The Regulation of Commissureless in the Embryonic CNS of Drosophila melanogaster

Dissertation

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Abstract

During development the precise wiring of the nervous system is dependent on the correct repertoire of axon guidance molecules being expressed at the right time and place. In the CNS of *D. melanogaster*, Comm and Robo play important roles in regulating axons at the midline. To date much has been studied about how Comm regulates Robo, but little is known about how Comm is regulated in the CNS. Comm is dynamically expressed in an off, on, and off again fashion in contralaterally projecting axons and is not expressed in ipsilateral projecting axons. Our hypothesis is the dynamic spatial and temporal regulation of Comm is at the transcriptional level.

In our attempt to understand the molecular basis of Comm expression, we have undertaken several approaches to identify the necessary *cis*-regulatory elements. Experiments using large P[acman] rescue constructs fail to restore proper Comm expression in the CNS, suggesting that the *cis*-regulatory regions for Comm may be quite large. To further our understanding of these potentially large *cis*-regulatory regions, we have taken advantage of site-specific recombination using the yeast enzyme FLP recombinase and FLP recognition target (FRT) sites to make molecularly defined deletions. A series of deletions that remove large portions of DNA, as far as 71 kb 5’ of Comm and deletions 45 kb 3’ of Comm, have been tested with existing *comm* alleles. Analysis of these deletions using monoclonal antibodies BP102 and 1D4 to reveal axon projections in the CNS show deletions 5’ of Comm have a Robo-like phenotype, where axons cross the midline inappropriately. Examination by fluorescent *in situ* hybridization for Comm transcript at single cell resolution using genetically defined markers to reveal a subset of (ipsilaterally projecting) neurons reveals Comm accumulation in the cell body where Comm is not normally expressed. In a subset of commissurally crossing eagle (Ew) neurons Comm accumulation is detected earlier than normal. In summary, we have identified the 5’ region of Comm as crucial for negatively regulating Comm expression in the CNS.
In addition to Comm, there are two Comm-like genes in *Drosophila*, Comm2 and Comm3. Comm3 is expressed too late in embryogenesis to play a role in midline guidance. However, we show that Comm2 is largely co-expressed with Comm in a similar pattern throughout embryogenesis. Unlike Comm, deletion of Comm2 has no detectable BP102 or 1D4 phenotype; a mild Robo-like gain-of-function phenotype is produced when Comm2 is pan-neurally expressed in the CNS. A screen for dominant modifiers of the *Netrin* deficiency phenotype has revealed that large deletions removing both Comm and Comm2 act as dominant enhancers of the *Netrin* mutant phenotype. Further analysis demonstrates that mutations in *comm* and *comm2* individually enhance the *Netrin* deficiency phenotype, but to a lesser extent than the deletion of both *comm* and *comm2*. This result strongly suggests that Comm2 is playing commissural axon guidance at the midline. Uncovering the role of Comm2 will contribute to our knowledge of axon guidance decisions at the midline.
Dedication

This document is dedicated Jenny Jowdy.
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I would like to thank Mark Seeger for his support in allowing me to work and grow as a scientist. I’m so grateful for the opportunity given to me in the Department of Molecular Genetics at The Ohio State University. I would like to acknowledge all of the love and support that has been given to me most of all by my wife Jenny and our children Matthias, Adeline and Ridley. Without your love and support, I would not have been able to accomplish all that I have. I would like to thank my Mom and Dad for all of their support and encouragement. I would like to thank members of the Seeger lab past and present. Dave Forsthofel, thank you for all the great ‘fly room’ discussions. Laura Carver, thank you for helping me when I couldn’t be in the lab, changing stocks, apple-juice plates, collecting virgins etc. So many experiments would have been ruined without you.
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Chapter 1: An Introduction to Axon Guidance

1.1. Cajal

Proper functioning of the adult nervous system is dependent upon neurons being “wired” correctly in the body. How this occurs has been the subject of intense inquiry over many centuries and at various levels. The modern synthesis of the field is most notably attributed to Santiago Ramón y Cajal (1852-1934), who was one of the first to describe the ‘growth cone’ as an amoeboid-shaped tip at the end of an extending axon. Cajal’s observations of the developing nervous system also allowed him to rule out the “reticular” theory of nervous system formation (Ramón y Cajal 1999 English translation). The reticular theory suggested that the tips of axons fused with one another, as opposed to the current modern theory of the neuron being a discrete unit of the nervous system (Finger 2000). Cajal’s contributions did not stop there. Not only did Cajal coin the term ‘growth cone’, but he was also one of the first to hypothesize that the ‘growth cone’ pushed its way through the developing embryo, by ‘sensing’ its environment, to reach its final target (Tessier-Lavigne and Goodman 1996). Experimental support for Cajal’s intuitive observations did not come until later, when Harrison (1910) and Speidel (1941) both showed that axons were able to grow out from the cell body (Reviewed by Purves
1988; Tessier-Lavigne and Goodman 1996). However, taken together, these observations were not conclusive proof that axons navigate toward a specific target. How axons find their target in the staggeringly complex nervous system has been the subject of intense study in modern experimental biology. This chapter is a review of some of the key concepts and ideas on how axons find their targets.

1.2. How do axons find their way? The “General Chemo-affinity Hypothesis”

An alternative hypothesis to that of directed migration was proposed in 1941 by Paul Weiss: the ‘resonance hypothesis’. Weiss suggested that specific connections were not made from directed migration to a target, but rather, a subset of random connections were retained after being formed (Reviewed by Purves 1988; Tessier-Lavigne and Goodman 1996). An analogy of the resonance hypothesis may be that of a tuning fork to a string, where the neurons grow undirected and eventually ‘resonate’ to their synaptic muscle targets. However, Weiss’s ‘resonance theory’ did not hold for long, as one of his former students, R.W. Figure 1.1. Directed axonal regeneration in the frog retina. After dissections, rotation and regeneration of the optic nerve, animals reacted in the opposite fashion by responding away from the prey instead of attacking it. Regenerating axons bypassed the more proximal side and re-innervated to their original targets now on the inverted side. Since axons navigated toward their target as predicted, resulting in an inverted image in the brain, Sperry interpreted this result as support for directed migration and the chemoaffinity hypothesis.
Sperry, challenged his mentor’s idea of highly malleable connections (Reviewed by Purves 1988; Meyer 1998). Sperry revisited the idea of a chemical relationship of nerve to target cell with a series of experiments. Taking advantage of the observation that the optic nerve could regenerate after severing the nerve in frogs and newts, Sperry tested the validity of Weiss’s hypothesis by dissecting the optic nerve and rotating the eye 180° (Fig.1.1). After recovery, the animals reacted in an opposite fashion when presented with stimuli, suggesting that the connections were restored to their original target. The correct targeting of these projections was confirmed by staining the regenerated axons to show their location (Sperry 1963). In addition, an important aspect for the revival of the chemoaffinity hypothesis was its exclusion of the resonance hypothesis. Sperry was able to show that axons regenerated toward specific targets as predicted by the chemoaffinity hypothesis therefore excluding the resonance hypothesis, which was unable to account for axons bypassing synaptic targets in favor of others. (Sperry 1963; and reviewed by Purves 1988; Meyer 1998).

The results of his experiments allowed Sperry to summarize: “The cells and fibers of the brain… must carry some kind of identification tags, presumably cytochemical in nature, by which they are distinguished one from another almost, in many regions, to the level of the single neuron; and further, that the growing fibers are extremely particular when it comes to establishing synaptic connections, each axon linking only with certain neurons to which it becomes selectively attached by specific chemical affinities” (Sperry 1963).
The renewal of interest in the chemoaffinity hypothesis and the use of different model organisms (more amenable to genetic analysis) have revealed many new insights and a more molecular view of axon guidance. Since Sperry, there have been several related hypotheses: the guidepost cell hypothesis (Bate 1976; Bentley and Caudy 1983; Molnar and Blakemore 1995), the blueprint hypothesis (Singer, Nordlander et al. 1979; Gottlieb 1980; Bastiani and Goodman 1986), and the labeled pathways hypothesis (Goodman, Raper et al. 1983). These refined hypotheses have driven the bulk of investigation over the last 30 plus years.

1.3. Axon guidance: extending the “Chemo-affinity Hypothesis”

The first axonal projections (a.k.a. pioneers) that form during development are guided by cues in the extra-cellular environment. These axons do not follow axon tracts laid down earlier in development, but are critical for the formation of pathways of other ‘follower’ axons later in development. The question, 'How do axons precisely navigate long distances toward their final target?' may be answered simply: each axon navigates to several discrete ‘choice-points’ en route to its target.

In 1976, Michael Bate took an embryological approach to determine how the peripheral neuron connections from the epidermis to the central nervous system (CNS) were made in the nascent limb bud of locust. Since it was observed in later developmental stages that newly differentiated axons followed pre-existing pathways, the question of how axons navigate without such established pathways was crucial to understanding how the
nervous system is formed. Bate observed that axons from the epidermis navigate toward the CNS in the developing limb buds of locusts and not visa-versa (Bate 1976). He also noted that these axons grew toward the CNS prior to any existing axon tracts. While some axons grow directly toward their target based on their orientation (e.g. antennal neurons), other projections, such as the limb bud axons, took indirect routes toward the CNS.

Based on these observations, Bate proposed that axons navigate toward their targets not based solely on orientation, but by extrinsic cues presented by “guidepost cells” in the limb bud. Bate hypothesized that the obvious candidates were the “sheathing cells” based on the consistently spaced intervals of these cells along the limb bud. Although Bate was not the first to make this observation, his work opened up a new experimental platform using the early embryo to test hypotheses about axon guidance mechanisms (Bate 1976). Later, Bentley and Caudy were able to ablate CT1 cells (presumptive guidepost cells) in the limb bud of embryonic grasshoppers and show disrupted pioneer axon trajectories, thus lending further support to the ‘guidepost cell’ hypothesis (Bentley and Caudy 1983).

The ‘blue print’ hypothesis, as proposed by Singer, advances the ‘guidepost cell’ hypothesis. He compared axon trajectories in embryogenesis of the newt spinal cord to axon trajectories in regenerating spinal cords of adult newts (Singer, Nordlander et al. 1979). Singer proposed that pathways on the neuroepithelium have “mechanical-
chemical itineraries’ which the neurites follow according to their individual affinities” (Singer, Nordlander et al. 1979). Although the identity of these hypothetical ‘tags’ were not known, this hypothesis did seem consistent with the stereotypical nature of axon pathways in the early embryo and regenerating axons of vertebrates and invertebrate embryos (Gottlieb 1980; Bastiani and Goodman 1986).

The previous hypotheses all suggest that axons find their way by following cues in the environment. However, how do axons find their way with such precision, given the diversity of choices that are presented to individual axons? In addition, how do axons, which are initially specified in the same location, have divergent final trajectories?

Detailed cellular studies on the behavior of both pioneer and follower axons in grasshopper embryos lead Goodman and colleagues to propose the ‘labeled pathways hypothesis’. A key difference in this hypothesis is an active, rather than passive, guidance mechanism--specifically, that the growth cone at the tip of the axon actively samples the cues present in the environment to navigate towards its target (Goodman, Raper et al. 1983). The ‘labeled pathway’ hypothesis is composed of three facets:

(i) pioneer neurons establish stereotypical axonal pathways;  
(ii) these axonal pathways are differentially marked, most likely on their cell surfaces; and  
(iii) later growth cones are differentially determined in their ability to make specific choices of which labeled pathway to follow. (Goodman, Raper et al. 1983)

The ‘labeled pathways’ hypothesis is also implicit in its predictions about the nature of the cues, their presence at the cell surface, and in the extracellular environment. Nevertheless, what are the molecular identity of these cues?
1.4. Molecular mechanisms of Axon Guidance

Using both vertebrate and invertebrate model organisms, many of the molecules important for axon guidance have been identified with both genetic and biochemical approaches. The mechanism of action for these molecules can be broken down into distinct categories: chemoattraction, chemorepulsion, contact-mediated attraction, and contact-mediated repulsion (Tessier-Lavigne and Goodman 1996; Dickson 2002). These ‘forces’ allow axons to navigate long distances to locate their final synaptic targets.

Axons navigate to their targets in a highly stereotypical fashion, making very few errors along the way. They accomplish this task by detecting molecules in the environment. What molecules are involved in exerting attractive and repulsive forces on an axon?

Several families of guidance molecules have been identified that play a role in the above mentioned categories and are conserved from invertebrates to vertebrates. These families of conserved signaling molecules include Semaphorins (Semas), and their receptors (Plexins (Plex) and Neuropilin), Netrins and their receptors (DCC/Frazzled, UNC5 and DSCAM), Slits and their Robo receptors, and the Ephrins and Eph Receptors (Reviewed by Huber, Kolodkin et al. 2003; O'Donnell, Chance et al. 2009). How do these seemingly few molecules produce precise control of a large diversity of neuron trajectories in a dynamic environment such as the developing embryo? Ultimately, these ligands and receptors exert their influence to promote local reorganization of the actin and microtubule cytoskeleton. Activation of specific pathways can promote either repulsion or attraction, which either results in the collapse of a growth cone or can increase the rate of which an axon extends toward its target. Ultimately, each individual
axon utilizes these molecules to navigate toward its final target to establish a functioning nervous system.

1.5. CNS midline guidance

Coordinated movement in bilateral animals is dependent upon the proper wiring of the two halves of the CNS. As the neurons on each side of the midline begin to send out axonal projections, they make the binary ‘choice’ to navigate across or away from the midline. Once commissural axons cross the midline, they do not re-cross, even though they encounter the same cues that guided them there initially.

Figure 1.2. Axon guidance at the midline. (A) *Drosophila* ventral nerve cord showing segmentally repeated anterior (AC) and posterior (PC) commissures in blue. Vertical dashed line represents secreted attractive and repulsive cues from midline glia cells. Individual neurons illustrate commissural (green) and ipsilateral (red) projections in the CNS. (B) Spinal cord section of a mouse embryo. Floor plate express both attractive and repulsive cues. Dorsal neurons project toward the floor plate. (C) “Open-Book” preparation looking dorsally on CNS projections. Anterior (A) to the left Posterior (P) to the right (Adapted from Dickson and Gilestro 2006).
In the *Drosophila* ventral nerve cord (Fig 1.2), axons that project across the midline make contralateral projections and contribute to the formation of the anterior and posterior commissures of each segment. Axons that do not cross the midline make ipsilateral projections in the CNS. (Kaprielian, Imondi et al. 2000; Kaprielian, Runko et al. 2001; Dickson and Gilestro 2006). These contralateral and ipsilateral projections make up the orthogononal pattern of the CNS in *Drosophila* and have served as an excellent assay to both identify and study the genes involved in axon guidance.

Likewise, axons in the CNS of vertebrates make similar types of projections in the spinal cord (Fig 1.2). Dorsal/lateral interneurons all begin by orienting ventrally toward the floorplate, where they either navigate across the floorplate, or project on the same side of the CNS that they originated (Kaprielian, Imondi et al. 2000; Kaprielian, Runko et al. 2001; Dickson and Gilestro 2006). Also, ipsilaterally projecting axons growing alongside the floorplate never cross (Farmer, Altick et al. 2008). The simple binary decision, whether or not to cross, has made the midline one of the most well studied ‘choice points’ in vertebrates and invertebrates (Tessier-Lavigne, Placzek et al. 1988; Jacobs and Goodman 1989; Klambt, Jacobs et al. 1991; Bernhardt, Nguyen et al. 1992; Seeger, Tear et al. 1993; Halloran and Kalil 1994; Kaprielian, Imondi et al. 2000; Kaprielian, Runko et al. 2001; Gilestro 2008; Evans and Bashaw 2010).
1.6. Guidance cues at the midline

As mentioned above axons at the midline in vertebrates and invertebrates can be grouped into two types of trajectories, ipsilateral (same side) and contralateral (opposite side). Conserved guidance cues secreted from the vertebrate floorplate and *Drosophila* midline glia serve similar roles. In addition, receptors on growth cones function in guiding axons either across or away from the midline in response to guidance cues. The following is a brief review of the key molecules important for both vertebrate and invertebrate midline guidance.

