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Department of Zoology

The University of British Columbia
Vancouver, Canada

Date 24 December 2002
ABSTRACT

Gliotactin is a non-catalytically active, serinesterase-like, transmembrane protein that is required for the formation of the blood-nerve barrier in Drosophila. Gliotactin is expressed by the peripheral nervous system glia, which ensheath motor and sensory axons. Pleated septate junctions present between the wraps of peripheral glia have previously been shown to be required for blood-nerve barrier formation, and are believed to have a role in cell-cell adhesion. In gliotactin mutants, peripheral glia display wrapping defects at the distal reaches of embryonic peripheral nerves. Paralysis and death occurs in gliotactin mutants, as the disrupted blood-nerve barrier exposes neurons to the high potassium concentration of the haemolymph which causes action potential blockade. It has been hypothesized that the blood-nerve barrier and ensheathment phenotypes observed in gliotactin mutants arises from abnormal pleated septate junction development, or from a defect in other cellular events during peripheral glial development.

Here, the role of Gliotactin in Drosophila pleated septate junction development was investigated. Through mutant analysis and cell biological techniques, it was found that Gliotactin is necessary for septate junction development, yet has a role that is unique from other previously identified pleated septate junction proteins. In addition to being expressed in the peripheral nervous system glia, Gliotactin was also found to be expressed in the epidermis, salivary glands, and a variety of other pleated septate junction containing tissues. Gliotactin in the epidermis and salivary glands is found concentrated at regions where three cells meet and is only partially overlapping with other septate junction proteins. At these tricellular junctions, Gliotactin physically associates with septate junction proteins, as demonstrated through co-immunoprecipitation experiments. Through dye injection assays, it was demonstrated that loss of Gliotactin results in the disruption of the integrity of the transepithelial barrier in the salivary gland, similar to other pleated septate junction mutants. A model is proposed in which Gliotactin at tricellular corners acts as a physical link between pleated septate junctions and specialized structures, termed tricellular plugs which have previously been identified at tricellular junctions.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>af</td>
<td>Anterior peripheral nerve fascicle</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens junction</td>
</tr>
<tr>
<td>AMA</td>
<td>Amalgam</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical protein kinase-C</td>
</tr>
<tr>
<td>Arm</td>
<td>Armadillo</td>
</tr>
<tr>
<td>ASIP</td>
<td>Atypical protein kinase-C isotype-specific interacting protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BNB</td>
<td>Blood-nerve-barrier</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>Caspr</td>
<td>Contactin associated protein</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CD-8</td>
<td>Cluster of differentiation-8</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COOH</td>
<td>Carboxyl group</td>
</tr>
<tr>
<td>Cor</td>
<td>Coracle</td>
</tr>
<tr>
<td>CRB1</td>
<td>Crumbs 1 (human)</td>
</tr>
<tr>
<td>DaPKC</td>
<td>Drosophila atypical protein kinase-C</td>
</tr>
<tr>
<td>Dlg</td>
<td>Lethal(1)discs-large</td>
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<tr>
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</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNL</td>
<td>Drosophila Neuroligin</td>
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<td>E.R.</td>
<td>Endoplasmic reticulum</td>
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<tr>
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<td>Embryonic day 15 of development (mouse/rat)</td>
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<td>E-Cad</td>
<td>E-cadherin</td>
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<td>E-face</td>
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<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
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<td>EMS</td>
<td>Ethylmethane sulfonate</td>
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<td>Fas II</td>
<td>Fasciclin II</td>
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<tr>
<td>Fas III</td>
<td>Fasciclin III</td>
</tr>
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<td>g</td>
<td>Grams</td>
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<td>GAL4</td>
<td>Yeast transcriptional activator GAL4</td>
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<tr>
<td>gapGFP</td>
<td>GAP4-43 (myristylation sequence of) GFP fusion protein</td>
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<td>GFP</td>
<td>Green Fluorescence Protein</td>
</tr>
<tr>
<td>Gli</td>
<td>Gliotactin</td>
</tr>
<tr>
<td>GPL</td>
<td>Glycosyl Phosphatidylinositol</td>
</tr>
<tr>
<td>GUK</td>
<td>Guanylate kinase</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>immuno-EM</td>
<td>Immunogold electron microscopy</td>
</tr>
<tr>
<td>IMP</td>
<td>Intermembrane particle</td>
</tr>
<tr>
<td>InaD</td>
<td>Inactivation no-after potential</td>
</tr>
<tr>
<td>IS</td>
<td>Instar</td>
</tr>
<tr>
<td>ISN</td>
<td>Intersegmental nerve</td>
</tr>
<tr>
<td>JAM</td>
<td>Junction Adhesion Molecule</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun Kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base pairs (nucleotide)</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo daltons</td>
</tr>
<tr>
<td>LacZ</td>
<td>Z gene of Lac operon (encodes β-Galactosidase)</td>
</tr>
<tr>
<td>LAP</td>
<td>Leucine-rich repeats and PDZ</td>
</tr>
<tr>
<td>Lch</td>
<td>Lateral chordotonal sensory neuron</td>
</tr>
<tr>
<td>Lgi</td>
<td>Lethal (2) giant-larvae</td>
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<td>LRRs</td>
<td>Leucine-rich repeats</td>
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<td>mAb</td>
<td>Monoclonal antibody</td>
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<td>MAGI</td>
<td>Membrane-associated guanylate kinase inverted</td>
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<td>Map kinase</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>p-Casper</td>
<td>P-element transformation vector Casper</td>
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<td>pf</td>
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<td>P-face</td>
<td>Protoplasmic face</td>
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<tr>
<td>PG</td>
<td>Peripheral glia</td>
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<tr>
<td>pH</td>
<td>Power of ten hydrogen</td>
</tr>
<tr>
<td>Pins</td>
<td>Partner of Inscuteable</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Post Synaptic Density protein 95</td>
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<tr>
<td>PSJ</td>
<td>Pleated septate junction</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue deleted on chromosome 10</td>
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<td>Pyd</td>
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<td>Rapsynoid</td>
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<td>Reversed polarity</td>
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<td>Serine</td>
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<td>Scrib</td>
<td>Scribble</td>
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<td>Secretory pathway mutant-6 (yeast)</td>
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<td>SH3</td>
<td>Src Homology region-3</td>
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<tr>
<td>SJ</td>
<td>Septate junction</td>
</tr>
<tr>
<td>SN</td>
<td>Segmental nerve</td>
</tr>
<tr>
<td>SSJ</td>
<td>Smooth septate junction</td>
</tr>
<tr>
<td>St.</td>
<td>Stage</td>
</tr>
<tr>
<td>Std</td>
<td>Stardust</td>
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<td>Description</td>
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<td>------------------------------------</td>
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<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>Tam</td>
<td>Tamou</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCDs</td>
<td>Tricellular channel diaphragms</td>
</tr>
<tr>
<td>TCPs</td>
<td>Tricellular plugs</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TJs</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream Activation Sequence</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>V</td>
<td>Valine</td>
</tr>
<tr>
<td>Vpg</td>
<td>Ventral peripheral glial cell</td>
</tr>
<tr>
<td>Wt</td>
<td>Wildtype</td>
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<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl β-D-galactoside</td>
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<tr>
<td>ZO-1,-2,-3</td>
<td>Zonula Occludins-1,-2 or -3</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>μm</td>
<td>Micro meter</td>
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</table>
Acknowledgements

I would like to thank my Ph.D. supervisor, Vanessa Auld, for giving me the opportunity to pursue my interest in cell biology and basic research, and for introducing me to the power of experimental analysis in the fruit fly. I appreciate the effort that Vanessa took to prepare me to become an independent researcher along the various stages of this degree. Early in the program Vanessa reviewed countless journal articles with me in preparation for the Ph.D. comprehensive exam. Later in the program, Vanessa encouraged me to apply for external funding, which was an invaluable educational experience. Throughout my studies, the door to Vanessa's office has always been open whether it was to discuss science or simply to make a sandwich! Vanessa selflessly offered to integrate the lab lunchroom with her office since no space was made available elsewhere in the Department for this purpose. Vanessa has been incredibly flexible and patient throughout my studies. I thank Vanessa for permitting me to baby-sit my parents' German Shepherd puppy Syla at the lab while they were away on vacation for several months. I am also grateful for the tolerance that Vanessa showed of my absence during annual back-country skiing trips, and mountaineering adventures.

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Lastly, I would like to thank the fruit flies as well as all the researchers that have preceded me for making it possible for me to investigate the role of Gliotactin in the formation of the Drosophila pleated septate junctions.
I. INTRODUCTION

Gliotactin (Gli) is a transmembrane protein that is expressed by the peripheral nervous system glia of Drosophila melanogaster during embryonic development (Auld et al., 1995). Homozygous gli mutants die during late embryogenesis and are paralyzed (Auld et al., 1995). Electrophysiological studies of these mutant embryos show that the high potassium concentration of the insect haemolymph comes into contact with axon fibers and disrupts the propagation of action potentials (Auld et al., 1995). The blood-nerve-barrier (BNB) is thus disrupted in gli mutants. From electronmicrographs of peripheral nerve cross-sections prepared from gliotactin mutant embryos, only subtle glial ensheathment defects are evident at the distal reaches of peripheral nerves (Auld et al., 1995). Pleated septate junctions are believed to constitute the BNB in Drosophila (Baumgartner et al., 1995). However, it has not been determined if these structures are intact in the peripheral nerves of gliotactin mutants. It remains to be clarified whether the leaky BNB observed in gliotactin mutants arises from the subtle glial ensheathment defects or from aberrant pleated septate junction development. The main goal of the current work was to extend the analysis of Gliotactin and determine if Gliotactin is localized to, and necessary for, pleated septate junction formation.

Gliotactin and the Electrotactin Protein Family

To understand what specific cellular functions Gli might have, it is useful to compare it to other structurally related proteins. Gli has an extracellular non-catalytically active serinesterase-like domain, that is also found in Drosophila Glutactin (a basement membrane glycoprotein), Drosophila Neurotactin (a cell adhesion molecule necessary for axon guidance), and the vertebrate Neuroligins which are cell adhesion molecules necessary for synapse development (Auld et al., 1995; Olson et al., 1990; and
Ichtchenko et al., 1995). Ligands have been identified for Neurotactin and the Neuroligins; however these ligands are structurally quite different. The ligand for Neurotactin, Amalgam (AMA), is a secreted protein and a member of the immunoglobulin superfamily (Fremion, et al., 2000). In contrast, Neuroligin-1 interacts with the β-Neurexins. These proteins do not have an immunoglobulin-like structure but instead are transmembrane glycoproteins that contain epidermal growth factor-like and Laminin A G-domain like repeats (Ichtchenko et al., 1995; Ushkaryov, et al., 1992). Interestingly, Glutactin and the Neuroligins have both been shown to bind calcium (Nguyen and Sudhof, 1997; Olson et al., 1990). Moreover, the interaction between Neuroligin-1 and the β-Neurexins is calcium-dependent (Ichtchenko et al., 1995).

Gliotactin, like the Neuroligins and other cholinesterases, contains a consensus calcium-binding EF-hand in its extracellular domain (Tsigelny et al., 2000). Calcium is probably required to maintain the structural integrity and functional properties of serinesterase-like proteins (Tsigelny et al., 2000). Of proteins that have been shown to have either catalytically active or non-catalytically active serinesterase-like domains, Gli shares the greatest DNA and amino acid sequence identity with the Neuroligins (Gilbert et al., 2001; Botti et al., 1998). Interestingly a PDZ recognition sequence is conserved within the cytoplasmic tail of Gli and the Neuroligins (see below).

Computer modeling studies based on the crystal structure of *Torpedo californica* acetylcholinesterase suggest that the three-dimensional structures of acetylcholinesterases, butyrylcholinesterases, Gliotactin, Neurotactin, and the Neuroligins are all very similar and these proteins have collectively been termed the electrotactins (Botti et al., 1998). All these proteins contain a deep narrow gorge, or α/β hydrolase fold, and an annulus of negative electrostatic potential at the gorge surface (Botti et al., 1998). It has been speculated that this peripheral anionic site may be necessary to initially engage ligand-receptor interactions through long-range, non-specific electrostatic interactions and that the α/β hydrolase fold may be important in a
subsequent, more specific 'docking step' (Botti et al., 1998). In vitro cell adhesion studies, using differential blockers of the active site gorge and the peripheral anionic site, however, suggest that the peripheral anionic site may be sufficient to endow the acetylcholinesterases with adhesive properties (Johnson and Moore, 1999).

At the extreme carboxy terminus of the intracellular tail of Gli, there is a three amino acid (Serine/Threonine-X-Valine) consensus binding motif for proteins containing PDZ domains (X represents any amino acid). The PDZ acronym refers to the first three proteins (PSD-95/Dlg/ZO-1) that were found to contain these domains. PDZ domains are protein-protein interaction domains that consist of about 100 amino acid residues (Harrison, 1996). PDZ domains are small β-barrels that interact with PDZ recognition sequences in a peptide-surface association, such that the bound peptide forms an additional and complementary β-strand in the β-barrel (Doyle et al., 1996; Harrison, 1996). PDZ containing proteins are often found at sites of cell-cell contact or at junctions (reviewed in Dimitratos et al., 1999; Sarah and Bedt, 1998). PSD-95, for example, is a synapse associated protein found in the brain, and it binds to the C-terminal tails of K+ channels, NMDA receptors, and to the Neuroligins (Irie, et al., 1997; Niethammer et al., 1996; Kornau et al., 1995; Kim et al., 1995). The Drosophila homologue of PSD-95, Dlg, localizes to the neuromuscular junctions in larvae, as well as to septate junctions (Thomas et al., 1997; Woods and Bryant, 1991). ZO-1, and more recently ZO-2, ZO-3, are associated with tight junctions in epithelial cells (Haskins et al., 1998; Jesaitis and Goodenough, 1994; Willott et al., 1993). PDZ domains, are also capable of binding to other PDZ domains, in a homo- or heteromeric fashion, and this property in certain cases enables PDZ proteins to drive protein clustering (reviewed in Fanning and Anderson, 1999; Xu et al., 1998). Proteins that contain PDZ domains usually have several copies, and there are many examples in which PDZ proteins are involved in signal transduction (reviewed in Dimitratos et al., 1999). The Drosophila inaD gene for example, encodes a protein containing five PDZ domains, and it serves as a
molecular scaffold to organize components of the rhabdomere phototransduction cascade including, light activated ion channels, Phospholipase C-β, and Protein Kinase C (Tsunoda, et al., 1997). Given the similarity between the Neuroligins and Gli, plus the fact that Dlg is the Drosophila homologue of PSD-95, it is foreseeable that Gli could interact with Dlg at septate junctions (see Dlg below).

Septate Junction Structure and Molecular Composition

Significant insight into the specific cellular function of Gli came with the discovery of Drosophila Neurexin-IV (Nrx-IV). This transmembrane protein was found to be required for septate junction development and homozygous nrx-IV mutant embryos displayed a leaky BNB phenotype equivalent to that seen in Gli mutants (Baumgartner et al., 1996). Moreover, Nrx-IV was shown to be expressed in the PNS glia, like Gli, as well as a number of other ectodermally derived tissues (Baumgartner et al., 1996).

Septate junctions are found in most invertebrates and are believed to function both in cellular adhesion and in the formation of cellular permeability barriers (reviewed in Lane et al., 1994). Septate junctions are characterized by a ladder-like array of cross-bridges (15-20nm) that span intercellular clefts in transmission electronmicroscope images of invertebrate tissue (reviewed in Lane et al., 1994). Septate junctions in epithelial cells, are positioned in the apical membrane domain, below actin-rich adherens junctions, and similarly encircle the cell as a belt (see Figure 1A) (reviewed in Lane et al., 1994). Septate junctions are also composed of multiple strands. In locust epithelial cells, ten or more strands typically compose a septate junction (Lane and Swales, 1982). The multi-stranded nature of septate junctions assists in blocking the paracellular flow of molecules. Although heavy-metal tracer dyes are often able to cross multiple strands in a SJ domain, these dyes are frequently unable to cross an entire stacked array of SJ strands (Swales and Lane, 1985). In Drosophila, two
types of septate junctions, smooth and pleated, have been observed (Tepass and
Hartenstein, 1994). Smooth septate junctions (SSJs) are found in endodermally derived
tissue such as the midgut and Malpighian tubules while pleated septate junctions (PSJs)
are found in ectodermally derived tissue such as the foregut, hindgut, tracheae, and glia
(Tepass and Hartenstein, 1994; Noirot-Timothee and Noirot, 1980; Dallai, 1976). In
transmission electronmicroscope images, the septa of PSJs are more evident than those
of SSJs, which are often obscured by the presence of electron-dense material in the
intercellular cleft (Tepass and Hartenstein, 1994). The morphological distinction
between SSJs and PSJs is most evident in freeze-fracture electronmicroscope replicas.
Undulating stacks of strands are visible on the protoplasmic (P) face of smooth SJ
replicas; while multiple rows of particles are seen on the P-face of pleated SJ replicas.
Despite morphological differences, SSJs and PSJs are believed to be functionally
equivalent structures (Lane et al., 1994).

Neurexin-IV

*Drosophila* Nrx-IV was identified as the first non-mammalian Neurexin.
Vertebrate Neurexins were discovered as neuronal receptors for α-Latrotoxin, a
component of the venom from black widow spiders which causes massive
neurotransmitter release (Ushkaryov et al., 1992). The vertebrate Neurexin genes (I, II,
and III) each produce a large α-, and a small β-transcript, which are believed to be
transcribed from different promoters and are subject to extensive alternative splicing
(Tabuchi and Sudhof; 2002; Ushkaryov, et al., 1992). From gene structure analysis, it is
estimated that more than 1000 Neurexin isoforms are generated in rat brain by
alternative splicing (Ullrich et al., 1995). Since the β-Neurexins have been shown to
interact with Neuroligin-1, it has been widely speculated that Nrx-IV might physically
interact with Gliotactin to form the BNB (Carlson et al., 2000; Baumgartner et al., 1996;
Ichtchenko et al., 1995). This hypothesis was tested in S2 tissue culture cell aggregation
Figure 1. Schematic diagrams, showing the position of septate junctions, tight junctions, paranodal junctions (among other cellular features) in various Drosophila and mammalian cell types.

(A) Cross section view of two adjacent, epidermal, Drosophila epithelial cells. These cells have two membrane domains: an apical and a basolateral. The apical membrane domain is composed of the plasma membrane that lies above the adherens junctions (encompassing the microvilli and marginal zone) while the basolateral membrane domain encompasses the plasma membrane below the adherens junctions. Pleated septate junctions (PSJs) are found in the basolateral membrane domain and are composed of multiple intermembrane particle (IMP) strands, which encircle each columnar epithelial cell. The IMP strands of neighboring epithelial cells are linked by PSJ septa. Overlying the microvilli of the Drosophila epidermis is a thick cuticle layer, and at the extreme basal surface there is a prominent basement membrane.

(B) En-face view of two mammalian epithelial cells. Tight junctions are evident in the apical membrane domain above the adherens junctions, but below the microvilli. Tight junctions are composed of a network of anastomizing intermembrane particle (IMP) strands. IMP strands are in register between adjacent epithelial cells, and contact each other at 'Kissing-Points'. The intermembrane space is obliterated at 'Kissing-Points'. At the extreme basal side of mammalian epithelial cells there is a basal lamina.

(C) A myelinated axon from a mammalian peripheral nerve. Three Schwann cells (each separated by a node) are shown to ensheathe a single axon. The majority of the myelinated fiber is viewed in cross-section to reveal various cellular features. A Schwann cell contains a number of cellular domains. At the paranode, Schwann cell paranodal Loops (which contain cytoplasm) anchor on the axolemma of the underlying neuron via paranodal junctions. Between paranodal loops, adherens and tight junctions are present. Flanking the paranode is the juxtaparanode, and the region between two juxtaparanodes (within a Schwann cell) is termed the internode. Compact myelin is found in the internodal region, and it lacks cytoplasm. Schwann cell microvilli protrude from paranodal loops near the node, and a basal lamina overlies the myelinated fiber.
**A**

Drosophila Epithelial Cell

- Cuticle
- Apical Microvilli
- Marginal Zone
- Adherens Junction
- Pleated Septate Junction
- PSJ IMP strand
- PSJ Septa
- Basement Membrane

**B**

Mammalian Epithelial Cell

- Apical Microvilli
- 'Kissing points'
- TJ IMP strand
- Tight Junction
- Adherens Junction
- Basal Lamina

**C**

Mammalian Peripheral Nerve

- Microvilli
- Juxtaparanode
- Paranodal Junction
- Tight Junction
- Adherens Junction
- Node

- Basal Lamina
- Compact Myelin
- Axon
studies, however no interaction between Nrx-IV and Gli was observed (Auld et al., 1995; Baumgartner et al., 1996). This result was not entirely unexpected since the structure of *Drosophila* Nrx-IV is more similar to the vertebrate α-Neurexins, than the β-Neurexins, and only the β-Neurexins have been shown to bind the Neuroligins. Since the discovery of Nrx-IV, two other vertebrate Neurexins: Contactin associated protein (Caspr) and Caspr2, have been identified that are more structurally similar to Nrx-IV than any of the other Neurexins (reviewed in Peles et al., 1997). Nrx-IV, Caspr, and Caspr2 all contain an extracellular Discoidin domain thought to be a lectin-binding domain, and a region similar to Fibrinogen that is not found in Nrx I, II, or III (reviewed in Peles et al., 1997). The mammalian Casprs localize to specialized junctional complexes that form during myelination and which are morphologically similar to the invertebrate septate junction (see below) (Poliak et al., 1999; Einheber et al., 1997; Menegoz et al., 1997). A summary of the pertinent molecular information regarding Nrx-IV as well as other junctional molecules discussed below is presented in Table I.

**Coracle**

*Drosophila* Coracle (Cor) is a membrane-associated cytoplasmic protein that is localized to and necessary for pleated septate junction development (Fehon et al., 1994). Cor, like Nrx-IV, is expressed during embryonic development in tissues that are ectodermally derived (Baumgartner et al., 1996; Fehon et al., 1994). It has not been determined if Cor has a role in the development of the BNB, although this is anticipated since Cor has been shown to be necessary to form transepithelial barriers, and the localization of Cor to pleated septate junctions is dependent on Nrx-IV and vice versa (Lamb et al., 1998; Ward et al., 1998; Baumgartner et al., 1996). Both cor and *nrx-IV* mutant embryos display large dorsal holes in cuticle preparations from late stage embryos (Lamb et al., 1998; Baumgartner et al., 1996). This arises because the
epidermis, which secretes the cuticle, fails to migrate adequately during a process known as 'dorsal-closure.' Many other mutants with dorsal-closure defects have been identified as components of the JNK signaling cascade which is equivalent to the mammalian stress-activated MAPK pathway (reviewed in Noselli, 1998). It remains to be determined if Nrx-IV or Cor have signaling activity or if they are simply structural components of pleated septate junctions.

Cor is a *Drosophila* homologue of the human protein band 4.1 which is a major component of erythrocyte cell membranes. Human band 4.1 is believed to stabilize the bi-concave structure of erythrocytes by interacting with the Actin/Spectrin cytoskeleton (Conboy et al., 1991; Marchesi, 1985). Both the human band 4.1 and *Drosophila* cor genes produce multiple splice variants. However, the region in human band 4.1 that is believed to be necessary for Actin/Spectrin interactions is not present in any of the cor splice variants (Fehon et al., 1994; Conboy et al., 1991). This suggests that Cor at SJs may not directly interact with Actin or Spectrin. However, immunohistochemical studies suggest that cytoskeletal components including Actin, alpha-Actinin and Vinculin are associated with SJs in *Manduca sexta*, and Actin microfilaments have been observed at SJs in several other invertebrates through electron microscopy (Colomb et al., 1993; Lane and Flores, 1988). Thus, although Cor may not directly link SJs to the actin cytoskeleton, other SJ components most certainly do (see Dlg/Lgl below). Aside from interacting with actin/spectrin, human band 4.1 was shown to interact with another erythrocyte protein, Glycophorin-C (Anderson and Lovrrien, 1984). The cytoplasmic tail of Nrx-IV shares 68% sequence identity with human Glycophorin C and through co-immunoprecipitation experiments and *in vitro* binding assays, Nrx-IV was shown to use its cytoplasmic tail to directly interact with Cor (Ward et al., 1998; Baumgartner et al., 1996). For this interaction, only the amino-terminal 383 amino acids of Cor are necessary (Ward et al., 1998).
Table 1. Summary of septate, tight, and paranodal junction proteins discussed.

A) Drosophila Septate Junction proteins

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein type</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurexin-IV (Nrx-IV)</td>
<td>Transmembrane</td>
<td>Contains Discoidin (lectin binding, domain and Fibrinogen-like region</td>
</tr>
<tr>
<td>Coracle (Cor)</td>
<td>Membrane-associated</td>
<td>Binds to Nrx-IV using last N-terminal 383 amino acids</td>
</tr>
<tr>
<td>Fasciclin III (Fas III)</td>
<td>Transmembrane</td>
<td>Immunoglobulin like cell adhesion molecule (IgCAM)</td>
</tr>
<tr>
<td>Discs Large (Dlq)</td>
<td>Cytoplasmic</td>
<td>Contains 3 PDZ domains, SH3 and GUK domain</td>
</tr>
<tr>
<td>Scribble (Scrib)</td>
<td>Cytoplasmic</td>
<td>Contains 4 PDZ domains and 16 leucine rich repeats</td>
</tr>
<tr>
<td>Lethal(2) giant-larvae (Lgl)</td>
<td>Cytoplasmic</td>
<td>Contains WD40 repeats</td>
</tr>
</tbody>
</table>

B) Vertebrate paranodal junction proteins.

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein type</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contactin</td>
<td>GPI-anchored membrane protein</td>
<td>IgCAM</td>
</tr>
<tr>
<td>Caspr</td>
<td>Integral membrane protein</td>
<td>Homologous to Nrx-IV</td>
</tr>
<tr>
<td>Neurofascin-155</td>
<td>Transmembrane protein</td>
<td>IgCAM binds to both Contactin and Caspr</td>
</tr>
</tbody>
</table>

C) Vertebrate tight junction proteins.

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein type</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occludin</td>
<td>Tetraspan transmembrane protein</td>
<td>Not essential for TJ formation</td>
</tr>
<tr>
<td>Claudins</td>
<td>Tetraspan transmembrane proteins</td>
<td>24 family members identified.</td>
</tr>
<tr>
<td>Junctional Adhesion Molecules (JAMs)</td>
<td>Transmembrane</td>
<td>IgCAMs</td>
</tr>
<tr>
<td>ZO-1, ZO-2, ZO-3</td>
<td>Cytosolic</td>
<td>Contain PDZ domains, GUK domain</td>
</tr>
<tr>
<td>MAGI-1, MAGI-2, MAGI-3</td>
<td>Cytosolic</td>
<td>Contain PDZ domains, GUK domain.</td>
</tr>
<tr>
<td>ASIP</td>
<td>Cytosolic</td>
<td>Contains 3 PDZ domains</td>
</tr>
<tr>
<td>Par-6</td>
<td>Cytosolic</td>
<td>Contains 3 PDZ domains</td>
</tr>
<tr>
<td>aPKC</td>
<td>Cytosolic</td>
<td>Complexes with ASIP and Par-6</td>
</tr>
<tr>
<td>Pals1</td>
<td>Cytosolic</td>
<td>Contains PDZ domain</td>
</tr>
<tr>
<td>PATJ</td>
<td>Cytosolic</td>
<td>Contains PDZ domain</td>
</tr>
<tr>
<td>CRB1</td>
<td>Cytosolic</td>
<td>Complexes with Pals1 and PATJ</td>
</tr>
</tbody>
</table>
Aside from Nrx-IV and Cor, which are bona fide pleated septate junction proteins, a number of other proteins have been shown to be associated with pleated SJs (and in certain cases also with smooth septate junctions). These include Fasciclin III (Fas III), Lethal(1)discs-large (Dlg), Scribble (Scrib), Lethal(2)giant-larvae (Lgl) and Polychaetoid (Pyd) (reviewed in Tepass et al., 2001). They are discussed below and summarized in Table 1A.

