NOTE TO USERS

DEVELOPMENT OF THE *DROSOPHILA* NERVOUS SYSTEM: ROLES FOR TGF-β SIGNALING AND THE GLYPICAN DALLY-LIKE

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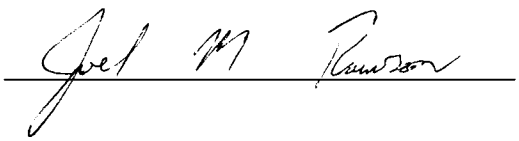
SIGNED: 

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ABSTRACT

Intercellular signaling is vital for the coordinated development of multicellular organisms. This is true for all tissue types including those that constitute the nervous system. The research described here explores the role of intercellular signaling in two vital steps of nervous system development, axon pathfinding and synapse maturation/modulation. The first chapter of this dissertation explores the role of the Transforming Growth Factor-Beta signaling pathway in the modulation of synaptic function and morphology at the *Drosophila melanogaster* neuromuscular junction. The second chapter explores the role of the glypican Dally-like in the process of axon outgrowth and axon guidance in the same organism. Glypicans are members of the heparan sulfate proteoglycan family of molecules and are required for the proper signal transduction of various intercellular pathways including TGF-β. Therefore both of the projects that compose this research explore the important role of intercellular signaling in development of the nervous system.

THE ROLE OF TGF-β SIGNALING IN THE DEVELOPMENT OF THE *DROSOPHILA* NEUROMUSCULAR JUNCTION

INTRODUCTION

Roles of TGF- ß

Growth factors serve as a principal means of intercellular communication important in both developing and mature tissues. The transforming growth factor-ß (TGF-β) superfamily is one of the largest and most widely expressed groups of secreted growth factors. There are open reading frames which encode 6 TGF-β ligands in *C. elegans*, 9 in *Drosophila melanogaster* and 42 in humans (Lander et al., 2001). These ligands can be placed into two main subfamilies by both sequence similarity and specificity of activation of downstream components. The first subfamily contains the bone morphogenetic proteins (BMPs), Muelerian inhibiting substance (MIS) and Growth and Differentiation Factor (GDF), while the second subfamily is made up of the TGF-β, Nodal and Activin type ligands (Shi and Massague, 2003). These ligands participate in a huge array of biological processes both during development and in adult tissues, in organisms ranging in complexity from the nematode *C. elegans* to mammals including humans (Blobe et al., 2000; Massague et al., 2000; Patterson and Padgett, 2000; Ten Dijke et al., 2002; Parker et al., 2004). In *Drosophila* TGF-β signaling is required for many developmental events including but not limited to dorsal/ventral patterning of the early embryo, development of the gut and patterning of larval imaginal tissue (Spencer et

al., 1982; Ferguson and Anderson, 1992; Staehling-Hampton et al., 1994). In other organisms TGF-β signaling also plays similarly important roles. For instance, in *Xenopus lavis*, the requirement of TGF-β signaling for dorsal/ventral patterning of the embryo is conserved (Dale et al., 1992). In the mouse, TGF-β superfamily members play a vital role in the patterning of limbs and various other developing tissues (Zhao, 2003). Moreover the TGF-β superfamily is critical for the proper development of complex multicellular organisms.

Roles of TGF-β Signaling in Nervous System Development

The TGF-β signaling system is known to serve versatile and vital roles in the assembly and maintenance of the nervous system (Mehler et al., 1997; Pratt and McPherson, 1997; Flanders et al., 1998; Krieglstein et al., 2002). In both vertebrates and invertebrates alike, TGF-β signaling early in development blocks the differentiation of ectoderm into neurogenic tissue while antagonists of TGF-β signaling serve as "neuralizing" factors (Wilson and Hemmati-Brivanlou, 1997; Raftery and Sutherland, 2003). Conversely, later during development TGF-β signaling acts as a positive factor in some contexts, serving to pattern distinct regions/cell types of the nervous system (Lee and Jessell, 1999). For example TGF-β signaling components are required in zebrafish for the specification of the neural crest and dorsal sensory neurons (Nguyen et al., 2000). In *Drosophila*, this vital intracellular signaling system is also required for the specification of some neuronal fates through its promotion of proneural gene expression

(Tomoyasu et al., 1998; Rusten et al., 2002) and it also plays a role in stimulating proliferation and migration of a subset of glial cells (Rangarajan et al., 2001).

In addition to these developmental processes, the TGF-β superfamily is also vital for nervous system developmental events that occur after initial patterning. Recent evidence has demonstrated that members of the TGF-β superfamily play an important role in axon outgrowth/guidance. For instance, mutations in *unc-129*, a TGF-β ligand, in *C. elegans* causes defects in axon guidance (Colavita et al., 1998). Similar roles for TGF-β signaling in vertebrate systems have been recently reported. BMP heterodimers expressed in the roof plate are required for commissural axon guidance fidelity in the mouse spinal cord (Augsburger et al., 1999; Butler and Dodd, 2003). In *Drosophila* there are currently no reported roles for TGF-β signaling in the process of axon guidance.

During the time in which this dissertation research was being undertaken there was emerging evidence that TGF-β signaling might also be important in the assembly, function, and plasticity of synapses. This evidence originates primarily from *in vitro* studies performed in the marine mollusk *Aplysia* and in research carried out in cultured hippocampal neurons. Experiments in *Aplysia* demonstrated an up regulation in a TGF-β signaling pathway component, *Aplysia tolloid*/BMP-1-like protein (apTBL-1), in response to manipulations known to produce long-term synaptic strengthening (Liu et al., 1997b). In *Aplysia*, long-term synaptic facilitation (LTF) of the pleural-pedal ganglia can be induced by application of tactile/electrical stimuli to the whole animal or by the addition of serotonin to the cultured ganglia. Similarly, artificial increase of TGF-β signaling levels in the cultured ganglia can induce LTF. Conversely, inhibition of

endogenous TGF-β signaling by addition of receptor specific antibody can block LTF induced by serotonin addition (Zhang et al., 1997; Chin et al., 1999). These results suggest that the developmentally important TGF-β signaling pathway may be required in the adult nervous system for the process of synaptic plasticity. The process of learning and memory is thought to require changes in synaptic efficacy which are sometimes coincident with morphological changes of existing synapses, formation of new synapses and a reorganization of synaptic machinery (Lamprecht and LeDoux, 2004). Consistent with this hypothesis, Bone Morphogenetic Protein-7 (BMP-7) increases dendritic elaboration of hippocampal neurons and promotes synapse formation (Withers et al., 2000). TGF-β ligands and their receptors are also found at the mature vertebrate neuromuscular junction (NMJ), suggesting that this signaling system could mediate cell-cell communication between motorneuron and muscle cells that modulate some aspect of synaptic strength or morphology (McLennan and Koishi, 1994; Jiang et al., 2000). Despite these data the physiological relevance of TGF-β signaling in the processes of synapse formation, maturation and plasticity was largely unknown at the onset of this study and therefore we proposed to use a genetically tractable synapse, the *Drosophila* NMJ, to ascertain the *in vivo* role of TGF-β signaling in these processes.

The *Drosophila* Neuromuscular Junction

Drosophila has served as a model for studying the genetic and molecular mechanisms of many aspects of nervous system development. These include developmental processes common to both the invertebrate and vertebrate nervous system

alike, including specification of neurogenic tissues, cell fate decisions leading to the generation of neuroblasts and mature neurons/glia, axon guidance, synaptogenesis and synaptic plasticity (Klambt et al., 1999; Skeath, 1999; Featherstone and Broadie, 2000; Koh et al., 2000; Broadie and Richmond, 2002; Jin, 2002; Stathopoulos and Levine, 2002; Dubnau et al., 2003; Huber et al., 2003; Skeath and Thor, 2003; Tayler and Garrity, 2003; Urbach and Technau, 2004). One of the most productive experimental systems exploited in the fly has been the NMJ. This synapse has allowed key discoveries in the mechanisms underlying how an axon recognizes its correct target and the process of synapse formation between a pre- and postsynaptic cell (Featherstone and Broadie, 2000; Rose and Chiba, 2000). Research performed on the NMJ has also contributed to the understanding of the processes of synaptic maturation and synaptic plasticity (Featherstone and Broadie, 2000). These studies have been successful for several reasons, not least of which is the relative ease of generating mutations in *Drosophila* genes and the accessibility of this synapse for morphological and functional (electrophysiological) evaluation (Brunner and O'Kane, 1997). The wealth of background knowledge about the development of the NMJ and the availability of powerful genetic tools make this synapse an ideal experimental system for exploring the role of TGF-β signaling in the processes of synaptic formation, maturation and plasticity.

Drosophila contains a stereotypic set of motorneurons that reside in the ventral ganglion and form synapses with a similarly stereotypic set of muscles in the abdominal body wall of a given segment (Johansen et al., 1989; Halpern et al., 1991). Research has shown that in order for this specific pattern of innervation to develop, many axon

guidance signaling pathways function to guide the motorneuron growth cone to the general target region (Huber et al., 2003). Once in the vicinity, growth cone filopodia sample the surface of many muscles in the immediate area as one method of finding the correct synaptic partner(s) (Rose and Chiba, 2000). Experimental evidence suggests that specific repulsive or attractive signals, such as homophilic molecules, expressed on select muscle and growth cone pairs play a role in allowing the growth cone to determine the correct synaptic partner with high fidelity. For instance the neural cell adhesion molecule Fasciclin III is expressed both on the RP3 motorneuron and its target muscles (abdominal muscles 6 and 7). Removal of FasIII from muscles 6 and 7 causes targeting errors in RP3. Likewise, misexpression of FasIII on other muscles can cause RP3 to mistarget to these muscles (Chiba et al., 1995). In summary, long and short range cues, both inhibitory and attractive in nature, guide motorneuron axons to their proper synaptic targets.

Synapse formation, or synaptogenesis, requires contact between the motorneuron and the muscle for successful completion, however certain aspects of this process can proceed in the absence of either the pre- or postsynaptic cell. For instance, when innervation is absent or delayed the muscles still develop normally and even localize certain synaptic proteins to the future site of synaptic contact (Broadie and Bate, 1993a). Likewise, in the absence of differentiated muscle cells, growth cones arrive in the correct target region and form normal presynaptic active zones (Prokop et al., 1996). However, coordinated synaptic development requires the close apposition of the motorneuron growth cone to the muscle membrane. Proper presynaptic localization of active zones and proper postsynaptic localization of glutamate receptors (the primary neurotransmitter

of the NMJ) require that pre and postsynaptic cells are in contact with one another (Broadie and Bate, 1993b; Keshishian et al., 1996). These results suggest both anterograde and retrograde signaling is necessary for the coordinated deposition of pre- and postsynaptic machinery.

After synapse formation is complete, the *Drosophila* NMJ is not a morphologically/functionally inert interface but rather remains quite plastic. The abdominal wall muscles increase over 150 fold in volume during larval development and motorneurons must provide appropriate matching input to drive muscle responses (Guan et al., 1996). In other words, as the muscle grows larger and its input resistance decreases the motorneuron must increase neurotransmitter release and the postsynaptic cell must express more glutamate receptors to maintain proper muscle activity. In practical terms this involves an elaboration of the synapse including an overall increase in the number of boutons (active zone containing varicosities). This elaboration, in conjunction with the recruitment of additional synaptic machinery accounts for the increase in neurotransmitter release observed during larval development. Therefore, throughout larval development the morphology and function of the NMJ continues to adjust in response to the overall growth of the muscle (Featherstone and Broadie, 2000).

Retrograde signaling from the muscle to the motorneuron may play a role in the process described above. The presence of a retrograde signal at the NMJ was unmasked by experiments which demonstrated compensatory changes in neurotransmitter release in response to alterations in muscle function. Reduction in the levels of glutamate receptors (GluR) expressed in the muscle produces an increase in the neurotransmitter released

from the presynaptic neuron. This presynaptic compensation for a decrease in postsynaptic (muscle) responsiveness allows the overall response of the muscle (excitatory junction potential-EJP) to remain constant (Petersen et al., 1997; Davis et al., 1998; DiAntonio et al., 1999). This result demonstrates the presence of a retrograde signal that can inform the neuron of the responsiveness of the muscle.

The level of neuronal activity can shape the morphology and function of the *Drosophila* NMJ (Koh et al., 2000). For example, mutations in ion channels that produce muscle hyperexcitability result in synapses with more boutons and synapse arborization (Budnik et al., 1990). cAMP-dependent signaling is involved in these activity-dependent changes at the NMJ in *Drosophila* (Zhong and Wu, 1991; Zhong et al., 1992). Mutations in several components of this pathway alter bouton number, axon branching, and the number of vesicles released per motorneuron stimulation (Koh et al., 2000). Much of the molecular machinery affecting plasticity of the NMJ also plays a role in learning and memory, both in *Drosophila* and in other systems including the mouse, suggesting that the molecular components governing plasticity at this relatively simple synapse are utilized broadly for controlling synapse change (Mayford and Kandel, 1999).

Mechanisms of TGF-β Signaling

The TGF-β signaling pathway has been well characterized and is largely conserved across species, including *Drosophila* (Massague, 1998; Raftery and Sutherland, 1999). Relatively few proteins are required to pass a TGF-β signal from the cell membrane to the nucleus, however, as might be expected for a pathway which controls a

large host of biological processes, TGF-β signaling is highly regulated at each step of signal transduction (Nakayama et al., 2000; Balemans and Van Hul, 2002; Shi and Massague, 2003). The introduction below gives an overview of the mechanism of TGF-β signaling and some of the regulatory systems known to control levels of signal transduction.

All ligands of the TGF-β superfamily are characterized by conserved cysteine residues which form disulfide bonds linking the protein into a tight three dimensional structure known as a "cysteine knot". TGF-β ligands are synthesized as monomer proproteins which dimerize and undergo cleavage by furin family members to form an active ligand. This dimerization is stabilized by both hydrophobic interactions and in most cases a disulfide bridge. Although the proprotein has undergone cleavage to the active form in at least some cases (the TGF-β subfamily) the cleaved N-terminal protein remains with the active subunit creating the latency-associated protein (LAP) (Koli et al., 2001; Annes et al., 2003). LAP can also be bound by latent TGF-β binding proteins (LTBPs) and this complex is then secreted as an inactive hetero/homodimer into the extracellular space where activation by thrombospondin-1 or possibly other means creates a free ligand which then can bind to its cognate receptors and activate the TGF-β signaling cascade (Koli et al., 2001; Annes et al., 2003; Petryk and O'Connor, 2004).

Prior to ligand/receptor binding however, the dimerized ligand can potentially interact with a plethora of extracellular binding partners which determine the movement and activity of the ligand (Balemans and Van Hul, 2002; Raftery and Sutherland, 2003; Petryk and O'Connor, 2004). There are several classes of extracellular ligand binding

proteins that modulate TGF-β activity. Antagonists include but are not limited to Chordin/Short Gastrulation (Sog), Twisted Gastrulation (Tsg), Noggin, Follistatin and members of the DAN family (differential screening-selected genes aberrative in neuroblastoma) (Nakayama et al., 2000; Balemans and Van Hul, 2002; Petryk and O'Connor, 2004). Chordin/Sog and Tsg are conserved between vertebrates and invertebrates and although primarily classified as antagonists they appear to have a more complex role in TGF-β signaling. Sog and Tsg bind to BMP ligands and inhibit ligand/receptor interactions but paradoxically can also serve to concentrate BMPs in certain regions and therefore can function to increase signaling levels (data from *Drosophila*). The Sog/Tsg/BMP complex is cleaved by Tolloid, a zinc metalloprotease, freeing the dimer from the inhibitory complex and allowing ligand/receptor binding (Marques et al., 1997). The exquisite interplay of extracellular antagonists and activators of TGF-β signaling is a major reason that this pathway is able to set up reliable activity gradients to pattern tissues (Raftery and Sutherland, 2003; Petryk and O'Connor, 2004).

Two receptor types, type-I and type-II, are required to transduce TGF-β signals across the cell membrane. Both receptor types are composed of an extracellular, transmembrane and cytoplasmic domain. Upon ligand binding to both receptor subtypes, the type-II receptor, which has a constitutively active serine/threonine kinase cytoplasmic domain, phosphorylates the type-I receptor in a conserved glycine/serine rich region (GS region). This phosphorylation in turn activates the serine/threonine kinase activity of the type-I receptor (Massague, 1998; Shi and Massague, 2003). In the absence of ligand, receptors from different subfamilies are found in varying basal states and differ slightly

in the mode of activation. TGF-β subfamily receptors are found in a homodimeric state in the absence of ligand (i.e. type-I/type-I and type-II/type-II homodimers). Binding of TGF-β subfamily ligands to the type-II homodimer initiates the formation of a heterotetrameric receptor complex and leads to activation of signaling (Yamashita et al., 1994; Massague, 1998). BMP receptor subtypes, on the other hand, can form homodimers or heterodimers in the absence of ligand (i.e. type-I/type-I, type-II/type-II and type-I/type-II homo/heterodimers) (Gilboa et al., 2000). BMPs have a higher affinity for the type-I receptor then the type-II receptor and this preassembled ligand/type-I receptor complex is more efficient at binding the type-II receptor then BMP alone (Kirsch et al., 2000).

TGF-β signaling is regulated at the cell surface by psuedoreceptors and co-receptors. BAMBI (BMP and Activin receptor membrane bound inhibitor) forms heterodimers with type-I receptors, however its lack of a kinase domain blocks TGF-β signal transduction. The expression of BAMBI can be up regulated by TGF-β signaling or by other signaling pathways such as the Wnt pathway, creating opportunities for both negative feedback and pathway crosstalk respectively (Onichtchouk et al., 1999; Tsang et al., 2000; Sekiya et al., 2004b; Sekiya et al., 2004a). In addition to type-I and type-II receptors a third class of co-receptor proteins have been identified. Sometimes referred to as type-III receptors these proteins have been shown to increase the binding affinity of the receptor for the ligand. These include betaglycan and other proteoglycans, as well as Endoglin (Shi and Massague, 2003; Petryk and O'Connor, 2004). In addition to affecting cellular responses to TGF-β, proteoglycans of the heparan sulfate variety, can also

modulate TGF-β distribution thereby influencing morphogen patterning (Filmus and Selleck, 2001; Fujise et al., 2003; Bornemann et al., 2004; Tabata and Takei, 2004; Takei et al., 2004).

The first direct substrate for the kinase activity of the type-I receptor, Mad (Mothers against Dpp), was identified in *Drosophila*, and a homologue, Sma (small body size), was quickly recognized in C. elegans (Raftery et al., 1995; Sekelsky et al., 1995; Savage et al., 1996). With the discovery of Mad/Sma homologues in the mouse the common descriptive term Smad was introduced (Derynck et al., 1996). Although the receptor-regulated Smad, or R-Smad, was the first to be identified it has since been recognized that there are actually three types of Smads, which include common Smads (Co-Smads) and inhibitory Smads (I-Smads) (Shi and Massague, 2003). R-Smad proteins are composed of two conserved domains known as the MH1 and MH2 domains (Shi et al., 1997; Shi et al., 1998). MH1, the N-terminal portion of the protein, is required for DNA binding and negative regulation of the MH2 domain. MH2, the C-terminal portion of the protein common to all Smad types, is required for type-I receptor interaction, formation of homo/heteromeric Smad complexes and nuclear shuttling (Shi and Massague, 2003). The MH2 domain of the R-Smads also contain a characteristic SSXS sequence, which is the site of type-I receptor mediated phosphorylation and regulates the activity/nuclear translocation of Smad (Kretzschmar et al., 1997b; Shi and Massague, 2003).

Phosphorylation of the SSXS sequence triggers binding of the R-Smad to co-Smad and the translocation of this complex to the nucleus where these proteins act to

modulate gene expression (Lagna et al., 1996; Liu et al., 1997a). In both the mouse and the fly specific TGF-β subfamilies preferentially act on distinct R-Smads. In flies for instance, BMPs signal though Mad, while the Activins signal through a second R-Smad, Smox (Smad on X) (Shi and Massague, 2003; Parker et al., 2004). Regulation of TGF-β signaling can occur at the level of R-Smad activation by the third class of Smads, the I-Smads (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997; Tsuneizumi et al., 1997). I-Smads act in several manners to suppress TGF-β signal transduction. Firstly, they can bind to type-I receptors and due to their lack of a SSXS motif they cannot be phosphorylated and released from the receptor. Secondly, they can act by competing with the activated R-Smad for binding to Co-Smad which decreases signal transduction (Hata et al., 1998). In addition these inhibitors can act at the level of transcriptional repression (Petryk and O'Connor, 2004). The function of R-Smads is further regulated at the level of degradation by selective targeting to the 26S proteosome after ubiquitination by Smurf (Smad ubiquitination regulatory factor), an E3 ubiquitin ligase (Zhu et al., 1999; Shi and Massague, 2003).

Although the MH1 domain of the Smads can directly bind promoter DNA this interaction is generally non-specific and interaction of the Smad complex with other DNA-binding cofactors is required for the transcriptional regulation of specific genes. Indeed, the Smad complex can interact with both members of many DNA-binding protein families (e.g. forkhead, homeobox, e-box, fos/jun), as well as members of the basal transcription machinery (transcriptional coactivators or corepressors) (Shi and Massague, 2003). For instance, R-Smad bound to SIP1 (Smad-interacting protein 1), a zinc-finger

protein which binds the promoter of *Brachyury* in *Xenopus*, represses the expression of this gene (Verschueren et al., 1999). Conversely, the activated Smad2/Smad4 complex can activate *Mix.2* expression with the help of the DNA-binding cofactor FAST1 (Forkhead activin signal transducer) (Chen et al., 1996; Chen et al., 1997). Interactions with a diverse group of transcriptional regulatory proteins allow TGF-β signaling to mediate both the repression and activation of gene transcription in a cell type/target gene specific manner (Shi and Massague, 2003; Petryk and O'Connor, 2004).

In some cellular contexts TGF-β signaling can apparently occur in a Smad independent manner. For instance, ectopic TGF-βs and BMPs can activate the MAPK kinase kinase, Tak1, which causes ventralization of *Xenopus* embryos in the presence of the apoptotic blocker p35. Expression of a kinase deficient form of TAK1 in this same system can partially block the effects of ectopic BMP signaling, suggesting a role for MAPK in the endogenous BMP signaling pathway (Yamaguchi et al., 1995; Shibuya et al., 1998). In addition to the direct activation of the MAPK pathway by TGF-β signaling, there is evidence that crosstalk can occur between the canonical TGF-β signaling pathway and canonical MAPK pathways. For instance the Erk subfamily of MAP kinases can phosphorylate Smad1 in the MH1/MH2 linker region causing Smad1 to be retained in the cytoplasm and thereby reducing TGF-β signaling (Kretzschmar et al., 1997a; Kretzschmar et al., 1999). Conversely, epithelial growth factor or hepatocyte growth factor activation of a receptor tyrosine kinase leads to phosphorylation of Smad2 which augments TGF-β signaling (de Caestecker et al., 1998). There is a growing body

of evidence that TGF-β signaling is involved in crosstalk with many other important intercellular signaling systems (von Bubnoff and Cho, 2001).

The overview presented here is meant to give a broad picture of the mechanisms of TGF-β signaling and some of the regulatory controls that modulate signal transduction. The immense complexity of the TGF-β pathway lends positively to the ability of this signaling system to regulate many vital aspects of biology including the potential for diverse roles in the developing and mature nervous system.

Summary of Results

Published studies preceding this project led us to hypothesize that TGF-β signaling might play a role in synapse development and/or plasticity. In order to test this hypothesis we examined the role of two of the three type-I receptors found in *Drosophila*, Saxophone (Sax) and Thickveins (Tkv), as well as the R-Smad, Mad, in the process of synapse development and plasticity/maturation. Sax, Tkv and Mad are responsible for transducing signals from the BMP subfamily of ligands in *Drosophila*, and therefore this study does not address the potential role of Activin signaling (also present in *Drosophila*) in the process of synapse development.

Here we show that the type I receptor Sax and the transcription factor Mad are vital for normal development of the *Drosophila* NMJ. *sax* and *Mad* mutants show decreases in synaptic strength and bouton number at the NMJ while retaining normal patterns of neuronal differentiation, axon pathfinding, and synapse formation. The synapse abnormalities found in *sax* and *Mad* mutants can in part, be phenocopied by

expression of dominant-negative type-I BMP receptors in neurons but not muscle. In addition, expression of an activated form of the type-I receptor Tkv in neurons but not muscle can increase synaptic strength and bouton density. These results demonstrate that the type-I receptor Sax, as well as the transcription factor Mad are vital for normal synaptic development of the *Drosophila* larval neuromuscular junction. The observed effects of expression of dominant negative and activated type-I receptors suggest that BMP signaling is required *in vivo* in neurons to establish a properly functioning and properly formed synapse.

RESULTS

BMP Signaling in the *Drosophila* Nervous System

TGF-ß signaling molecules, including those of the BMP subfamily are expressed in many tissues throughout development in *Drosophila*, including the central nervous system (CNS) and muscle (Lo and Frasch, 1999; Raftery and Sutherland, 1999). *In situ* localization of *sax* mRNA indicates this transcript is found in all tissue during development and in adults. *tkv* transcripts are readily detectable in the midgut and pharynx during late embryonic stages and in a complex pattern in larval imaginal discs, but were not detected in the embryonic nervous system (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994). Similar to the reports on the expression pattern of *sax,* an antibody that recognizes the inactive or unphosphorylated form of Mad, detects this protein in all tissues (Newfeld et al., 1996). Despite the presence of BMP signaling components in the *Drosophila* central nervous system (CNS) during development the state of BMP signaling was unknown. To establish whether BMP signaling is active in the CNS or peripheral effectors, particularly in motorneurons and abdominal muscles, we examined the distribution of phosphorylated/activated-Mad (P-Mad) using an anti-P-Mad antibody in the developing embryo. Mad is phosphorylated upon ligand activation of BMP receptors, an event that drives the formation of a complex between Mad and the co-Smad, Medea. As described in the introduction, this complex then translocates to the nucleus where it can modulate gene expression (Raftery and Sutherland, 1999; Shi and Massague, 2003). Previous work with this antibody revealed

that it is able to specifically detect BMP signaling in *Drosophila* embryos with accuracy, however the expression pattern had only been reported for larval imaginal tissue and in embryos through stage 11 (Persson et al., 1998; Tanimoto et al., 2000; Dorfman and Shilo, 2001). At this stage of embryogenesis, outgrowth of motorneuron axons and the formation of NMJs has not occurred. We found that P-Mad immunoreactivity first appears in the CNS in a few cells per segment of early stage 15 embryos (Figure 1A). The number of cells expressing P-Mad increases by stage 16 and immuno-positive cells were detected in clusters in the CNS (Figure 1B). In the final stage of embryogenesis, stage 17, these clusters have expanded further and their number corresponds roughly with the number of motorneurons per hemisegment (data not shown). P-Mad CNS staining persists through the wandering third larval instar (data not shown).

Immunoreactive cells are neurons and not glia since P-Mad co-localizes with the neuron-specific nuclear marker, ELAV (Embryonic lethal abnormal vision) (Figure 1C-E) (Campos et al., 1985; Homyk et al., 1985; Campos et al., 1987; Robinow and White, 1988). The nuclear localization of P-Mad immunoreactivity is consistent with the translocation of activated R-Smads to the nucleus. The appearance of P-Mad immunoreactivity coincides with the assembly of NMJ synapses which commences at around stage 15 and is complete by stage 17 (Broadie et al., 1993). Confocal serial sections through anti-P-Mad stained embryos demonstrated that P-Mad is also present in other embryonic tissues known to require TGF-β signaling for morphogenesis (e.g. the midgut for example) (Figure 1A, B) (Staehling-Hampton et al., 1994). Notably, P-Mad was not detectable in abdominal muscles (Fig. 1B, double arrowheads).

The identity of some of the neurons that show P-Mad immunoreactivity was determined by staining with antibodies that recognize identified groups of neurons, anti-Even skipped (Eve) and anti-Fasciclin III (FasIII) (Patel et al., 1987; Halpern et al., 1991; Landgraf et al., 1999). P-Mad co-localizes with Eve in a subset of motorneurons; aCC, RP2 and the U/CQ group (Figure 1F-H). Identified interneurons, pCC and the EL group, are devoid of P-Mad immunoreactivity. Colocalization of anti-P-Mad and anti-FasIII demonstrates that BMP signaling takes place in RP3, one of the two motorneurons that innervates abdominal wall muscles 6 and 7 (Figure 1I - K) (Broadie et al., 1993). This neuromuscular synapse shows activity-dependent changes and is the preparation we have used to evaluate the role of BMP signaling in synapse function. These findings demonstrate that BMP signaling is active in a limited number of CNS neurons of late stage embryos, including motorneurons.

Mad and *sax* Mutants Have Normal Nervous System Patterning and Axon Pathfinding

In order to evaluate the role of the type-I receptor Sax and the transcription factor Mad in NMJ assembly and function we have examined animals bearing mutations in the genes encoding these proteins. Both of these genes are essential for early embryonic viability but there is a large maternal contribution of mRNA for both *Mad* and *sax*, permitting animals to reach the last larval instar with loss of zygotic gene function only (Raftery et al., 1995; Sekelsky et al., 1995). Late stage embryos were stained with anti-Fasciclin II (FasII) and anti-Eve. These antibodies provide markers for subsets of axons

and differentiated neurons, respectively. Animals homozygous mutant for *sax* and *Mad* show wild-type staining patterns for anti-FasII (Figure 2A,C&E) and anti-Eve (Fig. 2B,D&F), demonstrating that by these measures, neuron fate specification and axon guidance occur normally. Indeed, Eve-expressing neurons RP2, aCC, and the U/CQ group, that are P-Mad positive in wild-type animals, are correctly specified in both *sax* and *Mad* mutants (Fig. 2B,D&F). In addition, BP 102 (Seeger et al., 1993), an antibody specific for axons within the CNS, revealed that the gross morphology of intersegmental tracts and the commissures of each abdominal segment was normal in both *sax* and *Mad* mutants (data not shown). These results indicate that *Mad* and *sax* zygotic mutations do not affect the initial events of neuron proliferation/differentiation and axon guidance. However, do to the maternal contribution of Mad and Sax mRNA for these proteins it is possible that BMP signaling could play a role in these developmental processes.

To evaluate the differentiation of the RP3 motorneuron and its axon projection (one of two neurons which contribute to the NMJ in question) in *sax* and *Mad* mutants we stained late stage embryos with anti-FasIII antibody. This antibody recognizes the cell body, process and synapse of the RP3 motorneuron, as well as a subset of other neuronal cell bodies and their processes. Confocal analysis of anti-FasIII stained, whole-mount embryos allowed us to view the RP3 motorneuron and other FasIII positive neurons and axons through serial optical sections of wild-type and mutant animals. The RP3 cell was present and in the correct position in both *sax* and *Mad* mutants and the trajectories of FasIII-positive processes were normal (wild-type and a *Mad* mutant represented in Figure 2G, J). For example, the FasIII-positive axons (small arrowheads) pass immediately

anterior to a set of FasIII-expressing cells (brackets) on their way to the periphery where they innervate multiple muscles (Figure 2G,J). Examination of serial optical sections permitted the identification of RP3 target muscles. We found that a FasIII-positive process terminated on muscles 6 and 7 in wild-type, *Mad* (Figure H, I - large arrowheads) and *sax* mutant embryos (data not shown). These data are consistent with muscles 6 and 7 receiving normal innervation from RP3 when the animal is mutant for *sax* or *Mad*.

In addition to RP3, there is a second motorneuron that provides innervation for muscles 6 and 7. Each of these cells has its own synapse comprised of many varicosities (boutons) which are spread across the surface of the muscles and contain sites of vesicle release known as active zones. The boutons of RP3 and the second motorneuron are different in size and contribute differentially to the electrophysiological response. RP3 boutons are larger and have been termed type Ib (big), while the synapse contributed by the second neuron is comprised of smaller boutons known as type Is. Visualization of type Is (small arrowheads) and type Ib (large arrowheads) boutons with anti-cysteine string protein (anti-CSP) antibody (Ranjan et al., 1998) showed both bouton types are found in *sax* and *Mad* mutants (Fig 3g-i). CSP is a component of the presynaptic neurotransmitter vesicle release machinery and therefore anti-CSP is a marker of the presynaptic terminal. Staining of third instar larval synapses with anti-CSP also revealed that the placement of motorneuron terminals on abdominal wall muscles is normal in *sax* and *Mad* mutants (data not shown). When muscles are innervated ectopically by incorrect motorneurons in the absence of native motorneuron innervation the placement

of the synapses is often abnormal (Chang and Keshishian, 1996). These findings suggest that correct innervation of muscles 6 and 7 is achieved in *sax* and *Mad* mutants.

Mad and *sax* Mutants Have Impaired NMJ Morphology and Function

The electrophysiological properties of the *Drosophila* NMJ have been well characterized throughout embryonic and larval development. Building on this knowledge we utilized available techniques to assay the functional consequences of zygotic loss of *sax* and *Mad*. We did so by obtaining intracellular voltage recordings from abdominal muscle 6 during artificial stimulation of the axons that innervate this muscle. The voltage response of the muscle to nerve stimulation, the excitatory junctional potential (EJP), and the number of neurotransmitter containing vesicles released upon stimulation (quantal content) were dramatically reduced in both *sax* and *Mad* mutant animals as compared to wild-type and heterozygous controls ($p<0.001$ Figure 3A, B).

