RICE UNIVERSITY

Control of Perineurial Glial Growth in *Drosophila melanogaster*

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ABSTRACT

Although intercellular communication within peripheral nerves is critical to the structure and function of the nervous system, it is incompletely understood. *Drosophila* peripheral nerves comprise motor and sensory axons bundled by peripheral glia (Schwann cells) and wrapped by perineurial glia (perineurium). I have shown that growth of the perineurial glia is controlled by signaling pathways involving six genes: *push*, which encodes a large Zn$^{2+}$ finger containing protein; *amn* which encodes a putative neuropeptide; *Axs*, which is suggested to encode a G-protein coupled receptor; *ine*, which encodes a putative neurotransmitter/osmolyte transporter; *eag*, which encodes a potassium channel; and *Nf1*, which encodes neurofibromin and is the *Drosophila* ortholog of the human gene responsible for Neurofibromatosis type 1. I provide evidence that neurofibromin, in accordance with its role as a Ras guanosine triphosphatase activating protein (Ras GAP), acts to down regulate Ras activity to control perineurial glial growth. My work suggests that loss of neurofibromin leads to an increase in Ras activity in the peripheral glia that, in conjunction with loss of either *Ine* or *Push*, introduces a cell-nonautonomous signal that promotes growth of the perineurial glia. I have also found that *Push* does not act through Ras to control perineurial glial growth. My working
hypothesis is that Amn acts through two separate pathways, one involving Push and the other involving neurofibromin, to inhibit perineurial glial growth. In this model, a separate pathway involving the substrate neurotransmitter of Ine promotes perineurial glial growth. I speculate that Ine may act to remove its substrate neurotransmitter from the extracellular space, thereby inhibiting the neurotransmitter from acting through its receptor to promote perineurial glial growth. Alternatively, Ine may control perineurial glial growth via its role as an osmolyte transporter. Eag may act to inhibit perineurial glial growth by repressing release of factors from the neurons or peripheral glia through maintaining these cells in a hyperpolarized state.
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<table>
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<th>Definition</th>
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<tbody>
<tr>
<td>push</td>
<td>pushover</td>
</tr>
<tr>
<td>amn</td>
<td>amnesiac</td>
</tr>
<tr>
<td>Axs</td>
<td>Aberrant X segregation</td>
</tr>
<tr>
<td>ine</td>
<td>inebriated</td>
</tr>
<tr>
<td>eag</td>
<td>ether a go-go</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromin 1</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase Activating Protein</td>
</tr>
<tr>
<td>gli</td>
<td>gliotactin</td>
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<tr>
<td>Nrv2</td>
<td>Nervana2</td>
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<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>mIPSCs</td>
<td>miniature Inhibitory Postsynaptic Currents</td>
</tr>
<tr>
<td>Dhh</td>
<td>Desert hedge hog</td>
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<tr>
<td>Hh</td>
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<tr>
<td>ana</td>
<td>anachronism</td>
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<tr>
<td>VIP</td>
<td>Vasoactive Intestinal Peptide</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary Adenyl Cyclase Activator Peptide</td>
</tr>
<tr>
<td>MGA</td>
<td>Medial Giant Axon</td>
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PDGF  Platelet Derived Growth Factor
MS    Multiple Sclerosis
FGF   Fibroblast Growth Factor
CNTF  Ciliary Neurotrophic Factor
NDF   Neu Differentiation Factor
TGF-β Transforming Growth Factor β
Dpp   Decapentaplegic
Tkv   Thick veins
DER   *Drosophila* Epidermal Growth Factor Receptor
EGF   Epidermal Growth Factor
EGF-R Epidermal Growth Factor Receptor
*rho* rhomboid
*rpr* reaper
*hid* *head involution defective*
MAPK  Mitogen Activated Protein Kinase
*nod* *non distributive disjunction*
PKA   Protein Kinase A
*rut* *rutabaga*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>TSP-1</td>
<td>Thrombospondin-1</td>
</tr>
<tr>
<td>MK</td>
<td>Midkine</td>
</tr>
<tr>
<td>MPNST</td>
<td>Malignant Peripheral Nerve Sheath Tumor</td>
</tr>
<tr>
<td>HBEC</td>
<td>Human Brain Derived Endothelial Cells</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
</tr>
<tr>
<td>FA1</td>
<td>Fetal Antigen 1</td>
</tr>
<tr>
<td><em>top</em></td>
<td><em>torpedo</em></td>
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<tr>
<td><em>spi</em></td>
<td><em>spitz</em></td>
</tr>
<tr>
<td>CyORoi</td>
<td>Curly Rough eye</td>
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<td>ORF</td>
<td>Open Reading Frame</td>
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Chapter 1

Introduction and Background

1.1 Structure of peripheral nerves

In *Drosophila*, the peripheral nerves contain both motor and sensory axons surrounded by peripheral glial cells, which are homologs of mammalian Schwann cells (Sepp et al. 2000). A perineurial glial layer (perineurium in mammals) wraps the axons and peripheral glia (Fig.1). The peripheral glia form the “blood-brain” barrier (Edwards et al. 1993). Little is known about the role of the perineurial glial layer, but it is thought to provide structural support to the peripheral nerve. The perineurial glial layer is not present in *twist* mutants, which lack mesoderm, suggesting that the perineurial glial layer is mesodermally derived (Edwards et al. 1993). This possibility is further supported by the observation that peripheral glia express *gliotactin* and *Nervana2* genes (Auld et al. 1995). These genes are not expressed in perineurial glia, suggesting that peripheral and perineurial glia are of different origins (Vannessa Auld and Mary Rheuben, Personal Communication).

1.2 Glial signaling

Recent discoveries have elucidated that glial cells play more than just a passive or structural role in the nervous system. It is becoming increasingly
Fig. 1. Transmission electron micrograph of a cross-section of a *Drosophila* peripheral nerve. Wandering third instar larvae were collected, dissected, fixed using paraformaldehyde and glutaraldehyde and stained with 0.5% OsO4 and 2% Uranyl acetate and embedded in an eponate 12-araldite resin. Cross-sectional slices were obtained and post-stained with Uranyl acetate and Reynolds lead citrate. The slices were analyzed using a Jeol or Hitachi transmission electron microscope at 60-80KV. Arrows indicate axons, peripheral glia and perineurial glia.
apparent that glial cells play an active role in neuronal function. Jahromi and colleagues found that electrical stimulation of the frog motor nerve or application of Acetylcholine (Ach) resulted in an increase in intracellular Ca$^{2+}$ levels in Schwann cells. To determine if the increase in intracellular Ca$^{2+}$ levels in the Schwann cells was due to the influx of extracellular Ca$^{2+}$ or release of Ca$^{2+}$ from internal stores, they applied Ach to the Schwann cells in the presence of the Ca$^{2+}$ chelator ethylene glycol bis(b-aminoethylether)N,N$^1$-tetraacetic acid (EGTA) and in the absence of extracellular Ca$^{2+}$. They observed an increase in intracellular Ca$^{2+}$ levels in the Schwann cells in the absence of extracellular Ca$^{2+}$ and in the extracellular presence of EGTA suggesting that the increase of intracellular Ca$^{2+}$ in the Schwann cells is due to release of Ca$^{2+}$ from the internal stores (Jahromi et al. 1992). The increase of intracellular Ca$^{2+}$ levels in the Schwann cells is suggested to be potentially achieved through the second messenger inositol 1,4,5-triphosphate (IP3) (Castonguay and Robitaille, 2001). Kang and colleagues have shown that direct electrical stimulation through a patch-clamping pipet of rat astrocytes causes miniature inhibitory postsynaptic currents (mIPSCs) in pyramidal neurons. They further noted that injection of the calcium chelator BAPTA into these astrocytes, or the application of glutamate antagonists blocked this response. Furthermore,
they reported that stimulation of interneurons cause nearby astrocytes to exhibit an increase in intracellular calcium. These results lead to the hypothesis that interneuron activity promotes an increase in intracellular calcium in the astrocytes, which subsequently potentiates the inhibitory signal (Kang et. al. 1998). Parpura and colleagues have studied neuron-astrocyte signaling in cell culture. They noted that when cultured astrocytes were exposed to bradykinin, they exhibited an increase in internal Ca$^{2+}$ levels and released glutamate. Application of bradykinin to glial cultures, or direct photostimulation of astrocytes resulted in an increase in calcium levels in the neurons in neuron-astrocyte co-cultures but not in cultures containing only neurons. Furthermore, glutamate receptor antagonists blocked this response. These results suggest that astrocytes send glutamate-mediated signals to neurons, which cause an increase in intracellular neuronal Ca$^{2+}$ levels (Parpura et al., 1994)

Several lines of evidence support release of a growth factor from peripheral glia. First, the observation of Ca$^{2+}$ dependent glutamate release from rat astrocytes, which suggests that Ca$^{2+}$/SNARE-dependent release of factors could be performed by peripheral glia (Araque et al. 2000). Second, it has been noted that proper formation of the perineurium in mammals requires the release of Desert hedgehog and other morphogens from the
Schwann cells (Parmantier et al. 1999). Dhh is homologous to *Drosophila* Hedgehog (Hh) protein, which is a secreted peptide that induces differentiation of lamina precursor cells into neurons and glia in the brain (Hammerschmidt et al., 1997; Huang et al., 1996). Mammalian Dhh and *Drosophila* Hh share 51% identity over the entire length of the processed proteins (Hammerschmidt et al. 1997). Third, it has also been shown that insulin-like growth factor, neurotrophin and platelet derived growth factor are released by Schwann cells in vitro (Meier et al. 1999). Fourth, *Drosophila* glial cells have been shown to control proliferation of neuronal precursors via secreted polypeptides. Ebens and colleagues identified a p-element induced mutation in the *anachronism* (*ana*) locus that leads to disorganization of the optic lobe. They noted an increase in the proliferation of optic lobe neuroblasts in *ana* mutants. Sequence analysis of *ana* predicted that *ana* encodes a secreted glycoprotein. Analysis of mRNA and reporter expression demonstrated that *ana* is expressed in glial cells, not neuroblasts, suggesting that glial cells release secreted factors to control growth of other tissues in *Drosophila* (Ebens et al. 1993).

### 1.3 Similarities in neuron-glia signaling in other systems

There are several potential similarities between neuron-glia signaling in squid and neuron-glia signaling in our system. It has been shown that
application of Vasoactive Intestinal Peptide (VIP), a PACAP-like
neuropeptide, causes long term hyperpolarization of the Schwann cell
membrane (Evans et al. 1986; Evans and Villegas 1988). It has also been
shown that glutamate application induces co-release of acetylcholine (Ach)
and a VIP-like peptide from these Schwann cells, which act in an autocrine
fashion, to cause a long-lived hyperpolarization of the Schwann cell
membrane (Evans et al. 1999; Evans et al. 1992; Villegas 1974; Villegas and
Villegas 1974). Taking into account the observation that rat astrocyte cells
release glutamate via a Ca^{2+}/SNARE-dependent pathway, it is possible that
the glutamate induced release of Ach and VIP from squid Schwann cells is
also accomplished via a Ca^{2+}/SNARE-dependent mechanism. Ach induces
this hyperpolarization via the nicotinic receptors on the Schwann cells,
which mediates its effect via activation of adenylate cyclase (Evans et al.
1985). In crayfish, the satellite cells, which are homologous to squid
Schwann and *Drosophila* peripheral glial cells, exhibit hyperpolarization in
response to treatment with Ach, carbachol and nicotine. Muscarine
treatment had no effect on the membrane potential of the satellite cells.
These observations suggest that nicotinic Ach receptors are present on these
cells (Lieberman et al. 1981). It has also been shown that electrical
stimulation of the crayfish medial giant axon (MGA) causes a change in the
membrane potential of the periaxonal glia. This glial response is blocked by non-N-methyl-D-aspartate glutamate receptor antagonists, and is extended by a nonspecific glutamate transporter inhibitor (Lieberman et al. 1994). In addition, it was noted that L-glutamate is taken up by periaxonal glia in crayfish central nerve fibers (Kane et al. 2000). This work suggests that glutamate plays a role in axon-glia signaling in crayfish, providing evidence that axon-glia communication has similar mechanisms in arthropods and squid. Amnesiac (Amn) is a putative neuropeptide in Drosophila that is a member of the VIP family, and the substrate neurotransmitter of Inebriated (Ine), a putative neurotransporter/osmolyte transporter, is probably a small molecule neurotransmitter. Therefore these studies may provide some insight into neuron-glia signaling in Drosophila (Fig. 2).