Fasciclin III

Fas III is a homophilic cell adhesion molecule and member of the immunoglobulin superfamily (Snow et al., 1989). In epithelial cells, Fas III is distributed along the entire length of the basolateral membrane domain, but it is enriched at pleated septate junctions (Woods et al., 1997). Fas III null mutants are viable, unlike nrx-IV and cor mutants which are embryonic lethal and display only subtle behavioral phenotypes (Baumgartner et al., 1996; Chiba et al., 1995; Fehon et al., 1994). This suggests that Fas III probably does not have an essential role in SJ formation.

Lethal(1)discs-large and Scribble

Two other genes whose products have been shown to localize to SJs are lethal(1)discs-large (dlg) and scribble (scrib) (Bilder and Perrimon, 2000a; Woods et al., 1996). Multiple splice variants of Dlg have been identified, and Dlg A is the most abundant variant. However, all variants appear to be localized to SJs (Woods and Bryant, 1991; Woods et al., 1996). Both DlgA and Scrib are cytosolic proteins, and each contains multiple PDZ domains (DlgA has 3, Scrib has 4) (Bilder and Perrimon, 2000a; Woods et al., 1996). The Dlg proteins were definitively shown to be SJ components through immuno-electronmicroscopy studies using an antibody that recognizes all Dlg variants (Woods and Bryant, 1991; Woods et al., 1996). Unlike Cor and Nrx-IV which appear to be specific to PSJs, the Dlg proteins localize to both SSJ and PSJs (Baumgartner
et al., 1996; Woods et al., 1996; Fehon et al., 1994). DlgA is a MAGUK (membrane-associated guanylate kinase homologue) and thus in addition to its PDZ domains, it also contains an SH3 domain, and a region similar to guanylate kinases (GUK domain) (Hough et al., 1997; Woods and Bryant, 1991). The second PDZ domain of DlgA is necessary to localize it to septate junctions, the SH3 domain is necessary for SJ function, but the GUK domain appears to be dispensable (Hough et al., 1997). Scrib is a LAP protein, containing 16 leucine rich repeats (LRRs) in addition to its PDZ domains (Bilder et al., 2000c). LRRs are thought to mediate protein-protein interactions (Bilder et al., 2000c). Scrib was shown to localize at PSJ in the epidermis through immunofluorescence microscopy (Bilder and Perrimon, 2000a). In these studies, Scrib co-localized with Cor (in the mature epidermis), but not with the adherens junctions marker, Armadillo (Arm), thus defining Scrib as a SJ protein (Bilder and Perrimon, 2000a). Since Gli contains a PDZ recognition sequence at the terminus of its intracellular tail, it is possible that Gli (as a putative SJ component) could physically interact with either Dlg or Scrib.

The role of Dlg and Scrib in SJ formation is complex. Although Dlg and Scrib are localized to SJs in the mature epidermis, they are probably functioning far before the first appearance of SJs to specify the region in the basolateral membranes where SJs are to be formed (Bilder and Perrimon, 2000a; Bilder et al., 2000b). Dlg and Scrib are present in the membrane during cellularization, in contrast to Nrx-IV and Cor which first appear in the epidermis during stage 12-13 of development (Bilder and Perrimon, 2000a, Bilder et al., 2000b; Woods and Bryant, 1991). During gastrulation (stage 8), Scrib is localized apically in the basolateral membrane at the future site of SJ biogenesis (Bilder et al., 2000a). At stage 12-13 when Nrx-IV and Cor are expressed in the basolateral membrane, they are diffusely distributed (Bilder and Perrimon, 2000a). Not until stage 14, when SJ biogenesis occurs, do Cor and Nrx-IV become restricted apically
to accompany Scrib (and Dlg) at the apical portion of the basolateral membrane (Bilder and Perimon, 2000a; Bilder et al, 2000b; Tepass and Hartenstein, 1994).

Electronmicroscopy analysis of *dlg* mutants has clearly shown that PSJs between salivary gland epithelial cells are poorly formed or absent (Woods et al., 1996). *nrx-IV, cor, and dig*, mutants all have dorsal closure phenotypes suggesting that they have similar functions (Baumgartner et al., 1996; Fehon et al., 1994; Perrimon 1988). However, in many respects, the mutant phenotypes of *dig* and *scrib* are quite unlike those of *cor* and *Nrx-IV*, suggesting that Dlg and Scrib have a unique role in SJ development. *dlg* and *scrib* are maternal effect genes, and mutants lacking both maternal and zygotic gene activity are embryonic lethal and have severe epithelial morphogenic phenotypes (Bilder and Perrimon, 2000a; Perrimon, 1988). The *dig* and *scrib* mutant phenotypes are essentially identical, and the epithelial cells of the embryonic epidermis are rounded rather than columnar in shape as in the wild type (Bilder and Perrimon, 2000a, Bilder et al., 2000b). Also, the localization of apical-basal polarity markers including the adherens junction protein, Arm, is severely disrupted in these mutants (Bilder and Perrimon, 2000a; Bilder et al., 2000b; Woods et al., 1996).

When only zygotic *dlg* or *scrib* gene activity is perturbed, the mutants survive to larval stages, but they develop imaginal disc and brain neoplasms (Bilder and Perrimon, 2000a; Bilder et al., 2000b; Woods et al., 1996; Woods and Bryant, 1991). These results suggest that SJs are necessary for the proliferative control of epithelial cells in *Drosophila*. In contrast, polarity and proliferative phenotypes do not occur in *nrx-IV* or *cor* mutants (Baumgartner et al., 1996; Lamb et al., 1998). One reason for this could be that these mutants die during embryogenesis (Baumgartner et al., 1996; Fehon et al., 1994). Since neoplastic overgrowth in *dlg* and *scrib* mutants are only apparent at larval stages, the *cor* mutants might die before neoplasms arise. However, when *cor* mutant clones are induced in wing imaginal discs, the mutant cells fail to proliferate adequately and they are eventually lost from adult tissue, indicating that SJs *per se* are not required to
attenuate cell proliferation (Lamb et al., 1998). Scrib and Dlg therefore have functionally distinct roles from Nrx-IV and Cor.

Lethal(2)giant-larvae

Lethal(2)giant-larvae (Lgl) is another protein that has been implicated in SJ development. This protein contains WD40 repeats, co-fractionates with cytoskeletal components, and physically associates with Non-Muscle Myosin II (Bilder et al., 2000b; Strand et al., 1994a; Strand et al., 1994b). Immuno-electronmicroscopy studies have shown that Lgl localizes to SSJs in the Drosophila proventriculus. However, it is also associated with other non-junctional regions of the plasma membrane and so it is not believed to be an exclusive junctional protein (Strand et al., 1994a). Like dig and scrib, lgl is a maternal effect gene as well as a tumor suppressor (Bilder et al., 2000b; Gateff and Schneiderman, 1969; Gateff and Schneiderman, 1974). Genetic epistasis experiments suggest that Lgl, Dlg and Scrib, act in a common genetic pathway (Bilder et al., 2000b). Yeast and vertebrate homologues of Lgl facilitate the fusion of cargo-carrying vesicles with target membranes, and so it has been speculated that septate junctions may act as target sites for the delivery of Golgi vesicles carrying basolateral membrane components (Bilder et al., 2000b; Lehman et al., 1999; Fujita et al., 1998; TerBush et al., 1996).

In addition to being involved in SJ development, Lgl, Dlg and Scrib may also have a role in adherens junction development, possibly in an indirect fashion. Adherens junctions (AJs) first form in embryos during cellularization (stage 5) and appear as spot adherens junctions, that later migrate apically and coalesce to form the mature zonula adherens during gastrulation (stage 8). Although AJ development has not been shown to be perturbed in dig, scrib or lgl mutants (lacking maternal and zygotic gene activity) through electronmicroscopy, the AJ markers Armadillo or E-Cadherin have been shown to be mislocalized in these mutants through confocal
analysis (Bilder and Perrimon, 2000a; Bilder et al., 2000b). Certain mutations that affect the biogenesis of AJs result in apical-basal polarity phenotypes that resemble those seen in the dlg, scrib, and lgl mutants. For example, in crumbs and stardust mutants, spot AJs are able to form, however they do not coalesce to form the zonula adherens (Grawe et al., 1996). The epithelium of these mutants is disorganized. The cells are rounded and apical-basal polarity is perturbed. Crumbs and Stardust physically interact, and together with another protein called Discs-lost, they form a complex that is localized apical to adherens junctions (but with some overlap) prior to and during zonula adherens development (Hong et al., 2001; Bauchmann et al., 2001; Bhat et al., 1999). This region is termed the marginal zone or subapical complex (see Figure 1A) (reviewed in Tepass et al., 2001). Interestingly, Discs-lost is a PDZ containing protein that was isolated through a yeast-two hybrid screen using the cytoplasmic tail of Nrx-IV as bait (Bhat et al., 1999). SJs, or at least Scrib, are necessary for the development of the subapical complex since Crumbs and Discs-Lost are mislocalized in Scrib mutants, and thus this supports the notion that Dlg, Scrib and Lgl are necessary for AJ formation (Bilder and Perrimon, 2000b). Reciprocally, the subapical complex is necessary for SJ formation since in stardust (std) mutants, the localization of Dlg and Scrib in the apical portion of the basolateral membrane becomes progressively compromised during development (Hong et al., 2001). Together these results suggest that Dlg, Scrib, and Lgl in some fashion interact with the proteins of the subapical complex, perhaps to establish the zonula adherens at an intermediate locale. Subsequently Dlg, Scrib, and Lgl recruit Nrx-IV and Cor to establish a mature SJ protein complex that is positioned basal to the zonula adherens.

Polychaetoid

The last molecule that to date, which has been described as being associated with SJs is Polychaetoid (Pyd), which is also known as Tamou (Tam) (Takahashi et al., 1998;
Pyd is a MAGUK and was originally reported as being associated with SJs in wing imaginal discs. However, it has since been shown to produce two splice variants (Pyd$^-$ and Pyd$^+$) and the expression profile of only one isoform (Pyd$^-$) overlaps with the SJ marker FasIII (Wei and Ellis, 2001; Katsube et al., 1998; Takahashi et al., 1998; Takahisa et al., 1996). While the Pyd$^-$ isoform is distributed along the lateral membrane, the Pyd$^+$ isoform is restricted apically and co-localizes with adherens junction markers (Wei and Ellis, 2001). Pyd$^-$ appears to be zygotically transcribed and Pyd$^+$ is maternally loaded (Wei and Ellis, 2001). Although pyd mutants are embryonic lethal and display defects in dorsal closure, it is not known if Pyd is required for SJ development (Chen et al., 1996; Takahisa et al., 1996). Rescue experiments have shown that only the Pyd$^+$ isoform (AJ isoform) is capable of rescuing the embryonic lethality of pyd null mutants, suggesting that Pyd is probably not an integral structural component of SJs (Wei and Ellis, 2001). Pyd could instead be involved at AJs to signal cell shape changes or epithelial cell movements during dorsal closure, since Pyd physically interacts with Cortactin (an F-actin binding protein) and pyd genetically interacts with hemipterous, a component of the JNK signaling cascade (Katsube, et al., 1998; Takahashi et al., 1998; Noselli, 1998; Wei and Ellis, 2001).

**Vertebrate Junctions**

There are two junction types in vertebrates that are functionally similar to invertebrate SJs: tight junctions and paranodal junctions (see reviews Bryant et al., 1997; Tepass et al., 2001). Up until the early 1980's, SJs were thought to be unique to invertebrates because of their distinct morphology. Nevertheless, SJs are frequently compared directly to TJs, especially by Drosophila biologists, since Drosophila unlike certain other insects, lack TJs (Tepass and Hartenstein; 1994). SJs are functionally analogous to TJs in that they both serve as permeability barriers and as adhesive...
contacts (Tsukita, 2001; Bryant, 1997; Lane et al., 1994; Noirot-Timothee et al., 1978). Recently, mutant analysis of *scrib*, has led to the hypothesis that SJs, like TJs, may also function as molecular fences (Bilder and Perrimon, 2000). With the identification of *Drosophila* Dlg as a vertebrate homologue of the TJ protein ZO-1, it was speculated that SJs and TJs may have had a common evolutionary origin, and over time they may have diverged structurally, but converged functionally (Woods and Bryant, 1991; Lane et al., 1994). Interestingly, in certain other insects, SJs and TJs often occur simultaneously in various epithelial tissues (Carlson et al., 2000; Lane et al., 1994). In these cases, both junction types appear to contribute to the formation of an effective permeability barrier (Carlson et al., 2000). It has been suggested that TJs have ultimately been more successful (than SJs) as permeability barriers, and thus they have been selected for in vertebrates over the course of evolution (Lane et al., 1994). However, in the early 1980's, septate-like paranodal junctions were reported to occur in vertebrates at axon-glial contacts, thus challenging the dogma that SJs are unique to invertebrates (Wiley and Ellisman, 1980). In recent years, homologues of *Drosophila* Nrx-IV have been found to be localized to paranodal junctions, and currently it is being debated that paranodal junctions are structurally and functionally more analogous to SJs than TJs (Tepass et al., 2001; Poliak et al., 1999; Einheber et al., 1997; Menegoz et al., 1997). Moreover, it is being suggested that the molecular composition of TJs (summarized in Table 1C) may in fact have greater similarity to the marginal zone of *Drosophila* epithelial cells than to SJs (see below) (Tepass et al., 2001).

**Tight Junctions**

*Morphology of Tight Junctions*

Tight Junctions (TJs) in vertebrate epithelial cells lie in the apical membrane domain above the zonula adherens (see Figure 1B) (Schneeberger and Lynch, 1992). In
transmission electron micrograph images of epithelial cells viewed in cross-section, TJs appear as a series of membrane fusions termed 'kissing-points' (Tsukita et al., 2001). At kissing points, the intermembrane space appears to be obliterated. In freeze-fracture replicas electron microscopic images of TJs, a network of anastomotic fibrils (intramembrane particle strands) is apparent on the protoplasmic (P) face and corresponding grooves are evident on the extracellular (E) face (Staehelin, 1973). TJ strands from one epithelial cell align or pair with those of an adjacent epithelial cell, such that kissing-points represent pairs of TJ strands that have been cross-sectioned (Tsukita et al., 2001). The number of intramembrane particle strands per TJ varies from cell type to cell type, as does the complexity of the anastomotic strand network (Schneeberger and Lynch, 1992). For example, TJs in the proximal tubule of kidney epithelial cells, have 1-2 TJ strands, while those in the distal tubule have 4-7 TJ strands (Claude and Goodenough, 1973). A positive correlation has been shown to exist in some cases between strand number and the tightness of a TJ (Claude and Goodenough, 1973; Madara and Dharmsathaphorn, 1985). Recently it has been suggested that aqueous pores are present in paired TJ strands, and that these pores impact the tightness of TJs (Tsukita and Furuse, 2000).

Molecular composition and function of Tight Junctions

Accumulating evidence suggests that the Claudin family of tetraspan transmembrane proteins are chiefly responsible for the formation of TJ strands. Twenty-four Claudin family members have been identified in mouse and humans and different Claudins are expressed in different tissues (Tsukita et al., 2001; Morita et al., 1999). For a given tissue type, usually greater than 2 types of Claudins are expressed, and these proteins have the capacity to interact in a homo- and heterotypic fashion (Tsukita et al., 2001; Furuse et al., 1999). Occludin is another tetraspan membrane protein that localizes to TJ strands and it is structurally related to the Claudins (Tsukita
et al., 2001; Furuse et al., 1993). Occludin was the first integral membrane protein found to be associated with TJ strands. However, it has since been shown to not be essential for TJ formation (Furuse et al., 1993; Saitou et al., 1998). Embryonic stem cells derived from Occludin deficient mice differentiate into polarized epithelial cells that have TJs (Saitou et al., 1998). The Claudins, in contrast, are essential for TJ formation. In vitro tissue culture reconstitution experiments using mouse L-fibroblasts (a culture line that lacks endogenous Claudins and Occludin) have shown that Claudin-1 or Claudin-2 can form well-developed networks of paired TJ-like intermembrane particle strands when these cells are transfected with them (Furuse et al., 1998). Interestingly, when Occludin is introduced into the Claudin-1 (or 2) transfected L-cells, it associates with the Claudin strands, suggesting that they are actual TJ strands (Furuse et al., 1998). Perhaps the most compelling evidence that Claudins are necessary for TJ strand formation has been the generation of the Claudin-11 knock out mouse (Gow et al., 1999). The myelin sheath of oligodendrocytes as well as the Sertoli cells only express Claudin-11 and not other variants (Gow et al., 1999). In the Claudin-11 knock out mice, TJ strands fail to form in these cell types, indicating that the Claudins are essential for TJ strand synthesis (Gow et al., 1999). In addition to being the backbone of TJ strands, it is also believed that the Claudins are responsible for the formation of aqueous pores in TJ strands. Specifically, it is believed that the number/type of Claudins and their mixing ratio in TJ strands affects the ability of paired TJ strands to associate 'tightly' with each other and to form effective permeability barriers (Furuse et al., 1999). This theory is supported by tissue culture experiments in which L cells were singly transfected with Claudin-1, -2, or -3 and then the transfected lines were co-cultured in pairs. Claudin-3 is able to form paired TJ strands with Claudin 1 or 2, but Claudin 1 can not pair with 2 (Furuse et al., 1999). In some tissues, it is important to have significant paracellular flow, such as in the kidney, where Mg²⁺ resorption occurs across TJ strands in the thick ascending loop of Henle. Recently, it has been determined that hereditary hypomagnesia is caused by
mutations in the claudin-16 locus, suggesting that this mutation causes TJ strands to pair more tightly in this tissue (Simon et al., 1999).

Another class of transmembrane proteins, the immunoglobulin-like Junctional Adhesion Molecules (JAMs), also localize to TJs (Itoh et al., 2001; Martin-Padura et al., 1998). Little is known about their role in TJ strand development although they are not able to form TJ strand networks independently in reconstitution assays (Itoh et al., 2001).

Aside from integral membrane proteins, a number of cytosolic proteins are associated with TJ strands. Interestingly, TJs are rich in PDZ-containing proteins. ZO-1, ZO-2, and ZO-3 were among the first TJ PDZ proteins to be identified and like the SJ protein Dlg, they are MAGUKs (Haskins et al., 1998; Hough et al., 1997; Jesaitis and Goodenough, 1994; Stevenson et al., 1986). These MAGUK proteins interact with Cingulin and Myosin and thus serve to cross-link TJs to the actomyosin cytoskeleton (Fanning et al., 1999; Cordenonsi et al., 1999).

Other PDZ-containing proteins associated with TJs include MAGI-1, MAGI-2, and MAGI-3, which are MAGUK variants that have a unique arrangement of protein-protein interaction domains (Wu et al., 2000a; Wu et al., 2000b; Dobrosotskaya et al., 1997). MAGI-2 and MAGI-3 interact with the PTEN tumor suppressor, suggesting that TJs may have a role in oncogenesis (Wu et al., 2000a; Wu et al., 2000b). This is in agreement with the observation that oncogenic Raf-1 can down-regulate Occludin, and the finding that TJ proteins are differentially expressed in ovarian cancers (Li and Mrsny; 2000; Hough et al., 2000). Recently, two protein complexes that contain PDZ proteins, and that are involved in the establishment and/or maintenance of epithelial polarity, have been shown to associate with TJ strands. The first is the ASIP/Par-6/aPKC complex (ASIP and Par-6 each contain 3 PDZ domains) (Gao et al., 2002; Izumi et al., 1998). The second protein complex is the Pals1/PATJ/CRB1 protein complex (Roh et al., 2002). Both Pals1 and PATJ contain PDZ domains (Roh et al., 2002).
Although PDZ proteins were initially reported to bind the S/T-X-V consensus recognition sequence, they are also capable of binding more diverse four amino acid consensus sequences which terminate in Val (Songyang et al., 1997). Since many of the Claudin and JAM integral membrane proteins terminate in Val, it is suspected that they may recruit PDZ proteins to TJ strands (Tsukita et al., 2001). In fact, it has been proposed that TJ strands, rich in Claudin and JAM cytoplasmic tails, may resemble toothbrush bristles which are available to sequester PDZ containing proteins (Tsukita et al., 2001). In support, ZO-1, ZO-2, and ZO-3 have been shown to interact with the Claudins via their first PDZ domains, ASIP has been shown to interact with the COOH terminus of JAM, and more recently the MUPP1 protein which contains 13 PDZ domains has been shown to interact with Claudin-1 via PDZ domain 10 and JAM via PDZ domain 9 (Hamazaki et al., 2002; Itoh et al., 2001; Itoh et al., 1999).

Aside from acting as PDZ protein scaffolds and permeability barriers that restrict the paracellular flow of extracellular fluids, TJs have also been proposed to function as molecular fences (reviewed in Tsukita et al., 2001). Although TJs are able to limit the lateral diffusion of plasmamembrane lipids between the apical and baso-lateral membrane domains, there is some controversy as to whether this is also true for integral membrane proteins (Jou et al., 1998; van Meer et al., 1986; Dragsten et al., 1981; Ziomek et al., 1980). Given that TJ strands form anastomotic networks, and obliterate the intermembrane space, it is foreseeable that they would have the capacity to restrict the lateral diffusion of integral membrane proteins through stearic hindrance. However, an increasing number of studies suggest that the localization of basolateral membrane proteins, but not apical proteins, are affected by loss of TJ integrity (Jou et al., 1998; Grindstaff et al., 1998). Insight into why this may occur has come from the study of the Sec6/8 protein complex.

In budding yeast, the Sec6/8 protein complex is restricted to the bud tip and it is necessary for vesicle exocytosis (TerBush et al., 1996; TerBush and Novick, 1995).
non-polarized MDCK cells, the Sec6/8 complex is cytosolic, but it is rapidly recruited to
the membrane following the addition of Ca$^{2+}$, which causes the formation of cell-cell
junctions and triggers cell polarization (Grindstaff et al., 1998). Since the Sec6/8
complex becomes enriched at TJs in MDCK cells following the addition of Ca$^{2+}$, and it
has been shown that apical and basolateral membrane proteins are sorted into distinct
post-Golgi transport vesicles, these results suggest that the Sec6/8 (and TJs) may serve
as a target for the delivery of vesicles carrying basolateral cargo (Grindstaff et al., 1998).

_Tight Junctions and the Marginal Zone of Drosophila Epithelial cells._

The marginal zone of _Drosophila_ epithelial cells, rather than SJs, may be the
_Drosophila_ anatomical analogue of TJs (Tepass et al., 2001; Müller, 2000) because the
marginal zone lies apical to adherens junctions, which is similar to the position of TJs in
mammalian cells (SJs lie basal to adherens junctions, see Figure 1 A,B). Moreover,
several TJ protein complexes have homologues in _Drosophila_ that localize to the
marginal zone. For example, the TJ protein complex ASIP/Par-6/aPKC corresponds to
the Bazooka/DmPar-6/DaPKC complex (Petronczki and Knoblich, 2000; Wodarz et al.,
2000), and the TJ protein complex Pals1/PATJ/CRB1 corresponds to Stardust/Discs-
Lost/Crumbs complex (Bachmann et al., 2001; Hong et al., 2001; Bhat et al., 1999). ZO-
1 is the only vertebrate TJ protein, to date, that has a _Drosophila_ homologue that
localizes to SJs (Dlg). However, ZO-1 may be homologous to Pyd since it is molecularly
similar and because mouse ZO-1 is able to rescue _Drosophila pyd_ gene function
(unpublished results of Togashi et al. reported in Katsube et al., 1998). It remains to be
determined if the expression profile of Pyd extends further apical than the AJs and if it is
a marginal zone protein component (Tepass et al., 2001). Recently, it has been reported
that two genes similar to the Claudins are present in the _Drosophila_ genome although no
Occludin-like genes are evident (Tepass et al., 2001). It will be interesting to determine if
these _Drosophila_ Claudin-like proteins are expressed in epithelial cells and if they localize
to the marginal zone or to SJs, especially since the vertebrate Claudins are able to form TJ strands in reconstitution assays, yet the marginal zone appears to be devoid of intermembrane particle-like strands.

**Paranodal Junctions**

In the last decade, invertebrate SJs have started to be compared more extensively to the paranodal junctions of mammalian myelinated fibers (and not exclusively to TJs). It is being suggested that although SJs are functionally analogous to TJs, SJs are functionally, morphologically, and molecularly analogous to paranodal junctions (see Figure 1C) (Tepass et al., 2001).

**Morphology of paranodal junctions**

Paranodal junctions are found between the paranodal loops of myelinating glia (CNS oligodendrocytes, and PNS Schwann cells) and the underlying axons (Peters et al., 1991; Wiley and Ellisman, 1980). In cross-section transmission electronmicroscopy images of paranodal junctions, a ladder like array of 'septa' span the intermembrane space between paranodal loops and the axolemma and thus morphologically, paranodal junctions are quite similar to invertebrate SJs (Wiley and Ellisman, 1980; Baumgartner et al., 1996).

**Molecular composition and function of paranodal junctions**

A number of proteins localize to paranodal junctions (summarized in Table 1B). Contactin and Caspr are neuronal paranodal junction proteins (Einheber et al., 1997; Menegoz et al., 1997). These two proteins interact with each other in cis, and function as a co-receptors for the glial paranodal junction protein Neurofascin-155 (NF-155) (Charles et al., 2002; Bhat et al., 2001; Boyle et al., 2001, Peles et al., 1997). Contactin is a
GPI-anchored neural Ig CAM, Caspr is an integral membrane protein homologous to *Drosophila* Nrx-IV, and NF-155 is an L1-like Ig CAM (Tait et al., 2000; Menegoz et al., 1997; Einheber et al., 1997; Ranscht and Dours, 1988). Like *Drosophila* Nrx-IV, rat Caspr physically interacts with Band 4.1 through its glycophorin-like cytoplasmic tail (Menegoz et al., 1997). A second vertebrate Caspr (Caspr2) has recently been identified. However, it localizes to the juxtaparanode which is the region flanking the paranode, and associates with K⁺ channels (Poliak et al., 1999). Thus, of the paranodal junction proteins identified to date, two (Caspr and Band 4.1) have *Drosophila* homologues that localize to SJs. In *contactin* and *caspr* mutant mice paranodal junctions fail to form, so these proteins are essential for paranodal junctions (Bhat et al., 2001; Boyle et al., 2001). Since the study of paranodal junctions is essentially in its infancy, it has not yet been determined if homologues of other SJ molecules such as Dlg, and Scrib, or Gliotactin for that matter have a role in the formation of paranodal junctions. Interestingly, a *Drosophila* Contactin-like protein has been identified in the genome sequence database (V. Auld, personal communication). However, it has not been investigated whether this protein localizes to SJs.

