. For all genotypes, n > 60 second instar larvae.
Figure 14. PAK transgene overexpression did not affect exploratory behavior.

For all genotypes, n > 60 second instar larvae. Added copies of GFP tagged PAK and myristoylated PAK did not cause an affect on average track length divided by displacement (Tukey HSD within a one-way ANOVA).
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</tr>
<tr>
<td>RetP2A-gal4 :: myrPAK</td>
<td>1.706207371</td>
<td>3.115381615</td>
<td>0</td>
<td>3.748958333</td>
<td>5.436304558</td>
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<td>RetP2B-gal4 :: myrPAK</td>
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<td>6.131838056</td>
<td>3.748958333</td>
<td>0</td>
<td>2.557697418</td>
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</table>

**Figure 15. PCA of altered behavioral parameters upon PAK overexpression.**

An added copy of myristoylated PAK had a greater effect on behavior than adding an extra copy of PAK (GFP tagged). The Euclidean distances between myristoylated PAK altered behaviors and wild type were significantly increased (see table). Yet, it is evident that the two SNS-specific divers also produced divergent results in overall behavior. The genotypes are 1) w₁₁₁₈, 2) RetP2A::PAK-GFP, 3) RetP2B::PAK-GFP, 4) RetP2A::myrPAK and 5) RetP2B::myrPAK.
Response to ethyl acetate odor

Figure 16. *Dscam1* overexpression did not alter RI to ethyl acetate odor.

For all genotypes, n > 60 second instar larvae. The average response index was between 0.20 and 0.25 for most genotypes. Although driving *Dscam1-FL* with *RetP2B* caused a decrease in RI (average RI was approximately 0.125), the change was not statistically significant (Tukey HSD within a one-way ANOVA).
Figure 17. *Dscam1-ΔC* overexpression increases number of bouts in the presence of ethyl acetate odor.

Average bout number in response to ethyl acetate. *Dscam1-ΔC* overexpression caused a significant increase in bout number compared to outcross control (*p*=0.000459, Tukey HSD within a one-way ANOVA). For all genotypes, n > 60 second instar larvae.
Figure 18. *Dscam1* overexpression disrupts run length in the presence of ethyl acetate.

Driving *Dscam1-FL* with *RetP2B* resulted in a significant increase in average run length in the presence of ethyl acetate (*p*=0.000026 compared to outcross control, Tukey HSD within a one-way ANOVA). Overexpression of *Dscam1-ΔC* through embryogenesis and larval stages caused significant decrease in average run length compared to *w^{1118}* and outcross controls (#p=0.000026). For all genotypes, n > 60 second instar larvae.
Figure 19. *Dscam1*-ΔC overexpression decreases run speed in the presence of ethyl acetate.

Much like run length, overexpression of *Dscam1*-FL with *RetP2B* resulted in an increase in average run speed (*p=0.000026, Tukey HSD within a one-way ANOVA). An extra copy of *Dscam1*-ΔC, however caused significant decrease (###*p=0.000026). For all genotypes, n > 60 second instar larvae.
Figure 20. Ethyl acetate did not elicit an affect on exploratory behavior.

Average track length divided by displacement of transgenic larvae was not significantly affected by the presence of ethyl acetate odor (Tukey HSD within a one-way ANOVA). For all genotypes, n > 60 second instar larvae.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>(1)</th>
<th>(2)</th>
<th>(3)</th>
<th>(4)</th>
<th>(5)</th>
<th>(6)</th>
<th>(7)</th>
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<tr>
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<td>3.1422</td>
<td>3.2909</td>
<td>2.5430</td>
<td>3.1244</td>
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<td>0.506820</td>
<td>0.996697</td>
<td>0.601227</td>
<td>0.126826</td>
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</tr>
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<td>0.999981</td>
<td>0.922380</td>
<td>1.000000</td>
<td>0.979687</td>
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</tr>
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<td>0.997886</td>
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<tr>
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</tr>
<tr>
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<td>0.999956</td>
<td>0.920657</td>
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<tr>
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<td>0.111438</td>
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<td>0.97886</td>
<td>0.405025</td>
<td>0.971523</td>
<td></td>
</tr>
</tbody>
</table>
Figure 21. *Dscam1* overexpression affects run ration.

Run ration is a measure of directionality (length toward odor / length away from odor). Driving *Dscam1-FL* with *RetP2B* had a very different affect than driving *Dscam1-ΔC* with the same SNS-specific driver.
Literature cited