1.4 Glial growth

Glial growth has been studied extensively in several demyelinating disorders. Multiple Sclerosis (MS) is a disease that affects 1 million people world wide. In patients with this disease, the immune system mounts an attack against glial cells that generate myelin sheaths. The resulting demyelination of neurons leads to debilitating motor system defects and in some cases death (Compston and Coles 2002). Although the major clinical
Fig. 2. Model of squid system as studied by the Villegas lab (Evans et al. 1985, 1986, 1988 and 1992; Villegas 1974; Villegas and Villegas 1974). This work suggests that signals from the neuron and peripheral glia act on the Schwann cells (peripheral glia) to increase K⁺ conductance of the Schwann cell (peripheral glial) membrane. VIP is in the same family as PACAP and Ann is a putative PACAP-like neuropeptide (Miyata et al. 1989; Feany and Quinn 1995). This work may provide some insight into neuron-glial signaling in Drosophila.
feature of MS is demyelination, Prineas and colleagues observed a limited amount of remyelination in the central nervous system of some MS patients (Prineas and Connell 1979). A limited amount of remyelination has also been noted to occur in other demyelinating disorders and has allowed researchers to elucidate the mechanisms of glial growth. It has been shown that astrocytes induce proliferation of bipotential progenitor cells that generate oligodendrocytes, which ultimately form myelin. This response is blocked in the presence of antibodies to platelet derived growth factor (PDGF) (Richardson et al. 1988). It has also been reported that injection of PDGF significantly improves remyelination in demyelinating lesions induced in rats (Allamargot et al. 2001). Redwine and colleagues investigated the possible role of PDGF in remyelination. They observed extensive remyelination in areas exhibiting demyelination in mice infected with murine hepatitis virus A59. Through in situ hybridization and immunocytochemical analysis, they found that expression of PDGF and its receptor were increased in oligodendrocyte progenitor cells that proliferate during remyelination. This observation suggests that PDGF plays a role in remyelination (Redwine and Armstrong 1998). In this same system, Messersmith and colleagues observed a significant increase in the levels of fibroblast growth factor 2 (FGF2) and FGF receptors 1,2 and 3 mRNA in the
spinal cord during remyelination suggesting that FGF2 also plays a role in remyelination (Messersmith et al. 2000).

Glial cell development has also been studied through mutagenesis. Murphy and colleagues generated a mouse line in which the zinc finger containing transcription factor Krox-20 is knocked out by insertion of the lacZ gene into the Krox-20 locus. In these mutants, Schwann cell differentiation into myelin-forming cells is blocked. They found that Krox-20/lacZ activity is induced in the satellite glial cells by application of diffusible growth factors including neu differentiation factor β (NDFβ), or a combination of basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF) (Murphy et al. 1996). This suggests that the input signal to the induction of myelination involves growth factors such as NDFβ, bFGF and CNTF that ultimately act through a transcription factor to mediate the output (induction of myelination) via promotion of the expression of specific genes that initiate myelination (Fig. 3).

1.5 Glial growth and proliferation in Drosophila

The Transforming Growth Factor - β (TGF-β) superfamily is a group of secreted proteins that mediate cell-cell signaling. Members of the TGF-β family have been found in Drosophila, vertebrates, nematodes and sea urchins (Raftery et al. 1999). The Drosophila gene Decapentaplegic (Dpp)
Fig. 3. Diagram of the factors involved in remyelination. Trophic factors such as platelet derived growth factor (PDGF), fibroblast growth factor (FGF), neu differentiatiation factor (NDF), and ciliary neurotrophic factor (CNTF) have been implicated in this process (Richardson et al. 1988; Allamargot et al. 2001; Redwine and Armstrong 1998; Messersmith et al. 2000). The zinc-finger containing transcription factor Krox-20 is also suggested to play a role in this process (Murphy et al. 1996). This suggests that the input signal to the induction of myelination involves growth factors that ultimately act through a transcription factor that mediates the output via promotion of the expression of specific genes that initiate myelination.
is a member of this family. *Dpp* induces non-cell autonomous overgrowth when expressed ectopically in the wing imaginal disks (Burke et al. 1996). Thick veins (Tkv) and Punt are type I and type II serine/threonine kinase receptors that are believed to be the main Dpp signal receptors (Ruberte et al. 1995). The type II receptors activate the type I receptors, which in turn activate the Smad Mad, which promotes specific gene expression (Reviewed Massague and Chen 2000). Rangarajan and colleagues expressed a dominant negative form of Tkv specifically in glia, and noted a reduction in the total number of glial cells in the eye disk. When Dpp was expressed ectopically, or a constitutively active form of Tkv was expressed in glial cells, an increase in glial cell density was observed in the optic stalk and eye disk. This increase in cell number is suggested to be due to increased proliferation (Rangarajan et al. 2001)(Fig. 4).

Extensive studies have been done on the regulation of midline glial cell number. Reviewing this work may be helpful in understanding the regulation of the thickness of the perineurial glia. The midline glia establish a path along which neurons are guided during embryogenesis. Between stages 13 to 16 of embryogenesis some of these cells undergo apoptosis. Several genes encode products, believed to play a role in epidermal growth factor-like signaling, that regulate the number of these glial cells. A
Fig. 4. Diagram of the control of optic stalk glia proliferation. Decapentaplegic (DPP) promotes proliferation of the optic stalk glia by activating Type II receptors that activate the Type I receptors that in turn activate the Smad Mad, which promotes specific gene expression (Raftery et al. 1999, Burke et al. 1996, Ruberte et al. 1995, Massague and Chen 2001, Rangarajan et al. 2001).
hypomorphic P-element insertion induced mutation in *argos, argos^{w11}*
confers a rough eye phenotype. Further analysis of the developing eye of
*argos^{w11}* mutants revealed enhanced neural recruitment in the imaginal disks
at the third instar stage. Sequence analysis suggested that *argos* encodes a
secreted protein with a region that bears resemblance to an epidermal growth
factor repeat (Freeman et al. 1992). Loss of function mutations in *argos*
exhibit an increase in wing vein material and thus phenotypically resemble
EGF receptor gain-of-function mutants. Overexpression of Argos
phenotypically resembles loss-of-function mutations in the *Drosophila*
epidermal growth factor-like receptor (DER): both overexpression of *argos*
and DER deficient mutants exhibit reduced wing veins. Through genetic
analysis, it was determined that reduction of *spitz*, which encodes an
activating ligand of the *Drosophila* EGF-like receptor (DER), suppressed the
*argos* eye and wing phenotypes. Schweitzer and colleagues measures DER
activation in S2 cells and noted that incubation with Spitz increased DER
activation considerably. They further observed that the presence of Argos
inhibited the activation of DER by Spitz. These results suggest that Argos
acts to inhibit EGF receptor signaling by either competing with Spitz for
DER binding, or by acting through another receptor to ultimately inhibit the
DER signal (Schweitzer et al. 1995).
Stemerdink and colleagues noted that a reduction in Argos, as exhibited in the \textit{argos}^{w11} mutant, increases the total number of midline glia. Ectopic expression of Argos lead to a decrease in the number of midline glial cells. In contrast, midline glial cell numbers are greatly reduced in \textit{rhomboid} (\textit{rho}), mutants: ectopic expression of \textit{rhomboid} results in an increase in the number of midline glia (Stemerdink and Jacobs 1997). The \textit{rho} gene encodes a transmembrane protein that is suggested to promote Spitz-DER signaling (Sturtevant et al. 1993). Reduction in expression of DER as exhibited in the hypomorphic \textit{flb}^{IIe07} results in a complete lack of midline glia, whereas the expression of a gain-of-function mutation in DER results in an increase in the number of midline glia. These results suggest that the Spitz-DER pathway acts to inhibit apoptosis, and Argos acts to promote loss of the midline glia (Stemerdink and Jacobs 1997).

Three genes, \textit{grim}, \textit{reaper} (\textit{rpr}) and \textit{head involution defective} (\textit{hid}) are intimately involved in apoptosis in \textit{Drosophila}. Loss of function mutations in \textit{grim}, \textit{rpr} or \textit{hid} result in embryos that do not exhibit programmed cell death. Immunocytochemical and \textit{in situ} analysis revealed that these genes are expressed in dying cells. Ectopic expression of \textit{hid} or \textit{grim} in the eye results in ablation of the eye. Co-expression of \textit{p35}, which encodes a baculovirus caspase inhibitor with anti-apoptotic activity, with \textit{grim}, \textit{rpr} or
*hid* prevents the cell death associated with the expression of these genes. Since p35 acts to inactivate ICE-proteases, Grim, Rpr and Hid may be acting through these proteases to promote cell death (Chen et al. 1996, Zhou et al. 1997, White et al. 1994). To assess the roles of these genes in midline glial cell death, Zhou and colleagues targeted expression of Rpr and Hid to the midline glial cells. Expression of either Rpr or Hid alone in the midline glia did not promote apoptosis. However, co-expression of Rpr and Hid in the midline glia resulted in a significant loss of midline glia. Through *in situ* analysis, they also observed that Rpr and Hid are expressed in dying glia. To determine if mitogen activated protein kinase (MAPK) plays a role in the inhibition of midline glial apoptosis, Bergmann and colleagues analyzed the midline glia in MAPK-deficient embryos. They noted that there were no midline glial cells left in the *mapk* mutants by stage 17, suggesting that MAPK is necessary to prevent apoptosis of these cells. To determine if MAPK prevents apoptosis by preventing Hid mediated cell death, they looked at embryos in which both *mapk* and *hid* were knocked out. The *mapk; hid* double mutant midline glia were rescued, suggesting that MAPK acts to control midline glial apoptosis by blocking the action of Hid (Bergmann et al. 2002). It has been shown that Spitz/DER signaling acts through Ras/MAPK in cell fate determination in the *Drosophila* leg (del
Alamo et al. 2002). Taken together these data support the hypothesis that Spitz/DER signaling acts through Ras/MAPK to block Hid mediated midline glial apoptosis (Fig. 5). These studies are of importance to our work because they implicate Ras in the control of glial cell number. We have shown that Ras activity also promotes an increase in perineurial glial thickness. These studies suggest that Ras acts to inhibit apoptosis of the midline glia. It is possible that inhibition of apoptosis prevents increased thickness of the perineurial glia.

1.6 Genes whose roles in perineurial glial growth I have examined

1.6.1 pushover (push)

The push gene has been cloned and encodes a 5,322 amino acid protein with 12 predicted transmembrane domains and two predicted Zn\(^{2+}\) binding regions (Yager et al., 2001). Pushover mutants exhibit neuronal hyperexcitability (Richards et al. 1996). Blast searches done by Stephen Richards revealed that genes homologous to push are present in C. elegans, Arabidopsis and humans. Previous work, performed by Stephen Richards and Daryl Hurd, has shown that the perineurial glial layer of push\(^{1}\) mutants is about 30 percent thicker than wild type. In contrast, ine\(^{1}\) mutants have normal perineurial glial thickness. However, ine\(^{1}\) push\(^{1}\) double mutants exhibit perineurial glial thickness about 3 times that of wild type. These
results suggest that ine$^l$ and push$^l$ are acting in redundant pathways in the control of perineurial glial growth (Fig. 6).

1.6.2 inebriated (ine)

The ine gene has been cloned and encodes at least two isoforms, of 658 amino acids and 943 amino acids, which bear similarity to a family of Na$^+$/Cl$^-$ dependent neurotransmitter transporters. Mutations in ine were isolated based on behavioral interactions with mutations in the K$^+$ channel gene Shaker (Sh). In the Sh mutant background, ine mutations cause indented thorax and down turned wings phenotypes (Stern et al. 1992). Mutations in ine alone confer a neuronal hyperexcitability phenotype, which is suggested to be the result of defective reuptake of the substrate neurotransmitter (Soehnge et al. 1996). Burg and colleagues independently identified mutations in ine based on electroretinogram (ERG) defects. The ine gene was cloned independently, and a heat shock ine rescue construct containing the long form of ine was generated (Burg et al. 1996). Ine is also a member of an osmolyte transporter family. Mutations in ine confer hypersensitivity to osmotic stress (Huang et al. 2001). Expression of the Manduca sexta ine homolog in Xenopus laevis oocytes leads to sensitivity to hyperosmotic stimulation and Ca$^{2+}$-activated Cl$^-$ currents (Chiu et al. 2000).
Fig. 6. Ine and Push act in separate pathways to control perineurial glial thickening. A. Electron micrographs of cross-sections of push<sup>1</sup>, inel<sup>1</sup>; push<sup>1</sup>, and inel<sup>1</sup> mutants. push<sup>1</sup> and inel<sup>1</sup>; push<sup>1</sup> exhibit thickened perineurial glial layers. Wild type indicates that isogenic parental control line for both inel<sup>1</sup> and push<sup>1</sup> mutants. All of the cells that comprise the segmental nerves appear to be present in all mutants. Analysis of inel<sup>1</sup>; push<sup>1</sup> nerves showed that they have a normal number of axons of normal cross-sectional area. The thickness of the perineurial glial layer of each nerve is determined by measuring the distance from the edge of the nerve to the edge of the axon containing lumen at 8 different positions. The eight measurements were used to determine the average perineurial glial thickness for each nerve. All statistical analysis was carried out using Statview v4.51 (Abacus concepts Inc. Berkeley, CA.). B and C. Scattergram and bar graph of glial thickness for wild type and mutant lines. push<sup>1</sup> p=0.0037 (twotailed unpaired t-test vs. wild type), inel<sup>1</sup>; push<sup>1</sup> p<0.0001 (twotailed unpaired t-test vs. wild type). Alleles used: inel<sup>1</sup> (transcript null mutation, push<sup>1</sup> (early nonsense mutation).
1.6.3 *ether a go-go* (*eag*)

The *eag* gene has been cloned and encodes a 1174 amino acid polypeptide containing seven transmembrane domains with similarity to known K$^+$ channel polypeptides of the *Shaker* (*Sh*) family (Warmke et al. 1991). Mutations in *eag* cause spontaneous action potentials in larval neurons. In a *Sh* mutant background, *eag* mutations also confer the downturned wings and indented thorax phenotypes (Ganetzky et al. 1983).