1.6.1. The midline repulsive cue Slit.

Slits are large (~190-kDa) multi-domain proteins secreted by midline cells in *Drosophila* and vertebrates (Rothberg, Hartley et al. 1988; Rothberg, Jacobs et al. 1990). The first *slit* mutation was identified in the classic screen for mutations affecting cuticle formation in *Drosophila* larvae (Nusslein-Volhard, Wieschaus et al. 1984). Subsequent genetic screens looking for mutations that affect formation of axon projections at the midline in *Drosophila* also identified mutations in *slit* (Seeger, Tear et al. 1993; Hummel, Schimmelpfeng et al. 1999a). There are three Slit orthologues in vertebrates (*Slit-1, Slit-2 and Slit-3*) (Yuan, Zhou et al. 1999). The *slit* mutant CNS phenotype in *Drosophila* has a characteristic collapse of all the axons onto the midline. Likewise, disruption of all three *slit* genes in mice leads to a similar phenotype (although less severe) with axons stalling and inappropriately crossing the midline (Sabatier, Plump et al. 2004).
The function of Slit at the midline was not realized until both genetic and biochemical approaches identified Slit as the ligand for the guidance receptor Roundabout (Robo) (Battye, Stevens et al. 1999; Brose, Bland et al. 1999; Piper, Georgas et al. 2000). Based on several lines of genetic and in vitro cell culture experiments, Slit has been shown to be a repulsive cue at the midline in both invertebrates and vertebrates (Brose, Bland et al. 1999; Kidd, Bland et al. 1999; Long, Sabatier et al. 2004; Farmer, Altick et al. 2008). Its function at the midline is to signal through its receptor, Robo, to prevent ipsilaterally projecting axons from crossing the midline and keep commissural crossing axons from re-crossing. Structure function analysis of Slit has shown that the leucine-rich repeat (LRR) domain is important to bind Robo family members and it is sufficient for chemotaxis in cell culture assays (Nguyen Ba-Charvet, Brose et al. 1999; Battye, Stevens et al. 2001; Chen, Wen et al. 2001; Howitt, Clout et al. 2004). Slit signaling through Robo receptors leads to decreased levels of active Cdc42 and increased RhoA and Rac activity. (Fan, Labrador et al. 2003; Huber, Kolodkin et al. 2003; O'Donnell, Chance et al. 2009). This has been shown to promote growth cone collapse and turning of the axon projection either towards its target or away from inappropriate targets (Wong, Ren et al. 2001; Lundstrom, Gallio et al. 2004).

1.6.2. The repulsive receptor Roundabout (Robo)

Robo was first identified in a large-scale mutagenesis screen for axon pathfinding defects at the midline of Drosophila embryos (Seeger, Tear et al. 1993). While Robo is expressed in all neurons in the CNS, Robo protein is enriched on the longitudinal
connectives of the left and right halves of the CNS and mostly absent from commissural portions of axon projections (Kidd, Brose et al. 1998; Kidd, Russell et al. 1998). Embryos mutant for robo in both vertebrates and invertebrates have too many axons crossing the midline (Seeger, Tear et al. 1993; Kidd, Brose et al. 1998; Zallen, Yi et al. 1998; Brose, Bland et al. 1999; Kidd, Bland et al. 1999; Jen, Chan et al. 2004; Long, Sabatier et al. 2004). Based on in vivo and in vitro analysis, the role of Robo is to mediate repulsive signaling through its ligand Slit (Kidd, Russell et al. 1998; Kidd, Bland et al. 1999; Bashaw, Kidd et al. 2000; Dickson and Gilestro 2006).

Since cloning Robo, three other homologues have been identified henceforth referred to as Robo1, 2, 3, and 4. The founding member of the family of Robo receptors is defined by the presence of an extracellular domain containing five repeats of an immunoglobulin-like (Ig) domain, and three fibronectin type III repeats. The cytoplasmic domain of Robo family members contains a combination of four conserved cytoplasmic motifs arbitrarily numbered 0-3 (Kidd, Brose et al. 1998; Chedotal 2007). Structure-function analysis of the Robo receptor has shown that the cytoplasmic domain is important for repulsive signaling at the midline (Bashaw and Goodman 1999; Wong, Ren et al. 2001; Fan, Labrador et al. 2003; Lundstrom, Gallio et al. 2004; Yuasa-Kawada, Kinoshita-Kawada et al. 2009; Evans and Bashaw 2010; Spitzweck, Brankatschk et al. 2010). All of the Robo receptors can bind Slit via their Ig domains, although it is not certain that Robo4 can bind Slit (Brose, Bland et al. 1999; Li, Chen et al. 1999; Sabatier, Plump et al. 2004; Chedotal 2007). Robo family members are all differentially expressed in the CNS when
axons are extending toward their target. What is the role of Robo family members in the CNS? Do they all play a role in repelling axons away from the midline or do structural differences in their cytoplasmic domain link them to other signaling pathways (Evans and Bashaw 2010; Spitzweck, Brankatschk et al. 2010)?

Unlike invertebrates where Commissureless (Comm) is known to antagonize Robo signaling to allow midline crossing (discussed in the next section 1.6.3), no Comm-like genes have been identified in vertebrates. However, Robo3 (aka Rig1) has been shown to negatively regulate Robo to allow axons to cross the midline (Marillat, Sabatier et al. 2004; Sabatier, Plump et al. 2004). Robo3 is expressed in commissural axons actively crossing the midline and mutants for Robo3 have commissural guidance defects where fewer axons than normal cross the midline (Marillat, Sabatier et al. 2004; Sabatier, Plump et al. 2004). Adding to the complexity of Robo3 regulation was the discovery that Robo3 is expressed in two isoforms, Robo3.1 and Robo3.2 arising from differential splicing of the pre-mRNA (Chen, Gore et al. 2008). Robo3.1 is translated in pre-crossing neurons and promotes midline crossing by suppressing Slit mediated signaling through Robo1 and Robo2 (Chen, Gore et al. 2008). After, crossing Robo3.1 is switched off and Robo3.2 is preferentially translated. Robo3.2 acts in cooperation with Robo1 and Robo2 to keep axons from re-crossing the midline (Chen, Gore et al. 2008). Interestingly, Robo3 mutations have been identified in human patients with horizontal gaze palsy with progressive scoliosis (HGPPS) (Jen, Chan et al. 2004). Mutations in human Robo3 lead
to defects in cortical and somatosensory axons that fail to cross the midline (Jen, Chan et al. 2004).

Experiments testing the structure and function of Robo family members by making chimeric receptors and genomic location swaps have led to some interesting conclusions about Robos and their role at the midline in *Drosophila* (Evans and Bashaw 2010; Spitzweck, Brankatschk et al. 2010). Genetic analysis in *Drosophila* has shown that Robo family members play distinct roles at the midline (Rajagopalan, Vivancos et al. 2000; Simpson, Kidd et al. 2000; Dickson and Gilestro 2006). Where Robo is the main gatekeeper to crossing the midline, Robo2 and Robo3 play a lesser role in keeping axons from crossing the midline (Rajagopalan, Nicolas et al. 2000; Rajagopalan, Vivancos et al. 2000). Robo2 and Robo3 are also expressed in different lateral domains that contribute to pathway selection once axons have crossed the midline (Rajagopalan, Nicolas et al. 2000; Rajagopalan, Vivancos et al. 2000; Simpson, Kidd et al. 2000).

By swapping Robo1, Robo2, and Robo3 into the respective genomic location of each gene, the Bashaw and Dickson labs addressed whether structural differences or differing expression patterns accounted for their specific roles in axon guidance (Evans and Bashaw 2010; Spitzweck, Brankatschk et al. 2010). They found that for midline crossing, both structure (specifically the cytoplasmic CC1 and CC2 domains) and expression of Robo1 are crucial for repulsive guidance decisions (Spitzweck, Brankatschk et al. 2010). Lateral pathway selection is determined mostly by expression
rather than unique structural differences among the Robos a.k.a. the “Robo code” (Rajagopalan, Vivancos et al. 2000; Simpson, Kidd et al. 2000; Dickson and Gilestro 2006). Additionally, Evans and Bashaw have shown in more detailed structure-function analyses that Ig1 and Ig3 of the Robo2 ectodomain are critical in determining lateral pathway selection in the absence of Robo and Robo3.

Another finding reported by both groups is that Robo2 plays a role in promoting axon guidance across the midline. Based on genetic dominant enhancement of the Netrin mutant phenotype Spitzweck et. al. found that Robo2 has a positive role in promoting commissural axons across the midline. Evans et. al. also arrived at the same conclusion, based on their experiments mis-expressing different levels of Robo2 in the CNS. This function was mapped by deletion analysis of different Ig domains in the Robo2 ectodomain. They found that deletion of Ig2 removed Robo2’s pro-midline crossing function; Deletion of other Ig domains in the presence of the Ig2 retained pro-midline crossing activity (Evans and Bashaw 2010).

In summary, Robo is critical for keeping axons from crossing the midline; its unique structure and expression pattern are key for its function. In addition, for Robo and its family members Robo2 and Robo3, expression is key to lateral pathway selection. Since Robo2 has a role in promoting guidance across the midline, one might speculate that this mechanism antagonizes Robo signaling in an analogous fashion to Robo3 in vertebrate midline crossing.
1.6.3. Commissureless: a regulator of Slit/Robo signaling

Comm is essential for axons to cross the midline in Drosophila and was identified in the same screen that identified Robo (Seeger, Tear et al. 1993). Embryos mutant for comm lack all commissural pathway formation, as axons fail to cross the midline (Seeger, Tear et al. 1993; Tear, Harris et al. 1996). When the comm mutant was first identified, it was proposed that a possible function for Comm was to attract commissurally-crossing axons to the midline either as a receptor or a signal (Seeger, Tear et al. 1993). However, after cloning Comm and analyzing comm; robo double mutants--which are indistinguishable from robo embryos--it was realized that Comm may in fact antagonize Robo repulsive signaling (Seeger, Tear et al. 1993; Tear, Harris et al. 1996).

Several other pieces of data fit in with this interpretation. First, Comm mis-expression in the CNS leads to a robo-like phenotype (Kidd, Russell et al. 1998). Second, where Comm levels are high, Robo levels are low, and vice versa (Kidd, Russell et al. 1998). Furthermore, in cell culture, the Robo receptor is localized to the cell surface when expressed alone. When Comm is co-expressed with Robo, Robo is re-localized into punctate vesicles (Georgiou and Tear 2002; Keleman, Rajagopalan et al. 2002; Choi 2003). Comm and Robo can also co-immunoprecipitate with one another, further suggesting that they exist in a complex together (Keleman, Rajagopalan et al. 2002; Choi 2003).
How does Comm regulate Robo? One key observation is that Comm is dynamically regulated in the embryo and specifically in the CNS. In those axons that do not cross the midline, Comm is not expressed, whereas Comm is expressed in those axons that do cross the midline (Keleman, Rajagopalan et al. 2002). Furthermore, Comm is dynamically expressed in commissurally-crossing axons in an off (pre-crossing), on (crossing), and off again (post-crossing axons) pattern (Keleman, Rajagopalan et al. 2002). Recently it has been shown that Frazzled plays a positive role in turning Comm expression on in commissurally-crossing axons (see section 1.6.5) (Yang, Garbe et al. 2009).

In summary, Comm plays an instructive role in midline guidance by regulating Robo so that axons can cross the midline. Several interesting questions that remain are: 1) how is the dynamic regulation of Comm controlled by Fra? 2) how is it turned off once axons have crossed the midline? 3) how is this regulation of Comm achieved? One simple and testable hypothesis is that the dynamic spatial and temporal regulation of Comm is at the transcriptional level.

1.6.4. The bifunctional guidance cue Netrins

Netrins are a family of axon guidance and cell migration cues conserved in vertebrates and invertebrates. Netrins are bi-functional cues that mediate both attractive and repulsive activities in a receptor-dependent context. Mutations in Netrin were first identified in a genetic screen for uncoordinated phenotypes in the invertebrate *C. elegans*
(Hedgecock, Culotti et al. 1990). The same study also proposed that Unc6/Netrin required Unc40/DCC/Fra to mediate chemoattraction and Unc5 to mediate chemorepulsion (Hedgecock, Culotti et al. 1990). It has been shown that this is the case in flies as well as vertebrates (Kolodziej, Timpe et al. 1996; Fazeli, Dickinson et al. 1997; Kolodziej 1997; Livesey and Hunt 1997; Culotti and Merz 1998; Keleman and Dickson 2001; Nishiyama, Hoshino et al. 2003).

When it comes to mediating attraction through its receptor, DCC/Fra Netrin signaling acts, in part, to regulate the cytoskeleton via Rho GTPases (Li, Meriane et al. 2002; Li, Saint-Cyr-Proulx et al. 2002; Gitai, Yu et al. 2003). Activation of these components leads to changes in growth cone morphology, an increase in cell surface area, and filopodial extensions—all are thought to lead to attraction toward the Netrin source (Li, Saint-Cyr-Proulx et al. 2002; Shekarabi and Kennedy 2002; Gitai, Yu et al. 2003). More recently, Netrins have been shown to mediate attraction at the midline through the receptor DSCAM (Andrews, Tanglao et al. 2008; Ly, Nikolaev et al. 2008; Liu, Li et al. 2009). Although less is known about the mechanisms downstream of DSCAM, Netrin-dependent DSCAM signaling may contribute to commissural axon pathfinding.

1.6.5. The guidance receptor Deleted in Colorectal Cancer (DCC), Frazzled (Fra)

DCC/Fra was originally identified as a potential tumor-suppressor gene (Fearon, Cho et al. 1990). However, it was later found that DCC encoded for a Netrin receptor in both vertebrates and invertebrates (Chan, Zheng et al. 1996; Keino-Masu, Masu et al. 1996;
Kolodziej, Timpe et al. 1996). Furthermore, detailed loss-of-function studies showed that DCC/Fra had no effect on the development of colorectal cancer. In fact, phenotypic analysis of DCC/Fra showed defects in 1) commissural axon guidance in mice mutant for DCC, 2) circumferential guidance in *C. elegans*, 3) commissural axon guidance in flies (Chan, Zheng et al. 1996; Kolodziej, Timpe et al. 1996; Fazeli, Dickinson et al. 1997).

DCC/Fra is a Netrin receptor (see 1.6.4. Netrins). In response to Netrin, signaling through DCC/Fra triggers a cascade of changes that in turn affect cytoskeleton rearrangement to promote outgrowth toward the Netrin source (de la Torre, Hopker et al. 1997; Culotti and Merz 1998; Li, Saint-Cyr-Proulx et al. 2002; Shekarabi and Kennedy 2002; Gitai, Yu et al. 2003; Meriane, Tcherkezian et al. 2004). Recent evidence suggests that DCC/Fra may regulate axon guidance and outgrowth through other mechanisms as well (Taniguchi, Kim et al. 2003; Parent, Barnes et al. 2005; Yang, Garbe et al. 2009). In vertebrates, DCC undergoes intramembranous proteolysis mediated by presenilin (PS1) (Taniguchi, Kim et al. 2003). In addition, a chimeric intra-cellular domain fused to GAL4 was able to translocate to the nucleus to activate a luciferase reporter in a PS1-dependent manner (Taniguchi, Kim et al. 2003). It has also been shown that Netrin/DCC can activate a transcriptional response through the mitogen-activated protein kinase (MAPK) and nuclear factor of activated T cells (NFAT) pathways (Forcet, Stein et al. 2002; Graef, Wang et al. 2003).
More evidence for DCC/Fra having a role in activating transcriptional pathways came from observations in *Drosophila* showing mis-expression of a version of Fra deleted for the cytoplasmic domain had a very robust Comm-like phenotype (Garbe, O'Donnell et al. 2007). This result is very striking since mis-expressing a Fra-cytoplasmic deletion was even more severe than loss-of-function mutations for *fra* in the CNS (Yang, Garbe et al. 2009). The result led them to test whether or not this was due to a reduction of Comm mRNA levels in commissural neurons (Yang, Garbe et al. 2009). This was in fact the case, as they showed a dramatic overall reduction of Comm mRNA. Furthermore, they were also able to demonstrate at single cell resolution that Comm mRNA accumulation was dramatically reduced in commissural neurons (Yang, Garbe et al. 2009). They demonstrated that Fra was sufficient to activate Comm mRNA expression by showing induction of Comm in a *fra* mutant background by expressing Fra in a subset of commissurally-crossing axons and by over-expressing Fra in ipsilaterally-projecting Ap neurons (Yang, Garbe et al. 2009).