Representative voltage traces showing the muscle responses to a single stimulation are shown in Figure 3A. All muscles recorded from, including wild-type, *sax* mutant and *Mad* mutant animals, showed a compound EJP response. This is an indication that the muscle has been properly innervated by two distinct motorneurons since the two axons arising from these neurons have different thresholds of artificial activation. Therefore, as one increases the stimulation voltage applied to the severed nerve, first a small muscle voltage response (~15 mV) and then a larger response (~40 mV) is observed. This is a direct effect of an action potential being stimulated in only one of innervating axons alone at lower stimulation strength and then in both innervating

axons simultaneously as stimulation voltage is increased. This second larger response is known as the compound response. In both *Mad* and *sax* mutants compound EJP amplitudes and quantal content were reduced to approximately 40% and 20% of control values respectively (Figure 3B). Quantal content is a measure of transmitter release in the form of the number of quanta or vesicles released upon nerve stimulation. Hence our findings indicate that *sax* and *Mad* mutants have compromised presynaptic function.

Neither mutant appears to have a decrease in postsynaptic response to neurotransmitter release. Indeed, *sax* mutants actually show an increase in the spontaneous or miniature EJP (mini) amplitude (*Df(2R)sax-H9/CyO*, mean mini amplitude = 0.75 mV; sax^3/CyO, mean mini amplitude = 0.76; $Df(2R)sax\text{-}H9/sax^3$ mean mini amplitude = 1.14 mV). This increase in mini EJP size is not statistically significant for sax^3/CyO compared to $Df(2R)sax\text{-}H9/sax^3$ ($p>.05$), but is significant when *Df(2R)sax-H9/CyO* control is compared to $Df(2R)sax\text{-}H9/sax^3$ ($p<.05$). Miniature EJPs are caused by the spontaneous fusion and release of neurotransmitter from a single vesicle. Since the amount of neurotransmitter loading/release from a single vesicle is generally constant in *Drosophila*, changes in mini amplitude are assumed to reflect alterations in postsynaptic responsiveness to neurotransmitter. Therefore the increase in mini amplitude in *sax* mutants may reflect a postsynaptic compensation for the decrease in presynaptic neurotransmitter release. *Mad* mutants, however, show no difference in miniature EJPs (data not shown), suggesting that in this genotype there is no compensation for decreased quantal content.

Visualization of both Ib and Is boutons with anti-CSP antibody revealed changes in the morphology of synapses in *sax* and *Mad* mutants (Figure 3C & G-I). Mutant *sax* and *Mad* synapses showed significantly reduced numbers of boutons (summation of both type Ib and Is boutons) compared to heterozygous controls ($p<0.01$) (*Df(2R)sax-H9/+*, mean bouton number = 110; sax^3/+, mean bouton number = 144; *Df(2R)sax-H9/*sax^3, mean bouton number = 62, Mad^1/+, mean bouton number = 113; Mad^{12}/+, mean bouton number = 129; Mad^1/Mad^{12}, mean bouton number = 52). Previously published research has demonstrated that there is a direct linear correlation between the size of the muscle and the number of the boutons at the synapse on that muscle (Lnenicka and Keshishian, 2000). Therefore it is vital to factor in possible differences in animal/muscle size when comparing the number of boutons between animals of different genotypes. After bouton number was corrected for muscle area, only *sax* mutant animals showed significantly fewer boutons when compared to wild-type ($p<0.01$) and heterozygous controls ($p<0.05$) (Figure 3C & I). *Mad* mutant larvae are overall smaller in size then controls and correspondingly have smaller muscles (Mad^1/+, mean muscle area = 52700 μm^2; Mad^{12}/+, mean muscle area = 45,000 μm^2; Mad^1/Mad^{12}, mean muscle area = 25,500 μm^2), therefore when the number of boutons was calculated as a function of muscle area there was no significant difference between *Mad* mutants and control animals. The small muscle found in *Mad* mutants raises the issue as to whether the reduced EJP responses could also be a consequence of the reduced muscle size. Analysis of endogenous EJP responses in animals with extremely small muscles, namely late stage embryos (21 hours after egg laying), revealed EJPs in this stage of development can be of the same or

greater amplitude than those observed in third instar larvae (Broadie et al., 1993), demonstrating that EJP amplitude, unlike bouton number, does not vary with muscle size. It is interesting to note that *sax* but not *Mad* mutant boutons show an altered pattern of anti-CSP staining. The *sax* type Ib (larger) boutons have lost the annular staining characteristic of wild-type boutons and appear larger than normal (Fig 3G & I). This result suggests that in addition to affecting the overall number of boutons, mutations in *sax* can alter the normal distribution of synaptic machinery within the presynaptic terminal.

We have also conducted electrophysiological and morphological analysis of third instar larvae bearing two different hypomorphic *tkv* mutations (tkv^1/tkv^4) and these animals did not show any significant abnormalities (data not shown). However, unlike *sax* or *Mad,* severe *tkv* alleles do not survive beyond the embryonic stage (Terracol and Lengyel, 1994). The existing *tkv* hypomorphic mutants that survive to third instar show modest wing patterning defects when compared to phenotypes produced with localized expression of dominant-negative Tkv constructs (see later section) in the developing wing (data not shown), suggesting that the lack of defects in these *tkv* mutant animals may be due to residual Tkv expression/activity in these hypomorphic animals. Conversely Tkv may not be required in the BMP signaling complex in motorneurons.

BMP Signaling is Reduced in the CNS of *Mad* and *sax* Mutants

The preceding results demonstrate that synapse morphology and function are compromised in *sax* and *Mad* mutants while neuronal differentiation and axon

pathfinding apparently occur normally in these animals. In order to determine if the level of BMP signaling in the nervous system is affected by mutations in *Mad* and *sax,* we examined the patterns of P-Mad immunoreactivity in animals homozygous mutant for *sax* or *Mad*. Analysis of *sax* and *Mad* mutants with anti-P-Mad antibody revealed that these animals are indeed compromised for TGF-ß activity by this measure. Mad^{1}/Mad^{12}, $sax^{3}/Df\text{-}sax^{H9}$, *Mad/CyO* and *sax/CyO* siblings were stained with anti-P-Mad antibody and stage 15-17 embryos were observed and imaged under identical conditions. *Mad* and *sax* mutants showed different levels of penetrance for loss of P-Mad staining in the midgut, while both showed almost complete penetrance for loss of P-Mad in the CNS (Figure 3D-F). 90% of Mad mutants showed either no staining or greatly reduced staining in both the midgut (small arrowheads) and CNS (large arrowheads) while 100% of their heterozygous siblings showed staining comparable to wild-type (Mad^{1}/Mad^{12} n= 10, *Mad/CyO* n= 21) (data not shown, Figure 3E). *sax* mutants showed selective reduction of P-Mad staining in the CNS compared to the midgut. In $sax^{3}/Df\ sax\text{-}H9$ 50% of animals showed greatly reduced or no staining in the midgut (Figure 3D & F, small arrowheads) while 100% of these same animals showed loss of staining in the CNS (Figure 3D & F, large arrowheads) ($sax^{3}/Df\text{-}sax^{H9}$ n= 16, *sax/CyO* n=31). Note that in the embryo shown in Figure 3F, while P-Mad is undetectable in the CNS (large arrowheads) some P-Mad remains in the midgut (more anterior small arrowhead). In comparison, 97% of *sax/CyO* animals showed P-Mad antibody staining levels comparable to wild-type (Figure 3D). These results indicate that zygotically transcribed Mad and Sax are required to produce normal levels of P-Mad in the nervous system and

midgut during late embryonic stages. The differential decrease of P-Mad in the CNS verses the midgut in *sax* mutants suggests that *sax* plays a more indispensable role in CNS BMP signaling than it plays in midgut signaling.

Tissue-specific Requirements for the Type-I Receptors Tkv and Sax in Specifying NMJ Function

Our analysis of *sax* and *Mad* mutants demonstrate that these BMP signaling components play an important role in NMJ development. The presence of P-Mad in motorneuron and not muscle nuclei strongly suggest that this pathway is primarily required on the presynaptic side of the NMJ. In order to further determine if this pathway must be intact in neurons, muscle or both of these cell types to specify proper NMJ development, we directed expression of dominant-negative or constitutively activated type-I receptors to these tissues using the GAL4/UAS system (Brand and Perrimon, 1993). These mutant receptors can either block or enhance signaling when expressed in wild-type cells (Holley et al., 1996). Electrophysiological evaluation of synapses altered by expression of dominant-negative (DN) type-I receptors revealed that expression of TkvDN in neurons, but not in muscles, had a significant effect on evoked responses and quantal content when compared to wild-type and control animals (*tkvDN/+*, *GAL4 driver/+* (data not shown)) (Figure 4A). TkvDN did not disrupt the NMJ position and both type Is and type Ib boutons were present on muscles 6 and 7, suggesting that innervation with the native motorneurons had occurred (data not shown).

Electrophysiological evaluation of animals expressing dominant-negative Sax (SaxDN) in the neurons, but not muscle, had slightly reduced EJP amplitude and quantal content as compared to controls (animals reared at 25°C, Figure 4B). However, statistical analysis revealed that there was no significant effect on evoked responses and quantal content when compared to wild-type and control animals (*saxDN/+*, *GAL4 driver/+* (data not shown)). These results may reflect the partial blockade of signaling that is afforded by expression of dominant-negative constructs.

To further explore the tissue-specific requirements for the BMP signaling pathway in synapse development we expressed a constitutively active form of the type-I BMP receptor Tkv (TkvA) (Holley et al., 1996; Hoodless et al., 1996) in either neurons or muscle. This mutant form of the receptor is known to activate BMP signaling in other developmental contexts (Adachi-Yamada et al., 1999). The signaling capacities of three different *UAS-tkvA* inserts were assessed by examining their effects on wing development using a *GAL4* line that directs expression in the wing pouch. One of these, *P[w$^+$; UAS::tkvA*]B3 (tkvA)*, showed significantly greater effects on wing patterning than the other two. Expression of the strongest of the three *UAS-tkvA* inserts had a dramatic effect on electrophysiological measures when expressed in neurons. EJP amplitude was significantly increased only in animals bearing both *UAS-tkvA* and the neuron-specific *elav-GAL4* driver compared to wild-type ($p<0.001$), *UAS-tkvA/+* ($p<0.02$) or *elav-GAL4/+* (data not shown, $p<0.02$) (Fig. 4C). Quantal content was significantly increased in animals with neuron-directed expression of *UAS-tkvA* compared to wild-type ($p<0.01$) and *elav-GAL4/+* (data not shown, $p<0.05$) but was not significantly different from

animals bearing *UAS-tkvA/+* ($p>.05$) (Fig. 4C). Ectopic expression of *UAS-tkvA* in the muscle actually inhibited evoked EJP and QC responses compared to control groups ($p<0.05$), suggesting that excessive BMP signaling in the muscle can have deleterious effects (Olson et al., 1986; Salzberg et al., 1995). Two other less active *UAS-tkvA* inserts and one *UAS-saxA* insert did not produce the *GAL4*-directed enhancements in synaptic function we observed with *UAS::tkvA***B3 (tkvA)* (data not shown).

Tissue-specific Requirements for the Type-I Receptors Tkv and Sax in Specifying NMJ Morphology

Our electrophysiological analyses of both dominant negative and activated forms of the type-I receptors Tkv and Sax, suggest that activation of the BMP signaling pathway in neurons is essential for the normal development of NMJ electrophysiological responses. In order to gain additional insight into the tissue-specific requirements for BMP signaling in the development of the NMJ, we evaluated the morphological consequences of neural or muscle expression of dominant negative or activated forms of the type-I receptors Tkv and Sax. Neuron-specific expression of TkvDN in animals reared at 30°C, but not 25°C (data not shown, *GAL4* activity shows temperature dependence) significantly reduced bouton number compared to controls (wild-type, *elav-GAL4/+* or *UAS-tkvDN/+*, $p<.05$) (Figure 5A). Muscle-specific expression of TkvDN did not significantly affect bouton number when compared to controls (wild-type, *muscle-GAL4/+* or *UAS-tkvDN/+*, $p>.05$) (Figure 5A). These observations support the

hypothesis that an intact BMP signaling pathway must be present in neurons for normal bouton elaboration.

When the *saxDN* construct was expressed in neurons, animals reared at 30°C showed a reduction in bouton number that was statistically significant in comparison to *saxDN/+* bearing animals (no GAL4 driver, $p<.05$) but not in comparison to *elav-GAL4/+* bearing animals (no *saxDN*, $p>.05$) (Figure 5B,C&D). In addition to a reduction in the number of boutons, the overall structure of the boutons was also affected in *saxDN; elav-GAL4* animals (neuron-specific SaxDN expression) as compared to controls. In these animals the boutons did not have their characteristic round shape, instead they were elongated (insets in 5C,D). Animals with *saxDN* expression directed in muscles did not show statistically significant reduction in bouton number compared to controls (wild-type, *UAS-saxDN/+*or *muscle-GAL4/+*, $p>.05$). Collectively, these findings suggest that expression of Sax in neurons participates in normal NMJ morphological development.

Ectopic activation of BMP signaling in neurons, but not in muscle, had effects on synapse morphology. In 90% of the animals with neuron-directed TkvA expression (using the "strong" *UAS::tkvA*B3* transgene), boutons at the NMJ were closely packed in some segment of the synapse, without distinct spaces between them (Figure 5E, F). In *UAS-tkvA/+* animals and *muscle-GAL4; tkvA* animals these morphological defects were only observed in 36% of synapses examined. The effects of *UAS-tkvA* on synapse structure without a *GAL4* driver could be due to a low level of TkvA expression from the UAS construct in the absence of a GAL4 transcriptional activator. Two other less active

UAS-tkvA inserts and one *UAS-saxA* insert did not produce the GAL4-directed morphological changes we observed with the *UAS::tkvA***B3 (tkvA)* line (data not shown).

These results, taken together with the electrophysiological experiments from the preceding section, demonstrate that neuron-specific expression of dominant negative or constitutively activated forms of type-I receptors is sufficient to effect NMJ morphology and function. While these findings support the conclusion that the TGF-ß pathway must be intact in neurons for normal synapse development, they do not address which type-I receptors participate in this developmental process *in vivo*, as it is possible that the dominant negative or activated forms of these receptor can influence the signaling of complexes that normally do not contain that receptor.

DISCUSSION

BMP Signaling is Essential for Normal NMJ Development

The studies reported here demonstrate that the normal morphology and function of the *Drosophila* larval neuromuscular synapse requires the BMP type-I receptor Sax and the BMP-activated transcription factor Mad. Our data also implicate the type-I receptor Tkv in BMP-directed modulation of the *Drosophila* neuromuscular synapse. *sax* and *Mad* mutants, while showing reduced number of boutons and quantal content nonetheless form synapses, and do not show any apparent innervation abnormalities of muscles 6 and 7. Differentiation of the RP3 motorneuron and extension of RP axons appear to occur normally despite the marked reduction in P-Mad levels in these cells. All told, these results indicate that *sax* and *Mad* are required for synaptic maturation/modulation (Rawson et al., 2003). Despite the lack of defects in cell differentiation, axon pathfinding or synapse formation, our results do not preclude a role for TGF-ß signaling in these processes as we have examined animals that are only zygotically mutant for *sax* and *Mad*. Therefore, it is possible that maternal contribution of mRNAs for these proteins could be sufficient to carry out earlier TGF-ß-dependent developmental events.

Two other groups also discovered the important role of TGF-ß signaling during the development of the *Drosophila* NMJ, each by different experimental avenues. While our analysis of TGF-ß signaling had primarily begun as a result of perusal of relevant literature, Mike O'Connor and Corey Goodman's groups discovered the role of BMP

signaling through the utilization of reverse and forward genetic strategies respectively (Aberle et al., 2002; Marques et al., 2002). The O'Connor group, whose primary focus has been on TGF-ß signaling mechanisms and the role of this pathway in development, recognized the existence of a second TGF-ß type-II receptor in *Drosophila*, *wishful thinking* (*wit*). Subsequent mutational analysis of this protein revealed that it serves as a type-II receptor important for transmitting BMP-type signals in larval motorneurons. Mutations in *wit*, like those in *sax* and *Mad*, cause defects in both NMJ morphology and function (Marques et al., 2002). Conversely, the Goodman group, whose primary focus has been the study of nervous system development, isolated mutations in *wit* in an elegant forward genetic screen for mutants that had either overly elaborated or reduced NMJs. In this screen, the NMJs of mutagenized animals were observed *in vivo* through the transparent larval cuticle, utilizing a synaptically-localized green fluorescent protein (GFP). *wit* mutants isolated in this manner were also observed to have reduced synapses that had defects in function (Aberle et al., 2002).

Our findings with tissue-specific activation or inhibition of TGF-β signaling as well as the expression pattern of P-Mad suggest that activation of this signaling pathway in motorneurons and not muscles is essential for specifying normal bouton number and physiological function of the NMJ. Consistent with this hypothesis, we see inhibition or enhancement of electrophysiological responses upon neural-specific expression of dominant-negative or constitutively activated forms of Tkv respectively. Though the results with neural-specific expression of this same type-I receptor on synapse morphology were less clear, these findings also suggest a requirement for the BMP

signaling pathway in neurons during synapse elaboration (Rawson et al., 2003). Similarly both the O'Connor and the Goodman groups found that motorneuron-specific expression of Wit is able to rescue the viability and NMJ abnormalities of *wit* mutants (Aberle et al., 2002; Marques et al., 2002). Together our results strongly suggest that BMP signaling in the motorneurons is essential for the proper elaboration of the *Drosophila* NMJ during larval development.

While the requirement for TGF-ß signaling in the nervous system for the normal assembly and function of the NMJ is firmly established by our research and the efforts of the O'Connor and Goodman groups, these findings do not directly address the origin and identity of the TGF-ß ligand/s involved. At the time of publication we hypothesized several possible sources for the unknown TGF-ß ligand. The ligand could be secreted onto the presynaptic motorneuron from a distant cell through the circulating hemolymph, by the muscle at the NMJ in a retrograde fashion, by neighboring CNS neurons or glia, or by the motorneuron itself in an autocrine manner. Further work was required to assess the likelihood of these various possibilities.

Source and Identity of the BMP Ligand

Collaborative efforts between the Goodman and O'Connor groups have made progress in identifying the BMP ligand and its source (McCabe et al., 2003). Glass bottom boat (Gbb) encodes one of three BMP-type ligands identified in *Drosophila* (Newfeld et al., 1999). Gbb is expressed in the abdominal body wall muscles and to a lesser extent in the CNS, and loss of this protein causes phenotypes largely similar to

those seen in *sax*, *Mad* and *wit* mutants. Muscle specific Gbb expression provides almost full rescue of *gbb* mutant NMJ morphological defects and motorneuron P-Mad accumulation while expression of Gbb in neurons rescues these defects to a lesser extent. Blockade of retrograde transport in neurons mimicks the phenotypes observed in BMP mutants and causes the disappearance of nuclear P-Mad in motorneurons. These effects are presumably due to the requirement of retrograde transport of synaptically activated BMP components from the NMJ to the cell body. Taken together these results are consistent with Gbb being provided to the motorneuron from the muscle in a retrograde fashion (McCabe et al., 2003).

Additional results however suggest the role of Gbb may be more complex. Full rescue of P-Mad accumulation in motorneurons requires simultaneous Gbb expression in muscles and motorneurons. Furthermore, rescue of *gbb* mutant electrophysiological defects is achieved to a similar but incomplete level when Gbb is expressed in the motorneurons or muscles alone, but interestingly, Gbb expression in all neurons rescues *gbb* electrophysiological defects fully (McCabe et al., 2003). These somewhat confounding results are still generally consistent with a retrograde role for Gbb but also suggest that CNS specific sources of Gbb may play a role in modulating the function of the NMJ (more on this later).

Control of the Homeostatic Signal at the NMJ

The *Drosophila* NMJ is a robust synapse that is often able to sustain normal function despite various experimental insults (Schuster et al., 1996; Petersen et al., 1997).

This is presumed to be in part due to an unknown retrograde signal that serves to maintain synaptic homeostasis (Petersen et al., 1997; Davis et al., 1998; DiAntonio et al., 1999). This signal can be observed indirectly by experimental manipulations that decrease glutamate receptor activity, and consequentially postsynaptic responsiveness (quantal size). Despite this reduction in postsynaptic responsiveness, the EJP remains constant due to a compensatory increase in presynaptic neurotransmitter release (quantal content). Since the reduction in glutamate receptors only affects the postsynaptic cell (muscle) and the compensation is seen in the presynaptic cell (motorneuron), a retrograde signal is implied (Petersen et al., 1997; Davis et al., 1998; DiAntonio et al., 1999). It has subsequently been hypothesized that this retrograde signal may be part of the endogenous synaptic mechanism that ensures neurotransmitter release is properly matched with the rapidly growing muscle during larval development. In the case of mutations in *sax*, *Mad*, and *wit* there is a reduction in both bouton number and synapse function suggesting that mutations in the BMP pathway may compromise the function of the retrograde/homeostatic signal.

If the retrograde/homeostatic signal involves BMP signaling, mutations in pathway components should suppress the increase in quantal content observed subsequent to decreased glutamate receptor activity. Indeed, a recent study on the nature and control of this retrograde signal has demonstrated this point. Expression of a dominant negative glutamate receptor in the muscles is sufficient to both reduce quantal size and induce a compensatory increase quantal content (Davis et al., 1998). However in a *wit* mutant background expression of dominant negative glutamate receptors did not

cause an increase in quantal content despite the reduction of quantal size, demonstrating that BMP signaling is required for some aspect of the retrograde signal present at the *Drosophila* NMJ (Haghighi et al., 2003).

Whether or not Gbb is the true retrograde signal remains unknown. If Gbb played a direct and instructive role, then a theoretical detector of muscle activity could control the level of Gbb released at the synapse thereby instructing the presynaptic cell to modulate the level of neurotransmitter release. For instance a decrease in muscle activity might trigger the release of Gbb at the synapse which in turn could activate synaptic BMP receptors and lead to the transcriptional up regulation of vesicle release machinery in the motorneuron nucleus. Conversely, it is also feasible that BMP signaling at the synapse might be a constitutive and permissive signal. In this scenario BMP that is constantly released at the NMJ during larval development might be required to trigger the expression of motorneuron-specific proteins required for the reception/interpretation of the instructive retrograde signal. All of the data to date are consistent with both of these hypothetical models and further experimentation will be required to determine if BMP signaling at the NMJ is instructive or simply permissive (Haghighi et al., 2003).

How might an instructive BMP retrograde signal be modulated? Ca^{2+}/calmodulin-dependent kinase II (CaMKII), a kinase whose activity is sensitive to calcium levels and can affect the activity of a host of downstream proteins through phosphorylation, has recently been shown to be an intricate component of the *Drosophila* NMJ retrograde signaling pathway (Lisman et al., 2002; Haghighi et al., 2003). In the mammalian hippocampus (CA1) the activity of postsynaptic CaMKII is unregulated upon

Ca^{2+} entry through NMDA-type glutamate receptors (Malenka and Nicoll, 1999; Lisman et al., 2002). On activation, CaMKII phosphorylates glutamate-gated ion channels (glutamate receptors) and consequently causes an increase in single-channel currents (Barria et al., 1997; Derkach et al., 1999). In addition to direct modulation of ligand-gated ion channel conductance, activated CaMKII also recruits more receptors to the synapse, thus increasing excitatory postsynaptic potential amplitude (Hayashi et al., 2000; Lisman and Zhabotinsky, 2001; Nicoll, 2003). These combined effects contribute in large part to the induction of synaptic strengthening in the hippocampus known as long-term potentiation (Malenka and Nicoll, 1999; Nicoll, 2003). Similarly, it appears that CaMKII, although through a different mechanism, can regulate the strength of the invertebrate NMJ, as postsynaptic inhibition of this molecule causes an increase in presynaptic neurotransmitter release (Haghighi et al., 2003). Ectopic increase in postsynaptic CaMKII activity blocks the compensatory increase in quantal content associated with down regulation of glutamate receptor activity. Since CaMKII activity is sensitive to Ca^{2+} levels this protein may serve as the activity "sensor" of the NMJ. In this model, reductions in Ca^{2+} levels due to decreased glutamate receptor activity would lead to attenuation of CaMKII activity, which in turn could trigger the induction of the retrograde signal that boosts presynaptic neurotransmitter release. Intriguingly, BMP signaling is also apparently required for CaMKII dependent induction of presynaptic quantal content increases, as decreased CaMKII activity fails to induce an increase in neurotransmitter release in a *wit* mutant background (Haghighi et al., 2003). These results demonstrate that BMP signaling is required for CaMKII mediated retrograde

increases in quantal content, though it is still unclear if this signal is permissive or instructive in nature.

Roles for Endocytic Trafficking and the Ubiquitin-proteosome Pathway in Retrograde BMP Signaling

The Goodman laboratory's forward genetic screen for mutations affecting synapse morphology implicated many genes as important in shaping the NMJ. Interestingly, at least two genes isolated in this screen, *spinster* (*spin*) and *highwire* (*hiw*), which are not related to BMP signaling components, nonetheless appear to affect synapse size through modulation of BMP signaling (Wan et al., 2000; DiAntonio et al., 2001; Sweeney and Davis, 2002; McCabe et al., 2004). Mutations in *spin* were originally isolated in a screen for *Drosophila* mutations that caused defective courtship behavior (Nakano et al., 2001). However, *spin* alleles were separately isolated by the Goodman laboratory because these flies showed a severe NMJ overgrowth phenotype (Sweeney and Davis, 2002). In addition to defects in synapse morphology, these animals also demonstrated reduced quantal content and EJP amplitude. Detailed analysis of Spin localization revealed that this protein colocalizes to late endosomes/lysosomes in both the CNS and muscle, suggesting a possible role for Spin in the regulation of endocytic trafficking. Consistent with this hypothesis, examination of *spin* mutants revealed that these animals have severe defects in the size and number of late endosomes/lysosomes. Spin must be expressed both pre and postynaptically to rescue NMJ overgrowth in

mutants, oddly however, replacement of Spin in either the CNS or the muscles is sufficient to rescue *spin* mutant electrophysiological defects (Sweeney and Davis, 2002).

In addition to the role the endocytic pathway plays in the trafficking of certain proteins to the lysosome for degradation, experimental evidence has demonstrated that this process is inexorably linked to the signaling properties of activated transmembrane receptors (Entchev et al., 2000; Di Fiore and De Camilli, 2001; Dubois et al., 2001; Lloyd et al., 2002; Benmerah, 2004). It was first recognized that internalization of some receptors by clatherin-coated pits was a means of attenuating their ability to signal to downstream components. Subsequently, it has been demonstrated that in the case of some pathways, including TGF-βs (Hayes et al., 2002; Penheiter et al., 2002; Di Guglielmo et al., 2003), activated receptors are still able to signal from endosomes after internalization. Indeed, for some signaling events, such as neurotrophin receptor activation at synapses distant from the cell body, endocytosis is vital for proper signal transduction (Di Fiore and De Camilli, 2001; Benmerah, 2004). Given the role of endocytosis in modulating transmembrane signaling and the fact that mutations in *spin* have the opposite effect of BMP mutations on synaptic morphology, the authors reasoned that Spin, which is required for proper size and distribution of late endosomal/lysosomal compartments, might normally serve to down regulate synaptic BMP signaling (Sweeney and Davis, 2002). Consistent with this hypothesis, removal of one copy of *sax*, *tkv* or *wit* from a *spin* mutant background decreases the amount of synaptic overgrowth while double mutations in *spin* and one of these receptors cause complete suppression of the *spin* NMJ morphological phenotype. In addition, mutations in the I-Smad, Daughters

against Dpp (Dad), cause an increase in bouton number to an extent similar to that observed in *spin* mutants (Sweeney and Davis, 2002). These results suggest that the endogenous role of Spin may serve to down regulate BMP signaling at the NMJ, perhaps by directing a subset of activated receptors to the lysosome for degradation.

Despite these findings there remain some puzzling aspects to the phenotypes observed in *spin* mutants. For instance, if Spin normally serves to down regulate BMP signaling at the NMJ, why is there no increase in neurotransmitter release in *spin* mutants? Instead, these mutants have a very significant decrease in both EJP and quantal content consistent with additional roles for Spin beyond the regulation of BMP signaling. In addition, if Spin's only function were to regulate BMP signaling in the motorneuron, *spin* mutant phenotypes should be rescued by presynaptic expression of Spin. However, Spin expression is required both pre and postsynaptically to rescue the *spin* mutant increase in bouton number. These confounding results reveal that there is more work to be done to reveal all the roles of Spin in shaping the NMJ.

Like mutations in *spin*, mutations in the E3 ubiquitin ligase, Hiw, were identified as having massively overgrown NMJs (Wan et al., 2000). E3 ubiquitin ligases are cofactors required for the ubiquitination of specific proteins, a process which can serve to modulate the biological function of a protein in a multitude of manners. For instance, ubiquitination of a cytosolic/nuclear/endoplasmic reticulum membrane protein can target it to the 26S proteosome for degradation (requires polyubiquitination), direct a plasma membrane protein to be endocytosed (polyubiquitination) or direct proteins through the endocytic/late secretory pathway (monoubiquitination) (Hicke and Dunn, 2003). In the

search for targets of Hiw directed ubiquitination, McCabe et al. performed a yeast two-hybrid screen and found that the *Drosophila* co-Smad, Medea (Med), is a bound by Hiw (McCabe et al., 2004). Evaluation of *med* mutant synapses revealed that this protein is also required for BMP mediated specification of NMJ morphology and function. The binding interaction between Med and Hiw suggests that loss of *hiw* could potentially cause an overly elaborated synapse due to aberrantly high BMP signaling due to improper endocytic trafficking (McCabe et al., 2004). Consistent with this idea, it has been well documented that E3 ubiquitin ligases are vital for both the targeted degradation of TGF-β signaling components (e.g. Smads, receptors and negative regulators) by the 26S proteosome and the directed trafficking of TGF-β receptor containing endocytic vesicles to various fates (e.g. signaling endosomes vs. lysosome mediated degradation) (Hicke and Dunn, 2003).

Mutations in *wit* and *med* suppress the NMJ overgrowth in *hiw* mutants supporting a role for Hiw in the modulation of BMP signaling (McCabe et al., 2004). Similarly McCabe et al. demonstrated that ectopic activation of BMP signaling in a *hiw* mutant background could further increase the number of boutons. These results indicate that Hiw may indeed negatively regulate BMP signaling levels. However as in the case of *spin* mutants, *hiw* mutants have a reduction in quantal content, the opposite result that might be expected for a protein that normally suppresses BMP signaling at the NMJ. Therefore it is likely that Hiw also targets other proteins for degradation and therefore has roles beyond simple negative regulation of BMP signaling at the *Drosophila* NMJ.

These phenotypes in *hiw* and *spin* mutants demonstrate that, as in other contexts, TGF-β signaling is highly regulated at the *Drosophila* NMJ. It is interesting to consider the possibility that a retrograde signal, other then a BMP itself, could actually modulate the activity Spin or Hiw and in this manner serve to fine-tune the properties of the NMJ through BMP-mediated transcriptional changes. Further research will need to be undertaken to determine the exact nature and control of the retrograde signal required for homeostasis of the *Drosophila* NMJ.

Retrograde BMP Signals are Implicated at Other *Drosophila* Synapses

Since the first publications revealing the presence of a retrograde BMP signal that modulates NMJ morphology and function, several other studies have uncovered a similar signaling paradigm at other identified *Drosophila* synapses (Allan et al., 2003; Marques et al., 2003; Baines, 2004). Baines performed a study exploring the possible role of BMP signaling in the modulation of function at central synapses. Mutations in *dunce*, a phosphodiesterase that reduces the levels of cAMP, show an increase in interneuron induced motorneuron synaptic currents (Baines, 2003). Postsynaptic (motorneuron) overexpression of the adenylate cyclase Rutabaga (Rut), an enzyme responsible for cAMP synthesis, mimicks the *dunce* mutant increase in synaptic current (Baines, 2004). Taken together, these results suggest that increasing the level of postsynaptic (motorneuron) cAMP is sufficient to increase overall synaptic strength at this identified central synapse. Since the amplitude of spontaneous events (quantal size) at the synapse is unchanged after postsynaptic expression of Rut, the increase in synaptic current is a

result of presynaptic enhancement. These findings strongly imply the presence of retrograde signal between the motorneuron and interneuron similar to the signal known to exist at the NMJ (Baines, 2004).

Baines presents several lines of evidence that suggests that BMP signaling plays an important role in the retrograde signal at this central synapse. Gbb is expressed in the ventral nerve chord and removal of this protein by null mutations is sufficient to cause a decrease in interneuron/motorneuron synaptic currents. In a *gbb* mutant background, postsynaptic overexpression of Rut no longer causes the same increase in the observed synaptic currents. In addition, presynaptic (interneuron) expression of the activated type-I receptor Tkv or postsynaptic (motorneuron) expression of Gbb in a wild-type background is sufficient to cause an enhancement in synaptic currents (Baines, 2004). These results are consistent with a requirement for intact BMP signaling in the propagation of this central synapse retrograde signal.

Separate studies performed in the O'Connor and Thor laboratories revealed the importance of BMP retrograde signaling in *Drosophila* for the specification of neuropeptidergic cell identity. Loss of BMP signaling in *gbb* and *wit* mutants leads to loss of FMRFamide expression in the Tv neurons, two of which are present in each of the three thoracic segments of the ventral nerve cord (Allan et al., 2003; Marques et al., 2003). The bilaterally symmetric Tv neurons of a segment each project an axon to the midline which then turns dorsally and innervates the neurohemal organ (NHO). The NHO is composed of two glial cells and large varicosities that are formed by the termini of the two innervating Tv neuroendocrine neurons. The Tv neurons secrete FMRFamide

into the hemolymph through the NHO. FMRFamide-related peptides are conserved in both vertebrates and invertebrates and like other neuropeptides are known to have varied functions including but not limited to modulatory effects on cardioexcitation, learning, memory and electrical activity of central synapses (Nichols, 2003). In *Drosophil*a, FMRFa can increase NMJ efficacy when perfused into the recording saline (Hewes et al., 1998).

