BIOCHEMICAL PROTEIN INTERACTIONS OF GLIOTACTIN AT THE TRICELLULAR JUNCTION

by

JAIMMIE QUE

B.Sc., University of British Columbia, 1999

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In

THE FACULTY OF GRADUATE STUDIES

(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA

December 2006

Abstract

The septate junction is an occluding junction in invertebrates, similar in function to tight junctions, playing a role in epithelial barriers, and in apical/basal polarity. Septate junction interactions are still being characterized as new component proteins are discovered. One septate junction protein, Gliotactin, was discovered in Drosophila to correlate with the mislocalization of characteristic septate junction proteins in a Gliotactin null animal. However, Gliotactin is the only component found exclusively at the level of septate junctions at tricellular junctions in epithelia. The tricellular junctions are the structure at the convergence of three cells and a potential organizing factor of the septate junction. This led to the question, what is Gliotactin's role in the organization of the tricellular and septate junctions? To study this, we looked at Gliotactin interactors, and attempted to elucidate a model of tricellular and septate junction protein interactions. Previous attempts at finding Gliotactin interactors were made using in vitro systems or by using transgenic animals using an over-expressed epitope-tagged Gliotactin, that showed that Gliotactin interacts with known septate junction proteins in a calcium dependent manner. This thesis aims to further explore Gliotactin interactions with the hypothesis that native Gliotactin in Drosophila interacts in a calcium dependant manner with septate junction proteins, Discs Large, and Neurexin IV. Using co-immunoprecipitation and GST pulldowns, on native Gliotactin protein in Drosophila, I have shown that Gliotactin does not interact with Neurexin IV but does interact with Discs Large in a calcium-dependent manner. This is significant in that, to date, there has been no known interactor of Discs Large or of native Gliotactin at the tricellular junction. I also present data on unidentified potential Gliotactin interactors seen in a GST pulldown assay. The data presented in this thesis has contributed to a new working model of the tricellular junction and the role of Gliotactin.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Ab	Antibody
aHAJ	Apical hemi adherens junction
BLAST	Basic Local Alignment Search Tool
Band 4.1	see Ferm
bHAJ	Basal hemi adherens junction
CNS	Central Nervous System
Cor	Coracle
DE-cad	DE cadherin
Dlg	Discs Large
DTT	Dithiothreitol
ECM	Extracellumar matrix
EDTA	Ethylenediaminetetraacetic acid
EF hand	A helix-turn-helix protein domain which can bind calcium ions
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FasIII	Fasciclin III
Ferm	Protein domain named for 4.1, Ezrin, Radixin and Moesin
GFP	Green Fluorescent Protein
GJ	Gap junction
Gli	Gliotactin
GPI	Glycosylphosphatidylinisotol
GST	Glutathione-S-Transferase
GUK	guanylate kinase
HA	Hemaglutinin
IF6.3	Mouse monoclonal anti-Gliotactin antibody
Ig	Immunoglobulin
IgG	Immunoglobulin G
K+	Potassium ion
KDa	KiloDalton
mM	MilliMolar
MZ	Marginal zone
Na/K+	Sodium/Potassium ion
NAPS	Nucleic Acid and Protein Services
Nlg	Neuroligin
Nrg	Neuroglian
NrxIV	Neurexin IV

PBS	Phosphate Buffered Saline
PDZ	Protein interaction domain, short for PSD-95, Discs Large, ZO-1
PJ	Paranodal Junction
PMSF	Phenylmethylsulphonylfluoride
PNS	Peripheral Nervous System
PSJ	Pleated Septate Junction
PVDF	PolyVinylidine DiFluoride
Scrib	Scribble
SDS	Sodium Dodecyl Sulfate
SDS-	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SH2/3	Src Homology 2/3, binds phosphotyrosine residues
SJ	(pleated) Septate Junction
TBS	Tris Buffered Saline
TCJ	Tricellular Junction
TJ	Tight Junction
TKM	Tris, KCl (potassium chloride), $MgCl_2$ (magnesium chloride)
TRAF	TNF (tumor necrosis factor) receptor associated factor, a protein signalling domain
uL	MicroLitre
uM	MicroMolar
um	MicroMeter
WD-40	Beta-Transducin Repeats
ZA	Zonula adherens
ZO-1	Zonula Occludens 1

Acknowledgements

I would like to thank my supervisor, Dr. Vanessa Auld, for providing me with the opportunity to pursue a graduate degree in her lab at the University of British Columbia. I am truly grateful for all the help and support she provides with her open door policy to all the lab members. This work would not have been possible without her guidance, knowledge and kindness. Her positive support and encouragement have been invaluable.

My committee members, Dr. Linda Matsuuchi and Dr. Cal Roskelley have been a wealth of resources for ideas and suggestions as well as providing me with motivation and encouragement along the way.

To Dr. Murry Gilbert and the Auld lab past and present, your friendship, support and of course, ideas and research discussion are well and truly appreciated. To Dr. Jacob Hodgson, who provided me with so much help with protein biochemistry, again, this work would not have been possible without your valuable insight and knowledge in the field.

To friends made at UBC, thank you for your help, ideas, assistance and most of all your friendship throughout these years. To the Zoology Department staff, especially our graduate secretary, thank you.

Finally, much love and thanks to my family for everything.

1. Introduction

One of the global questions often addressed in development of organisms is how do the formation and maintenance of separate body cavities and structures arise. Integral to this question is the role of permeability barriers. These structures help to control and regulate the movement of molecules through a layer of cells. Permeability barriers are helped by the presence of occluding junctions located between cells. These junctions help to prevent the diffusion of most molecules across the intercellular space. Study of these occluding junctions is an ongoing task, and we are just beginning to understand how they work. In the invertebrate model organism, *Drosophila melanogaster*, we have an opportunity to study the structure of the pleated septate junction, a type of occluding junction exclusive to invertebrates, in a living organism.

1.1 Cell Junctions and Permeability Barriers

Cell attachment, communication, polarity, and the formation of permeability barriers, are all functions of cell junctions. Occluding junctions are a subset of cell junctions which help maintain the integrity of different body cavities; these junctions create a seal which results in the creation of permeability barriers. These are seen in the blood nerve barrier, epithelial and endothelial barriers, and they also help in creating and maintaining cell polarity (Cereijido et al., 2004; Staddon and Rubin, 1996).

Permeability barriers are integral to both vertebrates and invertebrates, though the different organism types also have different methods for creating their permeability barriers. In

invertebrates, septate junctions (SJ) function as permeability barriers, while in vertebrate systems, the tight junctions (TJ) are the functionally analogous structures in epithelial tissues. There are many differences between these two junction types, the most obvious is their apicobasal location relative to the adherens junctions, and their components. Septate junctions are located basally to, whereas tight junctions occur apical to adherens junctions (Tepass et al., 2001).

1.2 Tight Junctions

Tight junctions are a type of vertebrate occluding junction. Depending on cell type, there is quite a bit of variability in the junction, the similarity is in their structure, which are often described as "kissing points". Tight junctions are varied because of the number of different proteins thought to exist at these junctions. There are scaffold proteins such as the zonula occludins and ZO-1, signalling proteins, such as G proteins, involved in the different aspects of assembly and cell polarity, and the structural proteins such as occludins and the large family of claudins, proteins which are implicated in cell adhesion and tight junction assembly (Anderson et al., 1993; Tsukita et al., 2001).

The first tight junction protein discovered, occludin, has four transmembrane domains (Balda and Matter, 2000), and shows homophilic binding in the extracellular space, aiding in TJ strand formation (Anderson, 2001; Fanning et al., 1999). They are known to be differentially phosphorylated, and that overexpression shows an increased number of TJ strands (Denker and Nigam, 1998).

Like occludin, claudins are proteins characterized by four transmembrane domains, and have two extracellular loops. Different claudins are expressed based on tissue type and is thought to be directly involved in the formation of TJ strands and in the ability of TJ's to act as permeability barriers (Tsukita et al., 2001; Sonoda et al., 1999; Anderson, 2001). There is however, little sequence homology between the various known claudins (Anderson, 2001), and heterophilic binding between different types of claudins have been observed (Furuse et al., 2001).

Recently, another barrier protein in vertebrates had been identified called tricellulin. Tricllulin appears at the corners where three cells meet (the vertebrate tricellular junction) and has some structural similarity to occludin (Ikenouchi et al., 2005). This protein however does not resemble Gliotactin and does not appear to have a Drosophila homolog.

1.3 Paranodal Junctions

Another junction in vertebrates, which acts as an occluding junction, is the paranodal junction (PJ). These junctions resemble and show a high degree of conservation to the invertebrate septate junction. They are found at the paranodal region, flanking the nodes of Ranvier in myelinated axons (Scherer and Arroyo, 2002). They are thought to be similar to the invertebrate septate junction because of their morphology and molecular components (Scherer and Arroyo, 2002). Septate and paranodal junctions have some structural and molecular similarities that have not yet been fully determined. It has been suggested that insect septate junctions took a more specialized role in vertebrates, as the paranodal junction, with tight junctions replacing septate junctions in the epithelia (Hortsch and Margolis, 2003). It is with this idea that we can examine

paranodal junctions in vertebrates and compare the structure and function with that of the invertebrate septate junction.

1.3.1 Paranodal Junctions – Contactin, Caspr and Neurofascin

Contactin, an axonal protein found at paranodal junctions (PJ), is a GPI anchored protein belonging to the Ig superfamily (Ranscht, 1988). This protein is known to complex with another PJ protein called Caspr (contactin associated protein) (Rios et al., 2000), and its absence causes disrupted paranodal junctions, Caspr expression, and localization of K+ channels, Kv1.1 and Kv1.2 (Boyle et al., 2001).

Caspr is another axonal transmembrane protein required for proper septa formation at the paranodal junction (Poliak et al., 2003). It has a proline rich domain known to bind SH3 domains, and is similar to the Drosophila SJ Protein, Neurexin IV (NrxIV) (Peles et al., 1997). It was found that an intercellular region of Caspr has a Protein 4.1 binding domain that stabilizes it in a complex with Contactin (Gollan et al., 2002). It was also noted that Caspr does not have a PDZ binding domain (Arroyo et al., 1999). Another protein, Caspr 2 on the other hand, does have a PDZ binding domain on the C terminus tail, while also having a protein 4.1 binding domain (Poliak et al., 1999). Caspr 2 was found to also associate with K+ channels (Kv1.2, 1.2 and the beta 2 subunit) which are present at the juxtaparanode (the area before the paranode) (Arroyo et al., 1999).

Caspr and Caspr 2, homologues of the septate junction protein Neurexin IV, interact with Protein 4.1B and Protein 4.1R, which are similar to the invertebrate SJ protein, Coracle (Cor), at

paranodal junctions (Denisenko-Nehrbass et al., 2003; Einheber et al., 1997; Bellen et al., 1998). As Neurexin IV and Coracle are known to interact in septate junctions (Ward et al., 1998), Caspr2 has been shown to biochemically interact with Protein 4.1B (Denisenko-Nehrbass et al., 2003). As well, mutations in Caspr and Caspr2 show mislocalization of Protein 4.1B (Poliak et al., 2001). Recently, Caspr 3 and 4 have also been discovered in the central nervous system and appear to have the ability to bind PDZ domains (Spiegel and Peles, 2002).

Neurofascin, a cell surface glycoprotein, has a variety of splice variants like its invertebrate homolog, Neuroglian (Nrg) (Rathjen et al., 1987; Volkmer et al., 1992; Hortsch and Margolis, 2003). Like Neuroglian, Neurofascin has two isoforms, NF186, a neuronal Neurofascin, and NF155, the form expressed on glial cells (Tait et al., 2000). Neurofascin is implicated in axonal guidance (Rathjen et al., 1992; Shiga and Oppenheim, 1991), is found to colocalize with Caspr (Tait et al., 2000), is found to bind the Caspr/Contactin complex on the extracellular tail (Charles et al., 2002), and has ankyrin binding domains (Zhang et al., 1998). The binding of NF155 to Caspr and Contactin also seems to be important in the proper formation of paranodal junctions (Schafer et al., 2004; Charles et al., 2002).

1.4 Septate Junctions

Septate junctions are the invertebrate occluding junction. They are found in epithelial, trachea, gut, and glial cells (Carlson et al., 2000; Tepass et al., 2001). Two different varieties of septate junctions exist, differing in their morphology. Pleated septate junctions arise in tissue originating from ectoderm, and smooth septate junctions arise from tissue originating from the endoderm (Tepass and Hartenstein, 1994). Physically, pleated septate junctions are visible under

electron microscopy, while smooth SJ's are barely, if at all, visible (Tepass and Hartenstein, 1994). In this thesis, SJ will refer to the pleated variety.

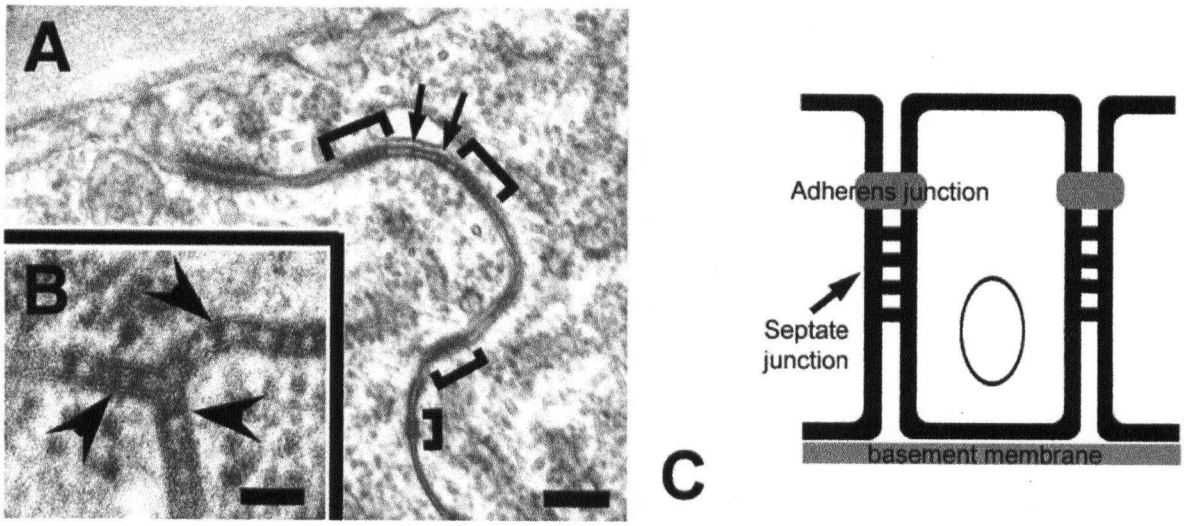
The invertebrate septate junction is often thought to be analogous to the vertebrate tight junction because of their role as barriers. However, unlike the tight junctions, septate junctions occur basal to adherens junctions. Septate junctions are named because of the ladder-like appearance of septa (Figure 1, panel A and B) which occur in spirals around cells (Tepass et al., 2001). As occluding junctions, septate junctions are involved in permeability (Lord and DiBona, 1976), such as in epithelial barriers, or in the blood-nerve-barrier (Knust and Bossinger, 2002). As well, they are also involved in cell polarity because they can block apical/basal migration of membrane proteins (Knust and Bossinger, 2002).

Figure 1: Septate Junction Transmission Electron Micrograph

Panel A shows pleated septate junctions show a "ladder-like" morphology at cell junction points, with clusters shown in brackets and single septa shown with arrowheads. Bar = 100nm. Figure adapted from (Schulte et al., 2003).

Panel B shows the septate junction at the tricellular junction (TCJ), with arrows showing the septa between two cells. Bar = 50nm. Figure adapted from (Schulte et al., 2003).

Panel C shows the basolateral position of the septate junction in relation to the adherens junction in invertebrates.



1.4.1 Septate Junction Development

In Drosophila, septate junction assembly occurs about two thirds through embryogenesis in anticipation of forming permeability barriers later in development. The SJ assembly takes place at approximately stage 14-15 when dorsal closure occurs, although some known SJ components such as discs large and scribble appear earlier, playing what is believed to be an unrelated role in the formation of apical/basal polarity (Tepass et al., 2001; Tepass and Hartenstein, 1994). As SJ components are being continuously discovered, the process of assembly of SJs, on a molecular level, is not yet fully understood. Additionally, at the corners of epithelial cells at the level of SJs, there is a specialized structure called the "tricellular plug", which forms at the convergence of three neighbouring cells to form a permeability barrier (Graf et al., 1982). This is important for the prevention of diffusion between compartments.