1.6.4 *NF1*

*NF1* is believed to be the gene responsible for the disease Neurofibromatosis type 1. Neurofibromin is approximately 280 kDa (DeClue et al. 1991). The *Drosophila* neurofibromin bears 60% homology to human neurofibromin (The et al. 1997). A 360 amino acid region of the human NF1 protein bears homology to the catalytic domain of mammalian guanosine triphosphatase-activating protein (GAP) (Xu et al. 1990). It has also been shown that both human and *Drosophila* NF1 act as GAPs, thus negatively regulating Ras activity *in vitro* by promoting the GTPase activity of Ras, thereby shifting Ras toward the GDP bound (inactive) state (The et al. 1997).
1.6.5 *amnesiac* (*amn*)

Mutations in the *amn* gene were isolated as enhancers of a weak mutation in *non distributive disjunction* (*nod*), loss of which leads to abnormal segregation of non-exchange chromosomes during female meiosis (Zitron and Hawley 1989; Zhang and Hawley 1990; Zhang et al. 1990; Whyte et al. 1993). Mutations in the *amn* gene have also been isolated based on defects in learning, memory and ethanol sensitivity (Feany and Quinn 1995; Moore et al. 1998).

1.6.6 *Aberrant X segregation* (*Axs*)

Mutations in *Axs* were isolated based on the observation that these mutants exhibited aberrant segregation of nonexchange chromosomes during female meiosis (Zitron and Hawley 1989). *Axs* is suggested to encode a G-protein coupled receptor (Scott Hawley, personal communication).

1.6.7 *Ras*

*Ras* is a member of a family of proteins that are highly conserved from yeast to humans. Mutations in *Ras* have been shown to promote the transformation of NIH 3T3 cells and have also been identified in tumors. These observations suggest a role for Ras in cell proliferation and differentiation. Ras protein is suggested to exist in a state of equilibrium between an active GTP bound and inactive GDP bound state. It has been
shown that Ras mutants defective in GTP binding exhibit reduced ability to transform 3T3 cells (reviewed, Santos and Nebreda 1989). Ras is believed to act as a switch in many signal transduction pathways and many effectors of Ras, including the mitogen activated protein kinases (MAPKs) have been identified (reviewed, Joneson and Bar-Sagi 1997, and Avruch et al. 1994).

1.7 Two signaling pathways in Drosophila mediated by Amnesiac

1.7.1 Control of segregation of non-exchange chromosomes during female meiosis

Mutations in push were isolated independently in Dr. Scott Hawley’s lab. They found that push mutants exhibited abnormal segregation of non-exchange chromosomes during female meiosis (Sekelsky et al. 1999). non distributive disjunction (nod) and Axs mutants also exhibited this phenotype (Zitron and Hawley 1989; Zhang and Hawley 1990; Zhang et al. 1990; Whyte et al. 1993). Mutations in the amn gene were isolated as enhancers of a weak nod mutation (Scott Hawley, personal communication). Mutations in the amn gene, which encodes a putative Pituitary Adenyl Cyclase Activator Protein (PACAP) – like neuropeptide, have also been isolated based on defects in learning, memory and ethanol sensitivity (Feany and Quinn 1995; Moore et al. 1998). These observations suggest that push is involved in a signaling pathway under control of the product of the amnesiac
gene and the *Aberrant X segregation* (*Axs*) gene which, due to its homology
to genes encoding G protein coupled receptors, is suggested to encode a
receptor for Amnesiac (Scott Hawley, personal communication). According
to Hawley, the ultimate target of this pathway is Nod, a kinesin-like protein,
which, when active, functions to hold homologous chromosomes together
until the appropriate time for segregation (Theurkauf and Hawley 1992). It
is speculated that the Amn pathway increases [cAMP], activates Protein
kinase A (PKA) and maintains Nod in the active phosphorylated state.
Mutations in genes that play a role in this pathway lead to inactivation of
Nod and abnormal segregation of the non-exchange chromosomes (Fig. 7).

1.7.2 Control of potassium channels in larval muscle

Treatment of *Drosophila* muscle with PACAP causes an increase in the
potassium conductance of the muscle membrane by activating one or more
*NFL*, *rutabaga*, which encodes a (Ca\(^{2+}\)/CaM)-sensitive adenylyl cyclase
(Levin et al. 1992), and *Ras* mutants do not exhibit this response to PACAP.
Treatment of the neuromuscular junction with cAMP analogs, in conjunction
with ectopic expression of a constitutively active form of Ras results in a
PACAP-like response (Zhong 1995; Zhong and Pena 1995; Guo et al.,
Fig. 7. Control of segregation of non-exchange chromosomes during female meiosis. Hawley suggests that this pathway is mediated by the Amn putative PACAP-like neuropeptide, released from follicle cells. Amn is suggested to act through its putative receptor, Axs located on the oocyte. This pathway acts through Push and PKA to maintain Nod in its active phosphorylated state. It is suggested that Nod holds the homologous non-exchange chromosomes together until the appropriate time for segregation. Elimination of members of this pathway leads to premature Nod inactivation and aberrant segregation of the non-exchange chromosomes (Zitron and Hawley 1989)(Zhang and Hawley 1990)(Zhang et al. 1990)(Whyte et al. 1993)(Theurkauf and Hawley 1992)(Scott Hawley, personal communication).
1997). These results suggested that this response requires two signaling
pathways, one involving *rutabaga* and *NF1*, the *Drosophila* ortholog of the
gene responsible for the disease Neurofibromatosis type 1, the other
involving Ras and Raf. Because the deficient PACAP response of *NF1*
mutants is rescued by ectopic expression of a constitutively active form of
PKA, the *rutabaga/NF1* pathway is proposed to act through [cAMP] and
PKA whereas the *ras/raf* pathway was proposed to act through MAP-kinases
(Guo et al. 1997). Further evidence that *NF1* acts through PKA includes that
observation that expression of a constitutively active form of PKA rescues
the small size defect of *NF1* mutants (The et al. 1997). In addition,
treatment of the muscle with forskolin, which activates adenylate cyclase,
restored the responsiveness of NF1 mutant muscle to PACAP (Guo et al.
1997) (Fig. 8).

1.8 Neurofibromatosis Type 1

NF1 is a genetic disorder that affects 1 in 3000 to 5000 people (Shen et
al. 1996). The three characteristic features of NF1 are café au lait spots,
neurofibromas (peripheral nerve sheath tumors) and lisch nodules (small
tumors) of the iris (Huson et al. 1988). Almost all adult patients with NF1
have neurofibromas (Riccardi et al. 1981). Neurofibromas consist mostly of
Fig. 8. Model of the response of Drosophila muscle to PACAP. Treatment of Drosophila muscle with PACAP causes an increase in the potassium conductance of the muscle membrane by activating one or more K+ channels. It is suggested that this response is mediated by two signaling pathways, one involving Rutabaga and NF1 and the other involving Ras and Raf. The Rut/NF1 pathway is suggested to act through PKA, whereas the Ras/Raf pathway is suggested to act through MAP Kinases (Zhong 1995; The et al. 1997)
Schwann cells (60-85%) and also perineurial cells, fibroblasts mast cells, vascular elements and pericytes (Peltonen et al. 1983). Although benign histologically, neurofibromas are disfiguring (Riccardi et al. 1981) and have the potential of transforming into malignant peripheral nerve sheath tumors (MPNSTs), which typically lead to death of the patient (Menon et al. 1990). It has been shown that the NF1 gene is disrupted in tumors from NF1 patients suggesting that NF1 is the gene responsible for the formation of these tumors (Wallace et al. 1990).

1.8.1 Function of NF1 in neurofibroma formation: RasGAP or activator of adenylate cyclase

A 360 amino acid region of the human NF1 protein bears homology to the catalytic domain of mammalian GTPase activating protein (GAP) (Xu et al. 1990). The Drosophila NF1 protein bears 60% homology to human NF1 (The et al. 1997). It has been shown that both human and Drosophila NF1 proteins act as GAP proteins regulating the activity of Ras in vitro. In addition, increased Ras-GTP levels have been observed in Schwann cells from human neurofibromas and NF1<sup>−/−</sup> mouse embryos (Sherman et al. 2000). In addition, analysis of Schwann cell populations from human neurofibromas has elucidated that a subpopulation of Schwann cells exhibit increased Ras activity suggesting that Ras activity plays a crucial role in the
formation of neurofibromas (Sherman et al. 2001). However, it has been shown that the size defect of *Drosophila NF1* mutants is not modified by altering Ras signaling but is suppressed by the expression of constitutively active PKA. Also, cAMP analogs have been observed to rescue the defective response of *NF1* mutant larval body wall muscle to pituitary adenyl cyclase activator protein (PACAP) suggesting that NF1 acts through PKA in this pathway (Guo et al. 1997). Furthermore, G-protein stimulated adenylate cyclase activity is lower in NF1<sup>−/−</sup> mouse brains (Tong et al. 2002). These observations suggest that NF1 acts through PKA in the control of the overall growth of *Drosophila* (The et al. 1997). There is still much controversy on the role of misregulation of Ras in neurofibroma formation. My studies address this question.

1.8.2 Cell nonautonomy of cell proliferation associated with the loss of NF1

Kluwe and colleagues cultured Schwann cells and fibroblasts from a neurofibroma from an NF1 patient. Using intragenic microsatellite markers they found complete loss of the NF1 allele in Schwann cells but not in fibroblasts from the neurofibroma. This observation suggests that loss of NF1 in Schwann cells is responsible for the formation of neurofibromas (Kluwe et al. 1999). Also, Zhu and colleagues used the cre/lox system
(Hamilton and Abremski 1984) to knock out NFI specifically in the Schwann cells of mice. They found that loss of NFI specifically in Schwann cells is sufficient to cause neurofibroma formation (Zhu et al. 2002). This conclusion is further supported by observations, through an immunocytochemical assay for active Ras, that Ras activity is increased in Schwann cells but not fibroblasts from neurofibromas form NF1 patients. It was also noted that this increase in Ras activity occurred only in a sub-population of Schwann cells (Sherman et al. 2000). Because fibroblasts and Schwann cells without altered Ras regulation also make up a portion of neurofibromas, it is likely that there is a non-cell autonomous effect of the loss of NF1. This is further supported by the observation through mosaic analysis that size reduction of the wings of NFI mutant flies is a cell-nonautonomous effect. This observation suggests that NF1 regulates a diffusible growth signal.

There is additional evidence that diffusible growth factors play a role in the development of neurofibromas. Mashour and colleagues noted that conditioned medium, from NF1+ cultured Schwann cells from murine dorsal root ganglia, promotes proliferation of human umbilical vein endothelial cells (HUVEC). Through differential expression screening via RT-PCR, they found that thrombospondin-1 (TSP-1), fibroblast growth factor-2 (FGF-
2), platelet derived growth factor β (PDGFβ) and midkine (MK) were expressed in NF1\(^{-/-}\) Schwann cells but not in NF1\(^{+/+}\) cells (Mashour et al. 2001). MK is a 13 kD secreted protein that has been implicated in neuronal differentiation and survival and tumor angiogenesis (Reviewed in Kurtz et al. 1995). When MK was applied to brain derived endothelial cells (HBEC) and a fibroblastoid cell line Hs422T, these cells were stimulated to proliferate (Mashour et al. 2001).

The gene product of the proto-oncogene kit is a member of the PDGF receptor tyrosine kinase family. (Yarden et al. 1987). Through western blot analysis, it has been shown that Kit is expressed at higher levels in neurofibromin deficient Schwann cell lines than in normal lines. It was also observed that application of the ligand for Kit, stem cell factor (SCF) promotes proliferation of a neurofibrosarcoma derived Schwann cell line (Badache et al. 1998). It has been shown through immunoblotting, Northern analysis and immunohistochemistry that malignant peripheral nerve sheath tumor (MPNST) cell lines from NF1 patients and derivatives of Schwann cells from mouse NF1 mutant embryos express the epidermal growth factor receptor (EGF-R). Fibroblasts that make up the perineurium but not Schwann cells, normally express EGF-R. It was also shown that growth of NF1 deficient MPNST lines and mouse embryo Schwann cells is
stimulated by EGF and inhibited by EGF-R antagonists in vitro (DeClue et al. 2000). The possibility that EGF plays a role in neurofibromatosis is further supported by the observation that Fetal antigen 1 (FA1), a member of the EGF superfamily, was found in significantly higher levels in serum from adult NF1 patients than in normal adults (Jensen et al. 1999).