Together these studies eloquently demonstrate that a retrograde BMP signal from the NHO to the Tv neuron is required for the Tv neuron to express FMFRamide (Allan et al., 2003; Marques et al., 2003). This indicates that BMPs can not only modulate synaptic morphology and function but can in at least some cases dictate the neuropeptide identity of a cell. This is not without precedence however, as TGF-β signaling in other contexts was previously known to drive the expression of a specific neuropeptide/neurotransmitter (Fann and Patterson, 1994; Lopez-Coviella et al., 2000; Nolan et al., 2002). Given the enhanced effect of FMFRamide perfusion on the efficacy of *Drosophila* NMJ neurotransmission, it stands to reason that loss of FMFRamide expression in BMP signaling mutants might partially account for ours and others observed defects in NMJ neurotransmission. Consistent with this hypothesis replacement of FMFRamide into the NHO in *wit* mutant animals is sufficient to partially rescue *wit* mutant lethality and wing inflation defects (Marques et al., 2003). Notably however, replacement of FMRF into *wit* mutants does not rescue the morphological defects at the synapse and only provides subtle rescue of the defects in synaptic transmission. These results indicate that BMP signaling in *Drosophila* can modulate the NMJ at both a local

and a global level. BMP signaling locally serves to modulate synaptic strength and morphology, while BMP signaling in neuropeptidergic cells serves to induce the expression of a FMRFamide that globally regulates the gain of body wall muscle activity.

CONCLUSION

Our results add to the growing understanding of the role of TGF-β signaling in the development of the nervous system. The *Drosophila* NMJ provides a unique window into the interplay between synaptic homeostasis and synaptic plasticity that is likely to be occurring at many synapses in the nervous systems of very diverse organisms (Turrigiano and Nelson, 2004). Evidence suggests that TGF-β signaling remains important to nervous system function even after nervous system patterning is complete (Flanders et al., 1998; Bottner et al., 2000; Buisson et al., 2003). Expression patterns of TGF-β signaling components in the adult rodent hippocampus and other brain regions suggest that here too, TGF-β signaling will remain important for adult mammalian nervous system function, perhaps in part by playing a vital role in learning and memory (Charytoniuk et al., 2000). Indeed investigation of TGF-β signaling in *Aplysia* and cultured mammalian hippocampal neurons, as well as recent studies in *Drosophila*, indicate that this pathway is capable of sculpting the form and function of synapses (Liu et al., 1997b; Zhang et al., 1997; Chin et al., 1999; Withers et al., 2000; Zheng et al., 2003). Our results add to the understanding of the important physiological roles the TGF-β signaling pathway plays in shaping the form and function of the *Drosophila* NMJ.

The diverse roles for the TGF-β pathway described above beg the question, how does an intercellular signaling pathway common to so many cells mediate such diverse effects. This is a fundamental question of development and one to which the answer is becoming better understood. There are several possibilities as to how the diverse and cell

specific affects might arise. One mechanism, which is at least in part important, is the intrinsic identity of an individual cell. For instance, BMP signaling alone must not be sufficient to induce FMRFamide expression in Tv neurons, as many cells of the developing CNS (e.g. motorneurons) are also undergoing active BMP signaling. Allan et al demonstrated that in order for BMP signaling to induce FMRFamide expression the transcription factors squeeze and apterous must be expressed in the same cell. Since this particular combination of transcription factors is only found in a small subset of cells, this is sufficient to mediate a specific identity for those cells (Allan et al., 2003). It is important to note, that the intrinsic cell identity itself was likely set up by earlier intercellular signaling events, indeed some of which may have been mediated by TGF-β signaling. Therefore intercellular signaling pathways serve to form a multi-tiered system to specify the ultimate form and function of the nervous system. It is possible, perhaps even likely, that in the case of a pathway as widely active as TGF-β, we are only beginning to understand the extent of its roles in shaping the development and adult function of the nervous system.

Lastly, the functions of TGF-β signaling in the development of the *Drosophila* NMJ provide further opportunity to explore how a process as potentially complex as synaptic homeostasis might be regulated. Modern techniques and experimental findings including microarray analysis and the completion of the *Drosophila* genome sequence, allow for the detailed examination of genes that might be transcriptionally regulated by a potential BMP retrograde signal. Understanding which genes are modulated by BMP signaling at the NMJ should prove useful in the pursuit of the molecular mechanisms

underlying synaptic plasticity and homeostasis. Additional research on the roles of TGF-β signaling in *Drosophila* and other model organisms should further enlighten our understanding of the role that this widespread signaling pathway plays in the developing and adult nervous system.

THE GLYPICAN DALLY-LIKE IS REQUIRED FOR AXON GUIDANCE IN *DROSOPHILA*

INTRODUCTION

Many researchers have been working to decipher the role of proteoglycans in nervous system development, including their role in the process of axon pathfinding. While there has been a significant amount of *in vitro* evidence generated that indicates that this diverse class of molecules is important for axon guidance, at the onset of our own work into this field there was no direct evidence as to the physiological role of these molecules in axon pathfinding. Therefore we hoped to contribute to the understanding of the *in vivo* role of these molecules in nervous system development. What follows is a brief and simplified introduction to the growing field of axon guidance research focusing on the extracellular ligands and transmembrane receptors required for this process. In addition, a brief introduction to proteoglycan form and function follows with a particular focus on members of the glypican family.

Mechanisms of Axon Guidance

Vertebrates and invertebrates alike share the developmental challenge of connecting distant neurons in a repeatable manner to form functioning neural circuits. In 1892 Santiago Ramon y Cajal first hypothesized the presence of a chemoattractive cue that would serve to direct axonal outgrowth toward the vertebrate midline during nervous

system development (Ramon y Cajal, 1909: 1952-1955; Andres-Barquin, 2002). Over 100 years later, research in many different organisms has significantly enlightened us as to the molecular mechanisms underlying axon guidance (Guan and Rao, 2003; Huber et al., 2003). Axon guidance is a specialized form of cell migration, and therefore it is perhaps not surprising that the molecular underpinnings of axon guidance and cell migration share some common mechanisms (Wu et al., 2001; Rao et al., 2002; Ward et al., 2003). In the case of axon guidance, a specialized structure on the tip of the axon, the growth cone, navigates its environment on course to an often distant target (Guan and Rao, 2003).

As predicted by Ramon y Cajal, the growth cone receives signals that attract it toward certain targets during outgrowth. In addition, it also receives repulsive cues as it navigates its environment. Intercellular transmembrane signaling pathways make up the molecular basis for attractive and repulsive axon guidance signals. In some cases the same signaling pathway is capable of mediating both the attraction of one growth cone and the repulsion of another growth cone (Guan and Rao, 2003; Huber et al., 2003). Transmembrane receptors expressed in neurons and localized to the growth cone transduce signals that are induced by the binding of specific ligands to these receptors. Ligands vary in their range of action; for example, secreted signals can act over long distances by diffusion from a source, while other ligands act at a more limited range. In some cases the growth cone must directly contact the source of a ligand (e.g. a membrane bound ligand) to activate the signaling cascade. In addition to receptor/ligand systems present in growth cones, cell adhesion molecules (which in some cases can also signal

into the neuron) play a vital role in axon guidance and mediate important events like fasciculation, defasciculation and target recognition (Kiryushko et al., 2004). Studies in diverse organisms have revealed that there is a high level of molecular conservation of axon guidance signaling pathways between all bilaterian organisms (Chisholm and Tessier-Lavigne, 1999). This fact makes studies performed in model organisms, such as *Drosophila* and *C. elegans*, extremely relevant to the general understanding of axon guidance in "higher" organisms.

The growth cone is composed of different regions that can be broken down by their morphological appearance and the composition of their cytoskeletal components. At the interface between the axon and the growth cone is a region that transitions from a cytoskeleton composed primarily of microtubules (axon) to a cross-linked network of actin filaments (lamellapodia). The lamellapodia makes up the broad flat structure that comprises most of the growth cone. Projecting from the lamellapodia are many finger-like extensions that sample the extracellular environment. These are known as filopodia which are made up of bundled filaments of F-actin. Polymerization of actin at the growth cone leading edge (lamellapodia and filopodia) contributes to the forward directional movement of this structure. Retrograde movement of F-actin bundles in the lamellapodia/filopodia halt and reverse growth cone advancement. In most cases, ligand/receptor signaling into the cell mediates the net movement of the growth cone by modulating actin polymerization, actin depolymerization and the retrograde movement of F-actin (Huber et al., 2003).

The journey of an axon to its target is broken down into smaller segments and as the growth cone navigates through its environment it has to make multiple guidance decisions along its course. The decision of whether or not to cross the midline is one such choice that many axons face. Genetic screens in *C. elegans* and *Drosophila* are responsible for the discovery of many proteins involved in the midline decision, as well as the discovery of proteins involved at other guidance choice points (Araujo and Tear, 2003). Uncoordinated-6 (Unc-6), also known as Netrin, was discovered in *C. elegans*, and animals carrying a mutation in this gene have defects in axon guidance at the midline (Hedgecock et al., 1990). Depending on the particular axon in question, Netrins can mediate both attraction towards and repulsion away from the midline (Chisholm and Tessier-Lavigne, 1999). This signaling pathway is conserved from worms to humans, and mutations in Netrin signaling components in several organisms have now been shown to cause similar defects in axon navigation at the midline (Hedgecock et al., 1990; Harris et al., 1996; Mitchell et al., 1996; Serafini et al., 1996).

Netrins are secreted ligands which are expressed at the midline and signal through transmembrane receptors found in the growth cone. Whether or not the response of the growth cone is one of attraction or repulsion to Netrin, depends on the type of receptors present in the growth cone. In general, growth cones expressing only members of the UNC-40/DCC (deleted in colorectal cancer) family of receptors are attracted to the Netrin source, whereas neurons expressing both DCC and UNC-5 receptor family members are repulsed from the Netrin source (Guan and Rao, 2003; Huber et al., 2003). Although secreted molecules, Netrins act at a relatively short range because their diffusion is

limited by binding to negatively charged molecules (e.g. proteoglycans) found on cell surfaces and in the extracellular matrix.

Slit is a midline secreted ligand that mediates axon repulsion. First discovered in *Drosophila*, Slit encodes a member of a conserved family of ligands that signals through the Roundabout (Robo) family of transmembrane receptors (Wong et al., 2002). In *Drosophila*, *robo* mutants have axons that cross the midline aberrantly (Seeger et al., 1993). *Drosophila slit* mutant embryos show a collapse of longitudinal axon tracts to the midline, indicating that Slit/Robo signaling also determines the distance away from the midline that a longitudinal axon will travel (Rothberg et al., 1988). In other organisms, including *C. elegans*, zebrafish, mouse and humans, Slit/Robo signaling is also required *in vivo* for midline axon guidance (Hao et al., 2001)}(Hutson and Chien, 2002; Plump et al., 2002; Jen et al., 2004; Long et al., 2004). Slit/Robo signaling can also mediate other important developmental events in the nervous system including neuronal migration and axon/dendrite branching (Wong et al., 2002; Huber et al., 2003).

During development ephrins can play many different roles, however, one of the primary functions of the ephrins and their transmembrane tyrosine kinase receptors (Ephs) in the nervous system, is to negatively regulate axon guidance by causing the collapse of the growth cone. Since both ligand and receptors are tethered to the membrane, signaling through this pathway is cell-cell contact dependent. In vertebrates, the Ephrins function *in vivo* to mediate topographic mapping of retinotectal/retinocollicular projections and can also direct other axon guidance events including midline crossing (Feldheim et al., 2000; Palmer and Klein, 2003). Likewise, in invertebrates, ephrins play a role in the

proper establishment of axonal architecture (George et al., 1998; Zallen et al., 1999; Bossing and Brand, 2002; Dearborn et al., 2002). Interestingly, in vertebrates the Ephrin ligands are themselves capable of signaling into the cell and therefore ephrin signaling is a bidirectional process.

The Semaphorins are a conserved family of ligands that can mediate both attraction and repulsion of growth cones (Huber et al., 2003). These ligands are found in both membrane-associated and secreted forms and in *Drosophila* mediate signaling through a receptor complex that contains Plexin and the transmembrane receptor Off-track (Yu et al., 1998; Winberg et al., 2001; Araujo and Tear, 2003). In flies, loss of Semaphorin signaling components causes defects in motor axon defasciculation and therefore defects in target muscle innervation (Kolodkin et al., 1993; Yu et al., 1998; Winberg et al., 2001). In vertebrates the Semaphorin receptor complex includes Plexin and Neuropilin, as wells as the hepatocyte growth factor receptor Met. In these organisms the Semaphorins regulate growth cone repulsion and collapse in several contexts (Huber et al., 2003).

Receptor protein tyrosine phosphatases (RPTPs) are important for a diverse group of developmental events including axon guidance (Huber et al., 2003). In *Drosophila* members of the RPTP family are required for motorneuron and photoreceptor axon guidance (Krueger et al., 1996; Clandinin et al., 2001; Maurel-Zaffran et al., 2001). This family of receptors has also been implicated in axon guidance in vertebrates (Huber et al., 2003). The ligands that modulate the phosphatase activity of RPTPs are poorly characterized but homophilic receptor dimerization regulates the activation state of these

receptors (Bilwes et al., 1996; Jiang et al., 1999; Huber et al., 2003). Recent evidence demonstrates that RPTPs in part regulate axon guidance by impinging on some of the signaling pathways discussed above (Chang et al., 2004).

The most recently classified signaling molecules required for axon guidance are the morphogens, including Hedgehog (Hh), Wnt and Transforming Growth Factor-β (TGF-β). These molecules are well known for their ability to pattern tissues during development and recent findings demonstrate that they have secondary activities in the process of axon guidance (Schnorrer and Dickson, 2004; Tabata and Takei, 2004). During tissue patterning these molecules largely affect cell fate by mediating changes in gene expression (Tabata and Takei, 2004). Experiments have now demonstrated that TGF-β family members, secreted from the dorsal side of the vertebrate spinal cord, not only serve to pattern dorsal tissue but also subsequently serve to repel commissural axons toward the ventral side of the spinal cord (Augsburger et al., 1999; Butler and Dodd, 2003). Similarly, Hh, secreted from the floor plate, not only serves to pattern ventral structures in the mammalian spinal cord but also functions to attract commissural neurons towards the ventral side of the spinal cord (Charron et al., 2003). In addition, Wnt signaling in both the vertebrate spinal cord and in the *Drosophila* ventral ganglion has been shown to direct axon guidance (Lyuksyutova et al., 2003; Yoshikawa et al., 2003). Although these morphogens are involved in *bona fide* cases of axon guidance, little is presently known about how these signaling systems, which generally direct cell patterning by modulating transcriptional changes, impinge on cytoskeletal dynamics and direct growth cone movement (Schnorrer and Dickson, 2004). In addition to the

morphogens, recent evidence has demonstrated that the insulin growth factor signaling pathway, a potent regulator of cell growth and division, is required cell autonomously for axon guidance in the developing *Drosophila* adult visual system (Song et al., 2003).

Structure and Function of Proteoglycans

The surface of nearly all cell types is highly decorated with proteoglycans, suggesting an important role for these unique proteins. Unlike other glycoproteins, these molecules bear unbranched repeating disaccharide side chains called glycosaminoglycans (GAGs), which typically have high levels of sulfation. These polymers are attached to serine residues found in the core protein and are generally 20 to 150 units long. Proteoglycans are grouped into classes by core protein sequence similarity and by the type of GAGs that they carry. The major groups of core proteins include the glypicans, syndecans and lecticans. Glypicans and syndecans are GPI-linked and transmembrane proteins respectively, while the majority of lecticans are secreted proteins. The types of GAGs a proteoglycan can bear include chondroiton/dermatan sulfate (CS/DS), heparan sulfate (HS) and keratin sulfate (KS). CS/DS is made up of repeating galactosamine and glucuronic/iduronic acid disaccharides. HS is composed of glucosamine and glucuronic/iduronic acid, and KS is composed of glucosamine and galactose. Glypicans and syndecans bear mainly HS, while the majority of lecticans bear CS (Bandtlow and Zimmermann, 2000; Perrimon and Bernfield, 2000).

The synthesis of GAGs is primarily carried out in the Golgi and is a process that involves many enzymes. Synthesis begins when a group of enzymes attach a

tetrasaccharide linker to a serine residue within the core protein. This serves as a common attachment site for all types of GAGs. Next, CS/HS/KS specific enzymes carry out polymerization of the disaccharide chain. Lastly, a third group of enzymes, each specific for a particular type of GAG, is responsible for modification of the completed GAG chain. This modification includes epimerization of some of the glucuronic acid to iduronic acid and the addition of sulfate groups to specific positions on individual saccharides. The variety of GAGs that exist coupled with their possible modifications and the particular core protein to which they attach create a hugely diverse population of molecules. This diversity is responsible for the variety of ligands these proteins can specifically interact with and it also contributes to the range of biological actions that proteoglycans can exert (Kramer and Yost, 2003; Sugahara et al., 2003). Some of these functions include, but are not limited to serving as structural components of the extracellular matrix, driving the formation of fluid filled spaces, functioning as co-receptors for intercellular signaling pathways and involvement in morphogen distribution (Filmus and Selleck, 2001; Hwang and Horvitz, 2002; Hwang et al., 2003; Sugahara et al., 2003; Tabata and Takei, 2004).

One of the best characterized functions of proteoglycans, particularly heparan sulfate proteoglycans (HSPGs), is their role as co-receptors for many intercellular signaling pathways. This requirement was first discovered in the case of fibroblast growth factor (FGF) signaling in cultured mammalian cells. Researchers demonstrated that repression of FGF/HSPG interactions decreased the binding affinity of FGF for its cell surface receptors. In addition, repression of FGF/HSPG interactions or removal of

HSPG sulfation blocked FGF dependent signaling events in cell culture assays. These results indicate that HSPGs are vital for FGF signaling (Rapraeger et al., 1991; Olwin and Rapraeger, 1992).

Subsequently, HSPGs were shown to be required for efficient signaling of TGF-βs (Lopez-Casillas et al., 1993), epidermal growth factor (EGF) (Aviezer and Yayon, 1994), hepatocyte growth factor (HGF) (Zioncheck et al., 1995), Wnts (Reichsman et al., 1996), and Hh (Bellaiche et al., 1998). All of these studies, save the last one involving Hh, were performed *in vitro* leaving the physiological relevance of these results unknown. However, subsequent experimentation in model organisms has demonstrated that most of these *in vitro* roles for HSPGs reflect an *in vivo* requirement for these molecules in multiple cell-cell signaling pathways. For instance, experiments performed in *Drosophila* have demonstrated that mutations in HSPG biosynthetic enzymes or core proteins compromise TGF-β, Hh, FGF and Wnt signaling *in vivo* (Jackson et al., 1997; Bellaiche et al., 1998; Lin et al., 1999; Lin and Perrimon, 1999; Tsuda et al., 1999; Bornemann et al., 2004; Han et al., 2004b; Takei et al., 2004). The requirement for HSPGs in vertebrate intercellular signaling is conserved, as similar HSPG-related mutations in these organisms also lead to signal transduction defects in TGF-β and Wnt signaling *in vivo* (Cano-Gauci et al., 1999; Paine-Saunders et al., 2000; Topczewski et al., 2001).

In addition to the role that HSPGs play as growth factor co-receptors, recent *in vivo* evidence from *Drosophila* demonstrates that these molecules also control Hh, Wnt and TGF-β gradient formation (The et al., 1999; Fujise et al., 2003; Bornemann et al.,

2004; Han et al., 2004b; Kirkpatrick et al., 2004; Takei et al., 2004). This is significant as these growth factors are known to serve as morphogens. A morphogen is a molecule that is produced at a source, forms a gradient over undifferentiated neighboring cells and consequently causes these cells to take on various fates in a concentration dependent manner. Therefore the alteration of Hh, Wnt and TGF-β gradients observed in HSPG mutants contributes to the overall patterning defects observed in these animals.

Chondroitin sulfate proteoglycans (CSPGs) along with HSPGs were traditionally recognized as major components of the extracellular matrix of cartilage. Indeed mutations in some CSPGs and CS modifying enzymes are linked with chondrodysplasias both in animal models and in humans alike, indicating that these molecules are important *in vivo* for the integrity of the skeletal system (Schwartz and Domowicz, 2002; Thiele et al., 2004). In addition there is recent evidence that CSPGs, like HSPGs, may act as co-receptors for certain growth factor signaling pathways. This has been demonstrated *in vitro* in the case of FGF and HGF, however the *in vivo* significance of these results is currently unknown (Bechard et al., 2001; Lyon et al., 2002; Trowbridge et al., 2002).

Roles for CSPGs in the Nervous System

Chondroitin sulfate proteoglycans are widely expressed in the nervous system and have been implicated in virtually all steps of development of this tissue (Bandtlow and Zimmermann, 2000; Bovolenta and Fernaud-Espinosa, 2000). One of the best characterized roles of CSPGs is their property as axon repellents (Margolis and Margolis, 1997). In many cases high levels of CSPGs are present in areas avoided by outgrowing

axons (Bandtlow and Zimmermann, 2000). For instance, CSPGs are normally expressed in areas of the mammalian retina that repel outgrowing retinal ganglion cell (RGC) axons, in addition, CSPGs can act to repel outgrowing RGC axons in *in vitro* axon guidance assays (Snow et al., 1991). Chondroitinase treatment (cleavage of CS from the core protein) of the mammalian retina causes RGC axons to deviate from their normal path towards the optic fissure and go into CSPG expressing areas that they would normally avoid (Brittis et al., 1992). These and other similar studies indicate that CSPGs may be required for axon guidance (Bandtlow and Zimmermann, 2000; Bovolenta and Fernaud-Espinosa, 2000).

Although the majority of studies on CSPGs have indicated that they serve as axon repellents there is some evidence that these molecules also have the capacity to mediate axon attraction in some contexts. Indeed, certain areas of the brain which have relatively high numbers of in growing axons also express high levels of CSPGs. Consistent with this, some chondroitin sulfate bearing proteins have been characterized as having both axon attractive and repulsive properties (Margolis and Margolis, 1997; Bandtlow and Zimmermann, 2000; Bovolenta and Fernaud-Espinosa, 2000). Despite a large amount of *in vitro* data concerning the roles of CSPG in axon guidance, the physiological relevance of these molecules has yet to be revealed by mutational analysis. In all cases explored thus far, the mutation of individual CS bearing core proteins or specific modifying enzymes in mouse did not cause any observable nervous system developmental defects (Harroch et al., 2000; Zhou et al., 2001; Brakebusch et al., 2002;

Uchimura et al., 2002). The lack of defects in these mutant mice is presumed to be due to functional redundancy between lectican family members/other modifying enzymes.

Consistent with their role as axon repellents CSPGs also appear to play a role in inhibiting axon regrowth after CNS injury. In "higher" vertebrates injury to the central nervous system (CNS), unlike that to the peripheral nervous system (PNS), is generally characterized by a lack of axon regeneration. This is in part due to inhibitory elements present in the CNS, as damaged axons from the CNS can grow into peripheral nerves much more efficiently. In further support of the presence of negative regenerative factors in the CNS, damaged PNS axons, which can readily regenerate in their native tracts, do not regrow effectively into CNS tissue (Morgenstern et al., 2002; Sugahara et al., 2003). One of the inhibitory constituents of the CNS appears to be CSPGs. Indeed, chondroitinase treatment of the damaged mammalian spinal cord or nigrostriatal tract is sufficient to allow much more efficient axonal regeneration in these structures (Moon et al., 2001; Bradbury et al., 2002). Similarly, data suggests that CSPGs are inhibitory to plasticity in the adult rat visual cortex as addition of chondroitinase is sufficient to reactivate ocular dominance plasticity in the mature animal (Pizzorusso et al., 2002).

Roles for HSPGs in the Nervous System

In the nervous system, HSPGs have been implicated in all steps of development including neuronal differentiation, cell migration, axon guidance, synaptogenesis, and synaptic plasticity (Yamaguchi, 2001). The breadth of the developmental processes that HSPGs are implicated in is mirrored by their widespread expression patterns (Ford et al.,

1994; Stipp et al., 1994; Watanabe et al., 1995; Hagihara et al., 2000; Ford-Perriss et al., 2003). For instance, perlecan is expressed in several areas of the mammalian CNS (Joseph et al., 1996; Winkler et al., 2002). This includes expression in the embryonic ventricular zone (VZ), an area in which massive neurogenesis occurs (Nurcombe et al., 1993; Joseph et al., 1996; Garcia-Verdugo et al., 2002). Interestingly, Perlecan, like some other HSPGs, is a potent co-receptor for FGF signaling and FGF has been characterized as being vital for neurogenesis occurring in the VZ (Aviezer et al., 1994; Ghosh and Greenberg, 1995; Reuss and von Bohlen und Halbach, 2003). These findings suggest Perlecan expressed here may play a role in promoting FGF driven neurogenesis. However, although Perlecan knockout mice show defects in brain development, these phenotypes are currently considered to be secondary to problems with basement membrane structure and are not consistent with defective neurogenesis in the ventricular zone (Arikawa-Hirasawa et al., 1999; Costell et al., 1999). The lack of neurogenic defects in perlecan nulls may reflect the ability for other HSPGs to substitute in this process (Yamaguchi, 2001).

Unlike mouse knockouts of *perlecan*, loss of the homologous gene in *Drosophila terribly reduced optic lobes* (*trol*) causes severe defects in the developing larval brain that are likely due to a more direct role of this protein in CNS-specific growth factor signaling (Voigt et al., 2002; Park et al., 2003). *trol* mutant animals show defects in larval neuroblast proliferation. These neuroblasts are responsible for populating the larval optic lobes which develop into the adult visual system. *trol* mutants show a genetic interaction with *hh* and *fgf* mutants in this system indicating that loss of *Drosophila* Perlecan may

affect endogenous signaling by these intercellular pathways. It is currently unknown whether this signaling occurs directly in neuroblasts or elsewhere (Voigt et al., 2002; Park et al., 2003).

There is a considerable amount of *in vitro* and expression data indicating that HSPGs may play a role in cell migration in several tissues including the nervous system, particularly the specialized form of migration that characterizes axon guidance (Yamaguchi, 2001; Toba et al., 2002; Feistritzer et al., 2004; Moon et al., 2004). For instance, syndecan-3 is one of several HSPGs expressed on actively outgrowing axon tracts (Kinnunen et al., 1998). Several of the glypicans are also expressed on axon tracts during outgrowth (see below for more details). The axonal localization of some HSPGs corresponds nicely with the results from *in vitro* experiments suggesting that these molecules are involved in axon guidance. Addition of exogenous HS or removal of endogenous HS leads to defects in axon guidance. For instance, application of exogenous HS or heparinase (cleaves HS from core protein) causes defects in pioneer axon guidance in cultured cockroach embryos. Conversely, addition of enzymes that cleaved other types of GAG chains had no effect on axon guidance here (Wang and Denburg, 1992; Rajan and Denburg, 1997).

Similar perturbations to HSPGs in a vertebrate system, *Xenopus*, also led to defects in axon guidance in the retinotectal system (Walz et al., 1997). Interestingly in both the *Xenopus* and cockroach experimental systems defects in FGF signaling cause similar pathfinding defects (McFarlane et al., 1995; McFarlane et al., 1996). Addition of exogenous FGF after heparinase treatment of the *Xenopus* retinotectal pathway partially

rescues defects in axon outgrowth (Walz et al., 1997). These results suggest that HSPGs in both vertebrates and invertebrates play an important role in axon guidance, perhaps in part by mediating FGF driven axon pathfinding. However these studies leave many questions about the role of HSPGs in axon guidance unanswered. For example, what is the physiological role of these molecules and which of the HSPG core proteins mediate these affects. Mutational analysis is required to definitively answer these queries.

HSPGs have also been implicated in the process of synaptogenesis. One of the best characterized HSPGs involved in this process is Agrin. Agrin is required *in vivo* for the stabilization of acetylcholine receptor clustering at the vertebrate NMJ and mice mutant for this protein fail to make normal NMJs (Gautam et al., 1996). HS attached to this protein is required for binding to certain ligands including FGF and laminin and therefore likely play an important role in the synaptic organization properties of Agrin (Martin, 2002).

At central synapses the majority of excitatory inputs are onto dendritic spines, or small stubby mushroom-like processes that protrude from the dendritic tree of a neuron (Harris and Kater, 1994). Syndecan-2 is expressed in neurons and localized to synapses (Hsueh and Sheng, 1999). In primary cultures of rat hippocampal neurons, Syndecan-2 is localized to dendritic spines concomitant with spine maturation (Ethell and Yamaguchi, 1999). Overexpression of Syndecan-2 in these neurons causes the premature maturation of dendritic spines suggesting that Sydecan may regulate changes in dendritic spine morphology, a process that is linked with the induction of long-term potentiation (LTP) (Ethell and Yamaguchi, 1999). LTP induction, the process believed to underlie learning

and memory, is suppressed upon addition of heparinase or exogenous heparan sulfate/soluble Syndecan-3 to hippocampal slices (Lauri et al., 1999). Additionally, Syndecan-3 mutant mice show enhanced LTP induction (Kaksonen et al., 2002). These findings, and those of the involvement of Agrin in synapse organization suggest that HSPGs are vital to both synaptogenesis and synaptic plasticity.

Glypicans in Nervous System Development

Dally-like, the molecule under primary consideration in this study, is one of two glypicans that have been identified in the *Drosophila* genome (Nakato et al., 1995; Khare and Baumgartner, 2000). All glypicans share in common several structural attributes including cell surface attachment by a glycophosphatidylinositol (GPI) linkage, the presence of heparan sulfate side chains and 14 conserved cysteine residues that through disulfide bonding probably confer a similar three dimensional structure on all glypicans (Filmus and Selleck, 2001). Similar to other HSPGs, glypicans serve as co-receptors for many growth factor signaling pathways, including FGF (Steinfeld et al., 1996; Hagihara et al., 2000; Grisaru et al., 2001; Galli et al., 2003), Wnt (Lin and Perrimon, 1999; Tsuda et al., 1999; Kirkpatrick et al., 2004), Hh (Desbordes and Sanson, 2003; Lum et al., 2003; Han et al., 2004a) and TGF-β signaling (Jackson et al., 1997; Grisaru et al., 2001). In addition, glypicans are required for the proper establishment of morphogen gradients (Fujise et al., 2003; Kirkpatrick et al., 2004). Therefore, it is not surprising that glypicans are necessary for diverse patterning events during development. The role of glypicans appear to be conserved between vertebrates and invertebrates as mutations in vertebrate

glypicans (including human) also cause developmental patterning defects (Pilia et al., 1996; Cano-Gauci et al., 1999).

There are six known glypicans in mammals. At least one of these glypicans is expressed in almost every tissue and five of six are highly enriched in the nervous system. Glypican-1 is expressed in the developing rodent brain, including expression on axon tracts. This protein continues to be expressed in the nervous system of adult animals (Litwack et al., 1998). Likewise, glypican-2 is also found in the CNS in a specific pattern including localization to axon tracts and growth cones at the time of axon outgrowth (Ivins et al., 1997). Glypican-4 (Veugelers et al., 1998), glypican-5 (Saunders et al., 1997) and glypican-6 (Paine-Saunders et al., 1999) are also expressed in the mammalian brain. The expression pattern of these genes, particularly that of Glypican-1 and 2, suggests a possible role for these molecules in the process of axon outgrowth/guidance.

Despite the expression pattern of these proteins there is currently no *in vivo* genetic evidence that these molecules are physiologically required for mammalian nervous system development. For instance, mouse knockouts in glypican-3 have no reported nervous system defects (Cano-Gauci et al., 1999; Chiao et al., 2002). However, in *Xenopus*, morpholino knockdown of glypican-4 causes defects in dorsal forebrain patterning strongly suggesting that glypicans are physiologically important for proper nervous system development in vertebrates (Galli et al., 2003). Since a drug-induced disruption of FGF signaling phenocopies Glypican-4 morpholino effects, Galli et al. has proposed a role for this glypican in FGF signaling during specification of the dorsal forebrain.

Unlike in vertebrates, there is direct evidence that glypicans are involved in the development of the invertebrate nervous system. In *Drosophila* the first glypican discovered, *dally*, was isolated because animals carrying a mutation for this gene had defects in phototaxis. Subsequent analysis of *dally* mutants revealed that these animals had defects in patterning of the developing adult visual system (Nakato et al., 1995). One of the defects observed in *dally* mutants was a failure of lamina precursor cells to proceed through mitosis in a normal fashion, a process that is driven by an intercellular signal from photoreceptor axons arriving from the eye. Like Dally, Dally-like (Dlp) has roles in nervous system development. In *dlp* mutant adult flies there are extra wing mechanosensory bristles and these animals also show disruption in the patterning of the eye (Kirkpatrick et al., 2004).

The findings in the invertebrate nervous system demonstrate that glypicans play a vital role in aspects of tissue patterning similar to their role in other body regions. However tantalizing evidence from *in vitro* experimental systems (cultured cockroach embryos and retinotectal projection in Xenopus) combined with the striking expression pattern of the mammalian glypicans on actively outgrowing axons suggest that these molecules might play a key role in the establishment of neuronal connectivity in the developing brain. Therefore using the powerful genetic tools available in *Drosophila* we set out to ascertain the physiological role of the glypican Dally-like in axon pathfinding.

Summary of Results

Here we demonstrate that Dlp is required to establish proper axonal architecture in *Drosophila*. Dlp is expressed on actively outgrowing embryonic axons as well as on outgrowing adult photoreceptor axons. In addition, Dlp is expressed in other regions of the developing adult visual system. While *dlp* mutations do not affect the patterning of neurons or glia in embryonic or larval stages, *dlp* mutants do have defects in embryonic and photoreceptor axon morphology. Functional analysis of the adult visual system revealed that *dlp* mutations also cause defects in the response to light stimuli.