1.4.2 Septate Junction Function

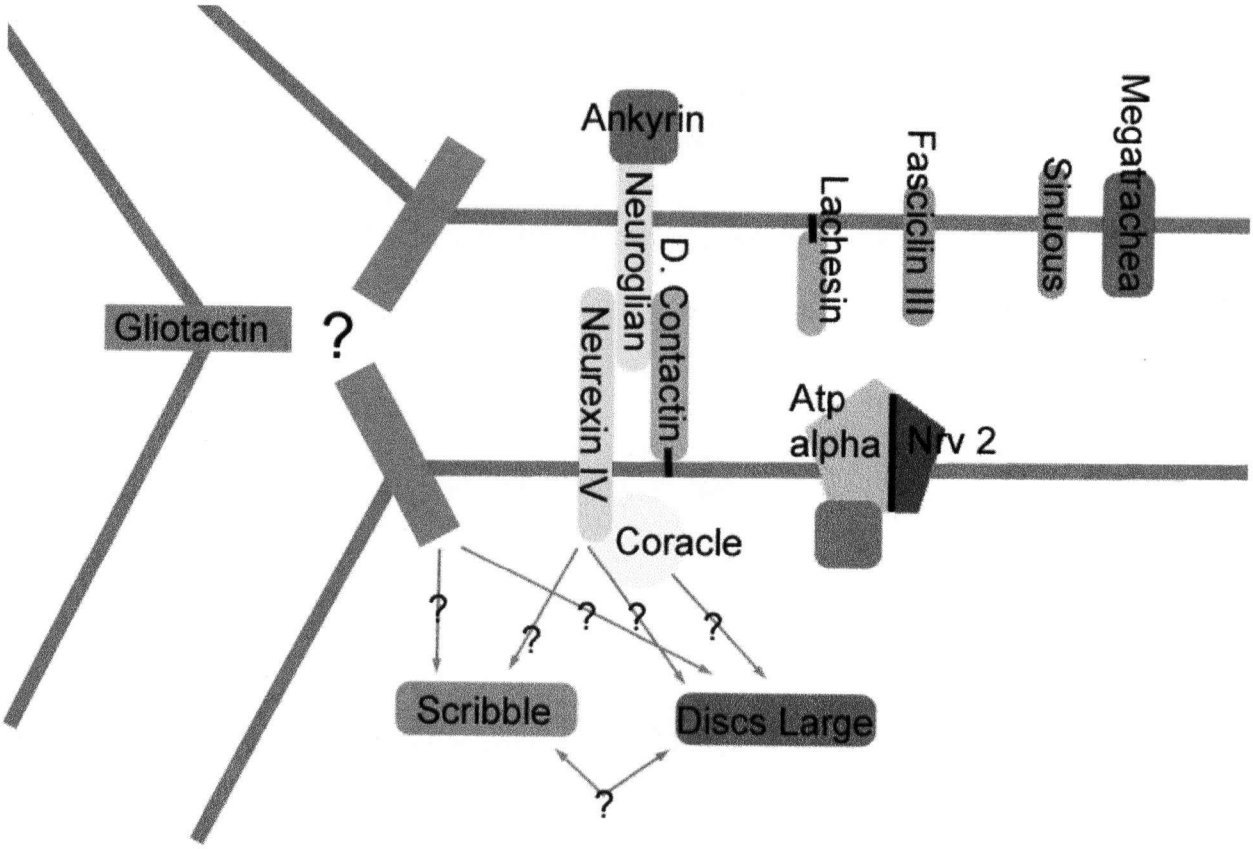
Because they act as a physical barrier, septate junctions are thought to function in cell adhesion, and have several functions in the formation and maintenance of apicobasal cell polarity (Tepass et al., 2001). Additionally, the tricellular junction (TCJ) is believed to be an organization centre for the SJ strands, since disrupting the TCJ shows a decrease in the number of septa, as well as increased permeability of the SJ, which results in compromised SJ's. (Schulte et al., 2003).

1.4.3 Septate Junction Components

While new components of SJ's are continuously being discovered, there are several characteristic proteins such as Neurexin IV, Coracle and Discs Large, as well as Scribble, Fasciclin III, Neuroglian, Gliotactin, and Ankyrins which are widely believed to be at the SJ due to known interactions (Hortsch and Margolis, 2003). In addition, there are the more recently discovered components of septate junctions such as the α and β subunits of the Na^+/K^+ pump, Lachesin, and the claudins, Megatrachea, Sinuous (Wu et al., 2004; Genova and Fehon, 2003; Llimargas et al., 2004; Behr et al., 2003). How these proteins all interact together is one aspect that is unknown and important for examining the mechanics of the septate junction. Please refer to Figure 2 for diagram.

Figure 2: Model of the Septate Junction

This shows a model of the possible septate junction components and possible interactions. Grey lines indicate potential interactors and the question mark indicates that the Gliotactin interaction is yet unknown. Note that Ankyrin is has not yet shown to be at the septate junction but is theorized to be present due to known interactors as shown in the figure.



Neurexin IV (NrxIV) is a transmembrane protein that has a discoidin domain, EGF domains and Laminin G domains, whose functions are yet to be determined, on the extracellular portion, while it has a PDZ binding domain and a Ferm binding domain on the intercellular portion (Einheber et al., 1997; Bellen et al., 1998). The Ferm binding domain (refer to NrxIV in Figure 2, black dot on intracellular domain) is known to interact with the SJ protein, Coracle (Baumgartner et al., 1996). Additionally, without NrxIV, Cor is mislocalized at the SJ (Baumgartner et al., 1996). Drosophila embryos, which have null mutations in NrxIV, have defective SJ's, leading to a disruption in barrier formation(Baumgartner et al., 1996). This leads to paralysis, and subsequent death of Drosophila embryos due to a leaky blood-nerve-barrier. NrxIV null mutants also show mislocalization of other septate junction proteins such as Coracle and Gliotactin (Baumgartner et al., 1996; Schulte et al., 2003).

Coracle, a cytoplasmic protein, is an invertebrate ortholog of Protein 4.1, a structural protein (Fehon et al., 1994). In vertebrates, Protein 4.1 is known to interact with the cytoskeleton, however, Cor seems to lack this function as it is missing the spectrin-actin binding domain present on Protein 4.1 (Ward et al., 2001; Woods et al., 1996a). Mutants of Cor have problems in dorsal closure, perhaps as Cor mutants have a defective septate junction and dorsal closure involves the fusion of epithelial cells (Fehon et al., 1994). As well, Cor mutants also dominantly suppresses ellipse (EGF receptor) mutations in Drosophila eye development (Fehon et al., 1994). Interestingly, a highly conserved domain on the Coracle protein (similar to Protein 4.1), the Ferm (Protein 4.1, Ezrin, Radixin, Moesin) domain, binds NrxIV, and was found to be sufficient for SJ barrier formation (Ward et al., 2001).

Fasciclin III (Fas III), is a transmembrane glycosylated protein, and while it has been a known SJ protein for some time, its function at the SJ is not yet known as null mutants have no discernable SJ phenotype (Patel et al., 1987). It is known that Fas III is a cell adhesion molecule with three Ig domains and that it binds in a homophilic manner (Snow et al., 1989; Patel et al., 1987). However, proteins that bind Fas III are not known, though it was found that Dlg and Lachesin are required for proper Fas III localization (Woods et al., 1996b; Llimargas et al., 2004).

One of the most recently discovered SJ components is the GPI (glycosylphosphatidylinositol) anchored protein, Lachesin (Llimargas et al., 2004; Karlstrom et al., 1993). Lachesin is first expressed during neurogenesis (Karlstrom et al., 1993), and has three Ig domains plus the GPI anchor, suggesting it may have a role in adhesion due to its similarities to Neuroglian and other cell adhesion molecules. Lachesin was later found to localize to the SJ and has a role in epithelial integrity and tracheal tube size control (Llimargas et al., 2004). An in vitro study showed homophilic properties of this protein, and co-localization experiments showed the mislocalization of known SJ proteins in Lachesin mutants, with Cor, Dlg, Scrib, NrxIV, Nrg and Fas III showing basolateral spreading and no longer being tightly localized to the SJ region (Llimargas et al., 2004).

Neuroglian is a transmembrane glycosylated protein that belongs to the Ig superfamily, similar to the vertebrate neuronal cell adhesion molecules (Bieber et al., 1989). It has six Ig domains and five fibronectin domains on the extracellular portion (Bieber et al., 1989). Neuroglian mutants have compromised septate junctions and do not survive past embryogenesis (Genova and Fehon, 2003). There are two splice variants of the Neuroglian protein which differ in their

cytoplasmic domains, and are either neuronal specific (longer splice variant) or expressed in a wide range of other tissues, including the epithelia (shorter splice variant) (Hortsch et al., 1990). Nrg, found at the SJ, has a highly conserved ankyrin binding site, and the non-neuronal Nrg interacts with echinoid, a cell adhesion molecule involved in EGFR signalling (Hortsch and Margolis, 2003; Bieber et al., 1989; Islam et al., 2003; Dubreuil et al., 1996). Interestingly, neurofascin, a vertebrate homolog of Nrg, does not show ankyrin binding when the highly conserved sequence on the intercellular tail of neurofascin, FIGQY (single aa code), is tyrosine phosphorylated, leading to a decrease in cell-cell interaction (Tuvia et al., 1997). However, this may not be the case for Nrg (Hortsch et al., 1995).

Ankyrins are membrane adapter proteins found in the cytoplasm, and are thought to be at the SJ because of known interactors. They contain many ankyrin repeats and have a spectrin binding domain, enabling it to cross link with actin and molecular scaffold proteins (Bennett and Baines, 2001). Ankyrins have the ability to bind to Na^+/K^+ pumps (Nelson and Veshnock, 1987; Zhang et al., 1998), and Neuroglian (Bouley et al., 2000). If ankyrins are at the SJ as suspected, it would indicate that the SJ could also play a role in cell structure as ankyrins would provide the link with the cytoskeleton.

One of the more recently discovered septate junction components are the α and β subunits of the sodium/potassium ATP-ase pump; the Na^+/K^+ ATP-ase pump components found at SJ's consists of the ATPα, and the Nervana 2 β subunit (Genova and Fehon, 2003). Null mutants of the Na^+/K^+ pump result in a disrupted barrier function of the SJ, missing septae, and tracheal tube size defects which are seen in many other mutations of known SJ components (Genova and

Fehon, 2003; Paul et al., 2003). The Na^+/K^+ pump is known to interact with ankyrin (Bennett and Chen, 2001), which leads to the speculation that the cytoskeleton is linked to the SJ.

Drosophila contactin is another SJ protein, with a lectin domain, six Ig domains, and four fibronectin domains (Hortsch and Margolis, 2003). This extracellular protein, like Lachesin, is covalently anchored to the membrane via a GPI anchor (Walsh and Doherty, 1991).

The next two septate junction proteins are unique because they are similar to claudins. These proteins contain four transmembrane domains, characteristic of tight junction claudins, although the septate junctions do not show any TJ-like strands despite these claudin-like proteins. The first claudin-like protein found at SJ's was Megatrachea (Mega), which has the four characteristic transmembrane domains(Behr et al., 2003). As well, Mega has a potential PDZ binding domain on the C terminus (Behr et al., 2003). Megatrachea mutants have compromised septate junctions (and thus epithelial barriers), as well as mislocalized SJ markers such as NrxIV, Cor, Sinuous, and FasIII, (Wu et al., 2004; Behr et al., 2003). The second claudin-like protein found at SJ's is Sinuous (Sin), also containing the characteristic four transmembrane domains and a potential PDZ binding domain on the C terminus (Wu et al., 2004). Sinuous is also required for proper SJ barrier formation, tracheal development, and localization of known SJ proteins, Cor, NrxIV, ATPα, and Dlg in the epidermis, hindgut and trachea, though strangely, not in the salivary gland (Wu et al., 2004). Which proteins these two claudins interact with have not yet been determined.

Discs Large (Dlg), was first discovered as a tumor suppressor gene expressed about half way through development (Watson et al., 1994). The protein was discovered to have at least ten

different isoforms, and localizes to the septate junction (Woods et al., 1996a; Woods and Bryant, 1991). Dlg is also necessary for proper localization of known SJ proteins, Coracle and Fasciclin III (Woods et al., 1996b). The Dlg protein is cytoplasmic, as it lacks a signal sequence, and has guanylate kinase (GUK) and SH3 domains (Woods and Bryant, 1991). Later, PDZ domains, known to be involved in protein-protein interactions, were found on Dlg, as well as a binding site for the Protein 4.1 family (Cho et al., 1992; Lue et al., 1994a; Marfatia et al., 1996; Morais Cabral et al., 1996). Dlg was also the first Maguk (membrane associated guanylate kinase homologs) protein found. Maguk proteins are involved in cell junctions and are known to have some importance in cell signalling; they are characterized as possessing GUK, SH3 and PDZ domains (Anderson, 1996; Caruana, 2002).

In 1997 the presence of three types of PDZ domains on Dlg, which were termed type 1, 2 and 3, were discovered (Hough et al., 1997). These PDZ binding domains are required for localization to the membrane, and the PDZ type 2 domain is required for SJ localization. The PDZ domains are particularly interesting as it makes Dlg a candidate for Gli binding, especially as Dlg appears to be enriched at the tricellular corners (Schulte et al., 2006).

Another protein with many PDZ domains, Scribble (Scrib), is also at the septate junction (Hortsch and Margolis, 2003). Scribble is a protein whose absence causes mislocalization of apical proteins, and is also a protein that has multiple PDZ type 1 domains on the carboxy terminus and many leucine rich repeats (also known to be involved in protein-protein interactions) on the amino end (Bilder and Perrimon, 2000). These potential protein interaction domains would seem to suggest that Scrib binds other proteins at the SJ, and while some have been identified (Discs Large, and Lethal Giant Larvae) (Bilder et al., 2000), it could potentially

have other as yet unknown interactions. Of course the presence of PDZ domains makes it a potential Gliotactin binding partner.

1.4.4 Septate Junction Component Interdependency

When one component of the septate junction is removed or mutated, often, the result is the disruption of other SJ components. For example, Coracle is mislocalized when Neurexin IV is disrupted (Baumgartner et al., 1996), Megatrachea is mislocalized in Cor and NrxIV mutant embryos, (Behr et al., 2003), and the mislocalization of FasIII is observed when Dlg and Lachesin are disrupted (Woods et al., 1996a; Llimargas et al., 2004). NrxIV, Cor, Sinuous and FasIII are dependant on Megatrachea (Behr et al., 2003), while Cor, NrxIV, ATPα, and Dlg are dependant on Sinouous (Wu et al., 2004). There is also mislocalization of Discs Large, Cor, NrxIV, Scribble, Fasciclin III and Neuroglian in Lachesin mutant embryos (Llimargas et al., 2004).

1.4.5 Gliotactin

Gliotactin (Gli), the protein examined in this thesis, is a transmembrane protein present exclusively at the tricellular junction, at the level of septate junctions (see figure 3) (Llimargas et al., 2004; Schulte et al., 2003). As the tricellular junction is thought to possibly organize the septate junction (Schulte et al., 2003), I am interested in understanding the role of Gli, and specifically address what proteins are bound to Gli. Characterization of Gli shows a type 1 PDZ recognition domain (Schulte et al., 2003), a non-functional choline-esterase-like domain on the extracellular portion of the protein, which may serve in cell adhesion, a putative EF hand

domain, which may bind calcium ions, a potential TRAF domain, which may be involved in signalling, potential SH2 or SH3 domains, known to be involved in protein-protein interactions, and possible tyrosine phosphorylation sites (V. Auld, personal communication). It was recently found that this intercellular domain of the protein lacks a fixed 3D structure (Zeev-Ben-Mordehai et al., 2003), though the implications of this has yet to be determined. As well, some genetic analysis shows the possibility that Gli may act as a dimer (Venema et al., 2004). Additionally, Gliotactin mutants show mislocalization of Cor, NrxIV and Dlg, septate junction markers that had spread more basally while still showing enrichment apically (Schulte et al., 2003). These mutants have delayed dorsal closure, the trans-epithelial barriers are compromised, and show a reduced number of septa, possible evidence for the tricellular junction as an organizing factor for septate junctions (Schulte et al., 2003). Dlg and NrxIV, invertebrate homologs of PSD-95, and Neurexin family member, Caspr (Bellen et al., 1998), have been tested for interactions with Gliotactin. NrxIV, Dlg and Cor showed calcium dependant binding to a transgenic Gli (Schulte, 2002 PhD thesis), while Dlg has been shown to complex with, though not directly binding to, Gliotactin (C. MacKinnon, M.Sc. Thesis, 2005).

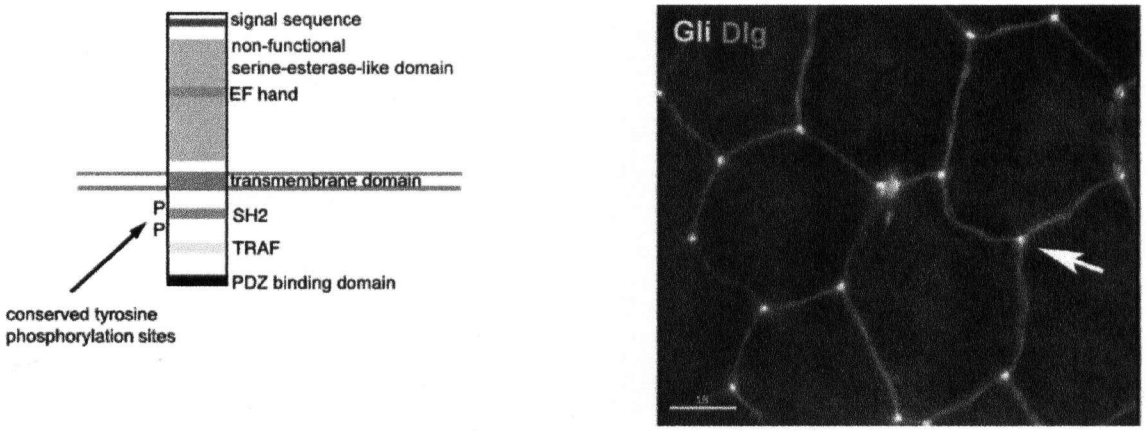
Gliotactin also shows homology (sharing a common developmental origin) to the Neuroligins, and more specifically, to Neuroligin 3 (Nlg3) (Gilbert et al., 2001). Nlg3 is expressed in a range of glia in the CNS and the PNS (Gilbert et al., 2001; Philibert et al., 2000), but interestingly, is not present at the septate-like paranodal junctions (M. Gilbert and A. Khurana, personal communication). Still, Nlg3 can potentially provide clues on molecular binding of its invertebrate homolog, Gliotactin.