The Drosophila torpedo (top) gene encodes a homolog of the vertebrate EGF-R (Price et al. 1989). The deduced amino acid sequence of a portion of the Torpedo protein bears 77% homology to the human EGF-R kinase domain (Wadsworth et al. 1985). Loss of function mutations in the top locus cause four distinct defects: zygotic embryonic lethality, zygotic pupal lethality, imaginal disk abnormalities and ventralization of the embryo and eggshell. Part of the eye-antennal disk that becomes the eye and the wing and haltere disks are smaller in size in top mutants than in wild type. This suggests that top is needed for cell proliferation in these structures (Clifford et al. 1989). Through RNA blot and in situ hybridization, it has been shown that top expression is correlated with mitotic activity in certain groups of cells during larval development, embryonic development and oogenesis (Kammermeyer et al. 1987). This also suggests an implication of top in the promotion of cell proliferation. The spitz (spï) gene encodes a predicted 26kD protein containing an EGF domain, and a putative transmembrane
domain. Spitz may be a ligand of Torpedo because weak *torpedo* mutants show phenotypic similarity to *spi* mutants (Rutledge et al. 1992). Though *spi* and *top* may be involved in the control of perineurial glial growth in *Drosophila*, testing this hypothesis is beyond the scope of this proposal. I have shown that there is a non-cell autonomous effect of NF1 in our system. The experimental details of this finding are addressed in the results section.

1.9 Questions that I would like to address

I would like to determine if NF1, Amn, Axs and Eag play a role in regulating perineurial glial thickness. I would also like to determine if these genes are acting in separate pathways to Ine and Push. I would like to determine if the increase in perineurial glial thickness exhibited by certain mutants is due to perineurial glial cell swelling, cell growth or an increase in cell number. I would like to determine if NF1 is acting to control perineurial glial thickening by down-regulating Ras activity. In addition, I would like to determine if increased ras activity has a cell-autonomous, or non-cell-autonomous effect on perineurial glial growth.
Chapter 2

Methodology

2.1 Maintenance of fly stocks and crosses

2.1.1 Stock maintenance

Fly stocks were maintained on standard cornmeal/agar (H₂O 1700 ml, Agar 18 g, Nipagin M (10% in 50 ml of 95% ETOH), Dextrose 150 g, Maize meal 170 g, Dry yeast 30 g) in either 1/2 pint bottles or vials, and maintained at room temperature (20-22°C) (Drosophila protocols, CSHL press). Stocks were transferred regularly every three weeks.

2.1.2 Heat shock

All lines that were heat shocked were subjected to 1hr daily treatment at 37°C.

2.1.3 Crosses

2.1.3.1 Second chromosome to second chromosome

In some instances we had to generate larvae that had more than one mutation or construct on the second chromosome. This was the case for ine¹ gli-Gal4, push¹ gli-Gal4 and ine¹ UAS-Ras⁺. All P-element insertions carry [w⁺] and were thus followed by the presence of red eyes. To follow the ine¹ mutation, we cross the line into the Shaker mutant background and check for the down-turned wings and indented thorax phenotype. Homozygous push¹
males are sterile. Thus mutations in *push* were followed by looking for male sterility in the homozygotes. To generate these stocks, the single mutant lines were crossed to each other. Recombination in *Drosophila* occurs only in females. In the gametes of the female progeny of this cross, recombinant second chromosomes were generated. These female progeny were isolated and crossed to the *CyORoi* (Curly Rough eye) balancer stock. Single red eyed progeny were then isolated and crossed to the *yw; CyO y*+ balancer stock. These stocks were then tested for the presence of *ine* or *push* as described above (Fig. 9).

### 2.1.3.2 Second chromosome to third chromosome

In some instances we had to generate larvae that contained mutations and constructs, or both mutations and constructs on the second and third chromosomes. This was the case for *ine*; *NF1* Ras**12A*/NF1 Ras**2F*, *ine*; *NF1*P2, *push*; *NF1*P2. The *NF1*P2 mutation is due to a p-element insert that carries [w+] and its presence can therefore be identified by red eye color. To generate these lines, each mutant line was crossed to the “double balancer” stock (*w*; *CyO/Sp; TM6/Pr Dr). The balanced progeny of these crosses were then crossed to each other to generate the balanced double mutant stock. Loss of the *CyO* (Curly wings) and *TM6* (Enlarged halteres) balancers in the progeny allow the generation of a homozygous stock. Since
w; ine\(^1\) \(\times\) w; gli-Gal4[w\(^+\)]

\[
\begin{array}{c}
\downarrow \\
\text{w; ine}\(^1\)  \\
\text{w; gli-Gal4}
\end{array} \quad \begin{array}{c}
\downarrow \\
\text{yw; CyO Roi}  \\
\text{y; Sco}
\end{array}
\]

\[
\begin{array}{c}
\text{(Single fly) CyO Roi}  \\
\text{rec [w\(^+\)]}
\end{array} \quad \begin{array}{c}
\downarrow \\
\text{yw or yw; CyO y\(^+\)}
\end{array}
\]

\[
\begin{array}{c}
\text{(Stock) w; CyO y\(^+\)}  \\
\text{yw; rec}
\end{array} \quad \begin{array}{c}
\downarrow \\
\text{Sh; CyO}  \\
\text{Sh; ine}\(^1\) bw
\end{array}
\]

(to test for the presence of ine\(^1\))

down turned wings and indented thorax phenotypes indicate that ine\(^1\) is in rec

Fig. 9. Diagram of a second chromosome to second chromosome cross.
*push* mutant males are sterile, the balanced stock was crossed to the \((CyO-TbTM6+/+)\). Homozygous larvae were identified by the loss of the Tubby \((Tb)\) marker \((\text{Short, fat larvae})\)(Fig. 10).

**2.1.3.3 X chromosome to second chromosome**

In some instances we had to generate larvae that contained mutations on both the X chromosome and the second chromosome. This was the case for \(amn^{x8}; \text{ine}^l\) and \(Axs^{R1}; \text{ine}^l\). To do this we made use of the attached X chromosome. When the attached X is introduced into a line, the males pass their X chromosomes on to their male progeny. We crossed \(Axs^{R1}\) males to attached \(X \text{ine}^l \text{bw}\) females. The \(\text{ine}^l \text{bw}\) mutation was followed by the presence of brown eye color. The male brown eyed progeny of this cross were crossed to attached \(X \text{ine}^l \text{bw}\) females. The male progeny homozygous for \(\text{ine}^l \text{bw}\) were then crossed to attached \(X \text{ine}^l \text{bw}\) females. Since brown eye color would not be detected in the \(w amn^{x8}\) background, we used \(w amn^{x8}\) males carrying the \(CyO Roi\) balancer on the second chromosome. In this case, homozygosity for \(\text{ine}^l\) was detected by loss of curly wings and rough eyes (Fig. 11).

**2.2 Gal4/UAS system**

The Gal4/UAS system is a method used, in \(Drosophila\), to express cloned genes in a specific tissue. Gal4, a yeast transcriptional activator, drives
Fig. 10. Diagram: Second chromosome to third chromosome cross.
Fig. 11. Diagram: X chromosome to second chromosome cross
expression of genes containing the UAS sequence. A specific promoter or enhancer element directs expression of Gal4 to a particular target tissue. The Gal4 gene and UAS-target gene are separated in 2 transgenic lines. In the Gal4 line, the activator is present, but there is no target gene to activate. In the UAS line, the target gene is present, but is not active because the activator is absent. The Gal4 line is crossed to the line containing the UAS-target gene construct. In the progeny, the target gene is expressed in the specific tissue expressing Gal4 (Phelps and Brand, 1998; Brand and Perrimon 1993; Brand and Dormand 1995)(Fig. 12).

2.3 Electron microscopy

Wandering third instar larvae were grown in uncrowded half pint bottles and collected 1 or 2 days after the appearance of the first third instar larvae. Larvae were pinned down and dissected in Schneider’s Drosophila medium (Life Technologies Inc, Gaithersburg, MD). Special care was taken to ensure the peripheral nerves were not stretched or damaged. The larvae were then cut open down the dorsal midline and the entrails were removed. The pelts were then washed three times with Schneider’s Drosophila medium. The pelts were then fixed in a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate pH 7.2-7.4 for 45 minutes (3 changes), unpinned and placed in a 1.5 ml eppendorf tube.
Fig. 12. The Gal4/UAS system is a method used in Drosophila, to express cloned genes in a specific tissue. GAL4, a yeast transcriptional activator, drives expression of genes containing the UAS sequence. A specific promoter or enhancer element directs expression of GAL4 to a particular target tissue. The GAL4 gene and UAS-target gene are separated in 2 transgenic lines. In the GAL4 line, the activator is present, but there is no target gene to activate. In the UAS line, the target gene is present, but is not active because the activator is absent. The GAL4 line is crossed to the line containing the UAS-target gene construct. In the progeny, the target gene is expressed in the specific tissue expressing GAL4 (Phelps and Brand, 1998; Brand and Perrimon 1993; Brand and Dormand 1995)(Adapted from, Phelps and Brand 1998).
The pelts were then post-fixed in 0.5% OsO₄, 0.8% K₃Fe(CN)₆ in 0.1M cacodylate, and then rinsed three times in double-distilled H₂O and placed at 4°C overnight. The following day, the pelts were incubated in 2% Uranyl acetate at 22°C for 2 hours in the dark and rinsed three times in double-distilled H₂O. The samples were then dehydrated in the following ethanol series: 30%, 50%, 75%, 95%, 100%, 100%, and 100% in ten minute increments. Samples were washed 2 times in 100% propylene oxide, 10 minutes each wash, then placed in 50% Epox with accelerator (Eponate 12 kit, Ted Pella) / 50% propylene oxide for 1 and 1/2 hours. Then the samples were placed in 100% Epox with accelerator, placed on stretched parafilm on a microscope slide and placed at 60°C overnight. Excess resin was cut away from the samples and they were placed in bullet molds, covered with Epox with accelerator and placed at 60°C overnight. Samples in the bullet molds were trimmed down and thick sections were taken using a glass knife and a Porter-Blum MT-2 ultramicrotome (Sorvall). Samples were further trimmed by hand using a razor blade. Sections were taken approximately 0.5 mm from the end of the ventral ganglion. Ultrathin cross-sectional slices (silver-pale gold, 75-125 nm thick) were collected on 75 mesh copper EM grids with formvar (Ted Pella) using a diamond knife (Micro Star Technologies Inc.) and an MT-6000 or MT-X ultramicrotome (RMC). Sections were
further stained in 1% uranyl acetate and Reynolds lead citrate (Cytochemical Staining methods for electron microscopy, Lewis and Knight). Micrographs were taken using a Jeol or Hitachi (Tokyo) transmission electron microscope at either 60, 80 or 100 kV. The thickness of the perineurial glial cell layer for a given nerve was determined by averaging the distance from the edge of the nerve to the boundary of the axon containing lumen at 8 different positions using Adobe photoshop. Statistical analyses were carried out by using STATVIEW v4.51 (Abacus concepts, Berkeley CA).

2.4 Nuclei counts

Wandering third instar larvae were grown in uncrowded half pint bottles and collected 1 or 2 days after the appearance of the first third instar larvae. Larvae were pinned down and dissected in 1X PBS (0.14 M NaCl, 7 mM Na₂HPO₄, 3 mM KH₂PO₄). Special care was taken to ensure the peripheral nerves were not stretched or damaged. The larvae were then cut open down the dorsal midline and the entrails were removed. The pelts were then washed three times with 1X PBS. Pelts were fixed in 5% paraformaldehyde, 0.1% Triton X-100 in 1X PBS for 1 hour. Samples were then treated with 400 ug/ml RNAse A in 1X PBS for 2 hours. Samples were stained with 10 ug/ml propidium iodide in 1X PBS for 20 minutes and then washed 3 times in 1X PBS (5 minutes each wash). Pelts were then pinned down and the
brain, ventral ganglion and nerves were removed and placed on a microscope slide in vectashield. Samples were analyzed using a Zeiss LSM 400 confocal microscope (Zeiss, Germany). The number of nuclei per µm length was calculated for each nerve, and averaged for each genotype.

2.5 X-Gal staining

Wandering third instar larvae were grown in uncrowded half pint bottles and collected 1 or 2 days after the appearance of the first third instar larvae. Larvae were pinned down and dissected in 1X PBS (0.14 M NaCl, 7 mM Na2HPO4, 3 mM KH2PO4). Special care was taken to ensure the segmental nerves were not stretched or damaged. The larvae were then cut open down the dorsal midline and the entrails were removed. The pelts were then washed three times with 1X PBS. The samples were then fixed in 1% glutaraldehyde in 1X PBS for 15 minutes, then rinsed 3 times in 1X PBS and placed in X-gal staining solution (10 mM NaPO4 pH 7.2, 150 mM NaCl, 1 mM MgCl2, 3 mM K4(FeII(CN)6), 3 mM K3(FeIII(CN)6), 0.3% Triton X-100, 0.27% X-gal) (Becker et al. 1995). Samples were then rinsed 3 times in 1X PBS and then placed in vectashield (Vector Laboratories Inc. Burlingame, CA) on a microscope slide.
2.6 List of mutations

\textit{ine}^1 - Transcript null mutation (Soehnge et al. 1996).