Two significant observations can be made from this study. One is the surprising result that activation of Comm expression in subset of commissurally crossing eagle neurons seems to be Netrin-independent (Yang, Garbe et al. 2009). This conclusion is drawn from the fact that Comm expression is normal in a *Netrin*-deficient background and that a myristilated form of the cytoplasmic domain of Fra is able to activate Comm expression (Yang, Garbe et al. 2009). Secondly, this is the first *in vivo* observation linking a receptor-mediated transcriptional event in post-mitotic neurons to ensure proper guidance
at the midline (Yang, Garbe et al. 2009). How is this event regulated, and what signal
triggers Fra to activate Comm transcription in commissural neurons? Is it ligand-induced
(presumably a cue secreted from midline cells) or a timing event regulated by a cellular
clock as axons project toward the midline? The answers to these questions will reveal
new insight into transcriptional mechanisms in axon guidance.

1.7. Significance of this work

Many of the genes important for axon guidance have been identified. How these genes
control proper guidance decisions remains the current challenge. Already much progress
has been made from structure function analyses of many of the receptors and their
ligands.

In addition, analysis of the signaling molecules that regulate the cytoskeleton to provide
motility and direction towards their final target are being dissected to uncover their
mechanism of action. The details of how these genes are regulated and the
transcriptional networks that are involved in specifying their expression are slowly
yielding progress in understanding how the nervous system is wired during development.
All of these mechanisms give rise to the amazing processing power of the adult nervous
system. Understanding how these mechanisms function during development may lead to
a greater understanding of diseases in the nervous system and treatments for those
affected.
Chapter 2: Dissection of Commissureless cis-regulatory Regions

2.1.1. Introduction

The nervous system of bilaterally symmetric organisms can be divided into two different axon trajectories: ipsilateral and contralateral. Ipsilateral projecting axons make synaptic connections on the same side that they originate, while contralateral projecting axons make synaptic connections on the opposite side of the CNS midline. The binary choice for axons to cross the midline or not is regulated by opposing attractive and repulsive cues that are secreted by specialized midline cells in both vertebrates and invertebrates (Thomas 1998; Kaprielian, Imondi et al. 2000; Garbe and Bashaw 2004; Dickson and Gilestro 2006; Evans and Bashaw 2010). Why do some axons cross while others do not? Once axons have committed to crossing the midline, what signals keep them from re-crossing after encountering the same signals on the mirror image side of the midline? This simple binary decision makes the midline an ideal model to identify the genes and their function in regulating midline axon guidance.

2.1.2. Gate keepers at the midline: Comm and Robo

The genes commissureless (comm) and roundbout (robo) are key regulators of guidance at the midline and are cell autonomously required for axons to correctly navigate an ipsilateral vs. a contralateral projection (Seeger, Tear et al. 1993; Tear, Harris et al. 1996;
Keleman, Rajagopalan et al. 2002; Keleman, Ribeiro et al. 2005). To date much is known about the repulsive receptor Robo and it downstream effectors that keep axons from inappropriately crossing the midline (Ratnaparkhi, Banerjee et al. 2002; Fan, Labrador et al. 2003; Hsouna, Kim et al. 2003; Hu, Li et al. 2005). All axons in the CNS express Robo and loss-of-function studies reveal inappropriate midline crossing for both ipsilateral and commissural axons in the embryonic CNS. Conversely, Robo gain-of-function studies show that elevated levels of Robo are able to repel axons away from the midline (Kidd, Russell et al. 1998; Simpson, Kidd et al. 2000). How is Robo repulsion regulated if it is expressed in all axons of the CNS? The hypothesis that Comm regulates Robo comes from both genetic and cellular studies. First, Comm and Robo have opposing phenotypes in the CNS (Fig. 2.1), yet comm, robo double mutant embryos exhibit a robo phenotype, suggesting that in the absence of Robo, Comm is not necessary for axons to cross the midline. Furthermore, in embryos, a comm gain-of-function caused by pan-neural expression of Comm phenocopies the robo-mutant phenotype. All of these observations taken together suggest that Comm may regulate Robo in vivo.

What is the molecular mechanism of Robo regulation by Comm? In cell culture Comm is localized in punctate vesicles while Robo accumulates at the cell surface. When both Comm and Robo are both expressed in cells, Comm and Robo localize together in punctate vesicles (Keleman, Rajagopalan et al. 2002; Myat, Henry et al. 2002; Choi 2003). It has also been shown in vivo that Robo tracks along the axon to the cell surface of the growth cone. When Comm is expressed, Robo no longer tracks along the axon,
but rather is diverted into endosomes (Keleman, Rajagopalan et al. 2002; Keleman, Ribeiro et al. 2005). Comm and Robo co-immunoprecipitate from both embryos and cell culture extracts suggesting that they can exist in a complex with one another. The above-mentioned experiments suggest that Comm regulates Robo by regulating the level of Robo cell surface accumulation.

2.1.3. Transcriptional regulation of guidance molecules

Axons extending toward their final target must be able to ‘interpret’ their environment. For each individual axon to reach its final destination, it must express the proper repertoire of receptors at the appropriate time and place (Thor, Andersson et al. 1999; Yu and Bargmann 2001; Crowner, Madden et al. 2002; Labrador, O'Keefe et al. 2005; Polleux, Ince-Dunn et al. 2007; Colomb, Joly et al. 2008; Lee, Petros et al. 2008; Yang, Garbe et al. 2009). Over the past two decades, much work has been done elucidating the mechanisms of receptor mediated signaling in the growth cone, but less is know about the transcription factors and transcriptional networks important for the precise regulation and establishment of neuronal circuits. An important aspect of axon guidance that needs further study is how transcriptional factors specify and determine axon trajectories. More specifically, is the repertoire of receptor molecules provided to the neuron during neurogenesis, or are additional molecules activated as the axon moves toward its target navigating specific ‘choice-points’ before arriving at its final target (Butler and Tear 2007)?
Recently, the Netrin receptor, Frazzled (Fra), has been shown to cell autonomously activate Comm expression in a subset of commissural projecting neurons (EW neurons) (Yang, Garbe et al. 2009). Furthermore, when expressed in ipsilaterally projecting neurons (Ap neurons), Fra is sufficient to activate Comm transcription (Yang, Garbe et al. 2009). Activation of Comm by Fra requires the Fra cytoplasmic domain. This is shown by expression of a dominant negative form of Fra (Fra ΔC) that fails to activate Comm expression in EW neurons (Yang, Garbe et al. 2009). Interestingly, pan-neural expression of FraΔC has a strong comm-like phenotype (Garbe, O'Donnell et al. 2007).

In contrast, the fra null mutant phenotype is less severe, with approximately 30% of segments affected (Forsthoefel, Liebl et al. 2005). This result suggests that other signals are required to activate Comm expression in commissurally crossing axons. What are the other signals that activate Comm expression?

The ability of Fra to activate Comm is independent of midline-secreted Netrins, the ligand for Fra (Yang, Garbe et al. 2009). This result is somewhat surprising given the tight window of Comm expression in commissurally crossing axons. Since Fra does not require Netrin signaling to activate Comm, does another cue, such as Slit signal through Fra? If not Slit, what other midline signals could be responsible for activation of Comm?

In addition, what receptors, besides Fra, are required to activate Comm expression? For instance, does DSCAM or Robo2, which are both known to play a role in promoting guidance across the midline, play a role in activating Comm expression (Garbe, O'Donnell et al. 2007; Andrews, Tanglao et al. 2008; Spitzweck, Brankatschk et al. 2008).
2010)? Comm is dynamically expressed in commissural projecting axons in an off, on, and off again manner, and Comm is not expressed in ipsilateral projections (Fig. 2.2). Identifying the signals by which Fra activates Comm in its spatial and temporal pattern is of great importance to further our understanding of how axons differentiate between crossing and not crossing the midline.

The above example is the first reported receptor mediated signaling event coupled to a transcriptional response in ensuring proper axon guidance decisions at the midline. Another approach to understanding transcriptional regulation of axon guidance molecules is to identify DNA cis-regulatory regions that are necessary and sufficient for proper expression. Previous attempts to define the Comm cis-regulatory regions where done using P-element based transgene rescue of the comm phenotype (McGovern 2003). Two potential drawbacks to using this approach are size limits of genomic rescue constructs (~35kb) and random integration sites of P-elements with potential varying position effects. As mentioned before Comm is dynamically expressed in the CNS, but Comm is also expressed in a variety of other tissues throughout embryogenesis; including expression in somatic muscles. Interestingly comm mutants have a synaptogenesis phenotype in addition to the midline crossing defects (Wolf, Seeger et al. 1998). In comm mutant embryos roughly 67% of the SNb motor neurons fail to initiate proper synaptogenesis (McGovern 2003).
With the advent of BAC-recombineering technology and the application of site-specific 
\( \phi C31 \) mediated germline transformation previous size limitations associated with 
germline transformation are largely eliminated. In addition, the ability to analyze all of 
the transgene insertions at the same genomic location eliminates concerns about varying 
position effects.

Our approach to understanding how Comm expression is restricted to commissural axons 
is to dissect Comm cis-regulatory region using two techniques. The first approach is to 
use molecularly defined genomic deletions around Comm to identify regions required for 
proper Comm expression. The second approach is to revisit previous attempts by 
McGovern (2003) to define cis-regulatory regions with large epitope-tagged genomic 
transgenes to identify sequences that are necessary and sufficient for normal Comm 
expression. Our working hypothesis is that the dynamic regulation of Comm is at the 
transcriptional level. The results of these experiments have led us to a region 5’ of Comm 
that is important for proper regulation in the CNS.

2.2. Materials and Methods

2.2.1. Custom deletions in and around Comm using FRT bearing transposon stocks.

All stocks were kept at room temperature (~22° C) and raised on standard cornmeal, 
sucrose, yeast, and agar medium. Crosses were performed in humidified incubators at 
25° C on standard medium supplemented with yeast. Custom deletions were made by 
choosing two transposons containing FRT sites in the proper orientation for the desired
region to be deleted (Table 2.1 and Fig. 2.4). Crosses were carried out as described by (Parks, Cook et al. 2004; Thibault, Singer et al. 2004). Briefly, males were selected that harbor hs-FLP recombinase on the X and one of the FRT containing transposons on the 3rd chromosome. These males were then crossed to females with the second FRT containing transposon. After 3 days of egg laying, adults are removed and the progeny heat shocked for 1 hour at 37°C. This 1-hour heat shock was repeated daily for 4 days. Virgin females heterozygous for flanking FRT transposons were collected and crossed to males carrying appropriate balancer chromosomes. All deletion candidates were verified by PCR from single fly genomic DNA preps, (Gloor 1992) using primer sets as described in (Parks, Cook et al. 2004) (Table 2.1). Once a specific molecularly defined deletion was identified, individual balanced stocks were selected and maintained over marked balancers TM6B, actin-lacZ or TM3, actin-lacZ for further analysis.

2.2.2. Detection of Comm transcript accumulation

Comm transcript was detected by fluorescence in situ hybridization using either Digoxigenin-11-UTP-labeled or Biotin-16-UTP-labeled single-stranded RNA anti-sense probes. RNA was synthesized from either full-length cDNA or PCR generated template with either T7 or T3 RNA polymerase promoters. Primary antibodies used to detect haptens were sheep anti-DIG (Roche), mouse anti-biotin (Invitrogen) or streptavidin-HRP (Invitrogen). For fluorescence detection of primary antibodies, secondary antibodies conjugated to Alexa Flour 488, 555 or 647 were used. Confocal microscopy was done on a Leica TCS SL. Images were scanned at a resolution of 1024 x 1024
pixels. Protocols and strategy follow those developed by Dave Kosman of the Bier Lab (Kosman, Mizutani et al. 2004); http://www-biology.ucsd.edu/~davek/).

2.2.3. Embryo collection and immunohistochemistry

Adults used for collections were homozygous for the deletion when possible; otherwise, deletions or single insertion heterozygous embryos were identified by expression of beta-galactosidase driven by the actin promoter inserted on the balancing chromosome (e.g. TM6B). Zero to twenty-four hour embryo collections were made on apple juice agar plates supplemented with yeast paste. Antibody staining was carried out using standard procedures (Patel, Snow et al. 1987). mAb BP102 (used at 1:20) and mAb 1D4 (used at 1:10) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). mAb anti-β-galactosidase (Promega) was used at 1:500 to detect lacZ expression from balancer chromosomes. Horseradish-peroxidase conjugated or fluorescent secondary antibodies (Jackson or Molecular Probes) were used to detect primary antibodies.

2.2.4. Fly stocks and germline microinjection

Germline transformation was performed using standard protocols with minor modifications (Venken, He et al. 2006; Bischof, Maeda et al. 2007; Venken and Bellen 2007). Embryos for injection were collected from apple-juice agar plates supplemented with yeast paste. DNA used for injections was EtOH precipitated after midi prep DNA isolation (Invitrogen) and resuspended in injection buffer at 750 to 1000 ng/µl. DNA was
diluted to 500ng/µl in injection buffer for constructs larger than 50 kb in order to increase survivability after injection into embryos. A stock with both ΦC31 integrase and a second chromosome attP integration site was used for injection.

2.2.5. λ RED recombineering with galK positive/negative selection

The ~160kb BAC, 20JO4 containing Comm is from the RPCI-98 Drosophila melanogaster BAC Library BACPAC Resource Center (BPRC) at Children's Hospital Oakland Research Institute in Oakland, California (CHORI http://bacpac.chori.org/home.htm). All λ mediated recombination cloning was done in the bacterial strain SW102 (which is a derivative DY380) to allow for selection of positive clones using galK positive and negative counter selection using protocol #3 http://recombineering.ncifcrf.gov/; (Warming, Costantino et al. 2005). Briefly, primer sequences to amplify the 1.2kb galK ORF were designed with approximately 60 to 80 bp of homology to the desired sequence. After selection on minimal media plates containing only galactose as a carbon source and verification on MacConkey media to indicate fermentation of galactose by the galK gene, five candidate constructs were selected for molecular characterization. Restriction fingerprinting with BamHI and EcoRI was compared to control DNA to confirm that no non-specific rearrangements were generated. PCR confirmed the presence of the right sized galK cassette in the proper location by using primers specific to the endogenous flanking sequences. After verification of the galK insertion, one clone was chosen for replacement of the galK cassette with the desired epitope tag cassette. After choosing an appropriate tag,
approximately 60bp homology arms are designed into primers appropriate to amplify target DNA. Approximately 5 µl of 200 ng/µl of PCR product is electroporated in SW102 cells as before only this time cells are plated on minimal media DOG counter-selection plates. Only those that incorporate the new modification that efficiently remove galK will survive. Any bacteria that still encode galK in the plasmid will die off. At this point, care must be taken in selecting candidates. Although rare, in/dels can result in a false positive by removing or disrupting galK allowing the bacteria to grow without incorporation of the desired modification. Careful examination with restriction digests, PCR, and sequencing of the desired modification were all done to ensure the correct plasmid was obtained. To distinguish transgenic expression of Comm from endogenous Comm, constructs were tagged with either a 2x myc epitope at the C-terminus of Comm just before the stop codon, or the 1st exon was replaced with the open reading frame of GAL4 starting at the initiating methionine and ending with a stop codon approximately 30 bp from the end of the 1st exon. Comm2, which is approximately 23 kb 3’ of Comm, was tagged with a 3X HA epitope (the 119 kb construct is the only one that contains both Comm and Comm2). These modifications were made seamlessly into wild type sequences using galK counter selection (Warming, Costantino et al. 2005). Once the modifications were made, the plasmids were then ready to be cloned into the p[acman]attB transformation vector (Venken and Bellen 2005; Venken, He et al. 2006).
2.2.6. P[acman] constructs

Comm genomic rescue constructs were cloned from the appropriate epitope-tagged versions of the BAC 20J04 cm$^R$ into P[acman]attB amp$^R$ using $\lambda$ recombination mediated cloning in the bacterial strain SW102 (Warming, Costantino et al. 2005). The P[acman]attB vector is from the Bellen lab (Venken, He et al. 2006) and available at the Drosophila Genomics Resource Center DGRC (https://dgrc.cgb.indiana.edu/). Three constructs were made: 28 kb, 50 kb and 119 kb in size (Table 2.2 and Fig. 2.8). First, primers were made to amplify ~500bp fragments flanking the region to be cloned (Table 2.2). Primers have a unique BamHI site that will be used to join two PCR cloned pieces together into a hybrid element by PCR. Primers also have an Ascl or PacI sites at the corresponding ends to allow restriction digest and subsequent ligation into the P[acman] vector (Table 2.3). By PCR overlap extension, the two-homology arms are joined into one piece with the unique BamHI site at the center and unique flanking Ascl and PacI sites at the 5’ ends. The joined PCR product was gel purified to obtain the correct size band. Purified DNA was then digested with Pac I and Asc I for 2 hours at 37° C. After heat inactivation of the digest, 150 ng/µl was used in a ligation reaction with Asc I and Pac I digested P[acman] vector. Positive clones were tested with the appropriate primers (Table 2.2). BamH I and either Pac I or Asc I double digests were performed to confirm proper annealing of the two-homology arms. Gap repair and retrieval of modified Comm clones was done by restriction digest of P[acman] vectors with BamH I to linearize the vector and electroporated into SW102 cells with the modified BAC. Cells were allowed to recover for one hour at 32° C in 1ml of LB before being plated on LB amp plates to
select for recombinants. Plates were then incubated overnight at 32°C. Ten candidate colonies were selected and grown overnight and plasmid DNA was isolated from 5ml overnight cultures using an alkaline lysis mini-prep protocol. Candidates were confirmed by PCR for intact right and left homology arms and restriction digest with BamHI, and EcoRI to confirm appropriate sized clones. Correct clones were then electroporated into the EPI300 strain (Epicentre) and induced with 1000x copy control solution per Epicentre protocols. High copy number DNA was isolated with Invitrogen Purelink™ Hipure plasmid midi or maxi kit.