Other known interactors of the Neuroligins are S-SCAMs (synaptic scaffolding molecule), binding Nlg via one of its five PDZ domains (Hirao et al., 1998), and CIPP (channel interacting PDZ domain), which also contains several PDZ domains (Kurschner et al., 1998). The S-SCAM interaction hints at Gliotactin binding partners, as a GST pulldown showed Magi, an S-SCAM homologue, potentially interacting with the C terminus end of Gli (C. MacKinnon, MSc. thesis, 2005).

Figure 3: Gliotactin and Gliotactin Localization in Epithelial Cells

Panel A shows a model of Gliotactin with domains of interest labelled. Note that the second phosphotyrosine is a potential SH2 domain.

Panel B shows Discs Large, a SJ marker in red (anti-Dlg primary antibody with fluorescent secondary antibody) and Gliotactin in green (anti-Gli primary antibody with fluorescent secondary antibody) in Drosophila larval epidermal cells. Note that Gli is shown to exist at the tricellular junction (appears yellow) and shows the characteristic pattern on Gli at the SJ in epithelial cells. Sizebar is 15 um. Adapted from Schulte et al., 2006.



It remains to be seen which other known, or unknown SJ proteins interact with Gli. Though PDZ containing proteins are likely candidates, Dlg, while present in a complex with Gli, does not appear to directly bind (C. MacKinnon, M.Sc. Thesis, 2005). It remains to be seen if Scribble, which has multiple type 1 domains (Bilder and Perrimon, 2000), binds to the intercellular portion of Gli.

1.5 Neuroligins

In vertebrates, the Neuroligins are a family of transmembrane cell adhesion proteins that are most closely related to the invertebrate septate junction protein, Gliotactin (Gilbert et al., 2001). They bind some α-Neurexins and β-Neurexins, neuronal cell surface proteins which play a role in adhesion and signalling, in a calcium dependant manner (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Nguyen and Sudhof, 1997; Boucard et al., 2005). They also bind PSD-95, a homolog of Drosophila protein Discs Large, at a PDZ binding domain (Irie et al., 1997; Nguyen and Sudhof, 1997). Neuroligin 1 (Nlg1) was specifically found to form synaptic junctions through interactions with β-Neurexins (Song et al., 1999). In a significant finding, Nlg 1 and 2 with their receptor, β-Neurexin, were found to initiate the formation of presynaptic structures showing that neuroligins are able to initiate and are required for synaptic development (Scheiffele et al., 2000).

At first, the Neuroligins (1-3) were thought to occur only in the CNS (Ichtchenko et al., 1995; Ichtchenko et al., 1996). However, this concept was revised when the discovery of a novel human Neuroligin, Nlg4, was found in many different tissue types, including the heart, liver,

pancreas, skeletal muscle, but only at low levels in the brain (Bolliger et al., 2001). Furthermore, Nlg3 was later found in glia both in the CNS and PNS (Gilbert et al., 2001).

Neuroligin 3 was first believed to be at the paranodal junction, but was found to not be present (M. Gilbert, unpublished data). However, this does not exclude the other Neuroligins such as 2 and 4, which could be at the paranodal junction. Although there are obvious differences in the vertebrate paranodal junctions when compared to the invertebrate septate junctions, it isn't unreasonable to believe that analogous components can provide clues to the interactions in both systems.

Recently, Nlg3 and Nlg4 have been implicated in a human disease, autism and Asperger syndrome (Jamain et al., 2003). The families analyzed either had a shortened Nlg4, or a point mutation in Nlg3 which converted an arginine to a cysteine in a section of the non-functional serine-esterase-like domain which is thought to show calcium binding properties (Jamain et al., 2003). This is interesting as the Drosophila septate junction counterpart, Gliotactin has shown calcium dependent binding to NrxIV and Coracle (Schulte, 2002 PhD thesis).

1.6 Specific Aim of the Thesis

Gliotactin is currently the only known protein present at in septate junctions that localizes exclusively to the tricellular junction. We know that when Gliotactin is disrupted, other septate junction components become mislocalized, and the barrier function of the SJ becomes compromised. Loss of Gliotactin leads to a mislocalization of Neurexin IV, Coracle and Discs Large, as well as a reduced number of septa, and septa spread down the basolateral edge

(Schulte et al., 2003). This indicates that Gliotactin may have an important role in either organization or structure of the pSJ, and specifically the TCJ by perhaps organizing or stabilizing the core SJ complex (Schulte et al., 2003). Additionally, an overexpressed HA tagged Gliotactin was shown to biochemically interact with known SJ proteins, Cor, NrxIV and Dlg in a calcium dependant manner (Schulte et al., 2003).

It is the aim of this thesis is to examine if Gliotactin interacts with other septate junction components, and to discover other potential Gliotactin protein interactors in an effort to elucidate further, the role of Gliotactin at the TCJ.

Hypothesis: Gliotactin interacts in a calcium dependent manner with other septate junction proteins.

Objectives of the thesis:

1. Look for Gliotactin protein interactors by testing potential known septate junction protein candidates.
2. Look for unknown Gliotactin protein interactors.

Methods to achieve thesis objectives:

1. Use immunoprecipitation of membrane protein extracts of *Drosophila melanogaster* to purify the protein of interest and then probe the resulting blots with various antibodies to known septate junction proteins.

2. Use glutathione-agarose beads bound to a protein construct of GST fused to the C terminus of Gliotactin in a GST pulldown using membrane protein extracts of Drosophila melanogaster. Probing of the resulting blots with various antibodies to known septate junction proteins will give supportive in vitro results of co-immunoprecipitation experiments.

3. Use glutathione-agarose beads bound to a protein construct of GST fused to the C terminus of Gliotactin in a GST pulldown using membrane protein extracts of Drosophila melanogaster. Resulting blots would be stained and prominent proteins that had bound to the C terminus of Gliotactin would be sent for sequencing to attempt to identify unknown proteins capable of binding Gliotactin.

These methods for identifying Gliotactin interactors have the limitations of a biochemical system. Immunoprecipitations show us entire organism protein interactions of Gli, not solely the Gli interactors in the TCJ, and GST pulldowns show only potential interactors of the C terminus. However these biochemical experiments are intended to complement other current research on Gliotactin. Additionally, the use of membrane protein extracts from the collection of adult and embryo Drosophila should help show native interactions occurring in the animal itself, with less risk of showing artifacts of a purely in vitro system of identifying protein-protein interactions.

2. Materials and Methods

2.1 Fly Strains Used

Oregon R – a wild type lab strain (OrgR)

w^{1118} – Drosophila lacking eye pigment, making the eye appear to be white in colour. Used here as a pseudo wild type strain, lab stock #107

(w^{1118} P{PTT-GA}Nrg^{G00305} [Nrg^{G305}]) – expresses GFP tagged Neuroglian (NrgGFP), lab stock # 138, Bloomington Stock # 6844. Drosophila strain from: (Morin et al., 2001).

w; UAS-$gli^{PDZ\Delta\#1\text{-}HA}$; da.G32 – expressing HA-tagged gliotactin with the last three amino acids truncated to exclude the last PDZ domain (HA-gliΔPDZ). Drosophila strain from: Schulte et al., 2003.

w; UAS-$gli^{wt3\text{-}HA}$; ga.G32 – expressing HA tagged gliotactin (HA-gli). Drosophila strain from: Schulte et al., 2003.

2.2 Fly Growth Conditions

Flies were raised at 22°C, on standard fly food media (12.6% agar, 31 mM potassium sodium tartrate, 5 mM calcium chloride, 350 mM dextrose, 175 mM sucrose, 3.2% w/v yeast, 7.6% w/v cornmeal, 2.5% ethanol, 480 mM tegosept, 0.5% ampicillin, 0.15% tetracycline). Adult collection involved exposing flies to carbon dioxide and collecting unconscious adults into eppendorf tubes. Embryo collection involved growth of flies in a population chamber on apple juice plates: apple juice agar media (2.6% agar, 25% apple juice, 73 mM sucrose, 10 mM Methyl 4-hydroxybenzoate) in large (Falcon, #351058, Franklin Lakes, NJ, USA) Petri dishes. Flies laid eggs on apple juice media, and apple juice plates were collected after 12 hours. Embryos were collected by dechorination through applying a 50% bleach solution to apple juice plates, which were allowed to sit for five minutes. Collection proceeded by filtering the media through fine nylon mesh, then washing off remaining embryos on apple juice plates with 0.05%

Triton X-100 solution. Embryos were then suspended in a small amount of 0.05% Triton X-100 solution and collected with a Pasteur pipette into eppendorf tubes.

2.3 Membrane Preparations

Membrane preparations were made from the following strains: w; UAS-*gli*$^{wt3\text{-}HA}$; ga.G32 adults, w; UAS-*gli*$^{PDZ\Delta\#1\text{-}HA}$; da.G32 adults, w^{1118} adults, OregonR embryos, and NrgGFP adults. Membrane extracts were prepared using the method described by Zhang and Hsieh (2000). This method involved the extraction of membrane and membrane bound proteins by centrifugation through a density gradient, and all steps were performed on ice or at 4°C. Approximately 4-6 mL of Drosophila adults or embryos were homogenized using a handheld 15 mL glass homogenizer with 2-4 mL of detergent-free lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 0.25 M Sucrose, 0.1 mM DTT, 1 mM PMSF, 2 ug/mL leupeptin, 2 ug/mL pepstatin), chosen because it would not dissociate integral proteins from cell membranes. Centrifugation at 1000G followed, to sediment particulates. The resulting supernatant was mixed with 15.2 mL of 2.5 M Sucrose in TKM (50 mM Trizma Base, 150 mM potassium chloride, 5 mM magnesium chloride, pH 7.5), and subsequent layers of 12.5 mL 2.0 M and 7.0 mL 0.5 M Sucrose solutions (of the 2.5 M Sucrose in TKM diluted with lysis buffer) were layered on top. A 100,000 G spin for 2.5 hours followed and the result was isolated membranes appearing at the interface of the 0.5 M and 2.0 M layers. This was collected using a Pasteur pipette and mixed with 2 volumes of lysis buffer. A final centrifugation to wash the isolated membranes was performed at 30,000 G for 30 minutes. The membrane pellet was resuspended in 200 uL of lysis buffer and total protein concentration was then determined using the Bradford dye-binding assay.

The Bradford assay is based on coomassie dye binding of proteins in a solution. More protein present results in a stronger blue colour, and thus protein concentration is tested against a standard curve of absorbance at 595 nm against known protein concentrations (usually ranging from 0-100 ug/100 uL). In this case, Bradford reagent was prepared using Bio-Rad Protein Assay Reagent (#500-0006, Hercules, CA, USA) by dilution of the concentrate in double distilled water at 1 in 5, and then filtered to remove particulates. Five standards of 0, 20, 60, 80 and 100 ug/uL bovine serum albumin were prepared and 5 mL of Bradford reagent was added. Similarly, the sample(s) tested were usually diluted as appropriate, typically 1 in 10, with 5 mL Bradford reagent added. Standards and samples were mixed by brief vortexing, and the colour was allowed to develop for 5 minutes. Following colour development, absorbance was measured at 595 nm, and a standard curve of absorbance versus protein concentration was generated to compare absorbance of the sample(s) and determine the protein concentration. The membrane extract was then flash frozen with liquid nitrogen and stored at –80°C. Membrane extracts were thawed on ice immediately prior to use.

2.4 GST Pulldowns

Glutathione agarose beads, (Sigma, cat #G4510, St. Louis, MO, USA), 30 uL in a 50:50 slurry in PBS (1.86 mM $NaH_2PO_4*H_2O$, 8.41 mM Na_2HPO, 174.9 mM NaCl, pH 7.2-7.6), were prebound overnight with 75 ug GST-Gli-cter (a construct designed by C. MacKinnon containing the intracellular portion of Gliotactin fused to GST - C. MacKinnon, 2005, MSc. Thesis) were incubated in 250 uL lysis buffer (10 mM Tris-HCl, 4 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, 2 ug/mL leupeptin, 2 ug/mL pepstatin) with varying calcium concentrations of either 0.0, 0.05, 0.1 or 0.2 uM. Calcium concentration was determined by the

on-line chelator calculator which was located at:

http://www.stanford.edu/~cpatton/webmaxCS.htm. After the overnight incubation, beads were washed three times with lysis buffer, then incubated with 150 ug w1118 adult or OregonR embryo membrane preparations for 3 hours at 4°C with gentle agitation. Following incubation, beads were washed 3-5 times with lysis buffer. After the final wash, 30uL of Laemmli loading buffer (2% SDS, 100 mM DTT, 50 mM Tris-Cl pH 6.8, 10% glycerol, 0.05% Bromophenol blue, 2% 2-mercaptoethanol) was added, and the beads heated for 15 minutes at 65°C. The loading buffer/protein mixtures were then loaded onto 8% 0.75 mm SDS-PAGE gels and run at 80-110V with Laemmli running buffer (10X running buffer: 250 mM Trizma base, 1.92 M glycine, 1%SDS) in a C.B.S. Scientific Co. mini gel box (#M6V-202, Del Mar, CA, USA).

2.5 Immunoprecipitation

Calbiochem Protein G agarose beads (cat #539207, San Diego, CA, USA), 30 uL in a 50:50 slurry in PBS (1.86 mM $NaH_2PO_4*H_2O$, 8.41 mM Na_2HPO, 174.9 mM NaCl, pH 7.2-7.6), were prebound overnight with 715 ug of 1F6.3 (mouse monoclonal anti-Gliotactin, hydridoma cell line rederived by ImmunoPrecise Antibodies Ltd., Victoria, BC, Canada) antibody in 250 uL PBS (10X PBS is: 13.7 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate, 1.4 mM potassium phosphate). Negative control was 20 ug mouse IgG (Jackson Immunoresearch #015-000-001) in 250 uL PBS. Follwing the overnight incubation, bead-Ab complexes were incubated in 250 uL lysis buffer (10 mM Tris-HCl, 4 mM EDTA, 1 mM EGTA, 150 mM NaCl or 300 mM NaCl where specified, 0.5% Triton X-100, 1mM PMSF, 2 ug/mL leupeptin, 2 ug/mL pepstatin) with a final calcium concentration of 0.0, 0.05, 0.1 or 0.2 uM and 150 ug w1118 adult, Nrg-GFP, or OregonR embryo membrane preparations for 3 hours

at 4°C with gentle agitation. Following incubation, beads were washed 3-4 times with appropriate lysis buffer. Laemmli loading buffer was added, and the beads heated for 15 minutes at 65°C. Loading buffer with immunoprecipitated proteins were then loaded onto 8% 0.75 mm SDS-PAGE gels and run at 80-110 volts with Laemmli running buffer in a C.B.S. Scientific Co. mini gel box.

2.6 Western Transfer and Immunoblotting

SDS-PAGE gels were transferred to nitrocellulose (Amersham BioSciences Hybond #RPN303D) or PVDF (Amersham BioSciences Hybond-P #RPN1416F, Piscataway, NJ, USA) using semi-dry transfer. SDS-PAGE gel, membrane and thick blotting paper (Bio-Rad Protean XL #1703969, Hercules, CA, USA) were soaked for 5 minutes in transfer buffer (25 mM Trizma base, 150 mM glycine) before being arranged on the Trans-Blot SD semi-dry transfer cell (Bio-Rad #170-3940, Hercules, CA, USA), as per manufacturer's directions, and then allowed to seperate at 10V for 15 minutes followed by 15V for 15 minutes. Blots were then blocked overnight with 5% skim milk in TBS (10X TBS is: 200 mM Trizma base, 1.37 M NaCl, HCl to pH 8.0) solution. Primary antibodies were allowed to bind for 2 hours at 4°C with gentle agitation. Primary antibodies of rabbit anti-Neurexin IV (gift from M. Bhat) and 4F3 (from University of Iowa Hybridoma Bank), mouse anti-Discs Large were used at a 1 in 500 dilution, while 1F6.3, mouse anti-Gli was used at 1 in 100 dilution, HA.11 mouse anti-HA (Covance Research #MMS-101R, Princeton, NJ, USA) was used at 1 in 1000 dilution, and BP104 (from University of Iowa Hybridoma Bank), mouse anti-Nrg, was used at 1 in 10 dilution. After incubation, blots were washed three times in TBS for 15 minutes. Membranes were then incubated for an hour with secondary antibodies Peroxidase-conjugated AffiniPure Goat Anti-

Mouse IgG (Jackson Immunoresearch #115-035-003, West Grove, PA, USA) or Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson Immunoresearch #111-035-045, West Grove, PA, USA) at an end dilution of 1 in 5000. Membranes were again washed three times with TBS for 15 minutes, then washed twice for 5 minutes, first in TBS with 0.5% Tween-20, then again with TBS before developing with Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer, cat #NEL105, Wellesley, MA, USA) according to manufacturer's directions.

2.7 Coomassie Staining

Proteins transferred to PVDF membrane from SDS-PAGE gels were coomassie stained using Gel Code Blue (Pierce, cat #24590, Rockford, IL, USA) according to manufacturer's directions.