Mosaic experiments in the visual system demonstrate that Dlp is required in the eye for photoreceptor axon pathfinding. We also demonstrate that *dlp* mutant photoreceptor phenotypes can be rescued by expression of a Dlp transgene. Tests for genetic interactions between *dlp* and *slit/robo* mutants did not reveal an interaction. Lastly we also examined photoreceptor axon development in flies mutant for another HSPG, *Drosophila* Syndecan (Sdc), and demonstrate that these animals have phenotypes largely distinct from *dlp* mutant animals. These experiments demonstrate that distinct core proteins, especially the glypican Dlp, are physiologically required for the axon guidance.

RESULTS

Dally-like Expression in the Embryonic Nervous System

We used a monoclonal antibody raised against the *Drosophila* glypican Dlp to ascertain the expression pattern of this molecule during the development of the embryonic nervous system (Lum et al., 2003). Detection of Dally-like by this antibody is specific as there is no detectable immunoreactivity in *dlp* mutant embryos or clones in imaginal disks (data not shown)(Kirkpatrick et al., 2004). Staining of embryos reveals that Dlp is expressed in the CNS in a segmentally repeating pattern beginning around stage 12, the time at which pioneer axon outgrowth begins (Figure 6A, arrowheads). These axons are the first to establish the tracts that many other axons will follow later in development as they grow towards their respective targets (Lin et al., 1994). By stages 13 and 14 it is apparent that Dlp is expressed in neurons and localized to their axons. This is first readily observable in stage 13 embryos as Dlp immunoreactivity is very high in both the anterior and posterior commissures of each segment, structures composed of axons crossing the midline (Figure 6B, labels). It is notable that at this developmental time point Dlp expression is rather low in the longitudinal tracts that connect each segment. However, by stage 14 these longitudinal tracts connecting each segment begin to show Dlp immunoreactivity (Figure 6C, arrow). Dlp is also detectable on peripheral axons that may be either exiting or entering the CNS (Figure 6D, arrowheads). By stage 16 of embryonic development Dlp is highly expressed on axons running in both the longitudinal tracts and in the anterior and posterior commissures (Figure 6D). Therefore,

similar to the expression pattern of Glypican 1 and 2 in mammals, the glypican Dlp is expressed in the developing *Drosophila* nervous system and is particularly localized to actively outgrowing axons (Ivins et al., 1997; Litwack et al., 1998).

dally-like Mutations Cause Embryonic Axon Guidance Defects

In collaboration with Karl Johnson of David Van Vactor's group we examined the implications of zygotic mutation of *dlp* on embryonic nervous system development. Recent reports have demonstrated the removal of both maternal and zygotic Dlp by either RNAi or the production of germ line clones leads to severe defects in Hh signaling in the early embryo (Desbordes and Sanson, 2003; Han et al., 2004a; Kirkpatrick et al., 2004). One of the consequences of defective Hh signaling in the early embryo is a segment polarity defect which could severely affect the development of the nervous system (Hatini and DiNardo, 2001). In addition, disruption of *dlp* and *dally* can cause defects in Wingless signaling (Wg, a Wnt family member), a pathway that is required for certain nervous system patterning events (Lin and Perrimon, 1999; Tsuda et al., 1999; Patapoutian and Reichardt, 2000; Baeg et al., 2001; Melton et al., 2004). Therefore we examined *dlp* mutant (zygotic only) embryos to determine if there were defects in the patterning of cell populations of the CNS. We stained with anti-Wrapper, a protein expressed in midline glia, and found no patterning defects in this cell population (Noordermeer et al., 1998) (Figure 6E,F). In addition staining with anti-Engrailed, a protein expressed in a subset of CNS neurons, revealed no patterning defects in this cell

population (Figure 6G,H) (Siegler and Jia, 1999). By these measures, our results indicate that zygotic loss of Dlp does not cause defects in the patterning of the cells of the CNS.

Staining of *dlp* mutants with anti-FasciclinII antibody revealed that these animals had defects in axon architecture indicative of aberrant axon guidance. FasciclinII (FasII) is a neural cell adhesion molecule that is important for various aspects of nervous system development including axon guidance (Lin et al., 1994). This molecule is expressed on three distinct fascicles, or tracts, in stage 17 embryos and has been used for identifying mutations in axon guidance (Figure 7A). While wild-type animals have three continuous independent FasII-positive tracts on either side of the midline (Figure 7A, dashed line), *dlp* mutants show breaks in the third fascicle, mingling of the fascicles (especially 2-3, numbered away from the midline) and occasional fusion of the second and third fascicle (Figure 7A-D) [250<n<380 for wild-type, dlp^1/dlp^1 and dlp^2/dlp^2]. Notably *dlp* mutants show a very low penetrance of aberrant midline crossing, a phenotype sometimes seen in conjunction with mutations affecting fasciculation (Figure 7E) (Guthrie, 2001). These results demonstrate that zygotically transcribed *dlp* is vital for the establishment of normal axonal architecture of the embryonic nervous system.

Dally-like Expression in the Developing Adult Visual System

In addition to our observations made in the embryonic nervous system in collaboration with the Van Vactor laboratory, our own laboratory explored the role of Dlp in the developing adult visual system which is comprised of the larval eye imaginal disks and the optic lobes of the larval brain. The adult *Drosophila* compound eye is

composed of approximately 750 units or ommatidia. Each of the ommatidium is composed of a hexagonal lens flanked on every other corner by a mechanosensory bristle (Figure 11A). The lens of each ommatidium overlies the light sensing machinery. This machinery consists of eight photoreceptor cells and many support cells which include cone and pigment cells (Figure 11C). The eight photoreceptor cells in each ommatidium project axons to a stereotypic position within the optic lobe of the brain. In particular, photoreceptor cells 1-6 (R1-6) project directly to the lamina, the distal most portion of the optic lobe, while photoreceptor cells 7 and 8 (R7&8) project through this layer to the more proximal portion of the optic lobe, the medulla. The pattern of axon projection is also retinotopographic in nature (Clandinin and Zipursky, 2002).

The crystalline structure of the eye and the projection of the photoreceptor axons to the optic lobe are established during larval/pupal development. The eye imaginal disk, which gives rise to the adult eye, is patterned in a wave-like fashion. Cell division and differentiation begins in the most posterior portion of the disc and proceeds to the presumptive anterior edge of the adult eye. This wave of cell division is characterized by a temporary shortening of the imaginal disc cells which causes a physical depression in the eye disc known as the morphogenetic furrow. As cells differentiate into photoreceptors they send out axons toward their respective target regions in the optic lobe (Clandinin and Zipursky, 2002).

Using the anti-Dlp antibody described above we tested for Dlp immunoreactivity in the developing visual system. In the third instar eye disc, we detected Dlp on differentiated photoreceptor cell bodies and axons, as well as on cells undergoing

division within the morphogenetic furrow (Figure 8.1A,B). In the optic lobe, Dlp immunoreactivity can be detected on the surface of a subset of photoreceptor projections and on expanded R1-6 growth cones that make up the lamina plexus, as evidenced by Dlp colocalization with a photoreceptor axon specific marker (Figure 8.1/2C-E). In particular, although Dlp is expressed on all differentiated photoreceptors the localization of this protein to axons in the optic lobe is restricted to those that make up the boundary between the photoreceptor projections and adjacent tissues (Figure 8.1D). Dlp is also expressed on the medulla glia, as well as the medulla neuropil glia (Fig. 8.2E,F). High levels of Dlp were found in the mushroom body neuropil and on neuroblasts of the inner and outer proliferative centers (Figure 8.2G,H). The mushroom body is a brain region that is vital for at least some forms of associative learning and memory and the proliferative centers give rise to various cell types that populate the brain lobes (Dumstrei et al., 2003; Siwicki and Ladewski, 2003). This staining pattern suggests that Dlp may play a vital role in the development of multiple aspects of the *Drosophila* adult visual system including the guidance of photoreceptor axons to their target regions.

Dally-like is Required for Photoreceptor Axon Guidance

In order to evaluate the pattern of photoreceptor projections from the retina to the optic lobe, *dlp* mutant and control third instar larvae and pupae (40 hours post pupariation) were stained with a photoreceptor membrane-specific antibody (MAb24B10) [dlp^1/dlp^2 n=25 optic lobes, $dlp^1/Df(3L)$ n=24, $dlp^1/+$ n=44, $Df(3L)/+$ n=46, wild-type n=40]. As described above, photoreceptor axons from R1-6 terminate in the lamina, the first optic

ganglion, while R7 and R8 project to the medulla (Figure 9A). In approximately 50 percent of *dlp* mutant third instar larvae the R1-6 termini, which form the lamina plexus, were irregular and thickened (*dlp*^{1}*/Df(3L)*, Figure 9A,B&E). This thickening may reflect a defect in the targeting of axons or defects in growth cone expansion which occurs during this stage of development. In addition, approximately 80 percent of *dlp* mutant animals we examined had other abnormalities in photoreceptor axon projections, including fibers that aberrantly crossed between ommatidial bundles (Figure 9B inset) and/or photoreceptor process expansions outside the normal termination zone of the lamina plexus (*dlp*^{1}*/Df(3L)*, data not shown, Figure 9E).

Despite the lamina defects observed in *dlp* mutants there does not appear to be any major problems in the termination pattern of R7/8 termini in the medulla during the third instar stage. After *Drosophila* larvae pupate, the whole animal, including the nervous system, undergoes major metamorphic changes (Tissot and Stocker, 2000). In the visual system the morphology of the photoreceptor axons change and further elaboration of neural circuitry yields the adult visual system. Evaluation of 24B10 staining in 40 hour (40h) old pupae revealed at this stage *dlp* mutants now have defects in R7/8 termini in the medulla [*dlp*^{1}*/dlp*^{2} n=23 optic lobes, *dlp*^{1}*/Df(3L)* n=14, *dlp*^{1}/+ n=34, *Df(3L)*/+ n=36, wild-type n=25] (Figure 9C,D & F). In greater than 80 percent of the mutant pupal optic lobes, we observed irregularities in the R7 and R8 termini, such that these two populations of endings no longer segregate into two precisely arrayed layers (Figure 9C,D&F). In a subset of mutants we also observed crossover of R7 axons to neighboring medulla cartridges (~50 percent) and misrouting of R7/8 axons on their

course to the medulla (~20 percent) similar to phenotypes we observed in *syndecan* (*sdc)* mutants ($dlp^{1}/Df(3L)$, data not shown, Figure 9F & Figure 18D-G). Since the field of R7/8 termini are normal during the third instar stage, these defects presumably arise later in development and may be due to defects in the cells that interlace their axons with those of the photoreceptors. For example, failure of medulla neurons to differentiate or send out axons into the medulla could result in the spacing abnormalities observed in the R7/8 layers. The defects observed in third instar and 40h *dlp* mutant pupae indicate that in the developing adult visual system, as in the embryonic nervous system, Dlp plays a role in the specification of correct axon architecture.

Dally-like is Required for Visual System Function

We tested *dlp* mutants for defects in the function of the adult visual system by classic electroretinogram (ERG) recording from the eyes of adult flies. This technique allows for extracellular recording of the photoreceptor population's response to a light stimulus. Following exposure of the retina to light, depolarization of photoreceptors produces a large negative deflection in extracellular voltage. In wild-type flies this sustained voltage deflection is preceded by a positive "on-transient". When the light stimulus is terminated, there is an "off transient" deflection followed by a restoration of the extracellular voltage to the resting level (Figure 10B). The "on" and "off-transients" reflect currents associated with synaptic transmission from photoreceptors to postsynaptic lamina and medulla neurons (Heisenberg, 1971). *dlp* adult escapers showed statistically significant defects in the photoreceptor response and in both "on" and "off-transients"

[*dlp*^{1}/*Df(3L)* n=11 animals, *dlp*^{1}/+ n=10, *Df(3L)*/+ n=10, wild-type n=18] (Figure 10A,B). The substantial reduction in "on" and "off-transients" appears not to be solely secondary to a reduction in the photoreceptor response since *dlp* mutants with a photoreceptor response in the control range (8-13 mV) also showed markedly reduced transients (36 percent of *dlp*/+ average value, Figure 10B) [*dlp*^{1}/*Df(3L)* n=3, *dlp*^{1}/+ n=10, *Df(3L)*/+ n=10]. These defects in photoreceptor depolarization and transients indicate *dlp* mutants have reduced photoreceptor currents and synaptic transmission.

Photoreceptors in *dally-like* Mutants are Patterned Properly

Mutations in *dally-like* cause defects in the development of the adult eye (Figure 11A&B). Several screens have been carried out in *Drosophila* to isolate genes that are required for photoreceptor axon pathfinding. Mutations isolated from these screens that affect both eye patterning and axon guidance have typically been ignored by researchers for the purpose of axon guidance studies. This has been done primarily to avoid the complication of studying axon guidance defects that might be secondary to retinal patterning defects. It is possible however those mutations that are ignored by these criteria are involved directly in axon guidance and their defects are not simply secondary to retina patterning defects. However, since it is possible that defects in photoreceptor patterning could cause defects in axon guidance we examined the fate of the photoreceptor cells in the *dlp* mutant eye.

Examination of *dlp* mutant eyes by scanning electron microscopy (SEM) revealed that these animals had reduced [388 ommatidia, n=6, compared to 620 ommatidia in

wild-type, n=4] and roughened eyes (Figure 11A,B) (Kirkpatrick et al., 2004). Mutant ommatidium often had four or five sides as opposed to the wild-type hexagonal ommatidia. Mutant ommatidium also showed both losses and duplications of mechanosensory bristles (Fig. 11A,B). These phenotypes are consistent with defects in accessory cell patterning and do not necessarily indicate photoreceptor patterning is affected. In order to examine the patterning of photoreceptors in *dlp* mutants, serial thin sections of the eye were produced and examined. Careful inspection of these sections revealed that while *dlp* eyes contained ommatidia that were irregularly shaped and larger than wild-type [27 percent larger, n=25, compared to wild-type, n=27], all photoreceptors were present in each ommatidium. *dlp* mutant ommatidia also had normal polarity and symmetry across the dorsal-ventral (D-V) axis of the retina (Fig. 11C,D).

These results suggest that the roughening in *dlp* mutant eyes is caused by defects in accessory cell specification. To confirm that the differentiation program of *dlp* mutant photoreceptors was intact, we visualized specific photoreceptor cell types in the developing eye disc. Pan-neural (anti-Elav), R8 (anti-Senseless) and R7 (anti-Prospero)-specific markers were expressed correctly in *dlp* mutants (Figure 11E,E',F&F') [dlp^1/dlp^2 n=17 eye discs, $dlp^1/Df(3L)$ n=9, wild-type n=20] (Robinow and White, 1988; Frankfort et al., 2001; Cook et al., 2003). These findings suggest that axon projection defects in *dlp* mutant larvae and pupae are not secondary to photoreceptor patterning abnormalities.

Glial and Neural Patterning is Intact in *dally-like* Mutants

Correct photoreceptor axon projection requires proper specification and placement of glial cells (Tayler and Garrity, 2003). At least two populations of glia direct the guidance of photoreceptor axons. Glia that migrate into the eye from the optic stalk are required to direct photoreceptor axons to grow out of the eye imaginal disc and into the optic stalk (Rangarajan et al., 1999). In addition, glial cells that reside adjacent to the lamina plexus are required for instructing the R1-6 axons where to terminate (Poeck et al., 2001). To determine if *dlp* affects axon projection via failure of glial cell differentiation or migration we examined the organization of glial cells in the retina and optic lobe of *dlp* mutant third instar larvae. Staining with the glial specific anti-Reversed Polarity (anti-Repo) antibody revealed that the retinal and lamina glia required for photoreceptor axon guidance were found in the correct number and location in *dlp* mutant animals, indicating that patterning defects of these critical cells cannot account for axon guidance defects (Figure 12A-D) [dlp^1/dlp^2 n=6 larvae, $dlp^1/Df(3L)$ n=8, wild-type n=10]. In particular the glia of the eye are correctly specified and migrate to the proper position behind the morphogenetic furrow in *dlp* mutant larvae (Figure 12A,B). In addition, the three layers of glia, the epithelial, marginal and medulla glia, that flank the termination zone for R1-6, as well as the glia that are adjacent to the R7/8 termination zone in the medulla, the medulla neuropile glia, are properly specified in the mutants (see labels, Figure 12C,D).

We also examined the specification of lamina neurons in *dlp* mutants. These cells are not required for axon ingrowth but their presence indicates proper retinal assembly

and growth factor signaling as their proliferation and specification depends on the growth factors (Hh and EGF) that are delivered on the surface of ingrowing photoreceptor axons (Selleck and Steller, 1991; Huang and Kunes, 1996). Lamina neurons, detected with anti-Dachshund (anti-Dac) antibody staining, were present in *dlp* mutant third instar larval brains (Fig. 12E,F), confirming that photoreceptor-induced patterning of the lamina occurred correctly [$dlp^{1}/Df(3L)$ n=9 larvae, wild-type n=5].

These results are significant in light of the previously described role of maternally and zygotically contributed *dlp* in Hedgehog signaling in the early embryo. Hh signaling defects in the developing visual system result in the failure of proper morphogenetic furrow initiation and lead to aberrant glial cell migration (Ma et al., 1993; Rangarajan et al., 2001; Hummel et al., 2002). In particular, glial cells of the eye, which under normal circumstances only migrate to the portion of the eye disc where photoreceptor cells have differentiated, migrate to more anterior positions along Bolwig's Nerve (larval photoreceptor axons) (Hummel et al., 2002). This aberrant anterior migration of glia triggers photoreceptor axons to grow anteriorly instead of posteriorly towards the optic stalk. In addition, Hh delivered on the surface of ingrowing photoreceptor axons is required for the final cell division that the lamina precursor cells undergo as they develop into the mature lamina neurons (Huang and Kunes, 1996). Hence, mutations which abolish these axons or Hh signaling result in the absence of lamina neurons (Selleck and Steller, 1991; Huang and Kunes, 1996). We observed normal eye glia migration and intact lamina neuron populations in *dlp* mutant third instar indicative of intact Hh

signaling in the visual system. These results demonstrate that zygotically transcribed *dlp* is dispensable for Hh signaling during visual system development.

Analysis of Axon Morphology in *dally-like* Mosaics

Dlp is expressed on photoreceptor cells/axons, glia, and neuroblasts. To determine what cell types require Dlp for visual system assembly and function we conducted somatic mosaic studies. Mosaic tissue analysis is characterized by the generation of cells homozygous mutant for a particular gene in an animal that is otherwise primarily composed of cells heterozygous (in the case of a recessive gene "wild-type") for that mutation. This type of analysis allows one to determine which cells require a protein for a given process. In addition, mosaic analysis allows the researcher to examine the function of a lethal gene in a tissue-specific manner at a post-lethal developmental stage. The mosaic system we chose to utilize was developed in order to screen for genes involved in photoreceptor axon pathfinding that might otherwise cause pre-visual system development lethality if mutated in the entire animal.

This technique allows the generation of an eye primarily composed (70-90 percent) of mutant cells that are present in an otherwise heterozygous animal and is achieved using a variation of the *FLP/FRT* system (Newsome et al., 2000). Flippase (FLP), a protein that drives mitotic recombination at FRT sites found on both the mutant and non-mutant homologous chromosomes, is expressed under the control of the *eyeless* promoter, and therefore, mitotic recombination will occur only in cells of the developing eye (site of *eyeless* expression). Successful recombination and subsequent cell division

will yield two daughter cells, one that carries no *dlp* mutation and one that is homozygous *dlp* mutant. In the system that we utilized, the non-*dlp* mutant chromosome bears a mutation in the *minute* locus. This serves a two-fold purpose. Firstly, mutations in *minute* are homozygous lethal so cells that get two non-*dlp* mutant chromosomes and therefore receive two copies of the *minute* containing chromosome consequently die. Secondly, cells that do not undergo mitotic recombination and remain heterozygous for both the *minute* and *dlp* mutant loci divide much more slowly then the homozygous *dlp* mutant cells owing to the fact that heterozygousity at the *minute* locus causes retardation of cell growth and division. All told, this mosaic system yields an eye composed primarily of photoreceptors mutant for *dlp* that project axons to a heterozygous ("wild-type") brain. This will allow us to determine if removal of Dlp only from the eye is sufficient to cause defects in photoreceptor axon projection.

dlp homozygous mutant photoreceptors projecting to a heterozygous (normal) brain showed a number of morphological abnormalities. Photoreceptor-specific antibody staining (24B10) of animals with *dlp* mutant eyes revealed that sixty-seven percent of the optic lobes examined had crossover of axons between ommatidial bundles and/or photoreceptor process expansions outside the normal termination zone of the lamina plexus, defects that were also common in *dlp* homozygous mutants (Fig. 13A,B) [n=33 brain hemispheres]. Comparably, only 8 percent of hemispheres from animals with wild-type "control" clones in the eye showed similar defects in photoreceptor axon morphology [n=37]. Interestingly, there was equal penetrance (approximately 25 percent) of the lamina thickening phenotype in optic lobes from animals bearing *dlp* mutant eyes

or control eyes, suggesting that loss of Dlp in the eye does not cause the lamina thickening phenotype in homozygous *dlp* mutant animals. Overall, these results indicate that Dlp is required in the retina for some aspects of correct photoreceptor axon guidance.

In addition to larval photoreceptor axon morphological evaluation, we examined the effect of eye-specific *dlp* mutations on axon morphology in the 40 hour pupal stage. While homozygous *dlp* mutant animals have defects in the spacing and separation of the R7/R8 layer in the medulla at the 40h pupal stage (Figure 9C,D & Figure 13C), these abnormalities were absent from animals with *dlp* mutant retinas and *dlp/+* optic lobes [n=30 lobes] (Figure 13D). These results demonstrate that Dlp expression in the brain is sufficient for proper R7/8 termini morphology.

Analysis of Visual System Function in *dally-like* Mosaics

In order to evaluate the tissue-specific requirement for *dlp* in the physiological function of the visual system, we employed a second FLP/FRT based somatic mosaic strategy that allows both the generation of *dlp* mutant photoreceptors while selectively killing non-mutant photoreceptors. This method removes the functional contributions from both heterozygous and +/+ photoreceptors (Stowers and Schwarz, 1999), allowing one to evaluate the functional contribution from only mutant photoreceptors. This is achieved in a manner similar to the method described above. However, in this case the non-*dlp* mutant chromosome bears a GMR-hid transgene instead of carrying a *minute* mutation. This transgene induces apoptosis in all non-*dlp* mutant photoreceptors, thereby creating an eye that is comprised solely of homozygous mutant photoreceptors. Animals

with *dlp* mutant photoreceptors [n=16] projecting to a heterozygous optic lobe did not show any statistically significant defects in ERG recordings as compared to the light response from control animals where recombination was induced with a dlp^{+} (control) chromosome [$p<.05$, n=10] (Figure 13E). These results demonstrate that the ERG defects found in *dlp* mutants are produced by loss-of-function in the optic lobe and not in the eye.

Dally-like Transgenic Rescue

The morphological and functional evaluation of *dlp* mosaic animals described above demonstrates that Dlp is required in the eye for proper axon guidance but is not required there for proper visual system function. In order to further evaluate the tissue-specific requirements of Dlp we performed transgenic rescue on *dlp* mutants. The *UAS/GAL4* system was used to express a *UAS-dlp* transgene in various tissue/temporal-specific patterns (different GAL4 lines) in a *dlp* mutant background (Brand and Perrimon, 1993). We generated *Drosophila* lines that carried both a mutation in *dlp* and one of three different *UAS-dlp* transgenes. In addition, we generated complementary lines carrying other *dlp* mutant alleles and a series of *GAL4* lines that would express Dlp in several developmentally relevant patterns when crossed to *UAS-dlp* fly strains. The GAL4 transgenes utilized included the ubiquitously expressed *daughterless-GAL4*, the neuronal-specific *elav-GAL4*, the glial-specific *repo-GAL4*, the photoreceptor-specific *GMR-GAL4* and lastly the eye-specific *eyeless-GAL4*.

By expressing Dlp in various cell types via this diverse group of GAL4 lines we hoped to gain further insight as to which cell types must express Dlp in order to rescue the various visual system phenotypes observed in *dlp* mutants. However, when we crossed the *UAS-dlp* transgene containing *dlp* heterozygous mutant flies to flies that bore only a different *dlp* mutant allele and no GAL4 driver there was a rescue of virtually all *dlp* mutant phenotypes. This occurred regardless of which *UAS-dlp* construct we utilized indicating that the low level of "leaky" expression that can occur from these transgenes is sufficient to rescue *dlp* mutants. This rescue included a partial rescue of lethality (data not shown), a full rescue of eye roughening in surviving animals (dissection microscope level of resolution, data not shown) and rescue of axon projection defects in both larval and pupal stages (Figure 14 A-F) [(larval - *UAS-dlp39.2/+; dlp^1/Df(3L)* n=18 optic lobes, *UAS-dlp39.3/+; dlp^1/Df(3L)* n=24, *UAS-dlp39.3/+; dlp^1/dlp^2* n=20, *UAS-dlp HA #5/+; dlp^1/dlp^2* n=14, all groups combined for phenotypic quantitation) (pupal - *UAS-dlp HA #5/+; dlp^1/dlp^2* n=14 optic lobes)]. Therefore we were unfortunately unable to use transgenic rescue to elucidate the sites of Dlp activity in visual system development. However, these results do verify that the phenotypes observed in *dlp* mutants are due to loss of this protein as *dlp* containing transgenes are sufficient to rescue these defects.

dally-like* Interactions with *slit/robo* and *syndecan

During the course of our research into the role of Dlp in visual system development, our collaborators in the Van Vactor laboratory, as well as a second group, published a description of the role of another HSPG, *Drosophila* Syndecan (Sdc), in

embryonic axon guidance (Johnson et al., 2004; Steigemann et al., 2004). The primary defect in *sdc* mutant embryos is one of aberrant midline crossing, a defect rarely observed in *dlp* mutants (Figure 15A & 16C,E) (Johnson et al., 2004; Steigemann et al., 2004). Further evaluation of *sdc* mutants by our collaborators revealed that these embryos also have fasciculation defects similar in penetrance and severity to those observed in *dlp* mutant embryos (Figure 15B). The previous studies on *sdc* demonstrated that these mutations showed a genetic interaction with mutations in components of the Slit/Robo signaling pathway. Removal of one copy of *sdc* and one copy of *slit/robo* in the same animal is sufficient to increase the penetrance of midline crossing defects seen in either heterozygote alone (Figure 15C) [all genotypes $130<n<350$] (Johnson et al., 2004). This type of genetic interaction can be interpreted as a result of heterozygous loss of two components functioning in the same signaling pathway. Johnson et al. also demonstrated that Sdc could physically interact with Slit and that mutations in *sdc* disrupt the Slit gradient found at the midline (Johnson et al., 2004). Johnson et al. therefore suggests that Sdc functions in the Slit/Robo signaling pathway by modulating the distribution of the ligand Slit.

The Van Vactor group also demonstrated that Dlp expressed in neurons under the control of the UAS/GAL4 system was sufficient to partially rescue *sdc* mutant phenotypes. This suggests that Dlp and Sdc are functionally redundant in Slit/Robo signaling and therefore implicate Dlp as part of the Slit/Robo signaling pathway as well. This hypothesis is consistent with other available data demonstrating that certain glypicans can physically interact with Slit (Liang et al., 1999; Hu, 2001; Ronca et al.,

2001; Zhang et al., 2004). Therefore to test this hypothesis our collaborators assayed for a genetic interaction between Dlp and Slit/Robo signaling in a manner similar to their technique for testing for Sdc/Slit genetic interactions. Removal of one copy of *dlp* and one copy of *slit* or *robo1, 2* is insufficient to augment the fasciculation defects or the midline crossing defects observed in either of the heterozygotes alone (Figure 15C,D) [all genotypes 130<n<350 segments]. These results suggest that Dlp is not as important for Slit/Robo signaling as Sdc is, but do not demonstrate conclusively that Dlp does not play a role in Slit/Robo signaling.

As mentioned above, the ability of neuron-specific Dlp expression to partially rescue *sdc* mutant embryonic axon pathfinding phenotypes suggests that these proteins are functionally redundant. Indeed these proteins are expressed in a similar pattern within the developing embryonic CNS (Figure 16A) (Johnson et al., 2004). Therefore, to genetically test for redundancy our collaborators created flies that were doubly mutant for *sdc* and *dlp*. These animals show a large increase in the penetrance of both midline crossing defects and fasciculation defects as compared to either single mutant animal alone (Figure 16B-H) [all genotypes 130<n<350 segments]. This is particularly interesting in the case of the midline crossing defects as *dlp* mutant animals alone show an extremely low penetrance of this phenotype (Figure 16B,D). The impressive genetic interaction between these HSPGs strongly suggests that they are involved in the same or parallel signaling pathways. While these results demonstrate that *dlp/sdc* double mutants cause severe axon pathfinding phenotypes it is currently unclear if this is a direct effect or if it is secondary to a nervous system patterning defect which occurs in these animals.

While neither homozygous mutant alone shows patterning defects we are currently unsure if the double mutants have such a defect. Further work is required to discover the nature of *dlp/sdc* double mutant axon guidance defects.

Syndecan Expression and Function in the Visual System

Given the role of Sdc in axon guidance in the embryonic nervous system we were interested to explore if this HSPG had a similar role in the developing visual system. An antibody directed against Sdc revealed that this proteoglycan, like Dlp, is expressed on photoreceptor cell bodies, a subset of photoreceptor axons and is expressed in the proliferative centers of the visual system (Figure 17A,A'-C,C'). Notably different from Dlp, Sdc immunoreactivity is largely absent from the medulla glia and medulla neuropile glia as well as the mushroom body (Figure 17A,A'-C,C'). The staining pattern for Sdc suggests that Dlp and Sdc may have some overlapping and some distinct functions during visual system development.

Analysis of *sdc* mutant third instar larvae and 40h pupae revealed a number of axon guidance abnormalities. 50 percent of mutant third instar hemispheres [n=28] showed defects in the organization of retinal projections to the lamina ranging from gaps (29 percent) to gross disorganization of the lamina plexus (21 percent, Figure 18A-C). Unlike *dlp* mutants, *sdc* mutants showed a lower penetrance of the lamina thickening (4 percent) and axon crossover phenotypes (Figure 18C). Some third instar *sdc* mutant larvae had R7/8 axon misrouting (corollary shown in the 40h *sdc* pupa, Figure 18C,F). This phenotype is not observed in *dlp* mutants in larval stages (Figure 18C).

40h *sdc* mutant pupae also showed defects in photoreceptor projection patterning. The major phenotypes observed included crossover of R7 to a neighboring medullary cartridge (100 percent, n=17, Figure 18D,E&G) and defective axon pathfinding to the medulla (86 percent, n=21, Figure 18D,F&G). Both the R7 crossover and pathfinding defects were observed in *dlp* mutants but occurred much more rarely (Figure 9F). s*dc* mutants showed a low penetrance of R7/8 termini disruption (~10 percent, n=21, Figure 18G), a phenotype common in *dlp* mutants (80-100 percent, Figure 9D,F). Overall, *dlp* and *sdc* mutants share similar phenotypes but each mutant has a largely distinct level of penetrance for each defect.

These results suggest that Sdc is required for photoreceptor axon guidance and that Dlp and Sdc may have some overlapping and some distinct functions during *Drosophila* visual system development. In order to better ascertain the relationship between Dlp and Sdc it would be informative to generate double mutants and evaluate photoreceptor projections in these animals. However, the lethality of *dlp/sdc* double mutant animals is prohibitive for this experiment (data not shown). Additionally, it is currently unknown if the defects in *sdc* mutants are secondary to other patterning defects.

DISCUSSION

Patterning, Axon Guidance and Function in *dally-like* Mutants

In vertebrates, glypicans are expressed in the developing nervous system, most notably in proliferating cells of the ventricular zone and on some axons and their growth cones (Yamaguchi, 2001). While previous findings have established a function for glypicans in the control of cell division and growth factor signaling (Filmus and Selleck, 2001; Nakato et al., 2002; Kirkpatrick et al., 2004), their role in nervous system development including axon guidance has not yet been reported. The data presented here demonstrate for the first time the *in vivo* requirement for a glypican in axon guidance. Similar to the expression patterns of several mammalian glypicans, Dlp is highly expressed in both the developing embryonic nervous system and in the developing adult visual system. In the embryonic nervous system Dlp appears to be localized only to axons while in the developing adult visual system Dlp is localized to several cell types including neurons and glia. Perhaps this variable expression pattern reflects a specialized role for Dlp in embryonic axon guidance, whereas in the developing visual system this glypican may carry out more diverse roles.

dlp mutants show defects in embryonic axon architecture indicating that this protein is required for axon guidance. Due to the previously described roles for this protein in Hh and Wnt signaling we assayed to determine if mutations in *dlp* cause axon pathfinding defects indirectly by affecting nervous system patterning (Han et al., 2004a; Kirkpatrick et al., 2004). In zygotic *dlp* mutants we saw no defects in the number or

position of midline glia or engrailed expressing neurons. While these antibodies label only a subset of cells in the nervous system, these results suggest that the gross patterning of the embryonic nervous system is intact in *dlp* mutants, therefore strongly suggesting that the role of Dlp in axon guidance is more direct in nature.

dlp mutants show defects in photoreceptor projections to the lamina and medulla, demonstrating that as in the embryo, Dlp is required for the establishment of proper axonal architecture in the developing adult visual system. *dlp* mutant adults also showed defects in the various components of the ERG. Photoreceptor response decreases observed in *dlp* mutants are likely due in part to the reduction in the overall number of ommatidia in the eye. In addition, it is also possible that the attenuation in the photoreceptor response is due to a reduction in the light triggered current flow into individual photoreceptors. While *dlp* flies do have an overall reduced number of photoreceptors and commensurate decreases in photoreceptor currents, these changes alone are unlikely to account for the marked reduction of the synaptic transients. Indeed, *dlp* mutant animals that have photoreceptor responses in the normal range still have "on" and "off-transients" that are greatly reduced. These results indicate that *dlp* may be required for either synapse formation or function in addition to its role in axon guidance.