\textit{push}^1 - Early nonsense mutation at codon 728 (Yager et al. 2001).

\textit{push}^2 - Early nonsense mutation at codon 883 (Yager et al. 2001).

\textit{eag}^1 - Loss of function not characterized molecularly (Ganetzky and Wu 1983).

\textit{Axs}^{R1} - Nonsense mutation, codon 270 (Whyte et al. 1993).

\textit{amn}^{X8} - Imprecise excision of the \textit{p(w^+)} element, resulting in a 0.8 Kb deletion, removing the open reading frame (Moore et al. 1998).

\textit{NF1}^{P2} - P-element insertion, into exon I, protein null mutation (The et al. 1997).

\textit{K33} - Parental control line of the \textit{NF1}^{P2} mutation (The et al. 1997)

\textit{Ras1}^{12a} - Hypomorph (Zhong 1995)

\textit{Ras1}^{e2F} - Hypomorph (Zhong 1995)

\textit{UAS Ras1}^{V12} - Gain of function mutation. The Valine substitution at position 12 eliminates GTPase activity (Lee et al. 1996).
Chapter 3

Results

3.1 Eag acts in a redundant pathway to Push to control perineurial glial growth

As described in the introduction, \(eag^1\) and \(ine^1\) mutants exhibit the down turned wings and indented thorax phenotypes in the \(Shaker\) mutant background, we were interested in determining if \(eag^1\) played a role in the control of perineurial glial growth. Dr. Stern, Vanathi Sundaresan and I analyzed perineurial glial thickness in \(eag^1, eag^1; push^1, eag^1; push^2\) and \(eag^1; ine^1\) mutants. I found that \(eag^1\) mutants had wild type perineurial glial thickness. The \(eag^1; push^1\) and \(eag^1; push^2\) mutants had perineurial glial thickness significantly greater than wild type, but to a lesser extent than \(ine^1\ push^1\) (Fig. 13). These results suggest that Eag, similarly to Ine, is acting in a redundant pathway to Push. The \(eag^1; ine^1\) double mutant perineurial glial layers were not significantly thicker than wild type (Fig. 14). This result suggests that Eag and Ine are acting in the same pathway to control perineurial glial growth.
Fig. 13. Eag acts in a redundant pathway to Push to control perineurial glial growth. Perineurial glial thickness for wild type (n=13), eag<sup>f</sup> (n=18), eag<sup>f</sup>; push<sup>l</sup> (n=25), and eag<sup>f</sup>; push<sup>2</sup> (n=14) larvae. The thickness of the perineurial layer for each nerve is determined by measuring the distance from the edge of the nerve to the edge of the axon containing lumen at 8 different positions. The eight measurements were used to calculate the average thickness of the perineurial glial layer. All statistical analysis was carried out using Statview v4.51 (Abacus Concepts Inc. Berkeley, CA.). The following pairwise combinations had statistically significant differences in perineurial glial thickness: push<sup>f</sup> vs. wt P=0.037, eag<sup>l</sup>; push<sup>f</sup> vs. wt P=0.0026, eag<sup>l</sup>; push<sup>f</sup> vs. eag<sup>l</sup> P=0.0045, eag<sup>l</sup>; push<sup>l</sup> vs. push<sup>f</sup> P=0.005, eag<sup>l</sup>; push<sup>2</sup> vs. wt P= 0.0007, eag<sup>l</sup>; push<sup>2</sup> vs. eag<sup>l</sup> P=0.039, eag<sup>l</sup>; push<sup>2</sup> vs. push<sup>f</sup> P=0.042, (two tailed unpaired t-tests). Alleles used: push<sup>f</sup> (early nonsense mutation), push<sup>2</sup> (early nonsense mutation), eag<sup>l</sup> (loss of function, not characterized molecularly).
Fig. 14. Eag and Ine act in the same pathway to control perineurial glial growth. Perineurial glial thickness mean (+/− standard error) for wild type (n=13), eag\(^{l}\) (n=18), ine\(^{l}\) (n=12), and eag\(^{l}\); ine\(^{l}\) (n=14) larvae. Measurements and statistical analysis were performed as described in Figure 13. Alleles used: ine\(^{l}\) (transcript null mutation), eag\(^{l}\) (loss of function, not characterized molecularly).
3.2 Expression of an ine\(^+\) rescue construct partially rescues the ine\(^1\) push\(^1\) glial phenotype

A heat shock rescue construct containing the long form of \(\text{ine} \) (called ine-RA) under control of the hsp 70 heat shock promoter was constructed previously by Burg and colleagues, which provided residual expression of \(\text{ine} \) at room temperature (Burg et al. 1996). The construct completely rescues the \(\text{ine} \) phenotype of photoreceptor potential oscillation, even without heat shock. Yan mei Huang showed rescue of neuronal hyperexcitability, down-turned wings, indented thorax and sensitivity to hypertonic stress phenotypes with this construct as well (Huang et al. 2002; Huang and Stern 2002). Dr. Stern generated lines that were \(\text{ine}^1 \ push^1; \text{hs-ine-RA} \). These larvae were not heat shocked. I analyzed the \(\text{ine}^1 \ push^1; \text{hs-ine-RA} \) larvae via electron microscopy and noted that they exhibited significant rescue of the perineurial glial phenotype (Fig. 15). This result further confirms that the thickened glia observed in \(\text{ine}^1 \ push^1 \) results from loss of \(\text{ine} \), rather than another mutation on the chromosome.

3.3 The ine\(^1\) push\(^1\) perineurial glial thickness is not due to cell swelling

I suspected the \(\text{ine}^1 \ push^1 \) phenotype may be due at least in part to the role of \(\text{Ine} \) as an osmolyte transporter. One could imagine how the loss of an osmolyte transporter could lead to tissue swelling. If cells cannot release
Fig. 15. Partial rescue of perineurial glial thickness phenotype with hs-ine-RA. Measurements and statistical analysis were performed as described in Figure 13. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), pushl (n=20), inel (n=12), inel pushl (n=14) and inel pushl; hs-ine-RA (n = 11). inel pushl; hs-ine-RA vs. wt (p= 0.0011), inel pushl; hs-ine-RA vs. inel pushl (P=0.008) (towtailed unpaired t-tests). Alleles used: inel (transcript null mutation), pushl (early nonsense mutation).
organic osmolytes, they might accumulate in the cells and promote water intake and cause the cells to swell. To resolve this, I assessed the number of mitochondria per \( \mu m^2 \) from cross sectional electron micrographs of wild type and \textit{ine} \textsuperscript{1} \textit{push} \textsuperscript{1} nerves. I found that there was not a significant difference between the number of mitochondria per \( \mu m \) of wild type versus \textit{ine} \textsuperscript{1} \textit{push} \textsuperscript{1} nerves (Fig. 16). This result suggests that the perineurial glial thickness exhibited in \textit{ine} \textsuperscript{1} \textit{push} \textsuperscript{1} double mutants is associated with an increase in overall cell material. Therefore, the perineurial glial thickness exhibited by \textit{ine} \textsuperscript{1} \textit{push} \textsuperscript{1} double mutants is not due to cell swelling, but rather increased growth.

3.4 Amn and Axs are involved in signaling pathways controlling perineurial glial growth

As noted earlier, Scott Hawley speculates that Push acts downstream in a signaling pathway under control of Amnesiac, a putative PACAP-like neuropeptide, and Axs, which is a putative receptor for Amn. This signaling pathway controls the segregation of non-exchange chromosomes during female meiosis. The ultimate target of the pathway is Nod, a kinesin-like protein which when phosphorylated holds the homologous chromosomes together. This pathway maintains Nod in the phosphorylated state.
Fig. 16. The ine$^1$ push$^1$ perineurial glial thickness phenotype is not due to cell swelling. Mitochondrial density for wt (n=10) and ine$^1$ push$^1$ (n=10). Alleles used ine$^1$ (transcript null mutation), push$^1$ (early nonsense mutation).
Premature inactivation of this pathway leads to premature and abnormal chromosome segregation.

3.4.1 Amn and Axs mutants exhibit thickened perineurial glial layers

To determine if Amn and Axs play a role in the control of perineurial glial growth, I analyzed the effect of Axs\textsuperscript{RI} and amn\textsuperscript{x8}, nonsense and deletion mutations respectively (Moore et al. 1998; Whyte et al. 1993), on perineurial glial growth. Both amn and Axs mutants exhibited thickened perineurial glia (Fig. 17). These results suggest that Amn and Axs play a role in the control of perineurial glial growth.

3.4.2 Expression of an amn rescue construct partially rescues the amn glial phenotype

A heat shock rescue construct, containing a 744 bp genomic fragment containing the amn ORF under control of the hsp70 heat shock promoter, was generated previously by Moore and colleagues (Moore et al. 1998). This construct was shown to completely rescue the amn\textsuperscript{chpd} ethanol sensitivity phenotype when administered daily 1 hour heat shock. Lines that were amn\textsuperscript{x8} hs-amn\textsuperscript{+} were provided to us by Ulrike Heberlein. These larvae were administered daily heat shock at 37\textdegree C for 1 hour and exhibited significant rescue of the perineurial glial phenotype (Fig. 18). This result
Fig. 17. *amnX8* and *Ax3R1* mutants exhibit thickened perineurial glial layers. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), *Ax3R1* (n=21) and *amnX8* (n=34), larvae. Measurements and statistical analysis were performed as described in Figure 13. *amnX8* P= 0.0175, *Ax3R1* P=0.0037 (twotailed unpaired t-tests vs wild type). Alleles used: *amnX8* (deletion mutation) and *Ax3R1* (nonsense mutation).
Fig. 18. Expression of an amn rescue construct partially rescues the amn<sup>X8</sup> glial phenotype. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), amn<sup>X8</sup> (heat shocked)(n=16) and amn<sup>X8</sup>; hs-amn<sup>+</sup> (heat shocked) larvae. Measurements and statistical analysis were performed as described in Figure 13. amn<sup>X8</sup> (heat shocked) p=0.0021, amn<sup>X8</sup>; hs-amn<sup>+</sup> (heat shocked) p=0.0708 (twotailed unpaired t-tests vs wild type). amn<sup>X8</sup> (heat shocked) vs amn<sup>X8</sup>; hs-amn<sup>+</sup> (heat shocked) p=0.0207 (twotailed unpaired t-test). Alleles used: amn<sup>X8</sup> (deletion mutation).
suggests that the thickened perineurial glial layer observed in \textit{amn}^{x8} mutants is a result of loss of \textit{amn} and not another mutation.

\textbf{3.4.3 Expression of an Axs rescue construct partially rescues the Axs\textsuperscript{RI} perineurial glial thickness phenotype}

Joe Kramer and Scott Hawley generated an Axs rescue construct that contained the genomic Axs gene under control of its endogenous promoter bearing several copies of the myc epitope. This construct partially rescued the Axs\textsuperscript{D} aberrant X segregation phenotype (Scott Hawley, Personal communication). Dr. Stern generated Axs\textsuperscript{RI}; \textit{P}\{Axs\textsuperscript{+}\}/+ larvae. I analyzed these larvae via electron microscopy and noted that they exhibited significant rescue of the Axs\textsuperscript{RI} perineurial glial phenotype (Fig. 19). This result further suggests that Axs plays a role in the control of perineurial glial growth.