2.8 Protein Sequencing Preparation

Proteins from w1118 adults or OregonR embryos were prepared by GST pulldown as described above, then transferred to PVDF membrane and stained with Gel Code Blue as described above. Bands of interest were indicated and the blot sent to NAPS (Nucleic Acid and Protein Sequencing) at the University of British Columbia for N-terminal protein sequencing based on the Edman degradation method. Alternatively, protein samples were also prepared by collecting 6-12 of the same protein bands from identical GST pulldowns, using a new razor blade (VWR Industrial Razor Blades #55411-050, West Chester, PA, USA) to cut out bands from PVDF membrane blots, and these bands were then sent to University of Victoria – Genome BC Proteomics Centre for N-terminal sequencing based on the Edman degradation method.

3. Results

3.1 Idenitfying Gliotactin Protein Interactors from Known Septate Junction Proteins.

Gliotactin is the only identified protein exclusively found at the TCJ at the level of septate junctions. Determining Gli interactors may help elucidate its role and function at the TCJ. A biochemical approach was used to determine which proteins interact with Gli, although this approach shows all Gli interactions in the animal. Previous preliminary immunoprecipitation work by Joost Schulte (2002, PhD. Thesis) with modified Gli proteins (HA-gli and HA-gliΔPDZ) had shown possible interactions of Gli with Discs Large, Neurexin IV and Neuroglian. Both immunoprecipitation and GST pulldowns were used as a test for biochemical protein interactions.

3.1.1 Overexpressed HA Tagged Gliotactin is Unstable in Drosophila in the Long Term

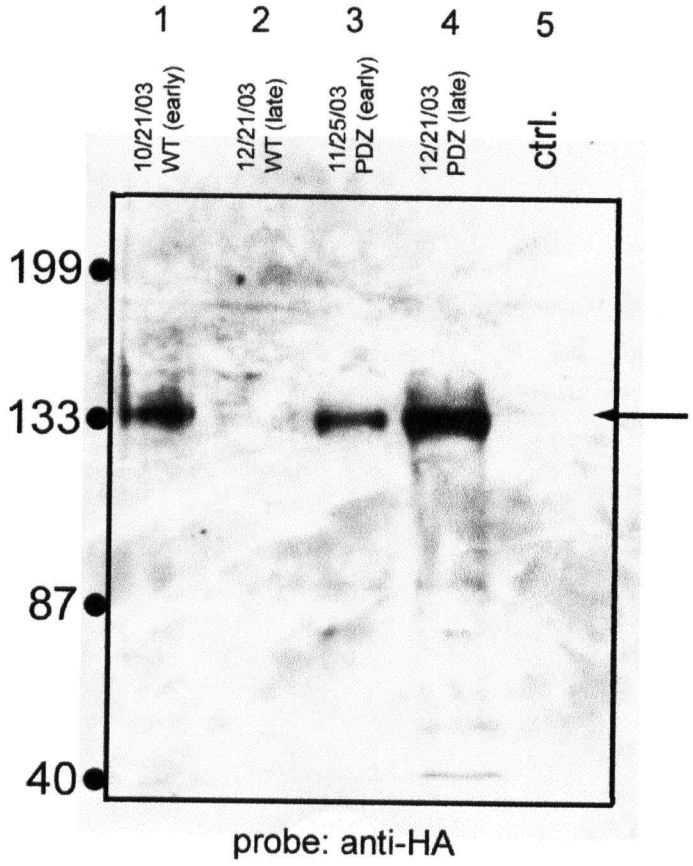
Previously, no reliable anti-Gliotactin antibody existed, therefore an epitope tagged Gliotactin was the only possible method for studying proteins interactions in Drosophila using immunoprecipitation. The constructed transgenic flies expressed HA-tagged, full length Gliotactin and Gliotactin with the PDZ recognition motif deleted (the last 3 amino acids were deleted) were initially used to test biochemical interactions. These lines expressing HA tagged versions of Gliotactin and a Gli mutant lacking the PDZ domain were generated by Joost Schulte (Schulte et al., 2003). The constructs were expressed in a wild type (w1118) background using the UAS/Gal4 system (Brand and Perrimon, 1993), using the ubiquitous daughterless-Gal4 driver. The UAS/Gal4 system is a yeast derived expression system, where the Gal-4

transcription factor causes expression of genes through binding with the upstream activating sequence (UAS). This system allows for strong ubiquitous expression of HA-tagged Gli, facilitating detection and biochemical work, though expressing the protein at higher than normal levels in the animal.

However, the expression of HA-tagged Gliotactin was found to be unstable when continually expressed, as HA-tagged Gli rapidly disappeared from the transgenic Drosophila lines in a span of as little as two months as seen in figure 4. The HA-tagged Gli ΔPDZ also proved to be unstable in the months following the disappearance of the HA-tagged Gli, though lasting longer in Drosophila than HA-tagged Gli, showing extensive degradation of the protein in the Drosophila lines (figure 4 does not yet show this degradation as it only became apparent in March-April of 2004). These Drosophila lines with the HA-tagged versions of Gli were not comparable to wild type in growth and reproduction as the transgenic animals had leg defects and did not grow at the same rate as wild type Drosophila (J. Schulte, PhD. thesis, 2003; D. Venema, PhD. thesis, 2003). It is thought that because of the disadvantages of carrying the transgene, overexpression of Gli may have created a selective pressure for the elimination of the transgenic protein. Transient expression may have helped to avoid this problem, however, it would have been an inconvenient system to use as large quantities of protein are required to perform biochemical studies. As well, transient expression would have required individual scoring of thousands of Drosophila. It is for these reasons that endogenous Gli was examined instead, despite the need to collect larger quantities of Drosophila adults and embryos to obtain enough Gli protein to study.

Figure 4: HA Tagged Gliotactin Expression is Unstable in Drosophila.

Whole adult extracts from strains expressing HA tagged Gli and HA tagged GliΔPDZ, driven by the daughterless-GAL4 line, probed with mouse anti-HA. Lane 1 shows the presence of HA tagged Gli while lane 2 shows the disappearance of HA tagged Gli from the same fly strain just two months later. Lane 3 and 4 shows HA tagged GliΔPDZ, not yet degraded. Lane 5 is the control lane, with whole adult extract from w1118 Drosophila, which does not show any HA-tagged proteins. Arrow indicates HA-tagged Gli and HA tagged GliΔPDZ (both approximately the same molecular weight), and size markers in kDa are shown at left.



3.1.2 Gliotactin Interacts with Discs Large

In previous work, it was shown that over-expressed HA-tagged Gliotactin interacts with Discs Large in a calcium dependent manner in immunoprecipitation experiments (Schulte, PhD thesis, 2003). My aim was to test if this was true with native Gli, using a rederived monoclonal antibody to Gli.

To test if Gliotactin and Discs Large are in the same protein complex, immunoprecipitation of membrane preparations from wild type Drosophila adults and embryos were carried out using a newly rederived monoclonal antibody raised to the C terminus of Gliotactin; the original antibody was first used by Auld et al. (1995). These immunoprecipitations were carried out in the presence or absence of calcium due to the calcium dependant interaction seen in previous experiments using the HA-tagged Gli (Schulte, PhD thesis, 2003). The resulting immunoprecipitations using the anti-Gli antibody was then probed with a monoclonal anti-Discs Large antibody, and shows Dlg in the same protein complex as Gli in both adult and embryo Drosophila (figure 5, panel B - adult, D - embryo). This was seen to occur in a calcium dependant manner (n=5 for adult, figure 5, panel B). Dlg occurs in multiple isoforms in Drosophila, with at least 10 known versions (Woods et al., 1996a). As expected, many distinct protein bands appear in the crude membrane protein lane ranging from about 90-133 kDa (figure 5, panel B, D). Multiple Dlg isoforms are able to complex with Gli, in particular proteins of 120, 110 and 97 kDa (Figure 5b, arrows).

With increasing free calcium in the assay, we observe increasing amounts of Dlg, seen in figure 5, panel B. The free calcium concentrations of 0.1 and 0.2 uM were used. At 0.1 uM free

calcium, we observe the presence of Dlg brought down by Gli, and this intensifies as free calcium increases to 0.2 uM (figure 5, panel B, compare lanes 5 and 6). This pattern is reflected in a probe of the same immunoprecipitation using anti-Gli antibody, showing increasing amounts of Gli with increasing calcium concentration from 0.1 uM to 0.2 uM (figure 5, panel C, compare lanes 5 and 6). We see the calcium dependant immunoprecipitation of Gli in the w1118 adult Drosophila.

Immunoprecipitations were repeated with membrane proteins isolated from wild type embryos (n=4), and similarly, Dlg was found in Gli immunoprecipitated complexes (figure 5, panel D). However, the embryo immunoprecipitation seems to be less calcium dependent (see figure 5, compare panel B and D), as anti-Gli probed embryo immunoprecipitations also did not appear to be calcium dependant. In the converse experiment, the immunoprecipitation of Discs Large failed to show the presence of Gliotactin in the subsequent immunoblots even with calcium.

Figure 5: Gliotactin Interacts Biochemically with Discs Large.

Panel A shows a predicted model of the immunoprecipitation, with the antibody binding the C terminus of Gli, and Gli then binding to an unknown intermediary (support for an indirect Gli and Dlg interaction is seen in C. MacKinnon's M.Sc. thesis, 2005), which then binds to Dlg.

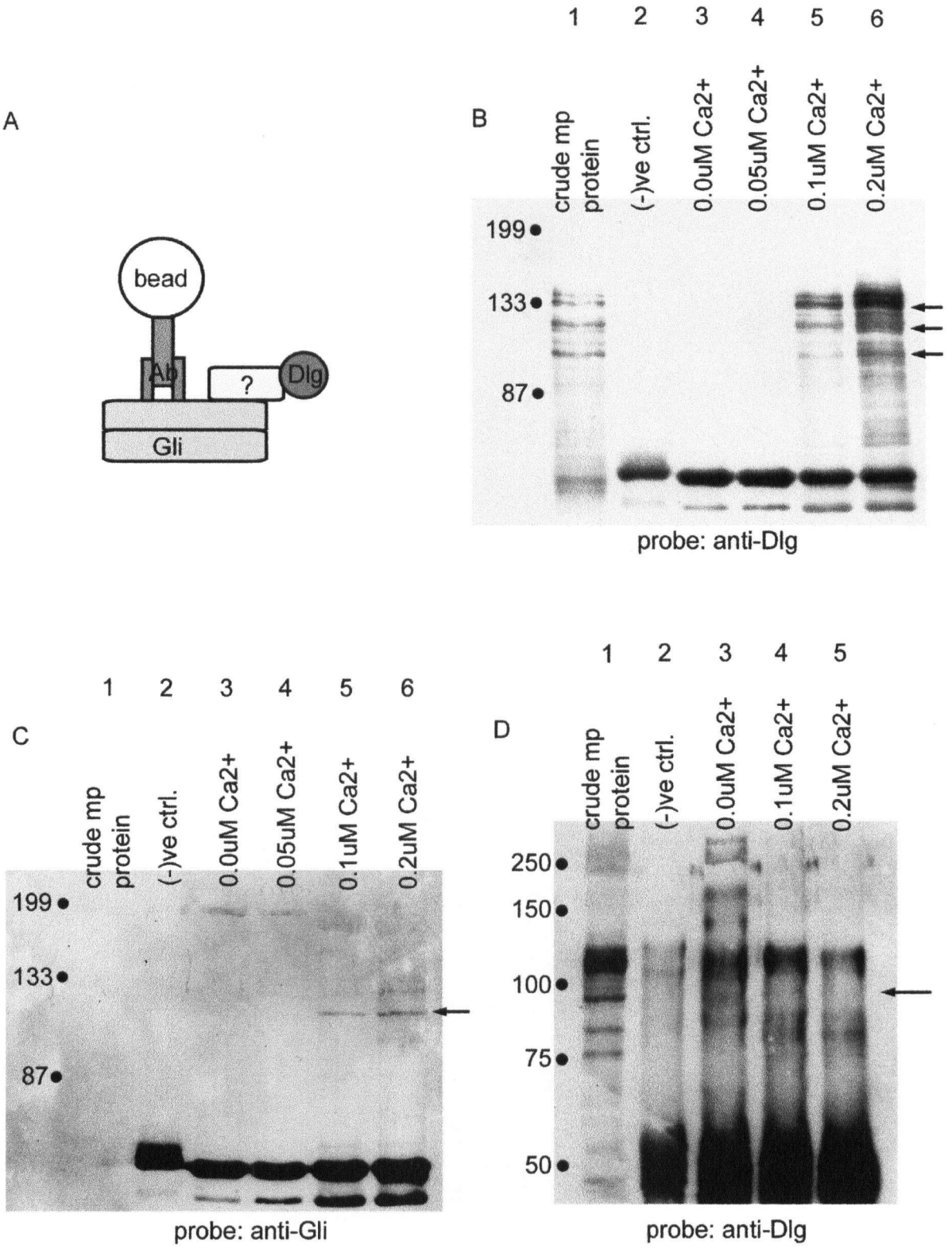
Panel B shows the immunoprecipitation of Gli from w1118 adult Drosophila membrane preparations, probed for Discs Large. Lane 1 shows crude membrane preparation protein, showing the presence of multiple isoforms of Dlg in the membrane preparation. Lane 2 is the negative control, an immunoprecipitation performed with mouse IgG and 0.05 uM free calcium (later replicates contained 0.1uM free calcium in the negative control). Lanes 3 and 4 shows an experimental lane with 0.0 and 0.05 uM free calcium, respectively, while lanes 5 and 6 show experimental lanes with 0.1 and 0.2 uM free calcium, respectively. Note that Dlg shows interaction with Gli in lanes 5 and 6, with possible dependency on free calcium concentration. Bands seen across the bottom at approximately 50 kDa are thought to be the antibody heavy chain, the band seen below the 50 kDa bands correspond with the dye front and are unknown. Arrows indicate known Dlg sizes of 120, 110 and 97 kDa, with size markers in kDa shown at left. Five replicates of this gel were performed and showed consistent results.

Panel C shows the same blot as in 2B, the immunoprecipitation of Gli from w1118 adult Drosophila membrane preparations, probed with anti-Gliotactin. Lane 1 shows crude membrane preparation protein, though no Gli protein is seen, this is likely due to insufficient loading of crude membrame prep. Lane 2 is the negative control, an immunoprecipitation performed with mouse IgG and 0.05 uM free calcium. Lanes 3 and 4 shows an experimental lane with 0.0 and

0.05 uM free calcium, respectively, while lanes 5 and 6 show experimental lanes with 0.1 and 0.2 uM free calcium, respectively. Note the presence of Gli in lanes 5 and 6. Bands seen across the bottom at approximately 50 kDa (variation may be due to difference in IgG types) are thought to be the antibody heavy chain, the band seen below the 50 kDa bands correspond with the dye front and are unknown. Arrow indicates Gli at 110 kDa, with size markers in kDa shown at left.

Panel D shows the immunoprecipitation of Gli from Oregon R embryo membrane preparations, probed for Discs Large. Lane 1 shows 20 ug of crude membrane preparation protein, showing the presence of multiple isoforms of Dlg in the membrane preparation. Lane 2 is the negative control, an immunoprecipitation performed with mouse IgG and 0.1 uM free calcium. Lane 3 shows an experimental lane with 0.0 uM free calcium, while lanes 4 and 5 show experimental lanes with 0.1 and 0.2 uM free calcium, respectively. Note that Dlg shows interaction with Gli in lanes 3-5. Bands seen across the bottom at approximately 50 kDa are thought to be the antibody heavy chain. Bands seen at 75 – 85 kDa (arrows) are possible Dlg isoforms. Size markers in kDa are shown at left. In contrast with the immunoprecipitation performed on adult membrane preparation, there appears to be less or no calcium dependency on the Gli-Dlg interaction in embryos. Four replicates of this gel were performed and shod consistent results.

Figure 5


A
bead
Ab
?
Dlg
Gli
B
1 2 3 4 5 6
crude mp protein
(-)ve ctrl.
0.0uM Ca2+
0.05uM Ca2+
0.1uM Ca2+
0.2uM Ca2+
199
133
87
probe: anti-Dlg
C
1 2 3 4 5 6
crude mp protein
(-)ve ctrl.
0.0uM Ca2+
0.05uM Ca2+
0.1uM Ca2+
0.2uM Ca2+
199
133
87
probe: anti-Gli
D
1 2 3 4 5
crude mp protein
(-)ve ctrl.
0.0uM Ca2+
0.1uM Ca2+
0.2uM Ca2+
250
150
100
75
50
probe: anti-Dlg

3.1.3 Gliotactin and Neurexin IV Do Not Show a Biochemical Interaction

In previous work, it was shown that HA-tagged Gliotactin interacts with Neurexin IV in a calcium dependant manner in immunoprecipitation experiments (Schulte, 2003, PhD thesis). However, in these experiments, my aim was to reproduce this with native Gli, which is only expressed at the tricellular junction. This would avoid any potential artifacts of an overexpression of the Gli transgene which causes the protein to spread throughout the SJ domain.