Photoreceptor axon pathfinding defects and visual system functional deficits are not secondary to patterning defects in photoreceptors. Although *dlp* mutant animals have a reduced and roughened eye several complementary assays demonstrate that there are no detectable defects in the patterning of photoreceptors. Rather the roughening/reduction of *dlp* mutant eyes appears to be the result of defects in the specification of accessory

cells such as mechanosensory bristle cells and pigment cells. As far as we know there are no published studies demonstrating that this type of defect directly leads to aberrant axon pathfinding. Consistent with this, accessory cells differentiate and undergo refinement by apoptosis well after photoreceptor axon outgrowth has already occurred (Brachmann and Cagan, 2003).

During the development of the visual system there is a high level of organizational interdependence between cells in different regions which in part serves to coordinate the development of these tissues. For instance photoreceptors induce the migration of more glia into the zone of the lamina plexus and in turn, these glia send a "stop" signal to younger photoreceptors specifying their termination zone (Poeck et al., 2001; Dearborn and Kunes, 2004). Similarly photoreceptors induce the proliferation and differentiation of their future synaptic partners in the lamina (Selleck and Steller, 1991; Huang and Kunes, 1996). To determine if the photoreceptor axon guidance defects were secondary to visual system patterning defects, we examined if neuronal or glial cell specification was deranged in *dlp* mutants. Lamina neurons as well as retinal and optic lobe glia were correctly specified in *dlp* mutant larvae. Therefore *dlp* mutant photoreceptor axon guidance defects, like embryonic axon defects, do not appear to be secondary to neuronal or glial patterning defects.

Since *dlp* is essential for Hh signaling in the embryo, it was important to determine if Hh-dependent events during adult visual system development were compromised by loss of *dlp* function (Han et al., 2004a; Kirkpatrick et al., 2004). Events known to require Hedgehog (Hh) signaling, initiation of the morphogenetic furrow,

specification of lamina neurons and the migration of glia into the eye, were unaffected in *dlp* mutants (Ma et al., 1993; Huang and Kunes, 1996; Hummel et al., 2002). Our observations that Hh-driven patterning in the visual system is unaffected by loss of *dlp* function suggest that Dlp is dispensable for Hh signaling in this tissue and are consistent with recent findings demonstrating that Dlp is not required for Hh signaling in other larval tissues (Han et al., 2004a).

Conversely, although we do not currently have direct evidence, there are indications that some but not all aspects of Wg (a Wnt family member) signaling in the eye may be affected by mutations in *dlp*. Previous work from our own laboratory has demonstrated that Dlp mediates Wg signaling in the wing imaginal disk in a bidirectional manner. In areas of the disc that contain high Wg levels, Dlp serves to decrease the amount of Wg signaling, while in areas with low levels of Wg, Dlp serves to augment Wg transduction (Kirkpatrick et al., 2004). Wg plays a role in many important aspects of eye imaginal disk development, including the determination of which tissue will develop into the retina and which tissue will develop into the surrounding head capsule, the positioning of the dorsal/ventral boundary, the polarity of ommatidia, the progression of the morphogenetic furrow and the induction of apoptosis in ommatidia at the edge of the eye during late development (Treisman and Rubin, 1995; Zheng et al., 1995; Heberlein et al., 1998; Baonza and Freeman, 2002; Lin et al., 2004). *dlp* mutants have intact furrow progression, dorsal ventral boundary specification and ommatidial polarity suggesting that these Wg-dependent events are largely unaffected in mutants.

However, the reduced size of *dlp* mutant eyes suggests that Wg mediated determination of retinal tissue or Wg directed apoptosis of ommatidia may be affected in *dlp* mutants. Early in the development of the eye imaginal disk, Wg serves to specify the border between the head capsule and retina such that loss of Wg leads to a larger eye while ectopic Wg leads to a reduction in the eye (Baonza and Freeman, 2002). Similarly, late in development Wg signaling refines the edge of the eye by inducing apoptosis in ommatidia at the boundary between the head capsule and retina. Here too activation of Wg, which serves to activate the expression of apoptotic genes, causes a reduction in the overall size of the eye (Lin et al., 2004). Therefore it is possible that mutations in *dlp* might cause an increase in Wg signaling at the edge of the retina early or late in eye development and thus lead to a reduction in the overall size of the eye. It is important to note, however, that there are no reports that reductions in eye size in and of itself affect photoreceptor axon guidance and therefore even if Dlp plays a role in the aforementioned processes this is most probably not the direct cause of photoreceptor axon guidance defects observed in *dlp* mutants.

Tissue-specific Requirements of Dally-like

In order to investigate the tissues-specific requirements of Dlp during axon guidance we used mosaic analysis. Generation of large *dlp* mutant clones in the eye alone was sufficient to produce photoreceptor projection defects to the lamina similar to those found in *dlp* homozygous mutant animals. However, animals bearing *dlp* mutant retinas did not show the larval lamina thickening phenotype nor did these animals show

aberrant R7/8 termini in the medulla of 40 h pupae. These findings indicate that loss of eye-specific Dlp results in some but not all of the axon guidance defects observed in homozygous *dlp* mutants, consistent with the pattern of Dlp immunoreactivity on both photoreceptors and glia and neurons of the optic lobe. Similarly, we explored the effect of removal of *dlp* from all photoreceptors on the physiological response of the eye to light stimulus. When Dlp is removed only from photoreceptors we observed that there were no longer defects in the components of the ERG, indicating that optic lobe-specific Dlp expression is sufficient to establish the proper light response of the visual system.

In addition to mosaic analysis, we addressed the tissue-specific requirements of Dlp using transgenic rescue. This technique allows the replacement of a protein into a mutant background and is particularly powerful in *Drosophila* because of the large number of different tissue/temporal-specific GAL4 drivers available in this organism. However, in the case of the three *UAS-dlp* constructs that we tested, *dlp* homozygous mutant phenotypes were rescued by the presence of the *dlp* transgene in the absence of a tissue-specific driver. Thus the "leaky" expression that can sometimes occur from these constructs is sufficient to rescue both larval and pupal *dlp* mutant phenotypes therefore making Dlp tissue-specific transgenic evaluation impossible. Despite our disappointment with this result, the finding indicates that the phenotypes observed in *dlp* mutants are specific to mutations at the *dlp* locus as transgenic replacement of this protein is sufficient to rescue mutant phenotypes.

dally-like Genetic Interactions

Johnson et al. recently published research demonstrating the role of another HSPG, Sdc, in *Drosophila* axon guidance (Johnson et al., 2004). Their work demonstrated that Sdc had defects in midline crossing in the embryonic nervous system. These defects are due, at least in part, to the role that Sdc plays in modulating the distribution of the midline derived axon repellent Slit. Antibody staining for Dlp revealed that Sdc and Dlp are expressed in roughly the same regions of the embryonic CNS. In addition, partial rescue of *sdc* mutations is afforded by transgenic overexpression of Dlp in neurons. These findings suggest that Dlp may also function in Slit/Robo signaling and in order to test this we assayed for a genetic interaction between components of the Slit signaling pathway and Dlp. Unlike in the case of *sdc*, removal of one copy of *dlp* and one copy of a Slit signaling component is insufficient to augment the defects seen in fasciculation or midline crossing in either heterozygote alone. Therefore it would appear that if Dlp does normally serve to mediate Slit/Robo signaling at the midline, remaining Dlp and Sdc protein is able to compensate for the loss of one copy of *dlp*.

If Dlp and Sdc are both truly important for Slit/Robo signaling one would predict that removal of all copies of both proteins might lead to a synergistic effect on the severity of Slit related defects observed in either mutant alone. Indeed, removal of both HSPGs leads to a large increase in the severity and penetrance of both midline and fasciculation defects observed in either single mutant. These results are particularly striking in the case of midline crossing defects as mutations in *dlp* do not cause detectable

midline crossing defects by themselves, suggesting that Dlp plays a redundant role with Sdc in some aspect of Slit/Robo signaling. However, we must still consider other possibilities. For instance, as of yet it is unknown if *dlp/sdc* double mutants have defects in patterning of the nervous system. If this were the case, the phenotypes observed in the double mutants could be due to Dlp/Sdc redundancy in some other signaling pathway important for nervous system patterning. Additionally, it is possible that Dlp could mediate signaling of a parallel pathway that also feeds into the fasciculation/midline crossing decisions known to be mediated by Slit signaling. However, it should be noted that there are no other major midline repulsive signaling pathways currently characterized in *Drosophila* and loss of Slit signaling appears to be sufficient to cause a total collapse of axons to the midline (Kidd et al., 1999; Kaprielian et al., 2001).

Visual System Syndecan Expression and Mutant Phenotypes

We explored the expression pattern of Sdc in the visual system as well as the effect of *sdc* mutations on photoreceptor axon projection to compare and contrast with our observations for Dlp. Like Dlp, Sdc is expressed on photoreceptor axons and proliferative centers of the optic lobe. Consistent with the expression pattern of Sdc, mutations in this protein lead to defects in photoreceptor axon guidance. Although similar phenotypes were observed in *dlp* mutants, *sdc* mutants produced a largely different distribution of penetrance for the various phenotypes, similar to the results observed in the embryo. In this developmental stage, while *dlp* and *sdc* mutants both have similar fasciculation defects, *dlp* mutants do not have the midline crossing defects

that are prevalent in the *sdc* mutant embryos. The visual system findings indicate that *dlp* and *sdc* might play largely separate roles in the process of photoreceptor axon outgrowth. Conversely, it may be the case, as in the embryonic nervous system, that loss of both proteins may cause a synergistic increase in the penetrance of all phenotypes observed in both single mutants indicative of redundant roles for these two HSPGs. Further experiments will be required to determine the relative contributions of these two proteins to photoreceptor axon guidance.

Other *in vivo* Evidence for the Role of HSPGs in Axon Guidance

During the course of our research on Dlp the first *in vivo* evidence for a physiological role of HS in axon guidance was published by the Yamaguchi laboratory (Inatani et al., 2003). This group demonstrated that brain-specific mutations in the mouse heparan sulfate co-polymerase, EXT1, cause defects in the development of the mammalian brain. HS co-polymerase is part of the enzymatic machinery that is responsible for HS disaccharide chain elongation (Sugahara and Kitagawa, 2002). EXT1 mutations in mice are sufficient to cause defects in the patterning of the midbrain and cerebellum and these mice also have deficits in cortical size due to a decrease in the number of neurons populating the cerebral cortex. Culture of cortical progenitor cells from EXT1 mutants demonstrated that these cells undergo much less proliferation upon FGF addition. These findings suggest that reductions in FGF stimulated ventricular zone cell proliferation, the process which populates the cerebrum, may account for the decreased cell numbers in this brain structure (Ghosh and Greenberg, 1995; Reuss and

von Bohlen und Halbach, 2003). Consistent with this hypothesis, there is a reduction in cell division in this brain region in EXT1 mutant mice. Brain-specific EXT1 mutations also cause major defects in axon tracts that include the total absence of major brain commissures and defects in retinal ganglion cell (RGC) axon projection. The patterning defects described above are perhaps not surprising given the known requirement of HSPGs in signaling by several pathways important for brain development (Kramer and Yost, 2003). For instance FGF and Wnt signaling, processes that require HSPGs *in vivo*, are vital for proper patterning of the mammalian brain (Patapoutian and Reichardt, 2000; Dono, 2003). Indeed some of the mutant phenotypes observed in brain-specific EXT1 mutants are similar to those observed in FGF and Wnt mutants (Thomas et al., 1991; Meyers et al., 1998).

The axon tract defects observed in EXT1 mutant animals could be due to a direct effect of loss of HS on axon guidance or be indirectly mediated by patterning defects. For instance, loss of HS may decrease the ability of the growth cone to respond to certain guidance cues or may cause a disruption of ligand gradient distribution. Conversely, axon guidance defects could be secondary to patterning defects that affect the fate and/or distribution of cells that provide guidance cues. The mechanism that underlies the loss of commissures in EXT1 mutants is unknown, however, the authors present evidence that the retinal axon guidance defects in these animals may be due to aberrant Slit/Robo signaling. While homozygous mutations in mouse Slit2 or heterozygous mutations in EXT1 do not cause defects in RGC axon guidance or patterning by themselves, the loss of one copy of EXT1 in a Slit2 -/- background leads to defects in retinal axon projections

similar to those seen in brain-specific EXT1 homozygous mutants or Slit1/Slit2 double mutants (Plump et al., 2002). This indicates that EXT1 may mediate retinal axon guidance by altering the transduction of the Slit signal in some manner. This study reveals the physiological importance of HS to several aspects of brain development, including the process of axon guidance, but many questions remain open. For instance, since the mutants lack EXT1 in all cells of the brain this study does not address whether HS is required in the cells sending out axons or in cells of the target region. Additionally, the relative contribution of different core proteins in the process of mammalian brain development remains to be elucidated.

Bulow et al. demonstrated that in addition to a physiological role for HS, modifications that occur to the disaccharide units after polymerization are important to axon guidance (Bulow et al., 2004). These modifications include N-deacetylation/N-sulfation, C5 epimerization and sulfation of C2, 3 and 6. These steps are carried out by the HS specific enzymes NDST, Epimerase and 2O, 6O and 3O-sulfotransferases (ST) respectively and occur in a modular/non-uniform manner along the length of the HS chain (Sugahara and Kitagawa, 2002). Previous studies have demonstrated that binding of particular growth factors to their cognate receptors and consequential activation require distinct HS modifications. For instance, activation of the FGF receptor requires 6-0 sulfation (Nakato and Kimata, 2002). Therefore, it is thought that removal of specific modifying enzymes may be sufficient to affect distinct growth factor signaling pathways. Indeed, in mouse and flies these modifications appear to be vital as mutations in C5 epimerase, 2O-ST or 3O-ST results in embryonic patterning defects or early lethality

(Perrimon and Bernfield, 2000; Forsberg and Kjellen, 2001; Shworak et al., 2002; Li et al., 2003). Intriguingly, in *C. elegans* removal of C5 epimerase, 6O-ST or 2O-ST yields viable and fertile animals with no apparent defects in either FGF or TGF-β signaling (Bulow et al., 2004). However, these animals have defects in neuron migration and axon guidance. Interestingly, these mutations can have differential effects on the guidance of a given axon. For instance midline guidance of the AVK interneurons is defective in C5 epimerase mutants but not in 6O-ST or 2O-ST mutants. Conversely, guidance of the PVQL/R interneuron is defective in C5 epimerase and 2O-ST mutants but is much less affected by 6O-ST mutations. These results indicate that specific modifications of HS differentially regulate axon guidance in this organism (Bulow et al., 2004).

Using genetic interactions, Bulow et al. demonstrated that mutations in the HS modifying enzymes C5 epimerase and 6O-ST cause axon pathfinding defects due to their role in the Slit/Robo signaling pathway. An animal homozygous for a null mutation in *slit* shows midline crossing defects in 17 percent of PVQ neurons while an animal homozygous for a null mutation in *6O-ST* shows a midline crossing defect in 41 percent of PVQ neurons. When Bulow et al. removed both copies of *slit* and both copies of *6O-ST* there was no increase in the penetrance of midline crossing defects compared to either single mutant alone. Therefore they concluded that 6O-ST mediates midline guidance via modulation of Slit signaling because if its affects on midline guidance were via a parallel pathway removal of both copies of *slit* and *6O-ST* should provide an additive effect on the penetrance of the midline defects (Bulow et al., 2004).

The two studies described above speak to the importance of HS side chains in the mediation of axon guidance. However HS is found attached to core proteins and work from model organisms has shown that removal of specific core proteins causes defects in signaling mediated by various growth factors, suggesting that specific core proteins may also be important for axon guidance (Kramer and Yost, 2003). Therefore the mutational analysis of the core proteins, such as the one performed by our laboratory on the glypican Dlp, are necessary to determine which core proteins bear the HS involved in the CNS developmental processes described above. Additionally, studies exploring the relative contribution of the core protein versus the HS in the mediation of axon guidance will further elucidate the roles of both proteoglycan components. Transgenic rescue of core protein mutants with a core protein that is unable to be GAG modified will be key in addressing the importance of HS in specific contexts.

Our collaborators in the Van Vactor laboratory, as well as a second group, have recently demonstrated the role of *Drosophila* Sdc in the process of embryonic axon guidance (Johnson et al., 2004; Steigemann et al., 2004). Sdc was found to be localized to axons in the developing embryonic nervous system and mutations in *sdc*, like those in Slit signaling components, cause defects in midline crossing. Biochemical and genetic evidence demonstrated that Sdc physically interacts with both Robo and Slit and that removal of one copy of *sdc* and one copy of a Slit signaling component is sufficient to disrupt midline axon guidance (Johnson et al., 2004). These results suggest that Sdc mediates Slit/Robo signaling, perhaps in a manner similar to the mechanisms by which other HSPGs are known to modulate growth factor signaling. Sdc might act as a Slit

binding protein that regulates the distribution of this guidance cue and/or serve as a co-receptor in Slit/Robo signaling. Johnson et al. demonstrate that mutations in *sdc* lead to defects in the Slit gradient found at the CNS midline supporting a role for Sdc in the distribution of Slit. The co-receptor function of Sdc for Slit signaling in this system is currently unknown.

Molecular Mechanisms of Dally-like Mediated Axon Guidance

What are the molecular mechanisms that underlie the axon guidance defects observed in *dlp* mutants? Thus far, the effects of mutation of heparan sulfate biosynthetic enzymes or heparan sulfate core proteins on axon pathfinding have been primarily considered in the context of classical axon guidance pathways, Slit-Robo signaling in particular. Genetic interaction data from mouse, *Drosophila* and *C. elegans* strongly suggests that the phenotypes observed in animals bearing mutations HS synthetic enzymes and Sdc occur as a result of signaling defects in this important midline repellent pathway (Inatani et al., 2003; Bulow et al., 2004; Johnson et al., 2004; Steigemann et al., 2004). Additionally, biochemical evidence from the Van Vactor laboratory demonstrates that Sdc can physically interact with Slit and Robo and mutations in *sdc* lead to defects in Slit distribution (Johnson et al., 2004). This suggests, that as in the case of the morphogens, HSPGs may serve both as co-receptors and regulate the distribution of axon guidance cues. Indeed evidence from cultured neurons has demonstrated that removal of HS by heparinaseIII significantly reduces the binding affinity of Slit to its cell surface receptor Robo and is sufficient to render axons unresponsive to Slit (Hu, 2001).

The signaling pathway through which Dlp mediates axon guidance is currently unknown. Our own genetic interaction experiments between Dlp and protein components of the Slit signaling pathway have not demonstrated significant interactions such as those observed between Slit and Sdc. However, lack of genetic interaction is not conclusive evidence that Dlp does not function in the Slit/Robo pathway. The huge increase in penetrance and severity in midline and fasciculation phenotypes observed in *dlp/sdc* double mutants suggests that Dlp and Sdc are involved in the same or parallel pathways during the process of midline guidance. Therefore, since Sdc functions in the Slit/Robo signaling pathway it stands to reason that Dlp might also function here during embryonic nervous system development. Consistent with a role for the glypican Dlp in Slit/Robo signaling, previous studies have demonstrated binding between mammalian Glypican-1 and Slit (Liang et al., 1999; Ronca et al., 2001; Zhang et al., 2004). This interaction requires the HS chains as binding of Slit to "naked" Glypican-1 core protein occurs at a binding affinity an order of magnitude lower then that of Slit binding to glycanated Glypican-1 (Liang et al., 1999). Additionally, Glypican-1 binding to Slit appears to require O-sulfated heparan sulfate (Ronca et al., 2001). Despite these findings, mutations in *dlp*, unlike those in *sdc*, do not cause appreciable defects in Slit distribution (data not shown). Therefore, the role of Dlp in Slit/Robo signaling still remains unknown.

Various evidence suggests that Dlp could mediate the signaling of one of the other well defined axon guidance pathways. Indeed there is evidence that HSPGs interact with Netrin, Ephrins and Integrins in addition to their interactions with Slit (Bennett et al., 1997; Woods and Couchman, 1998; Ethell et al., 2001). For instance Sdc, which can

stimulate dendritic spine maturation in cultured hippocampal neurons, forms a complex with and is phosphorylated by the Ephrin receptor. This interaction/phosphorylation is required for Sdc to induce spine maturation (Ethell et al., 2001). The search for additional binding partners of the Netrin receptor, DCC, led researchers to identify HS as a binding partner (Bennett et al., 1997). DCC was found to bind through a HS binding motif and addition of heparinase abolishes DCC binding to cultured neurons. Interestingly, in the mouse, brain-specific EXT1 mutations cause loss of the anterior commisure a phenotype also observed in Netrin mutants (Inatani et al., 2003). Therefore Itani et al. suggested that HS might modulate Netrin signaling as well as Slit signaling in the developing mouse brain. These studies suggest that a glypican might be important for mediating signaling via other axon guidance cues.

Despite the emphasis placed on the possible role of HSPGs in the classic axon guidance signaling pathways, recent studies demonstrating that the morphogens Wnt, Hh and BMP also play a *bona fide* role in axon guidance suggest the possibility that HSPGs govern axon guidance by affecting morphogen function during this process (Schnorrer and Dickson, 2004). In the case of Hh and TGF-β signaling, the role of these pathways in *Drosophila* axon guidance is currently unknown. However, given the role of these signaling pathways in the mammalian nervous system, there is a possibility that these morphogens are involved in *Drosophila* axon guidance. Our own data indicates that zygotic loss of TGF-β does not lead to axon guidance defects. Still, it is possible that maternal contribution of mRNA for TGF-β components is able to supply the needed protein to carry out axon guidance in these animals. The challenge with exploring the

axon guidance properties of these signaling pathways will be to separate patterning defects from direct effects on axon guidance as was elegantly performed in the case of the mammalian nervous system (Schnorrer and Dickson, 2004).

Wnt signaling is vital for many well characterized developmental events in *Drosophila*, including axon guidance. Wnt5 mutants have defects in commissural axon guidance (Yoshikawa et al., 2003). This function of Wnt5 is regulated through the atypical receptor tyrosine kinase Derailed (Drl) which acts as a Wnt5 receptor expressed on axons passing through the anterior commisure. Wnt5 expressed in the posterior commisure acts as a repellent of Drl expressing axons, confining these projections to the anterior commisure (Yoshikawa et al., 2003). In addition to commissural defects Wnt5 mutants, these animals have fasciculation defects very similar to the ones found in *dlp* mutants suggesting that Dlp may mediate the transduction of Wnt5 signals (Fradkin et al., 2004). While Dlp has not been shown to genetically interact with components of Wnt signaling, our group has shown that *wingless* (a *Drosophila* Wnt) signaling and distribution is compromised in the wing imaginal tissue of *dlp* mutants (Kirkpatrick et al., 2004). These results demonstrate that *dlp* has the ability to modulate *wingless* signaling in this developmental context. Given the phenotypic similarity of *dlp* mutants and *wnt5* mutants combined with our findings that Dlp is required for Wnt signaling in the wing, Wnt5 is a prime candidate for the signaling pathway through which Dlp mediates embryonic axon guidance.

Glypicans have been shown to bind to FGF receptors and ligands in biochemical and x-ray crystallography experiments (Bonneh-Barkay et al., 1997; Song et al., 1997;

Hagihara et al., 2000; Galli et al., 2003). Additionally, in *Xenopus* glypican-4 knockdown with anti-sense morpholinos demonstrate an *in vitro* role for this protein in FGF signaling (Galli et al., 2003). Experiments performed in various systems have demonstrated that FGF is required *in vitro* for axon pathfinding and in *Drosophila*, mutations in FGF signaling components lead to defects in peripheral axon guidance (McFarlane et al., 1995; Garcia-Alonso et al., 2000; Reuss and von Bohlen und Halbach, 2003). Given the important role of glypicans and other HSPGs in FGF signaling and the role of this pathway in axon guidance, it is possible that Dlp might mediate FGF dependent axon guidance in the embryonic nervous system or developing adult visual system.

Mutation of the *Drosophila* Insulin receptor (DInr) causes photoreceptor axon guidance defects similar to those observed in *dlp* mutants (Song et al., 2003). Therefore, Dlp might be involved in DInr signaling during the wiring of the visual system. Consistent with this hypothesis, unpublished genetic interaction data from our own laboratory suggests that *dlp* may function in the DInr signaling pathway in other developmental contexts. Additionally, Glypican-3 has been shown to physically interact with insulin growth factor (Pilia et al., 1996; Xu et al., 1998). Interestingly, mutations in human and mouse Glypican-3 (homologues of the other *Drosophila* glypican Dally) cause similar overgrowth phenotypes, which in humans are known as Simpson-Golobi-Bemel Syndrome (Hughes-Benzie et al., 1996; Pilia et al., 1996; Cano-Gauci et al., 1999). Mice that have increased insulin-like growth factor signaling also show an overgrowth syndrome that is related to human Beckwith-Wiedemann syndrome (Eggenschwiler et al.,

1997; Sun et al., 1997). Researchers therefore hypothesized that mutations in Glypican-3 might cause an aberrant increase in insulin signaling and hence lead to overgrowth, however *in vivo* genetic interaction experiments in mice did not reveal a role for Glypican-3 in Inr signaling (Pilia et al., 1996; Cano-Gauci et al., 1999). Thus, the physiological role of glypicans in insulin growth factor signaling in any context, including that of axon guidance, remains to be elucidated. However, it is still plausible that Dlp may mediate some of its affects on axon guidance through DInr signaling.

CONCLUSION

In conclusion, our study on Dlp and Sdc has contributed to the overall understanding of the role of HSPGs in axon guidance. Given the growing body of literature citing the physiological role of these molecules, it is likely that this type of proteoglycan will play a pivotal role in the process of axon guidance in many developmental contexts. More research is required to determine in which pathways Dlp functions during the guidance of embryonic and larval axons. It is possible, perhaps even likely given the current knowledge of glypicans, that Dlp may function in diverse guidance pathways in a context dependent manner.

MATERIALS AND METHODS

TGF-β

Drosophila strains. All flies were raised at 25°C on either instant food (Carolina Biological Supplies) or on a medium containing cornmeal, agar and yeast. The only exception to this is the larvae for the dominant negative experiment in which bouton number was assayed (some of these were raised at 30°C and are noted in the text). The wild-type controls were a Canton-S stock bearing a w^{1118} mutation (20X generation backcross to Canton-S, from Tim Tully). These were raised at 25°C. Flies expressing activated or dominant negative forms of the BMP type I receptors were created using the GAL4:UAS system (Brand and Perrimon, 1993). Activated *thickveins* (*tkvA*) receptor *w; P[w+; UAS::tkvA*]B3* was used (Holley et al., 1996; Hoodless et al., 1996) for pathway activation experiments. Activated receptors *w; P[w+; UAS::tkv^{Q199D}], w; P[w+; UAS::tkvAΔE]6B* and w; *P[w+; UAS::saxA (HA)]* were also tested. *w; P[w+; UAS-tkv ΔGSK]CA2* (*tkvDN*) and *w; P[w+; UAS-sax ΔI]11A; P[w+; UAS-sax ΔI]11A* (*saxDN*) were used for dominant negative experiments (Haerry et al., 1998). Both of these constructs are "strong" insertions. Relative strength of insertion was tested by crossing these UAS lines to the *P[GAL4]A9* line and looking at severity of the wing phenotype (Jackson et al., 1997). The nervous system specific GAL4 driver used was *P[GawB]elav^{C155}* (Lin et al., 1994) and the muscle specific driver was *P[GawB]how^{24B}* (Brand and Perrimon, 1993). Controls for transgene experiments were created by

outcrossing the UAS or GAL4 bearing animals to the w^{1118} Canton-S stock noted above. The only exception to this was UAS bearing animals in the 30°C bouton count experiment which were outcrossed to Oregon-R wild-type flies. sax^{P} bears a P-element insertion in the putative signal sequence and is a strong hypomorph (Nellen et al., 1994). *Df(2R)sax-H9* is a deficiency that deletes *sax* (Twombly et al., 1996). sax^{3} is a severe hypomorphic mutation (3% of adults escape in trans with deficiency lines) that has a premature stop codon in the extracellular domain (Gln 121 to stop, Twombly, Malnick & Gelbart, unpublished data). Mad^{1} is an EMS-induced mutant which acts as a genetic null (Gelbart, 1982; Sekelsky et al., 1995). Mad^{12} was isolated in a separate EMS screen, has a point mutation which causes a premature stop codon, and behaves as a genetic null (Sekelsky et al., 1995). *Df(2L)C28* is a small deficiency that deletes *Mad* (Raftery et al., 1995). Mutant embryos and larvae were identified by the absence of ß-galactosidase activity or GFP fluorescence respectively, derived from either *ftz-lacZ* or *ubiquitin-GFP*-bearing P-element inserts on the *CyO* balancer chromosome.

Larval neuromuscular preparation. Neuromuscular junction preparation was performed as described (Lin et al., 1994). Briefly, wandering third instars that were progeny of a given cross were selected and placed in a small cell culture dish that was filled halfway with polymerized Sylgard (Dow-Corning). The anterior portion of the larva was pinned with a minutien dissecting pin (Fine Science Tools) and the posterior fourth of the larvae was cut off with small Vannas-style angled spring scissors (Fine Science Tools). Ca^{++} free saline was then pipetted onto the larva (Estes et al., 1996), and the posterior end was

pinned down to the Sylgard through the opening. A cut was then made along the dorsal cuticle and the larva was pinned out on the four corners. Abdominal segment 2 was used in both morphological and electrophysiological experiments.

Immunohistochemistry and Confocal Microscopy. Embryos were fixed and stained using standard protocols (Sullivan et al., 2000). Phosphorylated-Mad (P-Mad) antibody (PS-1) was a generous gift from the lab of Peter ten Dijke. P-Mad staining was amplified using the VECTASTAIN ABC kit (Vector Laboratories) in conjunction with the TSA kit (Perkin-Elmer). Monoclonals anti-Eve (2B8), anti-FasIII (7G10), BP 102, anti-Elav (9F8A9), and FasII were also used for embryo staining (Iowa Hybridoma Bank). Secondary antibodies used to detect these monoclonals were from the Alexa series (Molecular Probes). Dissected larvae were processed as described (Estes et al., 1996; Sullivan et al., 2000). Primary antibody used for detection of presynaptic bouton structure was (mAb49) anti-cysteine string protein (gifts from Zinsmaier and Buchner) (Ranjan et al., 1998). Goat anti-mouse secondary antibody conjugated to Cy3 was used for immunofluorescence detection (Jackson Laboratories). A Nikon upright laser confocal microscope was used to acquire images of muscles 6 and 7 with either a 20x or 60x objective. The left hemisegment of A2 was selected for imaging unless damaged, at which point the right hemisegment was used for morphological assessment. Four to ten one micron horizontal sections were taken at 60x and the images were compressed into a Z-plane projection (Simple PCI). Bouton counts were done using NIH Image and normalized for the total dorsal area of both muscles 6 and 7.

Electrophysiology. Excitatory junction potential (EJP) recordings were taken from muscle 6 of the right A2 hemisegment of third instar larvae. Dissections were performed as described above in Ca^{++} free saline (Estes et al., 1996) and recordings were performed in HL3 which has a calcium concentration of 1.5 mM (Stewart et al., 1994). The CNS was removed and the motor nerves were stimulated with a glass suction electrode. Thin-walled glass intracellular recording electrodes with resistances from 10-20 MΩ were pulled (Sutter Instrument Company, Model P-97) and were backfilled with 2 M KAc. Intracellular recording electrodes were impaled into the muscle and EJPs were recorded after stimulating the nerve using a Grass Stimulator set to deliver ten 1 msec pulses at a frequency of 0.1 Hz and at an intensity ~1.5x that necessary to evoke the compound response. Recordings were acquired with an Axoclamp 2A amplifier and pClamp8 software (Axon Instruments). Only muscles with a resting potential of less than -60 mV and which demonstrated the compound response were recorded from and used for data analysis. Average resting membrane potentials (RMPs) for the different genotypes were as follows: *WT*= -69.9, n=10; *P[GawB]how*^{24B}/+ = -73.0, n=10; *P[GawB]elav*^{C155}/+ = -72.6, n=10; *Df(2R)Sax-H9/CyO*= -74.6, n=10; *sax*^{3}*/CyO*= -76.2, n=5; *Df(2R)Sax-H9/sax*^{3}= -74.4, n=9; *Mad*^{1}*/CyO*= -75.5, n=10; *Mad*^{12}*/CyO*= -74.6, n=10; *Mad*^{1}*/Mad*^{12}=-71.0, n=8; TkvDN/+ (control)=-74.3, n=10; TkvDN (muscle)=-75.8, n=10; TkvDN (neuron)= -69.8, n=10; SaxDN/+ (control)= -71.6, n=10; SaxDN (muscle)= -73.3, n=11; SaxDN (neuron)= -75.9, n=10; TkvA/+ (control)= -72.0, n=9; TkvA (muscle)= -74.0, n=10; TkvA (neuron)= -82.6, n=11. TkvA (neuron) is the only animal with an average

resting membrane potential that is significantly different from wild-type ($p<.05$). Since recordings of spontaneous/miniature EJPs were done in normal bridging mode differences in resting potentials have the potential to affect mini size and therefore the calculation of quantal content. The majority of experimental groups tested are not significantly different from their controls or wild-type therefore the slight differences in RMPs should not overtly affect the calculation of quantal content. In the case of TkvA (neuron) where the RMP is more negative this would tend to increase miniature size and decrease quantal content when compared to controls. To find the average EJP, 10 evoked responses were evaluated using MiniAnalysis software and averaged (Synaptosoft). Spontaneous events were recorded for one minute and the first 50 events were evaluated using MiniAnalysis software (Synaptosoft) to determine quantal size and frequency. The exception to this is the Mad^{1}/Mad^{12} mutant, where due to the decreased frequency of spontaneous events, an average number of 36 were evaluated for each animal. Quantal content was adjusted because spontaneous events and EJPs were recorded in saline with physiological Ca^{++} (Martin, 1955).