The analysis of the *contactin* and *caspr* mutant mice has also revealed functional similarities between paranodal junctions and SJs. Like SJs, paranodal junctions have adhesive properties, inferred from the observation that in *contactin* mutant mice, the intermembrane space between the axon and the paranodal loops is significantly greater than in wild-type mice (Boyle et al., 2001). In *caspr* mutants, the paranodal loops are everted such that the loops face away from, and not toward the axolemma (Bhat et al., 2001). Paranodal junctions also appear to function as molecular fences, a role that has also been suggested for septate junctions. In *Drosophila* SJs, Scrib is required for the maintenance of apical and basolateral membrane proteins (Bilder and Perrimon, 2001). In vertebrates, paranodal junctions separate different domains of ion channels. Specifically, in the axolemma of the nodal region, a mixed population of voltage gated
Na\(^+\) channels (Na\(_\text{v}1.2\), Na\(_\text{v}1.6\), Na\(_\text{v}1.8\), Na\(_\text{v}1.9\)) are concentrated, while in the axolemma of the internodal region (at the juxtaparanode), delayed rectifying K\(^+\) channels (K\(_\text{v}1.1\), K\(_\text{v}1.2\)) and their associated \(\beta\)-subunit (K\(_\text{v}\beta2\)) are concentrated (reviewed in Scherer and Arroyo, 2002). In contactin and caspr mutant mice, the distribution of these channels is disrupted (Bhat et al., 2001; Boyle et al., 2001). Na\(^+\) channels largely remain concentrated at the nodal region as in wild types, however, their localization domain is broadened. The localization of K\(^+\) channels in the mutant mice is more severely affected and the channels shift from the juxtaparanodal region to the paranodal region, such that they underlie the paranodal loops, and abut the Na\(^+\) channels. The mislocalization of these channels results in a marked decrease of axonal conduction velocity, but why this occurs is not known (Bhat et al., 2001; Boyle et al., 2001). Nevertheless, this data suggests that paranodal junctions are required to segregate the Na\(^+\) from the K\(^+\) channels and thus they function as molecular fences. Compared to wild-type, contactin and caspr mutant mice are highly sensitive to K\(^+\) channel blockers (Bhat et al., 2001; Boyle et al., 2001). This suggests that blockers have greater access to the channels, and thus that the paranodal junctions also function as permeability barriers in wild-type animals. Paranodal junctions thus share many similarities with invertebrate SJs.

**Peripheral nerves and formation of the Blood-Nerve Barrier**

Gli was originally identified as a peripheral nervous system glial transmembrane protein that was necessary for the formation of the blood-nerve barrier (Auld et al., 1995). Since a major hypothesis of this thesis is that Gli is involved in the formation of pleated septate junctions in the peripheral nerves, it is important to have a knowledge of the cellular architecture of *Drosophila* peripheral nerves. The anatomy of *Drosophila*
larval peripheral nerves has been best characterized and therefore they are described here.

*Anatomy of Drosophila peripheral nerves.*

The center of third instar larval peripheral nerves contains motor and sensory axons which are ensheathed by peripheral nervous system glia (Auld et al., 1996). These glia wrap neurons either singularly or in bundles (Auld et al., 1995). Myelination does not occur in *Drosophila* and thus the peripheral nervous system glia are most similar to the non-myelinating Schwann cells found in mammalian peripheral nerves (Auld et al., 1996; Auld et al., 1995; Peters et al., 1991). Overlying the peripheral glia is a single layer of cells that have a squamous epithelial morphology. These cells are termed perineurial cells (Carlson et al., 2000). Surrounding the exterior of the larval peripheral nerves is a thick neural lamella which is probably produced by the perineurial cells (Auld et al., 1996; Carlson et al., 2000).

*Formation of the blood-nerve barrier in Drosophila*

As aforementioned, the blood-nerve barrier (BNB) is formed in *Drosophila* by septate junctions (Carlson et al., 2000; Baumgartner et al., 1996). These junctions occur between the wraps of peripheral glial between abutting perineurial cells, and between the contacting surfaces of peripheral glia and the perineurial cells (Carlson et al., 2000). There has been some controversy as to whether the BNB in *Drosophila* is formed by the peripheral glia or the perineurial cells, however, it is likely that both cell types contribute to form a functional BNB (Carlson et al., 2000; Baumgartner et al., 1996; Auld et al., 1995). Some of the controversy stems from the lack of knowledge about exact timing of SJ maturation in these cell types, as well as from the timing of perineurial cell association with peripheral nerves. The blood nerve barrier is established in *Drosophila* at the end of embryogenesis (stage 17) (Carlson and Hilger 1998). During this period,
the SJs are maturing between the wraps of peripheral glia, and the perineurial cells are first observed to associate with the peripheral nerves (Tepass and Hartenstein, 1994). Although the perineurial cells initially lack SJs, by the end of stage 17 they have fully formed SJs and the BNB is intact (Carlson et al., 2000; Carlson and Hilger 1998; Tepass and Hartenstein, 1994).

Anatomy of mammalian peripheral nerves.

The organization of mammalian peripheral nerves is quite similar to that of Drosophila peripheral nerves. At the center of mammalian peripheral nerves are myelinated and non-myelinated axons (Olsson, 1990; Thomas and Olsson, 1984). These ensheathed nerve fibers are embedded in a collagen-rich matrix or endoneurium, and encased by a six to eight layers of flattened epithelial cells (the perineurium). On either side of the perineurium is a prominent basal lamina. Overlying the outer basal lamina of the perineurium is another dense layer of collagen fibers (the epineurium). The two collagen-rich domains, lying below and above the perineurial sheath, are produced by fibroblasts that are scattered throughout these matrices. The endo-, epi-, and perineurium together form a protective nerve sheath that enables peripheral nerves to withstand mechanical stresses.

The BNB in mammalian peripheral nerves is formed by the extensive tight junction networks that are present between the flat squamous cells of the perineurium (Thomas and Olsson, 1984). Tight junctions are also present between the paranodal loops of Schwann cells (Scherer and Arroyo, 2002). These tight junctions are necessary for normal nervous system function, since Occludin-11 knock out mice have slowed axonal conduction velocities, however, what role these TJs carry out is not fully understood (Gow et al., 1999). CNS myelination is able to proceed normally in occludin-11 mutant mice, including the development of paranodal junctions. However, it has not
been determined if these TJs contribute to the formation of the blood-nerve- or blood-
brain-barrier (Gow et al., 1999).

The development of the mammalian peripheral nerve also occurs in a similar
fashion to that of Drosophila peripheral nerves. Axons are not initially sheathed by the
Schwann cells, which are neural crest-derived, and subsequently encased by the
perineurial cells, which are mesodermally derived (Dong et al., 1999; Bunge et al., 1989).
Similarly, mammalian perineurial cells do not associate with ensheathed axons until late
in embryonic development (E15 in mouse), and the blood-nerve-barrier is not intact
until a few weeks postnatally (Bunge et al., 1989; Thomas and Olsson, 1984; Kristensson
and Olsson, 1971).

Gliotactin and the formation of the Blood-nerve barrier - a perspective

Taking into consideration all that is known about Gliotactin, septate junctions,
and the development of Drosophila peripheral nerves, it is possible to formulate a model
that predicts that Gli is part of a protein complex that is necessary for septate junction
development. A few observations in particular point to Gli having a role in SJ
development. First, gli and nrx-IV mutants have very similar mutant phenotypes with
respect to the blood-nerve barrier (Baumgartner et al., 1996; Auld et al., 1995). Second,
gli mutants die during stage 17 of embryonic development, when the BNB is being
established and septate junctions in the peripheral nerves are maturing (Carlson et al.,
2000; Carlson and Hilger 1998; Auld et al., 1995; Tepass and Hartenstein, 1994).

As a neuroligin-like molecule, Gli could interact through its extracellular domain
with a Drosophila Neurexin, possibly Nrx-IV, to form the BNB. However, Nrx-IV is not a
β-Neurexin (the binding partner of Neuroligins) but is more similar to Caspr and
Caspr2. Nrx-IV is thus more likely to interact with a Drosophila Ig CAM (like contactin
or NF-155) than with a cholinesterase-like molecule such as Gli (Peles et al., 1997; Poliak
et al., 1999). In wing imaginal discs of *Drosophila*, the IgCAM Neuroglian is expressed in the basolateral membrane, thus it could potentially be a binding partner for Nrx-IV (Woods et al., 1996). Although Gli is not likely to interact directly with Nrx-IV, it could still be part of a septate junction complex that includes Nrx-IV. Gli could also interact with other septate junction proteins, such as the PDZ containing proteins Scrib and Dlg because the cytoplasmic tail of Gli contains a PDZ recognition sequence.

The goal of this work has been to test whether Gli is involved in *Drosophila* SJ formation using *in vivo* mutant analyses in the peripheral glia and epidermis. Using genetic and cell biology techniques, it was determined that SJs markers are mislocalized in *gli* mutants. Dye permeability studies showed that the mislocalization of SJ markers were indicative of a loss of the integrity of SJ-based permeability barriers between epithelial cells. Next it was shown that Gli localizes to SJs, however, it has a novel distribution for a molecule needed for SJ development. Transgenic lines expressing wild type and Gli deletion constructs were generated to determine if the cholinesterase-like domain or the PDZ recognition sequence within Gli are necessary for Gli to become localized to SJs. The PDZ recognition sequence within the cytoplasmic tail of Gli appears to be necessary for it to become localized to SJs, but it is not essential for Gli function since the mutant construct is able to rescue the lethality of *gli* protein null mutants. Interestingly, Gli protein lacking the PDZ recognition sequence is less susceptible to proteolysis, suggesting that the PDZ recognition sequence may be required for the cellular turn-over of endogenous Gli. Immunoprecipitation studies utilizing the wild-type transgenic lines revealed that Gli is indeed a member of a complex of SJ proteins that includes Nrx-IV, Cor and Dlg. Together these results strongly suggest that the disrupted blood-nerve-barrier phenotype and subtle peripheral nerve glial wrapping phenotypes, observed in *gli* mutants arises from the lack of septate junctions.
II. EARLY STUDIES OF THE MECHANISM OF GLI ACTION DURING DROSOPHILA BLOOD-NERVE-BARRIER DEVELOPMENT.

(Chapter contains data published by Sepp, K.J., Schulte, J., and Auld, V.J. 2000. in Glia 30: 122-133)
Introduction

Gliotactin was identified as the first cellular marker for peripheral glia (Auld et al., 1995). Up until the generation of an antibody probe for Gliotactin, *Drosophila* embryonic peripheral glia had only been visualized through the use of nuclear lacZ enhancer trap lines (Klambt and Goodman, 1991), or through cross-section images of peripheral nerves obtained through electronmicroscopy studies (Jacobs and Goodman, 1989). These studies largely focused on CNS glia, thus little was known about extent of peripheral nerve ensheathment in the embryo. The initial publication of the expression pattern of Gli during embryonic development, thus provided the first glimpse of the nature of the peripheral nerve glial sheath in *Drosophila* embryos (Auld et al., 1995). In recent years, genetic cell labeling methods have been developed and these have permitted a thorough characterization of peripheral glial embryonic development (Sepp et al., 2000). An interesting observation from the studies of peripheral glial development, is that the distal reaches of the peripheral motor neurons are not ensheathed by glia by the end of embryogenesis.

The lack of glial ensheathment of the distal reaches of peripheral motor neurons in embryos is surprising, given that action potentials are blocked from propagation if neurons become exposed to the high K$^+$ concentration of the haemolymph (Auld et al., 1995). No other cell types that could shield the motor axons from the haemolymph have been observed to associate with the distal reaches of the peripheral nerves (Sepp et al., 2000). However, in locust it has been suggested the K$^+$ concentration of the haemolymph of embryos at day 11 is lower, and the Na$^+$ concentration is higher, than in the adult which would make the ionic environment of the embryo more favorable than that of the adult to action potential propagation (K. Schofield in Swales and Lane, 1985). It is possible that because distances are small in the embryo, an action potential
generated at the axon hillock of a motorneuron (within the CNS) would be able to reach the nerve terminus (in the periphery) and have sufficient magnitude to trigger synaptic vesicle release at the neuromuscular junction even without a full glial sheath. In the larva, neuron lengths increase exponentially, and it is doubtful that unensheathed axons could propagate action potentials to the neuromuscular junction. Thus it is possible that glial ensheathment is not complete by the end of embryogenesis and that there would be a second post-embryonic phase of glial process extension to bring about complete coverage.

Before carrying out an investigation of the role of Gli in the formation of septate junctions, which is the main hypothesis to be tested in this thesis, it was pertinent to carry out a characterization of larval peripheral glial development. Given the exponential cellular growth that occurs to transform a 0.5 mm long *Drosophila* embryo into a 0.9 cm long 3rd instar larva, it was reasoned that as a putative cell adhesion molecule, Gli might be essential in a 'second phase' of glial development.

Another issue concerning the *gli* mutants that needed to be more fully investigated before embarking on an analysis of septate junction development, was to determine the extent of the glial wrapping defect in the mutant embryos. It had previously been shown that although ventral regions of the intersegmental nerve (ISN) were appropriately ensheathed in *gli* mutants, the distal reaches of this nerve branch had aberrant glial wrapping in the mutants (Auld et al., 1995). For these studies, electronmicroscopy was employed and only a single nerve branch was examined at two cross sections. To obtain a better understanding of the extent of the ensheathment defect reported for *gli* mutants, it was important to carry out whole cell staining of the *gli* mutants, so that all the nerve branches that are normally wrapped by PNS glia could be visualized simultaneously. Six to eight peripheral glia normally ensheathe the peripheral nerves, and these are evenly spaced over the length of the two main nerve bundles in the peripheral nervous system: the anterior and posterior fascicles (Sepp et
al., 2000). With whole cell staining experiments, it would be possible to determine if any unensheathed 'gaps' between peripheral glia occur in the gli mutants. Such mutant phenotypes would be missed through the previous electronmicroscopy analysis that only looked at the peripheral glia at two positions along the nerve, and did not include a serial reconstruction of cross-section taken along the entire length of the peripheral nerve.

In this chapter, a description of wild type glial development during larval stages as well as a characterization of ensheathment defects in gli mutants is presented. From the larval studies, it was discovered that there is indeed a second, post-embryonic phase of glial development during which the distal reaches of motor neurons become ensheathed by the peripheral glia. Interestingly, this second phase of glial-ensheathment is accomplished without glial proliferation and occurs instead through the extension of embryonic glial processes. By monitoring the expression profile of Gli in peripheral glia during larval stages, it was also observed that during this second phase of glial growth, the distribution pattern of Gli becomes strikingly different. During embryogenesis, Gli has a diffuse distribution in peripheral glia, but during larval development, it becomes concentrated into ribbon-like strands that run the length of peripheral nerves. The possibility that these Gli 'ribbons' represent peripheral glial septate junctions is discussed.

In carrying out the analysis of the extent of peripheral nerve ensheathment in the gli mutants, it was discovered that loss of Gli only causes subtle wrapping defects and these are most evident in the ventral peripheral nerve fascicle, where glia are often observed to have a lamella-like morphology rather than being tightly associated with the peripheral nerves. The wrapping defect in turn leads to defasciculation of the axons within this fascicle. However, even subtle wrapping defects could cause a disruption of the blood-nerve barrier which would result in paralysis.
Materials and Methods

Fly Strains

The enhancer trap line rL82 was generated in a standard enhancer trap screen (Klämbt and Goodman, 1991). This line expresses a β-galactosidase protein that is fused with a nuclear localization signal. rL82 is a viable, P[lacZ, ry+] insertion in the gliotactin gene and it is expressed in all peripheral glia (Sepp et al., 2001; Auld et al., 1995). The rL82#29 stock is a GAL4 enhancer trap line that was generated by converting rL82 to the GAL4 system via targeted transposition (Sepp and Auld, 1999). The repoGAL4 line is an insertion into the repo locus and it was supplied by W. Leiserson. RepoGAL4 drives expression of GAL4 in all central and peripheral nervous system glia (Sepp et al., 2001). UAS-taulacZ stock was provided by A. Brand. The UAS-gapGFP stock was obtained from the Bloomington Stock Center (donated by A. Chiba). gli$^{AE2A45}$ is a P-element excision allele, and protein null (Auld et al., 1995). HA-tagged UAS-Gli$^{I0-HA}$ was generated as described in Chapter IV.

Fly crosses

To visualize the peripheral glial cellular processes, the rL82#29 or the repoGAL4 strains were crossed to either UAS-taulacZ or UAS-gapGFP. For the analysis of the peripheral glial cellular morphology in the gli mutants, w;gli$^{AE2A45}$,UAS-taulacZ/CyOenwglacZ females were crossed to w;gli$^{AE2A45}$/CyOenwglacZ;RepoGAL4/TM6,Tb males, and homozygous gli mutants were identified by staining for a lack of enwglacZ expression. For these studies, the RepoGAL4 driver was used to express UAS-taulacZ in the peripheral glia, rather than the rL82#29 enhancer trap that was used for the characterization of larval glial development. This was done for technical reasons, since the rL82#29 strain is a viable
insert in the *gli* locus and thus it can not be used to label peripheral glia while maintaining a homozygous *gli* mutant background. To examine the localization of HA- tagged Gli in the peripheral glia, *rL82#29* virgin females were crossed to UAS-*gli*<sup>UAS-HA</sup> males.

**Antibodies**

The primary antibodies and the dilutions that were used for immunohistochemistry are as listed: mouse 22C10, 1:2 (Fujita et al., 1982); mouse anti-β-galactosidase, 1:250 (Sigma BioSciences, St. Louis, MO); mouse anti-Fasciclin II (1D4), 1:2; mouse anti-Coracle (9C and C615-16B cocktail), 1/100 (Feohon et al., 1994); rabbit anti-β-galactosidase, 1:1000 (Cappel, ICN Pharmaceuticals Inc., Aurora, OH); and rabbit anti-repo, 1:200 (donated by Sarb Ner). Secondary antibodies, and dilutions used for chemical labeling were: alkaline phosphatase conjugated goat anti-rabbit, 1:1500; and horseradish peroxidase (hrp) conjugated goat anti-mouse, 1:300 dilution (both were obtained from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). For larval staining for brightfield microscopy, rabbit anti-hrp polyclonal and hrp-conjugated goat anti-rabbit secondary (Jackson) were used at 1:100 and 1:300 dilutions respectively. For larval confocal analysis, rabbit anti-GFP polyclonal IgG (Clontech, Palo Alto, CA) and goat anti-hrp (Jackson) primaries were both used at a 1:100 dilution. The donkey anti-rabbit FITC and donkey anti-goat Texas Red fluorescent secondaries (Jackson) were used at 1:100.

**Immunohistochemistry**

For brightfield microscopy, embryos and larvae were fixed and stained as described previously (Klambt et al., 1991; Ito et al., 1995). Embryos of the nuclear *lacZ* enhancer trap lines were stained with the anti-β-galactosidase antibody. The anti-repo antibody was used to label glial nuclei in wild type embryos. All embryos were also
double stained using either the 1D4 or 22C10 antibody. Larvae were stained with anti-hrp to detect neurons and then were stained with Xgal (American Biorganics, Niagara Falls, NY) to detect glia. Embryos were cleared in a series of 50%, 70%, and 90% glycerol (in PBS) and dissected. Stained and dissected larvae were also cleared in a glycerol series. All dissections were mounted with 90% glycerol and viewed with a Zeiss Axioskop microscope with Nomarski optics. 35 mm slides were made using Kodachrome ISO 64 film, digitized and assembled into figures using Adobe Photoshop 4.0.

For confocal analysis, larvae were prepared as for brightfield, substituting PBS with TBS (25 mM Tris-Cl, pH=7.4; 137 mM NaCl; 5 mM KCl; 0.6 mM Na₂HPO₄; 0.7 mM CaCl₂; 0.5 mM MgCl₂) and using antibodies for fluorescence as detailed above. Fluorescently labeled larvae were mounted with 90% glycerol, 2.5% DABCO (Sigma) in TBS and viewed under a Biorad MRC 600 confocal microscope. Digital images were processed using NIH Image 1.61 and Adobe Photoshop 4.0.
Results

Larval development of peripheral glia

*Drosophila* peripheral nerves are composed of sensory and motor neurons and glia. As observed previously, by the end of embryogenesis, sensory neurons are fully ensheathed by the peripheral glia but not motor neurons (Sepp et al., 2000). Two main motor neuron trunks, the intersegmental nerve (ISN) and the segmental nerve (SN) extend from the central nervous system out into the periphery where they innervate somatic muscle fields (Figure 2A). The ISN with its associated nerve branches (ISNb and ISNd) is found within the anterior peripheral nerve fascicle (af), while the SN and its associated nerve branches (SNa and SNc) are components of the posterior peripheral nerve fascicle (pf) (Figure 2A). Although motor neuron fibers are ensheathed by peripheral glia in the ventral region of the embryo (near the CNS) by the end of embryogenesis, the distal reaches of motor neurons are not wrapped (arrows, Figure 2B,C). Thus it is possible that glial nerve wrapping in the mature embryo is at an intermediate stage.

To investigate if there is continued glial growth following embryogenesis, such that distal reaches of motor tracts are eventually covered, the GAL4/UAS gene expression system (Brand and Perrimon, 1993) was used to label the peripheral glia so that they could be visualized using immunohistochemical techniques. The GAL4/UAS system is a *Drosophila* two gene expression system that makes it possible to express any gene of interest in a given target tissue. Here, the bacterial enzyme β-galactosidase (fused to the microtubule associated protein Tau) was selectively expressed in the glia so that they could be visualized through the use of standard immunohistochemical procedures. First, second and third instar larvae were dissected, fixed, and stained with Xgal, which is cleaved into a blue product by the β-galactosidase expressed in the glia.
Figure 2. Characterization of Larval Peripheral Glial Development.

(A) Diagram of the main motor neuron branches in the peripheral nervous system. Anterior is to the top, and dorsal is to the left. Motor neurons arise in the central nervous system (CNS) and project into the periphery where they innervate muscle fibers (not indicated). The anterior (af) and posterior (pf) peripheral nerve fasicles contain both motor and sensory neurons but for simplicity, only motor branches are indicated. The anterior fasicle contains the intersegmental nerve (ISN) trunk, and associated branches ISNb and ISNd. The posterior fasicle contains the segmental nerve (SN) branches SNa and SNC. (B,C) Extent of glial coverage (black) of motor neuron terminals (brown) is shown in an rL82#29:UAS-taulacZ late stage embryo double stained for β-Gal and Fas II. The distal reaches of all motor neuron branches are not ensheathed by the peripheral glia (arrows). Two focal planes of the same embryo are presented to show the distal reaches of all motor branches. (D-G) Glial coverage of peripheral nerves during larval development. rL82#29:UAS-taulacZ larvae were stained with Xgal (blue) to label glia and anti-hrp to label neurons (brown). At this magnification, only the peripheral glia are evident. (D) First instar larval staining of peripheral glia (blue). The ISNb branch is now covered by peripheral glia (arrowhead). Peripheral glia begin to extend along the ISN terminus (arrow) past the region where the ISN diverges from the anterior sensory fascicle. (E) A lower focal plane of the same PNS segment as in (A). Peripheral glia now begin to ensheathe SNC motor branches (arrowhead) and SNA motor branches (asterisk). (F) Second instar. Glial coverage of the ISN motor terminus is now far more extensive (arrow), as peripheral glia have grown well past the ISN/anterior fascicle branch point. The anterior fascicle is slightly anterior to the ISN terminus in this region. Similarly, the SNA root also has more extensive peripheral glial coverage (asterisk). SNC and ISNd are covered by peripheral glia at these stages but cannot be seen in this focal plane. (G) Third instar. Peripheral glia cover all motor branches of the PNS and have maintained their sensory projection coverage established in embryogenesis. Glial coverage of ISNd is visible (arrowhead) as well as coverage of the forked projections of the SNA terminus (asterisk). Peripheral glia are also observed reaching to the synapses of the ISN distal tip. Bars, B-E, 20 μm. Bars, F and G, 100 μm.
Larvae were counterstained with an anti-hrp neuronal marker (Bodmer and Jan, 1987). The hrp epitope is expressed strongly in all larval neuronal cells, and made it possible to monitor the extent of glial ensheathment in relation to motor terminals (Bodmer and Jan, 1987). Embryonic and larval neuronal circuitry is essentially analogous, and the only significant difference between larval and embryonic neuronal patterning is size. The larvae grow many times their initial body size as they mature over their three instar molts.

In first instar larvae, it was found that the peripheral glia further elongate and elaborate their processes such that distal motorneuron branches which were unensheathed in the embryo begin to acquire peripheral glial wrapping (Figure 2D,E). The basic pattern of glial sensory tract wrapping in the embryo is maintained, yet new cytoplasmic projections of glia that reach to neuromuscular junctions continue to appear. Typically, wrapping extends past the af/ISN divergence site, as well as on the ISNb, ISNd, SNa and SNC motor branches (arrows and asterisk, Figure 2D,E). For the shorter motor branches ISNb and ISNd, peripheral glia already appear to extend to the synapse at this stage. The remaining motor branches are typically not entirely covered in the first instar although coverage is more extensive than in the embryo.

The peripheral glia of the second instar larva also show more extensive coverage of the distal motor branches compared to the first instar. Glia wrap the entire lengths of SNa, SNC, ISNb, and ISNd at this stage (Figure 2F). The ISN distal tip also has greater glial coverage than in first instars, but still is not entirely ensheathed at this time.

The peripheral glial staining pattern of the third instar larva is very similar to the second instar but on a larger scale in accordance with overall growth of the larva. By third instar, all motor axon branches are entirely wrapped by peripheral glia, including the distal tip of the ISN (Figure 2G). Together, the data show that peripheral glia continue to extend sheaths along the peripheral nerves during larval stages. The
mature embryo therefore represents an intermediate stage of peripheral glial development.

With brightfield microscopy, glial processes were observed to extend very close to larval neuromuscular junctions (arrows Figure 3A-D in 3rd instar larva). To observe these structures in greater detail, laser scanning confocal microscopy was carried out. With confocal imaging, the tau-β-Galactosidase in the larval peripheral glia could clearly be seen to associate with microtubules as a rope-like network of fibers (Figure 3E). Since X-gal is able to diffuse away from the site of β-galactosidase localization, these structures had not been visualized with previous enzymatic labeling of the peripheral glia.

To observe the full extent of glial cell process extension at nerve terminals, gapGFP, which associates with cell membranes, was subsequently used to label the larval peripheral glia and to determine the extent of peripheral glia association with the boutons of the neuromuscular junction (NMJ). Fine structures of glial membranes associated with both type I and type II synapses. Type I synapses have large boutons which contain glutamatergic vesicles and are further subdivided into type Is (small) and Ib (big) according to size. Type II synapses are observed on long, thin axons and contain a mixed collection of vesicles including dense core vesicles (Gorczyca et al., 1993). The glial processes often extend up to and cover the first bouton of many NMJs (Figure 3F,G) similar to glial caps observed at the blowfly larval NMJ (Osborne, 1967). Labeling of peripheral glia is not always penetrant enough to determine what percentage of synapses have glial caps, however the glia almost always cover the first boutons of synapses at ventral muscles 6,7,12, and 13. It is not known whether the presence of peripheral glia at the larval NMJ is of functional significance.

The elaboration of peripheral nerve glial processes that occurs during larval development appears to occur without glial proliferation. The number peripheral glial nuclei does not increase as seen with the rL82 gliotactin enhancer trap which
Figure 3. Peripheral glia extend as far as the larval neuromuscular junction in the third instar.

(A-D) Serial focal planes of an rL82#29:UAS-taulacZ 3rd Instar larva doubled stained for horseradish peroxidase to label neurons (brown) and anti-βGal to label glia (black). With bright field microscopy, peripheral glial processes are observed to extend very near to the NMJ (arrows). (E) A third instar larva of the same genotype as in (A-D) but labelled for confocal microscopy. Peripheral glia are indicated in green, neurons in red and overlapping regions are in yellow. The rope-like network of microtubule strands and associated Tau-βGal is evident in the peripheral glia (arrow heads) and the microtubules extend to near the NMJ (arrow). (F,G) rL82#29:gapGFP 3rd Instar larva. Through gapGFP labelling of the peripheral glia, the cellular processes of the peripheral glia are observed to extend to the first bouton of Type I and Type II NMJs (arrows). Neurons were labeled with anti-hrp. Bars, 20 μm.
Figure 4. Ensheathment of larval peripheral nerves proceeds without glial proliferation.