Results 2.3.

2.3.1. Deletions 5’ of Comm result in a mild Robo-like phenotype

Previous results suggest that Comm cis-regulatory regions may be quite large. Rescue experiments with a cosmid clone containing 31.5kb of genomic DNA (18.5kb 5’ and 5.5kb 3’ of Comm) failed to rescue the Comm CNS phenotype, however the Comm synaptogenesis phenotype was rescued in mutant embryos containing this genomic rescue construct. We reasoned that this transgene was capable of rescue, but lacked the necessary cis-regulatory regions for full and proper expression in the CNS (McGovern 2003). Another result that suggests Comm cis-regulatory regions may be extensive was the phenotype of two P-element insertions located ~23kb 3’ of Comm. Both of these insertions when homozygous have a strong Comm-like phenotype, suggesting that these insertions may be affecting Comm expression (Choi 2003). Complicating the interpretation of this result is the fact that both of these insertions are near the 5’ UTR of
Comm2 and are potentially disrupting Comm2 expression. Comm2 is a Comm-family member with significant homology in regions important for Robo regulation. Although Comm2 is expressed in the CNS and potentially playing a role in axon guidance at the midline, we questioned whether these Comm-like phenotypes were the consequence of disrupting Comm2 expression for several reasons. First, Comm2 had never been identified in any large-scale mutagenesis screens looking for CNS phenotypes. Second, RNAi used to knock down Comm2 had no affect on the CNS, while RNAi specific for Comm produced a dramatic CNS phenotype (Forsthoefel and Seeger personal communication). Third, expression of Comm was disrupted in these two insertion lines (Choi 2003).

To resolve these observations we made a recombinant chromosome containing the P-elements \(comm^{K40}\) and \(comm_2^{PE4}\) to conclusively test whether or not the 3' P-element insertion was having a cis-effect on Comm expression or that the CNS defects were due to disruption of Comm2. When in trans to each other (\(comm^{K40}, +/+\), \(comm_2^{PE4}\)), 29% of the segments are defective; however, when in cis to one another, (\(comm^{K40}, comm_2^{PE4}/+\), +), only 3% of segments display defects (Fig. 2.3). This result suggests that the phenotypes resulting from these P-element insertions 23 kb 3' of Comm are due to disruption of Comm expression rather than disruption of Comm2.

With the results of the above experiments in mind, we took an approach that would allow us to examine large genomic regions both 5' and 3' of Comm. By using FRT-containing
transposable elements, we were able to make a series of molecularly defined deletions that cover approximately 72kb 5’ and 35kb 3’ of Comm (Fig. 2.4).

Our initial characterization was to assess, *in vivo*, phenotypes of embryos homozygous for each of the deletions 5’ and 3’ of Comm. Del 7 served as a positive control for the creation of these deletions as it has an obvious *comm* phenotype when deleted (Table 2.4). Our predictions for deletions 3’ of Comm were based on P-element insertions PE4 and PK7. We showed that these insertions are having a *cis*-affect on Comm expression (Fig 2.3). By deleting regions 3’ of Comm, we expected to characterize the extent of positive regulatory regions necessary for Comm activation. However, our largest deletion 3’ of Comm, Del 21, has a wild-type phenotype when examined with BP102 and 1D4 (Table 2.4). Further analysis of deletions 3’ of Comm heterozygous to Del 14 (removes over 110kb of genomic DNA) indicated that these deletions also do not display a phenotype (Table 2.5). This result is the same as with single insertions from the Exelixis collection near the same genomic site as PE4 or PK7 (Table 2.6). These results are in apparent contradiction to our previous observations with P-elements PE4 and PK7. At this point in our analysis, it is impossible for us to resolve the differences in effect between our 3’ deletions and the P-element insertions 3’ of Comm.

Interestingly, deletions 5’ of Comm have a mild *Robo*-like CNS phenotype as revealed with monoclonal antibodies (mAb) BP102 and 1D4 (Table 2.4 and Fig. 2.5). Of the five deletions 5’ of Comm, the two with the most dramatic CNS phenotypes, deletions 10 and
11, are 10kb and 18.5kb 5’ of Comm respectively (Table 2.4 and Fig. 2.4). A deletion closer to the promoter 5’ of Comm, deletion 8 has no phenotype in the CNS. This result is somewhat surprising since deletion 8 starts just 125 bp 5’ of the Comm transcriptional start site and 365 bp 5’ from the single insertion P-element $comm^{d04506}$ that has a strong Comm phenotype (Table 2.5). Deletion 9, a smaller deletion that is the same distance from Comm as Deletion 10 is, also has a mild Robo-like phenotype in the CNS. Deletion 12, which is the farthest from Comm at just over 40kb 5’, does not have any affect in the CNS even though it has considerable overlap with deletions 10 and 11. Deletions 5’ of Comm are rather large, with deletion 8 being ~5kb and the rest being over 30kb. In order to try to narrow down a region that might be influencing Comm expression in the CNS, we also looked at single transposon insertions heterozygous over a large deletion that includes both Comm and Comm2. Two independent single insertions, F02802 and F07213, in the same region as deletion 10 and 11 showed inappropriate midline crossovers (Table 2.5), although at a reduced frequency when compared with the deletions. All of the other insertion lines displayed background levels of CNS defects (<1%). The broad phenotypic results of assaying these mutations with mAb BP102 and 1D4 are informative, but to get a clearer idea of what is going on at the transcriptional level we need to look specifically at Comm expression in ipsilateral and commissural projecting axons at single cell resolution.
2.3.2. Single cell analysis of Comm expression in eagle (Eg) and apterous (Ap) positive neurons

How are 5’ deletions affecting Comm transcription? For instance, in embryos with 5’ deletions, is Comm being inappropriately expressed in ipsilateral projecting axons? In addition, how is Comm expression being affected by 5’ deletions in commissural axons? To address these questions, I assayed Comm mRNA accumulation using fluorescently labeled in situ probes at single cell resolution in a subset of ipsilateral projecting Ap neurons and a subset of commissural projecting Eg positive neurons. Comm is not normally expressed in Ap neurons except for late stochastic expression (stage 16) after axon projections are nearly complete in the embryo. Therefore, I looked at stage 14, when Ap neurons have already encountered the midline and turned to make ipsilateral projections (Keleman, Rajagopalan et al. 2002). In stage 14 embryos homozygous for 5’ deletion 10, Ap positive neurons all inappropriately cross the midline. Comm accumulation is detected in approximately 55% of Ap neurons at this stage (Table 2.8). In contrast, Comm transcript is not detected at this stage in wild-type Ap neurons (Fig. 2.6). Inappropriate midline crossing of Ap neurons is strongly correlated with expression of Comm (Keleman, Ribeiro et al. 2005; Yang, Garbe et al. 2009). Since all Ap neurons cross the midline in a Del 10 background, we would expect to detect Comm accumulation in these neurons. It is possible that Comm expression was present at an earlier stage (i.e. stage 12-13) when axons are beginning to ‘explore’ the midline. Assaying earlier stages in embryogenesis should reveal whether this is the case or not for Ap neurons.
Comm accumulation was also assayed in the EW lineage, which is a group of 4 cells, 3 of which make commissural projections across the midline in the posterior commissure of each segment. In wild-type cells, axons from the EW lineage do not start crossing the midline until stage 13. Before stage 13, EW neurons do not express Comm, but once they start crossing, Comm is up regulated and after crossing is quickly extinguished (Keleman, Rajagopalan et al. 2002; Yang, Garbe et al. 2009). In a 5′ deletion background Comm accumulation is detected before stage 13 and is present at stage 16 when Comm expression is not normally seen (Fig. 2.7). In addition, unlike ipsilateral projecting Ap neurons, which have obvious altered axon trajectories in the 5′ deletion background, EW neurons have a wild-type trajectory. The unaltered EW projection is somewhat surprising given the fact that the BP102 phenotype of Del 10 embryos is very strong (97% of segments have a fused/fuzzy commissure phenotype). One possible explanation for the unaltered EW projection is that the EW neurons synapse immediately with their target once they cross the midline (Garbe and Bashaw 2007), this is in contrast to other neurons that select longitudinal pathways once they cross the midline, and without high levels of Robo, re-cross the midline.

2.3.3. P[acman] rescue constructs

Because our previous rescue experiments were unsuccessful in identifying the necessary cis-regulatory regions for proper CNS expression and given that P-element based transgenesis has an upper limit in transgene size (35 ~ 40kb), we wanted to revisit our rescue experiments with larger DNA constructs. By using φC31 integrase to mediate
site-specific recombination between a pre-engineered site within the genome and a donor molecule it has been shown that transgenes over 100kb can be introduced into the *Drosophila* genome (Venken, He et al. 2006). With this new technology, we would presumably be able to scan larger areas of DNA in hopes of identifying and further characterizing Comm cis-regulatory regions. Using this approach, we were able to create three transgenic lines of 119kb, 50kb, and 28kb in size.

All three constructs were epitope tagged in order to distinguish endogenous Comm expression from transgenic Comm expression. Two different approaches were used to distinguish transgenic Comm from endogenous Comm. The first was a 2x myc tag at the C-terminus of Comm, which was used to successfully transform animals with a 28kb myc tagged construct (see materials and methods 2.2.4.). The second approach was slightly different and was based on three factors: First, microinjection of large DNA constructs can reduce the viability of progeny. Second, we had several failed attempts at generating transgenic lines of the large 119kb myc-tagged version of Comm. Third, results from our deletion analysis indicated that 5’ regions may be critical for proper repression of Comm expression, so we reasoned that mis-regulation of Comm from transgenes could be reducing our ability to recover transgenic animals. To overcome this potential problem we engineered a Gal4 reporter into the 1st exon of Comm. If mis-expression from these transgenes were a problem, the benign Gal4 reporter would alleviate any toxic affect from inappropriate expression of Comm. Two Gal4 transgenic lines were recovered,
50kb and 119kb respectively. With these constructs, we proceeded to analyze their expression in vivo.

We used two approaches to assay for Comm expression from these constructs, detection with antibodies and the ability to rescue a comm mutant CNS phenotype. For the 28kb myc tagged transgene, we tried to detect expression of myc-tagged Comm with anti-myc mAb 9E10. Although we were unable to visually detect any expression, it is possible that low levels of Comm from the transgene are capable of rescue in the CNS. We tested this by placing the 28kb construct in a comm mutant background. Embryos homozygous mutant for, (comm\textsuperscript{E39}/comm\textsuperscript{7}) and either heterozygous or homozygous for the 28kb Comm construct failed to rescue any commissural axon formation when assayed using mAb BP102. To test expression from the two larger Gal4 constructs we crossed flies to stocks that contain UAS-LacZ and then used anti-beta-galactosidase to detect expression of the LacZ reporter. We were unable to detect the presence of LacZ in embryos. We also tried to rescue the comm mutant phenotype using the Gal4 constructs, but this time we used the UAS containing transposon insertion allele comm\textsuperscript{d04506}. The comm\textsuperscript{d04506} allele when combined with a pan-neural driver like 1407-Gal4 generates robust comm-gof phenotype, showing that in the presence of Gal4 the UAS containing allele comm\textsuperscript{d04506} efficiently produces functional Comm. By itself, this allele produces a comm null mutant phenotype. In the same comm mutant background, neither the 50kb nor 119kb-Comm-Gal4 constructs are able to rescue commissure formation.
2.3.4. Rescue of comm SNb motor neuron defects

Even though our rescue constructs fail to restore proper Comm expression in the CNS, the proper regulatory regions may be present for expression in muscle. To address this question we assayed segmental nerve root (SNb) motor neurons in comm mutant embryos. The SNb is a subset of the intersegmental neurons (ISN) made up of neuron RP3, RP5, and RP1 that project from the CNS to innervate muscle groups 6 and 7 (RP3), muscles 12/13 (RP5), and muscles 13/30 cleft (RP1) (Bate and Martinez Arias 1993). In previous studies, McGovern was able to demonstrate that even minimal rescue constructs, ~2 kb 5' and 2 kb 3', (11 kb overall in size) were able to rescue the Comm synaptogenesis phenotype (McGovern 2003). This result suggests that our 28 kb myc tagged construct should be able to restore proper Comm expression in muscle and rescue the comm mutant synaptogenesis phenotype. This experiment could serve as a positive control in two aspects. First, it would demonstrate that our transgene can encode functional Comm protein in vivo, and secondly, it could show that our attP-landing site is permissive for expression of our Comm transgene (at least in muscle).

In a comm mutant background 67% of the SNb motor neurons fail to form proper synapses compared to 7% in wild-type controls (Fig 2.9 and Table 2.10). Motor neurons were scored based on two criteria: stopping short or extending past the muscle target. If any of the SNb projections were found to be defective for either, the segment was scored as defective. The 28 kb myc-tagged construct does not rescue the comm synaptogenesis defect (Fig. 2.9 and Table 2.10). This result is consistent with the lack of detectable
expression with the epitope tagged Comm in this transgenic line including no detectable expression in embryonic somatic muscles or that the genomic location is not conducive for expression of the transgene.

One potential problem with the reporter constructs is that the Comm-Gal4 construct might not produce a functional protein product. As a positive control to test whether functional Gal4 could be produced from our transgene, we cloned Gal4 from the Comm-Gal4 exon-1 with approximately 100bp of endogenous Comm sequence on either side into a pMT vector with a metallothionein inducible promoter (Bunch, Grinblat et al. 1988). Once transfected into *Drosophila* S2 cells and induced with CuSO4, the Comm-Gal4 transgene is able to activate a UAS-GFP reporter construct (Fig. 2.10). Neither the 50kb nor the 119kb Comm-Gal4 constructs appear to be expressed in embryos or are able to rescue the *comm* CNS phenotype. We also tested whether our 28kb myc 2x construct was able to localize correctly in S2 cells and re-localize Robo from the cell surface efficiently (Fig. 2.10). This construct when expressed in S2 cells is functional for both localization and for its ability to re-localize Robo in cell culture (Fig. 2.10).