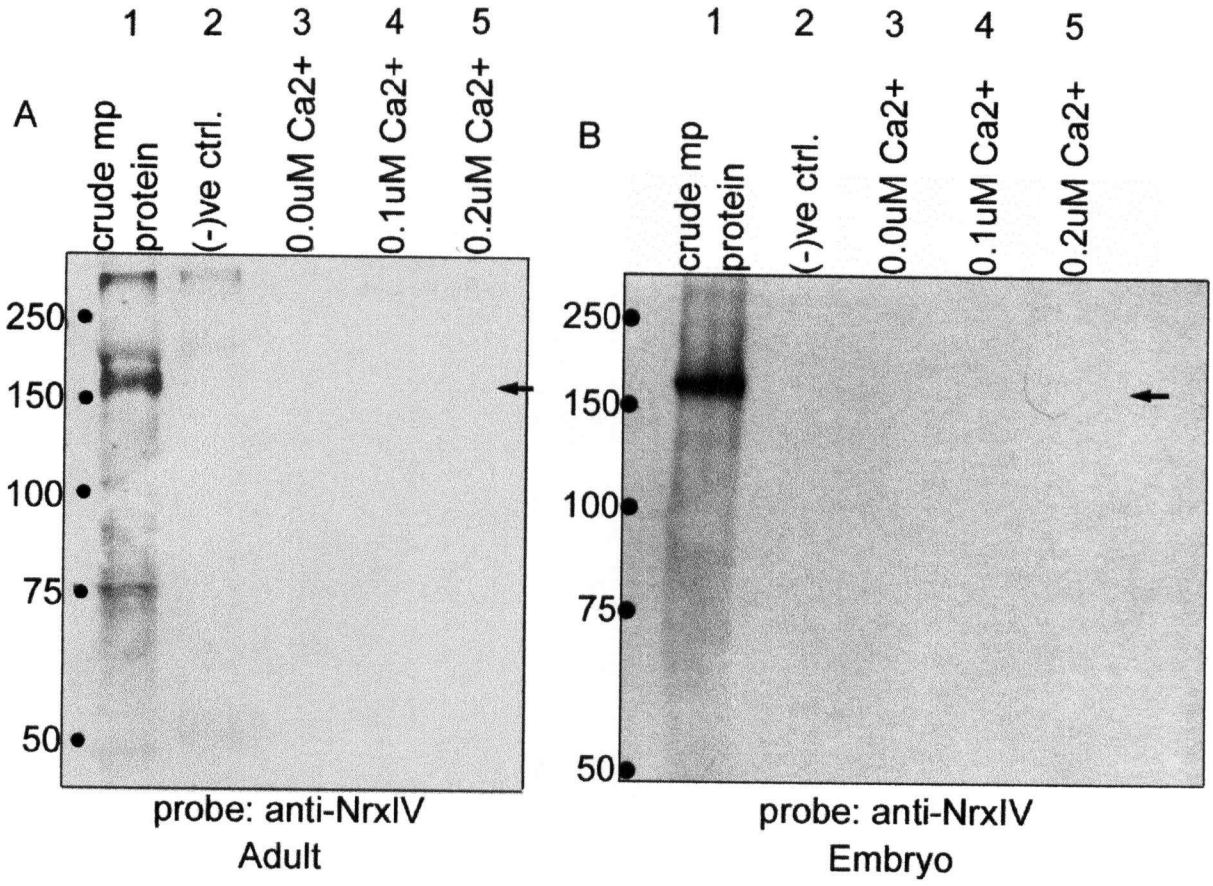
Immunoprecipitation of Gli from either adult or embryonic membrane preparations were carried out under the same conditions used for the Dlg immunoprecipitations, and was unable to show an interaction with Neurexin IV in membrane preparations of both adult and embryo Drosophila (n=4 for adult, n=2 for embryo). For both adult and embryo immunoprecipitation experiments, the resulting immunoblot, probed with rabbit anti-NrxIV, failed to show any NrxIV in the immunoprecipitation lanes (figure 6), while the crude membrane preparation protein lane shows a band corresponding to the expected range at approximately 155-160 kDa for NrxIV (figure 6, panel A, lane 1 and panel B, lane 1).

Figure 6: Gliotactin Fails to Show an Interaction with Neurexin IV.

Panel A shows the immunoprecipitation of Gli from w1118 adult Drosophila membrane preparations, probed with anti-NrxIV. Lane 1 shows crude membrane preparation protein, showing the presence of NrxIV in the membrane preparation at approximately 155-160 kDa (arrow). Lane 2 is the negative control, an immunoprecipitation performed with mouse IgG and 0.1 uM free calcium. Lanes 3, 4 and 5 show a experimental lanes with 0.0, 0.1 and 0.2 uM free calcium, respectively. Size markers in kDa are indicated at left. Note that we fail to see NrxIV interaction with Gli in any of the experimental lanes.

Panel B shows the immunoprecipitation of Gli from Oregon R membrane preparations, probed with anti-NrxIV. Lane 1 shows crude membrane preparation protein at pproximately 155-160 kDa (arrow), showing the presence of NrxIV in the membrane preparation. Lane 2 is the negative control, an immunoprecipitation performed with mouse IgG and 0.1 uM free calcium. Lanes 3, 4 and 5 show a experimental lanes with 0.0, 0.1 and 0.2 uM free calcium, respectively. Size markers in kDa are indicated at left. Note that we fail to see NrxIV interaction with Gli in any of the experimental lanes.

Figure 6


A
1
2
3
4
5
crude mp protein
(-)ve ctrl.
0.0uM Ca2+
0.1uM Ca2+
0.2uM Ca2+
250
150
100
75
50
probe: anti-NrxIV
Adult
B
1
2
3
4
5
crude mp protein
(-)ve ctrl.
0.0uM Ca2+
0.1uM Ca2+
0.2uM Ca2+
250
150
100
75
50
probe: anti-NrxIV
Embryo

In summary, Gliotactin immunoprecipitation experiments were able to show interaction with Discs Large in both Drosophila adult and embryo membrane protein preparations, but were not able to show interaction with Neurexin IV.

3.2 GST Pulldowns Confirm Immunoprecipitation Results and Validates GST Pulldowns as a Method for Studying Gliotactin Biochemical Interactors

To support the results seen from immunoprecipitation experiements, another biochemical approach using a GST pulldown was used to study the interaction of Gliotactin with Discs Large and Neurexin IV. The purpose of the GST pulldowns was two-fold, to attempt to replicate the results of the immunoprecipitation experiments, and to validate the GST pulldown as a method to study protein interactors of Gliotactin, to look for unknown Gli interactors. GST-pulldowns were performed using GST-Gli-Cter, a construct comprised of GST fused to the C terminus of Gli, the section of the protein following the transmembrane domain. This does differ from immunoprecipitatons in that the GST pulldowns would only reveal proteins that interact with the C terminus of Gliotactin while immunoprecipitations using the anti-Gli antibody would reveal proteins interacting with the entire protein. A diagram of the predicted mechanism of the GST pulldown with GST-Gli-Cter is shown in figure 7, panel A. In figure 7, panel D a probe of the GST pulldown using anti-Gli is shown. This shows a protein (arrow) that corresponds to native Gliotactin that appars to be non-Calcium dependent, and is the basis for the predicted GST pulldown mechanism which shows GST-Gli-Cter binding to native Gli (figure 7, panel A). It was thought that the GST-Gli-Cter might interact with the protein complex containing Discs Large but not directly to Dlg as Gli and Dlg have been shown to not directly interact (C. MacKinnon, M.Sc. thesis, 2005). Alternatively, it is possible that GST-Gli-Cter would interact

with native Gli and pull out any proteins bound to native Gli because the over abundance of GST-Gli-Cter would outcompete the native Gli. The GST-Gli-Cter/Glutathione agarose complex was allowed to incubate with the membrane preparations from adults and the resulting immunoblots reflected the results shown with the co-immunoprecipitation experiments. When probed with anti-Dlg, the immunoblots (n=2) showed similar results to the Gli immunoprecipitatios, showing a wide range of Dlg isoforms interacting with GST-Gli-Cter (compare figure 7 panel B, with figure 5, panel B). We see similar multiple Dlg isoforms in the experimental lanes, with increasing Dlg signal in 0.2 uM free calcium compared to the 0.1 uM free calcium lane (see figure 7, panel B, compare lanes 4 and 5). When GST pulldowns with GST-Gli-Cter were probed with rabbit anti-NrxIV, the immunoblots (n=2) again showed similar results to the Gli immunoprecipitation probed with rabbit anti-NrxIV; we failed to detect any NrxIV lanes in the experimental lanes of the immunoblot (compare figure 7, panel C, with figure 5, panel C).

Figure 7: GST Pulldowns Show a Similar Result to Immunoprecipitation Experiments.

Panel A shows a predicted model of the GST-pulldown, with the GST-Gli-cter binding Gli, and Gli then binding to an unknown intermediary, which then binds to Dlg.

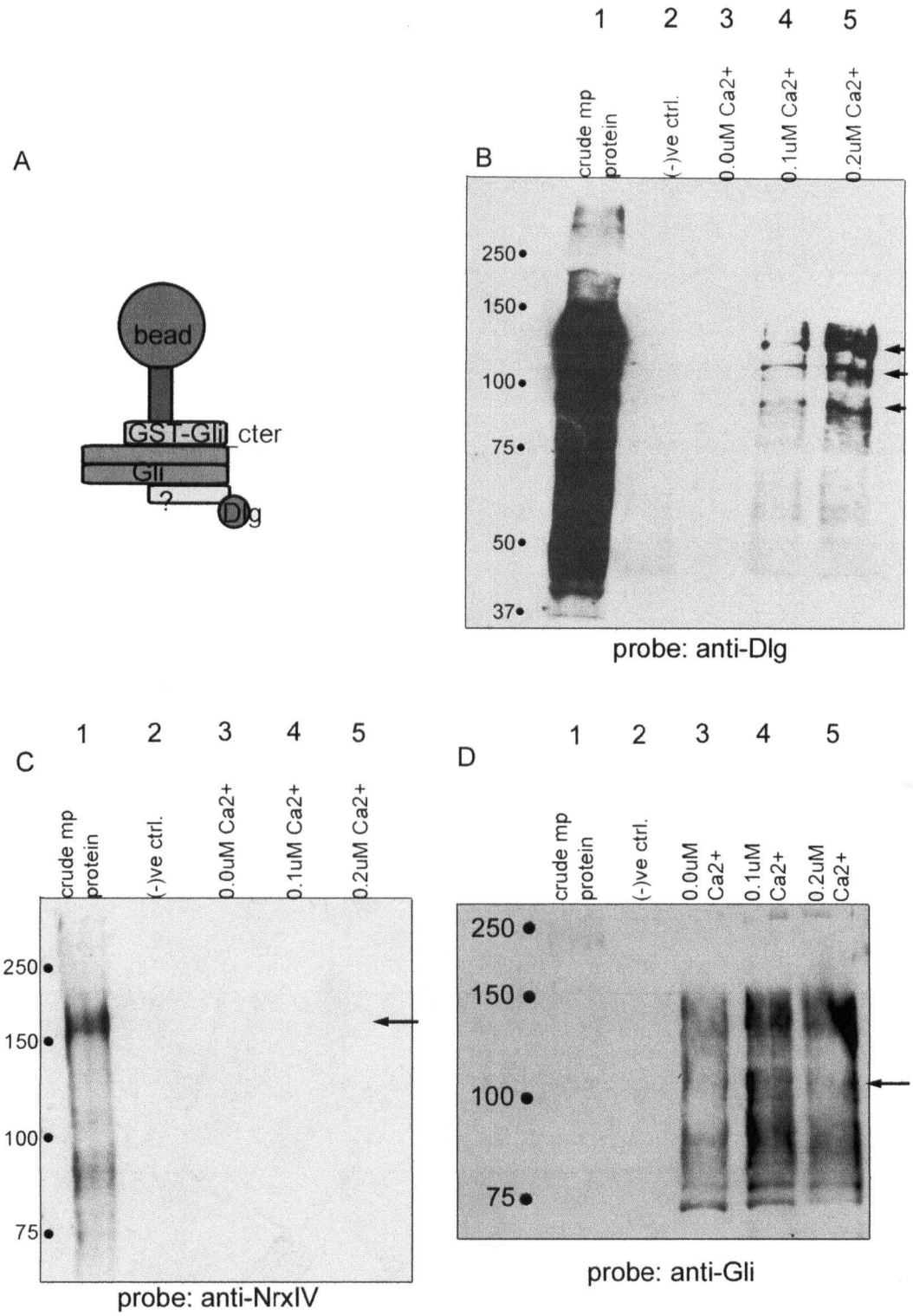
Panel B shows GST pulldowns performed using GST fused to the C terminus of Gliotactin, bound to glutathione agarose beads, and w1118 adult Drosophila membrane preparations. This blot was probed for Discs Large. Lane 1 shows 20 ug crude membrane preparation protein, showing the multiple isoforms of Dlg present in the membrane preparation. Lanes 2-5 includes 150 ug of crude membrane preparation input into the GST pulldowns. Lane 2 is a negative control using GST in place of GST-Gli-cter with 0.1 uM free calcium. Lanes 3-5 show experimental lanes with 0.0, 0.1 and 0.2uM free calcium concentrations, respectively. Note that Dlg shows an interaction with GST-Gli-cter in lanes 4-5 with possible dependency on free calcium concentration. Arrows indicate known Dlg sizes of 120, 110 and 97 kDa, with size markers in kDa shown at left. Faint bands seen in lnaes 4 and 5 at 50 – 75 kDa are thought to be possible Dlg isoforms.

Panel C shows GST pulldowns performed using GST fused to the C terminus of Gliotactin, bound to glutathione agarose beads, and w1118 adult Drosophila membrane preparations. This was probed with anti-NrxIV. Lane 1 shows 20 ug of crude membrane preparation protein, showing the presence of NrxIV in the membrane preparation (arrow at approximately 155-160 kDa, the predicted NrxIV size). Bands seen from 75 to 100 kDa in lane 1 are unknown. Lanes 2-5 includes 150 ug of crude membrane preparation input into the GST pulldowns. Lane 2 is a negative control using GST in place of GST-Gli-cter with 0.1 uM free calcium. Lanes 3-5 show

experimental lanes with 0.0, 0.1 and 0.2 uM free calcium concentrations, respectively. Size markers in kDa are shown at left. Note that we fail to see NrxIV interaction with GST-Gli-cter in any of the experimental lanes.

Panel D shows the same blot as in panel B, showing GST pulldowns performed using GST fused to the C terminus of Gliotactin, bound to glutathione agarose beads, and incubated with w1118 adult Drosophila membrane preparations. This was probed with anti-Gli. Lane 1 shows 20ug of crude membrane preparation protein, showing the presence of Gli in the membrane preparation. Lanes 2-5 includes 150 ug of crude membrane preparation input into the GST pulldowns. Lane 2 is a negative control using GST in place of GST-Gli_cter with 0.1uM free calcium. Lanes 3-5 show experimental lanes with 0.0, 0.1 and 0.2uM free calcium concentrations, respectively. Note arrow indicates Gli in lanes 1, 3-5 at approximately 110kDa, with size markers in kDa shown at left.

Figure 7


A
bead
GST-Gli
cter
Gli
?
Dlg
B
1
2
3
4
5
crude mp protein
(-)ve ctrl.
0.0uM Ca2+
0.1uM Ca2+
0.2uM Ca2+
250
150
100
75
50
37
probe: anti-Dlg
C
1
2
3
4
5
crude mp protein
(-)ve ctrl.
0.0uM Ca2+
0.1uM Ca2+
0.2uM Ca2+
250
150
100
75
probe: anti-NrxIV
D
1
2
3
4
5
crude mp protein
(-)ve ctrl.
0.0uM Ca2+
0.1uM Ca2+
0.2uM Ca2+
250
150
100
75
probe: anti-Gli

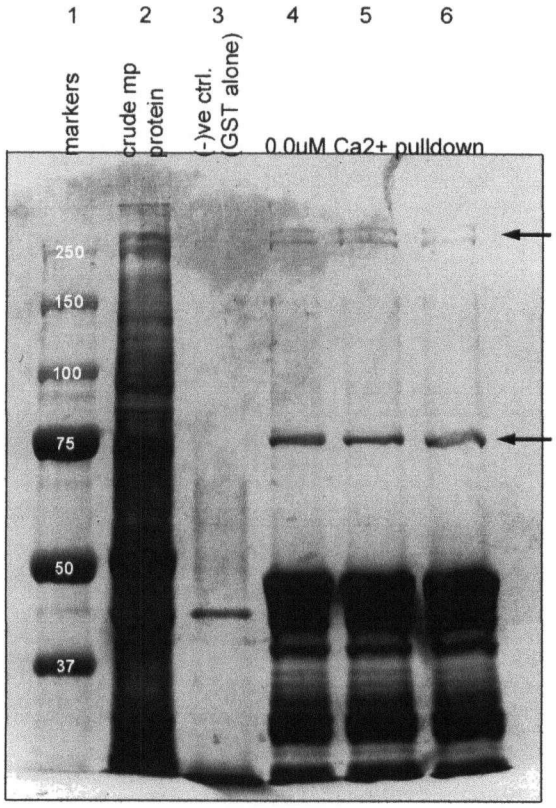
These results show us that GST pulldowns using GST-Gli-Cter can be used as a system to study Gliotactin protein interactions because it is consistent with results from Gli immunoprecipitation experiments (GST-Gli-Cter showing an interaction with Discs Large and not with Neurexin IV). In addition, this also supports the observation that Gli is able to interact with Dlg.