(A) Stage 16 embryo (rL82) stained with X-gal to reveal peripheral glial nuclei and to enable cell counting. Motor neurons are counter stained with Fas II. 6-8 peripheral glia (dark ovals) typically associate with the embryonic peripheral nerves in each hemisegment. (B) 3rd Instar larvae of the same genotype as in (A) double stained for horseradish peroxidase and X-gal, to reveal motor neurons and peripheral glial nuclei respectively. (C-E) High magnification of the peripheral glial nuclei located at the positions of the arrows labelled C,D,E in (B). Typically, 6 glial nuclei are evident along the larval peripheral nerves. (F,G) Oregon R wild-type embryos stained for presence of the glial-transcription factor Repo. (F) In late stage embryos, equivalent number of peripheral glial nuclei are evident with Repo staining as compared to X-gal (as in A). (G) 3rd Instars stained for Repo have many more Repo-positive nuclei associated with the peripheral nerves (arrows) than larvae stained for X-gal (as in B). In larvae, Repo is not glial specific as muscle nuclei are also evident (arrowheads). The many additional nuclei associated with larval peripheral nerves probably represent perineurial cells. Bars, A,C,D,E,F, 20 μm. Bars, B and G, 200 μm.
expresses a nuclear localized β-Galactosidase reporter (Figure 4A-E). To confirm this observation, embryos and larvae were also stained for the glial transcription factor, Repo (Figure 4F,G). There is a significant increase in the number of Repo-positive nuclei associated with the peripheral nerve in larvae. The extra nuclei associated with the peripheral nerves most likely represent perineurial cells which are known to proliferate and associate with peripheral nerves at the end of embryogenesis. In support, the morphology of the extra Repo-positive nuclei are different from nuclei identified in the rL82 enhancer trap. Peripheral glia nuclei have an elliptical shape, while those of the presumptive perineurial cells have a flat bar-like shape (Leiserson et al., 2000). Although glial expression of Repo is maintained, it appears that the tissue-specificity of this marker is broadened during larval stages. Repo is also observed to stain muscle fibers which is never observed in embryos (Figure 4G). It can thus be concluded that Repo is not a glial-specific marker in larvae.

**Gliotactin localization in larval peripheral nerves**

Since gliotactin mutants die during late embryonic stages, it is difficult to assess if Gli has an essential role in the glial morphogenic events occurring during larval development. By examining the expression profile of Gli during larval development, insight into the function of Gli during larval glial development could nevertheless be obtained. Larval glia are difficult to stain, and low signal intensity is typically observed with conventional antibody staining methods. Since the Gli mAb that is available has a very low signal to noise ratio, it stains larval peripheral glia poorly. To overcome this problem, a transgenic *Drosophila* line expressing hemagglutinin (HA) epitope-tagged Gli was generated and used to study the localization profile in larval peripheral glia. The HA antibody gives a robust signal at low dilution, and has little background staining. In embryos, the HA-tagged Gli in peripheral glia is equivalent to that of endogenous Gli, and it has a diffuse distribution (Figure 5 A,B).
Figure 5. Distribution of Gliotactin in the peripheral nervous system glia in embryos and larvae.

(A) 1F6 (Gli) monoclonal antibody staining of a stage 16 embryo, with silver/gold signal intensification. Gli is homogeneously distributed in the peripheral glia. (B) RepoGal4:UAS-Gli\textsubscript{wt}HA\#1 embryo (stage 16) stained for Gli using a monoclonal antibody specific to hemagglutinin. As in (A), HA-tagged Gli has a homogenous distribution in the peripheral glia. (C) 1\textsuperscript{st} instar RepoGal4:UAS-Gli\textsubscript{wt}HA\#1 larva stained for HA. In contrast to the distribution of Gli in the embryo, Gli in the peripheral glia of larvae becomes concentrated into 'ribbons' that span the length of the nerves (arrows). These 'ribbons' represent the mature distribution of Gli in the peripheral glia. Bars, 20 μm.
During the 1\textsuperscript{st} instar stage of larval development, HA-tagged Gli becomes concentrated into thin bands that run the length of peripheral nerves and it no longer has a diffuse distribution as in the embryo (Figure 5C). This is probably the mature distribution of Gli. The rapid concentration of membrane proteins into discrete domains is reminiscent of the formation of various junctional complexes. Both adherens and septate junctions in the \textit{Drosophila} epidermis form in short developmental time frames and many of the proteins that are associated with them initially have a diffuse distribution (Müller, 2000). The identification of these ribbon-like concentrations thus support the hypothesis that Gli plays a role in septate junction development. Furthermore, the redistribution of Gli between embryonic and larval stages suggests Gli could have a role in generating the mature nerve wrapping phenotype.

\textit{Whole cell staining of peripheral glia in gli mutant embryos}

As mentioned above, the initial characterization of peripheral glial ensheathment in \textit{gliotactin} mutants was carried out with electronmicroscopy analysis, at select positions along the peripheral nerve (Auld et al., 1995). To more thoroughly investigate the nature of the peripheral nerve glial ensheathment defect in \textit{gliotactin} mutants, whole cell staining was carried out to visualize glial ensheathment along the length of peripheral nerves. The GAL4/UAS system (Brand and Perrimon, 1993) was used to express tau-\textbeta-galactosidase in the glia of wild-type or \textit{gliotactin} mutant embryos. Embryos were double stained for tau-\textbeta-galactosidase and Fasciclin II (Fas II), which labels all peripheral motor neurons. During development, the peripheral glia are born at the lateral edge of the central nervous system where they arise from two glioblasts (1-3 and 2-5). At embryonic stage 12, they migrate into the periphery along Fas II-positive motor neuron fascicles and have largely completed their cellular migrations by stage 16 (Sepp et al., 2000; Schmidt et al., 1997). In homozygous \textit{gli}\textsubscript{AE2445} mutants, these migrations appear to be delayed. Early in development, the cellular processes
Figure 6. Glial ensheathement defects in the peripheral nervous system of gliotactin mutant embryos.

(A,C,E) Wild-type embryos of genotype: RepoGAL4:UAS-taulacZ. (B,D,F) Homozygous mutant gli embryos of genotype: RepoGAL4:UAS-taulacZ;gli \textsuperscript{AE245}. Glia are labeled blue (anti-β-Gal with alkaline phosphatase staining) and motor neurons are labeled brown (anti-FasII with horseradish peroxidase staining). (A) At stage 14 of embryogenesis, the peripheral glia have exited the CNS and migrated into the periphery, where they are associated with the peripheral nerves in a characteristic cone shaped array. (B) In gli mutant embryos, the migration of peripheral glia into the periphery is delayed, and the glial processes are very thin and linear, compared to the cone-shaped appearance of wild-type embryos. Compare glial processes along a peripheral nerve and between the pairs of concave arrows shown in (A) and (B). (C) At stage 15 of embryogenesis, the peripheral glia have extended along peripheral nerves up to the m-cell (labeled). (D) In gli mutants, at stage 15, lamella-like processes are observed to extend from the peripheral glia (compare arrows in C with D) and the peripheral glia do not appear to be as tightly associated with the peripheral nerves as in wild type. The migration of the peripheral glia is still delayed at this stage. Compare extent of nerve ensheathment between concave arrow pairs in (C) and (D). (E) At stage 16 of embryogenesis, the peripheral glia in wild types have ensheathed all but the distal motor termini of peripheral nerves. (F) In gli mutants, at stage 16 of embryogenesis, the overall ensheathment of peripheral nerves is very similar to wild-type, and mutants appear to have corrected early delays in glial migration (compare extent of glial wrapping between concave arrow pairs in (E) and (F)). However, in the mutants, lamella-like processes are still present and protrude predominantly from the ventral peripheral glial cell (vpg) (compare arrows in (E) with those in (F)). Bar, 20 μm.
that associate with peripheral nerves are thinner in the mutants than in wild-type (Figure 6A,B). By embryonic stage 15, the mutant glia have not associated as tightly with the motor neurons and they are observed to have large, lamella-like processes extending laterally from them (non-concave arrows, Figure 6C,D). By the end of embryogenesis (stage 17), the peripheral glia in \( \text{gli}^{AE2M5} \) mutants appear to have extended to ensheathe the peripheral nerves successfully. However, lamella-like cellular processes are still frequently observed to protrude from the peripheral glia associated with the posterior peripheral nerve fascicle (non-concave arrows, Figure 6E,F). Loss of \( \text{gli}^{AE2M5} \) thus only appears to have subtle defects on the overall peripheral glial morphology during development.

The peripheral glia preferentially associate with sensory neuron fibers during embryonic development, and these neurons become defasciculated in the absence of peripheral glia (Sepp et al., 2001). Moreover, these studies illustrate that during development, motor neurons are less dependent on peripheral glia for axon guidance than sensory neurons (Sepp et al., 2001). In the initial characterization of \( \text{gli} \) mutants, motor neuron development had been observed to proceed normally. However, sensory neuron development had not been characterized (Auld et al., 1995). Since the peripheral glia of \( \text{gli} \) mutants display subtle wrapping defects (particularly in the posterior sensory fascicle), it was speculated that subtle peripheral nerve defects could be observed through sensory neuron staining. Wild-type and \( \text{gli} \) mutants embryos were stained with the sensory neuron marker 22C10, and neuronal fasciculation of mature stage 17 embryos was observed. In \( \text{gli} \) mutants, sensory neuron defasciculation is evident along both the anterior and posterior fascicles, although the posterior fascicle is most severely affected (Figure 7 A,B). These results indicate that defects in glial wrapping lead to subtle secondary sensory neuronal defects in \( \text{gli} \) mutants.
Figure 7. *gliotactin* mutants have defasciculated peripheral nerves.

(A) Wild type Oregon R embryo at stage 16, stained for the sensory neuron antigen 22C10. The cell bodies and axons of the four sensory neuron clusters are evident: ventral (v), ventral' (v'), lateral chordotonal (lch), and dorsal (d). (B) In homozygous *gli* \(^{AE2445}\) mutant embryos the position and morphology of sensory neurons is normal. The sensory neurons project correctly to the CNS. The only visible sensory neuron defect is abnormal axon fasciculation (compare concave arrows between (A) and (B)). The lateral edge of the central nervous system (CNS) is marked with a vertical line. Horizontal bar, 20 μm.
Investigation into septate junction formation in peripheral glia

Although there has been interest in septate junction development in peripheral glia, particularly with respect to their role in the formation of the blood-nerve barrier, these junctions have previously only been visualized through electronmicroscopy. Reasons for this may have included that antibody probes to septate junction components have not been available until the early 1990's and the peripheral glia were not characterized until 2000 (Sepp et al., 2000; Woods and Bryant, 1991). Since electronmicroscopy requires extensive technical expertise, the feasibility of studying septate junction development in embryonic peripheral glia was investigated with antibody staining. As a putative septate junction molecule, Gli would be expected to localize to glial septate junctions along with known septate junction markers such as Coracle (Cor).

To test whether septate junction development is visible in peripheral glia using confocal microscopy techniques, the glia of wild type embryos were genetically labeled with tau-β galactosidase, and counter stained for the septate junction marker Cor. Coracle is expressed in peripheral glia (Figure 8B), however, by the end of embryogenesis there is no distinctive subcellular localization of Cor within the peripheral glia. Cor staining remains diffusely distributed along peripheral glial membranes (compare Figure 8A with 8B). The absence of a distinct localization pattern for Cor by the end of embryogenesis makes it difficult to study the role of Gli in septate junction development in peripheral glia via light microscopy. Although Cor may become localized in peripheral glia during larval development, similar to Gli, it would not be possible to investigate the effect of Gli loss on the localization of septate junction proteins in larval glia since gli mutants die during embryogenesis. Thus, it was concluded that the development of these junctions should be studied in another tissue.
Figure 8. Distribution of the septate junction protein Coracle in peripheral glia of embryos.

(A,B) Confocal images (red and green channel) of a stage 16, rL82#29;UAS-taulacZ embryo double stained for β-Gal to label the PNS glia (A), and Cor to visualize the septate junctions (B). Peripheral nerves (arrows) are observed to project from the central nervous system (CNS). Cor has a diffuse distribution in the peripheral glia at stage 16 (arrows in B) of embryogenesis. Bar, 20 μm.
Gliotactin expression in cell types, other than the peripheral glia

To determine if the role of Gli in septate junction development could be studied in another tissue besides the peripheral glia, wild-type stage 16 embryos that had been stained for Gli during the initial characterization of gli gene (Auld et al., 1995) were carefully re-examined for Gli expression in other tissues. Examination of these embryos showed that Gli is expressed in a number of cell types other than the peripheral glia, and this had not previously been reported (Figure 9). These cell types included the sensory chordotonal neurons (Figure 9E), the salivary gland epithelial cells (Figure 9D), and the epithelial cells of the epidermis (Figure 9C). All of these cell contain septate junctions (Tepass and Hartenstein, 1994). Some staining was also observed within the CNS longitudinal axon tracts, which probably corresponds to the axon termini of peripheral sensory neurons (Figure 9B). Septate junctions have been extensively characterized in the epidermis and salivary glands of Drosophila. In these tissues, septate junctions are established early in development (approximately embryonic stage 14), thus these tissues are ideal to determine the role of Gli in septate junction development.
Figure 9. Gliotactin is expressed in a variety of cell types.

(A-E) Wild type stage 16 embryo labeled with the 1F6 Gli monoclonal Ab. Robust Gli staining is black, while less intense Gli staining is brown (horseradish peroxidase staining with silver/gold intensification). (A) Gli expression in the peripheral glia (PG, arrows) and tracheae (TR, arrow heads). (B) Gli expression in the central nervous system, transverse nerve exit glia (EG), and in sensory neurons. The CNS neuronal labeling of Gli appears to be concentrated in sensory neurons at synapses with the CNS longitudinal neurons. (C) Gli expression the epidermis (tangential section). (D) Gli expression in the salivary gland (SG). (E) Gli expression in the sensory lateral chordotonal neurons (Ch), viewed here to emerge from the anterior peripheral nerve fascicle which is ensheathed by glia (arrowhead). Bars, 20 μm.
Discussion

By carrying out preliminary investigations into possible mechanisms by which Gli acts to form the blood-nerve barrier, important observations were made about peripheral nerve glial development and Gli function. First, by characterizing larval peripheral glial development, it was discovered that there is a second phase of peripheral nerve ensheathment. Second, it was determined that in gli mutant embryos, the peripheral glia are largely able to ensheathe peripheral nerves and there are no major gaps between the contacting cellular processes of the peripheral glia are distributed along the peripheral nerve. Gli is therefore likely to primarily have a role in the maturation of the peripheral nerve glial sheath. Third, it was realized that it would be best to study role of Gli in the development of septate junctions in a tissue other than the peripheral glia. Nevertheless, the observation that Gli becomes concentrated into 'ribbons' of intense staining in the peripheral glia during development supports the hypothesis that Gli has a role in junctional development.

Characterization of larval glial development

In the mature stage 17 embryo, glia extend and wrap all sensory axonal tracts entirely but leave many motor axon branches untouched. Perhaps glia do not reach up to the tips of motor axons during this time as they could interfere with muscle target recognition and synapse consolidation. However, during first instar larval development, peripheral glia continue to extend their cytoplasmic processes so that they reach towards the distal termini of motor axons. It will be interesting to determine what changes in peripheral glia or motor neuron gene expression are necessary for glia to become so highly attracted to motorneurons after embryo hatching.
Interestingly, just as in *Drosophila* embryos, much of mammalian peripheral nerve development occurs postnatally. For example in mice, although axons and Schwann cell precursors have extended into the limb at embryonic day 12, myelination and maturation of the protective nerve sheath including the development of tight junctions, is not complete until a few weeks postnatally (Dong et al., 1995).

One surprising observation was that glia appear not to proliferate during the rapid growth phase of the larva, but rather extend their processes along the elongating peripheral nerves to match their growth. Peripheral glial numbers therefore seem to be fixed early in embryonic development. This is in contrast to the perineurial cells which appear to proliferate rapidly during larval development (Leiserson et al., 2000). In vertebrates, Schwann cells are necessary to trigger the development of the overlying perineurium through the Hedgehog signaling pathway (Parmantier et al., 1999). Very little is known about perineurial cells in *Drosophila*, but it would be interesting to determine if the peripheral glia express the segment polarity gene *hedgehog* and if it is required for the development of the perineurial sheaths in flies. Given the similarity in mammalian and *Drosophila* peripheral nerve anatomy, *Drosophila* will likely be a very good model system to not only study neuron-glial interactions, but also glial-perineurial cell interactions.

In the larva, many glia were found to extend processes to the neuromuscular junction and cover the first synaptic bouton. These coverings are possibly similar to the glial cap of the blowfly larval NMJ (Osborne, 1967). To understand the significance of peripheral glial presence at the NMJ, more detailed analyses of glial structure must be performed. Staining of glia does not always penetrate the distal glial processes and detergents required for membrane permeabilization damage such fine membranous structures. In the future, double labeling live glia and neurons with GFP variants of different excitation and emission wavelengths should enable determination of the frequency of appearance of glia at the NMJ. Electron microscopy will also be essential.
to determine the fine structure of the peripheral glial/bouton interface, which should provide a basis for functional study of these structures. In vertebrates, Schwann cells cover the neuromuscular junction and these glia can modulate NMJ function (Robitaille, 1998). The Drosophila model system could be very useful to understand glial-synapse interactions as single bouton electrophysiological recordings can be made from the larval NMJ.

**Characterization of the peripheral nerve ensheathment defects in gliotactin mutants**

In gli homozygous mutants, the peripheral glia are able to exit the CNS early in development, and migrate distally along the anterior and posterior peripheral nerve fascicles. However, the lamella-like processes that are observed to protrude from glia suggest that they are not effectively ensheathing the peripheral nerves. This is supported by the sensory neuron defasciculation that was observed in the gli mutants. These observations are in agreement with the electronmicroscopy studies that were performed on the intersegmental nerve of gli mutants, which also identified glial ensheathment defects (Auld et al., 1995). The present findings, however, suggest that the posterior fascicle is more severely affected in gli mutants than the anterior fascicle (which contains the intersegmental nerve).

It is foreseeable that there are two independent developmental events that occur to bring about axon ensheathment by the peripheral glia. Peripheral glia probably migrate into the periphery by associating with motor neurons and then sensory neurons, using a repertoire of cell adhesion molecules that enables neuron-glial interactions (Sepp et al., 2001). Another set of molecules that are important for glial-glial interactions are likely to be employed tightly wrap nerve fascicles together. Gli may be a member of such a group of molecules. Since septate junctions are adhesive structures and are present between glial-glial wraps, it is possible that other members of this second group of molecules may include septate junction proteins. With this in
mind, it would be interesting to determine if cor and nrx-IV mutants have similar defects in peripheral glial cell morphology.

Since Cor has a diffuse distribution in late stage embryonic peripheral glia, electron microscopy is probably the only method that can be used to determine if Gli has a role in septate junction development in this tissue. However, since Gli is expressed in the epidermis, and septate junctions have been successfully analyzed with confocal microscopy in this tissue, it would be best to determine if Gli has an essential role in septate junction development by looking at this tissue and not the peripheral glia. The rest of the thesis therefore will investigate the role of Gliotactin in the formation of septate junctions in the epidermis and salivary glands.
III. THE ROLE OF GLIOTACTIN IN THE FORMATION OF PLEATED SEPTATE JUNCTIONS IN THE EPIDERMIS OF DROSOPHILA EMBRYOS
Septate junction development has been well characterized in the epidermis of *Drosophila*. Septate junctions in the epidermis are pleated, as the epidermis is ectodermally derived (Tepass and Hartenstein, 1994). The junctions form in the epidermis at stage 13 of embryogenesis, when the epithelial cells of the epidermis are undergoing cell-shape changes to bring about dorsal closure (Tepass and Hartenstein, 1994; Campos-Ortega and Hartenstein, 1985).

Following gastrulation and germband retraction, presumptive ectoderm has been set up in the head, ventral, and lateral regions of the developing embryo (Noselli, 1998; Campos-Ortega and Hartenstein, 1985). However, the dorsal region of the embryo lacks ectoderm and it is covered instead by a tissue termed the amnioserosa, which is transient and degrades late in embryonic development. At stage 14, the hexagonal-shaped cells of the lateral ectoderm elongate along the dorsal-ventral axis of the embryo and become columnar in shape. Through these cell-shape changes, the lateral ectoderm is 'stretched' dorsally to overlay the amnioserosa. Following dorsal closure, the epithelial cells secrete cuticle to form the exoskeleton of the 1st instar larva. In pleated septate junction (PSJ) mutants, the lateral ectoderm fails to elongate, but the amnioserosa degrades. Thus when the cuticle is secreted, the embryos develop dorsal 'blow-holes' (reviewed in Noselli, 1998). How PSJs are involved in the process of dorsal closure is not known, however, it has been suggested that they could act as adhesive structures or as signaling centers.

The epidermis is a convenient tissue to study PSJ development since the epithelial cells are relatively large. PSJs can easily be visualized at their stereotypic positions, in the apical half of the basolateral membrane (just below adherens junctions), using simple immunohistochemistry and laser scanning confocal microscopy techniques.
Stained embryos can be imaged as whole mounts, and since the epidermis is a sheet of columnar epithelial cells, mutant phenotypes can be scored accurately because many epithelial cells can be observed simultaneously. The majority of known septate junction proteins (including Dlg, Cor, Nrx-IV and Scrib) have been characterized primarily in the epidermis or the salivary gland, which arises from the epidermis (Campos-Ortega and Hartenstein, 1985).

Classically, pleated and smooth septate junctions have been described to encircle cells as a continuous multi-stranded 'belt', however, this view has been challenged. Electronmicroscopy studies of epithelial cells in insects have shown that the continuity of septate junctions is interrupted at sites of tricellular contact by 'channels' that span the length of the epithelium (Graf et al., 1982; Noirot-Timothee, 1982; Fristrom, 1982). It has been proposed that diaphragms associated with these channels in *Drosophila* and other insects may serve as anchors for septate junction strands and may function in association with septate junction strands to maintain trans-epithelial permeability barriers (Graf et al., 1982; Noirot-Timothee, 1982; Fristrom, 1982). Interestingly, none of the known septate junction proteins have been found to localize specifically to these specialized structures.

It has frequently been speculated that Gliotactin (Gli) is necessary for PSJ development (Carlson et al., 2000; Trapp and Kidd, 2000; Littleton et al., 1997; Baumgartner et al., 1996). To address this hypothesis, we analyzed wild type Gli expression and gli mutants in the *Drosophila* epidermis and other PSJ containing tissues. In gli mutants, localization of SJ markers Neurexin-IV, Discs-large, and Coracle are disrupted in both the epidermis and salivary glands. Furthermore, PSJ permeability barrier function is lost as determined by dye fill assays. Together, the data show that Gli is necessary for SJ formation. Surprisingly, wild type Gli expression in epithelial tissue only overlaps with other PSJ markers at tricellular junctions suggesting that Gli has a unique function in PSJ development. The localization of Gli at tricellular junctions is
dependent on wild-type PSJ development, since Gli is mislocalized in the PSJ mutant Nrx-IV. In this chapter, a model whereby Gli functions at epithelial tricellular junctions to bind, anchor, and compact SJs apically during a terminal maturation phase of PSJ development is proposed.
Materials and Methods

Fly Strains

The wild-type stock, Oregon R, was used as a control in all experiments except for the transepithelial barrier assay (see below). $gli_{AE2A45}$ and $gli_{AE2A4b}$ are P-element excision alleles, and protein nulls (Auld et al., 1995). $gli_{DV3}$ and $gli_{CQ1}$ are EMS alleles and both contain stop codons in the extra-cellular domain of Gli (unpublished data, D. Venema, C. Queano, V. Auld). The SJ mutant $nrx^{46}$ is a strong EMS allele and putative protein null (Baumgartner et al., 1996). The GAL4 drivers $da.G32-GAL4$ (Wodarz et al., 1995), $hsp-GAL4 303-7$ (Leiserson et al., 2000) and $repo-GAL4$ (Sepp et al., 2001) have previously been described. The Gliotactin-GAL4 driver $J29GAL4#2$ and the $UAS-gli^{wt}$ reporter line were generated as outlined below. $UAS-gapGFP$, (Bloomington Stock Center, Indiana University, IN) was used to label cell membranes. $UAS-DNL$ was obtained from G. Boulianne (Hospital for Sick Children, Toronto, ON). Marked balancer chromosomes used for mutant analysis were $CyO, P\{ry^{+}, enwg lacZ\}$ and $CyO, P\{w^{+}, actinGFP\}$ and $TM6B, P\{w^{+}, iab-2 (1.7) lacZ\}, Tb$.

Generation of Transgenic Lines

For the $J29GAL4#2$ driver, 3.7 Kb of the $gli$ 5' regulatory sequence (Bam H1-Xba1 fragment of J29LamdaG5 genomic clone (Auld et al., 1995)) was subcloned upstream of the $GAL4$ cDNA in a pCasper2A $^{+}$ P-element transformation vector (Brand and Perrimon, 1993). For the $UAS-gli^{wt}$ line, the 3.9 Kb $gli$ cDNA (AE2 7.41, Auld et al., 1995) was subcloned into the Eco R1 site of the pP[UAST] transformation vector (Brand and Perrimon, 1993). Engineered constructs (200 ng/μL) were injected into $w^{118}$ embryos together with the pP25.7wcA2-3 (400 ng/μL) using standard techniques (Rubin and Spradling, 1982), and insertion lines were isolated and balanced.
Immunohistochemistry

Antibody staining of embryos was carried out as described by Halter et al. (1995). Homozygous gli and nrx-IV mutants were identified with marked balancers (blue or GFP) by staining for a lack of β-Gal or GFP expression. Embryos were staged according to Hartenstein (1993). Stained embryos were mounted in Vectashield (Vector Laboratories, Burlingame, CA), and imaged with a Bio-Rad Radiance Plus confocal microscope (40X oil and 63X oil objective lenses). Single, 2 μm optical slices were recorded in all experiments. Confocal files were processed with Image-J 1.24 and Adobe Photoshop 5.5. Primary antibodies and the dilutions used for embryo staining were: guinea pig anti-Discs-large at 1/300 (Woods and Bryant, 1991), mouse anti-Gliotactin (1F61D4) at 1:1 (Auld et al., 1995), mouse anti-Drosophila α-Spectrin (3A9) at 1/5 (Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-Armadillo (N2 7A1) at 1/5 (Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-β-Gal at 1/500 (Sigma BioSciences, St. Louis, MO), mouse anti-Coracle (9C and C615-16B cocktail) at 1/100 (Feohon et al., 1994), rabbit anti-Neurexin-IV at 1/200 (Baumgartner et al., 1996), rabbit anti-β-Gal at 1/400 (Cappel, ICN Pharmaceuticals Inc., Aurora, OH), rabbit anti-GFP at 1/200 (Abcam Ltd., Cambridge, UK), and rat anti-DE-Cadherin at 1/50 (Oda et al., 1994). All the following secondary antibodies (Molecular Probes, Eugene, OR) were used at 1/300 dilution: goat anti-guinea pig A488, goat anti-mouse A488 and A568, goat anti-rabbit A488 and A568, and goat anti-rat A488. All the secondary antibodies were highly cross-adsorbed, except for the rat secondary. The 1F61D4 monoclonal antibody was preadsorbed prior to use, by adding 100 μl of 4 hr old embryos (fixed, and blocked) to 900 μl of 1F61D4 containing 10% normal goat serum (Sigma, St. Louis, MO) and then incubating at 4°C overnight.
**Transepithelial Barrier Assay**

Dye injections were carried out as previously reported, with some minor changes (Lamb et al., 1998). The salivary glands of wild type and gli mutant embryos were labeled with gapGFP to facilitate detection using the GAL4/UAS expression system (Brand and Perrimon, 1993). For wild type embryos, Da.G32 females were mated to w;UAS-gapGFP/CyO males. Labeled gli mutant embryos were obtained by crossing w; gli^{AE2A45},UAS-gapGFP/CyO females to w;gli^{AE24A5}/CyOactinGFP; da.G32/TM6,Tb males. The da.G32 GAL4 driver is strongly expressed in salivary gland tissue. Crosses were carried out at 21°C. Eggs were collected at 1 hr intervals, then aged for 24 hrs to obtain mature embryos. Embryos were then dechorionated and sorted using a GFP dissecting scope. Mutant gli embryos of the genotype w; gli^{AE2A45},UAS-gapGFP/gli^{AE2A45}; da.G32/+ were identified by their brightly fluorescing salivary glands, but lacking CyOactinGFP expression. Rhodamine labeled dextran (10,000 MW, Molecular Probes, Eugene, OR) was reconstituted in ddH2O to 3 mM.