2.4. Discussion

The experiments outlined in this chapter are beginning to yield insights into the complex regulation of Comm transcription. Analysis of large regions with molecularly defined deletions revealed that regions 5’ of Comm are important for negatively regulating expression of Comm in Ap neurons and for precise temporal regulation of Comm in
commissural EW neurons. Deletions 3’ of Comm have no detectable affect on Comm expression and none of the deletions analyzed, either 5’ or 3’ have any detectable affect on positive expression of Comm. To complement our deletion analysis and to revisit previous genomic rescue experiments, several large genomic rescue constructs were made. These constructs were analyzed in vivo and in vitro cell culture assay. In contrast to previous results showing that Comm rescue constructs were capable of rescuing the Comm synaptogenesis phenotype, none of our transgenes expresses detectable levels of Comm or reporter gene in any of the in vivo assays tested. However, Comm and the reporter gene used in these experiments behaved as expected in vitro. Taken together these results suggest Comm regulatory regions are potentially quite large and complex. Further analysis is required to refine 5’ regulatory regions and to identify regions required for positive expression of Comm.

What is the molecular nature of deletions 5’ of Comm? Examples in the literature show that insulator/chromatin boundaries are important in regulating enhancer/promoter interaction (Drewell, Brenton et al. 2000; Majumder and Cai 2003; Akbari, Bousum et al. 2006; Akbari, Schiller et al. 2007). For example, the domain boundary element Fab-7 is critical for regulating Abd-B expression in the CNS of Drosophila (Mihaly, Hogga et al. 1998). Deletion of Fabl-7 in the Bithorax complex leads to a dominant gain-of-function phenotype in para-segment 11 (PS11) (Mihaly, Hogga et al. 1998). Antibody staining in Fab-7 mutants confirm inappropriate expression of Abd-B in PS11 (Mihaly, Hogga et al.)
1998). This example is similar to what we see with deletions 5’ of Comm where Comm is mis-expressed in ipsilateral Ap neurons (Fig 2.6).

It is also interesting to point out that even though there are complex regulatory regions around Fab-7 that have discreet and important regulatory functions these regulatory elements may also be somewhat redundant since the larger the deletion the stronger the gof phenotype (Mihaly, Hogga et al. 1998). This result suggests that chromosome pairing in this region maybe critical for proper function and expression of Abd-B. In comparison, deletions 5’ of Comm behave similarly. Deletion 10, the largest deletion, has the strongest phenotype in the CNS (Table 2.7 and Table 2.6). Deletion 11 has a less dramatic phenotype (Table 2.7 and Table 2.6). Deletion 9 and deletion 12 remove portions of the same region that are removed by deletions 10 and deletion 11 yet have no detectable phenotype in the CNS (Table 2.7 and Table 2.6). A higher resolution approach to define 5’ regions of Comm would be to clone smaller genomic fragments (2-3 kb) into reporter constructs; this would allow for this region to be assayed to define more discreet sequences required for regulation of Comm. This approach may be more difficult given that our data suggests repression of expression is a key mechanism for Comm regulation.

It is tempting to speculate that regions upstream of Comm represent an insulator/boundary element. However, two simple alternative hypotheses could be made from our deletions 5’ of Comm. The first Del 10 region is critical for a trans-acting
factor to bind a repressor element, which acts as an on/off switch for Comm. The second
is, this region is a boundary element that is critical in regulating proper
enhancer/promoter interaction between Comm and enhancer elements required for proper
expression. Distinguishing between these two mechanisms will be crucial to understating
Comm regulation in the CNS.

One possible approach is to search for candidate transcription factors expressed in the
CNS that have repressor activity. One intriguing candidate is the transcription factor
longitudinals lacking (Lola) (Seeger, Tear et al. 1993; Giniger, Tietje et al. 1994;
Crowner, Madden et al. 2002). *Lola* mutants have thin longitudinal connectives and
fused commissures similar to *robo* and *comm*-gof phenotypes. Although Lola has been
shown to regulate Slit expression and interact genetically with Robo, it is not clear that
Lola directly influences Robo transcription. One could argue, based on the
transcriptional repressor activity of Lola, that the *lola* mutant phenotype is due in part to
inappropriate expression of Comm. This hypothesis could be tested directly by looking
in Ap neurons of *lola* mutants for Comm accumulation. If *lola* does play a role in
suppressing Comm transcription, we would expect to see accumulation of Comm
transcript in Ap neurons.

An important piece of data we do not have thus far is expression from genomic rescue
constructs or deletions that remove positive regulatory elements for Comm. Data from
these sources are critical for a more complete understanding of the regulation of Comm
expression. Based on our deletion analysis and our large rescue/reporter constructs, our
data suggests one of two things: 1) Since none of our deletions disrupt the normal
activation of Comm, cis-regulatory regions required for positive expression of Comm are
very compact (i.e. intron) or 2) the necessary cis-regulatory regions lie outside of the
range of our deletions.

The latter interpretation seems more likely given all that we know to date. If cis-
regulatory elements are compact, then why do we not get proper expression from these
transgenes-the largest over a 100kb in size? One area of concern that we addressed is
whether our P[acman] constructs can express functioning protein. We have shown that
Gal4 from our constructs is capable of activating a UAS-GFP reporter in S2 cell cultures
(Fig. 2.10 and Table 2.11). Also, our Comm myc 2x construct was able to both localize
correctly and re-localize Robo from the cell surface (Fig. 2.11 and Table 2.12). These
controls further suggest that regions critical for positive regulation of Comm expression
are outside of the regions of our rescue constructs.

An alternative hypothesis is that chromatin and chromosome context is critical for Comm
regulation. This possibility is particularly intriguing given the differences in rescue of the
synaptogenesis phenotype between the P[acman] 28 kb construct and the 31 kb cosmid
construct used by McGovern (2003). It is possible that the particular requirements of
Comm expression are dependent on genomic location and its particular chromosomal
context (Yasuhara and Wakimoto 2006; Venken, Carlson et al. 2009). Venken et al has
reported rescue of a mutation in the heterochromatic gene light (lt) with a 92 kb genomic fragment inserted in euchromatin chromosomal DNA. This result is notable, since proper expression of heterochromatic genes has been reported to be dependent on their genomic context (Yasuhara and Wakimoto 2006). Interestingly, only 1 of 3 genomic constructs complemented lt lethality. All three were inserted into the same genomic location, and differed in the region around the lt gene with sizes of 92 kb, 92 kb and 78 kb (Venken, Carlson et al. 2009), suggesting DNA within the construct that rescues possesses essential cis-regulatory elements or sufficient genomic context that were absent in the other two clones.

Given the fact that there is little data in the literature from this particular genomic location and from our own data showing that our Comm myc tagged construct can function in S2 cell culture the latter scenario could be a possibility. Although ϕC31 mediated, transgenesis is a one-way recombination reaction that destroys the recognition sites, the P[acman]attB vector contains P-inverted repeats flanking the construct. This gives us another approach to further investigate our transgenes. For instance if after mobilizing our 28kb construct into a more ‘suitable’ genomic location we can assay the SNb motor neuron again in a comm background. If we get rescue we can then address whether or not our larger constructs would reveal any positive regulatory elements in a different genomic context.
One aspect of Comm regulation that currently is lacking is positive regulatory sequence information, necessary for activating Comm expression. As previously mentioned, the Bashaw lab has shown that Comm expression is switched on in commissural axons by the Netrin receptor Fra/DCC and is sufficient to activate Comm expression in ipsilateral projecting Ap neurons (Yang, Garbe et al. 2009). It may be possible to use Fra to activate Comm expression in S2 cells by transfecting Fra in with our P[acman] epitope-tagged version of Comm in order to dissect necessary cis-regulatory regions for Fra dependent activation of Comm (de la Torre, Hopker et al. 1997; Taniguchi, Kim et al. 2003; Yang, Garbe et al. 2009). With this approach, we may be able to scan larger areas of Comm regulatory regions necessary of activation of Comm. We could then use this information to address these sequences in the embryo via reporter constructs.

The dynamic expression of Comm is highly regulated and complex. With further experimentation we will be able to understand how Comm expression is regulated. This will not only contribute to our understanding of axon guidance at the midline choice point but also to the cis-regulatory logic of Comm regulation.
Figure 2.1. CNS phenotypes. The monoclonal antibody BP102 recognizes an unknown epitope on all of the axons in the CNS. Embryos are filleted dorsally and laid flat to reveal the ventral nerve cord (VNC). (A) Wild-type (wt) embryo reveals the ladder-like structure of the VNC. The longitudinal bundles are connected by repeated segments made up of an anterior and posterior commissure. Axons that make up the commissural bundles at the midline only cross once. Axons that project ipsilaterally don’t cross the midline. (B) Embryos homozygous mutant for *commissureless* lack all commissural bundles. (C) In embryos homozygous mutant for *roundabout* too many axons cross the midline. Axons that normally cross once, re-cross and axons that make ipsilateral projections inappropriately cross. This same phenotype is observed in Comm gain-of-function mutants were Comm is expressed in all neurons.
Figure 2.2. Model of Comm function at the CNS Midline. Ipsilateral projecting axons: High levels of Robo are on the cell surface of the growth cone in cells that do not express Comm. Contralateral projecting axons: Comm re-localizes Robo from the cell surface as axons are crossing the midline. Post crossing: Comm expression is extinguished in post-crossing axons allowing high levels of Robo to accumulate at the cell surface.
Figure 2.3. Cis/trans analysis of P-elements comm$^{K40}$ and comm$^{2PE4}$. The P-element PE4 is approximately 29 kb 3′ of the Comm transcription start site. The K40 insertion is in the 5′ UTR of Comm. Embryos homozygous for either K40 or PE4 display a Commissureless-like phenotype. (A) In trans to each other, these two mutations have a milder affect on CNS commissure formation as revealed by mAb BP102. Some commissures are thin or missing. In embryos of the genotype (comm$^{K40}$,+/+, comm$^{2PE4}$, 29% of segments are defective. (B) In comparison, a recombinant chromosome was made that harbored both mutations (comm$^{K40}$,comm$^{2PE4}$/+,+) in cis to each other 3.2% of segments are defective.
Figure 2.4. Molecularly defined deletions. Bracketed lines represent deletions. Triangles represent insertion sites of FRT-containing transposable elements. Orientation is indicated as + orientation (triangle down), and – orientations (triangle up). Map is to scale.
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<th>Distal transposons</th>
<th>Proximal transposons</th>
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<th>Element remaining after recombination</th>
<th>PCR product size</th>
<th>UAS site remaining after recombination</th>
<th>Su(Hw) Site remaining after recombination</th>
<th>Primer pairs used to detect deletion</th>
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Table 2.1. FRT containing stocks for molecularly-defined deletions. Columns left to right: Deletion name, Distal transposons relative to centromere, Proximal transposons relative to centromere. Eye color selection scheme for identification of candidate recombinants. Eye color is white when mini-white dominant eye marker is deleted after recombination between FRT sites. Some deletions leave the mini-white marker intact. Depending on orientation, recombination can create a new hybrid element or ‘re-create’ an intact element. Predicted size of hybrid element for PCR detection. Some combinations leave an intact UAS site. Su(Hw) sites remain after recombination that ‘re-creates’ an intact transposable element. Primer pairs used for detection (51A, 5F1, 3R2, and 5R2 are all unique to transposable elements). Primer named after the transposons are designated either 5’ or 3’ accordingly.
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<td>RA F primer BamHI CGCGCGGATCCGGCCGC</td>
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</tbody>
</table>

Table 2.2. Primers for gap repair mediated cloning from BAC20J04. Restriction sites are added to the 5’ corresponding left (LA) or right (RA) homology arm. After individual amplification, the LA and RA are annealed together in a PCR reaction and then cloned into the Pacman attB vector via the AscI and PacI sites. The Pacman attB vector is then linearized through restriction digest by BamHI. Electrocompetent cells are made out of the recombinegenic strain SW102 that harbors the target BAC (target BAC should have a different antibiotic selection marker to that of the Pacman vector). Approximately 250 ng of linear DNA is electroporated into 40 µl of cells. Following recovery for one hour at 32 °C in 1 ml of LB media, dilutions of 100 µl, 50 µl and 20 µl were plated onto LB plates containing ampicillin. Positive clones are molecularly characterized by PCR and restriction digest.
<table>
<thead>
<tr>
<th>COMM HOMOLOGY ARMS FOR ADDITION OF galK AND HA EPITOPE</th>
<th>COMM2 HOMOLOGY ARMS FOR ADDITION OF galK AND HA EPITOPE</th>
<th>COMM2 galK F END</th>
<th>COMM2 GAL4 (galK) (RFPI ACTS) EXON 1</th>
<th>COMM GAL4 (galK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.3. Primers for \textit{galK} and λ mediated recombineering to introduce seamless epitope and reporter constructs into BAC 20J04. Primers have between 60-80 bp of homology to target DNA. The 2X Myc tag and 3X HA tag oligo are synthesized 100 bp oligos (Invitrogen) with unique ends to amplify by PCR the flanking homology arms. The galK and Gal4 cassettes were PCR amplified from plasmid templates.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.4. BP102 and 1D4 phenotypes of embryos homozygous for deletions. Each segment is scored as a whole (both anterior and posterior commissure) Del 7 removes Comm and serves as a positive control given the unambiguous phenotype. Deletion 8, 9, 10, 11 and 12 are all 5’ of Comm. Deletion 21 is 3’ of Comm. See Fig 2.4 location and size of the deletion. BP102 phenotypes include thin/missing commissures (Del 7) or a fused/fuzzy commissure phenotype (Del 9, Del 10, Del 11). 1D4 phenotypes are scored as abnormal crossovers of 1D4+-nerouns across the CNS midline in late stage 16 and stage 17 embryos.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>BP102 Phenotype</th>
<th>1D4 Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Del 7/Del 7</td>
<td>Commisureless</td>
<td>N/A</td>
</tr>
<tr>
<td>Del 8/Del 8</td>
<td>0/121</td>
<td>0/99</td>
</tr>
<tr>
<td>Del 9/Del 9</td>
<td>8/99 8% defects per segment</td>
<td>4/132 3% defects per segment</td>
</tr>
<tr>
<td>Del 10/Del 10</td>
<td>118/121 97% defects per segment</td>
<td>25/121 20% defects per segment</td>
</tr>
<tr>
<td>Del 11/Del 11</td>
<td>80/121 66% defects per segment</td>
<td>26/99 26% defects per segment</td>
</tr>
<tr>
<td>Del 12/Del 12</td>
<td>0/99</td>
<td>0/88</td>
</tr>
<tr>
<td>Del 21/ Del 21</td>
<td>0/110</td>
<td>0/110</td>
</tr>
</tbody>
</table>
Table 2.5. Single transposon insertions heterozygous to Del 14. Top to bottom order of insertions is from distal to proximal the Comm2 and Comm genomic region. Insertions D06978, D08181 are inserted near the 5' UTR of Comm2. Insertion D04506 is in the 5' UTR of Comm. Insertion F07146 is 25bp from the Comm transcription start site. Insertion names starting with C, E or F are *piggyBac* based elements. Insertion names starting with D are P-element based (Parks, Cook et al. 2004; Thibault, Singer et al. 2004)
<table>
<thead>
<tr>
<th>Genotype</th>
<th>BP102 Phenotype</th>
<th>1D4 Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Del 1/Del 14</td>
<td>0/144</td>
<td>0/121</td>
</tr>
<tr>
<td>Del 2/Del 14</td>
<td>commissureless</td>
<td>N/A</td>
</tr>
<tr>
<td>Del 3/Del 14</td>
<td>0/110</td>
<td>0/110</td>
</tr>
<tr>
<td>Del 4/Del 14</td>
<td>0/132</td>
<td>0/110</td>
</tr>
<tr>
<td>Del 5/Del 14</td>
<td>0/135</td>
<td>0/121</td>
</tr>
<tr>
<td>Del 6/Del 14</td>
<td>0/121</td>
<td>0/110</td>
</tr>
<tr>
<td>Del 7/Del 14</td>
<td>commissureless</td>
<td>N/A</td>
</tr>
<tr>
<td>Del 8/Del 14</td>
<td>0/155</td>
<td>0/155</td>
</tr>
<tr>
<td>Del 9/Del 14</td>
<td>0/146</td>
<td>4/132 3% of segments</td>
</tr>
<tr>
<td>Del 10/Del 14</td>
<td>127/132 96% of segments</td>
<td>44/164 26% of segments</td>
</tr>
<tr>
<td>Del 11/Del 14</td>
<td>44/108 41% of segments</td>
<td>45/165 27% of segments</td>
</tr>
<tr>
<td>Del 12/Del 14</td>
<td>0/121</td>
<td>4/198-(Wavy fascicles)</td>
</tr>
<tr>
<td>Del 14/Del 14</td>
<td>commissureless</td>
<td>N/A</td>
</tr>
<tr>
<td>Del21/Del14</td>
<td>0/121</td>
<td>0/121</td>
</tr>
</tbody>
</table>