3.3 Identifying Unknown Proteins as Gliotactin Interactors.

Previous results had indicated that GST pulldown was a valid method for finding Gliotactin interactors, as GST pulldowns reflected the result of co-immunoprecipitations for Discs Large and Neurexin IV. It was hypothesized that unknown proteins could also be brought down in this assay, and thus this method might be employed to discover unknown proteins complexed with the C terminal tail of Gli. GST pulldowns using the GST-Gli-cter fusion were carried out using membrane preparations from w1118 adults (n=3) or Oregon R embryos (n=1). Following SDS-PAGE protein separation, the protein bands were transferred to PVDF membrane. Gel Code Blue (coomassie based dye) staining of blotted PVDF membranes revealed several unknown proteins, one prominent band at approximately 75 kDa, and a fainter doublet at approximately 250 kDa, seen in figure 9, lanes 4-6. There are multiple bands at 50 kDa and below in the GST-Gli-Cter pulldown (figure 8, lanes 4-6), and they are most likely background bands with the most prominent band at 50 kDa corresponding to GST-Gli-Cter, and the smaller bands likely degradation of the fusion protein. A negative control containing just GST protein, without the C terminal tail of Gliotactin was run as a negative control (figure 8, lane 3), and did show a background band at 45 kDa. Interestingly, the proteins seen on the Gel Code Blue stained PVDF membranes did not appear to be calcium dependent as they appeared in all lanes equally in an attempt (not shown) that included a free calcium concentration range of 0.0 uM to 0.2 uM.

Figure 8: GST Pulldown Using GST Fused to Gliotactin C Terminus Shows the Presence of Unidentified Proteins.

GST pulldowns were performed using GST fused to the C terminus of Gliotactin, bound to glutathione agarose beads, and embryo OregonR membrane preparations. Lane 1 shows size markers with corresponding sizes in kDa indicated. Lane 2 shows crude membrane preparation protein, showing all detectable proteins in the membrane preparation. Lane 3 is a negative control using GST in place of GST-Gli-cter with 0.0 uM free calcium, note that GST protein alone is approximately 26 kDa and was mostly run off the gel. Lanes 4-6 show experimental lanes with 0.0 uM free calcium concentrations. Note the doublet bands seen at approximately 250 kDa, and the robust band at approximately 75 kDa, seen in the experimental lanes.



Attempts at sequencing the unknown 75 kDa band have yielded unsatisfactory results. Samples from GST pulldowns using the w1118 adult membrane preparations were sent for protein sequencing to the NAPS protein sequencing facility at UBC. Two attempts yielded two different amino acid sequences. A third attempt was made from samples from GST pulldowns using OregonR embryo membrane preparations, sent to the protein sequencing facility at the University of Victoria. This yielded yet another amino acid sequence appearing unrelated to the previous sequencing attempts (see table 1).

Sequencing of the 250 kDa doublet from embryonic tissue, yielded more interesting results as both bands of the doublet yielded similar sequences (see table 1). After performing a short-sequence amino acid BLAST search using the sequence ENENDXXXDN (single amino acid code), a potential gene (CG31652) and gene product were identified for this 250 kDa doublet protein. The candidate contains the matching short amino acid sequence 17 amino acids into the predicted 2148 amino acid sequence, placing the candidate's potential molecular weight at a predicted 248 kDa (http://www.ebi.ac.uk/servicestmp/saps-20060523-14280096995539.html).

Table 1: Comparison of N-terminal Sequence Data of Unknown Proteins Seen in a GST Pulldown of GST-Gli-cter.

Sequence comparison of four sequencing attempts of the unknown protein seen at 75 kDa which associated with GST-Gli-cter, seen in rows 1-4 Sequence comparison of the 250 kDa protein doublet (named A and B), which are associated with GST-Gli-cter seen in rows 5-6.

Sequence sample	N-terminal peptide sequence
UBC NAPS 75kDa adult unknown – trial 1	S/T/?, P/Q, E/N/I, V/P/M, D/T/?, Q/V/I, F, T/A, N/I, E
UBC NAPS 75kDa adult unknown – trial 2	H/G/?, Q/K/L/?, I/V, I/Q, G, Q/P/I/V, D, T/L/K, G, T, T/M/A
UVic Protein Sequencing 75kDa adult unknown	P/W/I, K, I, I, A, I, H, L, L, L
UVic Protein Sequencing 75kDa embryo unknown	D/G, L, A, I, D, D, S
UVic Protein Sequencing 250kDa A	E/D/N, N, E, N, D, ?, ?, ?, D/S/?, N
UVic Protein Sequencing 250kDa B	E/D/N, ?, D/N/?, E/S/?, N/S/?, E, D/S/?

Figure 9: Amino Acid Sequence of CG31652

Amino acid sequence of potential Gliotactin interactor, CG31652 with matching N-terminal sequence of unknown 250 kDa protein underlined.

MDTTQESDLLGEEEELENENDVSFPRDIVSVEHSFGYDCKKLFNLVLVDSDTLVFASGNF
LNYFSISRQEISFQETVYGCGVGFITKNEHPDYTNLFTVGENGARPTVFIYEYPSLNVRV
KLLNAAQNCFTAGSYNKTGELFASQAGYPDFIITIWRWENAEVVLRAKSFQSDILFVHFS
EHNPILLCSSGLSHIKFWKMANTFTGLKLKGDLGRFGKTDFSDIYAMYMLQDENVISGSD
WGNMLLWQAGLIKFEICRKGRKPCHTKPITRITMKNGEVTTVGMDGYVRVWYWETVDLAD
PPEDDLFVEIDPIYEFKIADVELRCMQKIHPFDESDFTHYAQDGNGGIWFCDINTYDVPQ
KPRKLYSCIGGKVLAAQMSPVSPHFLCMSESGKLFVYQYDEQRLILEKEFPAEGVDVIWL
DTNISVKGTELVAAFKDGILRQMYLDLSNGERPKMTRVRAFKAHTSPITALTVSRNSSLL
LTGSADKSIFIYQLSRDEHQMVDMRPLGFVQFAAIPNCFYWHETEPTVVLVGCKSGDLYE
YNIRTEVTDHETYLSYNITENSRMRHTKFASIKSRIRRDIHRENVKKRKERKRERKMNEV
EKLKKANPGLQIDIESALADSEPDEEEEPLHIPTVPNAIIWLRYTLRDTIWVSMAGYDAG
YIYELEFNAAEPTRATIIAEADDIEIHSYCIIDEFIIFGLHNGRIRINRINPDNFTDLSE
FRIYPMHDGLKGTIPKIDISYSGEHLLTIGYDGNVFLHKWNGPKIVRNKVRDKLPPLPEG
VTQIPDIEPEACSLEQEKINAELRRQQEAADAHDRDVLSKIGALQNVYFDIIRINEELPP
GLRAKDTDLLLDDRITRQIRNELQAELDDVREDLAYDLEFAQVGHTKLYNHFLKELVQVP
FTVAPLRANVPGVSTFRLQALGEEHAQIKQDIEDRLKREHDMGLHDYVAPERSDESIGEP
PPESFFFGRDPKSIEPRFSKKMMRLLTRYRKRQIFEVRRIFDWDKLERQKPDPNRNHPDD
DAQIEDAKRNLGDFKLKIGSDYEPKSSETLTQKYIEVVECREQYFAMVDAFNQMVLDLRE
RKGELDQFITAKRNRLAVIHNYLPEPDRQPLDPIQEIDMDLEYPELNLIEHYTPGCGVEI
DDILTLEHSVDQVIARMAPMRSTANVSLFSLHGLDVPELHEHTAFTMLQISKLNRSLPNA
ELDEELLKLPPKSDPAYFELNDDGQVPYMMETRHRWLRQLLVEQNAIRTEVDEEVRQFDK
ALEKLQVRRFHVKMDAEFMTAFMNSLNQELYILRDSEEIETQLLLNAKTAMSTRNDLQMV
INSTNRQLDELRRNIDKLGEQIVALQVLFATTVKGHKFFDFLRRIFKKKWRPPKVSRGDD
ESSSSSSSSSSSEDEQADNKSLDSLDMTTIRLDEATCPTGLDRSLYDLAFSMRSDRHDL
ERNVRELTREVENKRKEIAEMQIKMKFHEEVYQREKNALLQFRRNRQQEVNKVHISAILR
MDQLQHFYDGDDYRDLSKAILFDADMLVDLRRRADQLSEETLATKRWHRINFIHLRRMNT
DIKFMRFEITRLEEEIRQAMMKKFGIIVNLDELEEEVLRRYIFDLETNAEDELMALEKEL
LEKQKELARCEEELVLETQNNTEKVNIMTVLREENNILRTLLDIQNKNYAKWANPNALNL
SYDIEKLRGIEKSLLDQIECLEREICALRLKSKPLQINYEFEVDPNAQPQVVTSEIMPND
DAMVCPAILNVDAYMLPPMPDEFIMDRVQTIVQKSFNRFFGKSTTPENVRKFAQRSSLYL
CQAAYSFQGRYTDRIAECITEHLQTFVPKKYFIHISNEELRKLFNEVVAVFDYERSDVNT
EELISGIFDHAKDSLCAHVHMGDSEVVNRTHFIVAHMFKELIEVLPLEEFQADETLRMIV
DVLEREPMVDPRAIAVEQLIDSTLQHARENLIDGITAVPIRSLGSSIQRDLIKRRQYKQP
SCESPIRDKMSKPSKAGPPSEGSAPAYDYRHSGDDMAMRAPPPTYEESQRNGGTGAGAYG
YPQGGGAPPSQNQYYGMISHQQQQMPFSGQPNYGMTHGSGFGLAGPSTSAGAAAAQVLHL
DSRAEVRTNSNGNVVIPPPPPGCLPTPAQWAAMQGQPVVLKQKKRSFF

4. Discussion

4.1 Gliotactin May Be In A Separate Protein Complex Than Other Currently Known Septate Junction Proteins

It has been previously seen that the majority of known SJ proteins are interdependent and a loss of one protein results in the mislocalization of the other SJ proteins (Hortsch and Margolis, 2003; Genova and Fehon, 2003; Llimargas et al., 2004) . It has also been seen that a loss of Gliotactin leads to a mislocalization of Neurexin IV, Coracle and Discs Large (Schulte et al., 2003). In this case, characteristic SJ septa are still seen, though spread down the basolateral edge, suggesting that perhaps Gli plays an integral role in organizing or stabilizing the core SJ complex just under the adherens junction (Schulte et al., 2003). When overexpressed, an HA-tagged version of Gli spread throughout the SJ domain and was able to biochemically interact with Discs Large, Neurexin IV and Coracle (Schulte et al., 2003). In an effort to study other potential Gli protein interactors, an attempt was first made to replicate Schulte's (Ph.D. thesis, 2003) immunoprecipitation experiments, which showed the biochemical interaction of HA-tagged Gli with NrxIV, Cor and Dlg. This was done to verify if I was able to reproduce the same results, demonstrating an ability to successfully immunoprecipitate HA-tagged Gli. When it was discovered that the Drosophila strains used were unstable and had lost the HA-tagged Gli, we looked to other methods of studying Gli protein interactions including immunoprecipitation and GST pulldown of essentially wild type Drosophila membrane preparations. This approach also allowed the study of endogenous protein complexes, rather than those formed with overexpressed protein in transgenic flies, thus, reducing the potential for artifacts due to the modification of the protein sequence and expression,

Through both co-immunoprecipitation experiments and GST pulldown experiments, it has been shown that Gliotactin, a protein localized to the septate junctions at the tricellular junction, does not appear to interact with Neurexin IV (figure 6) in both adult and embryo membrane preparations. However, Gliotactin does appear to be in a complex with Discs Large in both adult and embryo membrane preparations. The only difference seen between embryo and adult preparations being that there seems to be no calcium dependence in the binding in the embryos. This is possibly due to changes in the cell and protein interactions during embryo to adult development. As identical detergent conditions were used to in immunoprecipitations from both adult and embryo membrane preparations, it is possible that the detergent conditions would extract different proteins in adult and embryo membrane preparations.

To date, Dlg is the only known SJ protein with which Gli interacts. Interestingly, this interaction in adults is only seen in the presence of calcium at 0.1 or 0.2 uM. It is possible that the Gli epitope recognized by the anti-Gli antibody requires calcium to be properly recognized, as we see in the calcium dependency of Gli immunoprecipitation (figure 5, panel C). Alternatively, this may indicate that Gli requires calcium for proper protein interactions, protein conformation, or as a factor in dimer stabilization. Gli genetically appears to act as a dimer due to some combinations of different Gli mutations being able to show rescue of the mutation (Venema et al., 2004). In addition, the candidate Gli vertebrate homologs, the Neuroligins are known to bind Neurexins in a calcium dependent manner (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Nguyen and Sudhof, 1997), possibly through a putative EF-hand domain on Neuroligin (Gilbert and Auld, 2005). This may point to a similarity in the function of calcium in the Gli-Dlg interaction, and this is supported by the possibility of a calcium binding EF-hand domain on the

extracellular portion of Gli (Gilbert and Auld, 2005). Alternatively, the immunoprecipitation of Gliotactin itself may be calcium dependant (figure 5, panel C), if the Gli epitope requires calcium for proper binding of the anti-Gli antibody, a property also seen in the antibody binding to CD29 and to leukocyte function-associated molecule-1 (Levesque et al., 2002; van Kooyk et al., 1991).

The data showing Discs Large as the only protein seen to interact with Gliotactin, had led to the idea that perhaps Gli may be independent of other known septate junction proteins, existing in a unique complex at the tricellular junction (Schulte et al., 2006), illustrated in figure 10. Discs Large is known as a SJ protein, occurring around the entire cell and additionally, showing enrichment at the TCJ (Woods and Bryant, 1991). Gli is known to only occur at the TCJ (Schulte et al., 2003; Woods and Bryant, 1991). As there are many known Dlg isoforms (Mendoza et al., 2003), perhaps Gli interacts with different isoforms present at the TCJ, which are different from those in the known septate junction protein complex that occur around the cell (Hortsch and Margolis, 2003). Which isoforms of Dlg interact with Gli is a more difficult question to answer as the anti-Dlg antibody recognizes the PDZ 2 domain of Dlg, and this domain occurs on many different Dlg isoforms. It would be interesting to have seen if indeed different Dlg isoforms associate with Gli as with the SJ by performing immunohistochemistry. It is interesting to note that the interaction of Gli and Dlg does not appear to be PDZ dependant in in GST pulldowns using a Gli construct with a truncated PDZ binding domain (Schulte et al., 2006).

Interestingly, human Discs Large (hDlg) also has several different isoforms (so far all contain PDZ domains 1 to 3), and 5 have been identified (McLaughlin et al., 2002). They have a wide

variety of locations and functions including cell-cell contacts interacting with other known structural proteins human Scribble, and the vertebrate ortholog of Coracle, Protein 4.1 (Lue et al., 1994b).

Recent cell biological work has pointed to a possible link between Gliotactin and Discs Large. In 3rd instar larve columnar epithelial cells of the imaginal wing disc and the peripodial cells, Dlg shows enrichment at the TCJ, as well as Gli and Dlg showing strong colocalization at the TCJ. As well, the overexpression of Gli and its subsequent spreading of Gli into the entire SJ domain, appears to correlate with the down regulation of Dlg in the immaginal discs of 3rd instar larvae (Schulte et al., 2006; Charish, personal communication). This would make sense if Gli was indeed somehow bound to Dlg, perhaps the overexpression of Gli provides competition for the usual Dlg ligand at the septate junction.

However, there is the possibility that Gliotactin and Discs Large do not work through a direct interaction, but an indirect one, as supported by work by C. MacKinnon (2005, M.Sc. thesis), which included experiments involving GST pulldowns of GST-Gli-Cter expressed in E. coli, with in vitro translated Dlg. Her work showed no evidence of direct physical interaction between the two proteins, though the experiments did not involve testing the effect of free calcium (C. MacKinnon, 2005, M.Sc. thesis). As we see some calcium dependence of the Gli-Dlg interaction in immunoprecipitations and GST pulldowns, this could be taken as Gli needing calcium to show binding to Dlg, that Gli itself is calcium dependant or that the TCJ as a whole is somehow calcium dependant.

There may also be other proteins in the Gli-Dlg complex, that act as intermediate proteins, linking Gli and Dlg, perhaps transiently because a Dlg immunoprecipitation fails to show Gli. Since there is an abundance of Dlg in Drosophila, and only a small subset of Dlg might be bound to Gli at any one time, perhaps we were unable to detect what would be very small amounts of Gli in a Dlg immunoprecipitation.