\textbf{3.4.4 Mutations in ine fail to enhance the amn and Axs mutant phenotypes}

To determine if \textit{ine} enhanced the phenotype caused by these mutations, I analyzed Axs\textsuperscript{RI}; \textit{ine}\textsuperscript{l} and amn\textsuperscript{x8}; \textit{ine}\textsuperscript{l} double mutants. I found that \textit{ine} did not enhance Axs\textsuperscript{RI} or amn\textsuperscript{x8} (Fig. 20). This result seems to suggest that Amn and Axs are acting in the same pathway as Ine. However, I speculate that this is not the case based on evidence that Ine is a putative small molecule
Fig. 19. Expression of an Axs rescue construct partially rescues the Axs\textsuperscript{R1} glial phenotype. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), Axs\textsuperscript{R1} (n=12) and Axs\textsuperscript{R1}; P/\textit{Axss}+/+ (n=25) larvae. Measurements and statistical analysis were performed as described in Figure 13. Axs\textsuperscript{R1} vs. Axs\textsuperscript{R1}; P/\textit{Axss}+/+ P=0.041 (two-tailed unpaired t-test). Alleles used: Axs\textsuperscript{R1}(nonsense mutation).
Fig. 20. Mutations in *ine* fail to enhance the *ann* and *Axs* mutant glial phenotypes. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), *annX8* (n=34), *AxsR1* (n=21), *annX8; ine1* (n=21), and *AxsR1; ine1* (n=16) larvae. Measurements and statistical analysis were performed as described in Figure 13. *annX8* P=0.0175, *AxsR1* P=0.0037, *annX8; ine1* P=0.0325, *AxsR1; ine1* P=0.0204, (twotailed unpaired t-tests vs wild type). *AxsR1* vs *AxsR1; ine1* P=0.2114, *annX8* vs *annX8; ine1* P=0.2614 (twotailed unpaired t-tests). Alleles used: *annX8* (deletion mutation), *AxsR1* (nonsense mutation), *ine1* (transcript null mutation).
neurotransmitter transporter and that Amn is a putative neuropeptide. Perhaps there is another signal from the neurons that acts through Push and NF1 to control perineurial glial growth but is independent of Amn and Axs. In that case, *in e* mutations would be expected to enhance the *push* and *NF1* mutant phenotypes, but would not enhance the *amn* and *Axs* mutant phenotypes. This possibility must be tested further. Analyzing *amn*\(^{x8}\); *push, amn*\(^{x8}\); *NF1, Axs*\(^{R1}\); *push and Axs*\(^{R1}\); *NF1* mutants may help to resolve this issue since mutations in *push* and *NF1* would be expected to enhance the *amn* and *Axs* mutant phenotypes if there is another pathway acting through Push and NF1 to control perineurial glial growth.

### 3.4.5 Amn may be released from either the neurons or peripheral glia

Sequence analysis of the *amn* gene demonstrated that it encodes a putative neuropeptide bearing similarity to the mammalian PACAP neuropeptide (Feany and Quinn 1995). One would expect a neuropeptide to be released from the neurons. However there are several lines of evidence suggesting that neuropeptides may be released from Schwann cells, the mammalian and squid homolog of peripheral glia. Astrocytes cultured from the rat hippocampus have been observed to release glutamate through a SNARE dependent mechanism (Araque et al. 2000). In squid, glutamate application induces co-release of acetylcholine (Ach) and a VIP-like peptide
from Schwann cells acting in an autocrine fashion, resulting in a long-lived hyperpolarization of the Schwann cell membrane (Evans et al. 1999; Villegas 1974; Villegas and Villegas 1974). It thus seems feasible that Amn may be released from the peripheral glia, acting in an autocrine fashion to control perineurial glial growth. Preliminary results support this possibility.

3.4.5.1 Expression of $amn^+$ in the neurons does not rescue the $amn^{x8}$ glial phenotype

The $elavGal4$ driver drives expression of UAS targeted genes to neurons (Robinow and White 1991; Luo et al. 1994). Larvae were generated that were $elavGal4$ UAS-$amn^+$ in the $amn^{x8}$ background. The expression of $amn$ in the neurons was not sufficient to rescue the perineurial glial phenotype (Fig. 21). This result does not necessarily mean that Amn is not released from the neurons to control perineurial glial growth since the $elavGal4$ driver may not express $amn$ at the appropriate time in development to prevent aberrant growth of the perineurial glial layer. Another possibility is that Amn must be released by both neurons and peripheral glia to prevent perineurial glial growth.

3.4.5.2 $amn$ is expressed in peripheral glia

Waddell and colleagues determined, through molecular cloning of the $P\{Gal4\}$ element ends, that $P\{Gal4\}$ is inserted into the amn gene in the enhancer trap
Fig. 21. Expression of amn\(^+\) in the neurons does not rescue the amn\(^X8\) glial phenotype. Perineurial glial thickness mean (± standard error) for wild type (n=13), amn\(^X8\) (n=34) and amn\(^X8\) elav-Gal4/+; UAS-amn\(^+\)/+ (n=12) larvae. Measurements and statistical analysis were performed as described in Figure 13. Alleles used: amn\(^X8\) (deletion mutation).
line C651 (Waddell et al. 2000). This line expresses the Gal4 transcriptional activator under control of endogenous promoters and enhancers and should express Gal4 in the same cells as *amn* is normally expressed. I decided to use this line to determine which of the three cell types, present in the segmental nerves, normally express *amn*. I crossed this line to a line containing a *UAS-LacZ* construct with a nuclear localizing sequence. The larval progeny of this cross exhibited B-Galactosidase activity in the nuclei of cells that normally express *amn*. My strategy was based on the fact that only three cell types comprise the peripheral nerve. All of the neuronal cell bodies are present in the ventral ganglion, therefore the neuronal nuclei are not situated along the length of the nerves. Only 6-8 peripheral glial cells constitute the peripheral glial layers along the length of each nerve, therefore only 6-8 peripheral glial nuclei are present along the length of each segmental nerve. There are 30 or more perineurial glial cells that contribute to the perineurial glial layer along the length of each nerve, accordingly there are 30 or more perineurial glial nuclei along the length of each nerve. The possible outcomes of this experiment are as follows: If 30 or more nuclei are stained along the length of each nerve, the perineurial glia normally express *amn*. This result would not rule out the possibility that the neurons and peripheral glia might also express *amn*. If 6-8 nuclei are stained
along the length of each nerve, the peripheral glia express *amn*, and the perineurial glia do not express *amn*. This result would not rule out the possibility that neurons express *amn*. If no nuclei are stained along the length of the nerve then neither the peripheral glia nor the perineurial glia express *amn*. Again I could not rule out the possibility that neurons normally express *amn*. I observed that 6-8 nuclei were stained along the length of each segmental nerve suggesting that the peripheral glia express *amn* (Fig. 22). However, one drawback of the Gal4/UAS system is that it has been noted that occasionally mosaicism occurs. This leaves open the possibility that *amn* is also expressed in the perineurial glia and that only a subset of the perineurial glial nuclei expressed the reporter. In light of this preliminary data, the observation that expressing *amn* in the neurons via the *elavGal4* driver does not rescue the *amn* glial phenotype, the sequence similarity of Amn to a neuropeptide and consideration of the findings in mammals and squid, I suggest that *amn* is released from either the neurons or the peripheral glia acting on the peripheral glia to control perineurial glial growth. Studies are currently in progress to resolve this issue. Peripheral glial Gal4 drivers including *gli-Gal4*, MZ317 and MZ709 are being employed to determine if peripheral glial expression of *amn* is sufficient to rescue the *amn* glial phenotype.
Fig. 22. *amn* is expressed in the peripheral glia. C651 (*amn* enhancer trap line) crossed to UAS-LacZ (nuclear localized). Third instar larvae were dissected and stained for B-Galactosidase activity.
3.5 NF1 acts to control perineurial glial growth in a redundant pathway to the one involving Ine

3.5.1 ine\(^1\); NF1\(^{P2}\) double mutants exhibit thickened perineurial glial layers

It has been shown that PACAP treatment of Drosophila muscle leads to the activation of one or more potassium channels. This activation requires two signaling pathways. One pathway requires NF1, the Drosophila ortholog of the gene responsible for type 1 neurofibromatosis in humans (Zhong 1995; Zhong and Pena 1995). This observation was intriguing because neurofibromatosis is characterized by the formation of benign tumors in peripheral nerves. This phenotype appears on the surface to be very similar to the perineurial glial phenotype we have been observing in the mutants we studied previously. Therefore I decided to investigate whether NF1 is required in the signaling pathways that control perineurial glial growth. I found that NF1\(^{P2}\) mutants have perineurial glial layers that are slightly thicker than wild type. However, ine\(^1\); NF1\(^{P2}\) double mutants have extremely thick glia, comparable to ine\(^1\); push\(^1\) and eag; push\(^1\). This suggests that NF1 acts in a redundant pathway to Ine to control perineurial glial growth. The mutation in eag did not significantly enhance the NF1\(^{P2}\) phenotype (Fig. 23). This result seems to suggest that NF1 and Eag are
Fig. 23. inel; NF1P2 double mutants exhibit a thickened perineurial glial layer. Perineurial glial thickness mean (+/− standard error) for wild type (n=13), NF1P2 (n=10), eag1 (n=18), inel (n=12), inel; NF1P2 (n=12) and eag1; NF1P2 (n=22). Measurements and statistical analysis were performed as described in Figure 13. NF1P2 vs. wt P=0.244, inel; NF1P2 vs. wt P<0.0001, inel; NF1P2 vs. inel P<0.0001, inel; NF1P2 vs. NF1P2 P=0.0004, eag1; NF1P2 vs. wt P=0.0018, eag1; NF1P2, eag1 P=0.3365, eag1; NF1P2, NF1P2 P=0.0571 (two-tailed unpaired t-tests). Alleles used: NF1P2(protein null mutation), eag1 (loss of function, not characterized molecularly), inel (transcript null mutation).
acting in the same pathway. However, I speculate that Ine and Eag may both be controlling the Ca\(^{2+}\) dependent release of the growth factor. Because mutations in eag do not enhance the push mutant phenotype as much as mutations in ine, I suggest that mutations in eag are less effective than mutations in ine in promoting release of this growth factor. Because ine\(^1\); NF1\(^{p2}\) mutants do not exhibit perineurial glial layers as thick as ine\(^1\); push\(^1\) mutants, it seems that if eag and NF1 mutations do not have as much of an effect on perineurial glial growth as ine or push mutations, then eag; NF1 mutants might not have significantly thickened perineurial glial layers.

More work will have to be performed to sort this out.

3.5.2 Expression of an NF1 rescue construct rescues the ine\(^1\); NF1\(^{p2}\) glial phenotype

A heat shock rescue construct containing NF1 cDNA under control of the hsp70 heat shock promoter was generated previously by The and colleagues (The et al. 1997). This construct was shown to completely rescue the NF1 small size phenotype when administered daily heat shock at 37\(^{0}\)C for 30 minutes (The et al. 1997). Dr. Stern generated lines which were ine\(^1\) hs-NF1/ine\(^1\) +; NF1\(^{p2}\). These animals were heat shocked throughout development, daily at 37\(^{0}\)C for one hour. I analyzed the peripheral nerves of these larvae via electron microscopy and noted that they exhibited complete
rescue of the perineurial glial phenotype (Fig. 24). This result further suggests that effects in glia are due to the \textit{NF1} mutation.

\subsection*{3.5.3 \textit{ine}^{1}; \textit{NF1}^{P2} double mutant nerves contain twice as many perineurial glial nuclei as wild type nerves.}

The increase in thickness of the perineurial glial layers of \textit{ine}^{1}; \textit{NF1}^{P2} double mutants may be due either to an increase in the number of perineurial glial cells or due to an increase in size of the perineurial glial cells. To resolve this question, I stained wild type and \textit{ine}^{1}; \textit{NF1}^{P2} mutant nerves with propidium iodide, which stains DNA and allows visualization of nuclei. I analyzed these mutants via confocal microscopy. I observed that \textit{ine}^{1}; \textit{NF1}^{P2} mutant larvae had twice as many nuclei per \textmu m of nerve than wild type (Fig. 25). This result suggests that perineurial glial growth in \textit{ine}^{1}; \textit{NF1}^{P2} double mutants is accompanied by an increase in perineurial glial cell number.

\subsection*{3.5.4 \textit{NF1} and Push act in separate pathways to control perineurial glial growth}

If \textit{NF1} and Push act in the same pathway to control perineurial glial growth, \textit{NF1} mutations should not enhance the \textit{push} perineurial glial phenotype. However if Push and \textit{NF1} are acting in different pathways, \textit{NF1} mutations will enhance the perineurial glial phenotype of \textit{push} mutants.
Fig. 24. Expression of an NF1 rescue construct rescues the inel; NF1P2 glial phenotype. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), inel (n=12), NF1P2 (n=10), inel; K33 (n=16), inel; NF1P2 (n=12), inel; NF1P2 (heat shocked) (n=15) and inel hs-NF1+/inel +; NF1P2 (heat shocked) (n=12) larvae. Measurements and statistical analysis were performed as described in Figure 13. inel; K33 p=0.0132, inel; NF1P2 (heat shocked) p<0.0001, inel hs-NF1+/inel +; NF1P2 (heat shocked) p=0.3793 (two-tailed unpaired t-tests vs wild type). inel; K33 vs inel; NF1P2 (heat shocked) p=0.0033, inel; K33 vs inel hs-NF1+/inel +; NF1P2 (heat shocked) p=0.1204, inel; NF1P2 (heat shocked) vs inel hs-NF1+/inel +; NF1P2 (heat shocked) p=0.0003 (two-tailed unpaired t-tests). Alleles used: inel (transcript null mutation), NF1P2 (protein null mutation), K33 (parental control line of the NF1P2 mutation).
**Fig. 25.** *ine^1*; *NFIP^2* double mutant nerves contain twice as many perineurial glial nuclei as wild type nerves. A. Confocal image of third instar wt segmental nerves. B. Confocal image of third instar *ine^1*; *NFIP^2* segmental nerves. C. Mean number of nuclei per um nerve for wt and *ine^1*; *NFIP^2* larvae. D. Nuclei per um for each nerve. Alleles used: *ine^1* (transcript null mutation) and *NFIP^2* (protein null mutation). wt vs. *ine^1*; *NFIP^2* P=0.0041 (two-tailed unpaired t-test).
push\(^1\); \(NF1^{p2}\) mutant larvae have been analyzed via electron microscopy.