Table 2.6. Phenotypic analyses of deletions heterozygous to Del 14 (deletes ~116kb of genomic DNA). BP102 and 1D4 phenotypes are scored as a whole (both anterior and posterior commissure) of each segment. Deletions that remove Comm lack all commissure formation. Deletion 10 and Deletion 11 have a Robo-like phenotype. A fused/fuzzy commissure formation phenotype as visualized with the mAb BP102 and ectopic crossovers of FasII-positive fascicles as visualized with mAb 1D4. Deletions 3’ of Comm have no detectable phenotype.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>BP102 Phenotype</th>
<th>% defects per segment</th>
<th>1D4 Phenotype ectopic crossovers</th>
<th>% defects per segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Wt</td>
<td>0/114</td>
<td>0%</td>
<td>0/130</td>
<td>0%</td>
</tr>
<tr>
<td>B. Del 7/Del 10</td>
<td>31/110</td>
<td>28%</td>
<td>3/110</td>
<td>2.7%</td>
</tr>
<tr>
<td>C. Del 8/Del 10</td>
<td>1/120</td>
<td>&lt;1%</td>
<td>0/110</td>
<td>0%</td>
</tr>
<tr>
<td>D. Del 9/Del 10</td>
<td>0/121</td>
<td>0%</td>
<td>0/110</td>
<td>0%</td>
</tr>
<tr>
<td>E. Del 10/Del 10</td>
<td>118/121</td>
<td>97%</td>
<td>26/121</td>
<td>21%</td>
</tr>
<tr>
<td>F. Del 11/Del 10</td>
<td>24/121</td>
<td>18%</td>
<td>7/110</td>
<td>6.5%</td>
</tr>
<tr>
<td>G. Del 12/Del 10</td>
<td>0/121</td>
<td>0%</td>
<td>0/110</td>
<td>0%</td>
</tr>
<tr>
<td>H. Del 21/Del 10</td>
<td>0/110</td>
<td>0%</td>
<td>0/110</td>
<td>0%</td>
</tr>
<tr>
<td>+/-Del 10</td>
<td>0/110</td>
<td>0%</td>
<td>0/110</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 2.7. BP102 and 1D4 scored phenotypes of deletions heterozygous to 5' deletion 10. A. Wt BP102 and 1D4. Deletion 10 homozygous has the strongest phenotype with either BP102 or 1D4. Letter next to genotypes correspond to panels in (Fig. 2.5).

Figure 2.5. Deletions 5' of Comm have a mild Robo-like phenotype. Arrows highlight ectopic crossovers of 1D4 positive fascicles and fused/fuzzy commissures of BP102 positive axons. See Table 2.7 for scored phenotypes.
Figure 2.6. Ap neurons cross midline in a Comm 5’ deletion background. Stage 14 embryos. Ap neurons magenta. Comm *in situ* probe green. Embryos are dissected dorsally to allow the nerve cord to lay flat for imaging. Embryos are staged by head and gut involution before dissection and confirmed by extent of AP axonal projections. (A) Wt stage 14 embryos. (B) Stage 14 embryos homozygous for 5’ Del 10. All Ap positive axons cross the midline in the Del 10 mutant background.
<table>
<thead>
<tr>
<th>genotype</th>
<th>St. 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>0/35 hemi-segments + for Comm</td>
</tr>
<tr>
<td>Del 10/Del 10</td>
<td>24/43 hemi-segments + for Comm</td>
</tr>
</tbody>
</table>

Figure 2.7. Comm accumulation in EW neurons. Comm accumulation was assayed at three developmental time points. Stage 12-13 pre-crossing EW neurons. Stage 14-15 crossing EW neurons. Stage 16-17 post-crossing neurons. Magenta EW positive neurons. Green Comm \textit{in situ} probe. Top panel WT. Bottom panel homozygous for Del 10. (A,D) pre-crossing (B,E) crossing. (C,F) post-crossing. Black and white images to show projections. Colocalization finder plug-in (ImageJ software) turns pixels ‘white’ when there is significant overlap of two signals.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>St. 12-13 Pre-crossing</th>
<th>St. 14-15 Crossing</th>
<th>St. 16-17 Post crossing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>0/60 hemi-segments + for Comm</td>
<td>44/46 hemi-segments + for Comm</td>
<td>5/28 hemi-segments + for Comm</td>
</tr>
<tr>
<td>Del 10/Del 10</td>
<td>48/52 hemi-segments + for Comm</td>
<td>36/36 heme-segments + for Comm</td>
<td>12/32 hemi-segments + for Comm</td>
</tr>
</tbody>
</table>

Table 2.9. Hemi-segments scored for + expression of Comm. Single stack images processed with imageJ software. Plugin “Colocalization Finder” used to correlate cell body (magenta) with Comm transcript (green). Positive ‘white’ color used to score Ew neurons as + for Comm accumulation.
Figure 2.8. P[acman] rescue constructs. All of the P[acman]attB constructs where integrated into the 51C attP containing site on the second chromosome using φC31 mediated transgenesis. (A) Size of constructs and coverage of the Comm genomic region. (B) Tagged versions of Comm used to detect transgenic expression of Comm.
Figure 2.9. SNb motor neurons in Comm mutant embryos with and without Comm 28 kb 2x myc tagged genomic rescue construct. Stage 17 embryos. mAb 1D4 recognizes the SNb motor neuron projections. (A) Wild type, individual muscles are labeled with numbers 12, 13, 6, and 7. The RP3 neurons innervates between muscles 6/7. RP1,4 innervates muscles 13/30 cleft and the RP5 neuron innervates the 12/13 cleft. (B) (comm null embryos) SNb fails to initiate synaptogenesis in 67% of hemi-segments scored. (C) Rescue construct Comm 28 kb 2x myc construct fails to rescue the synaptogenesis phenotype of comm mutant embryos.
### Table 2.10

Quantification of SNb synaptogenesis defects. Each hemisegment is scored as a single unit. Defect in any of the three projections was scored as defective. Stop short refers to the axon extension not making it to the target field. Extend past indicates that the axon has passed the target field. The total # of defects is divided by the # of hemisegments scored to express as a percentage.

<table>
<thead>
<tr>
<th>Genotype</th>
<th># hemi-segments</th>
<th>Stop short</th>
<th>Extend past</th>
<th>Total</th>
<th>% defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>60</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>7%</td>
</tr>
<tr>
<td><em>comm</em>(^{Del17})</td>
<td>52</td>
<td>27</td>
<td>8</td>
<td>35</td>
<td>67%</td>
</tr>
<tr>
<td>28kb/28kb; <em>comm</em>(^{Del17}) /<em>comm</em>(^{E39})</td>
<td>28</td>
<td>20</td>
<td>3</td>
<td>23</td>
<td>82%</td>
</tr>
</tbody>
</table>
Figure 2.10. Controls demonstrating that GAL4 reporter rescue constructs can produce functional protein in S2 cell culture. Three representative fields are shown for each combination for transfected plasmids. (A) pMT-Comm-GAL4/pUAS-GFP. (B) Positive control Ubi-GAL4/pUAS-GFP. (C) Negative control pUAS-GFP.
Table 2.11. S2 Cells expressing GFP. S2 Cells expressing GFP when co-transfected with Comm-Gal4 and UAS-GFP. Positive control UBI-Gal4 transfected with UAS-GFP. pUAS-GFP alone does not express when transfected into S2 cells.
Figure 2.11. Comm 2x myc relocalizes Robo from cell surface into punctate vesicles in S2 cell culture. (A) Comm 2x Myc cloned from transgenic flies inserted into pMT vector and expressed in S2 cells (magenta). (B) Comm 2xMyc can re-localize Robo (green) from the cell surface. (C) Robo alone at the cell surface. (D) Comm HA tagged also re-localizes Robo from the cell surface and serves a positive control.
Chapter 3: The role of Comm2 in the CNS of Drosophila melanogaster

3.1. Introduction

Axon guidance decisions at the Drosophila CNS midline are an intensely studied model. Several large-scale mutagenesis screens have been performed looking for genes that affect the patterning of axon trajectories in the embryonic ventral nerve cord (Seeger, Tear et al. 1993; Hummel, Schimmelpfeng et al. 1999; Hummel, Schimmelpfeng et al. 1999a; McGovern, Pacak et al. 2003). Two key regulators of guidance decisions at the midline are Comm and Robo, which were both identified in the same screen (Seeger, Tear et al. 1993). Mutations in either gene result in dramatic axon guidance defects in the CNS. In comm mutant embryos, too few axons cross the midline, leading to a lack of commissure formation (Seeger, Tear et al. 1993). In robo mutant embryos, too many axons cross the midline, leading to thinner longitudinal connectives and fuzzy commissures (Seeger, Tear et al. 1993).

Robo is broadly conserved in vertebrates and invertebrates (Zallen, Yi et al. 1998; Brose, Bland et al. 1999; Challa 2003). Many organisms encode multiple Robo-family members: Robo2, Robo3, and Robo4 (Kidd, Brose et al. 1998; Zallen, Yi et al. 1998; Challa, Beattie et al. 2001). Robo family members have also been implicated in axon

Analysis of mutant phenotypes shows that *Drosophila* Robo family members play different roles in the CNS (Evans and Bashaw 2010; Spitzweck, Brankatschk et al. 2010). Robo plays the main role in keeping axons from crossing the midline. Robo2 and Robo3 play a role in specifying lateral pathway selection after midline crossing. Interestingly, Robo2 plays a positive role in promoting commissural midline crossing (Evans and Bashaw 2010; Spitzweck, Brankatschk et al. 2010). This result was very surprising, considering loss-of-function mutations have the opposite phenotype with inappropriate axons crossing the midline (Rajagopalan, Vivancos et al. 2000; Simpson, Kidd et al. 2000; Spitzweck, Brankatschk et al. 2010). Additionally, mutations in *robo2* enhance the *robo* lof phenotype, suggesting that Robo2 plays a role in repelling axons away from the midline (Simpson, Kidd et al. 2000).

Although Comm homologues are not as broadly conserved across species, there are two additional Comm family members (Comm2 and Comm3) in *Drosophila* (Choi 2003). In the CNS, Comm3 is expressed too late in embryogenesis to play a role in commissural pathfinding (Choi 2003). However, Comm2 is expressed at a time and pattern in the developing CNS such that it could be playing a role in midline guidance decisions. Although not with the same efficiency as Comm, Comm2 can re-localize each of the Robo family members from the cell surface into punctate vesicles in an S2 cell culture.
Comm2 can also complex with Comm in S2 cells as revealed by co-immuno-precipitation experiments (Choi 2003). This raises the possibility that Comm and Comm2 heteromeric complexes exist in vivo (Choi 2003).

Comm2 has a similar function to Comm in S2 cell assays; however, the in vivo role of Comm2 in the CNS has been difficult to identify. No EMS-induced alleles have been identified for Comm2 in any of the large-scale mutagenesis screens targeting the CNS. Two independent insertions in the 5’ UTR have a strong comm-like phenotype (Choi 2003); however, these two particular insertions have a cis-affect on Comm expression, which is located is approximately 23 kb upstream of Comm2 (see chapter 2). In addition, RNAi against Comm2 has no obvious affect on the CNS when assayed with the BP02, while Comm has a robust RNAi-induced BP102 phenotype (David Forsthoefel personal communication).

What might the role of Comm2 be if any in the CNS? To further investigate the role of Comm2, we addressed several aspects. Comparison of Comm and Comm2 mRNA accumulation suggest that both genes are expressed in similar patterns. To investigate the extent to which their pattern of expression overlap and differ during embryogenesis I used specific fluorescently labeled in situ probes. Next, the CNS was assayed for mutant phenotypes, using mAbs 1D4 and BP102, in a molecularly defined deletion background of comm2. To complement the loss-of-function analysis, comm2 was mis-expressed using the pan-neural driver 1407-GAL4. Furthermore, I characterized the effect of
comm, comm2, and comm, comm2 double mutants that dominantly enhance the Netrin deficiency phenotype.

3.2. Materials and Methods

3.2.1. Exelixis deletions of Comm2

All stocks were kept at room temperature (~22°C) and raised on standard cornmeal, sucrose, yeast, and agar medium. Crosses were performed in humidified incubators at 25°C on standard medium supplemented with yeast. Custom deletions were made by choosing two transposons containing FRT sites in the proper orientation for the desired region to be deleted (Table 3.1 and Fig. 3.1). Crosses were carried out as described by (Parks, Cook et al. 2004; Thibault, Singer et al. 2004). Briefly, males were selected that harbor hs-FLP recombinase on the X and one of the FRT-containing transposons on the 3rd chromosome. These males were then crossed to females with the second FRT-containing transposon. After 3 days of egg laying, adults were removed and the progeny heat shocked for 1 hour at 37°C. This 1-hour heat shock was repeated daily for 4 days. Virgin females heterozygous for flanking FRT transposons were collected and crossed to males carrying appropriate balancer chromosomes. All candidates were verified by PCR from single-fly genomic DNA preps, (Gloor 1992) using primer sets as described in (Parks, Cook et al. 2004) (Table 3.1) Once a specific molecularly-defined deletion was identified, individual balanced stocks were selected and maintained over marked balancers TM6B, actin-lacZ or TM3, actin-lacZ for further analysis.
3.2.2. Embryo collection and immunohistochemistry

Adults used for collections where homozygous for the deletion when possible; otherwise, deletions or single insertions heterozygous embryos were identified by expression of LacZ driven by the actin promoter inserted on the balancing chromosome (e.g. TM6B). 24 hour collections were made on apple juice agar plates supplemented with yeast paste. Antibody staining was carried out using standard procedures (Patel, Snow et al. 1987).

mAb BP102 (used at 1:20) and mAb 1D4 (used at 1:10) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). mAb anti-β-galactosidase (Promega) was used at 1:500 to detect lacZ expression from balancer chromosomes.  Horseradish-peroxidase conjugated or fluorescent secondary antibodies (Jackson or Molecular Probes) were used to detect primary antibodies.

3.2.3. Detection of Comm2 transcript accumulation

Comm2 transcript was detected by fluorescence in situ either by hybridizing Digoxigenin-11-UTP-labeled or Biotin-16-UTP-labeled single-stranded RNA anti-sense probes. RNA was synthesized from PCR generated template using the T7 polymerase promoter. Primers used to create DNA template: Comm2 T7 promoter

CTAATACGACTCACTATAGGGAGATGTCGGCAGAACATTTACGCTAACACA and Comm2 T7 R 1.9kb TCATTACGCGGTGCGCAG. Primary antibodies used to detect haptens were sheep anti-DIG (Roche) (1:400), mouse anti-biotin (Invitrogen) (1:200) or streptavidin-HRP (Invitrogen) (1:200). For fluorescence detection of primary antibodies, secondary antibodies conjugated to Alexa Flour 488, 555 or 647 were used. Confocal
microscopy was done on a Leica TCS SL. Images were scanned at a resolution of 1024 x 1024 pixels. Protocols were followed with a few minor exceptions as developed by Dave Kosman of the Bier Lab (Kosman, Mizutani et al. 2004) http://www-biology.ucsd.edu/~davek/.