**Rescue Experiment**

GAL4 drivers capable of rescuing Gli lethality were identified by crossing gli^{AE2A45},UAS-gli^{wt}/CyOactinGFP females with gli^{AE2A45}/CyOactinGFP; GAL4 driver/TM6,Tb males at 23°C. Progeny were screened with a GFP dissecting scope for non-GFP 1st instar larval escapers. For each rescue experiment approximately 2000 (mixed genotype) embryos were screened. To tabulate the frequency at which w; gli^{AE2A45},UAS-gli^{wt}/gli^{AE2A45}; da.G32/+ embryos were able to hatch, all progeny of the parental rescue cross were dechorionated and screened with a GFP scope to identify homozygous gli mutants. Embryos of the correct genotype were then arrayed on apple juice plates, overlaid with halocarbon oil, and incubated at 23°C until hatching occurred. Percent survival was tabulated as the number of 1st instar larval escapers/total number mutant eggs arrayed.
Results

The expression profile of Gliotactin in the epidermis is novel

To investigate the role of Gli in the formation of PSJs in the epidermis, co-localization experiments with Gli and PSJ proteins were carried out. Gli is strongly expressed in the epidermis (Figure 10A). Embryos were double labelled for Gli and the PSJ marker, Nrx-IV, and temporal and spatial overlap of these two markers was examined with confocal analysis. Gli first appears in the epidermis at stage 11 of embryogenesis, after Nrx-IV, and persists throughout embryonic development. Gli expression at this stage appears to be due to zygotic gene activity as no maternal gli mRNA is detected in Northern blots of 0-6 hr embryos (stage 1-10) (V. Auld, unpublished data). The distribution of Gli and Nrx-IV protein is similar in the epidermis at stage 11, and both molecules are distributed evenly over the surface of epithelial cells (Figure 10B). At stage 13, the localization patterns of Nrx-IV and Gli becomes different. In en face views of the epidermis, Gli becomes concentrated at the tricellular corners of abutting epithelial cells, while Nrx-IV remains distributed around the cell circumferences (Figure 10C, arrowheads). Interestingly, in epithelial cells immediately flanking the amnioserosa during dorsal closure, the distribution of Gli is different than elsewhere in the epidermis. Gli is not localized at tricellular junctions but instead it is concentrated in patches around the circumference of epithelial cells (Figure 10C, solid arrow), or it diffusely labels the leading edge (Figure 10C concave arrow). This suggests that these cells are less mature than those in more ventral positions. Following Stage 13, Gli remains concentrated at the tricellular corners of epithelial cells until the end of embryogenesis (Figure 10E).
Figure 10. Gliotactin is expressed in the epidermis and it is localized to the tricellular corners of abutting epithelial cells.

(B-H) Whole mount embryos of different developmental stages are shown double stained for Gli (green), and the PSJ protein Nrx-IV (red). The overlap of Gli and Nrx-IV is yellow. Images (A-G) are of wild type embryos, while (H) is of a gli$^{AE2A45}$ null mutant. (A) A stage 16 filleted embryo, stained for Gli only. Gli is expressed in the peripheral nervous system glia (arrows) as well as the underlying epidermis. (B) High magnification view of the epidermis (cross section) of a Stage 11 embryo taken at the position of the arrow in the low-magnification inset. At this stage the distribution of Gli is quite uniform around the surface of the epithelial cells and similar to that of Nrx-IV. (C) En face view of a stage-13 embryo undergoing dorsal closure. The localization profile for Gli is variable. At the leading edge (concave arrow), Gli diffusely labels epithelial cell membranes as they make contact with the underlying amnioserosa (asterisk). Epithelial cells in more ventral positions have Gli concentrated in patches around their circumferences (solid arrow), or localized to tricellular corners (arrowheads). Gli distribution is distinct, although overlapping, with that of Nrx-IV. The amnioserosa lacks both Gli and Nrx-IV expression (asterisk). (D) Cross-section of a stage-13 embryo. Gli expression is restricted to the basolateral membrane of epithelial cells (region between arrowheads), and is concentrated in patches. Gli staining in the tracheae underlying the epidermis is also evident (asterisk). (E) En face view of epidermis of a stage-15 embryo. Gli remains concentrated at tricellular junctions (arrow). (F) Cross-section of the epidermis of a stage 15 embryo. Gli localization is restricted to the apical portion of the basolateral membrane domain and lies within the Nrx-IV positive pleated septate junction domain (arrowheads). Gli expression does not extend to the bottom of epithelial cells (arrow). Gli is only sporadically seen in the basolateral membrane of epithelium, because the plane of section only occasionally transects a tri-cellular corner. Gli expression in tracheae persists (asterisk). (G) Stage 15 embryo. Sensory neurons (lateral chordotons) also express both Nrx-IV and Gli; however, like in the epidermis, their distributions are only partially overlapping (arrows). (H) En face view of a stage-15 gli$^{AE2A45}$ null mutant, double stained for Gli and Nrx-IV. In the absence of Gli protein, the Gli monoclonal antibody does not stain the tri-cellular corners of the epidermis (arrow) and thus it is specific to a Gli epitope. Some diffusion of Nrx-IV away from the epithelial lateral membrane is evident (arrowhead). Bars: 50 μm (inset B), and 10 μm.
During the development of the epidermis, Gli also undergoes a redistribution along the apical-basal axis of epithelial cells. At stage 13 of embryogenesis Gli labels the entire length of the basolateral membrane, similar to Nrx-IV. However, unlike Nrx-IV, Gli is typically concentrated into multiple discrete patches within the basolateral membrane (Figure 10D). By stage 15 of embryogenesis, Gli expression is restricted to the apical half of the basolateral membrane and it co-localizes with Nrx-IV at the presumptive PSJ domain (Figure 10F).

In addition to the epidermis, Gli appears to be expressed in most tissues that express Nrx-IV, and in which PSJs have been observed at the EM level (Baumgartner et al., 1996; Tepass and Hartenstein, 1994). This includes, but is not restricted to the tracheae (Figure 10F, asterisk), salivary glands (see below), PNS glia (Figure 10A) and gut (Auld et al., 1995), plus the support cells of the lateral chordotonal sensory neurons (Figure 10G). As in the epidermis, Gli in the gut, tracheae, and salivary glands is also restricted to the tricellular corners. In the homozygous gli mutants gli, gli, gli, and gli, no Gli staining was observed (Figure 10H) demonstrating that the Gli 1F61D4 monoclonal antibody is specific to a Gli epitope. In the wild type tissues in which Gli and Nrx-IV staining is observed, different distribution patterns are seen, suggesting that if they are both involved in PSJ development, they are likely to have distinct roles.

**Pleated septate junction markers are mislocalized in gliotactin mutants**

To investigate the effect of Gli loss on epithelial cell development, homozygous gli mutants were stained for a variety of epithelial markers. The epidermis of stage 15 embryos was analyzed in cross section, using confocal microscopy. At this stage, the epithelium is mature (AJs and PSJs have formed) and the cells are larger than at later stages, thereby providing optimal imaging conditions. For these experiments, four
different strong gli loss of function alleles: gli, gli, gli, gli, were tested and they all gave identical results.

α-Spectrin was used as a marker for general epithelial morphology. In gli mutants, the epithelial cells within abdominal segments are slightly taller and have a more uniformly columnar appearance than wild type (Figure 11A, 11B). The localization of α-Spectrin to the apical and basolateral membrane domains of epithelial cells, however, is normal in gli mutants.

Next, embryos were double stained for AJ and PSJ markers. In stage 15 gli mutants, the PSJ markers Dlg, Nrx-IV and Cor are all mislocalized (Figure 11C-H). Rather than being confined to the apical half of the basolateral membrane, these PSJ markers extend to the extreme basal side (dotted lines in Figure 11D, F, and H) of epithelial cells as compared to wild-type. In contrast, the localization of the AJ markers, Arm and E-cadherin are correctly localized in gli mutants (Figure 11C-F and Figure 11G, H, respectively). The generally normal columnar morphology of epithelial cells in gli mutants, and the correct apical localization of AJ markers suggests that Gli does not have a significant role in specifying apical-basal epithelial polarity. However, the mislocalization of PSJ markers in gli mutants indicates that Gli has a specific role in the development of PSJs.

PSJs form in the epidermis during stage 13 of development (Tepass and Hartenstein, 1994). Prior to this (stage 12), Dlg, Nrx-IV and Cor (as well as Gli) label the entire length of the basolateral membrane. During PSJ development, these markers redistribute to the apical half of the basolateral membrane. In gli mutants the distribution of Dlg, Nrx-IV and Cor appear wild-type at stage 12. However they fail to localize apically during stage 13. These results suggest that Gli is necessary for the maturation of PSJs.

To date, all PSJ mutants identified (cor, nrx-IV, dlg and scrib) have defects in cuticle formation. Cuticles isolated from dlg and scrib mutants (lacking both maternal
Figure 11. Pleated septate junction markers are mislocalized in gliotactin mutants.

All images are of stage 15 embryos and the epidermis is viewed in cross section. (A, C, E, G) Wild type. (B, D, F, H) Homozygous gli$^{AE2A45}$ mutants. (A, B) $\alpha$-Spectrin staining reveals general epithelial morphology. Wild type epithelial cells have columnar morphology, and $\alpha$-Spectrin labels both the apical (arrow) and basolateral membrane domains (arrowhead) (A). In gli mutants, epithelial cells are slightly taller and have a more uniform columnar shape, while the localization pattern of $\alpha$-Spectrin is wild-type (B). (C, D) Embryo stained for the PSJ marker, Dlg (green) and the AJ marker; Armadillo (red). The dotted line marks the basal surface of epithelial cells in a single abdominal segment. The stereotypic organization of AJs positioned apical to PSJs can be observed in wild type embryos (C). In gli mutants, Dlg localization is disrupted and has diffused basally, while Arm localization is normal (D). (E,F) Embryos doubled stained for the PSJ marker Nrx-IV (green) and Armadillo (red). Nrx-IV is also mislocalized in gli mutants (F). (G,H) Embryos stained for the PSJ marker Cor (green) and the AJ marker E-Cad (red). Just like Dlg, and Nrx-IV, the PSJ marker Cor is mislocalized in gli mutants; however, the localization of E-Cad is unaffected (H). Bar, 10 $\mu$m.
and zygotic transcript) are grossly abnormal and resemble a haphazard meshwork (Perrimon, 1988; Bilder and Perrimon; 2000); while those of nrx-IV and cor mutants have large holes or scabs on the dorsal surface (Baumgartner et al., 1996; Lamb, et al., 1998). The cuticles of gli mutants, on the other hand appear wild-type (data not shown), and this reflects the generally normal morphology of the underlying epidermis (Figure 11B). However, in staining gli mutants for this study, small dorsal ‘blow-holes’ were observed at a low frequency in the epidermis of mature stage 15-16 embryos. These results suggest that dorsal closure is delayed in some homozygous gli mutant embryos; however, it is completed successfully prior to cuticle deposition.

*Localization of gliotactin at epidermal tri-cellular corners is dependent on pleated septate junction formation*

To determine if PSJ formation is necessary for Gli to become localized to the tricellular corners of epithelial cells, Gli distribution in nrx46 homozygous mutants was analyzed. The nrx46 allele is a severe Nrx-IV loss-of-function mutation and PSJs fail to form their characteristic septal-cross bridges in these mutants (Baumgartner et al., 1996). We found that Gli does not correctly localize to the tricellular corners of epithelial cells in mature nrx-IV mutant embryos (Figure 12). Most nrx-IV mutants have severe defects in dorsal closure, and so mature (stage 15 or older) embryos were identified by the morphology of the anterior of the embryo (as compared to wild type). In en-face views of the epidermis of stage 15 nrx-IV mutants displaying a severe dorsal closure phenotype, Gli is inappropriately distributed around the circumference of cells (Figure 12C). Similarly, in cross-section views of these mutants, Gli is not restricted to the apical half of basolateral membrane, but instead extends basally (Figure 12D). In stage 15 nrx-IV mutants with mild dorsal closure phenotypes, Gli is still mislocalized albeit less severely (Figure 12E, F). There is a direct correlation between the severity of the
Figure 12. Gli localization at epithelial tricellular corners is dependent on the formation of pleated septate junctions.

All embryos are stained for Gli and are at stage 15 of development. (A, B) Wild type embryos. (C–F) nrx\textsuperscript{46} homozygous mutant embryos. (A) Gli localizes to the tricellular corners (arrow) of epithelial cells (en face view) (B) Gli, at tricellular corners, is restricted to the apical-half of the basolateral membrane domain (cross section view). The apical (a) and basal (b) surfaces of an epithelial cell are marked. (C,D) Severe nrx\textsuperscript{46} homozygous mutant embryo. (C) Gli fails to localizes to the tricellular corners of epithelial cells (arrow) and is distributed around the circumference (en face view). In this late stage embryo, dorsal closure has failed (asterisk). (D) Cross-section view of the epidermis in (C). Rather than being restricted to the apical-half of the basolateral membrane, Gli extends to the basal surface. (E, F) nrx\textsuperscript{46} homozygous mutant embryo with a weak dorsal closure phenotype. (E) En face view of the epidermis. In these mutants, the epidermis appears more wild-type, but Gli does not localize to tricellular corners (arrow). (F) Cross section view of the epidermis in (E). As in (D) Gli is not concentrated to the PSJ domain at tricellular corners but diffuses basally. Bar, 10 μm.
dorsal closure phenotype in the \textit{nrx-IV} mutants and the severity of Gli mislocalization in the epithelium. The localization profile of Gli in stage 15 \textit{nrx-IV} mutants, resembles that of Gli in wild type embryos at stage 13 of development (compare Figure 10C with 12C, 12E, and Figure 10D, with 12D, 12F). These results show that there is a reciprocal dependence between Gli and Nrx-IV for localization, and suggests that PSJs must develop to enable Gli to localize to tricellular junctions.

\textit{Pleated septate junction transepithelial barriers are not functional in gliotactin mutants}

The mislocalization of Dlg, Nrx-IV and Cor in epidermis of \textit{gli} mutants suggests that the PSJs in these embryos can not form effective transepithelial barriers. To test this hypothesis a salivary gland based transepithelial barrier assay was used (Lamb et al., 1998). For the assay, the salivary glands of wild type controls and \textit{gli} mutants were labeled with Green Fluorescence Protein (GFP) using the GAL4/UAS system to facilitate their detection (Brand and Perrimon, 1993). A 10 kDa Rhodamine-dextran conjugate was injected into the posterior of live embryos (to fill the hemocoel) and the movement of the tracer dye across the salivary gland epithelium was monitored using confocal microscopy. If PSJ impermeability is intact, the tracer should not leak into the salivary gland lumen.

As in the epidermis, Gli in the salivary gland is localized to the tricellular corners of epithelial cells (Figure 13A,B). Gli staining in the salivary gland is almost identical to that of the PSJ marker Cor when the gland is viewed in cross-section (Figure 13 C). Both Gli and Cor localize to the apical portion of the salivary gland’s basolateral membranes. In the salivary gland, the apical membrane domain faces the gland lumen as this tissue arises through the invagination of ectoderm (Campos-Ortega and Hartenstein, 1985). The PSJ markers Dlg, Cor, and Nrx-IV are all mislocalized in the salivary glands of \textit{gli} mutants; however, they are less severely affected than in the epidermis (data not shown).
To test the integrity of the PSJ transepithelial barrier in salivary glands, the tracer dye was injected into the hemocoel of wild type (n=23), and homozygous gli mutant (n=37) embryos. In these studies mature stage 16/17 embryos which have fully formed PSJs were selected for dye injections. In wild-type animals, the Rhodamine-dextran dye was effectively blocked from passing into lumen of the salivary gland for up to 40 minutes following injection (Figure 13D-F), after which the animals would hatch and crawl away. In these wild types, the dye was observed to pass between salivary gland epithelial cells, in a basal to apical direction, up to the presumptive PSJ boundary where it stopped without penetrating into the lumen (Figure 13E, arrowhead). In contrast, the tracer dye was found to pass into the salivary gland lumen of gli mutants within 5 minutes of injection (Figure 13G-I). In the gli mutant animals, the dye penetrates the PSJ domain around the entire circumference of salivary gland epithelial cells and it does not solely pass down tricellular channels to fill the gland lumen. These results show that Gli is required for the formation of functional PSJ transepithelial barriers.

Rescue of the embryonic lethality of gliotactin mutants

Gli is a member of a large family of cholinesterase-like molecules called the electrotactins, which are structurally very similar to one another (Botti et al., 1998). Three-dimensional modeling studies strongly suggest that these proteins contain an annulus of negative electrostatic potential at the surface of a cleft homologous to that of cholinesterases (Botti et al., 1998). It has been suggested that the annular motifs endow the electrotactins with adhesive properties (Botti et al., 1998).

Drosophila Neuroligin (DNL), has the highest sequence similarity to Gli, of the electrotactins in Drosophila (Gilbert et al., 2001), and so it was tested if DNL might be functionally similar enough to Gli be interchangeable. To carry out this experiment, the embryonic lethal phenotype of homozygous gli mutants was used as a basis for a
Figure 13. The integrity of the salivary gland transepithelial barrier is disrupted in gliotactin mutants.

(A-C) Wild type, stage 16, Da.G32:UASgapGFP embryos, fixed and stained for gapGFP (red) which marks salivary gland epithelial membranes, and Gli (green, A, B) or Cor (green, C). Co-localization of gapGFP with Gli or Cor is yellow. (A) Gli is localized to the tricellular corners of salivary gland epithelial cells (en-face view) as in the epidermis (arrow). (B) Longitudinal section of the salivary gland shown in (A) reveals the gland lumen (asterisk) and the localization of Gli to the PSJ domain at the apical portion of epithelial basolateral membranes (arrow). (C) The PSJ marker Cor, labels the apical portion of the basolateral membrane similar to Gli (compare arrows in A and C). (D-I) Live, wild type (D-F) and gli homozygous mutant (G-H) embryos whose hemocoels have been injected with a 10 kDa M.W. Rhodamine dextran to evaluate the integrity of the salivary gland transepithelial barrier, and whose salivary glands express gapGFP. Embryos were imaged within ten minutes of tracer injection. (D-F) Da.G32:UASgapGFP wild type embryo (merge, red and green channels respectively) (D) Rhodamine dextran (red) is excluded from the lumen (asterisk) of the salivary gland (green). (E) Red channel shows that the Rhodamine dextran is able to penetrate between epithelial cells in an apical (a) to basal (b) direction, but only as far as the presumptive PSJ domain (x). Compare the staining profile for Cor in C, with the dye block site (x) in E. (F) Green channel showing the dye block site in E, superimposed on the salivary gland epithelial cell profiles. (G-I) gli^{AE2A45}:Da.G32:UASgapGFP mutant embryos (merge, red and green channels respectively) (G) In homozygous gli mutant embryos, the transepithelial barrier function of the salivary gland (green) is impaired and Rhodamine dextran (red) fills the lumen of the salivary gland (asterisk). Bar, 20 µm.
rescue assay to determine if Gli and DNL are functionally interchangeable. The GAL4/UAS system (Brand and Perrimon, 1993) was used to express either wild-type Gli (UAS-Gli<sup>wt</sup>) or DNL (UAS-DNL) in homozygous <i>gli<sup>AE z45</sup></i> mutant embryos and then the ability of these rescued embryos to hatch into 1<sup>st</sup> instar larvae was scored.

In developing the rescue assay, a variety of GAL4 drivers were screened in combination with UAS-Gli<sup>wt</sup>. A rescue was obtained with the ubiquitously expressed daughterless-GAL4 (da.G32) driver (the data is summarized in Table 2). This result indicates that there are no second site lethal mutations in the <i>gli<sup>AE z45</sup></i> mutant strain. In da.G32:UAS-Gli<sup>wt</sup> rescued embryos, Gli localizes correctly to the tricellular corners of epithelial cells in the epidermis (Figure 14C). 78% (n=39) of rescued embryos hatch and these survive to adulthood (Table 2). Rescued adults are fertile and can be maintained as a stable stock however 58% (n = 51) have severe leg defects. The metatarsus and tibia are typically bent at 45° towards the midline, and necrotic tissue is often present on the medial aspect of the limbs and limb joints. These phenotypes are also seen in adult escapers of a gli hypomorphic strain (unpublished data, D. Venema and V. Auld), and all SJ mutants for which hypomorphic alleles have been isolated (Baumgartner, 1996; amb et al., 1998). These results further suggest that Gli has an essential role in SJ development.

It was not possible to rescue the lethality of homozygous gli mutants with various other GAL4 drivers including: repoGAL4 (glial specific), hsp-G303-7 (semi-ubiquitous) or J29GAL4-2 (a gli-GAL4 driver generated using 3.7 kb of gli's 5' regulatory sequence) (Table 2). The inability of these drivers to rescue gli lethality is probably because their GAL4 expression patterns do not adequately match that of endogenous Gli. Since both the RepoGAL4 and J29GAL4-2 lines are strongly expressed in glia, but can not rescue, it is unlikely that gli mutants are lethal solely because of a disrupted glial-based Blood-Nerve-Barrier (Auld et al., 1995). Rather, these results
Figure 14. Wild type Gliotactin, when expressed in gli homozygous mutant embryos, localizes correctly to the tricellular corners of epithelial cells.

(A-C) Confocal images of the epidermis of stage 16 embryos. (A) *En face* view of a wild type embryo, double stained for Gli (*green*) and Nrx-IV (*red*). Co-localization of the two molecules is yellow. Gli localizes to the tricellular corners of epithelial cells (*arrow*) and is excluded from bicellular contacts (*arrowhead*). (B) Gli$^{AE2A45}$ homozygous mutant embryo double stained as in (A), lacks Gli (*green*) staining. (C) Gli expressed ubiquitously in homozygous Gli$^{AE2A45}$ mutant embryos, using *da.G32* and the GAL4/UAS System, is able to localize correctly to the tricellular corners of epithelial cells (arrows). In many cells Gli labels the entire cell surface, due to differential expression levels, and the heterogeneous nature of the GAL4/UAS system (*arrowheads*). (D) Schematic diagram of Gli and DNL showing various protein features. Both Gli and DNL contain extracellular cholinesterase-like domains with putative Ca$^{2+}$ binding EF hands, and contain PDZ recognition peptides at the termini of their cytoplasmic tails. Consensus phosphotyrosine residues and a TRAF binding site are present in Gli but not in DNL. Bar, 20 μm.
### Table 2. Rescue of gli^{AE2A45} Embryonic Lethality with the Gal4/UAS Expression System using various tissue-specific Gal4 drivers.

<table>
<thead>
<tr>
<th>GAL4 driver</th>
<th>Embryonic Expression pattern</th>
<th>UAS construct (cDNA)</th>
<th>Genotypes of Rescued Embryos</th>
<th>Rescue?</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>no driver</td>
<td>-</td>
<td>giotactin</td>
<td>w; gli^{AE2A45},UAS-gli^{wt82} / gli^{AE2A45}</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>repoGAL4</td>
<td>all glia except midline</td>
<td>giotactin</td>
<td>w; gli^{AE2A45},UAS-gli^{wt82} / gli^{AE2A45}; repoGAL4/+</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>J29GAL4-2</td>
<td>giotactin-like</td>
<td>giotactin</td>
<td>w; gli^{AE2A45},UAS-gli^{wt82} / gli^{AE2A45}; J29GAL4-2/+</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>hsp-G303-7</td>
<td>ubiquitous (patchy)</td>
<td>giotactin</td>
<td>w; gli^{AE2A45},UAS-gli^{wt82} / gli^{AE2A45}; hspG303-7/+</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>daughterless-GAL4</td>
<td>ubiquitous</td>
<td>giotactin</td>
<td>w; gli^{AE2A45},UAS-gli^{wt82} / gli^{AE2A45}; daG32/+</td>
<td>Yes</td>
<td>-escapers survive to adulthood and are fertile -58% of adult escapers have severe leg phenotypes</td>
</tr>
<tr>
<td>daughterless-GAL4</td>
<td>ubiquitous</td>
<td>D-neuroligin</td>
<td>w; gli^{AE2A45}/ gli^{AE2A45}; daG32/UAS-NL</td>
<td>No</td>
<td>-</td>
</tr>
</tbody>
</table>
suggest that Gli has a vital role in a large number of tissues, and is consistent with the notion that Gli plays a general role in PSJ development.

Since it was possible to rescue the lethality of gli mutants with the da.G32 driver, it was next tested if DNL with its many structural and sequence similarities (Figure 14D) could rescue gli lethality. Gli and DNL share 40% amino acid identity across their extracellular serinesterase-like domains. They both contain putative extracellular calcium binding EF hands, and cytoplasmic recognition motifs for PDZ containing proteins. However, Gli contains putative signaling motifs in its intracellular tail that are not found in DNL (Figure 14D).

Despite sequence similarity between the Gli and DNL it was found that DNL could not functionally replace Gli. Due to the lack of a DNL antibody it was not possible to test if DNL localizes to the tri-cellular corners of epithelial cells similar to Gli. These results suggest that Gli and DNL are not functionally redundant electrotactin molecules. The putative signaling modules found within Gli’s intracellular tail (such as the predicted TRAF recognition sequence and the phosphotyrosine residues) are potentially responsible for the functional differences between Gli and DNL, and may be important for Gli function in PSJ development.
Discussion

Three lines of evidence clearly show that Gli is necessary for PSJ formation in *Drosophila*. First, the PSJ markers Dlg, Cor, and Nrx-IV are mislocalized in the epidermis and salivary glands of *gli* mutants, however the localization of other cellular markers (Spectrin, E-cadherin, and Armadillo) are normal. This indicates that Gli loss directly impacts PSJ formation and that the mislocalization of these PSJ markers is not secondary to gross morphological defects. Secondly, *gli* mutants do not form effective transepithelial barriers as determined through a salivary gland dye exclusion assay. This result is significant because the salivary gland transepithelial barrier is formed by PSJs in *Drosophila*. Third, Gli is mislocalized in the epidermis of *nrx-IV* mutants. This indicates that Gli is dependent on PSJ formation for its own localization. Together the results provide convincing evidence that Gli is required for PSJ development. Our finding that Gli is localized to the tricellular corners of epithelial cells in the epidermis is unprecedented for a PSJ molecule and suggests that Gli has a novel role in PSJ formation.

*Not all pleated septate junction molecules are created equal.*

If the phenotypes of all PSJ mutants are compared they can be organized into three phenotypic classes. Those that have defects in: 1) epithelial polarity, dorsal closure, and PSJ structure (e.g. *scrib, dlg*), 2) dorsal closure and PSJ structure (e.g. *cor, nrx-IV*) and 3) PSJ structure only (e.g. *gli*). Why is it that some PSJ mutants have more extensive phenotypes than others do? This can be explained, if different PSJ molecules carry out different roles at different times during epithelial development.