These mutants exhibited thickened perineurial glial phenotype significantly greater than either single mutant alone, suggesting that push and \(NF1\) act in separate pathways to control perineurial glial growth (Fig. 26).

3.6 Ras activity is both necessary and sufficient to control perineurial glial growth in the \(ine^1\) background

3.6.1 Reducing Ras activity rescues the \(ine^1\); \(NF1^{p2}\) perineurial glial phenotype

If NF1 normally acts to down regulate Ras activity via its GAP function, I would expect reduction of Ras activity to compensate for the loss of Ras regulation and thus suppress the \(ine^1\); \(NF1^{p2}\) nerve phenotype. The transgenic combination \(Ras1^{12a}/Ras1^{e2F}\) is viable and reduces Ras activity and PACAP signaling at the neuromuscular junction (Zhong, 1995; Zhong and Pena 1995). \(Ras1^{12a} NF1^{p2}/Ras1^{e2F} NF1^{p2}\) has been constructed in the \(ine^1\) background. These larvae exhibit reduced perineurial glial thickness compared to \(ine^1\); \(NF1^{p2}\) double mutants (Fig. 27). This result suggests that NF1 acts to down regulate the activity of Ras to control perineurial glial thickness.
Fig. 26. NF1 and Push act in separate pathways to control perineurial glial growth. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), push<sup>1</sup> (n=20), NF<sup>1P2</sup> (n=10) and push<sup>1</sup>; NF<sup>1P2</sup> (n=13) larvae. Measurements and statistical analysis were performed as described in Figure 13. push<sup>1</sup>; NF<sup>1P2</sup> vs. push<sup>1</sup> or NF<sup>1P2</sup> (*, P=0.002 and 0.005 respectively).
Fig. 27. Reducing Ras activity rescues the \textit{ine}\textsuperscript{1}; \textit{NF1}\textsuperscript{P2} perineurial glial phenotype. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), \textit{ine}\textsuperscript{1} (n=12), \textit{NF1}\textsuperscript{P2} (n=10), \textit{ine}\textsuperscript{1}; \textit{NF1}\textsuperscript{P2} (n=12), \textit{ine}\textsuperscript{1}; \textit{Ras1}\textsuperscript{12A NF1}\textsuperscript{P2/+ NF1}\textsuperscript{P2} (n=8), \textit{ine}\textsuperscript{1}; \textit{Ras1}\textsuperscript{2F NF1}\textsuperscript{P2/+ NF1}\textsuperscript{P2}, and \textit{ine}\textsuperscript{1}; \textit{Ras1}\textsuperscript{12A NF1}\textsuperscript{P2/Ras1}\textsuperscript{2F NF1}\textsuperscript{P2} (n=24) larvae. Measurements and statistical analysis were performed as described in Figure 13. \textit{ine}\textsuperscript{1}; \textit{NF1}\textsuperscript{P2}, \textit{ine}\textsuperscript{1}; \textit{Ras1}\textsuperscript{12A NF1}\textsuperscript{P2/Ras1}\textsuperscript{2F NF1}\textsuperscript{P2} P<0.0001.
3.6.2 Ectopic expression of a constitutively active form of Ras in the peripheral glia promotes perineurial glial growth in the ine' background

If NF1 is acting to down regulate Ras activity to control perineurial glial growth, increasing Ras activity should promote perineurial glial growth. Sweet and colleagues expressed in *Escherichia coli* (*E. coli*) a form of Ras that contains a valine substitution at position 12. They noted that this form of Ras exhibited a 10-fold decrease in GTPase activity. They also observed that injection of this form of Ras into rodent fibroblasts caused these cells to divide (Sweet et al. 1984). Valine-12 substitutions have also been identified in *Ras* genes in tumor cell lines. Capon and colleagues found through sequence analysis that the *Ras* gene exhibits a single base pair change in codon 12 that results in a valine substitution at position 12 in the SW480 human colon carcinoma cell line (Capon et al. 1983). *Ras1\textsuperscript{V12}* is a gain-of-function allele of *Ras1*, in *Drosophila*, that has a valine substitution at position 12. *UAS-Ras1\textsuperscript{V12}* was generated by Tzumin Lee and Denise Montell (Lee et al. 1996). This construct contains the constitutively active *Ras1\textsuperscript{V12}* gene under control of the UAS sequence. This construct was observed to delay border cell migration when specifically expressed in those cells prior to migration using a border cell specific Gal4 driver (Lee et al. 1996). *ine\textsuperscript{l} gli-Gal4/ine\textsuperscript{l} +; UAS-Ras1\textsuperscript{V12}/+* mutant larvae exhibited thickened
perineurial glial layers, suggesting that constitutively active Ras activity in the peripheral glia is sufficient to promote perineurial glial growth in the ine- background. To be certain that this effect was not due to the presence of either the gli-Gal4 driver or the UAS-Ras1^{V12} construct alone, I analyzed ine^{l} gli-Gal4/ine^{l} + and ine^{l}; UAS-Ras1^{V12}/+ and noted that ine^{l} gli-Gal4/ ine^{l} +; UAS-Ras1^{V12}/+ exhibited significantly thicker perineurial glial layers than either control. I also observed that the perineurial glial thickness of ine^{l} gli-Gal4/ ine^{l} +; UAS-Ras1^{V12}/+ larvae was significantly greater than that of gli-Gal4/++; UAS-Ras1^{V12} demonstrating that both loss of ine and expression of constitutively active Ras in the peripheral glia are necessary to result in perineurial glial growth (Fig. 28). Felix Karim and Gerald Rubin generated a UAS-Ras1^{+} construct that contains the wild type Ras gene under control of the UAS sequence (Karim and Rubin 1998). I wanted to determine if driving the expression of an extra copy of Ras specifically in the peripheral glia is sufficient to promote perineurial glial growth in the ine- background. I generated and analyzed ine^{l} gli-Gal4/ine^{l} UAS-Ras1^{+} . Ectopic expression of another copy of normal Ras did not increase perineurial glial thickness significantly in the ine- background suggesting that Ras must be freed from regulation by NF1 GAP activity to effectively regulate growth of the perineurial glia (Fig. 29).
Fig. 28. Ectopic expression of a constitutively active form of Ras in the peripheral glia promotes perineurial glial growth in the ine background. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), ine\textsuperscript{1} gli-Gal4/ine\textsuperscript{1} +; UAS-Ras\textsuperscript{V12} (n=25), ine\textsuperscript{1}; UAS-Ras\textsuperscript{V12} (n=27) and ine\textsuperscript{1} gli-Gal4/ine\textsuperscript{1} + (n=17) larvae. Measurements and statistical analysis were performed as described in Figure 13. ine\textsuperscript{1} gli-Gal4/ine\textsuperscript{1} +; UAS-Ras\textsuperscript{V12} vs. ine\textsuperscript{1}; UAS-Ras\textsuperscript{V12} (p=0.0027, two-tailed unpaired t-test), ine\textsuperscript{1} gli-Gal4/ine\textsuperscript{1} +; UAS-Ras\textsuperscript{V12} vs. ine\textsuperscript{1} gli-GAL4/ine\textsuperscript{1} + (p=0.0056, two-tailed unpaired t-test).
Fig. 29. Ectopic expression of another copy of wild type Ras does not increase perineurial glial thickness significantly in the ine- background. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), ine1 gli-Gal4/ine1 + (n=17) and ine1 gli-Gal4/ine1 UAS-Ras1wt (n=11) larvae. Measurements and statistical analysis were performed as described in Figure 13.
3.6.3 NF1 controls perineurial glial growth by regulating Ras activity

To further determine if NF1 acts through Ras to control perineurial glial growth, I analyzed larvae expressing constitutively active Ras in the peripheral glia in the NF1$^{P2}$ mutant background. If NF1 and Ras regulation are acting separately to control perineurial glial growth, gli-Gal4/+; NF1$^{P2}$ UAS-Ras$^{V12}$/NF1$^{P2}$ + larvae should exhibit a thickened perineurial glial layer. If NF1 is acting to down-regulate Ras activity, these larvae will not exhibit thickened perineurial glial layers. I have found that gli-Gal4/+; NF1$^{P2}$ UAS-Ras$^{V12}$/NF1$^{P2}$ + larvae do not exhibit thickened perineurial glial layers, providing further evidence that NF1 acts to regulate Ras activity to control perineurial glial growth (Fig. 30).

3.6.4 Amn controls perineurial glial growth partially by regulating Ras activity through NF1

If Ras regulation is a downstream effect of the Amn signal, I would expect Ras activity to be increased in amn mutant larvae, and that increase in Ras activity to contribute to the perineurial glial growth defect observed in amn$^{x8}$ mutants. I have observed that amn$^{x8}$; Ras$^{12A}$/Ras$^{e2F}$ mutants exhibit partial rescue of the perineurial glial thickness phenotype of amn$^{x8}$ mutants (Fig. 31). This suggests that Amn acts to control perineurial glial growth by down regulating Ras activity through NF1.
Fig. 30. Ectopic expression of a constitutively active form of Ras in the peripheral glia does not promote perineurial glial growth in the \textit{NFIP2} background. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), \textit{NFIP2} (n=10), \textit{gli-Gal4/+; UAS-Ras1V12/+} (n=11) and \textit{gli-Gal4/+; NFIP2 UAS-Ras1V12/NFIP2 +} (n=22). Measurements and statistical analysis were performed as described in Figure 13. Alleles used: \textit{NFIP2}(protein null mutation).
Fig. 31. Reducing Ras activity rescues the \( amn^{X8} \) perineurial glial phenotype. Perineurial glial thickness mean (± standard error) for wild type (n=13), \( amn^{X8} \) (n=34) and \( amn^{X8}; Ras^{12α}/Ras^{12β} \) (n=20) larvae. Measurements and statistical analysis were performed as described in Figure 13. \( amn^{X8} \) vs \( amn^{X8}; Ras^{12α}/Ras^{12β} \) (p=0.0157 two-tailed unpaired t-test).
3.6.5 Push does not act to regulate Ras activity to control perineurial glial growth

I have shown that push; NFI1p2 mutants exhibit thicker perineurial glial layers than either single mutant alone, suggesting that Push and NF1 act in separate pathways to control perineurial glial growth. I have also shown that NF1 acts to down regulate Ras activity to control perineurial glial growth. It therefore seems unlikely that Push is acting to control perineurial glial growth by regulating Ras activity. I found that expression of RasV12 in the peripheral glia promotes growth of the perineurial glial layer in the push mutant background by analyzing push gli-Gal4/push +; UAS RasV12 mutant larvae. These mutants exhibit thickened perineurial glial layers (Fig. 32) suggesting that Push activity and Ras regulation are acting in separate pathways to control perineurial glial growth. I have also analyzed ine push; Ras112a/Ras112p triple mutant larvae. These mutants exhibit perineurial glial layers as thick as ine push mutants (Fig. 33). If Push acts to down regulate Ras activity to control perineurial glial growth, I would expect that reducing Ras activity would rescue the ine push phenotype. Reduction of Ras activity does not rescue the ine push perineurial glial thickness phenotype. Taken together, these data suggest that Push does not act to regulate Ras activity to control perineurial glial growth.
Fig. 32. Ectopic expression of a constitutively active form of Ras in the peripheral glia promotes perineurial glial growth in the push<sup>l</sup> background. Perineurial glial thickness mean (+/- standard error) for wild type (n=13), gli-GAL4/+; UAS-Ras<sup>V12</sup>/+ (n=11) and push<sup>l</sup> gli-GAL4/push<sup>l</sup> +; UAS-Ras<sup>V12</sup>/+ (n=19) larvae. Measurements and statistical analysis were performed as described in Figure 13. gli-GAL4/+; UAS-Ras<sup>V12</sup>/+ vs. push<sup>l</sup> gli-GAL4/push<sup>l</sup> +; UAS-Ras<sup>V12</sup>/+ (p=0.0026 two-tailed unpaired t-test).
Fig. 33. Reducing Ras activity does not rescue the \textit{inl} \textit{push}^{1} perineurial glial thickness phenotype. Perineurial glial thickness mean ( +/- standard error) for wild type (n=13), \textit{inl} \textit{push}^{1} (n=14) and \textit{inl} \textit{push}^{1}; \textit{Ras}^{12A}/\textit{Ras}^{12F} (n=11) larvae. Measurements and statistical analysis were performed as described in Figure 13.
Chapter 4
Discussion

4.1 Summary of findings

We have shown that eag; push double mutants exhibit thickened perineurial glial layers, suggesting that Eag and Push act in separate pathways to control perineurial glial thickening. We have also shown that ine\(^1\); push\(^1\) double mutants exhibit the same perineurial glial mitochondrial density as wild type suggesting that the increased perineurial glial thickness exhibited by these mutants is not due to cell swelling. We have shown that both Axs\(^{R1}\) and amn\(^{x8}\) mutants exhibit thickened perineurial glial layers suggesting that Axs and Amn play a role in the control of perineurial glial thickness. We have found that both ine\(^1\); NF1\(^{P2}\) and push\(^1\); NF1\(^{P2}\) double mutants exhibit thickened perineurial glial layers suggesting that NF1 acts in separate pathways to both Ine and Push.