Statistics. Data found in text and graphs are shown +/- standard error of the mean. Statistical significance was determined using the Student's t-test ($p<0.05$ = statistical significance).

DALLY-LIKE

Drosophila strains. "Wild-type" refers to *Oregon-R* except for larval anti-Syndecan staining which was performed on w^{1118} [Bloomington Stock Center]. dlp^{1} st^{1} ry^{506}/*TM6B Tb* was crossed to *Df(3L)fz D21, st th/TM6B Tb* [Bloomington Stock Center] or dlp^{2} *ru h st ry e/TM6B Tb* to generate *dlp* mutants(Kirkpatrick et al., 2004). dlp^{1} is a 504 bp deletion that extends two bp beyond the ATG of the presumed *dlp* initiation codon. dlp^{2} is an EMS allele that has not been sequenced, but lacks all detectable Dlp protein (data not shown). *Oregon-R* was crossed to above *dlp* heterozygotes to generate *dlp/+* larvae, pupae and adult controls. *P[ry*$^{+t7.2}$*; ey-FLP.N]2; RpS17*^{4} *P[ry*$^{+t7.2}$*; neoFRT]80B/TM6B,* Tb^{1} [Bloomington Stock Center] flies were crossed to dlp^{1} st^{1} ry^{506} *P[ry*$^{+t7.2}$*; neoFRT]80B /TM6B, Tb* or *P[ry*$^{+t7.2}$*; neoFRT]80B* flies to generate flies with mosaic *dlp* mutant or mosaic "wild-type control" eyes (Newsome et al., 2000). Mosaic animals for ERG recordings were generated by crossing *P[w*$^{+m*}$*; GAL4-ey.H]3-8, P[w*$^{+mC}$*; UAS-FLP1.D]JD1/CyO; P[w*$^{+mW.hs}$*; FRT(w*^{hs}*)]2A P[w*$^{+mC}$*; GMR-hid]SS3, l(3)CL-L*^{1}*/TM6* flies [Bloomington Stock Center] to dlp^{1} st^{1} ry^{506} *P[w*$^{+mW.hs}$*; FRT(w*^{hs}*)]2A/TM6B, Tb* or *P[w*$^{+mW.hs}$*; FRT(w*^{hs}*)]2A* flies (Stowers and Schwarz, 1999). *P[w*$^{+}$*; UAS-GFP nls]* or *P[w*$^{+mC}$*; UAS-mCD8::GFP.L]LL4* flies were crossed with either *P[w*$^{+}$*; GAL4-repo]/TM3, Sb* or *P[w*$^{+}$*; GAL4-GMR]CyO* flies for the purpose of marking glia or photoreceptors respectively with GFP [Bloomington Stock Center]. Dlp rescue constructs *UAS-dlp39.2; Df(3L)/ TM6B Tb, UAS-dlp39.3; Df(3L)/ TM6B Tb, UAS-dlp39.3;* dlp^{2}*/ TM6B Tb* and *UAS-dlp HA #5;* dlp^{2}*/ TM6B Tb* were crossed to dlp^{1} st^{1} ry^{506}*/TM6B* to generate rescued

animals. *Df48*$^{ubi\text{-}Sara}$ and *Sdc*^{10608} alleles balanced over *CyO P[w*$^{+}$*; actin-GFP]* were crossed to each another or *Oregon-R* to generate homozygous *syndecan* mutants or heterozygous controls (Johnson et al., 2004). *slit*^{2} and *robo*^{GA285} were obtained from the Bloomington Stock Center. *Robo*^{GA285}, *robo3*^{1} and *robo*^{GA285}, *robo2*^{8} double mutants were obtained from B. Dickson.

Immunohistochemistry. Larvae and pupae were fixed and stained using standard procedures (Sullivan et al., 2000). Monoclonal antibodies anti-Repo (8D12), anti-Elav (7E8A10), anti-Prospero (MR1A), anti-Chaoptin (24B10) and anti-Dachshund (mAbdac2-3) were used for immunocytochemistry (Iowa Hybridoma Bank). Anti-senseless (guinea pig) was used for R8 identification (Nolo et al., 2000). Anti-Dlp was provided by Phil Beachy and anti-Sdc was provided by David VanVactor (Lum et al., 2003; Johnson et al., 2004). Secondary antibodies used for detection were from the Alexa series (Molecular Probes). Embryonic antibody staining was carried out as described below. Staining was conducted using the following antibodies: Anti-Wrapper (Noordermeer et al., 1998) and Anti-Engrailed (Patel et al., 1989). Anti-Sdc immunostaining was conducted on stage 14-16 live-dissected embryos as described previously (Johnson et al., 2004). 1D4 (FasII) immunohistochemistry was performed as previously described (Vactor et al., 1993). After extensive washing in PBS, embryonic pelts were incubated for 1-2 hours in 1:500 dilutions of goat-anti-mouse Alexa488 and goat-anti-rabbit Alexa568 (both from Molecular Probes) or of peroxidase conjugated goat-anti-mouse and goat-anti-rabbit (both from Jackson Immunoresearch). Following

fluorescent immunohistochemistry, pelts were washed in PBS and mounted in SlowFade (Molecular Probes). Embryos stained with peroxidase-conjugated antibodies were reacted with a DAB peroxidase substrate kit (Vector Laboratories) and mounted in 70% glycerol.

Confocal Microscopy, Scanning Electron Microscopy (SEM) and Thin Sections. A Nikon upright laser confocal microscope was used to acquire images for larval and pupal studies. SEM and thin sections of the adult eye were performed following standard procedures (Sullivan et al., 2000). For embryos analysis of antibody staining was conducted using a Nikon E800 microscope and a Bio-Rad Radience confocal.

Electrophysiology. Electroretinograms (ERGs) were recorded using standard techniques described in (Sullivan et al., 2000). Thin-walled glass recording electrodes with resistances from 10-20 MΩ were pulled (Sutter Instrument Company, Model P-97) and were backfilled with 1M KCl. One glass electrode was placed into the thorax as a reference electrode while the other electrode was placed just under the surface of the fly's eye. The recording equipment was inside of a curtained area to prevent the entry of light. After electrode placement, the enclosure was sealed and the animal was dark adapted for 5 minutes. After dark adaptation, a 1 minute recording was performed during which a manual shutter was opened for ~1 second intervals with ~2 seconds of recovery time in between light stimuli. Recordings were acquired with an Axoclamp 2B amplifier and pClamp9 software (Axon Instruments). To find the average "on-transient", "off-

transient" and photoreceptor response amplitudes from a single animal, approximately 13 events per animal were measured with ClampFit and averaged. Values from each animal of a given genotype were averaged to give the amplitudes shown in the Result section.

Statistics. Error bars in graphs are +/- SEM. Statistical significance was determined using the Student's t-test ($p<0.05$ = statistical significance).

APPENDIX A. FIGURE LEGENDS

Figure 1. P-Mad is expressed in the nervous system of late stage embryos.

All panels show a projection of multiple confocal sections of wild-type embryos, ventral up, anterior to the left. (A) Early stage 15 embryo, projection of ventral half. Cells in the CNS begin to show P-Mad reactivity (large arrowheads). Cells in the midgut also express P-Mad (small arrowheads) (scale bar = 100 μm). (B) Stage 16 embryo, projection of ventral half. More cells in each segment of the CNS show anti-P-Mad reactivity (large arrowheads). Cells in the midgut continue to express P-Mad (small arrowheads). Conversely note lack of P-Mad staining in abdominal muscles (double arrowheads). (C-E) Stage 16 embryo, projection of dorsal part of CNS and part of midgut. (C) Anti-P-Mad (red) staining in CNS (large arrowheads) and midgut (small arrowheads) (scale bar = 50 μm). (E) Anti-Elav staining marking neurons of the CNS (green). (D) Merge of C & E demonstrates co-localization of P-Mad to Elav positive nuclei (neurons). (F-H) Stage 16 embryo, projection of whole CNS. (F) Anti-P-Mad (red) staining in CNS (large arrowheads) and midgut (small arrowheads). (H) Anti-Eve staining (green) of a characterized set of neurons (see labels). (G) Merge of F & H demonstrates co-localization of P-Mad to a subset of Eve-expressing neurons. (I-K) Stage 17 embryo, projection of dorsal part of CNS. (I) Anti-P-Mad (red) staining of neurons (large arrowheads). (K) Anti-FasIII staining (green), RP3 neurons are labeled. (J) Merge of I & K demonstrates co-localization of anti-P-Mad with anti-FasIII staining (RP3) neurons.

Figure 2. *sax* and *Mad* mutant embryos have phenotypically normal nervous systems. (A,B&C) Ventral-lateral view of stage 16 embryos stained with anti-FasII shows a subset of axons in the CNS, projections of whole embryos, insets are magnifications of area outlined by small rectangles. (A) Wild-type FasII staining pattern (scale bar = 100 μm). (B) Mad^{1}/Mad^{12} and (C) $Df(2R)Sax\text{-}H9/sax^{3}$ mutants do not have defects in axon architecture indicating pathfinding occurs normally. (D,E&F) Ventral view of stage 16 embryos stained with anti-Eve, left panel is projection of dorsal portion of the nervous system, right panel is projection of ventral portion of the nervous system in the same embryo, characterized Eve-expressing neurons are labeled. (D) Wild-type Eve expression pattern (scale bar = 50 μm). (E) Mad^{1}/Mad^{12} and (F) $Df(2R)Sax\text{-}H9/sax^{3}$ mutants do not have defects in specification of Eve expressing neurons. (G,J) Anti-FasIII stained stage 16 embryos showing RP3 cell body, RP processes, and synapse on muscles 6 and 7. These images are projections of multiple confocal sections that allow FasIII positive axons (one is presumably the RP3 axon, small arrowheads) to be followed from the CNS to the periphery. The black bars mark segmental boundaries observed with anti-FasIII staining of epidermal cells (scale bar = 50 μm). (H,I) Higher magnification view of single confocal section revealing a FasIII positive axon (small arrowheads) and termini (large arrowheads) at muscles 6 and 7 in wild-type and *Mad* mutant embryos.

Figure 3. *sax* and *Mad* mutants have morphological and electrophysiological defects. * indicates statistical significance of mutant group compared to control groups. (A) Representative voltage traces showing evoked responses (EJPs) following a single

stimulation in wild-type, *Df(2R)Sax-H9/sax*^{3} and *Mad*^{1}*/Mad*^{12} third instar larvae. (B) Average EJP amplitude (dark bars) and quantal content (light bars) for *sax* and *Mad* mutants and controls. [For EJP and QC measures: WT, n=16, n=10; *Df(2R)Sax-H9/CyO*, n=10, n=10; *sax*^{3}*/CyO*, n=5, n=5; *Df(2R)Sax-H9/sax*^{3}, n=15, n=9; *Mad*^{1}*/CyO*, n=10, n=10; *Mad*^{12}*/CyO,* n=10, n=10; *Mad*^{1}*/Mad*^{12}, n=10, n=8.] (C) Bouton counts of wild-type, *sax* and *Mad* mutants and heterozygous controls, represented as % of wild-type, normalized for muscle area. [WT, n=14; *Df(2R)Sax-H9/+*, n=10; *sax*^{3}*/+*, n=8; *Df(2R)Sax-H9/sax*^{3}, n=9; *Mad*^{1}*/+*, n=10; *Mad*^{12}*/+*, n=9; *Mad*^{1}*/Mad*^{12}, n=10.] (D-F) Anti-P-Mad and anti-βGal staining of control (heterozygotes bear balancer chromosome with *wingless-lacZ* insert) and mutant stage 16 embryos. Small arrowheads point to the normal location of wild-type midgut anti-P-Mad staining and large arrowheads point to the normal location of wild-type CNS anti-P-Mad staining. (D) *sax/CyO*. (E) *Mad*^{12}*/Mad*^{1}. (F) *Df(2R)Sax-H9/sax*^{3}. (G-I) anti-CSP staining of third instar larval synapses. Type Ib boutons are indicated by large arrowheads and type Is boutons are indicated by small arrowheads. (G) Wild-type (scale bar = 50 μm). (H) *Mad*^{1}*/Mad*^{12}. (I) *Df(2R)Sax-H9/sax*^{3}.

Figure 4. Expression of dominant negative or activated type-I receptors affect NMJ function.

* indicates a statistically significant difference for control animals (wild type, *UAS-construct/+* or *tissue-specific GAL4/+*) compared to experimental animals with tissue specific expression of *UAS-construct*. (A) Average EJP amplitude (dark bars) and quantal content (light bars) from control animals and experimental animals with tissue-specific

expression of *UAS-tkvDN*. [For EJP and QC measures: WT, n=16, n=10; TkvDN/+ (control), n=16, n=10; TkvDN (muscle), n=14, n=10; TkvDN (neuron), n=10, n=10.] (B) Average EJP amplitude (dark bars) and quantal content (light bars) from control animals and experimental animals with tissue-specific expression of *UAS-saxDN*. (C) Average EJP amplitude (dark bars) and quantal content (light bars) from control animals and experimental animals with tissue-specific expression of *UAS-tkvA*. [For EJP and QC measures: WT, n=16, n=10; TkvA (control), n=13, n=9; TkvA (muscle), n=10, n=10; TkvA (neuron), n=16, n=11.]

Figure 5. Expression of dominant negative or activated type-I receptors affect NMJ morphology.

* indicates a statistically significant difference for control animals (wild type, *UAS-construct/+* or *tissue-specific GAL4/+*) compared to experimental animals with tissue specific expression of *UAS-construct*. (A) Normalized bouton counts for animals bearing various transgenes. [muscle GAL4 (control), n=10; neuron GAL4 (control), n=10; TkvDN (control), n=10; TkvDN (muscle), n=10; TkvDN (neuron), n=8.] (B) Normalized bouton counts for animals bearing various transgenes. [muscle GAL4 (control), n=10; neuron GAL4 (control), n=10; SaxDN (control), n=10; SaxDN (muscle), n=9; SaxDN (neuron), n=10.] (C-F) Anti-CSP staining of third instar larval synapses at abdominal wall muscle 6 and 7, segment 2. (C) *saxDN/+* NMJ. (D) NMJ with neuron-specific expression of SaxDN. (E) Wild-type NMJ. (D) NMJ with neuron-specific expression of TkvA.

Figure 6. Expression of Dlp in the embryonic nervous system and patterning of the embryonic nervous system in *dlp* mutants.

(A-D) Wild-type *Drosophila* embryos stained with anti-Dlp antibody, ventral view, anterior left. (A) Nervous system specific Dlp expression begins at stage 12 embryo (arrowheads). (B) Dlp expression is found on axons crossing the midline at the anterior and posterior (AC & PC) commissures in stage 13 embryos. (C) Dlp is found on longitudinal axon tracts in stage 14 embryos (arrow). (D) Dlp is expressed strongly on longitudinal axon tracts, commissures and on peripheral nerves (arrowheads) in stage 16 embryos. (E-H) Dissected *Drosophila* embryos, partial view of ventral nerve cord. (E) Wild-type embryo stained with midline glia-specific anti-Wrapper antibody shows normal distribution of these cells in the repeating abdominal segments of the ventral nerve cord. (F) dlp^1/dlp^1 mutant embryo stained with anti-Wrapper demonstrates these cells are patterned normally. (G) Wild-type embryo stained with anti-Engrailed antibody shows normal distribution of a subset of neurons in the repeating abdominal segments of the ventral nerve cord. (H) *dlp* mutant embryo stained with anti-Engrailed demonstrates these cells are patterned normally.

Figure 7. Axon guidance defects in *dlp* mutant embryos.

(A-C) Stage 17 filleted embryos stained with anti-FasciclinII (FasII) antibody, anterior is to left. (A) Wild-type embryos have three distinct FasII expressing longitudinal tracts on each side of the midline (dashed line). (B&C) dlp^1/dlp^1 mutants show defects in the

fasciculation of these FasII positive axon tracts, particularly gaps in the most lateral fascicle (arrows) and fusion of the lateral and intermediate fascicles (arrowheads). (D) Graph showing quantitation of percent animals showing fasciculation defects in *dlp* mutants. (E) Graph showing quantitation of average number of segments per animal with midline crossing defects in *dlp* mutant animals.

Figure 8. Dlp expression in the developing adult visual system.

All images are of wild-type preparations. (A) Eye-antennal disc stained with anti-Dlp antibody (red), anterior to left. Dlp is expressed in the morphogenetic furrow (arrow) and in differentiated photoreceptors (arrowhead). Dlp immunoreactivity is found in photoreceptor axons exiting at optic stalk (asterisk). (B-F) Middle panel is a merge of red and green channels. (B) View of posterior portion of eye-antennal disc. Dlp (red) is present on photoreceptors as evidenced by colocalization with photoreceptor specific expression of GFP (*GMR-GAL4* driving *UAS-GFP*) (green) (arrowheads). (C-H) Dorsal/posterior view of right hemisphere of third instar larval brain, anterior to left. (C) Dlp is observed on R1-6 termini in lamina plexus (arrow) and in cells just below the photoreceptor R1-6 termini, consistent with the position of a group of glial cells (arrowhead) (see E below). (D) Dlp is present on a subset of photoreceptor projections to the optic lobe (arrows). Dlp colocalizes with R7/8 termini in the medulla (arrowheads) but is also present broadly in this region (see F below). (E) Dlp (red) is expressed in medulla glia (arrowhead) but not lamina epithelial or marginal glia (arrow), as demonstrated by Dlp colocalization with GFP expressed under control of a glial-specific

GAL4 line (*repo-GAL4* directed expression of *UAS-GFP*). Dlp is present on lamina plexus (asterisk). (F) Dlp is also expressed by medulla neuropil glia (arrowhead) in the region of R7/8 termination zone (arrowhead). (G) Dlp is present on neuroblasts of both the inner (arrows) and outer (arrowheads) proliferative centers of the optic lobe, and is highly expressed in the mushroom body neuropil (G-MB, H-arrow). Scale bars are 50 microns.

Figure 9. *dlp* mutations affect photoreceptor axon morphology.

(A-B) Photoreceptor specific antibody staining (24B10) of third instar brains, dorsal/posterior view, anterior to left. (A) *dlp* heterozygote showing wild-type 24B10 staining pattern of lamina plexus (arrow) and medulla (arrowhead). (B) *dlp* mutant 24B10 staining reveals a thickening of the lamina plexus (arrow) and crossovers between ommatidial axon bundles (see inset), while the pattern of R7&8 termini in the medulla remains unaffected (arrowhead). (C-D) 24B10 staining of 40 hour pupal brains. (C) Wild-type optic lobe. Note the two distinct layers of R7 and R8 termini (labels) in medulla. (D) *dlp* mutant pupae show defects in R7/8 (labels) termini in medulla. Scale bars are 50 microns. (E) Quantitation of photoreceptor axon defects in third instar larvae. (F) Quantitation of photoreceptor axon defects in 40 hour pupae.

Figure 10. *dlp* mutations affect physiological function of the visual system.

(A) Voltage measurements of ERG components in *dlp* heterozygote controls and *dlp* homozygous mutant animals. Asterisk denotes statistically significant difference of

mutant compared to control (P<.05). (B) Representative ERG traces from *dlp* heterozygous control and *dlp* homozygous mutant. Scale is 3mV, 200ms.

Figure 11. *dlp* mutations affect patterning of retina accessory cells but not photoreceptors. (A-B) SEM of adult eyes. (A) Wild-type eye demonstrating repeating pattern of hexagonal ommatidia that have mechanosensory bristles flanking every other corner (arrowhead). (B) *dlp* mutations disrupt the hexagonal structure of ommatidia (arrows) and also cause defects in the placement and number of bristles (arrowheads). (C-D) Toluidine Blue stained thin sections of an adult eye. Scale bar is 10 microns. (C) Wild-type eye with hexagonal ommatidia and seven photoreceptors (one not visible in section; arrowheads mark rhabdomeres, the photosensing apparatus of the cell). Note mirror symmetry of ommatidia across D/V boundary (dashed line). (D) *dlp* mutant eye shows defects in the hexagonal structure of ommatidia, but retains correct photoreceptor specification (see rhabdomeres marked with arrowheads). Mirror symmetry of ommatidia across D/V boundary is also intact (dashed line). (E-F) anti-ELAV (pan-neural marker-red), anti-Senseless (R8 marker-blue) and anti-Prospero (R7 marker-green) staining of third instar eye-antennal disc, anterior to left. Scale bar is 50 microns. (E-E') Wild-type disc. (F-F') *dlp* mutant disc retains wild-type staining patterns of anti-ELAV stained ommatidia (outlined with dashed red line) and R7/R8 photoreceptors (labels). Scale bar is 20 microns.

Figure 12. *dlp* mutations do not cause defects in glial cell or lamina neuron specification.

(A-B) anti-Repo (glial) staining of eye-antennal discs from third instar larvae, anterior to left. (A) Wild-type disc showing normal pattern of glial migration terminating posterior to morphogenetic furrow (arrow). (B) *dlp* mutant disc with proper specification and migration of glia posterior to morphogenetic furrow (arrow). (C-D) anti-Repo staining of third instar optic lobes, dorsal/posterior view, anterior to left. (C) Wild-type larval optic lobe showing the three layers of glia that flank the lamina: epithelial (eg), marginal (mg) and medulla glia (meg). Medulla neuropile glia (mng) are also shown. (D) *dlp* mutant larvae have proper specification of all four populations of glia (labels). (E-F) anti-Dachshund staining of third instar lamina neurons in optic lobe, dorsal/posterior view. (E) Wild-type brain showing lamina neurons (arrow). (F) *dlp* mutant brain shows correct number and specification of lamina neurons (arrow). Scale bars are 50 microns.

Figure 13. Removal of Dlp from the eye is sufficient to produce some but not all *dally-like* mutant phenotypes.

(A-B) 24B10 staining of larvae (single optical sections) with retinas composed of ~80 percent *dlp* homozygous mutant cells projecting to *dlp* heterozygous (wild-type) brain, dorsal/posterior view of optic lobe. Scale bar is 20 microns. (A) Homozygous *dlp* mutant photoreceptors projecting to a heterozygous (wild-type) brain show defects including fibers crossing between ommatidial axon bundles (arrowheads). (B) These larvae also show abnormal photoreceptor process expansion outside of lamina plexus (arrowheads). (C-D) Single optical sections from 24B10 stained 40 hour pupal optic lobes, dorsal view.

Scale bar is 50 microns. (C) *dlp* homozygous mutant pupae have defects in R7/8 termini in medulla (see labels). (D) Homozygous *dlp* mutant photoreceptors projecting to heterozygous brain do not have the defects in R7/8 termini seen in homozygous mutants (see labels). (E) Graph of ERG component amplitudes for control mosaic and *dlp* mosaic animals. Animals with a *dlp* homozygous mutant eye projecting to heterozygous (normal) brain have normal ERG components.

Figure 14. *dlp* mutant transgenic rescue.

(A-B) Photoreceptor specific antibody staining (24B10) of third instar brains, dorsal/posterior view, anterior to left. (A) *dlp* mutant 24B10 staining reveals a thickening of the lamina plexus and other axon abnormalities. (B) *UAS-dlp* transgene present in a *dlp* mutant background is sufficient to rescue axon abnormalities without a *GAL4* driver. (C-D) 24B10 staining of 40 hour pupal brains. (C) *dlp* mutant pupae show defects in R7/8 (labels) termini in medulla. (D) *UAS-dlp* transgene present in a *dlp* mutant background is sufficient to rescue R7/8 termini abnormalities without a *GAL4* driver. (E) Quantitation of transgene rescue of photoreceptor axon defects in *dlp* mutant third instar larvae. (F) Quantitation of transgene rescue of photoreceptor axon defects in *dlp* mutant 40h pupae.

Figure 15. *dlp* mutations do not show a genetic interaction with mutations in Slit/Robo signaling components.

(A) Quantitation of midline crossing defects in *dlp* and *sdc* mutant embryos. Note lack of defects in *dlp* mutants. (B) Quantitation of fasciculation defects in *dlp* and *sdc* mutant

embryos. Note similar severity and penetrance of defects in animals carrying mutations in either gene. (C) Quantitation of genetic interactions between *dlp/slit* or *sdc/slit* pertaining to midline crossing defects. Note genetic interaction of *sdc/slit* but lack of interaction between *dlp* and *slit*. (D) Quantitation of genetic interactions between *dlp/slit* or *sdc/slit* pertaining to fasciculation defects. Note lack of genetic interaction between either *slit* and *sdc* or *slit* and *dlp*.

Figure 16. *dlp* mutations show a genetic interaction with mutations in *sdc*.

(A,C-H) Stage 17 filleted embryos, anterior is to left. (A) Wild-type *Drosophila* embryo stained with anti-Dlp and anti-Sdc antibodies, ventral view, anterior left. Note colocalization of Dlp and Sdc to both commissures (c) and longitudinal tracts (L). Midline is marked (dashed line). (B) Table showing quantitation of midline and fasciculation defects in *sdc*, *dlp* and *sdc/dlp* mutants. Note strong genetic interactions between *sdc* and *dlp* (*sdc;dlp* compared to single mutants) for both midline crossing and fasciculation defects. (C-H) FasII staining of embryos. (C) Wild-type embryos have three distinct FasII expressing longitudinal tracts on each side of the midline (dashed line). (D) dlp^1/dlp^1 mutants show defects in the fasciculation of these FasII positive axon tracts, particularly gaps in the most lateral fascicle (arrows) and fusion of the lateral and intermediate fascicles (arrowheads). (E) *sdc/sdc* mutants show defects in the fasciculation of FasII positive axon tracts including gaps in the most lateral fascicle (arrows), fusion of the lateral and intermediate fascicles (arrowheads) and aberrant midline crossing

(asterisk). (F-H) *sdc/sdc; dlp/dlp* double mutants have much more severe fasciculation (asterisk) and midline crossing (arrows) defects than observed in either mutant alone.

Figure 17. Sdc is expressed in the developing adult visual system.

(A-C) Anti-Sdc (red) and 24B10 (green) staining of wild-type third instar larval eye-antennal disc and brain, anterior to left, dorsal/posterior view of brain. (A & A') Wild-type eye disc shows colocalization of anti-Sdc and 24B10 on photoreceptors (arrowhead). (B & B') Wild-type optic lobe shows presence of Sdc on a subset of photoreceptor axons (arrows) and (R1-6) termini in lamina plexus (arrowhead). (C & C') Sdc is expressed within the inner and outer proliferative centers (arrows). Conversely, note lack of Sdc expression on photoreceptor axons or other tissue in R7/8 termination zone in medulla (arrowheads).

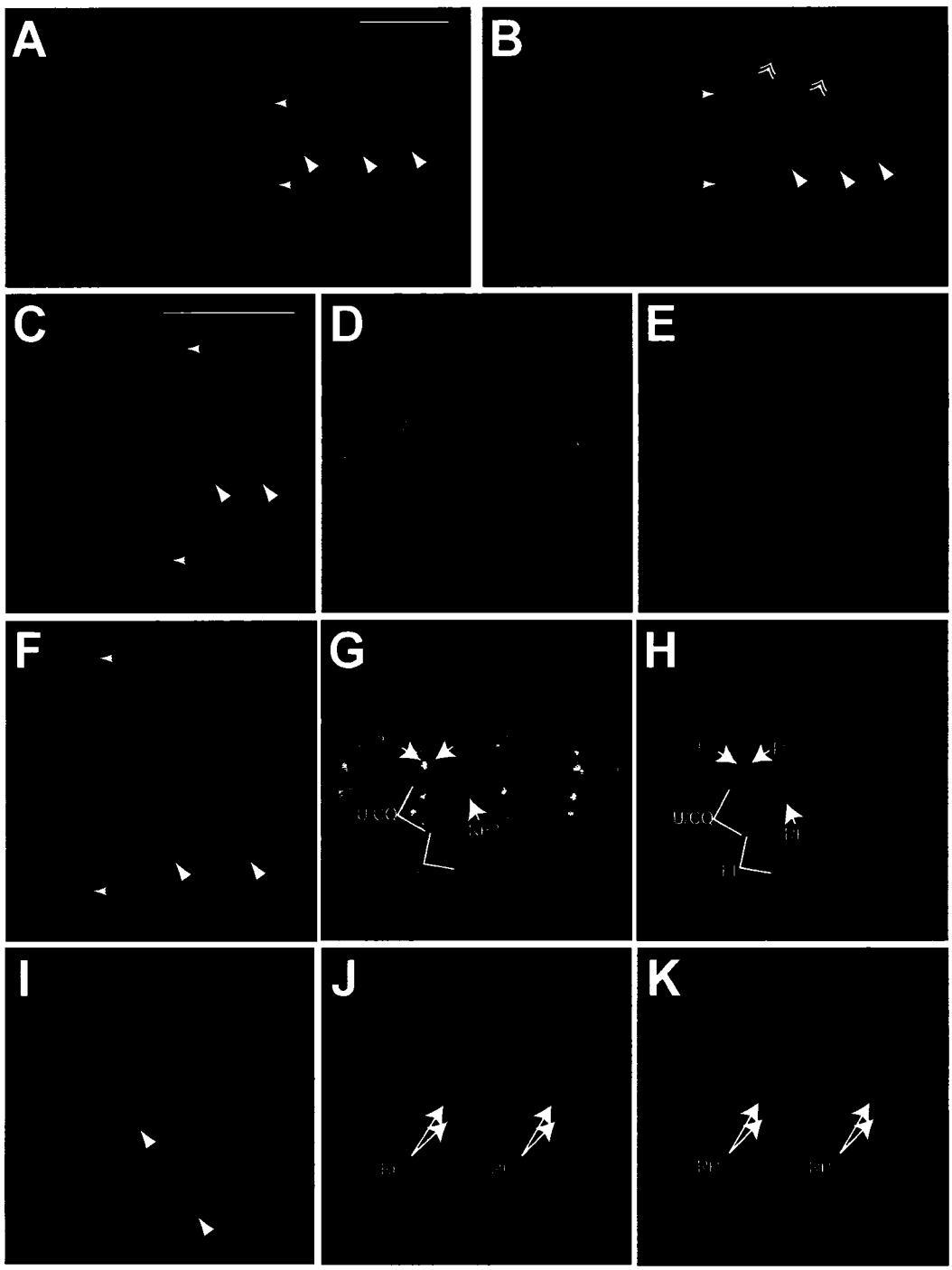
Figure 18. Sdc is required for the specification of proper photoreceptor axon morphology.

(A-B) 24B10 staining of third instar larval brains. (A) *sdc* heterozygote has wild-type 24B10 staining pattern; lamina plexus (arrow) and medulla (arrowhead). (B) *sdc* mutant 24B10 staining pattern shows severe disruption of lamina plexus morphology (arrow) while the pattern of R7/8 endings in medulla remains relatively unaffected (arrowhead). (C) Quantitation of phenotype penetrance in *sdc* and *dlp* mutant larvae. (D-F) 24B10 staining of 40 hour pupal brains. (D) Wild-type optic lobe shows the projections of R7 & R8 from a single ommatidium (arrowhead on inset) remain in the same medulla cartridge. (E) *sdc* mutant pupae show crossover of R7 termini to neighboring cartridges (arrowhead

on inset). (F) In *sdc* mutant pupae some R7/8 axon projections deviate (arrows) from the proper route (arrowheads) on course to the medulla. (G) Quantitation of phenotype penetrance in *sdc* mutant pupae. Scale bars are 50 microns.