Early in development (stage 11-12), Scrib and Dlg may have an important role to specify a target for the delivery of membrane vesicles from the Golgi to the basolateral

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membrane (Bilder and Perrimon, 2000). Indeed, Lecuit and Wieschaus (2000) have shown that targeted delivery of membrane to the apical-lateral membrane during cellularization is likely to be an important means of establishing polarity in the cell. Later in development (stage 12-13), epithelial polarity may be quite stable, due to the presence of AJs, and nascent PSJs that could act as diffusion barriers to prevent the mixing of apical and basolateral membrane components. PSJs may have a more important role in facilitating cell adhesion (Lane and Swales, 1982) during this stage of development, as there are large-scale epithelial morphogenic events occurring to bring about dorsal-closure. Nrx-IV may have an integral adhesive role during this phase of PSJ development since as a transmembrane protein it has the capacity to adhere to other cell membranes or extracellular matrices, and it is anchored to Cor (a membrane skeletal protein) via its cytoplasmic tail (Ward et al., 1998; Baumgartner et al., 1996). In the last phase of PSJ development, which occurs during late embryonic development (stage 14-16), the transepithelial barrier becomes mature, and this is likely to involve the formation of specialized structures at tricellular corners of epithelial cells. Gli probably has a key role in this last phase of PSJ development.

**EM analysis of epithelial tricellular corners in insects**

The work of several electron microscopists studying smooth and pleated septate junction development in the epidermis of various insects (including *Drosophila*) has shown that there are specialized structures at the tricellular corners of epithelial cells that are linked to SJs (Graf et al., 1982; Fristrom, 1982; Lane and Swales, 1982). Graf et al. (1982) performed a detailed freeze-fracture EM analysis of epithelial tissue in crustaceans and cockroaches and identified channels at the tricellular corners of abutting epithelial cells. These channels span the length of the cells, and run parallel to their basolateral axis. In the region of the SJ domain, the channels are filled with what appears to be a series of diaphragms that are stacked on top of each other. The
diaphragms make contact with the basolateral membranes of all three epithelial cells comprising a channel. On the intracellular side of each epithelial cell, in the vicinity of the tricellular corners, SJ strands run parallel to the axis of the channel. This organization is different from that of SJ strands elsewhere in the cell. SJ strands typically run parallel to the axis of the apical membrane domain. Graf et al. (1982) presented a model to explain their observations in which they suggested that SJ strands anchor on the stacked arrays of diaphragms at tricellular corners. Other researchers have also observed these structures and referred to them as ‘tricellular plugs (TCPs).’ These plugs may serve as occlusive devices during transepithelial barrier formation in addition to acting as SJ anchors (Lane and Swales, 1982; Fristrom, 1982).

**Gliotactin, Septate Junction Development and the Tricellular Plug Model**

Our observations that Gli is localized to the tricellular corners of epithelial cells, and that Gli is necessary for the formation and function of PSJs is consistent with the TCP model. Moreover, the EM images of Graf, et al. (1982) which show that TCP diaphragms are associated with SJ strands in the apical half of tricellular channels, is consistent with our observations that Gli is restricted to the apical half of tricellular corners (when the epidermis is viewed in cross-section). This data suggests that Gli is an integral component of TCPs; however, it will be necessary to carry out immuno-EM experiments in the future with a Gli antibody to definitively show this. Currently, these experiments are not feasible as the binding affinity of the 1F61D4 Gli monoclonal antibody is not sufficient for immuno-EM studies.

TCPs are probably a mature feature of SJs, and do not play a significant role in early SJ development. This notion is supported by EM studies documenting the development of SJs in moth and locust (Lane et al., 1982). Early in SJ development, intermembrane particles (IMPs), the building blocks of SJ strands, are homogeneously distributed throughout the basolateral membrane. They then polymerize at random
sites in the basolateral membrane to form short SJ strands. These in turn lengthen, and 'stack' to form SJ placodes which eventually anchor on TCPs. SJ strands do not appear to associate with TCPs early in SJ development suggesting that TCPs only have a significant role in late SJ development. This is in agreement with the idea that Gli is a TCP component and that it is necessary for the maturation of PSJs.

Combining the TCP model with the EM observations of Lane et al. (1982), and our analysis of Gli in the epidermis, a model to suggest how Gli is involved in the formation of SJ junctions can be generated (Figure 15). As a transmembrane protein Gli could function to anchor PSJ strands to the tricellular channel diaphragms late in embryonic development. Presumably, loss of Gli would not interfere with the polymerization of PSJ strands early in development. Nrx-IV and Cor (and possibly Dlg and Srib) may be integral components of SJ strands and may represent the SJ IMPs reported by Lane et al. (1982). Moreover, as a transmembrane protein, Nrx-IV may be an integral component of the paracellular SJ septa that are observed in EM cross-sections of SJs. The homogeneous distribution of Dlg, Nrx-IV and Cor in the epidermal basolateral membranes of wild type embryos during stage 12 of embryogenesis, followed by their subsequent concentration at the apical half of the basolateral membrane domain is consistent with these proteins being SJ IMPs. Gli may anchor PSJ strands to tricellular channel diaphragms by binding either Nrx-IV through its extracellular domain, or Dlg, and Scrib (or possibly some other unidentified SJ PDZ containing protein) through its intracellular PDZ recognition sequence (Figure 14D).

The results presented above are consistent with this model, as Dlg, and Nrx-IV (plus its cytosolic binding partner Cor) are all observed to diffuse throughout the basolateral membrane in stage 15 gli mutant embryos. The hypothesis that Gli anchors PSJ strands to TCP diaphragms via its PDZ recognition sequence is favored for a number of reasons. First, the vertebrate Gli-like Neuroligins have been shown to bind
Figure 15. Model of Gliotactin in pleated septate junction maturation in the epidermis.

PSJ junction development is depicted at three stages of embryogenesis (Stage 11, 13, and 15). For each stage, a tricellular corner is shown with one cell shifted off to the left (dotted lines in A) in order to reveal the paracellular space. The apical membrane domain of the epithelium is denoted with its characteristic projections of microvilli. (Stage 11) Early in PSJ development, PSJ strand intermembrane particles (IMPs) are randomly distributed in the basolateral membrane and begin to polymerize into short PSJ strands. IMPs may represent protein complexes containing the transmembrane protein Nrx-IV and various cytosolic proteins (Cor, Dlg, and Scrib). Gli has affinity for IMP components and associates with nascent PSJ strands (Stage 13) Gli’s ligand appears at the tricellular corners of epithelial cells. The ligand may be a secreted protein and a component of tricellular channel diaphragms (TCD). As a secreted molecule, Gli’s ligand diffuses down tricellular channels (arrow) and this draws Gli and associated PSJ strands apically (curved arrows) (Stage 15) PSJ strands are compacted apically, and anchored to a stacked array of TCDs through Gli. Together, TCDs, Gli and PSJ strands form an effective transepithelial barrier.
the Dlg-like protein PSD-95 via their intracellular PDZ recognition sequence (Ichtchenko, K., 1995; Irie, M., 1997). Second, Nrx-IV is similar to the vertebrate Capr proteins which bind the immunoglobulin superfamily protein NF-155, and Gli is a serinesterase-like protein. Last, other researchers have not been able to detect a direct interaction between Gli and Nrx-IV in S2 cell aggregation assays (Baumgartner et al., 1996; Auld et al., 1995). Rather than binding Nrx-IV with its extracellular domain, it is speculated that Gli may interact with other components of tricellular channels. In order for Gli to be drawn apically in epithelial cells, and to the tricellular corners during development, it is proposed that the extracellular ligand for Gli is soluble. Early in development, when septate junctions have not formed, such a soluble ligand would be able to diffuse freely throughout the paracellular space to interact with Gli. Later in development, as nascent PSJ strands form, the soluble ligand would only be able to diffuse down tricellular junctions and this would draw Gli to the tricellular corners, and apically in epithelial cells. The extracellular ligand for Gli could also simply be a component of the tricellular channel diaphragms.

Many interesting questions remain to be answered regarding the specific role of Gli in TCP development. Is Gli only necessary to anchor SJ strands to TCP diaphragms, or is Gli also directly involved in the formation of the TCP diaphragms? These experiments may prove difficult to perform as few images of TCPs have been obtained through conventional TEM analysis and the structures appear to be best studied with freeze-fracture EM analysis.

Gliotactin, Tricellular channels and Tight Junctions in Vertebrates

Tricellular channels are not features unique to the insect epithelium. Walker et al., (1985) carried out a freeze-fracture EM analysis of vertebrate epithelial tissue, and found the organization of TJs at tricellular corners to be strikingly similar to that of SJs in insects. Interestingly, recent studies on human umbilical vein cultures have suggested
that the localized disruption of TJs at endothelial tricellular corners is important during acute immune responses as it enables neutrophils to migrate across capillaries and reach sites of inflammation or infection (Burns et al., 2000).

Given the sequence and structural similarities between Gli and the vertebrate Neuroligins it will be interesting to determine if any of the Neuroligins are localized to the tricellular corners of epithelial cells, and if they have a role in TJ maturation similar to Gli’s role in PSJ development. Of particular interest are human Neuroligins 3 and 4, and rat Neuroligins 2 and 3, which are not nervous system specific and which have a broader tissue distribution than other vertebrate neuroligins (Bolliger et al., 2001; Glibert et al., 2001; Philibert et al., 2000).
IV. GLI AT TRICELLULAR JUNCTIONS PHYSICALLY INTERACTS WITH SEPTATE JUNCTION COMPONENTS
Introduction

In the last chapter, it was established that Gli is necessary for the formation and proper function of pleated septate junctions in the epidermis and salivary glands. Unprecedented for other septate junction molecules, Gli was found to be localized at tricellular junctions rather than being distributed throughout the pleated septate junction domain of epithelial cells. This led to the proposition that Gli is necessary to anchor pleated septate junction strands to specialized 'tricellular plug diaphragms' at tricellular junctions. This hypothesis is in accordance with the previously described Tricellular Plug model of pleated septate junction development (Fristrom, 1982; Graf et al., 1982; Lane and Swales, 1982). If the hypothesis is correct, Gli stands to be the first molecular component of tricellular junctions identified. Certain protein-protein interactions must occur between Gli and pleated septate junction strands, and between Gli and tricellular plug diaphragms, if Gli is to serve as a physical link between these two structures. In the current chapter, the nature of these interactions is investigated. Two specific questions are addressed. First, does Gli physically interact with pleated septate junction constituents, and secondly, what domains within Gli could facilitate interactions with either the tricellular plug diaphragms or with pleated septate junction strands.

For addressing the first question, whether Gli is physically tethered to pleated septate junction intermembrane particle strands, overexpression studies were carried out using the GAL4/UAS system (Brand and Perrimon, 1993). Specifically, if Gli protein has an intrinsic binding affinity for pleated septate junction molecules, and its expression level is strongly elevated, it would be likely that its domain of localization would spread laterally outside of the tricellular junction to where pleated septate junction strands exist. Thus Gli could adopt a localization pattern equivalent to other
pleated septate junction molecules (such as Cor or Nrx-IV). A complementary approach to investigate physical interaction of Gli with pleated septate junction components was carried out using co-immunoprecipitation experiments.

For the second aim, which was to determine what domains of Gli could mediate interactions, it was essential first to determine whether Gli has any known protein interaction domains using knowledge of Gli sequence. Indeed, Gli sequence contains two known protein-protein interaction domains that could enable it to interact with either the tricellular plug diaphragms or pleated septate junction strands. These domains are the extracellular serinesterase-like domain of Gli and the PDZ recognition sequence within the cytoplasmic tail. To test if these domains are necessary for Gli to interact with either the tricellular plug diaphragms or with pleated septate junctions, transgenic Drosophila strains were generated that expressed either wild-type Gli or mutant Gli in which these domains were deleted. Cell biology and rescue of viability studies were then carried out to test if these mutant proteins are able to interact with tricellular junctions or with pleated septate junction strands.

In this chapter, numerous lines of evidence are shown which support the Tricellular plug model, and the notion that Gli is a physical link between pleated septate junctions and tricellular plug diaphragms. Overexpression of wild-type Gli in the epidermis causes it to adopt a pleated septate junction-like distribution such that it is no longer exclusively localized at tricellular corners, suggesting that Gli has affinity for pleated septate junction proteins. In addition, Gli was found to be associated with pleated septate junction proteins through the co-immunoprecipitation experiments. Interestingly, by investigating what protein-protein interaction domains are important for Gli function, it was found that the PDZ recognition sequence within Gli renders it susceptible to protein degradation. The implications of these findings are discussed as are new observations that suggest the extracellular ligand for Gli may be a soluble protein as proposed by the Tricellular Plug model presented in Chapter 3 (Figure 15).
Materials and Methods

Fly Stocks

Oregon R was used as the wild type strain in all control experiments. The GAL4 driver da.G32-GAL4 (Wodarz et al., 1995) was used to express UAS reporter lines in the epidermis. The 5' regulatory element of this GAL4 driver is derived from the daughterless gene which is involved in cell-cycle regulation and thus it is ubiquitously expressed in Drosophila at all developmental stages (Hassan and Vaessin, 1997). UAS-gli\textsuperscript{tut\#2}, UAS-gli\textsuperscript{tut\#3-HA}, UAS-gli\textsuperscript{EC\#2-HA}, and UAS-gli\textsuperscript{Sec\#1-HA} are 2\textsuperscript{nd} chromosome insertion lines (homozygous viable), while UAS-gli\textsuperscript{PDZ\#1-HA} is a 3\textsuperscript{rd} chromosome insertion line (homozygous viable). EP(3)0604 contains a P[EP] inserted into 5' UTR of the nrx-IV locus (1\textsuperscript{st} exon), and the element is oriented to transcribe the sense strand (Berkeley Drosophila Genome Project accession number AQ025125). gli\textsuperscript{AE2\#45} is a P-element excision allele, and a protein null (Auld et al., 1995).

Molecular Biology and Transgenic Strain Production

To generate the UAS-gli\textsuperscript{tut\#3-HA} line, complementary oligonucleotides [(HATagPflM1a) 5' TAT CCA TAT GAT GTG CCA GAT TAT GCC - TAC CCG TAC GAC GTC CCG GAC TAC GCC/CTG 3' and (HATagPflM1b) 3' GAC/ATA GGT ATA CTA CAC GGT CTA ATA CGG - ATG GGC ATG CTG CAG GGC CTG ATG CGG 5'] encoding two repeats of the haemagglutinin (HA) epitope tag (amino acid sequence: YPYDVPDYA) and tailed with PflM1 restriction sites, were annealed to generate a linker, and this was subcloned into the gli cDNA (AE2 7.41, Auld et al., 1995). The linker was inserted into a unique PflM1 restriction site at the 3' end of the gli open reading frame. The HA-tagged gli cDNA (AE27.41-HA) was subcloned as an EcoR1 fragment into pP[UAST] transformation vector (Brand and Perrimon, 1993) to generate pP[UAST-
AE27.41-HA). UAS-\textit{gli}^{PDZ\Delta}\textit{HA} construct generation: to delete the S/T-X-V PDZ consensus binding site at the terminus of the cytoplasmic tail of Gli, PCR was used. 1.5 Kb of the 3' end of the AE27.41-HA cDNA was amplified with the primer pairs: AE VII (5' GGA ATT CCA TTG GTA CGA GGG ATG GCG C 3' - binds upstream of an internal Kpn I site in \textit{gli} cDNA), and Gli-PDZ (5' GGT ACC / TTA / GGC GTA GTC CGG GAC GTC GTA CGG GTA 3' - contains a KpnI tail, a stop codon, and it is complementary to the HA tag linker). The resultant PCR product (lacking the last nine amino acids of the C-terminus of Gli, including the PDZ recognition sequence) was subcloned into pGEM-T and sequenced to detect errors in Taq polymerase fidelity. The clone was digested with KpnI to release a 1.1 Kb g\textit{gli}-HA-PDZA C-terminal fragment, and then exchanged for the 1.1 Kb KpnI insert of pP\{UAST-AE27.41-HA\} to produce pP\{UAST-AE27.41-HA-PDZA\}.

To generate the UAS-\textit{gli}^{\text{Sec#1-HA}} line, the vector pCR2.1-AE27.41-Sec (E. Ackerman and V. Auld, unpublished data), containing the 2.5 Kb extracellular domain of Gli was modified to introduce an HA tag (X2) at its 3' terminus. To achieve this, complementary oligonucleotides coding for the HA tag repeat (HATagSnaBla: 5' TAT CCA TAT GAT GTG CCA GAT TAT GCC – TAC CCG TAC GAC GTC CGG GAC TAC GCC 3' and HATag SnaBlb: 3' ATA GGT ATA CTA CAC GGT CTA ATA CGG – ATG GGC ATG CTG CAG GGC CTG ATG CGG 5') were annealed to produce a linker. This linker was subcloned into the blunted HindIII site of pBS, and the extracellular domain of Gli (AE27.41-Sec cDNA) was inserted as an EcoRI fragment, upstream, and in frame with it. PCR was used to amplify 0.9 Kb of the resultant AE27.41-SEC-HA construct (using primer pairs AE VII and Gli-PDZ) so as to introduce a stop codon and KpnI site downstream of the HA tag. The PCR product was then subcloned into pGEM-T and sequenced to identify Taq polymerase errors. The clone was digested with KpnI to release a 0.5 Kb AE27.41-SEC-HA fragment, and swapped for the 1.1 Kb KpnI insert of pP\{UAST-AE27.41-HA\} to produce pP\{UAST-AE27.41-SEC-HA\}. UAS-\textit{gli}^{EC\Delta#2-HA}: 1.3 Kb of the extracellular domain of \textit{gli} (encompassing the serinesterase-like domain) was
deleted from the AE27.41-HA cDNA through a BsrBI and SnaBI double digest. The resultant 2.6 Kb truncation product (AE27.41-ECΔ-HA), was sequenced across the BsrBI–SnaBI junction to ensure that the correct reading frame was maintained, and then it was subcloned as an EcoRI fragment into the pP[UAST] transformation vector to generate pP[UAST-AE27.41-ECΔ-HA]. Engineered constructs (200 ng/ul) were injected into \textit{w} \textsuperscript{1118} embryos together with the pPI25.7wcΔ2-3 (400 ng/ul) using standard techniques (Rubin and Spradling, 1982), and insertion lines were isolated and balanced.

\textit{Ectopic Expression and Rescue Studies}

For all overexpression and rescue studies, the \textit{da.G32-Gal4} driver was used. Overexpression studies were carried out at 26°C, while rescue experiments were carried out at 23°C. For overexpression studies involving a wild-type background (with respect to Gli), \textit{da.G32-Gal4} virgin females were crossed to isogenic \textit{UAS-gli} or \textit{UAS-nrx-IV} males. To study the localization of engineered (mutant or wild-type) Gli proteins in a \textit{gli} null background, the transgenic UAS strains were placed into a \textit{gli}AE2A45/CyOactinGFP background and then crossed to \textit{w;gli}AE2A45/CyOactinGFP;daG32 males. Progeny were fixed and stained with antibodies specific to GFP (to identify homozygous mutants), and with HA specific antibodies to follow the localization of engineered Gli proteins. For rescue experiments, the progeny of this cross were screened with a GFP dissecting microscope to identify non-GFP 1\textsuperscript{st} instar larval escapers. Approximately 2000 (mixed genotype) embryos were screened for each rescue experiment. 1\textsuperscript{st} Instar larval escapers were transferred to fresh food vials and allowed to mature to adulthood.

\textit{Embryo Staining}

The immunohistochemistry protocol of Halter et al. (1995) was followed.
to stain embryos. Embryos were staged according to Hartenstein (1993). Stained embryos were mounted in Vectashield (Vector Laboratories, Burlingame, CA), and imaged with a Bio-Rad Radiance Plus confocal microscope (63X oil objective lens). Single, 2 μm optical slices were recorded in all experiments. Confocal files were processed with Image-J 1.24 and Adobe Photoshop 5.5. Primary antibodies and the dilutions used for embryo staining were: mouse anti-Gliotactin (1F61D4) at 1:1 (Auld et al., 1995), mouse anti-Armadillo (N2 7A1) at 1/5 (Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-HA 1/100 (BAbCO, Richmond CA), rabbit anti-Neurexin-IV at 1/200 (Baumgartner et al., 1996), rabbit anti-GFP at 1/200 (Abcam Ltd., Cambridge, UK). All the following secondary antibodies (Molecular Probes, Eugene, OR) were highly cross-adsorbed and used at 1/300 dilution. These included: goat anti-mouse A488 and A568, and goat anti-rabbit A488 and A568. The 1F61D4 monoclonal antibody was preadsorbed prior to use, by adding 100 μl of 4 hr old embryos (fixed, and blocked) to 900 μl of 1F61D4 containing 10% normal goat serum (Sigma BioSciences, St. Louis, MO) and then incubating at 4°C overnight.

**Immunoprecipitation and Western Blot Analysis**

1.0-1.5 g of 0-24 hr w;UAS-gli^{w#3-HA};da.G32 embryos were collected from population cages at 25°C, and a membrane protein extract was prepared according to Zhang and Hsieh (2000). Membrane proteins were solubilized in 200 μl of lysis buffer (10 mM TRIS-CL pH 7.4, 50 mM NaCl, 1mM EDTA, 1.0% NP-40, 1mM PMSF, 2 μg/mL leupeptin, 2 μg/mL pepstatin), and quantified with a PIERCE BCA Protein Assay Kit (MJS BioLynx Inc., Brockville, ON). For each immunoprecipitation experiment, 20 μg of membrane protein was incubated overnight (at 4°C, and rotating) with 4 μg of mouse anti-HA antibody (BAbCO, Richmond CA) or control mouse pre-immune serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), in 200 μl of lysis buffer (containing either 0.0, 0.1, 0.5 or 20.0 μM free Ca^{2+}). Free Ca^{2+} concentrations were set
at 0.0, 0.1, 0.5 or 20.0 μM, through the addition of EGTA to a final concentration of 4 mM, and CaCl₂ to 0, 2.9, 7.3 and 11 mM respectively, as described by Bazbek and Sudhof (1993). Immunoreactive complexes were precipitated by centrifugation following a 1 hr incubation (rotating at 4°C) with 50 μl of a 50% Protein-A agarose slurry (Invitrogen Life Technologies, Burlington, ON), that had been rinsed with lysis buffer containing the appropriate free Ca²⁺ concentration. Precipitated complexes were washed 4X in 200 μl of lysis buffer (containing 0.2% NP-40, and the appropriate free Ca²⁺ concentration), and then subjected to SDS-PAGE (8% gel), and Western blot analysis. Primary antibodies used for Western blots included mouse anti-HA (1/1,000), rabbit anti-Nrx-IV (1/2,000), guinea-pig anti-Cor (1/2,000) and rabbit anti-Dlg (1/2,000). Secondary antibodies used included HRP-conjugated goat anti-mouse (1/10,000), HRP-conjugated goat anti-rabbit (1/5,000), and HRP-conjugated goat anti-guinea pig (1/10,000). Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer™ Life Sciences, Boston, MA) was used as the HRP substrate for westerns, and blots were stripped according to the manufacturers protocol. Autoradiographic exposure times were increased with each consecutive blot stripping (5 secs - initial exposure, 30 min second, 60 min third).
Results

Overexpression of Gliotactin in the epidermis causes it to adopt a distribution very similar to other pleated septate junction molecules

To test the prediction that Gli physically associates with pleated septate junction strands, Gli overexpression studies were carried in the epidermis using the GAL4/UAS system (Brand and Perrimon, 1993). The da.G32-GAL4 strain, which is strongly expressed in the epidermis, was used to drive the ectopic expression of wild-type Gli (Hassan and Vaessin, 1997; Wodarz et al., 1995). The UAS-Gli\(^{wt\#2}\) strain was used for these studies since it is capable of rescuing the embryonic lethality of gli homozygous mutants and thus has wild type activity (see Chapter III, Table 2). The distribution of Gli in the epidermis was followed in stage 15 embryos, which have mature pleated septate junctions, using the Gli 1F61D4 monoclonal antibody and confocal microscopy. Embryos were counterstained for Nrx-IV to visualize pleated septate junctions. For comparison, overexpression studies were also carried out with Nrx-IV since it is a well documented PSJ molecule. For these studies, the EP[0604] strain was used which contains a GAL4 responsive UAS P-element inserted into the nrx-IV locus and oriented to produce a sense product. In the overexpression studies, varied levels of Gli or Nrx-IV expression were observed throughout the epidermis due to the heterogeneous nature of the GAL4/UAS system (Sepp et al., 2001; Lee and Luo, 1999).

In wild type embryos at stage 15 of development, Gli is localized to the tricellular corners of epithelial cells (Figure 16C, arrow), and its expression is partially overlapping with Nrx-IV, which labels cellular circumferences when the epidermis is viewed en-face (Figure 16A,E). Overexpression of Gli results in a lateral spread of Gli away from tricellular corners, such that it labels the circumference of
Figure 16. Overexpressed Gliotactin diffuses laterally away from tricellular corners and adopts a pleated septate junction-like distribution.

Confocal microscopy images of a wild-type (A-F), Gliotactin overexpressing (G-L) and a Nrx-IV overexpressing embryo (M-R). Embryos are double stained for Gliotactin with 1F61D4 monoclonal antibody (green) and with anti-Nrx-IV polyclonal antibody (red). Merge, red and green channels are indicated. (A,C,E,G,I,K,M,O,Q) En-face view of the epidermis. (B,D,F,H,J,L,N,P,R) Cross-section view of the epidermis. In the cross-section images, the apical and basal surfaces of the epidermis are indicated with arrows, while the midpoint is indicated with a horizontal bar. (A-F) In Oregon R embryos, Gli localizes to the tricellular corners (arrow in C). In cross section views of the epidermis, Gli at the tricellular corners falls within the pleated septate junction domain in the apical half of the basolateral membrane (arrowhead in D). UAS-Gli Overexpression. Gli diffuses laterally away from the tricellular junction and labels the circumference of epithelial cells (represented by open circle in I). (H,J,L). Gli is enriched in the apical half of the basolateral membrane (arrowhead in J), although some diffusion basolaterally is also evident. Overexpression of Gli does not severely alter the localization of Nrx-IV (compare L with F). UAS-Nrx-IV overexpression. In en-face views of the epidermis, no significant concentration of Nrx-IV is apparent around epithelial cell circumferences (open circle in Q, compare to E). (N,P,R). Cross-section images of strong Nrx-IV overexpressing cells (arrowhead in R), show homogeneous Nrx-IV distribution throughout the cell. Severe mislocalization of Gli is evident (compare O,P, with C,D). Bar, 10 µm.
epithelial cells (Figure 16I). This expression profile is very similar to Nrx-IV in wild type embryos (compare Figure 16I to 16E). When the epidermis is viewed in cross-section, Gli localization in the overexpressing lines is evident in the majority of epithelial cells and within the pleated septate junction domain in the apical half of the basolateral membrane, where it co-localizes with Nrx-IV (Figure 16, H,J,L). This is in contrast to the distribution of Gli in the wild type epidermis, where Gli is only sporadically observed in the pleated septate junction domain, and only when the plane of section falls across a tricellular junction (compare Figure 16D, with 16J). The lateral diffusion of Gli away from tricellular junctions in the Gli overexpressing lines, but contained within the PSJ domain, strongly suggests that Gli has affinity for PSJs.