3.3 Results

3.3.1. Comparison of Comm2 to Comm accumulation in embryogenesis

Previous in situ hybridization results show that Comm2 mRNA accumulation is similar to that of Comm mRNA accumulation (Choi 2003). Throughout embryogenesis, Comm and Comm2 are dynamically expressed (Fig. 3.2). Comparison of Comm and Comm2 mRNA accumulation shows similar patterns of expression as determined by in situ hybridization with antisense riboprobes (Fig. 3.2). However, the stage specific extent of overlap is difficult to determine in side-by-side comparisons between Comm and Comm2 mRNA accumulation. A direct approach is to assay each transcript in the same embryo using fluorescently labeled riboprobes and confocal microscopy.

To ensure no cross hybridization occurs between Comm riboprobes and Comm2 transcript and vice versa, control hybridizations were performed for each riboprobe. Any cross hybridization would be unexpected since the overall amino-acid identity is 20% between Comm and Comm2. Comm riboprobe was hybridized to embryos in a comm null background and in a comm2 null background. As expected Comm riboprobe hybridized to heterozygous embryos for comm Del 7 over a marked balancer and
embryos homozygous for *comm2* Del 1 (Fig. 3.3 and Table 3.2). Comm riboprobe did not hybridize to transcript in embryos homozygous for *comm* Del 7. The reciprocal experiments were done with Comm2 riboprobe. Comm2 riboprobe hybridized to heterozygous embryos for *comm2* Del 1 and embryos homozygous for *comm* Del 7. Comm2 riboprobe does not hybridize to embryos in a *comm2* Del 1 homozygous background (Fig 3.3 and Table 3.2).

To quantify the *in situ* hybridization result, stage 12-15 embryos were scored for the presences or absence of hybridized probe. To determine heterozygotes from homozygous null embryos for either *comm* or *comm2*, embryos were scored for the presences or absence of beta-galactosidase (Table 3.2). Additionally, mAb BP102 was used to help stage and unambiguously identify *comm* null embryos. In experiments with Comm riboprobe 68% of embryos scored positive for the presence of beta-galactosidase and positive for hybridization of Comm riboprobe. The remaining 32% of embryos were negative for beta-galactosidase and presence of hybridized probe (Table 3.2). *In situ* hybridizations using a Comm riboprobe in a *comm2* null background, all of the embryos hybridized with the Comm riboprobe.

In control experiments using the Comm2 riboprobe, 72% of the embryos were positive for the presence of hybridized probe and for beta-galactosidase. The remaining 28% of embryos negative for the presence of hybridized riboprobe and negative for beta-
galactosidase. In summary, these experiments demonstrate the specificity of these probes with no detection of cross hybridization to the other transcript.

The overlapping pattern for Comm and Comm2 mRNA accumulation is revealed by co-localization of signals in embryos labeled for both transcripts (Fig. 3.4). In stage 4-6 embryos, both Comm and Comm2 are detected in seven overlapping segmental stripes. As embryogenesis continues, the stripes double with Comm and Comm2 mRNA accumulation continuing to overlap in a segmentally repeated pattern, as seen in stage 7-8, and 9-10 embryos (Fig. 3.4). By stage 11 the repeated segmental stripes have become more continuous while the ventral most portions of expression fades in a manner so that now Comm and Comm2 are expressed in stripes lateral to the midline. At stage 11 there are some differences in mRNA accumulation between Comm and Comm2 as shown by arrows (Fig. 3.2 panel D).

In later stages of development, specifically in the CNS (stage 12-13) when axons are beginning to send out projections, Comm and Comm2 mRNA are detected in midline glia and in neurons (Fig. 3.4). At this stage Comm mRNA accumulation is robust compared to Comm2 mRNA accumulation (Fig. 3.4 arrows panel E). This difference in level between Comm and Comm2 continues when the majority of neurons in the CNS are crossing the midline around stage 14 (Fig. 3.4 arrows panel H). Additionally, during stages 12-15, Comm and Comm2 are expressed in developing muscle (Fig. 3.4 panel F,G and H). In later stages of embryogenesis after the majority of axons have made their
projections in the CNS (late stage 15-16 embryos) Comm and Comm2 mRNA accumulation is dramatically reduced, except for low levels detected in midline glia (Fig. 3.4 panel I). Comm and Comm2 have similar patterns of expression as revealed by extensive co-localization mRNA accumulation. There are some differences between the two. Overall, there is a broad low level of Comm2 mRNA detected throughout embryos (Fig 3.2). In comparison, Comm mRNA accumulation patterns are robust and exhibit greater sharpness in the CNS than Comm2 (Fig. 3.4).

3.3.2. BP102 and 1D4 CNS phenotypes of Comm2: loss of function and mis-expression

In the CNS, Comm regulates Robo to allow axons to cross the midline. Since Comm2, like Comm, can re-localize Robo in cell culture (Choi 2003), we addressed whether Comm2 is necessary in the CNS for midline crossing by assaying commissural projections in comm2 mutant embryos with mAb BP102 and ipsilateral pathway formation with mAb 1D4. Comm2 is expressed early enough in embryogenesis, when axons are sending out their projections, to play a role in axon guidance decisions at the midline (Fig 3.4).

Because of previous results with two P-element insertions just upstream of the Comm2 transcription start site (PE4 and PK7) that affect Comm expression, (Choi 2003, Fig 2.3) we made a precise Comm2 deletion using FRT containing transposable elements (Table 3.1 and Fig 3.1). Analysis of this defined deletion indicates that deletion of Comm2 does
not result in any significant commissural or ipsilateral axon projection defects in the CNS, as assayed with either BP102 or 1D4 (Fig 3.5 and Table 3.3).

To further characterize Comm2 *in vivo*, we used two P-element insertions (D08181 and D06978) near the 5’ UTR of Comm2. Both of these elements have fourteen copies of the UAS sequence (14x UAS) in the correct orientation to drive Comm2 expression in the CNS. In addition, we compared pUASTattB Comm HA1x and Comm2 HA1x constructs, each inserted by site-specific recombination into the same attP genomic location (Table 3.4) (Bischof, Maeda et al. 2007). We assayed gain-of-function phenotypes by ipsilaterally projecting axons for ectopic midline crossovers with mAb 1D4. By mis-expressing Comm2, using the pan-neural driver 1407-Gal4, we observed a mild phenotype with few axons inappropriately crossing the midline (Fig. 3.6 and Table 3.4) this was seen with D08181, D06978 and pUASTattB Comm2-HA line. In comparison, mis-expressing Comm with 1407-Gal4 has a robust Robo-like phenotype where every segment is affected (Fig 3.6 and Table 3.4).

3.3.3. Dominant enhancement of the Netrin Deficiency phenotype by Comm2

In a screen looking for dominant enhancers of the Netrin deficiency phenotype (Forsthoefel and Seeger, personal communication), two large chromosomal deficiencies (*Df(3)fz-M21* and *Df(3)XG5*) on the 3rd chromosome were identified that dramatically enhance the Netrin phenotype (Fig. 3.7 and Table 3.5). Interestingly, these deficiencies remove both Comm and Comm2, in addition to many other genes. Analysis using *comm*
alleles shows that Comm dominantly enhances the \textit{Netrin} deletion phenotype in the CNS, but not to the extent of \textit{Df(3)fz-M21} and \textit{Df(3)XG5}. Since Comm2 is so close to Comm and removed by the same two deficiencies, we reasoned that the more dramatic disruption of the CNS could be due to the additional deletion of Comm2 or by other genes removed by the deficiencies. To test this hypothesis we used several Comm2 alleles (both transposable element insertions and molecularly deletions). Mutations in Comm2 also dominantly enhance the \textit{Netrin} deficiency phenotype, comparable to the enhancement by mutant \textit{comm} alleles (Table 3.5). All of the \textit{comm2} alleles tested resulted in enhancement of CNS commissure defects (Table 3.5). In addition, a recombinant chromosome with insertions in both Comm and Comm2 enhances the \textit{Netrin} deficiency to the same degree as the two large deficiencies (\textit{Df(3)fz-M21} and \textit{Df(3)XG5}).

We further tested whether the strong enhancement of the \textit{Netrin} deficiency phenotype was due to removal of both Comm and Comm2 by using several molecular defined deletions (Fig. 3.7 and Table 3.5). Del 14, which deletes only Comm and Comm2, recapitulates the strong enhancement seen in the larger deficiencies and the \textit{comm^{2PE4},comm^{K40}} double mutant recombinant chromosome. In total, these results demonstrate that Comm2 does play a role in promoting axon guidance across the midline.

3.4. Discussion

Of all the mutant phenotypes revealed thus far in the \textit{Drosophila} CNS, Comm and Robo are the most dramatic. Nevertheless, what is the role of other genes that contribute to axon guidance decisions at the midline? We have addressed Comm2 mRNA
accumulation throughout embryogenesis and have used Comm as a reference to gauge their overlap. Analyses with mAb BP102 and mAb 1D4 reveal no discernable CNS phenotype in \textit{comm2} mutant embryos. However, mis-expression with a pan-neural driver has a mild affect on CNS development with some axons ectopically crossing the midline. Interestingly, mutations in Comm2 have a dramatic dominant enhancement of the \textit{Netrin} mutant phenotype, suggesting that Comm2 positively contributes to midline crossing. Uncovering, the exact role of Comm2 in the CNS will yield new insights into axon guidance.

Based on our \textit{in situ} data, Comm2 is expressed at the right time and place to be playing a role in midline guidance. Comm2 expression is correlated with Comm throughout embryogenesis (Fig 3.2 and 3.4). Their close proximity in the genome and tight regulation of expression suggest that Comm and Comm2 promoters can respond to similar or possibly even the same enhancer(s). A more thorough analysis of Comm2 expression would include fluorescent detection of accumulation at single cell resolution in ipsilateral and contralateral projecting axons at multiple stages during CNS development. Is Comm2 expressed in commissurally crossing axons in the CNS in an off, on and off again fashion similar to Comm? Knowing the precise accumulation of Comm2 will aid in interpreting CNS phenotypes in the context of mutant backgrounds such as the \textit{Netrin} deficiency.
On a more speculative note, an interesting question to address that may help us understand more about the cis-regulatory mechanisms in this region would be to determine how Comm2 is regulated. For instance, Comm2 regulatory regions may be more compact than Comm and BAC transgenes might therefore recapitulate Comm2 expression allowing for detailed dissection of Comm2 regulatory regions. This would not only be a useful in understanding regulation of Comm2 and it role in midline crossing, but also may be a useful tool in identifying a positive regulatory region(s) for Comm.

From our analysis using mAbs BP102 and 1D4 as markers, comm2 mutations have no effect on embryonic CNS development. Mis-expressing Comm2 throughout the CNS has a mild 1D4 phenotype (Table 3.4). Two UAS P-elements near Comm2 (100 bp and 200 bp from the transcription start site) show some activity (3-5% segments affected, Table 3.4), but this result is difficult to interpret on its own given that the same UAS P-element inserted into the Comm 5’ UTR has a very robust robo-like phenotype (Fig 3.6). The more compelling mis-expression data comes from the comparison of 1D4 phenotypes of pUASTattB-Comm2 and pUASTattB-Comm constructs, driven by the pan-neural 1407-Gal4. Both are being expressed by the same Gal4 driver line and from the same genomic location (Table 3.4). By comparing these two constructs, we see that the activity of Comm2 is mild with approximately 7% of the segments showing ectopic crossovers (Table 3.4), while Comm, under the same conditions affects 75% of the segments (Table 3.4).
Clearly the most convincing evidence to date that Comm2 is playing a role in the CNS is the dominant enhancement of the Netrin deficiency phenotype. Interestingly, comm2 is not dominantly enhanced by heterozygosity of comm (Fig 3.5 and Table 3.3). So, by what mechanism are comm and comm2 enhancing the Netrin deficiency? Both Comm and Comm2 enhance the Netrin deficiency to a similar degree (Table 3.3). Yet, homozygous mutations in comm2 do not have a BP102 phenotype, while homozygous mutations in comm produce dramatic commissure loss phenotypes. Is the enhancement of the Netrin deficiency phenotype due to a loss in the balance of attractive vs. repulsive guidance forces? For instance, loss of one copy of Comm enhances the Netrin deficiency background by an increase of repulsive Robo signaling. If this hypothesis is true, what does it suggest about Comm2? In this case, if Comm2 is required to antagonize Robo signaling, then why does it not have a BP102 phenotype? We know that Comm2 can re-localize Robo in S2 cell culture (Choi 2003). Additionally, Comm2 can also co-immunoprecipitate with Comm (Choi 2003). Does a ‘helper’ role for Comm2 make sense in light of what we know? This interpretation would fit nicely given our current data. Comm is the main regulator of repulsive signaling allowing axons to cross the midline and Comm2 plays a role by promoting Comm function, perhaps by either binding to Robo or Comm.

Other hypotheses could be proposed as well. Recently, Robo2 has been shown to play a role in promoting guidance across the midline (Spitzweck, Brankatschk et al. 2010). Interestingly, Robo2 has a mild Robo-like loss of function. However, the authors
uncovered a similar dominant enhancement of the Netrin deficiency phenotype similar to what we describe for Comm and Comm2. Could Robo2 and Comm2 be interacting in some specific ways? We know from S2 cell culture assays that Comm2 shows activity in re-localizing Robo2 into punctate vesicles (Choi 2003). Without further data, it is more difficult to propose a specific mechanism of how Robo2 and Comm2 function together to promote midline crossing. The next step will be to look for genetic interactions between these two genes.

Are there other contexts in which Comm2 functions? In addition to its phenotype in the CNS, Comm mutants have a synaptogenesis phenotype (Wolf, Seeger et al. 1998). Comm2, like Comm, is also expressed in muscle (Fig 3.4). A relevant hypothesis to test is whether Comm2, like Comm, has a role in synaptogenesis. If it does, this may give us an opportunity to test other aspects of Comm2 function.

The function of Comm2 is an intriguing question for several reasons. One, it could give us a better understanding of the complex cis-regulatory mechanisms in this region. Two, how Comm2 is functioning in the CNS will lead to a greater understanding of midline guidance. Finally, Comm2 may have functions outside the CNS, perhaps a role in synaptogenesis. Overall, many questions remain to be answered to further our understanding of Comm2 function in the CNS and elsewhere during Drosophila development.
Table 3.1. FRT-containing stocks used to create molecularly defined *comm2* deletions. Columns right to left. Deletion name. Distal transposon relative to the centromere. Proximal transposons relative to the centromere. Eye color selection scheme for some combinations of transposable elements. Eye color is white when the mini-white dominant marker is deleted after recombination between FRT sites. Some deletions leave the mini-white marker intact. Depending on orientation, recombination can create a new hybrid element or ‘re-create’ an intact element. Predicted size of hybrid elements for PCR detections scheme indicated. Some combinations leave a remaining UAS site. Su(Hw) sites remain after recombination that ‘re-creates’ an intact transposable element. Primer pairs used in detection of desired deletion (51A, 5F1, 3R2, and 5R2 are all unique to transposable elements). Primer names are designated either 5’ or 3’ accordingly.
Figure 3.1. Comm2 deletions. To scale map of genomic location of each deletion that removes Comm2 or Comm. For individual FRT containing insertions names see Table 3.1. Deletions, 1, 16, and 21 each remove Comm2. Deletion 14 and 2 remove both Comm and Comm2. Deletions 3, 4, 5, 6 and 17 remove portions of the intergenic region between Comm and Comm2. Deletion 7 removes Comm.
Figure 3.2. Comparison of Comm and Comm2 mRNA accumulation throughout embryogenesis. Left column Comm probe. Right column Comm2 probe. (A, E) Stage 4-6 embryos. Comm and Comm2 accumulation is detected in 7 stripes. (B, F) Stage 7-8 embryos. Comm and Comm2 stripes double to 14 stripes from 7 stripes in earlier stages. (C, G) Stage 9-10 embryos. Segmental stripes continue before fading at the end of stage 10, beginning stage 11. (D, H) Stage 12-13 embryos. Comm and Comm2 accumulation is detected in developing muscle and in the CNS (midline glia and neuronal accumulation not see in lateral view). At this stage in the CNS neurons have differentiated and just beginning to send out their axonal projections.
Figure 3.3. Riboprobe control for Comm and Comm2 specificity. Left column Comm riboprobe. Right column Comm2 riboprobe. Stage 12-14 embryos were staged and scored (Table 3.2) (A, E) Wt embryos (B, F) LacZ positive embryos identify heterozygotes (C, G) Del 7 (Comm null) and Del 1 (Comm2 null) respectively (D, H) Comm probe in \textit{comm2} null background. Comm2 probe in \textit{comm} null background. Comm and Comm2 riboprobes show strong specificity to their respective transcripts.
% embryos expressing