APPENDIX B. FIGURES

Figure 1



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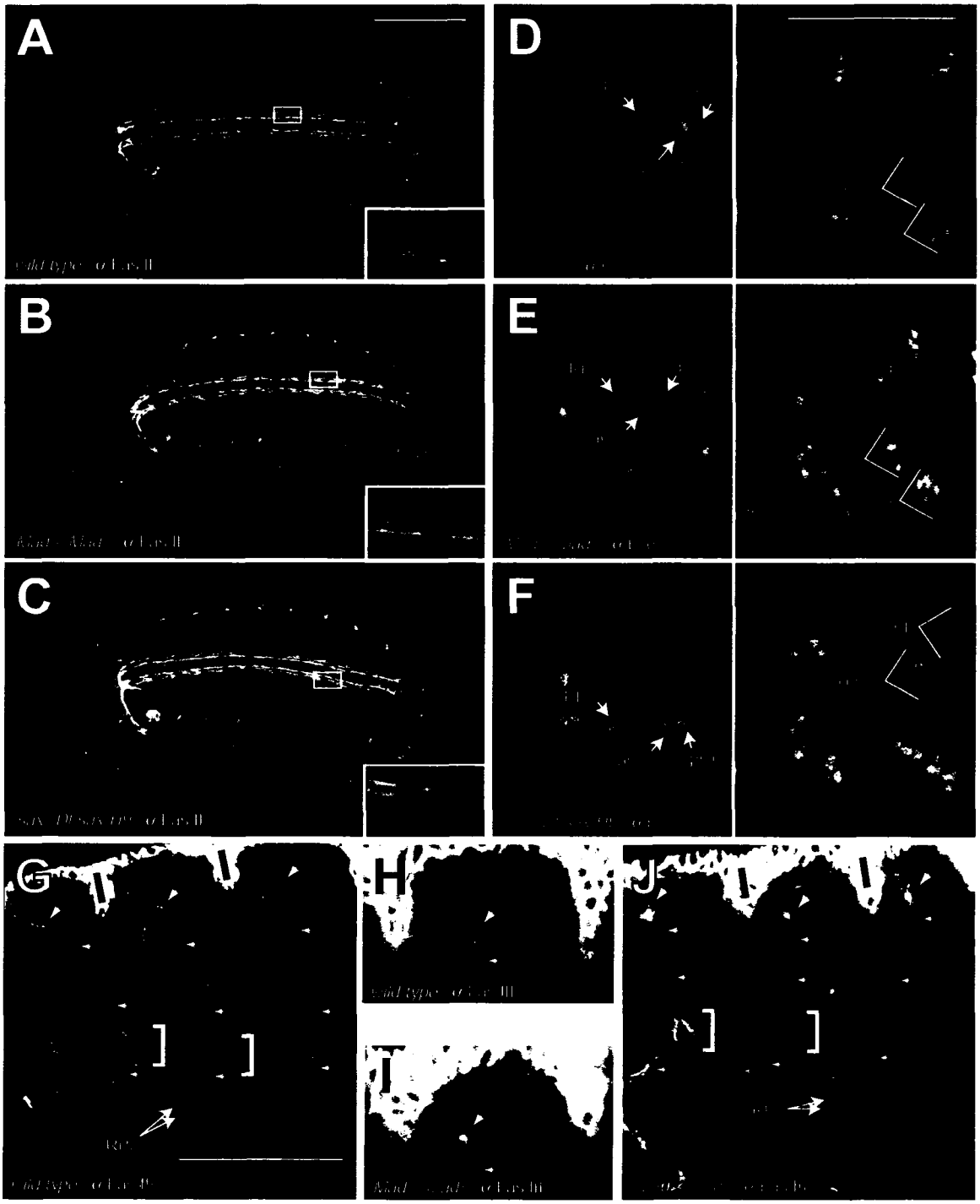

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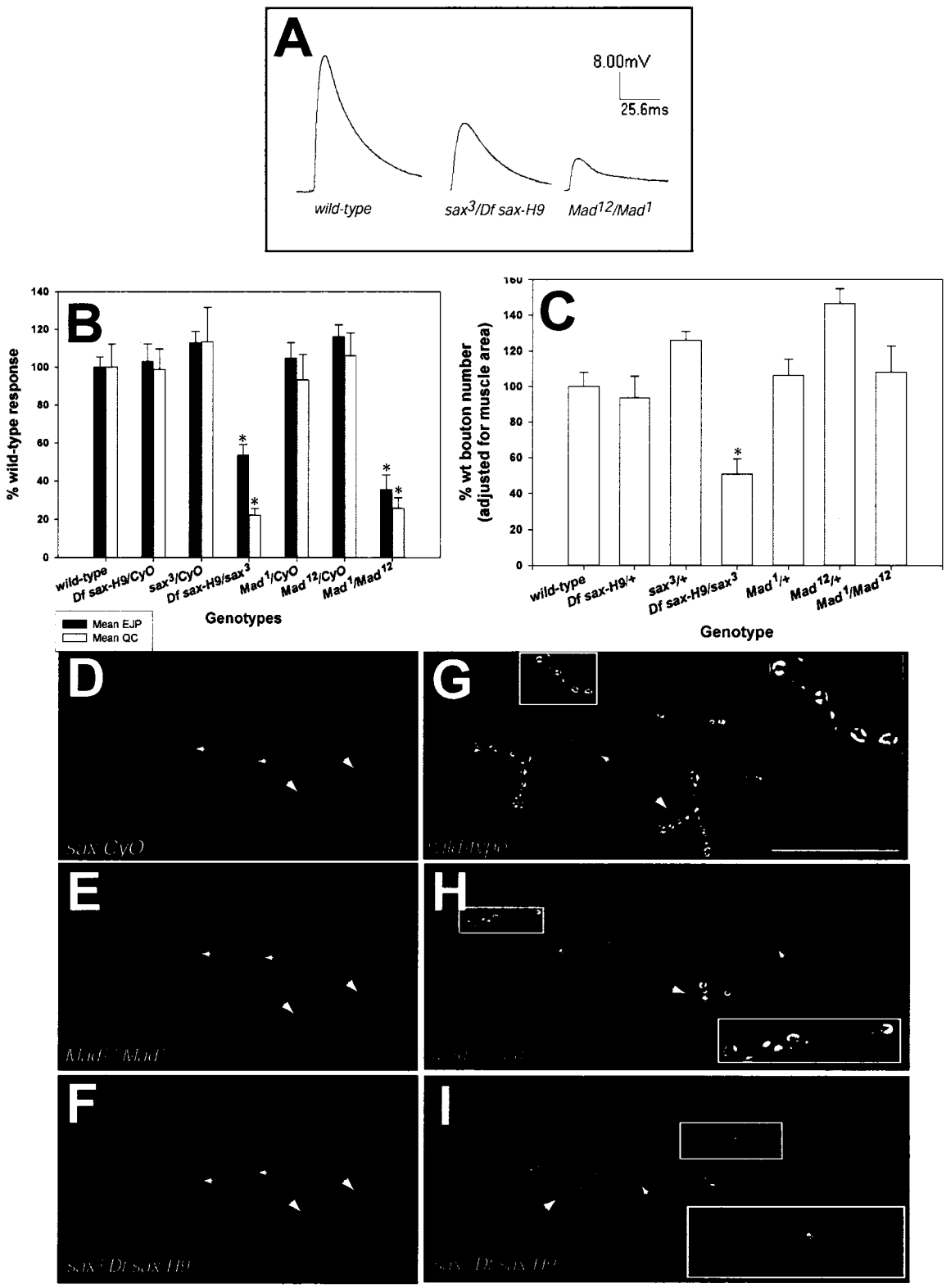
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sax3/CyO
Df sax-H9/sax3
Mad1/CyO
Mad12/CyO
Mad1/Mad12
Mean EJP
Mean QC
Genotypes
C
% wt bouton number (adjusted for muscle area)
wild-type
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E
H
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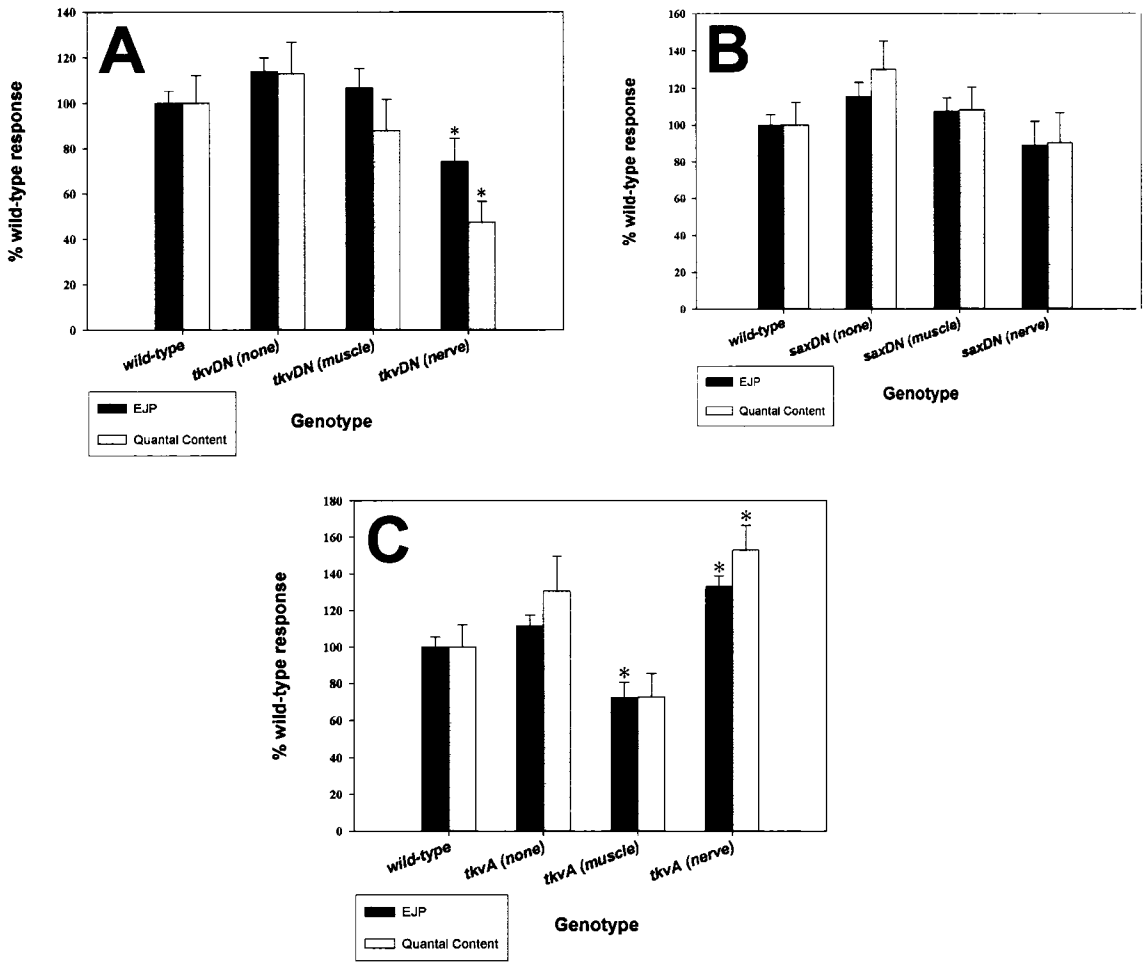
I

Figure 4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EJP
Quantal Content
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EJP
Quantal Content
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160
140
120
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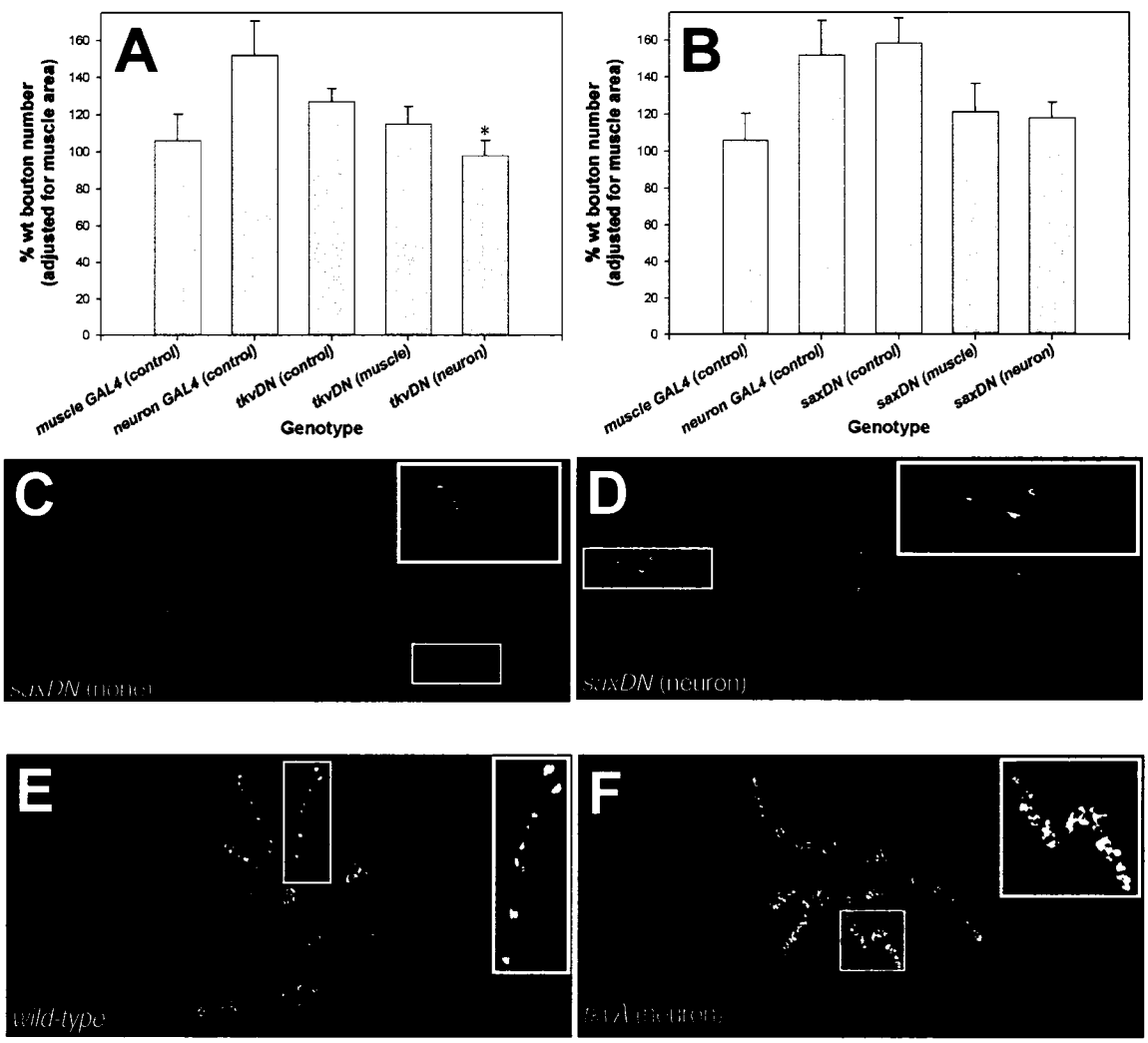
Quantal Content
Genotype

Figure 5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100
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140
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tkvDN (control)
tkvDN (muscle)
tkvDN (neuron)
Genotype
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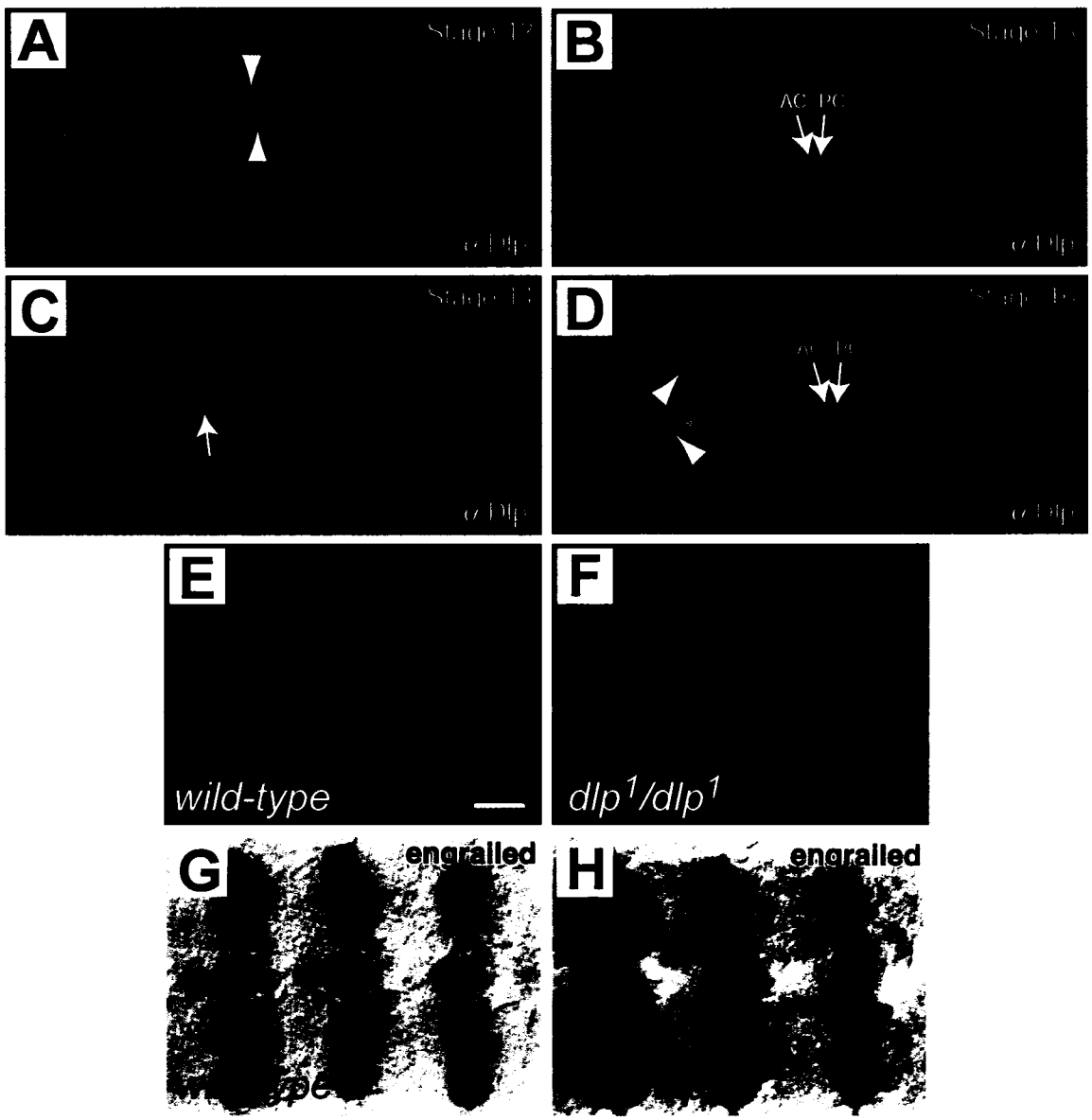
Figure 6



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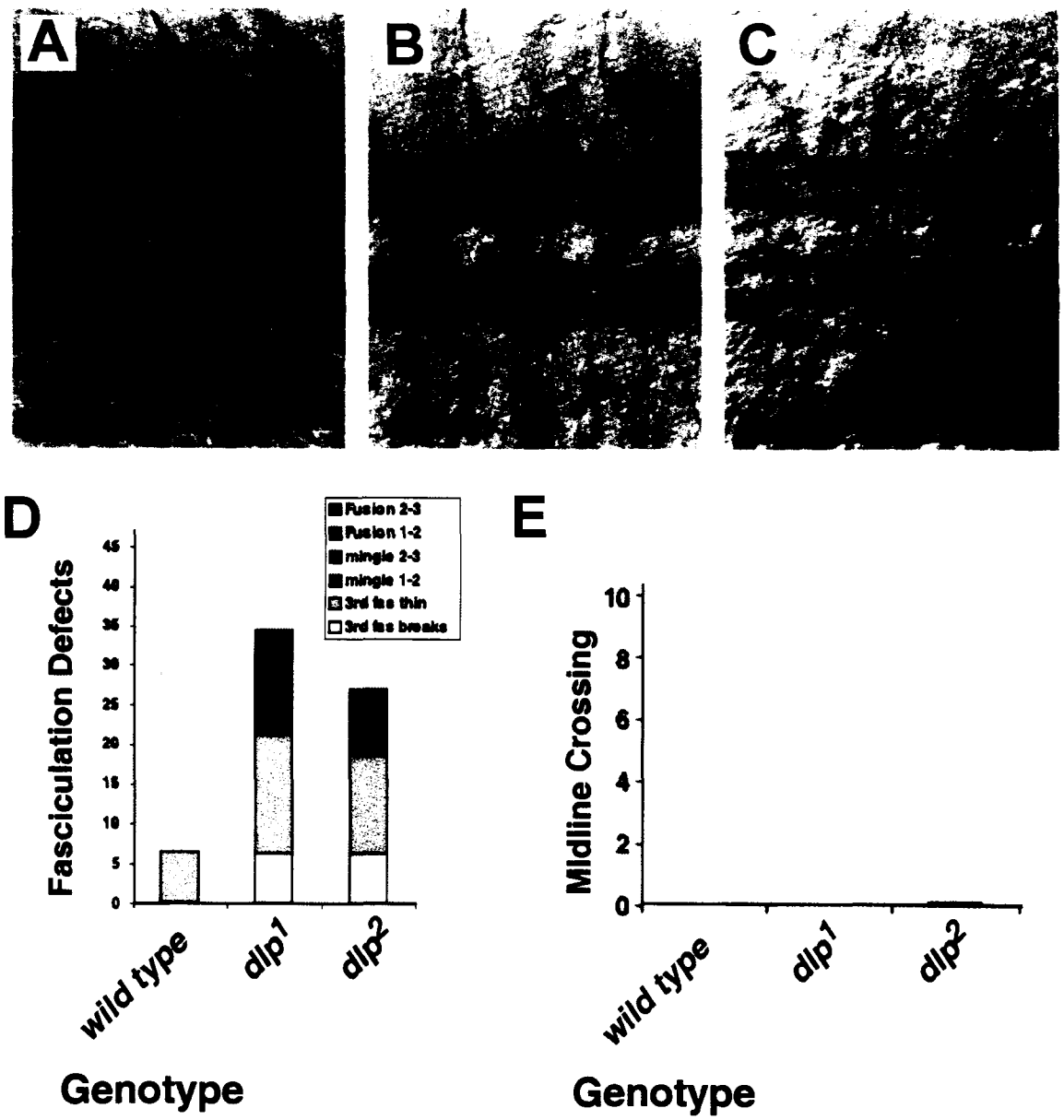
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5
10
15
20
25
30
35
40
45
wild type
dlp1
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dlp2
Genotype

Figure 8.1

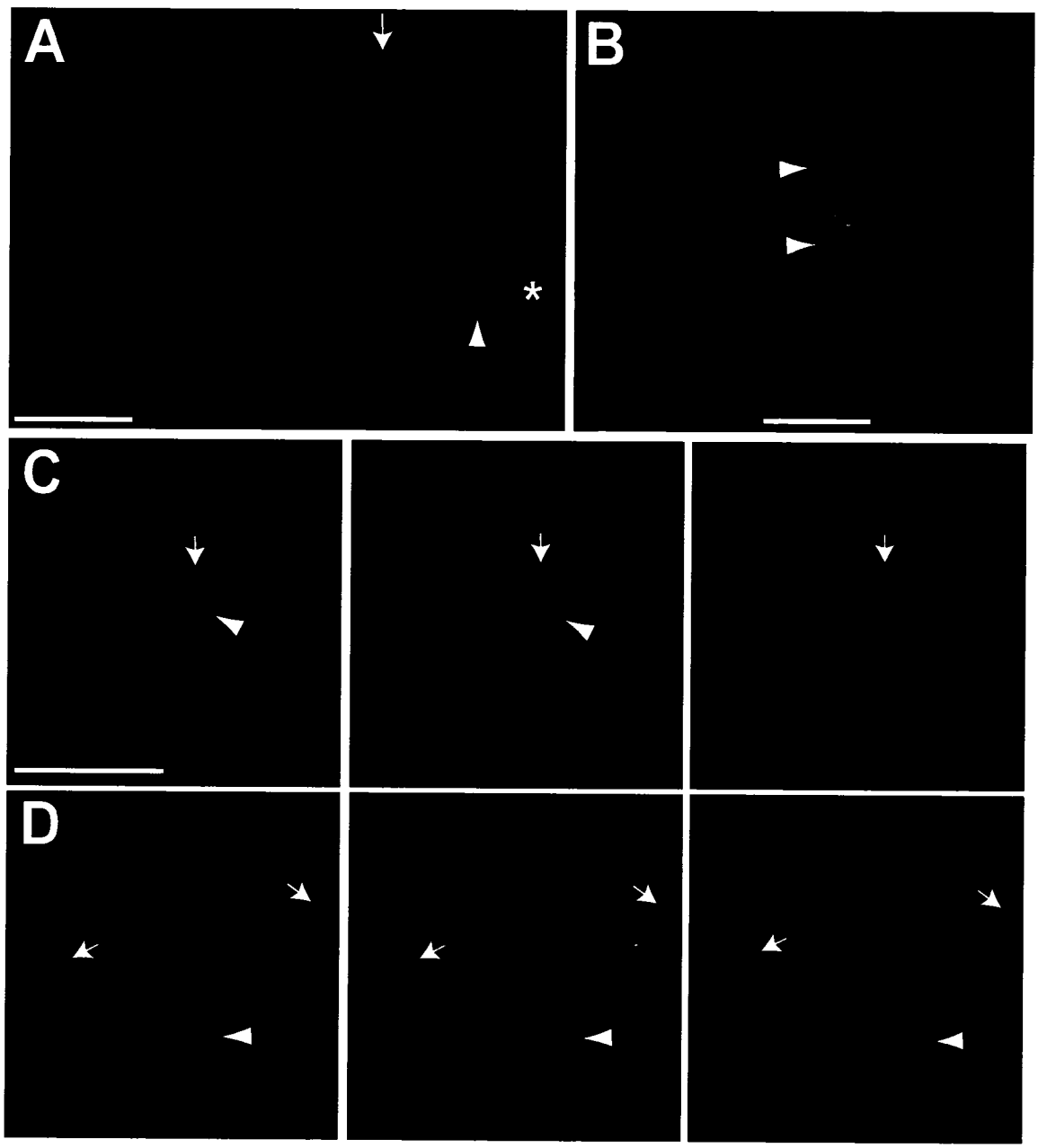


Figure 8.2

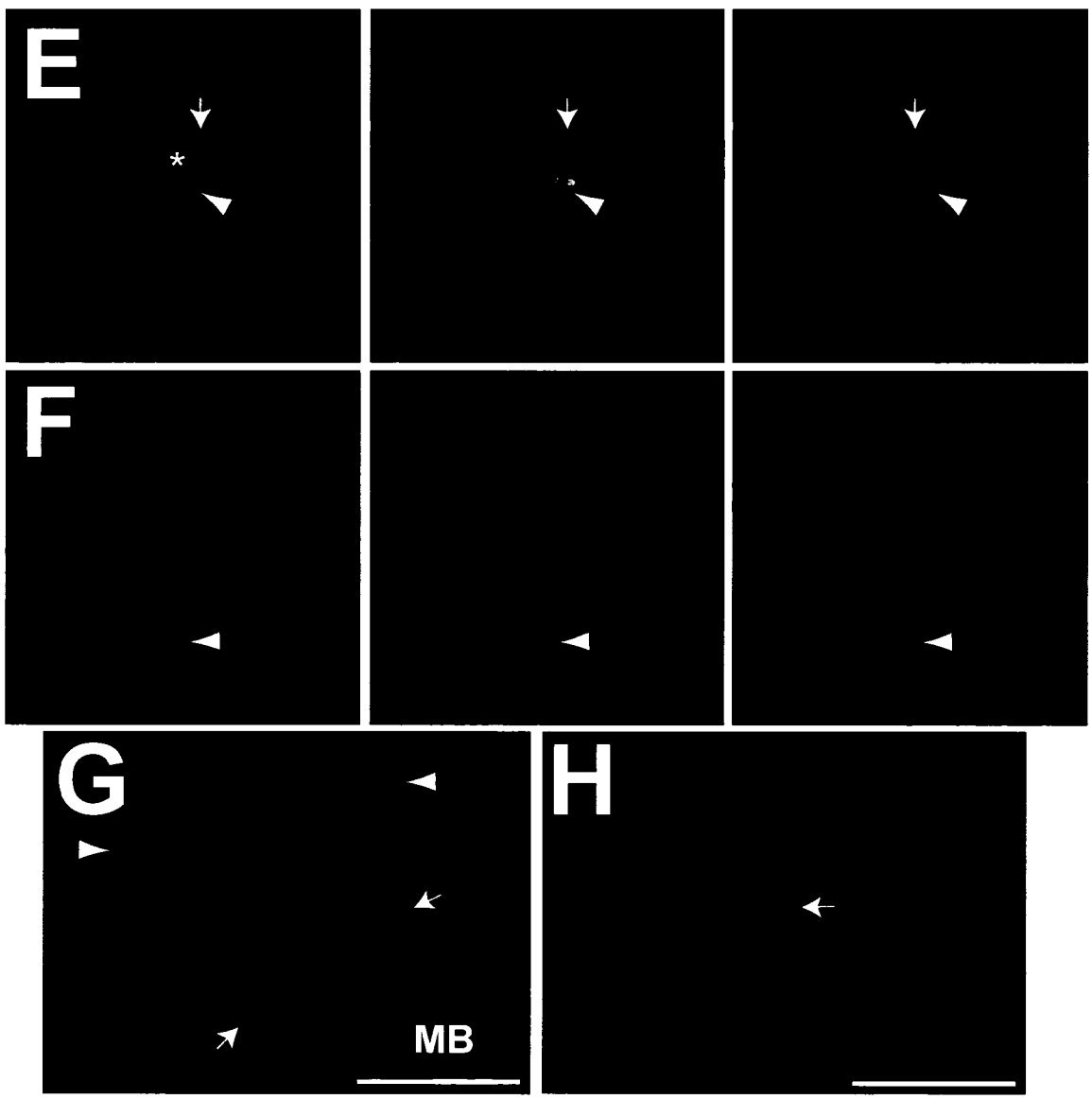
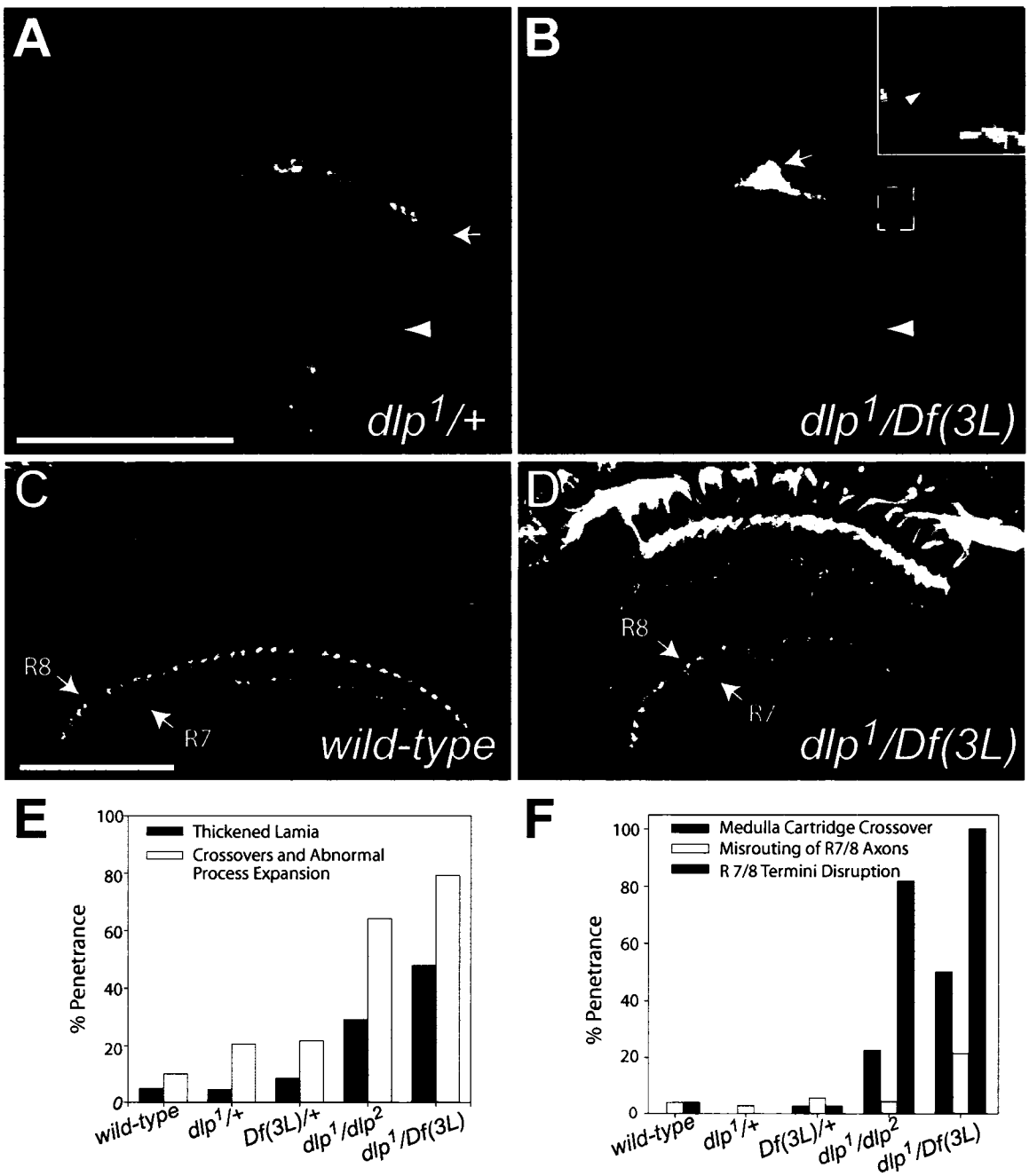


Figure 9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R8
R7
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80
60
40
20
0
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dlp1/+
Df(3L)/+
dlp1/dlp2
dlp1/Df(3L)
F
Medulla Cartridge Crossover
Misrouting of R7/8 Axons
R 7/8 Termini Disruption
% Penetrance
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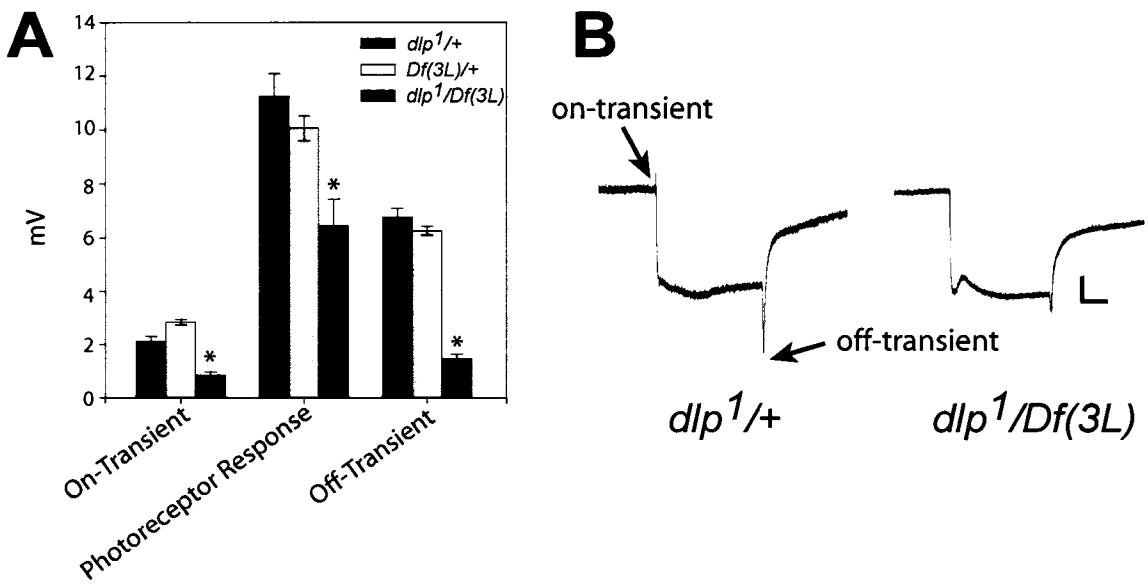
Df(3L)/+
dlp1/dlp2
dlp1/Df(3L)