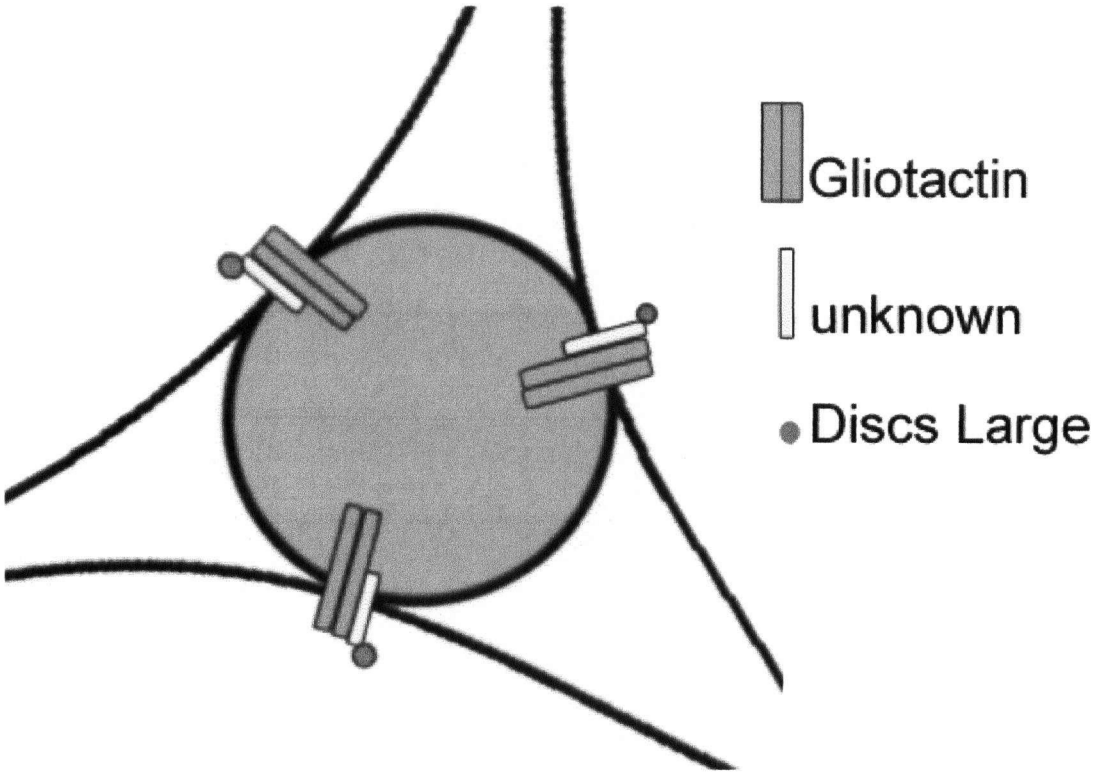
As Gli does not bind to NrxIV, it is likely that this Gli-Dlg complex is separate from the known septate junction complex. From the data gathered so far, we can arrive at a model of the workings of the tricellular junction at the level of septate junctions. We believe that the TCJ does indeed act as an organizing centre for the septate junction strands, and that the tricellular plug is where SJ strands are anchored at the TCJ by the Gliotactin-Discs Large complex. Gli, a transmembrane protein, might be responsible for linking the tricelular plug, an extracellular structure, to the cells at the TCJ via Dlg, an intracellular protein. This concept would support the observation that the loss of Gli, and thus the destabilization of this TCJ complex, leads to the basolateral diffusion of the septate junction strands –the tricellular plug is no longer being anchored to the TCJ.

Recently, a new vertebrate protein had been identified, Tricellulin, a protein with some structural similarity to occludin and which exists in the area of tight junction strands at tricellular contacts in vertebrate cells (Ikenouchi et al., 2005). However, there does not appear to be any homology between Tricellulin and Gliotactin though they appear to both be required for epithelial barrier integrity (Ikenouchi et al., 2005; Schulte et al., 2003). In fact, there is no homology between Tricellulin and any Drosophila protein, nor are there any known Tricellulin interactors to date, including PDZ containing proteins as Tricellulin does not appear to have a PDZ binding domain. Though the functional similarities between Tricellulin and Gliotactin are

intriguing, the similar functions demonstrated by both proteins may simply be an example of convergent evolution.

Figure 10: Model of Protein Interactions at the Tricellular Junction.

The tricellular plug (blue), a structure comprised of unknown components, is shown with Gliotactin, at the tricellular junction, as a component of a unique TCJ protein complex. Shown with Gli are unknown protein(s) (yellow) and Discs Large (red). It is believed that the Gliotactin complex is helping to organize the septate junction and anchor the tricellular plug.



Gliotactin is a possible homolog of a class of vertebrate proteins called the Neuroligins, with the closest relation being to Nlg3 (Gilbert et al., 2001). Neuroligin 1 binds to α and β Neurexin in the vertebrate system, only in the presence of calcium (Gilbert et al., 2001; Nguyen and Sudhof, 1997). Additionally, neuroligins are thought to function as dimers (Dean et al., 2003) and have been found to bind to the third PDZ domain of PSD-95, a vertebrate Dlg homolog (Bolliger et al., 2001; Irie et al., 1997; Willott et al., 1993). Perhaps the calcium dependence observed in the binding of Gli and Discs Large in Drosophila reflects the binding activity of its vertebrate homologs, since Neuroligins also bind to the Neurexins in a calcium dependant manner (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Nguyen and Sudhof, 1997; Boucard et al., 2005).

More recently, further links between neuroligins and PSD-95 have been determined. It had been found that PSD-95 has some role in the clusetering of Nlg2 and Nlg3 (Levinson et al., 2005), and there is suggestion that Nlg can regulate PSD-95 (Iida et al., 2004). There has been no evidence of interaction between Neuroligin 3 and Caspr (Gilbert and Khurana, unpublished data), a class of proteins thought to be vertebrate homologs of Neurexin IV (Peles et al., 1997). Again, this reflects our biochemical results in the Drosophila system, where we fail to observe an interaction between Gliotactin and NrxIV. Such vertebrate proteins with invertebrate septate junction homologs as Caspr, Neurofascin and Contactin are found at the paranodes of myelinated nerves, often believed to have septate-like junctions (Hortsch and Margolis, 2003; Bhat et al., 2001). Surprisingly, Neuroligin 3, the closest homolog to Gliotactin, does not appear to be present in the paranode (Gilbert, personal communication). This may be consistent with the idea that Gliotactin is in a separate TCJ protein complex, and thus its vertebrate homolog, are in separate complexes from the septate and septate-like protein complexes, respectively.

4.2 Unknown Gliotactin Interactors

The next step in searching for potential candidates for Gliotactin binding partners was to use a biochemical screen. This approach isolated membrane preparations and looked for proteins able to bind to a fusion protein: GST fused with the C terminal domain of Gli, in a GST pulldown. Since GST pulldowns had replicated the results seen in immunoprecipitations for Gliotactin and Dlg, as well as Gli and NrxIV, it was surmised that GST pulldowns would be a valid method of locating other Gli interactors.

Using this GST pulldown method from both adult and embryonic membrane preparations, we are able to observe several unknown proteins, a doublet appearing at about 250 kDa, a faint band at about 150 kDa and a prominent band at 75 kDa. The intensities may be an indication of the prevalence of the Gliotactin-unknown protein interaction. All bands appeared to be independent of calcium concentration. As the most prominent, the 75 kDa unknown protein was sequenced using N-terminal degradation. The first two attempts at sequencing the 75 kDa protein resulted in two completely different sequences. The third attempt at sequencing the 75 kDa protein again resulted in a different sequence. It is suspected that the 75 kDa band may be a non-specific cleavage or degradation product as there was a lack of an N-terminus methionine, or a collection of different proteins of similar molecular weight.

Other work had since been done on GST pulldowns of adult and embryo membrane preparations using GST-Gli-Cter, as well as GST-Gli-CterΔPDZ, a similar construct though missing the last PDZ domain on the C terminus of Gliotactin. It was found that in the presence of high salt (300 mM NaCl), the 75 kDa band persisted, as well, it was also present in pulldowns performed with

GST-Gli_cterΔPDZ (Alan Gillet, unpublished data). This shows that the binding of the unknown 75 kDa protein to the C terminus of Gli is a non-PDZ interaction.

The 250 kDa doublet, alternatively, resulted in two similar sequences, an indication that they may be isoforms of the same protein. BLAST searches revealed the most likely candidate for this 250 kDa band is as yet, an unknown and uncharacterized gene sequenced from cDNA, CG31652. This gene appears to be highly conserved across various species, including the human gene AAI01583. This gene product contains potential WD-40 repeats, also known as beta-transducin repeats, which are associated with a variety of functions including signal transduction and transcription regulation to cell cycle control and apoptosis (information from The European Bioinformatics Institute, http://www.ebi.ac.uk/Information/). However, all proteins with WD-40 repeats are associated with the coordination of multi-protein complexes as the WD-40 domains act as a scaffold (European Bioinformatics Institute, electronic communication). As well, WD-40 domains bind to phosphoserine residues, which are potentially present on Gliotactin. A Drosophila line with an insertion of a transposable element in the candidate gene has been examined, though it failed to show any mislocalization of Gli in the embryo (Julia Wierzchwski, personal communication).

4.3 Summary and Future Studies

Curently, there is some evidence that the Gliotactin and Discs Large interaction is somehow dependant on calcium. We also see some unknown proteins which appear to biochemically interact with the C terminus of Gli. The question remains, what other proteins are present in this unique TCJ complex?

It would be interesting to see if any of the unknown proteins discovered in the GST pulldown experiments may potentially be the missing link between Gliotactin and Discs Large, as they are proteins that interact with the cytosolic domain of Gli. We can begin to examine this by looking to see if any SJ defects, and mislocalization of Dlg are present in the Drosophila line (flybase id: FBgn0051652) with an insertion in the gene of the 250 kDa protein candidate. We already do not see a mislocalization of Gli, which would indicate that perhaps the unknown protein is not required for Gli localization at the TCJ, or that it does not play a role at the TCJ, or that the insertion into the gene did not cause a mutation. It is of course, equally likely that they may be interacting in other ways or in another location. We can pursue this candidate protein in the future by intentionally making a deletion mutation in this gene, in case the transposon insertion did not cause a mutation. This could be done by injecting embryos with the transposase gene, which might cause an inaccurate excision of the insert, and screening for mutant phenotypes. The easier route to look for an interaction with Gliotactin would be to simply take the CG31652 gene and in vitro translate it to perform a GST pulldown with Gli. If this proves fruitful, it would be possible to raise an antibody to the CG31652 gene product and attempt to do antibody staining of the gene product and Gli to look for colocalization and subsequently, perform immunoprecipitation experiments looking for the CG31652 gene product with Gliotactin.

To further examine Gliotactin interactions, we would in the future, need to be able to verify potential candidates both biochemically and through co-localization studies in vivo in Drosophila as outlined above. If potential candidates co-localize in vivo, and also interact in immunoprecipitation or pulldown assays, then they would be suspected of interacting with Gli. Additional support would arise if mutations in potential candidates had a visible effect on Gli, and vice versa in both cell biological and biochemical studies. It would also be intriguing to see

if any Gli interactors discovered in the future would interact with other known SJ components (for example, through immunoprecipitation), as this may provide a link between the TCJ and the SJ.

4.4 Conclusions

This thesis has shown the first known Gliotactin protein interaction with another known septate junction protein, Discs Large through biochemical assays including immunoprecipitation and GST-pulldowns. Additionally, this interaction appears to be independent of the currently known pSJ complex, which contains Neurexin IV as an integral part of the complex. This observation points to a new, unique pleated SJ complex, perhaps existing at the tricellular junction. Additionally, new unknown Gli interactors have been found, which may help in future endeavours in locating other proteins of this potential new TCJ complex.

References

Al Awar,O., Radhakrishna,H., Powell,N.N., and Donaldson,J.G. (2000). Separation of membrane trafficking and actin remodeling functions of ARF6 with an effector domain mutant. Mol. Cell Biol. *20*, 5998-6007.

Anderson,J.M. (1996). Cell signalling: MAGUK magic. Curr. Biol. *6*, 382-384.

Anderson,J.M. (2001). Molecular structure of tight junctions and their role in epithelial transport. News Physiol Sci. *16*, 126-130.

Anderson,J.M., Balda,M.S., and Fanning,A.S. (1993). The structure and regulation of tight junctions. Curr. Opin. Cell Biol. *5*, 772-778.

Arroyo,E.J., Xu,Y.T., Zhou,L., Messing,A., Peles,E., Chiu,S.Y., and Scherer,S.S. (1999). Myelinating Schwann cells determine the internodal localization of Kv1.1, Kv1.2, Kvbeta2, and Caspr. J. Neurocytol. *28*, 333-347.

Balda,M.S. and Matter,K. (2000). Transmembrane proteins of tight junctions. Semin. Cell Dev. Biol. *11*, 281-289.

Baumgartner,S., Littleton,J.T., Broadie,K., Bhat,M.A., Harbecke,R., Lengyel,J.A., Chiquet-Ehrismann,R., Prokop,A., and Bellen,H.J. (1996). A Drosophila neurexin is required for septate junction and blood-nerve barrier formation and function. Cell *87*, 1059-68.

Behr,M., Riedel,D., and Schuh,R. (2003). The claudin-like megatrachea is essential in septate junctions for the epithelial barrier function in Drosophila. Dev. Cell *5*, 611-620.

Bellen,H.J., Lu,Y., Beckstead,R., and Bhat,M.A. (1998). Neurexin IV, caspr and paranodin--novel members of the neurexin family: encounters of axons and glia. Trends Neurosci. *21*, 444-449.

Bennett,V. and Baines,A.J. (2001). Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues. Physiol Rev. *81*, 1353-1392.

Bennett,V. and Chen,L. (2001). Ankyrins and cellular targeting of diverse membrane proteins to physiological sites. Curr. Opin. Cell Biol. *13*, 61-67.

Bhat,M.A., Rios,J.C., Lu,Y., Garcia-Fresco,G.P., Ching,W., St Martin,M., Li,J., Einheber,S., Chesler,M., Rosenbluth,J., Salzer,J.L., and Bellen,H.J. (2001). Axon-glia interactions and the domain organization of myelinated axons requires neurexin IV/Caspr/Paranodin. Neuron *30*, 369-383.

Bieber,A.J., Snow,P.M., Hortsch,M., Patel,N.H., Jacobs,J.R., Traquina,Z.R., Schilling,J., and Goodman,C.S. (1989). Drosophila neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. Cell *59*, 447-460.

Bilder,D., Li,M., and Perrimon,N. (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. Science *289*, 113-116.

Bilder,D. and Perrimon,N. (2000). Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. Nature *403*, 676-80.

Bolliger,M.F., Frei,K., Winterhalter,K.H., and Gloor,S.M. (2001). Identification of a novel neuroligin in humans which binds to PSD-95 and has a widespread expression. Biochem. J. *356*, 581-588.

Boucard,A.A., Chubykin,A.A., Comoletti,D., Taylor,P., and Sudhof,T.C. (2005). A splice code for trans-synaptic cell adhesion mediated by binding of neuroligin 1 to alpha- and beta-neurexins. Neuron *48*, 229-236.

Bouley,M., Tian,M.Z., Paisley,K., Shen,Y.C., Malhotra,J.D., and Hortsch,M. (2000). The L1-type cell adhesion molecule neuroglian influences the stability of neural ankyrin in the Drosophila embryo but not its axonal localization. J. Neurosci. *20*, 4515-4523.

Boyle,M.E., Berglund,E.O., Murai,K.K., Weber,L., Peles,E., and Ranscht,B. (2001). Contactin orchestrates assembly of the septate-like junctions at the paranode in myelinated peripheral nerve. Neuron *30*, 385-397.

Brand,A.H. and Perrimon,N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Carlson,S.D., Juang,J.L., Hilgers,S.L., and Garment,M.B. (2000). Blood barriers of the insect. Annu. Rev. Entomol. *45*, 151-174.

Caruana,G. (2002). Genetic studies define MAGUK proteins as regulators of epithelial cell polarity. Int. J. Dev. Biol. *46*, 511-518.

Cereijido,M., Contreras,R.G., and Shoshani,L. (2004). Cell adhesion, polarity, and epithelia in the dawn of metazoans. Physiol Rev. *84*, 1229-1262.

Charles,P., Tait,S., Faivre-Sarrailh,C., Barbin,G., Gunn-Moore,F., Denisenko-Nehrbass,N., Guennoc,A.M., Girault,J.A., Brophy,P.J., and Lubetzki,C. (2002). Neurofascin is a glial receptor for the paranodin/Caspr-contactin axonal complex at the axoglial junction. Curr Biol *12*, 217-20.

Cho,K.O., Hunt,C.A., and Kennedy,M.B. (1992). The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein. Neuron *9*, 929-942.

Dean,C., Scholl,F.G., Choih,J., DeMaria,S., Berger,J., Isacoff,E., and Scheiffele,P. (2003). Neurexin mediates the assembly of presynaptic terminals. Nat. Neurosci. *6*, 708-716.

Denisenko-Nehrbass,N., Oguievetskaia,K., Goutebroze,L., Galvez,T., Yamakawa,H., Ohara,O., Carnaud,M., and Girault,J.A. (2003). Protein 4.1B associates with both Caspr/paranodin and Caspr2 at paranodes and juxtaparanodes of myelinated fibres. Eur. J. Neurosci. *17*, 411-416.

Denker,B.M. and Nigam,S.K. (1998). Molecular structure and assembly of the tight junction. Am. J. Physiol *274*, F1-F9.

Dubreuil,R.R., MacVicar,G., Dissanayake,S., Liu,C., Homer,D., and Hortsch,M. (1996). Neuroglian-mediated cell adhesion induces assembly of the membrane skeleton at cell contact sites. J. Cell Biol. *133*, 647-655.

Einheber,S., Zanazzi,G., Ching,W., Scherer,S., Milner,T.A., Peles,E., and Salzer,J.L. (1997). The axonal membrane protein Caspr, a homologue of neurexin IV, is a component of the septate-like paranodal junctions that assemble during myelination. J. Cell Biol. *139*, 1495-1506.

Fanning,A.S., Mitic,L.L., and Anderson,J.M. (1999). Transmembrane proteins in the tight junction barrier. J. Am. Soc. Nephrol. *10*, 1337-1345.

Fehon,R.G., Dawson,I.A., and Artavanis-Tsakonas,S. (1994). A Drosophila homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the coracle gene. Development *120*, 545-57.

Furuse,M., Furuse,K., Sasaki,H., and Tsukita,S. (2001). Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. J. Cell Biol. *153*, 263-272.

Genova,J.L. and Fehon,R.G. (2003). Neuroglian, Gliotactin, and the Na+/K+ ATPase are essential for septate junction function in Drosophila. J Cell Biol *161*, 979-89.