<table>
<thead>
<tr>
<th>Genotype (n)</th>
<th>Comm mRNA + LacZ</th>
<th>Neither</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comm Del 7 (128)</td>
<td>(0) BP102 + commissureless -LacZ</td>
<td>(87) 68%</td>
</tr>
<tr>
<td>Comm2 Del 1 (57)</td>
<td>(57) 100%</td>
<td>(57) 100%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comm2 mRNA + LacZ</th>
<th>Neither</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comm2 Del 1 (152)</td>
<td>(0) BP102 + -LacZ</td>
</tr>
<tr>
<td>Comm Del 7 (60)</td>
<td>(60) 100%</td>
</tr>
</tbody>
</table>

Table 3.2. in situ hybridization controls for Comm and Comm2 probes. To ensure specificity of probes to each transcript null alleles for either Comm (Del 7) or Comm2 (Del 1) were used over marked balancer expressing LacZ. Embryos were also labeled with mAb BP102 to reveal CNS phenotype and unambiguously identify comm null embryos. Embryonic stages 12-15 were scored based on BP102 phenotype, head and gut morphology.
Figure 3.4. Comm and Comm2 accumulation through embryogenesis. Comm (green); Comm2 (magenta); Merge (white). Embryos are hybridized with a Comm biotin-labeled anti-sense probe and a Comm2 digoxegenin-labeled anti-sense probe. (A) Stage 4-6 (lateral view): Comm and Comm2 accumulation is detected in seven segmentally repeated stripes. (B) Stage 7-8 (ventral view): both transcripts are detected in 14 segmentally repeated ectodermal stripes. (C) Stage 9-10 (lateral view): stripes continue through the end of germ band extension. (D) Stage 11 (dorsal view): ectodermal expression consolidate; into lateral stripes relative to the midline and clusters adjacent to the midline. Arrows point to differences in Comm and Comm2 mRNA accumulation. (E) Stage 12 (ventral view): midline glia and lateral neuronal expression. Arrows point to Comm mRNA accumulation that is detected in neurons that Comm2 is not. (F) Stage 12 (lateral view): muscle expression. (G) Stage 13 (lateral view): muscle expression, midline expression and neuronal expression just adjacent to midline cells. (H) Stage 14 (ventral view): muscle and CNS midline expression and increased neuronal accumulation of Comm mRNA (arrow) as compared to Comm2 mRNA accumulation (arrow). (I) Stage late 15 early 16 (ventral view): Comm and Comm2 accumulation diminishes; however, midline glia expression persists for both transcripts at this later stage (arrow).
Figure 3.5. Comm2 CNS phenotypes. Embryos are filleted dorsally and laid flat to reveal the ventral nerve cord (VNC). (A) Wild-type (wt) stained with the mAb BP102 reveals the ladder-like structure of the VNC. The longitudinal bundles are connected by repeated segments made up of an anterior and posterior commissure. (B) Wt embryo—stained with mAb 1D4, which recognizes FasII, and is expressed on a subset of longitudinal fascicles on each side of the nerve cord in stage 17 embryos. (C) Comm2 Del/ Del14. (D) Comm2 Del 1/Del 14.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>BP102 Phenotype</th>
<th>1D4 Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># of scored</td>
<td># of scored</td>
</tr>
<tr>
<td></td>
<td>segments</td>
<td>segments</td>
</tr>
<tr>
<td>Del 1/Del 14</td>
<td>0/144</td>
<td>0/121</td>
</tr>
<tr>
<td>Del 2/Del 14</td>
<td>comissureless</td>
<td>N/A</td>
</tr>
<tr>
<td>Del 3/Del 14</td>
<td>0/110</td>
<td>0/110</td>
</tr>
<tr>
<td>Del 4/Del 14</td>
<td>0/132</td>
<td>0/110</td>
</tr>
<tr>
<td>Del 5/Del 14</td>
<td>0/135</td>
<td>0/121</td>
</tr>
<tr>
<td>Del 6/Del 14</td>
<td>0/121</td>
<td>0/110</td>
</tr>
<tr>
<td>Del 7/Del 14</td>
<td>comissureless</td>
<td>N/A</td>
</tr>
<tr>
<td>Del 14/Del 14</td>
<td>comissureless</td>
<td>N/A</td>
</tr>
<tr>
<td>Del 21/Del 14</td>
<td>0/121</td>
<td>0/121</td>
</tr>
<tr>
<td>Wt</td>
<td>0/140</td>
<td>0/132</td>
</tr>
</tbody>
</table>

Table 3.3. BP102 and 1D4 phenotypes of *comm2* mutant embryos. Deletions 1 and 21 remove Comm2 completely. Deletions 3,4,5,6 remove the intergenic region between Comm and Comm2. Deletions 2 and 14 remove both Comm and Comm2. Deletion 7 removes Comm alone.
Figure 3.6. Mis-expression of Comm2 generates a subtle CNS phenotype. Insertions D08181, D06978 (both near Comm2) and D04506 (in the 5’ UTR) of Comm contain 14x UAS sites. (A) Wt. (B, C) Mis-expression of \textit{comm2} has a mild phenotype with few ectopic crossovers. (D) Mis-expression of \textit{comm} has a robust phenotype in the CNS.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Crossovers per segment</th>
<th>Percent crossovers per segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1407-Gal4/comm2&lt;sup&gt;208181&lt;/sup&gt;</td>
<td>4/128</td>
<td>3%</td>
</tr>
<tr>
<td>1407-Gal4/comm2&lt;sup&gt;206978&lt;/sup&gt;</td>
<td>6/107</td>
<td>5%</td>
</tr>
<tr>
<td>1407-Gal4/comm&lt;sup&gt;d04506&lt;/sup&gt;</td>
<td>132/1/32</td>
<td>100%</td>
</tr>
<tr>
<td>1407-Gal4/p[UAS-Comm1-HA.13]&lt;sup&gt;*&lt;/sup&gt;</td>
<td>60/80</td>
<td>75%</td>
</tr>
<tr>
<td>1407-Gal4/p[UAS-Comm2-HA.5]&lt;sup&gt;*&lt;/sup&gt;</td>
<td>6/80</td>
<td>7.5%</td>
</tr>
<tr>
<td>1407-Gal4/p[UAS-Comm2-HA.3]&lt;sup&gt;*&lt;/sup&gt;</td>
<td>7/88</td>
<td>7.95%</td>
</tr>
<tr>
<td>1407-Gal4/+</td>
<td>0/112</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 3.4. Mis-expression of UAS-Comm2. The 1407-Gal4 line is a pre-mitotic (st. 8-9) pan-neural CNS driver that is expressed until the end of embryogenesis (Luo, Liao et al. 1994). P-elements D08181 and D06978 are inserted near the 5' UTR of Comm2 and have 14x UAS sites. The P-element insertion D04506 is inserted in the Comm 5' UTR. *Personal communication Laura Carver 1x HA tagged pUASTattB constructs. All inserted into attP-22A (Bischof, Maeda et al. 2007).
Table 3.5. Dominant enhancement of the Netrin deficiency phenotype by mutations in comm, comm2, and comm,comm2/+,+ double mutants. Df(1)NP5 removes NetrinA and NetrinB. Df(3)fz-M21 and Df(3)XG5 both remove Comm and Comm2 as well as other genes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th># of defects per segment</th>
<th>% segments defective</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(1)NP5/Y</td>
<td>32/127</td>
<td>25%</td>
</tr>
<tr>
<td>Df(1)NP5/Y; Df(3)fz-M21/+</td>
<td>114/144</td>
<td>79%</td>
</tr>
<tr>
<td>Df(1)NP5/Y; Df(3)XG5/+</td>
<td>117/150</td>
<td>78%</td>
</tr>
<tr>
<td>Df(1)NP5Y; commP/+</td>
<td>59/115</td>
<td>51%</td>
</tr>
<tr>
<td>Df(1)NP5/Y; comm^e39/+</td>
<td>52/120</td>
<td>43%</td>
</tr>
<tr>
<td>Df(1)NP5/Y; comm^K40/+</td>
<td>58/135</td>
<td>36%</td>
</tr>
<tr>
<td>Df(1)NP5/Y; comm Del 7/+</td>
<td>58/109</td>
<td>58%</td>
</tr>
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<td>52/140</td>
<td>37%</td>
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<td>87/156</td>
<td>56%</td>
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<tr>
<td>Df(1)NP5/Y; comm2^d08181/+</td>
<td>58/101</td>
<td>58%</td>
</tr>
<tr>
<td>Df(1)NP5/Y; comm2^d06978/+</td>
<td>60/122</td>
<td>49%</td>
</tr>
<tr>
<td>Df(1)NP5/Y; comm2 Del 1/+</td>
<td>75/119</td>
<td>47%</td>
</tr>
<tr>
<td>Df(1)NP5/Y; Del 14/+</td>
<td>117/144</td>
<td>81%</td>
</tr>
<tr>
<td>Df(1)NP5/Y; comm2^PE4, comm^K40/+,+</td>
<td>142/180</td>
<td>79%</td>
</tr>
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</table>
Figure 3.7. Dominant enhancement of the Netrin deficiency phenotype by $Df(3)fz-M21$ (removes Comm and Comm2) and dominant enhancement by $comm^P$. Defective segments were identified as displaying thin or missing commissures (arrowheads highlight defective segments).
Chapter 4: Summary and Discussion

How the nervous system functions is one of the great marvels in the living world. To function properly it must be correctly ‘wired’ into functional circuits, allowing actions such as thoughts, senses, and coordinated movements. It has been over a century since Santiago Ramón y Cajal postulated that the tip of the axon (termed the ‘growth cone’) rams its way through a developing brain to make connections with its target cell. Since that time, hypothesis-driven research has given us an ever-expanding view of the processes and molecules directing axons toward their synaptic partners.

Since the revival of the “General Chemoaffinity hypothesis” and the abounding refinements that have followed, many of the hypothesized ‘chemical tags’ that Sperry proposed to exist have been identified. In fact, the broad and circumstantial evidence from both in vitro and in vivo experiments has come to a point where currently there is little debate over the veracity of the “General Chemoaffinity hypothesis”. This statement hardly means that all aspects have been solved, but rather points out that many fields of study have arisen in light of the work done by Sperry and researchers that followed him. Many disciplines, such as the study of growth-cone motility, neural circuits, limb
regeneration, animal behavior, formation of synapses and neurological disorders all have some overlap with the “Chemoaffinity hypothesis”.

Perhaps one of the best examples and most studied aspects of how the nervous system is formed are from studies of the midline choice point in both vertebrates and invertebrates. The simplicity to study the binary choice for axons to cross or not to cross makes the midline an excellent place in which to test hypotheses, identify genes through mutation, and study individual neurons as they navigate toward their final target.

My study of axon guidance at the midline was directed at addressing two questions. First, how is Comm regulated at the transcriptional level? Second, does Comm2 play a role in midline guidance?

Comm regulatory regions are certainly large and complex. We have identified regions 5’ of Comm that are important for negatively regulating expression and prevent Comm from being transcribed in ipsilaterally-projecting cells and for tight temporal regulation in commissurally-projecting axons. Interestingly, both Comm and Comm2 are expressed in the CNS together (Fig.3.2). Since they are close in proximity to each other (~30 kb) in the genome, it will be interesting to see whether Comm2 is also affected by the same 5’ deletions (Fig. 2.1). In addition, it may be important to address Comm2 regulation in order for us to better understand cis-regulatory mechanisms in this region.
To clarify, do both Comm and Comm2 promoters respond to the same distant enhancer or are there separate enhancers that control the expression of each? Potentially it could be either, depending how this region originated, presumably by gene duplication. Comm and Comm2 promoters may have retained the ability to respond to the same enhancer, or possibly the enhancer was duplicated as well in the initial event that originally led to Comm and Comm2 diverging. If they are in fact co-regulated by the same enhancer(s), it maybe possible to use Comm2 as readout to identify regions important for positive expression that have proved to be elusive so far using our current methods. For instance, we could revisit our rescue constructs with the ability to scan an even larger area of regulatory regions 3’ of Comm2.

Perhaps the most analogous region to compare cis-regulation of Comm and Comm2 comes from the gene abdominal-B (Abd-B) in the bithorax complex. The bithorax complex (BX-C) is a large and complexly regulated region (Celniker and Drewell 2007). Cis-regulatory control regions are scattered over 300 kb in this region. To summarize briefly, Abd-B gene expression is regulated by enhancers, silencers, promoter targeting sequences (PTS), and boundary elements (Zhou, Barolo et al. 1996; Akbari, Bousum et al. 2006; Mihaly, Barges et al. 2006; Celniker and Drewell 2007). Positive elements of regulation respond to gap and pair-rule genes (Casares and Sanchez-Herrero 1995). Negative regulation includes interaction with Polycomb response elements (PREs) (Kassis 2002). Interestingly, in the absence of negative regulatory regions, activation of gene expression is initiated but proper expression is not maintained and leads to an
expansion of Abd-B expression (Mihaly, Hogga et al. 1997). This example may help us not only in understanding the mechanism of Comm and Comm2 regulation, but also, as we dissect this region it will potentially give us molecular details—such as DNA element structure and sequence information.

A different approach to understanding Comm at the transcriptional level could be through Fra activation (Yang, Garbe et al. 2009). It will be interesting to determine downstream signaling events of Fra that lead to the activation of Comm in commissurally-crossing axons. For instance, does the Fra cytoplasmic domain translocate to the nucleus (Taniguchi, Kim et al. 2003)? In light of the fact that Fra activation of Comm is Netrin independent, is there another ligand that signals through Fra (Yang, Garbe et al. 2009)? It is reasonable to speculate that Slit could be acting as a bifunctional guidance cue similar to that of Netrin acting as an attractive cue to the receptor Fra and repulsive cue to the receptor Unc5 (Hedgecock, Culotti et al. 1990). Furthermore, we could attempt to use transgenes to identify regions necessary for Fra activation of Comm.

Another topic to address is the function of Comm2. Although on its own Comm2 has no detectable loss-of-function phenotype in the embryonic CNS and only a mild gain-of-function phenotype, we have shown that Comm2 dominantly enhances the Netrin deficiency, strongly suggesting it does play a role in promoting midline crossing (Fig. 3.5 and Table 3.4). How Comm2 promotes midline crossing will be important to address for further understanding of how the CNS is properly wired during development.
In addition, Comm2 could be playing a more specific role in the CNS, which is not evident with mAbs BP02 or 1D4 markers. For example, Comm2 could contribute to regulating commissural pathway selection (i.e. anterior vs. posterior commissure selection). Analysis at single cell resolution will be important for clarifying whether or not Comm2 is playing such a role. For example, Robo2 and Robo3 play a role in specifying lateral pathway selection, once commissurally crossing axons have crossed the midline, as further revealed by analysis with a subset of neurons marked by Sema2b in the CNS (Rajagopalan, Vivancos et al. 2000; Spitzweck, Brankatschk et al. 2010).

An intriguing set of experiments, to address the function of Comm family members, may be similar to the approach taken by Spitzweck et. al. (2010), where they swapped Robo family members into each other’s genomic location with homologous recombination. A similar approach could be taken with Comm family members. Not only would this experiment potentially yield important data about gene expression requirements for each of the Comm family members, but it would also reveal important structural requirements specific to each Comm-family member.

In all, my analyses of Comm and Comm2 have contributed to the refinement and characterization of axon guidance at the midline of Drosophila melanogaster. Future goals in understanding Comm regulation will include defining cis-regulatory regions important for activation of Comm, as well as further dissecting regions 5’ of Comm important for negative regulation of Comm. Furthermore, understanding the role of
Comm2 in the CNS will lead to further insights into the critical regulation of axons crossing the midline.
References


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