When Nrx-IV is overexpressed in the epidermis, it has a very different distribution within epithelial cells than overexpressed Gli. In da.G32:EP{0604} epithelial cells expressing the most elevated levels of Nrx-IV, Nrx-IV is homogeneously distributed throughout the cell and labels the entire basolateral membrane (Figure 16Q,R). There is little or no concentration of Nrx-IV within the apical half of the basolateral membrane at pleated septate junctions in the da.G32:EP{0604} embryos (Figure 16 Q,R). The mislocalization of Nrx-IV in these mutant embryos severely perturbs the localization of Gli at tricellular junctions (Figure 16 O,P). The mislocalization of Gli observed in these mutant strains is very similar to the Gli mislocalization phenotype observed in nrx-IV homozygous mutant embryos (see Chapter III, Figure 3 D,F) suggesting that Nrx-IV overexpression interferes with pleated septate junction formation.

*Generation of Gliotactin deletion mutants reveals that the cytoplasmic tail of Gliotactin renders it susceptible to proteolysis*

The results from the Gli overexpression studies support the notion that Gli is physically linked to septate junctions. Since the extracellular domain of Gli is likely to
project into the paracellular space, it was hypothesized that the serinesterase-like domain found within the extracellular domain of Gli, might be necessary for Gli to become localized to tricellular junctions. The cytoplasmic tail of Gli contains a PDZ recognition sequence and it is possible that Gli at tricellular corners is linked to pleated septate junction strands through PDZ-containing pleated septate junction molecules such as Dlg or Scrib (Bilder and Perrimon, 2000; Woods and Bryant, 1991).

To test if the serinesterase-like domain or PDZ recognition sequence are necessary for Gli to associate with tricellular plugs, and pleated septate junctions respectively, the DNA sequences corresponding to these protein regions were deleted from wild type Gli cDNAs (AE2 7.41). The mutant Gli cDNAs (gli\textsuperscript{ECΔ}, gli\textsuperscript{PDZΔ}) were then reintroduced into Drosophila through P-element mediated transformation to generate transgenic strains. In generating the transgenic strains, the UAS P-element transformation vectors were used such that the GAL4/UAS system (Brand and Perrimon, 1993) could be used to express the engineered Gli proteins in the epidermis. The mutant proteins were tagged with a Haemagglutinin (HA) epitope tag so that they could be distinguished from endogenous Gli protein in immunohistochemistry studies using a HA-specific antibody (Figure 17A). In addition to the gli\textsuperscript{ECΔ} and gli\textsuperscript{PDZΔ} mutant constructs, a third mutant Gli construct was also generated that lacks the entire cytoplasmic and transmembrane domain of Gli (gli\textsuperscript{Sec}) (Figure 17A). This mutant Gli protein was designed to be secreted from epithelial cells, and thus to act as an \textit{in vivo} 'antibody' to investigate the nature of the extracellular ligand of Gli. To control for potential deleterious effects associated with the HA-epitope tag, a transgenic strain carrying a wild-type Gli protein with an HA-tag inserted in the same position as in the Gli\textsuperscript{ECΔ} and Gli\textsuperscript{PDZΔ} mutant proteins was also generated (Figure 17A).

To ensure that mutant Gli proteins of the expected molecular weight were produced by the transgenic strains generated, Western blots were carried out on proteins from 0-24 hr embryos collected from the transgenic strains. The calculated
Figure 17. The PDZ recognition sequence within the cytoplasmic tail of Gliotactin renders it susceptible to protein degradation.

(A) Schematic cartoon of various wild-type and mutant Gliotactin proteins used to identify domains necessary for Gliotactin localization to tricellular corners. The extracellular serinesterase-like domain of Gliotactin (yellow), its PDZ recognition sequence (orange), as well as other domains of interest are indicated (see Key). Of the two wild-type constructs generated, one (wt-HA) contains a Haemagglutinin epitope tag flanking the PDZ recognition sequence. The three mutant Gliotactin constructs produced contain different deletions. The ECA construct contains a deletion that removes the majority of the serinesterase-like domain. The region that was deleted from the wild-type molecule is indicated by the horizontal bars to the right of wt-HA. The Sec construct contains a deletion that removes the entire cytoplasmic and transmembrane domain of Gli. The PDZA construct only lacks the PDZ recognition sequence at the extreme terminus of Gli. All of the mutant constructs contain a Haemagglutinin epitope tag, and for the ECA and PDZA constructs, the position of this tag is equivalent to that of the wt-HA construct. (B) Western blots of transgenic strains prepared from these da.G32-GAL4:UAS-Gli strains, and subjected to SDS-PAGE and Western blotting. Blots were probed with anti-HA monoclonal antibody. Lane 1: negative control (da.G32 driver only). Lane 2: da.G32-GAL4:UAS-Gli\textsuperscript{wt\#HA} membrane protein extract prepared from a crude embryonic lysate. Lane 3: da.G32-GAL4:UAS-Gli\textsuperscript{ECA\#HA} crude embryonic extract. In the crude extract a ~130 kDa Gli\textsuperscript{wt\#HA} band (arrow) as well as several Gli degradation products (arrow heads) are present, while in the membrane protein extract the majority of the Gli degradation products have been removed so that largely undigested Gli remains. Lane 4: da.G32-GAL4:UAS-Gli\textsuperscript{ECA\#2HA} crude embryonic lysate. A ~75 kDa Gli\textsuperscript{ECA} band (arrow) is evident as well as several degradation products (arrowheads). Lane 5: da.G32-GAL4:UAS-Gli\textsuperscript{Sec\#1HA}. A single 100 kDa Gli\textsuperscript{Sec} band is present and no degradation products. Lane 6: da.G32-GAL4:UAS-Gli\textsuperscript{PDZA\#1HA}. A single ~130 kDa Gli\textsuperscript{PDZA} band appears with very few degradation products. (C) Schematic diagram of Gli\textsuperscript{wt\#HA} and Gli\textsuperscript{ECA} showing the positions of the proteolysis sites (horizontal bars to the right of the proteins) as calculated from the sizes of the degradation products in B. The Gli\textsuperscript{ECA} protein is also shown in an expanded form to show the position of the degradation sites relative to the Gli\textsuperscript{wt\#HA} molecule. The majority of the proteolytic sites within Gli\textsuperscript{wt\#HA} and Gli\textsuperscript{ECA} fall in the extracellular domain, but there appears to be little conservation in the position of these sites between the two molecules.
expected molecular weights for the engineered proteins without taking into consideration the additional 2 kDa for the HA epitope tags are as listed: Gli<sup>wt-HA</sup> (109 kDa), Gli<sup>ECΔ</sup> (59 kDa), Gli<sup>Sec</sup> (86 kDa), and Gli<sup>PDZΔ</sup> (108 kDa). The observed molecular weights for each of these constructs was somewhat larger than expected and are: Gli<sup>wt-HA</sup> (130 kDa), Gli<sup>ECΔ</sup> (75 kDa), Gli<sup>Sec</sup> (100 kDa) and Gli<sup>PDZΔ</sup> (130 kDa) (Figure 17B). Interestingly, in embryos collected from the transgenic strains expressing either Gli<sup>wt-HA</sup> or Gli<sup>ECΔ</sup>, additional protein bands immuno-reactive to the HA-antibody were evident (Figure 17B, Lane 3 and 4 respectively, arrowheads). Embryonic poly(A)<sup>+ </sup>RNA Northern blots, prepared from Oregon embryos, and probed with the full length Gli cDNA (AE27.41) produce only a single ~6 kb transcript (Auld et al., 1995) suggesting that these smaller products are not alternative splice variants of Gli, but rather Gli degradation products. Similar degradation products have been observed in Western blots of wild-type Oregon R embryos, using the 1F6 monoclonal antibody, suggesting that these results are not an artifact of ectopic Gli expression (V. Auld, unpublished data). To test if any of the smaller Gli<sup>wt-HA</sup> products are membrane-associated, membrane protein extracts, were prepared from da.G32-GAL4:UAS-Gli<sup>wt-HA##</sup> embryos and subjected to Western blot analysis (Figure 17, Lane 2). The majority of the smaller HA-tagged Gli products are no longer observed in membrane protein preparations, although the 130 kDa Gli<sup>wt-HA</sup> band persists. This suggests that wild-type Gli is subject to protein degradation and that the degradation products are not membrane-associated. Since Gli degradation products are only observed in embryos expressing Gli<sup>wt-HA</sup> or Gli<sup>ECΔ</sup> and no (or very few) degradation products are observed in embryos expressing Gli<sup>Sec</sup> or Gli<sup>PDZΔ</sup> (Figure 17, lanes 5 and 6 respectively) the results suggest that wild-type Gli must be membrane associated, and have an intact PDZ recognition sequence for it to be targeted for degradation.

By calculating the size of the of the protein degradation products observed in the transgenic strains expressing Gli<sup>wt-HA</sup> or Gli<sup>ECΔ</sup>, the position of the proteolytic sites
within these proteins was mapped (Figure 17C). Interestingly, the majority of the proteolytic sites in Gli<sup>wt-HA</sup> or Gli<sup>ECΔ</sup> were calculated to fall in the extracellular domain of these proteins. In addition, the positions of the proteolytic sites were not found to be consistent between the two proteins (Figure 17C, compare wt-HA with ECΔ expanded). This suggests that the conformations of these proteins are different. That is, the proteases acting at the surface of Gli<sup>wt-HA</sup> and Gli<sup>ECΔ</sup> may have access to different target sites. Together with the observation that Gli must be membrane-associated (and contain a PDZ domain) to be degraded, these results suggest that Gli could be internalized through endocytosis for subsequent degradation.

The PDZ recognition sequence present within the cytoplasmic tail of Gli is necessary for the association of Gli with PSJs but not with tricellular junctions.

Since the western blots of the transgenic strains indicated that the strains were producing the engineered wild type and mutant HA-tagged Gli proteins, confocal microscopy was next used to study the localization of these proteins in vivo. Acetylcholinesterases have been shown to form dimers or tetramers (Bourne et al., 1999; Talesa et al., 1997) and given the conservation of structure between Gli and the acetylcholinesterases (Botti et al., 1998) it was considered a possibility that the engineered mutant proteins might associate with endogenous Gli. Therefore, the engineered proteins were expressed in a gli<sup>AE2A45</sup> mutant background so that no interference from endogenous Gli would occur. The transgenic embryos expressing either wild type or mutant Gli protein were double stained with an HA antibody to follow the localization of Gli, and with Nrx-IV to monitor the development of PSJs (Figure 18).

When overexpressed in the epidermis using the GAL4/UAS system, the HA-tagged wild-type Gli was found to diffuse laterally away from tricellular corners, such that it adopted a pleated septate junction-like distribution (Figure 18 A-F). This
construct was found to behave essentially identical to untagged wild type Gli (compare Figure 16 G-L, with Figure 18 A-F). This result suggests that the HA-epitope tag does not interfere with the ability of Gli to associate with pleated septate junctions, and thus it has wild-type activity.

The engineered Gli^{PDZΔ} mutant protein, unlike wild type Gli (tagged or untagged), is not able to adopt a PSJ-like distribution when overexpressed in the epidermis (Figure 18 I). Interestingly, the Gli^{PDZΔ} mutant protein is able to localize to tricellular corners (Figure 18I, arrow), however substantial Gli^{PDZΔ} is also observed within the cell (Figure 18I, arrowhead). The PDZ recognition sequence is thus not essential for Gli localization to tricellular corners. When Gli^{PDZΔ} is overexpressed in a wild type background, it behaves almost equivalently as when it is expressed in a gli mutant background. In a wild type background Gli^{PDZΔ} is however, capable of diffusing somewhat laterally within the PSJ domain and away from tricellular corners (compare arrows in Figure 18 O and Figure 18 I). Interestingly, Nrx-IV is mislocalized when Gli^{PDZΔ} is overexpressed in either a gli mutant or wild type background (compare Figures 18 L,R, with Figure 18F). Furthermore, when Gli^{PDZΔ} is overexpressed in either background, the distribution of Nrx-IV is far more diffuse compared to the wild type (compare Figures 18L,R to Figure 16F) which leads to a poorly staining, less distinct Nrx-IV profile. In a gli mutant background, Nrx-IV has some apical localization, however weak compared to wild type (Figures 16F, 18L). Therefore, in a gli mutant background, Gli^{PDZΔ} may be unable to compact PSJ strands, while in a wild-type background, Gli^{PDZΔ} may act as a dominant negative and block PSJ strand compaction. Together, the Gli^{PDZΔ} overexpression results suggest that PSJs are not forming correctly in these embryos. Also, these results suggest that the Gli^{PDZΔ} protein is incapable of associating with pleated septate junction strands.
Figure 18. The PDZ recognition sequence within the cytoplasmic tail of Gliotactin is necessary for it to adopt a pleated septate junction-like distribution in overexpression studies.

(A-Z) Confocal images of the epidermis of various transgenic lines (as indicated) expressing either wild-type or mutant Gli proteins. (A,C,E,G,I,K,M,O,Q,S,U,W,Y) are en face sections of the epidermis. (B,D,F,H,J,L,N,P,R,T,V,X,Z) are cross-sections of the epidermis. In cross-sections, arrows mark the apical and basal surface of the epidermis, and the midpoint is represented by a horizontal bar. Embryos are stage 15, and double stained for Gli with an HA-specific antibody (green) and for Nrx-IV (red). For each transgenic strain, the merge of the red and green channel is presented at the top, followed by the green channel (Gli), and then the red channel (Nrx-IV). Overexpression studies were carried out in a gli^AE2;^d45 homozygous mutant background unless indicated otherwise with a '(wt)' designation (as in M-R and Y,Z). UAS-Gli-HA overexpression. Gli^HA labels the circumference of epithelial cells (represented by open circle in C), but some diffuse labeling throughout the cells is also evident (arrowhead in C). Gli^HA is enriched in the apical half of the basolateral membrane (arrowhead in D). UAS-Gli-PDZAHA overexpression. Gli^PDZAHA does not label cell circumferences, but is only localized at tricellular junctions (arrow in I), or is homogeneously distributed throughout the cell (arrowhead in I). Nrx-IV is severely mislocalized in epithelial cells (compare K,L with E,F). UAS-Gli-PDZAHA overexpression (wt background). As in the gli null mutant background, Gli^PDZAHA in a wild-type background does not label the cell circumferences, but is largely concentrated at tricellular junctions (arrow in O). Some lateral spread of Gli^PDZAHA in a wild type background is however, apparent (compare arrow in O with arrow in I). Gli^PDZAHA in a wild type background, is homogeneously distributed throughout the epithelium (P). Gli^PDZAHA interferes with Nrx-IV localization such that its distribution spreads throughout the basolateral membrane and it is no longer concentrated in the apical-half of the basolateral membrane (arrowhead in R, compare with Figure 16F). UAS-Gli-SecHA overexpression. Gli^Sec has a punctate staining pattern that is evenly distributed over the epidermis. Gli^Sec does not appear to be enriched at tricellular junctions (arrowheads). Gli^Sec is secreted from the apical membrane domain of epithelial cells and is concentrated below the cuticle (arrowhead in V). UAS-Gli-ECAHA (wt) overexpression. (Y,Z). Gli^ECAHA strongly labels the center of epithelial cells in en face sections (arrowhead in Y), which corresponds to perinuclear labeling in cross-sections (arrowhead in Z). Bar, 10 μm.
Secreted Gliotactin forms aggregates in the extracellular space

The gli^ECΔ mutant strain (Figure 17A) was designed to investigate the requirement of the extracellular domain of Gli for its localization to tricellular junctions. When this construct was expressed in the epidermis in a wild type background, it was found to be concentrated in regions surrounding the nucleus (arrowheads, Figure 18 Y,Z), and was not observed to be targeted to the cell membrane. This result suggested that the mutant protein was retained in the endoplasmic reticulum or Glogi. This construct could therefore, not be used to determine if the extracellular domain of Gli is necessary to target Gli to tricellular junctions. Moreover, this result suggests that the protein folding of the extracellular domain of Gli is under strict cellular quality control and thus may not be amenable to deletion analysis.

An alternative means by which data can be obtained about the nature of the extracellular ligand of Gli is through the use of the secreted Gli construct. This Gli construct, when expressed in the epidermis, in a gli homozygous mutant background, is successfully secreted from the apical membrane domain of epithelial cells and is observed to concentrate between the epidermis and the overlying cuticle (Figure 18T,V). Interestingly, secreted Gli forms protein aggregates in the extracellular space (Figure 18U). These aggregates are homogeneously distributed and do not appear to overlie or associate specifically with tricellular junctions (arrowheads, Figure 18S,U,W), as might be expected if the extracellular ligand of Gli is immobilized at tricellular junctions. These results suggest that the extracellular ligand for Gli may be a secreted protein or alternatively that Gli is self-aggregating.

The PDZ recognition sequence within the cytoplasmic tail of Gli is not necessary for viability.

Each of the engineered Gli constructs was next tested for its ability to rescue the embryonic lethality of homozygous gli^AEΔAS mutant embryos. For each of the engineered constructs that could successfully rescue the embryonic lethality of gli
mutants, 50 rescued embryos were collected, transferred to *Drosophila* fly food, and scored for their ability to develop to adulthood. Since hypomorphic escapers of the pleated septate junction mutants, *nrx-IV* and *cor*, have leg malformation defects, the penetrance of leg malformation defects in these rescued adults was also scored. Of the engineered constructs, the HA-tagged wild-type construct as well as the Gli^{PDZΔ} were able to rescue the embryonic lethal *gli^{AE2Δ45}* mutants, but not the Gli^{ECΔ}, Gli^{Sec} mutant proteins (Table 3).

The HA-tagged wild-type Gli construct was able to rescue the embryonic lethal phenotype of *gli^{AE2Δ45}* homozygotes to a comparable level as the untagged UAS-Gli wild-type control (Table 3). For both of these strains, all rescued embryos were able to develop to adulthood. Moreover, adults that had been rescued with either the HA-tagged or untagged *gli* transgenes presented a similar frequency of leg malformation defects. 58% (n=50) adults that had been rescued with the wild-type untagged Gli transgene had leg defects, as compared to 49% (n=51) of adults that had been rescued with the HA-tagged Gli transgene. Adults that were rescued with either wild type transgene could be maintained as a stable stock. Together, these results indicate that HA-epitope tag does not significantly interfere with Gli function.

Of the mutant HA-tagged *gli* transgenic constructs used to rescue the embryonic lethality of homozygous *gli^{AE2Δ45}* , only the Gli^{PDZΔ} mutant construct was successful. Interestingly, 42/50 of the mutant embryos carrying the *gli^{PDZΔ}* rescue transgene were able to develop to adulthood. These adults, however, had severe locomotor difficulties. Although most adults were able to eclose from their pupal cases, they could not walk, crawl, or inflate their wings. The Gli^{PDZΔ} rescued adults were only able to survive 2-3 days following eclosure. 100% (n=42) of the rescued adults had severe leg malformation defects. These results suggest that although the PDZ recognition sequence within the cytoplasmic tail of Gli is needed for protein degradation, it is not essential for development to the adult stage.
Gliotactin interacts biochemically with known septate junction proteins

To test if Gli physically interacts with PSJ components, co-immunoprecipitation experiments were carried out. For these experiments, purified membrane protein extracts were prepared from 0-24 hour transgenic embryos expressing wild type HA-tagged Gli protein (da.G32-GAL4:UAS-Gli^w^H^HA^ strain). Membrane protein extracts, and not crude lysates were used for these studies due to the presence of Gli degradation products in crude embryonic lysates. Such degradation products would compete with full-length Gli, for primary (HA) antibody in immunoprecipitation reactions, which would reduce the assay sensitivity.

Since Gli contains a putative calcium binding EF-hand, and it is structurally very similar to the Neuroligins, which require calcium to interact with their ligand Neurexin-1β (Ichtchenko et al., 1995; Nguyen and Sudhof, 1997), it was reasoned that Gli might also require calcium to interact with PSJ components. Thus, calcium was included as a variable in the co-immunoprecipitation experiments. CaCl_2/EGTA buffers were used to set free calcium concentrations within a physiological range of 0.0, 0.1, 0.5 and 20.0 μM. A calcium concentration of 0.5 μM was identified as being optimal for Neuroligin/Neurexin interactions (Nguyen and Sudhof, 1997).

When HA-tagged Gli, was immunoprecipitated from purified embryonic lysates in the presence of calcium, the septate junction markers Nrx-IV, Dlg and Cor were observed to be associated with the HA-tagged Gli complexes (Figure 19 A-C). HA-tagged Gli was found to have an optimal association with these PSJ proteins at a free calcium concentration of 0.5 μM, while no interaction was detected in the absence of calcium. The interaction between HA-tagged Gli and the PSJ proteins could not be detected in the negative control, where pre-immune serum was used to carry out
Table 3. Rescue of gli\(^{AE2Δ45}\) Embryonic Lethality with the Gal4/UAS Expression System using various UAS-gli constructs.

<table>
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<th>UAS-gli construct</th>
<th>Genotypes of Rescued Embryos</th>
<th>Rescue of Embryonic Lethality</th>
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<th>Cellular Distribution</th>
<th>% with leg defect</th>
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mut, mutant; E.R., endoplasmic reticulum
the immunoprecipitation experiments (in the presence of 0.5 μM free calcium). These results suggest that Gli interacts with PSJ proteins in a calcium dependent fashion. In the future it will be necessary to confirm this result through reciprocal co-immunoprecipitation experiments.
Figure 19. Gliotactin co-immunoprecipitates with pleated septate junction proteins in the presence of Ca2+.

(A-C) Western blots of co-immunoprecipitation experiments. Membrane protein extracts were prepared from $w^t$;UAS-gli$^{UAS-HA};daG32$ embryos (0-24 hr) via a sucrose-density gradient, and were immunoprecipitated with mouse anti-HA monoclonal antibody and Protein-A agarose (lanes 4-7) or with mouse pre-immune serum and Protein-A agarose (lane 3), or with Protein-A agarose only (lane 2). For mouse-anti HA immunoprecipitations (lanes 4-7), free calcium concentration was varied over the range of 0-20 μM as indicated, and for the pre-immune control calcium was included to 0.5 μM (lane 3). Purified membrane protein extract used for immunoprecipitation experiments is shown in lane 1. Molecular weight markers are indicated at the left of blots. (A) Western blot of immunoprecipitation experiment that has been probed with rabbit anti-Nrx-IV. Nrx-IV appears as a single band of 150-155 kDa in the membrane prep sample (lane 1) (Baumgartner et al., 1996). Nrx-IV is able to co-immunoprecipitate with Gli only in the presence of Ca$^{2+}$ (lanes 5-7). Optimal free calcium concentration for this interaction occurs at 0.5 μM. No Nrx-IV is detected in the negative controls (lanes 2 and 3). (B) Same blot as in A, stripped and re-probed with rabbit anti-Dlg. Dlg appears as two prominent bands in the pure extract (lane 1) at 116 and 97 kDa (Woods et al., 1996). Dlg co-immunoprecipitates with Gli only in the presence of Ca$^{2+}$ (lanes 5-7). (C) Same blot as in B, stripped and re-probed with guinea pig anti-Cor. In the pure membrane extract (lane 1), Cor appears as a prominent band at 180 kDa with several lower molecular weight splice variants (Fehon et al., 1994). Cor only co-immunoprecipitates with Gli in the presence of Ca$^{2+}$ (lanes 5-7).
Discussion

The tricellular plug model of septate junction development suggests that pleated septate junction strands are physically linked to the diaphragms of tricellular plugs. In the current chapter, it was investigated whether Gli serves as the physical link between these two structures. A variety of different experimental approaches were used to test this hypothesis including overexpression studies, Gli deletion analysis, and co-immunoprecipitation experiments.

Two results strongly suggest that Gli physically interacts with pleated septate junction strands. First, the finding that overexpression of Gli in the epidermis results in the lateral diffusion of Gli away from tricellular junctions, but within the pleated septate junction domain. Second, the observation that Gli can be co-immunoprecipitated with various septate junction proteins including Nrx-IV, Cor and Dlg.

Does Gli interact with PSJ strands through the PDZ recognition sequence?

Through the generation of a Gli deletion construct that lacked the S/T-X-V Type I PDZ recognition sequence, the hypothesis that this sequence is necessary for Gli to interact with PSJ strands was tested. The observations that the Gli^{PDZA} construct is not able to adopt a PSJ-like distribution when overexpressed in a wild type or gli mutant background, and that Gli^{PDZA} construct is unable to rescue the mislocalization of the PSJ marker Nrx-IV when expressed in a gli mutant background, support this hypothesis. However, an unexpected result is that the Gli^{PDZA} construct is able to rescue the lethality of homozygous gli embryos. If the Gli^{PDZA} construct is unable to associate with PSJ strands to drive the apical compaction of these strands during PSJ development (as the tricellular plug model predicts) how would the Gli^{PDZA} rescue the transepithelial barrier...
defect of gli mutants? These results can be explained if one assumes that there are two permeability barriers in a field of epithelial cells that must remain ‘water-tight’ if the overall transepithelial barrier is to function. Pleated septate junctions block the flux of extracellular fluids along the basolateral membranes of epithelial cells, and tricellular plugs block the passage of extracellular fluids at tricellular junctions. It is possible that in gli mutants, tricellular plugs fail to form and as a consequence PSJ strands fail to compact apically to form a mature PSJ barrier. In gli mutants, PSJ strands may form however. Extracellular fluids in gli mutants would be expected to freely pass down tricellular channels and cause lethality. With PSJ strand still being presumably intact but just not compacted in gli mutants, extracellular fluids may still be partially restricted from passage across the basolateral surfaces of epithelial cells. In the Gli\textsuperscript{PDZΔ} rescue animals, the Gli\textsuperscript{PDZΔ} mutant protein is able to aggregate at tricellular corners. Thus, the Gli\textsuperscript{PDZΔ} may rescue the tricellular plug defect in the transepithelial barrier, and the flux of paracellular fluid across the uncompacted PSJ domain may not be sufficient to cause lethality. Thus in this model, it is suggested that the PDZ domain of Gli is necessary for association with and compaction of PSJ strands. It is possible that compaction of PSJ strands by Gli is somewhat dispensible, but blocking of tricellular channels by Gli is not.

Gli localization to Tricellular Corners

In the tricellular plug model, it was proposed that the ligand for Gli is soluble molecule (Chapter 3, Figure 15). During the development of septate junctions, a soluble ligand secreted from the apical membrane domain of epithelial cells late in development would be restricted from passage between epithelial cells (due to the presence of nascent PSJ strands) and would thus preferentially pass down tricellular channels. The apically secreted soluble ligand could thus provide a means to drive the apical compaction of PSJ strands by interacting with Gli at tricellular corners. The observation that the secreted construct forms Gli protein aggregates indeed suggests that the
extracellular ligand for Gli is soluble. In this case, when secreted Gli associates with its soluble ligand, it is likely that an insoluble precipitate forms which appears as a protein aggregate.

Given that the extracellular domain of Gli contains a consensus Ca\(^{2+}\) binding EF hand, it is tempting to speculate that Ca\(^{2+}\) may be essential for the formation of the observed aggregates. From the co-immunoprecipitation experiments, it is clear that Ca\(^{2+}\) is necessary for Gli association with PSJ components. Ca\(^{2+}\) may simply be necessary to ensure the proper 3D structure of the extracellular domain.

If the ligand is soluble, and secreted apically, how do the phenotypes observed in this study fit in with the model? Presumably, in a \(gli^{AEz\Delta 45}\) mutant background, the ligand would preferentially pass down tricellular channel since the model predicts that PSJ strands still form and function as permeability barriers in the absence of Gli, although they are not compacted. The Gli\(^{PDz\Delta}\) mutant construct expressed in a \(gli^{AEz\Delta 45}\) mutant background, could thus interact with the soluble ligand passing down tricellular channels and Gli\(^{PDz\Delta}\) could localize correctly to tricellular corners, as was observed in the experiments. In a \(nrx-IV\) mutant background, Gli fails to localize to tricellular junctions (Chapter 3, Figure 12C-F). Presumably, Nrx-IV is a component of PSJ strands, and thus in the absence of Nrx-IV, PSJ strands do not form. Indeed in \(nrx-IV\) mutants, no PSJ septa are observed in EM images of various cell types (Baumgartner et al., 1996). Presumably, in a \(nrx-IV\) mutant background, the soluble ligand for Gli is able to pass across the PSJ domain, and obtains a homogenous distribution throughout the paracellular space, such that Gli is not drawn to tricellular corners, as was observed in the experiments.