Gilbert,M., Smith,J., Roskams,A.J., and Auld,V.J. (2001). Neuroligin 3 is a vertebrate gliotactin expressed in the olfactory ensheathing glia, a growth-promoting class of macroglia. Glia *34*, 151-164.

Gilbert,M.M. and Auld,V.J. (2005). Evolution of clams (cholinesterase-like adhesion molecules): structure and function during development. Front Biosci. *10*, 2177-2192.

Gollan,L., Sabanay,H., Poliak,S., Berglund,E.O., Ranscht,B., and Peles,E. (2002). Retention of a cell adhesion complex at the paranodal junction requires the cytoplasmic region of Caspr. J. Cell Biol. *157*, 1247-1256.

Graf,F., Noirot-Timothee,C., and Noirot,C. (1982). The specialization of septate junctions in regions of tricellular junctions. I. Smooth septate junctions (=continuous junctions). J. Ultrastruct. Res. *78*, 136-151.

Hirao,K., Hata,Y., Ide,N., Takeuchi,M., Irie,M., Yao,I., Deguchi,M., Toyoda,A., Sudhof,T.C., and Takai,Y. (1998). A novel multiple PDZ domain-containing molecule interacting with N-methyl-D-aspartate receptors and neuronal cell adhesion proteins. J. Biol. Chem. *273*, 21105-21110.

Hortsch,M., Bieber,A.J., Patel,N.H., and Goodman,C.S. (1990). Differential splicing generates a nervous system-specific form of Drosophila neuroglian. Neuron *4*, 697-709.

Hortsch,M. and Margolis,B. (2003). Septate and paranodal junctions: kissing cousins. Trends Cell Biol. *13*, 557-561.

Hortsch,M., Wang,Y.M., Marikar,Y., and Bieber,A.J. (1995). The cytoplasmic domain of the Drosophila cell adhesion molecule neuroglian is not essential for its homophilic adhesive properties in S2 cells. J. Biol. Chem. *270*, 18809-18817.

Hough,C.D., Woods,D.F., Park,S., and Bryant,P.J. (1997). Organizing a functional junctional complex requires specific domains of the Drosophila MAGUK Discs large. Genes Dev *11*, 3242-53.

Ichtchenko,K., Hata,Y., Nguyen,T., Ullrich,B., Missler,M., Moomaw,C., and Sudhof,T.C. (1995). Neuroligin 1: a splice site-specific ligand for beta-neurexins. Cell *81*, 435-443.

Ichtchenko,K., Nguyen,T., and Sudhof,T.C. (1996). Structures, alternative splicing, and neurexin binding of multiple neuroligins. J. Biol. Chem. *271*, 2676-2682.

Iida,J., Hirabayashi,S., Sato,Y., and Hata,Y. (2004). Synaptic scaffolding molecule is involved in the synaptic clustering of neuroligin. Mol. Cell Neurosci. *27*, 497-508.

Ikenouchi,J., Furuse,M., Furuse,K., Sasaki,H., Tsukita,S., and Tsukita,S. (2005). Tricellulin constitutes a novel barrier at tricellular contacts of epithelial cells. J. Cell Biol. *171*, 939-945.

Irie,M., Hata,Y., Takeuchi,M., Ichtchenko,K., Toyoda,A., Hirao,K., Takai,Y., Rosahl,T.W., and Sudhof,T.C. (1997). Binding of neuroligins to PSD-95. Science *277*, 1511-1515.

Islam,R., Wei,S.Y., Chiu,W.H., Hortsch,M., and Hsu,J.C. (2003). Neuroglian activates Echinoid to antagonize the Drosophila EGF receptor signaling pathway. Development *130*, 2051-2059.

Jamain,S., Quach,H., Betancur,C., Rastam,M., Colineaux,C., Gillberg,I.C., Soderstrom,H., Giros,B., Leboyer,M., Gillberg,C., and Bourgeron,T. (2003). Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. Nat. Genet. *34*, 27-29.

Karlstrom,R.O., Wilder,L.P., and Bastiani,M.J. (1993). Lachesin: an immunoglobulin superfamily protein whose expression correlates with neurogenesis in grasshopper embryos. Development *118*, 509-522.

Knust,E. and Bossinger,O. (2002). Composition and formation of intercellular junctions in epithelial cells. Science *298*, 1955-1959.

Kurschner,C., Mermelstein,P.G., Holden,W.T., and Surmeier,D.J. (1998). CIPP, a novel multivalent PDZ domain protein, selectively interacts with Kir4.0 family members, NMDA receptor subunits, neurexins, and neuroligins. Mol. Cell Neurosci. *11*, 161-172.

Levesque,J.P., Takada,Y., Puzon-McLaughlin,W., and Simmons,P.J. (2002). A novel monoclonal antibody recognizing a cation-dependent epitope within the regulatory loop of human beta(1) integrin (CD29). Hybrid. Hybridomics. *21*, 253-260.

Levinson,J.N., Chery,N., Huang,K., Wong,T.P., Gerrow,K., Kang,R., Prange,O., Wang,Y.T., and El Husseini,A. (2005). Neuroligins mediate excitatory and inhibitory synapse formation: involvement of PSD-95 and neurexin-1beta in neuroligin-induced synaptic specificity. J. Biol. Chem. *280*, 17312-17319.

Llimargas,M., Strigini,M., Katidou,M., Karagogeos,D., and Casanova,J. (2004). Lachesin is a component of a septate junction-based mechanism that controls tube size and epithelial integrity in the Drosophila tracheal system. Development *131*, 181-90.

Lord,B.A. and DiBona,D.R. (1976). Role of the septate junction in the regulation of paracellular transepithelial flow. J Cell Biol *71*, 967-72.

Lue,R.A., Marfatia,S.M., Branton,D., and Chishti,A.H. (1994b). Cloning and characterization of hdlg: the human homologue of the Drosophila discs large tumor suppressor binds to protein 4.1. Proc. Natl. Acad. Sci. U. S. A *91*, 9818-9822.

Lue,R.A., Marfatia,S.M., Branton,D., and Chishti,A.H. (1994a). Cloning and characterization of hdlg: the human homologue of the Drosophila discs large tumor suppressor binds to protein 4.1. Proc. Natl. Acad. Sci. U. S. A *91*, 9818-9822.

Marfatia,S.M., Morais Cabral,J.H., Lin,L., Hough,C., Bryant,P.J., Stolz,L., and Chishti,A.H. (1996). Modular organization of the PDZ domains in the human discs-large protein suggests a mechanism for coupling PDZ domain-binding proteins to ATP and the membrane cytoskeleton. J. Cell Biol. *135*, 753-766.

McLaughlin,M., Hale,R., Ellston,D., Gaudet,S., Lue,R.A., and Viel,A. (2002). The distribution and function of alternatively spliced insertions in hDlg. J. Biol. Chem. *277*, 6406-6412.

Mendoza,C., Olguin,P., Lafferte,G., Thomas,U., Ebitsch,S., Gundelfinger,E.D., Kukuljan,M., and Sierralta,J. (2003). Novel isoforms of Dlg are fundamental for neuronal development in Drosophila. J. Neurosci. *23*, 2093-2101.

Morais Cabral,J.H., Petosa,C., Sutcliffe,M.J., Raza,S., Byron,O., Poy,F., Marfatia,S.M., Chishti,A.H., and Liddington,R.C. (1996). Crystal structure of a PDZ domain. Nature *382*, 649-652.

Morin,X., Daneman,R., Zavortink,M., and Chia,W. (2001). A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in Drosophila. Proc. Natl. Acad. Sci. U. S. A *98*, 15050-15055.

Nelson,W.J. and Veshnock,P.J. (1987). Ankyrin binding to (Na+ + K+)ATPase and implications for the organization of membrane domains in polarized cells. Nature *328*, 533-536.

Nguyen,T. and Sudhof,T.C. (1997). Binding properties of neuroligin 1 and neurexin 1beta reveal function as heterophilic cell adhesion molecules. J. Biol. Chem. *272*, 26032-26039.

Patel,N.H., Snow,P.M., and Goodman,C.S. (1987). Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in Drosophila. Cell *48*, 975-988.

Paul,S.M., Ternet,M., Salvaterra,P.M., and Beitel,G.J. (2003). The Na+/K+ ATPase is required for septate junction function and epithelial tube-size control in the Drosophila tracheal system. Development *130*, 4963-74.

Peles,E., Joho,K., Plowman,G.D., and Schlessinger,J. (1997). Close similarity between Drosophila neurexin IV and mammalian Caspr protein suggests a conserved mechanism for cellular interactions. Cell *88*, 745-746.

Philibert,R.A., Winfield,S.L., Sandhu,H.K., Martin,B.M., and Ginns,E.I. (2000). The structure and expression of the human neuroligin-3 gene. Gene *246*, 303-310.

Poliak,S., Gollan,L., Martinez,R., Custer,A., Einheber,S., Salzer,J.L., Trimmer,J.S., Shrager,P., and Peles,E. (1999). Caspr2, a new member of the neurexin superfamily, is localized at the juxtaparanodes of myelinated axons and associates with K+ channels. Neuron *24*, 1037-1047.

Poliak,S., Gollan,L., Salomon,D., Berglund,E.O., Ohara,R., Ranscht,B., and Peles,E. (2001). Localization of Caspr2 in myelinated nerves depends on axon-glia interactions and the generation of barriers along the axon. J. Neurosci. *21*, 7568-7575.

Poliak,S., Salomon,D., Elhanany,H., Sabanay,H., Kiernan,B., Pevny,L., Stewart,C.L., Xu,X., Chiu,S.Y., Shrager,P., Furley,A.J., and Peles,E. (2003). Juxtaparanodal clustering of Shaker-like K+ channels in myelinated axons depends on Caspr2 and TAG-1. J. Cell Biol. *162*, 1149-1160.

Ranscht,B. (1988). Sequence of contactin, a 130-kD glycoprotein concentrated in areas of interneuronal contact, defines a new member of the immunoglobulin supergene family in the nervous system. J. Cell Biol. *107*, 1561-1573.

Rathjen,F.G., Norenberg,U., and Volkmer,H. (1992). Glycoproteins implicated in neural cell adhesion and axonal growth. Biochem. Soc. Trans. *20*, 405-409.

Rathjen,F.G., Wolff,J.M., Chang,S., Bonhoeffer,F., and Raper,J.A. (1987). Neurofascin: a novel chick cell-surface glycoprotein involved in neurite-neurite interactions. Cell *51*, 841-849.

Rios,J.C., Melendez-Vasquez,C.V., Einheber,S., Lustig,M., Grumet,M., Hemperly,J., Peles,E., and Salzer,J.L. (2000). Contactin-associated protein (Caspr) and contactin form a complex that is targeted to the paranodal junctions during myelination. J. Neurosci. *20*, 8354-8364.

Schafer,D.P., Bansal,R., Hedstrom,K.L., Pfeiffer,S.E., and Rasband,M.N. (2004). Does paranode formation and maintenance require partitioning of neurofascin 155 into lipid rafts? J. Neurosci. *24*, 3176-3185.

Scheiffele,P., Fan,J., Choih,J., Fetter,R., and Serafini,T. (2000). Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. Cell *101*, 657-669.

Scherer,S.S. and Arroyo,E.J. (2002). Recent progress on the molecular organization of myelinated axons. J. Peripher. Nerv. Syst. *7*, 1-12.

Schulte,J., Charish,K., Que,J., Ravn,S., Mackinnon,C., and Auld,V.J. (2006). Gliotactin and Discs large form a protein complex at the tricellular junction of polarized epithelial cells in Drosophila. J. Cell Sci.

Schulte,J., Tepass,U., and Auld,V.J. (2003). Gliotactin, a novel marker of tricellular junctions, is necessary for septate junction development in Drosophila. J Cell Biol *161*, 991-1000.

Shiga,T. and Oppenheim,R.W. (1991). Immunolocalization studies of putative guidance molecules used by axons and growth cones of intersegemental interneurons in the chick embryo spinal cord. J. Comp Neurol. *310*, 234-252.

Snow,P.M., Bieber,A.J., and Goodman,C.S. (1989). Fasciclin III: a novel homophilic adhesion molecule in Drosophila. Cell *59*, 313-323.

Song,J.Y., Ichtchenko,K., Sudhof,T.C., and Brose,N. (1999). Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. Proc. Natl. Acad. Sci. U. S. A *96*, 1100-1105.

Sonoda,N., Furuse,M., Sasaki,H., Yonemura,S., Katahira,J., Horiguchi,Y., and Tsukita,S. (1999). Clostridium perfringens enterotoxin fragment removes specific claudins from tight junction strands: Evidence for direct involvement of claudins in tight junction barrier. J. Cell Biol. *147*, 195-204.

Spiegel,I. and Peles,E. (2002). Cellular junctions of myelinated nerves (Review). Mol Membr Biol *19*, 95-101.

Staddon,J.M. and Rubin,L.L. (1996). Cell adhesion, cell junctions and the blood-brain barrier. Curr. Opin. Neurobiol. *6*, 622-627.

Tait,S., Gunn-Moore,F., Collinson,J.M., Huang,J., Lubetzki,C., Pedraza,L., Sherman,D.L., Colman,D.R., and Brophy,P.J. (2000). An oligodendrocyte cell adhesion molecule at the site of assembly of the paranodal axo-glial junction. J Cell Biol *150*, 657-66.

Tepass,U. and Hartenstein,V. (1994). The development of cellular junctions in the Drosophila embryo. Dev. Biol. *161*, 563-596.

Tepass,U., Tanentzapf,G., Ward,R., and Fehon,R. (2001). Epithelial cell polarity and cell junctions in Drosophila. Annu. Rev. Genet. *35*, 747-784.

Tsukita,S., Furuse,M., and Itoh,M. (2001). Multifunctional strands in tight junctions. Nat. Rev. Mol. Cell Biol. *2*, 285-293.

Tuvia,S., Garver,T.D., and Bennett,V. (1997). The phosphorylation state of the FIGQY tyrosine of neurofascin determines ankyrin-binding activity and patterns of cell segregation. Proc. Natl. Acad. Sci. U. S. A *94*, 12957-12962.

van Kooyk,Y., Weder,P., Hogervorst,F., Verhoeven,A.J., van Seventer,G., te Velde,A.A., Borst,J., Keizer,G.D., and Figdor,C.G. (1991). Activation of LFA-1 through a Ca2(+)-dependent epitope stimulates lymphocyte adhesion. J. Cell Biol. *112*, 345-354.

Venema,D.R., Zeev-Ben-Mordehai,T., and Auld,V.J. (2004). Transient apical polarization of Gliotactin and Coracle is required for parallel alignment of wing hairs in Drosophila. Dev. Biol. *275*, 301-314.

Volkmer,H., Hassel,B., Wolff,J.M., Frank,R., and Rathjen,F.G. (1992). Structure of the axonal surface recognition molecule neurofascin and its relationship to a neural subgroup of the immunoglobulin superfamily. J. Cell Biol. *118*, 149-161.

Walsh,F.S. and Doherty,P. (1991). Glycosylphosphatidylinositol anchored recognition molecules that function in axonal fasciculation, growth and guidance in the nervous system. Cell Biol. Int. Rep. *15*, 1151-1166.

Ward,R.E.t., Lamb,R.S., and Fehon,R.G. (1998). A conserved functional domain of Drosophila coracle is required for localization at the septate junction and has membrane-organizing activity. J Cell Biol *140*, 1463-73.

Ward,R.E.t., Schweizer,L., Lamb,R.S., and Fehon,R.G. (2001). The protein 4.1, ezrin, radixin, moesin (FERM) domain of Drosophila Coracle, a cytoplasmic component of the septate junction, provides functions essential for embryonic development and imaginal cell proliferation. Genetics *159*, 219-28.

Willott,E., Balda,M.S., Fanning,A.S., Jameson,B., Van Itallie,C., and Anderson,J.M. (1993). The tight junction protein ZO-1 is homologous to the Drosophila discs-large tumor suppressor protein of septate junctions. Proc Natl Acad Sci U S A *90*, 7834-8.

Woods,D.F. and Bryant,P.J. (1991). The discs-large tumor suppressor gene of Drosophila encodes a guanylate kinase homolog localized at septate junctions. Cell *66*, 451-64.

Woods,D.F., Hough,C., Peel,D., Callaini,G., and Bryant,P.J. (1996b). Dlg protein is required for junction structure, cell polarity, and proliferation control in Drosophila epithelia. J Cell Biol *134*, 1469-82.

Woods,D.F., Hough,C., Peel,D., Callaini,G., and Bryant,P.J. (1996a). Dlg protein is required for junction structure, cell polarity, and proliferation control in Drosophila epithelia. J. Cell Biol. *134*, 1469-1482.

Wu,V.M., Schulte,J., Hirschi,A., Tepass,U., and Beitel,G.J. (2004). Sinuous is a Drosophila claudin required for septate junction organization and epithelial tube size control. J Cell Biol *164*, 313-23.

Zeev-Ben-Mordehai,T., Rydberg,E.H., Solomon,A., Toker,L., Auld,V.J., Silman,I., Botti,S., and Sussman,J.L. (2003). The intracellular domain of the Drosophila cholinesterase-like neural adhesion protein, gliotactin, is natively unfolded. Proteins *53*, 758-767.

Zhang,Z., Devarajan,P., Dorfman,A.L., and Morrow,J.S. (1998). Structure of the ankyrin-binding domain of alpha-Na,K-ATPase. J. Biol. Chem. *273*, 18681-18684.