Figure 10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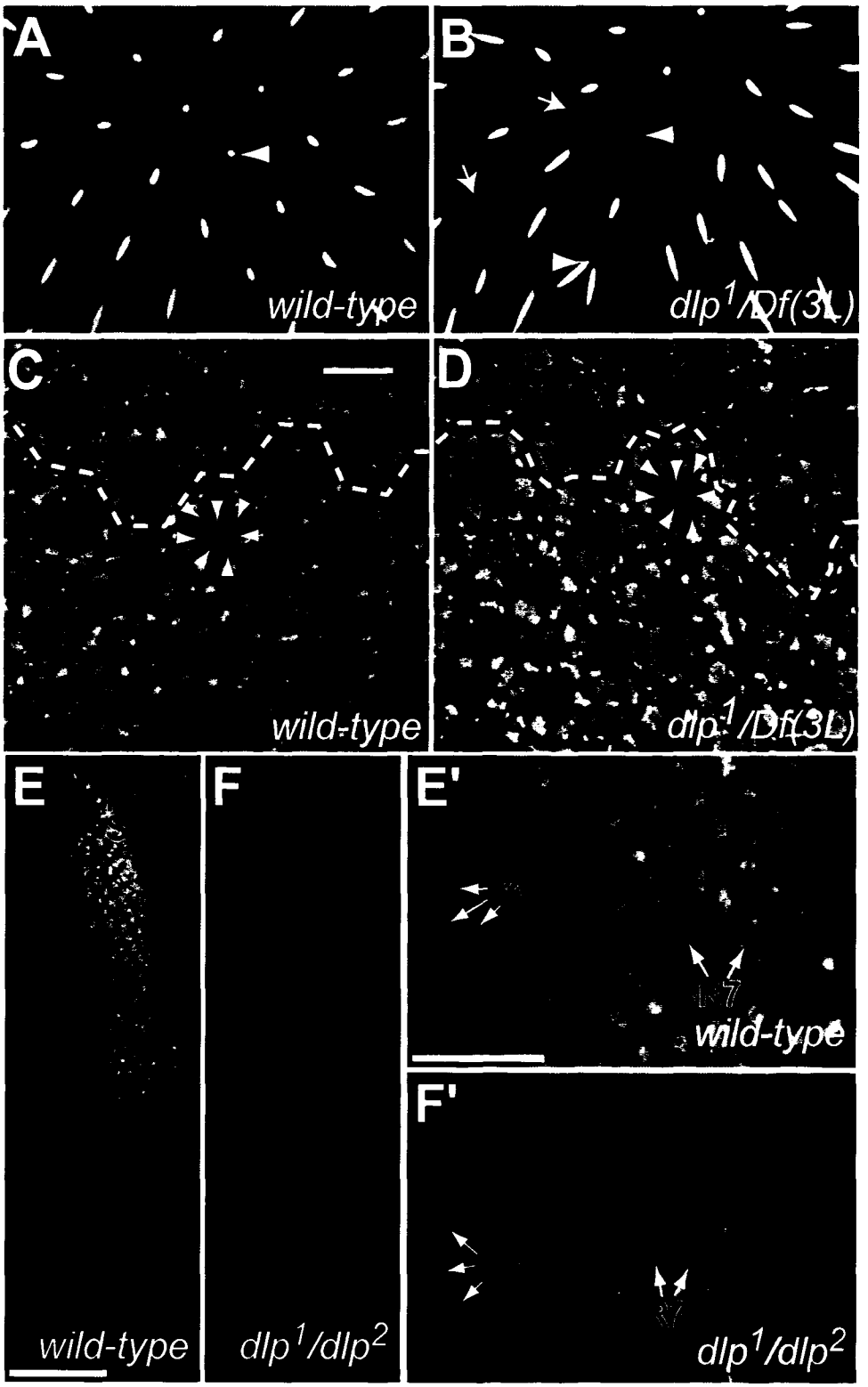
dlp1/Df(3L)

Figure 11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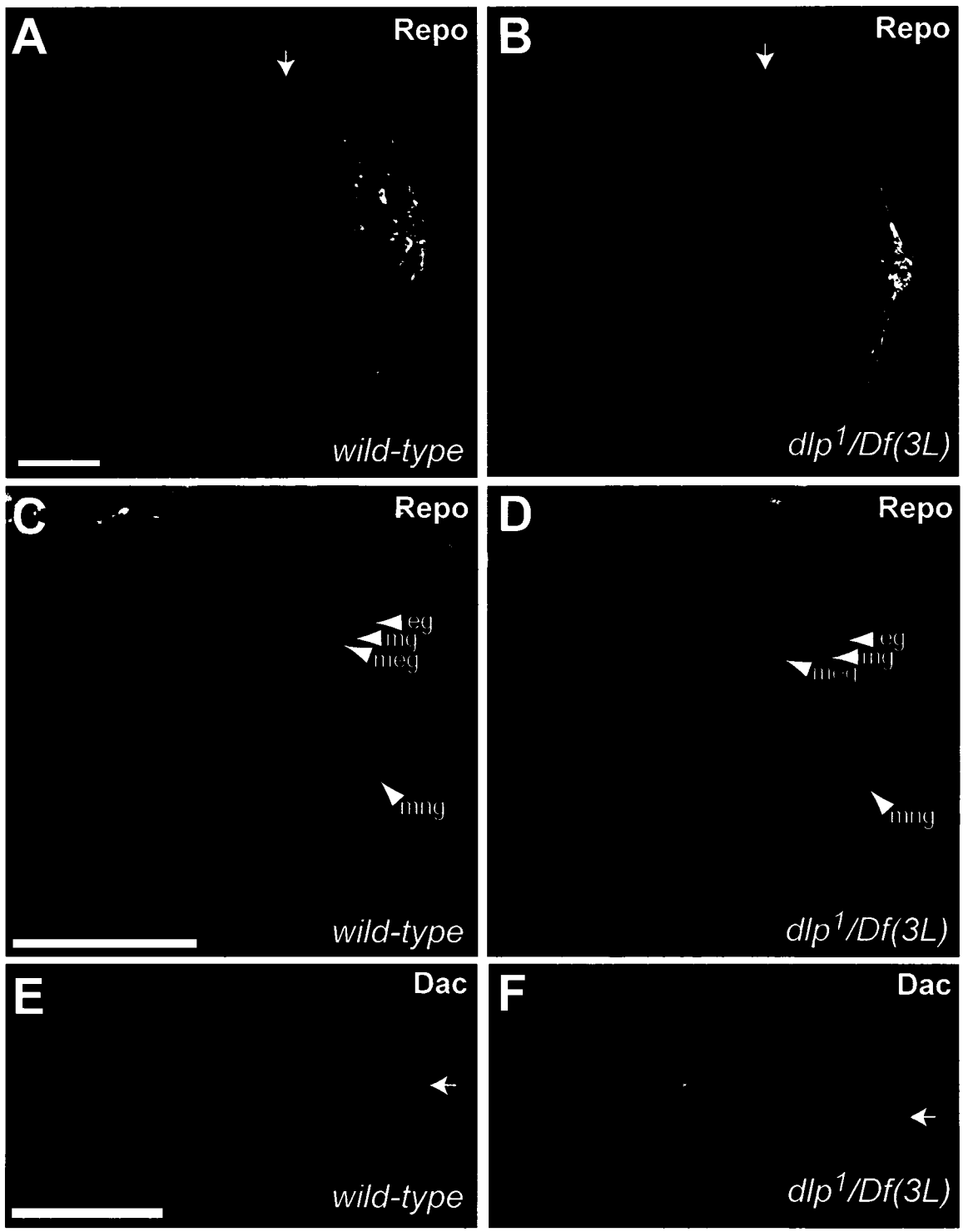
dlp1/dlp2

Figure 12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wild-type
B
Repo
dlp1/Df(3L)
C
Repo
eg
mg
meg
mng
wild-type
D
Repo
eg
mg
meg
mng
dlp1/Df(3L)
E
Dac
wild-type
F
Dac
dlp1/Df(3L)

Figure 13

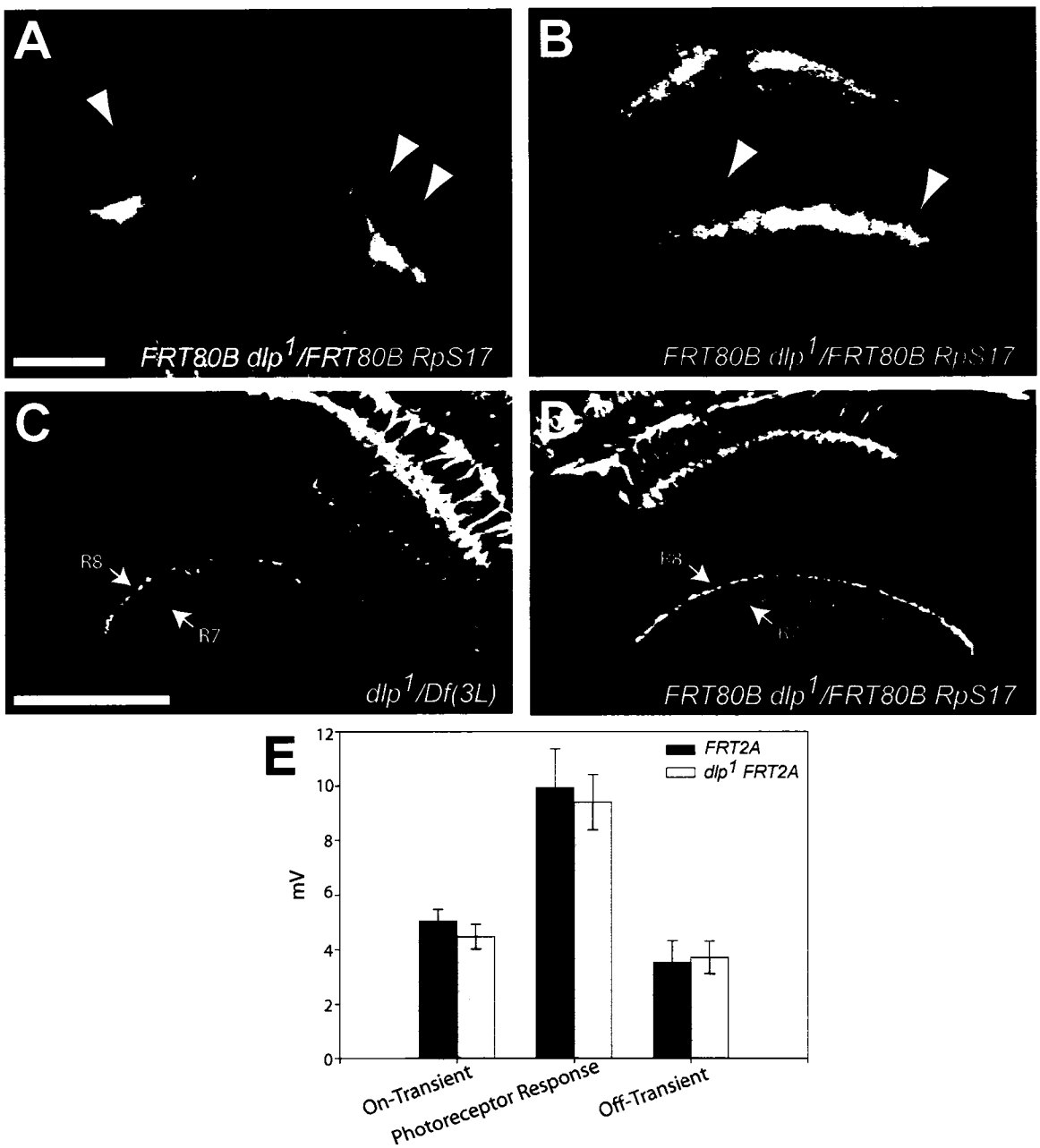

A
FRT80B dlp1/FRT80B RpS17
B
FRT80B dlp1/FRT80B RpS17
C
R8
R7
dlp1/Df(3L)
D
R8
FRT80B dlp1/FRT80B RpS17
E
FRT2A
dlp1 FRT2A
mV
12
10
8
6
4
2
0
On-Transient
Photoreceptor Response
Off-Transient

Figure 14

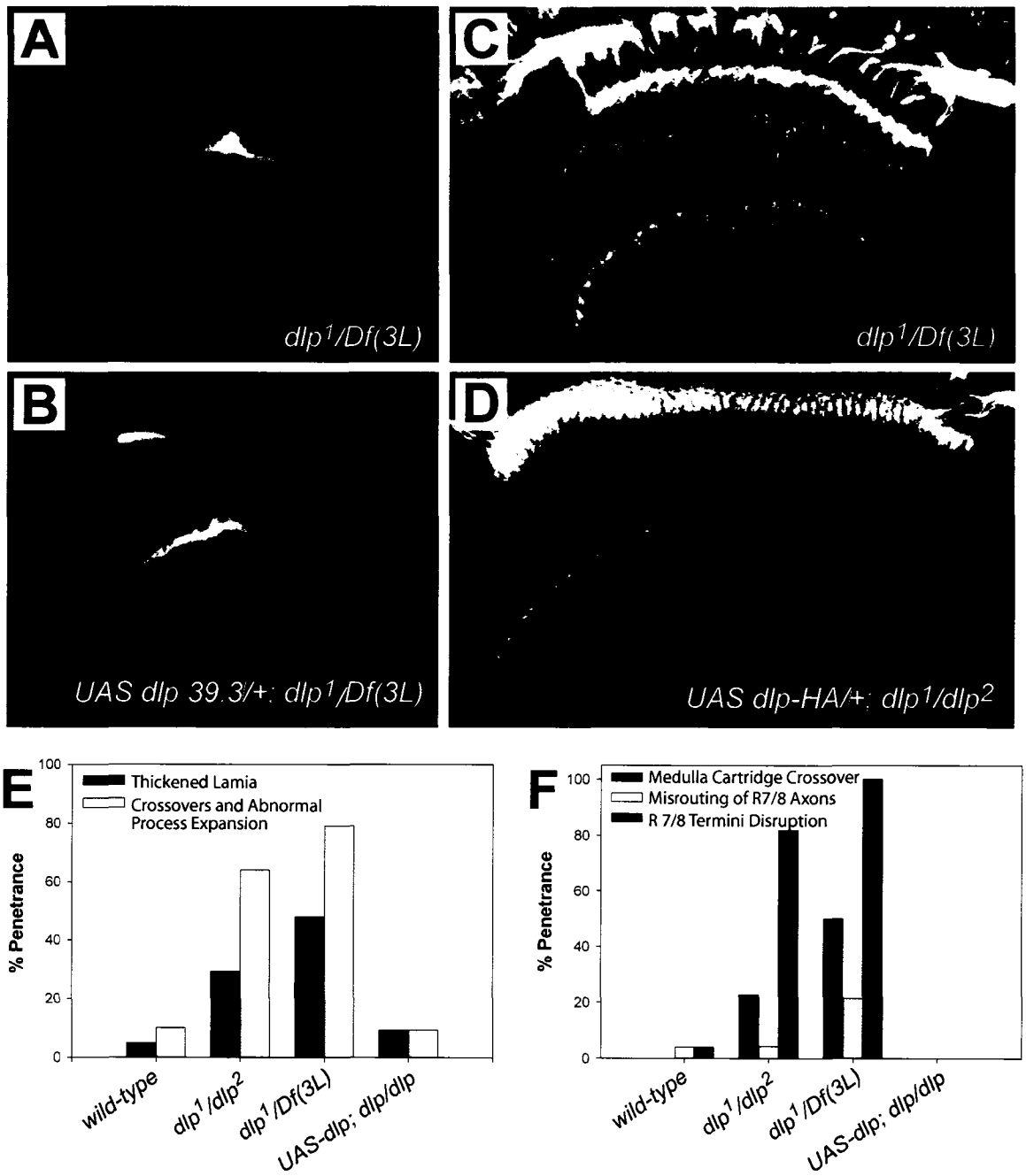

A
dlp1/Df(3L)
C
dlp1/Df(3L)
B
UAS dlp 39.3/+; dlp1/Df(3L)
D
UAS dlp-HA/+; dlp1/dlp2
E
Thickened Lamia
Crossovers and Abnormal Process Expansion
% Penetrance
100
80
60
40
20
0
wild-type
dlp1/dlp2
dlp1/Df(3L)
UAS-dlp; dlp/dlp
F
Medulla Cartridge Crossover
Misrouting of R7/8 Axons
R 7/8 Termini Disruption
% Penetrance
100
80
60
40
20
0
wild-type
dlp1/dlp2
dlp1/Df(3L)
UAS-dlp; dlp/dlp

Figure 15

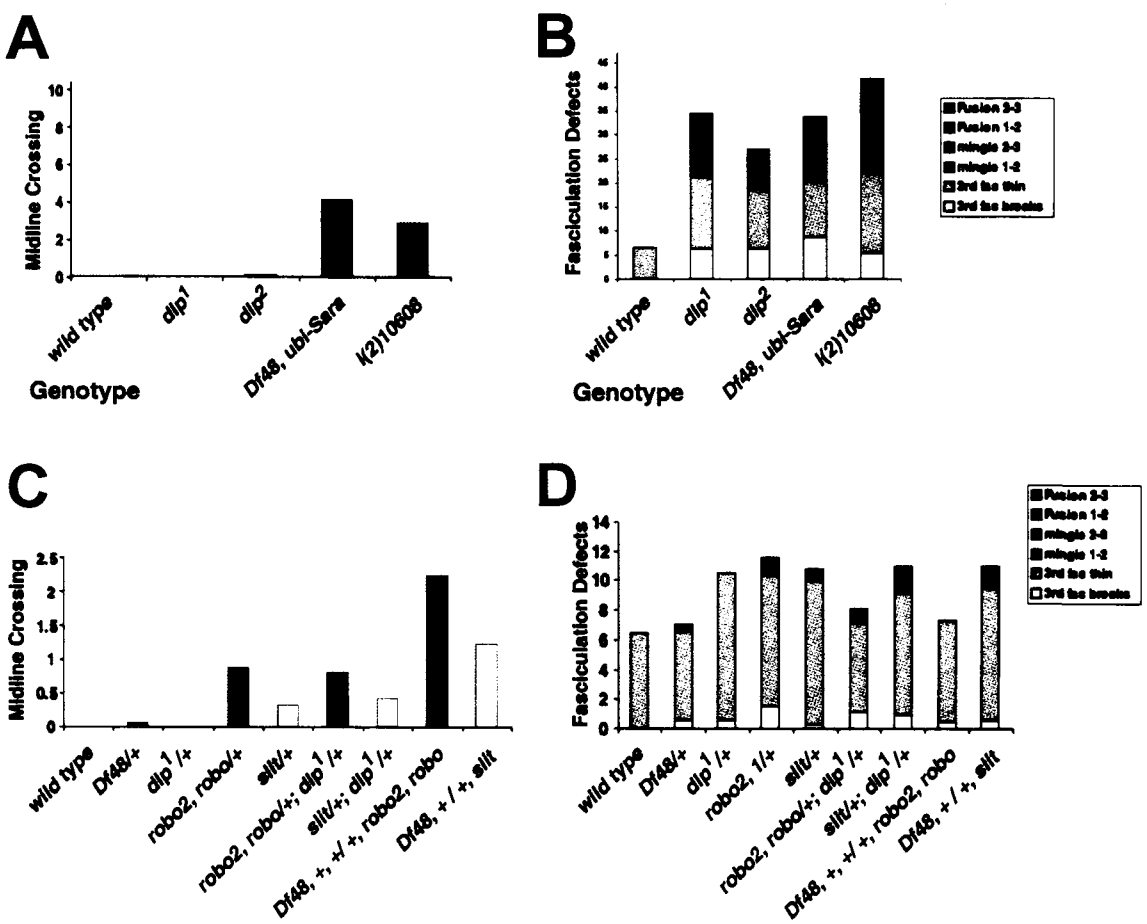

A
Midline Crossing
10
8
6
4
2
0
wild type
dlp1
dlp2
Df48, ubi-Sara
k(2)10606
Genotype
B
Fasciculation Defects
Fusion 2-3
Fusion 1-2
mingle 2-3
mingle 1-2
3rd fas thin
3rd fas beaded
wild type
dlp1
dlp2
Df48, ubi-Sara
k(2)10606
Genotype
C
Midline Crossing
2.5
2
1.5
1
0.5
0
wild type
Df48/+
dlp 1 /+
robo2, robo/+
slit/+
robo2, robo/+; dlp 1 /+
slit/+; dlp 1 /+
Df48, +, +/ +, robo2, robo
Df48, + / +, slit
D
Fasciculation Defects
14
12
10
8
6
4
2
0
Fusion 2-3
Fusion 1-2
mingle 2-3
mingle 1-2
3rd fas thin
3rd fas beaded
wild type
Df48/+
dlp 1 /+
robo2, 1/+
slit/+
robo2, robo/+; dlp 1 /+
slit/+; dlp 1 /+
Df48, +, +/ +, robo2, robo
Df48, + / +, slit

Figure 16

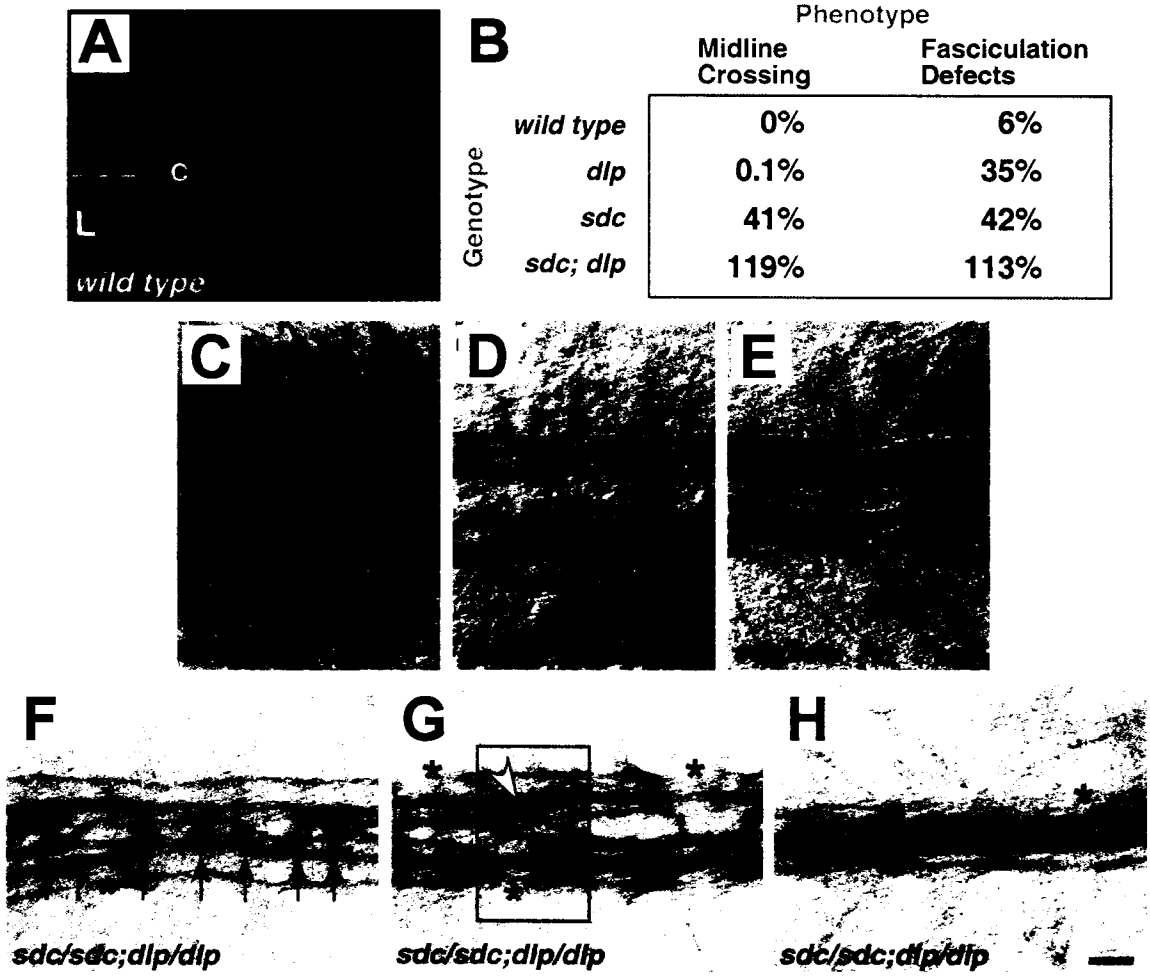

A
C
L
wild type
B
Phenotype
Midline Crossing
Fasciculation Defects
Genotype
wild type
0%
6%
dlp
0.1%
35%
sdc
41%
42%
sdc; dlp
119%
113%
C
D
E
F
G
H
sdc/sdc;dlp/dlp
sdc/sdc;dlp/dlp
sdc/sdc;dlp/dlp

Figure 17

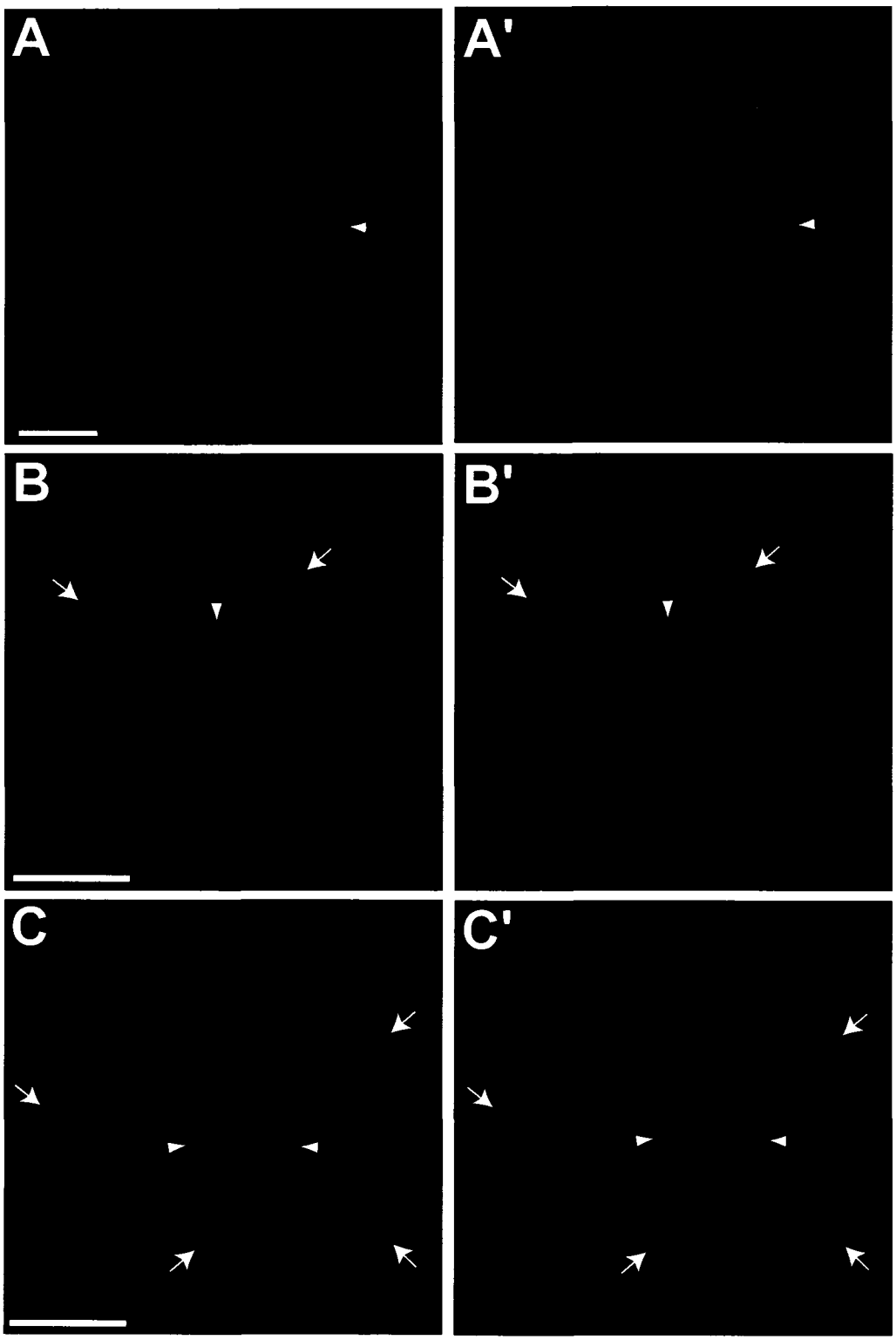
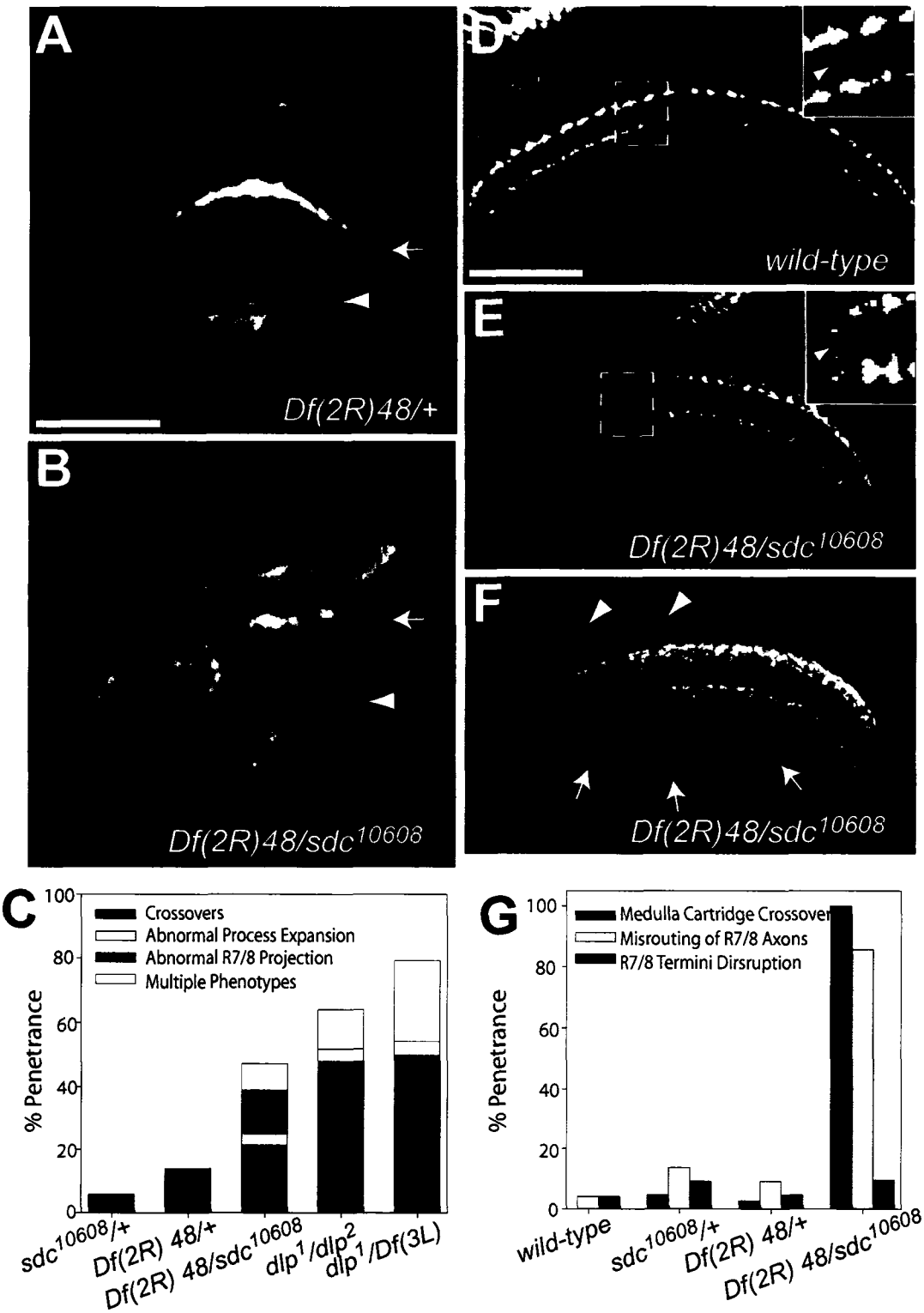


Figure 18


A
Df(2R)48/+
B
Df(2R)48/sdc10608
D
wild-type
E
Df(2R)48/sdc10608
F
Df(2R)48/sdc10608
C
% Penetrance
Crossovers
Abnormal Process Expansion
Abnormal R7/8 Projection
Multiple Phenotypes
sdc10608/+
Df(2R) 48/+
Df(2R) 48/sdc10608
dlp1/dlp2
dlp1/Df(3L)
G
% Penetrance
Medulla Cartridge Crossover
Misrouting of R7/8 Axons
R7/8 Termini Dirsruption
wild-type
sdc10608/+
Df(2R) 48/+
Df(2R) 48/sdc10608

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