**Proteolytic degradation of Gli**

Western blot analysis of the various Gli wild type and mutant transgenic strains used in this study, identified that Gli is susceptible to proteolysis, and the PDZ
recognition sequence within the cytoplasmic tail of Gli is required for Gli degradation to occur. Interestingly, the Neuroligins, which are structurally related to Gli, are also susceptible to proteolysis (Song et al., 1999). Moreover, they also contain a PDZ recognition sequence within their cytoplasmic tail (Itchtchenko et al., 1995). Although it is curious that Gli is proteolytically degraded, it is probably not of fundamental importance for embryonic development and Gli function since the Gli^{PDZA} transgenic strain can reach adulthood.

One common means of targeting proteins for proteolytic degradation is through ubiquitin-dependent pathway (reviewed in Laney and Hochstrasser, 1999). Ubiquitin is a 76 amino acid, 8 kDa, protein that can be attached to target proteins via ubiquitin ligases (via the side chain amino group of lysine residues), or removed via deubiquitinating proteases. For cytosolic proteins, the addition of polyubiquitin chains often leads to its degradation through the ATP-dependent 26S proteasome complex (reviewed by Mimnaugh et al., 1999). This complex is present in many copies throughout the cytosol and they serve as molecular "wood-chippers" to degrade target proteins into small peptides. Polyubiquitination of membrane proteins, in contrast, often leads to endocytosis and degradation via the lysosomal pathway (Garner et al., 2000; Longva et al., 2002).

If Gliotactin is targeted for protein degradation via a ubiquitin-dependent pathway, one would expect to see a smear for Gliotactin on Western blots owing to the addition of ubiquitin chains of varied lengths. Indeed, this is often the case with other poly-ubiquitinated proteins (Traweger et al., 2002; Longva et al., 2002; Nakagawa and Huibregtse, 2000). However, Gliotactin bands on Western blots are invariably 'tight' unlike proteins known to be ubiquitinated, suggesting that Gliotactin may be degraded through a ubiquitin-independent proteolytic pathway.
Gliotactin and the tricellular plug model

Results obtained in this chapter support the hypothesis that Gli acts as a physical link between tricellular plugs and pleated septate junction strands. Along with validating the tricellular plug model of pleated septate junction development, these results provide strong evidence that Gliotactin has a role unique from other identified PSJ proteins in the development of pleated septate junctions. In the ensuing chapter, various experiments are discussed that could offer further insight into the role of Gliotactin in pleated septate junction and tricellular plug development.
V. GENERAL DISCUSSION

Gliotactin was originally identified as a *Drosophila* glial transmembrane protein that is necessary for the development of the blood-nerve barrier (Auld et al., 1995). The main goal of this thesis has been to test the hypothesis that Gliotactin is necessary for the formation of pleated septate junctions. These junctions are present between the glial wraps of peripheral nerve glial sheaths, and they are functionally analogous to tight junctions (reviewed in Carlson et al., 2000; Bryant, 1997). In this thesis, the characterization and analysis of *gliotactin* mutants has been extended to investigate how the loss of Gliotactin results in a breakdown of the blood-nerve barrier. Through an analysis of Gliotactin function in the epidermis, it was established that Gliotactin is necessary for the development of *Drosophila* pleated septate junctions. Thus the disrupted blood-nerve barrier phenotype previously reported for *gliotactin* mutants is likely to arise from defective pleated septate junction development in the peripheral glia as well.

The role of Gliotactin in septate junction development, however, appears to be unique as compared to other known pleated septate junction molecules. In the epidermis, and salivary glands, the localization of Gliotactin to the position where three cells meet - termed the tricellular junction, is unprecedented for a septate junction protein. Nevertheless, Gliotactin was found to associate with pleated septate junction proteins through co-immunoprecipitation experiments and overexpression studies. The data presented in this thesis support a model in which Gliotactin acts as a physical linkage between highly specialized structures termed tri-cellular plugs, at tricellular junctions, and between individual pleated septate junction strands. It is foreseen that the septate junction molecules Dlg and Scrib, act early in septate junction development to identify the future site at which septate junctions form. Next, Nrx-IV and Cor are involved in the formation of pleated septate junction strands, and finally Gli plays a
critical role in the final maturation of pleated septate junctions and development of tricellular plugs. The formation of an intact transepithelial barrier is thus likely to require both pleated septate junctions and tri-cellular plugs, and Gliotactin could be the first identified molecular component of tri-cellular plugs.

In the future, to better understand how Gliotactin at tricellular junctions is involved in pleated septate junction development, it will be necessary to carry out electronmicroscopy studies. Although the function of transepithelial barrier and the localization of pleated septate junction molecules is compromised in _gliotactin_ mutants, it will be of interest to determine if pleated septate junction strands form in _gliotactin_ mutants, or if they simply do not compact, as the tricellular plug model might predict. If the tricellular plug model is correct, one would expect to find pleated septate junction septa present, but spaced further apart, in cross-section images of epithelial cells from _gliotactin_ mutants.

Freeze-fracture electronmicroscopy studies should also be carried out in conjunction with transmission electronmicroscopy analysis of wild type and _gliotactin_ mutants. It needs to be determined if the stacked arrays of tricellular plug diaphragms that are believed to act as anchors for septate junction strands are present at the tricellular corners of epidermal cells. In the past, tricellular plugs have been best imaged with freeze-fracture analysis (Graf et al., 1982).

Although it would be ideal to carry out immuno-electronmicroscopy (immuno-EM) studies or freeze-fracture immuno-EM to determine if Gliotactin is directly localized at tricellular plug diaphragms, such experiments will have to await the generation of a Gliotactin antibody that has sufficient binding affinity for immuno-EM. For immuno-EM studies, primary antibodies that can be used at a very low titration (1/300-1/500) are required (K. MacDonald, personal communication). The difficulty in generating a Gli antibody that gives a robust immunogenic response in rodents, may arise from the fact that cholinesterase-like proteins are well conserved throughout
evolution (Gilbert et al., 2001; Botti et al., 1998; Oakeshott et al., 1999). Although it is possible to carry out immuno-EM on the daG32-GAL4:UAS-Gli\textsuperscript{vot}

HA strains, since available anti-HA antibodies give a high signal to noise ratio at low concentrations such studies may not be informative. In this strain, the HA-tagged Gli is likely to be ectopically expressed, and thus immuno-EM analysis of Gli subcellular localization should await the generation of a suitable mono- or polyclonal antibody.

Structure-Function Analysis of Gliotactin

By comparing the amino acid sequence of Gliotactin to other protein sequences in the BLAST data base, certain regions within Gliotactin have been identified that may be of functional importance. Through deletion analysis, the importance of two such regions: the extracellular serinesterase-like domain, and the cytosolic PDZ-recognition sequence was investigated in Chapter IV. In addition to these two regions of interest, Gli also contains a Ca\textsuperscript{2+} binding EF-hand motif in its extracellular domain, and in its cytoplasmic tail a consensus TRAF recognition sequence, plus putative serine-threonine and tyrosine phosphorylation sites in its intracellular tail. The functional importance of each of these other protein regions to Gliotactin should be investigated in the future. However, a few follow-up experiments should be conducted to clarify the results obtained in Chapter IV, before a functional dissection of some of these other regions within Gliotactin is pursued.

The ectopic expression studies with Gli\textsuperscript{PDZ\Delta}, suggest that the PDZ recognition sequence is necessary for Gli to associate with pleated septate junction strands, since the mutant protein is not able to diffuse laterally away from tricellular junctions to adopt a Nrx-IV like distribution. To conclusively show that the PDZ recognition sequence is necessary for Gli to interact with pleated septate junction strands, co-immunoprecipitation experiments as carried out in Chapter IV, should be performed with the daG32-GAL4:UAS-Gli\textsuperscript{PDZ\Delta\#1HA} strain (in the presence of 0.5 \textmu M Ca\textsuperscript{2+}). As a
positive control the \textit{da.G32-GAL4:UAS-Gli^{wt\#3HA}} strain should naturally be included. The \textit{da.G32-GAL4:UAS-Gli^{PDZA\#1HA}} strain is unhealthy, thus it will take a significant period of time to generate sufficient flies to carry out this study.

Another finding from the Gli^{PDZA} ectopic expression studies is that the PDZ recognition sequence is not important for targeting Gli to tricellular junctions. The tricellular plug model predicts that the extracellular domain of Gli is necessary to localize Gli to tricellular corners. However, since the Gli^{ECA} deletion mutant was retained in the Golgi or endoplasmic reticulum, an alternative approach that could be used to investigate this hypothesis is to generate Gli-CD8 chimeric proteins. CD8 is a vertebrate lymphocyte transmembrane protein that has previously been used to generate chimeric proteins in \textit{Drosophila} (Zito et al., 1997). When ectopically expressed in \textit{Drosophila}, CD8 is targeted to the membrane and is diffusely distributed. Two Gli-CD8 chimeric constructs should be generated. In one construct the extracellular and transmembrane domains of Gli should be fused to the cytoplasmic domain of CD8. In the second transgenic construct, the extracellular domain of CD8 should be fused to the transmembrane and cytoplasmic domain of Gli. Using the GAL4/UAS system (Brand and Perrimon, 1993), these constructs would be expressed in the epidermis and the ability of these constructs to localize to tricellular corners in the epithelial cells of the epidermis could be assayed. The engineered proteins could be tracked either through the introduction of an HA tag, or through the use of CD-8 antibodies. It would also be of interest to determine if these constructs are capable of rescuing the lethality of \textit{gliotactin} homozygous null mutants in the rescue assay used in Chapter III and IV. These experiments should conclusively show if the extracellular domain of Gli is sufficient for targeting of Gli to the tricellular junctions.

\textbf{Gliotactin and Calcium}

From the co-immunoprecipitation experiments, it is clear that \(\text{Ca}^{2+}\) is
necessary for Gli to interact with pleated septate junction components. It would be interesting to know what role Ca\(^{2+}\) plays in this interaction. It is possible that Ca\(^{2+}\) is simply required to maintain the correct conformation of Gli. To investigate this idea, two experiments could be conducted: Gli \(^{45}\text{Ca}^{2+}\) Western blots and Circular Dichroism spectrum analysis of Gli. To carry out the Gli \(^{45}\text{Ca}^{2+}\) Western blots, Gli could be immunoprecipitated from \textit{da.G32-GAL4:UAS-Gli}^{PDZ\Delta1HA} embryonic lysate membrane preparations, separated on an SDS-PAGE gel, and then transferred to nitrocellulose. The Western blots could then be incubated in a saline solution containing radiolabelled \(^{45}\text{Ca}^{2+}\), and then imaged via autoradiography. As a positive control, the \textit{Ca}^{2+} binding protein Calbindin could be included. The Gli \(^{45}\text{Ca}^{2+}\) Western blots would demonstrate that \textit{Ca}^{2+} is able to associate with Gli. As a complementary approach, circular dichroism (CD) spectrum analysis would help establish if the protein folding of Gli is dependent on \textit{Ca}^{2+}. These experiments have been conducted in the past to determine if the interaction between the Neuroligins and the \(\beta\)-Neurexins was \textit{Ca}^{2+} dependent (Nguyen and Sudhof, 1997). To perform these studies, highly purified (HPLC grade) and concentrated Gliotactin protein (20 mg/mL) would be required as well as a spectropolarimeter. The CD spectra of Gliotactin in the presence or absence of \textit{Ca}^{2+} would then be plotted over a range of temperatures (e.g. from 20-70\(^{\circ}\text{C}\)). If \textit{Ca}^{2+} is necessary for the correct protein folding of Gli, a shift in the CD spectra of Gli would occur at a much lower temperature in the absence of \textit{Ca}^{2+} than in the presence.

In chapter IV, the engineered Gli\textsuperscript{Sec} protein was found to be secreted from the apical membrane domain of epithelial cells. Since embryos stained for Gli\textsuperscript{Sec} had a very punctate staining profile, it was suggested that the Gli\textsuperscript{Sec} protein was forming protein aggregates by either interacting with itself (homotypic interaction) or by binding a unknown secreted ligand (heterotypic interaction). Using cell aggregation assays, one could test if Gli interacts in a homotypic fashion and thus one could differentiate between these two possible hypotheses.
To test if Gliotactin interacts in a homotypic fashion, Gliotactin would be expressed under the control of an inducible promoter (such as metallothionine) in a suitable cell line (see below). Expression of Gliotactin would then be induced, and the cells would be subsequently monitored to determine when Gli expression occurs (through Western blot analysis) and when or if cell aggregates occur. Cells that have not been transfected with a gliotactin transgene, but were otherwise subjected to identical experimental manipulation should be carried along as a negative control. For these studies, Ca^{2+} should be included as a variable, given the possibility that Ca^{2+} may affect the protein folding of Gliotactin. In choosing a cell line for these studies, it is obviously important to select a cell line that does not aggregate readily in the presence of Ca^{2+}. Also, a mammalian and not a Drosophila cell line should be used to carry out the homotypic interaction studies, since it is possible that Gli binds a secreted ligand and one would not want this ligand to be present in the cells used for the aggregation assay as it could generate false positives for homophilic binding.

**Screens to identify Gliotactin interacting proteins**

Seeing as Gliotactin has such a novel distribution in epithelial cells, it will be important to carry out various genetic and biochemical screens in the future to identify Gliotactin interacting proteins. Independent screens could be carried out to identify proteins that specifically interact with the extracellular domain of Gli, or with the intracellular domain.

Perhaps one of the most powerful screens that could be carried out is a genetic suppressor screen. A gliotactin hypomorphic allele (gli^{d05}) has recently been isolated through EMS mutagenesis (D. Venema and V. Auld, unpublished data) and it could be used to carry out such a screen. Homozygous gli^{d05} mutant adult escapers eclose and have leg malformation defects, among other phenotypes (D. Venema and V. Auld, unpublished data). The gli^{d05} allele has been sequenced and a substitution mutation has
been identified in the extracellular domain that is predicted to affect the peripheral anionic site of Gli in computer modeling studies (D. Venema, S. Botti, and V. Auld, unpublished data). By subjecting the gli dv5 mutants to a second round of EMS mutagenesis, and screening for mutations that make the gli dv5 mutants healthier with fewer leg defects (which are easy to score), it may be possible to identify the ligand of Gliotactin. That is, it is conceivable that a point mutation in the ligand for Gli may be generated that would enable the mutant Gli dv5 protein to interact better with it. Before embarking on this suppressor screen, it would be important to shown that the mutant Gli dv5 protein is being targeted to the cell membrane in the epidermis. That is, since the engineered Gli ECA mutant protein discussed in Chapter IV, was found to be retained in the Golgi or endoplasmic reticulum (E.R.), it is possible that the Gli dv5 protein has the same fate. If the Gli dv5 protein is retained in the Golgi or E.R., the screen would likely identify little more than chaperone proteins. If the Gli dv5 protein is retained in the E.R., it would be important to identify other hypomorphic EMS alleles of gli that could be used for a suppressor screen.

As an alternative to a genetic screen, a yeast-two hybrid biochemical screen could be carried out to identify Gli interacting proteins. It would be best to use the cytoplasmic tail of Gli and not the extracellular domain as bait for such a screen. It is possible that the extracellular domain of Gli is glycosylated, given that the sizes of the engineered Gliotactin proteins are larger than expected on Western blots (Chapter IV). Neurotactin, another cholinesterase-like molecule in Drosophila, has been shown to be glycosylated (Hortsch, 1990). If Gli is glycosylated, it may be important for ligand-receptor interactions, and glycosylation of Gli would not occur in a yeast two hybrid screening system, thus carrying out a yeast-two hybrid using the cytoplasmic tail of Gli as bait may prove more successful.

A second biochemical screen that could be carried out, which may enable to identification of the extracellular ligand for Gli, is to purify Gli protein complexes from
embryos and then use reverse genetics to identify the components of the complex. For these studies, membrane preparations of embryonic lysates prepared from the \textit{da.G32GAL4-UASGli\textsuperscript{tot\#3HA}} strain (in the presence of \(\text{Ca}^{2+}\)) could be solubilized in detergent and then passed over a protein-A-sepharase immunoaffinity column containing covalently coupled anti-HA antibody. The bound protein would then be eluted (with a \(\text{Ca}^{2+}\) free buffer, or high salt buffer), and subjected to SDS-PAGE electrophoresis. Individual protein bands could then be cut out of the SDS-PAGE gel, trypsinized, and peptide-sequenced. One problem that is foreseen with this approach is the presence of high levels of contaminant protein in the preparations that are eluted from the anti-HA column. One way to determine which proteins may be of interest is to use the Gli\textsuperscript{Sec} protein as an 'antibody' to probe a Western blot prepared from wild-type embryos, and then only peptide-sequence proteins of the appropriate molecular weight. To obtain Gli\textsuperscript{Sec} protein to use as a probe for these studies, \textit{Drosophila} tissue culture S2 cells could be grown under serum-free conditions, and Gli\textsuperscript{Sec} could be isolated and concentrated from the supernatant using Amnicon filters and centrifugation.

\textit{What might come out of the screens?}

It is difficult to predict what types of proteins may interact with the extracellular domain of Gli, since as mentioned in the introduction the ligands that have been identified for other electrotactins are quite diverse. Amalgam, an Immunoglobulin-like protein, was found to interact with Neurotactin, while the \(\beta\)-Neurexins were found to interact with the Neuroligins (Fremion et al., 2000; Ichtchenko et al., 1995). Nevertheless, one can be almost certain that PDZ containing proteins would be identified as Gli interactors in the biochemical screens proposed. Possibly Dlg and Scrib will come out of the screens, as well as other PDZ containing proteins. Of the proteins that are identified in any of the genetic screens, it will be important to do RNA \textit{in-situ} analysis to see if the genes of interest are expressed in epidermis. Of the genes that are
expressed in the epidermis, those genes for which there are P-element insertions lines available in the Bloomington Stock Center should be followed up first since P-element excision mutagenesis could be used to rapidly generate null mutations in these genes. Once null mutants are generated, the lines that are embryonic lethal or have defects in dorsal closure as determined by cuticle preparations should be examined to see if they have defects in septate junction development. Antibodies could then be generated to determine the protein localization profile of the genes of interest.

_Gliotactin and Rapsynoid (Partner of Inscuteable)_

Recently, Rapsynoid (Raps), which is also known as Partner of Inscuteable (Pins), has been reported as a Discs-Large interacting protein that is concentrated at tricellular junctions (Bellaiche et al., 2001; Bryant Lab web page, 2002). Raps and Dlg, are necessary to establish planar polarity in the sensory organ precursor cell of the dorsal thorax (notum) in pupa. It is proposed that Raps and Dlg function to remodel the apical-basal axis in order to establish planar polarity through a process that requires Frizzled signaling (Bellaiche et al., 2001). The mechanism by which this occurs is not clear. However, it does appear that both Raps and Dlg are necessary to orient the mitotic spindle. Dlg may be part of a scaffold that could localize molecules involved in spindle rotation (Bellaiche et al., 2001). In a gain-of-function screen to identify genes that affect the development of the adult external sensory organs, Gliotactin has previously been identified, suggesting that Gli may also have a role in sensory organ precursor development (Abdelilah-Seyfried, 2000). In the future it would be very interesting to determine if the protein distributions of Rapsynoid and Gli are indeed similar in the epidermis of embryos and pupa. Moreover, it would be worth investigating if Gli has a role in planar polarity and mitotic spindle orientation in the sensory organ precursors of the notum similar to Dlg and Rapsynoid.
Conclusions

Pleated septate junctions in *Drosophila* function as transepithelial barriers. Through cell biological techniques, and mutant analysis, Gliotactin has been identified to be necessary for the normal development of pleated septate junctions. The subcellular protein distribution of Gliotactin is not equivalent to other pleated septate junction proteins, although Gliotactin and pleated septate junction proteins display spatial overlap at the region where three cells meet in the epidermis. Highly specialized structures termed tricellular plugs are found at tricellular junctions, and Gliotactin is likely to serve as a physical link between pleated septate junctions and tricellular plugs.
VI. REFERENCES


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# VII: APPENDIX I. DROSOPHILA MELANOGASTER STRAINS USED FOR EXPERIMENTS CARRIED OUT IN THIS STUDY

<table>
<thead>
<tr>
<th>Genotypic Class</th>
<th>Allele</th>
<th>Affected Chromosome</th>
<th>Phenotype</th>
<th>Comments/Source of Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutants</td>
<td>gli^AE2645</td>
<td>2^nd</td>
<td>St. 16/17 embryonic lethal, PSJ markers (Cor, Dlg and Nr1-IV) mislocalized, transepithelial barrier permeable.</td>
<td>gliotactin P-element excision allele, and protein null, removes approximately 1kb of 5' UTR (Auld et al., 1995).</td>
</tr>
<tr>
<td></td>
<td>gli^AE264b</td>
<td>2^nd</td>
<td>as for gli^AE2645</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td>gli</td>
<td>2^nd</td>
<td>as for gli^AE2645</td>
<td>gliotactin P-element excision allele, and protein null, removes approximately 1kb of 5' UTR (Auld et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>gli^DV3</td>
<td>2^nd</td>
<td>as for gli^AE2645</td>
<td>gliotactin EMS allele, premature stop just 3' to signal sequence, generated by D. Venenma.</td>
</tr>
<tr>
<td></td>
<td>gli^CQ1</td>
<td>2^nd</td>
<td>as for gli^AE2645</td>
<td>gliotactin EMS allele, premature stop near the transmembrane domain of Gli, putative secreted protein, generated by C. Queano.</td>
</tr>
<tr>
<td></td>
<td>nrX^36</td>
<td>3^rd</td>
<td>St. 16 embryonic lethal, PSJ septa not present at EM level, Cor mislocalized, dorsal closure defects, scolopale cells associated with sensory neurons are rounded (Baumgartner et al., 1996).</td>
<td>neurexin-IV EMS allele, donated by Bellen Lab.</td>
</tr>
<tr>
<td></td>
<td>w^118</td>
<td>X</td>
<td>white eye</td>
<td>_</td>
</tr>
<tr>
<td>UAS reporters</td>
<td>UAS-NL#17</td>
<td>3^rd</td>
<td>Not determined</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td>UAS-taulacZ</td>
<td>2^nd</td>
<td>^beta-Gal is targeted to microtubules</td>
<td>Obtained from Bloomington Stock Center</td>
</tr>
</tbody>
</table>

white EMS allele, obtained from Bloomington Stock Center

G. Boulianne

Obtained from Bloomington Stock Center
<table>
<thead>
<tr>
<th>UAS-gapGFP</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt;</th>
<th>GFP is targeted to the cell membrane by myristylation sequence of GAP-43</th>
<th>Obtained from Bloomington Stock Center</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAS-gld&lt;sup&gt;my2&lt;/sup&gt;</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>Behaves as wild-type</td>
<td>Untagged, generated by J. Schulte</td>
</tr>
<tr>
<td>UAS-Gli&lt;sup&gt;my2-HAP1&lt;/sup&gt;</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>Behaves as wild-type</td>
<td>HA-tag positioned 5' to PDZ recognition sequence at C-terminus, generated by J. Schulte</td>
</tr>
<tr>
<td>UAS-gli&lt;sup&gt;my2-HA&lt;/sup&gt;</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>Behaves as wild-type</td>
<td>HA-tag positioned 5' to PDZ recognition sequence at C-terminus, generated by J. Schulte</td>
</tr>
<tr>
<td>UAS-gld&lt;sup&gt;ECSFR-HA&lt;/sup&gt;</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>Retained in the endoplasmic reticulum</td>
<td>HA-tag positioned 5' to PDZ recognition sequence at C-terminus, generated by J. Schulte</td>
</tr>
<tr>
<td>UAS-gli&lt;sup&gt;Src-HA&lt;/sup&gt;</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>Secreted</td>
<td>HA-tag at C-terminus, generated by J. Schulte</td>
</tr>
<tr>
<td>UAS-gli&lt;sup&gt;PDZART-HA&lt;/sup&gt;</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>Putative dominant negative</td>
<td>HA-tag positioned 5' to PDZ recognition sequence at C-terminus, generated by J. Schulte</td>
</tr>
<tr>
<td>EP(3)0604</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>Putative dominant negative</td>
<td>P[EP] inserted into 5' UTR of the nrx-IV locus (1&lt;sup&gt;st&lt;/sup&gt; exon), and the element is oriented to transcribe the sense strand, Bloomington Stock Center</td>
</tr>
</tbody>
</table>

**GAL4 drivers**

<p>| repoGAL4 | 3&lt;sup&gt;rd&lt;/sup&gt; | GAL4 expressed in all glia except midline, semi-lethal insertion (Sepp et al., 2001) | Donated by Keshishian Lab |
| J29GAL4-2 | 2&lt;sup&gt;nd&lt;/sup&gt; | GAL4 expressed in apodemes, neurons and glia of the CNS and PNS, and the epidermis. | 3.7 Kb of the gli 5' regulatory sequence cloned upstream of GAL4, generated by J. Schulte |</p>
<table>
<thead>
<tr>
<th>Strain/Marker</th>
<th>Development Stage</th>
<th>Description</th>
<th>Source/Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp-G303-7</td>
<td>3rd</td>
<td>GAL4 expressed ubiquitously but has a patchy distribution in the embryo.</td>
<td>Donated by Leiserson et al., 2000</td>
</tr>
<tr>
<td>daughterless-</td>
<td>3rd</td>
<td>GAL4 expressed ubiquitously, throughout development (Wodarz et al., 1995)</td>
<td></td>
</tr>
<tr>
<td>GAL4</td>
<td></td>
<td></td>
<td>Obtained from Bloomington Stock Center</td>
</tr>
<tr>
<td>rL82#29</td>
<td>2nd</td>
<td>GAL4 expressed in the peripheral glia among other tissues. Viable insert into 5' UTR of gli locus</td>
<td>Generated through targeted transposition of rL82 strain (Sepp and Auld, 1999).</td>
</tr>
<tr>
<td>Balancers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CyO, P[ry, enwglacZ]</td>
<td>2nd</td>
<td>β-gal expressed in prominent bands in embryo beginning at approx. St12 of embryogenesis, homozygous lethal during mid-embryogenesis, adults have curly wings.</td>
<td>Excellent marker throughout embryogenesis (stages 10-17), obtained from Bloomington Stock Center</td>
</tr>
<tr>
<td>CyO, P[w, actinGFP]</td>
<td>2nd</td>
<td>GFP expressed ubiquitously in embryo, homozygous lethal mid-embryogenesis, adults have curly wings.</td>
<td>Good marker for late but not early embryogenesis, obtained from Bloomington Stock Center</td>
</tr>
<tr>
<td>TM6,Tb</td>
<td>3rd</td>
<td>3rd instar larvae are tubby, homozygous lethal.</td>
<td>Obtained from Bloomington Stock Center</td>
</tr>
<tr>
<td>TM6B,P[w, iab-2 (1.7)lacZ],Tb.</td>
<td>3rd</td>
<td></td>
<td>Good marker for late embryonic development, obtained from Bloomington Stock Center</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oregon R</td>
<td></td>
<td>wild-type strain</td>
<td>Obtained from Bloomington Stock Center</td>
</tr>
<tr>
<td>rL82</td>
<td>2nd</td>
<td>nuclear enhancer trap, labels peripheral glia</td>
<td>P[lacZ, ry+] insertion in the gliotactin gene (Auld et al., 1995)</td>
</tr>
</tbody>
</table>