We have shown that lowering overall Ras activity rescues the ine\(^1\); NF1\(^{P2}\) and amn\(^{x8}\) glial phenotypes. In addition, ectopic expression of a constitutively active form of Ras specifically in the peripheral glia promotes thickening of the perineurial glial layer in the ine\(^1\) background. Ectopic expression of a wild type copy of Ras specifically in the peripheral glia is insufficient to promote increased perineurial glial thickness in the ine\(^1\)
background suggesting that Ras must be freed from down-regulation by NF1 to promote perineurial glial growth. These results suggest that NF1 is acting to regulate Ras activity via its RasGAP activity to prevent perineurial glial thickening.

Lowering overall Ras activity does not rescue the *ine* *push* glial phenotype. In addition, ectopic expression of a constitutively active form of Ras specifically in the peripheral glia promotes perineurial glial thickening in the *push* background. These results suggest that Push does not act to regulate Ras activity to control perineurial glial thickening.

4.2 Working Hypothesis: Neurotransmitter-mediated signaling pathways regulate perineurial glial growth

The identification of Amn, a putative neurotransmitter, and Ine, a member of a family of neurotransmitter transporters, as proteins that control perineurial glial growth suggests that the initiation of signaling pathways involved in regulating perineurial glial growth are neurotransmitter-mediated. My results are consistent with a model in which perineurial glial growth is controlled by two interacting pathways, one mediated by Amn acting separately through Push and NF1, another controlled by the substrate neurotransmitter of Ine. Taking into account Yi Zhong’s discovery that a PACAP-like neurotransmitter acts through NF1 to regulate K+ conductance
at the neuromuscular junction, Scott Hawley’s implication of Amn, Axs and Push in proper non-exchange chromosome segregation and sequence similarity of Axs to a G-protein coupled receptor, I hypothesize that Amn is acting through Push and NF1 in our system. I have shown that Amn is expressed in the peripheral glia. Therefore it is possible that the Amn neuropeptide is released either from the neurons or peripheral glia and acts via the Axs encoded putative Amn receptor, which I predict is expressed in the peripheral glia. This may initiate a signaling pathway in the peripheral glia involving Push and NF1. I have shown that both amnX8 and inel; NF1P2 perineurial glial phenotypes are rescued by reducing Ras activity, and Drosophila NF1 has been shown to have Ras-GAP activity in vitro (The et al. 1997). These results suggest that the Amn signal acts through NF1 to ultimately regulate the activity of Ras. I observed that increased Ras activity in the peripheral glia acts cell-nonautonomously to promote perineurial glial growth, suggesting that activated Ras leads to an intercellular growth signal from the peripheral glia acting on the perineurial glia.

I have shown that the Push pathway does not act through Ras to control perineurial glial growth. The downstream targets of Push have not been identified. However, since signaling through Amn has been shown to activate potassium channels (Guo et al. 1997) and since push mutants are
hyperexcitable (Richards et al. 1996), a phenotype that may be due to decreased $K^+$ channel activity, I hypothesize that peripheral glia may respond to the Amn signal by activating $K^+$ channels through Push, which would maintain the peripheral glia in a hyperpolarized state. Based on the observation that Schwann cells can release factors such as Desert Hedge Hog (DHH) that promote growth of the perineurium (Parmantier et al. 1999) and that rat astrocytes release glutamate in a $Ca^{2+}$/SNARE dependent fashion, I hypothesize that maintenance of the peripheral glia in a hyperpolarized state could inhibit the $Ca^{2+}$ dependent release of a growth factor that acts on the perineurial glia to promote growth of this tissue. I hypothesize that another pathway promotes an intercellular growth signal from the peripheral glia to the perineurial glia. Ine and Eag may act to prevent the initiation of this intercellular signal (Fig. 34)
Fig. 34. Working Hypothesis: Neurotransmitter mediated signaling controls perineurial glial thickness. My work suggests that perineurial glial growth is controlled by two interacting pathways. The first initiated by Amn, acting separately through Push and NF1, inhibits the release of the trophic factor. Amnesiac is a putative neuropeptide (Feany and Quinn 1995) and our results have shown that Amn is expressed in the peripheral glia, therefore Amn is likely released from either the neurons or peripheral glia. Scott Hawley has implicated Push, Amn and Axs in the control of chromosome segregation during female meiosis. Hawley has also found that Axs bears similarity to a G-protein coupled receptor. The fact that Amn and Axs have extremely similar chromosome segregation and perineurial glial phenotypes and their putative roles as neuropeptide and G-protein coupled receptor respectively lightly suggests that Axs may be a receptor for Amn (Scott Hawley, personal communication). The implication of Push Amn and Axs in both chromosome segregation and perineurial glial growth suggests that they may be acting in the same pathway. Yi Zhong has shown that NF1 acts downstream of an Amn-like neuropeptide at the Drosophila neuromuscular junction. In addition we have found that NF1 plays a role in perineurial glial growth therefore we speculate that NF1 acts downstream of Amn and Axs to control perineurial glial growth. We have not yet determined specifically whether Amn and Axs act upstream or downstream of Push or NF1 in our system. The ultimate target of the NF1 pathway is Ras. We have shown that lowering Ras activity rescues both the ine; NF1 and the amn perineurial glial phenotype suggesting that both NF1 and Amn act through Ras to control perineurial glial growth. In addition, expression of a constitutively active form of Ras in the peripheral glia does not enhance the NF1 perineurial glial phenotype. The ine; push perineurial glial phenotype was not rescued by reducing Ras activity and expression of a constitutively active form of Ras in the peripheral glia enhances the push perineurial glial phenotype suggesting that Push does not act to regulate Ras activity to prevent perineurial glial growth. The target of the Push pathway may be K⁺ channels distinct from Eag. We have shown that expression of constitutively active Ras in the peripheral glia is sufficient to promote perineurial glial growth in the ine background demonstrating that there is a cell-nonautonomous effect of increased Ras activity strongly suggesting that an intercellular growth signal from the peripheral glia acts on the perineurial glia. The second pathway may also promote an intercellular growth signal from the peripheral glia acting on the perineurial glia. Ine and Eag may act to prevent the initiation of this intercellular signal.
4.3 Alternative Hypothesis: Deficient osmoregulation may play a role in perineurial glial growth

The implication of Ine in perineurial glial growth begs the question: does osmoregulation play a role in perineurial glial growth? The \textit{ine} gene bears similarity to an osmolyte transporter (Burg et al. 1996; Soehnge et al. 1996). In addition, mutations in \textit{ine} have been shown to confer hypersensitivity to osmotic stress (Huang et al. 2002). I have shown that the increase in perineurial glial growth exhibited by \textit{ine}\textsuperscript{1}; \textit{NF1}\textsuperscript{r2} mutant larvae is due to an increase in cell number as opposed to an increase in perineurial glial cell size. In addition, I noted that there was not a significant difference between the number of mitochondria per \(\mu\text{m}^2\) between wt and \textit{ine}\textsuperscript{1} \textit{push}\textsuperscript{1} double mutant perineurial glial layers. These results suggest that the increase in perineurial glial layer thickness is not directly due to cell swelling. However, deficient osmoregulation may initiate a signaling pathway that promotes growth of the perineurial glial layer. Fischer and colleagues noted that hypo-osmotic treatment of cultured rat astrocytes causes an increase in \(\text{Ca}^{2+}\) levels in the astrocytes. They found that this increase in intracellular \(\text{Ca}^{2+}\) was due to both influx from the extracellular fluid as well as \(\text{Ca}^{2+}\) release from internal stores (Fischer et al. 1997). These findings support the hypothesis that Ine might act to regulate the osmolyte levels of the
extracellular fluid surrounding the peripheral glial cells. In the absence of
Ine, the extracellular fluid may become hypo-osmotic. The resulting hypo-
osmotic extracellular environment might induce an increase in intracellular
Ca\textsuperscript{2+} concentration in the peripheral glia, leading to the Ca\textsuperscript{2+}/SNARE
dependent release of a growth factor that promotes growth of the perineurial
glia. This possibility is supported by the observation that rat astrocytes
release glutamate in a Ca\textsuperscript{2+}/SNARE dependent fashion (Araque et al. 2000)
(Fig. 35).

Another possibility is that a hypo-osmotic environment may affect the
perineurial glia directly to promote growth of the perineurial glial layer.
Schliess and colleagues determined through mobility shift assays that the
mitogen activated protein kinases (MAPKs), extracellular signaling-
regulated protein kinase (Erk) 1 and 2 are activated in rat astrocytes placed
in hypo-osmotic environments. Activation of Erk-1 and Erk-2 was blocked
by removing extracellular Ca\textsuperscript{2+} suggesting that activation of Erk-1 and 2
occurs in response to the increase in Ca\textsuperscript{2+} in the astrocytes that occurs as a
result of Ca\textsuperscript{2+} influx from the extracellular fluid (Schliess et al. 1996).
Erk/MAPK activity has been observed at high levels in astrocytic tumors
suggesting this pathway plays a role in cell proliferation (Mandell et al.
1998). Taken together, these results could be used to generate an alternative
Fig. 35. Alternative hypothesis: Osmoregulation may play a role in perineurial glial growth. Ine might act to regulate the osmolyte levels of the extracellular fluid surrounding the peripheral glial cells. In the absence of Ine, the extracellular fluid may become hypo-osmotic. The resulting hypo-osmotic extracellular environment might induce an increase in intracellular Ca\textsuperscript{2+} concentration in the peripheral glia, leading to the Ca\textsuperscript{2+}/SNARE dependent release of a growth factor that promotes growth of the perineural glia.
hypothesis of how deficient osmoregulation may promote perineurial glial growth. Loss of Ine might cause the extracellular fluid surrounding the perineurial glia to become hypo-osmotic. This hypo-osmotic effect may result in an increase of Ca^{2+} levels in the perineurial glia that causes activation of the Erk/MAPK signaling cascade. Ultimately, activation of this pathway results in overproliferation of the perineurial glia. The possible role of osmoregulation in perineurial glial growth will be tested further. To determine if osmoregulation plays a role in perineurial glial growth, we could test how high and low NaCl concentrations in the food affect perineurial glial thickness of push' or NFI' mutant larvae (Fig. 36).

4.4 My contribution to what is known about Neurofibromatosis type 1

My data can be used to reinterpreted a confusing observation about the role of NFI in neurofibroma formation. In general, it has been difficult to detect loss of NFI^+ from cells in neurofibromas. Kluwe and colleagues detected the loss of the NFI^+ allele in Schwann cells from human neurofibromas composed partially of fibroblasts and other cell types (Kluwe et al. 1999). In addition, analysis of Schwann cell populations from human neurofibromas has elucidated that a subpopulation of Schwann cells exhibit increased Ras activity (Sherman et al. 2001). These findings are puzzling because we would expect those cells exhibiting increased Ras activity to
Fig. 36. Alternative hypothesis: Osmoregulation may play a role in perineurial glial growth. Loss of Ine might result in the extracellular fluid surrounding the perineurial glia to become hypo-osmotic. This may result in an increase of Ca^{2+} levels in the perineurial glia that causes activation of the Erk/MAPK signaling cascade that results in overproliferation of the perineurial glia.
proliferate abundantly and be the exclusive contributors to the tumor. In light of these discoveries, it has been suggested that a core of Schwann cells exhibiting increased Ras activity release growth factors thus promoting the incorporation of cells with unaltered Ras activity into the tumor. My observation that increasing Ras activity in the peripheral glia promotes growth of the perineurial glia provides further evidence that increased Ras activity acts cell-nonautonomously to promote neurofibroma formation.

My data also helps clarify some confusion about the role of NF1 in *Drosophila*. The *Drosophila* NF1 protein bears 60% homology to human NF1 (The et al. 1997). A 360 amino acid region of the human NF1 protein bears homology to the catalytic domain of mammalian guanosine triphosphatase-activating protein (GAP) (Xu et al. 1990). It has also been shown that both human and *Drosophila* NF1 act as GAPs, regulating Ras activity in vitro (The et al. 1997). In addition, increased Ras-GTP levels have been observed in Schwann cells from human neurofibromas and NF1−/− mouse embryos (Sherman et al. 2000). However, *Drosophila* NF1 has been observed to mediate its affects mostly through Protein Kinase A. For example, the size defect of *Drosophila* NF1 mutants is not modified by altering Ras signaling, but is suppressed by the expression of constitutively active Protein Kinase A (PKA) (The et al. 1997). Also, cAMP analogs have
been observed to rescue the defective response of NF1 mutant larval body wall muscle to pituitary adenyl cyclase activator protein (PACAP) suggesting that NF1 acts through PKA in this pathway (Guo et al. 1997). Furthermore, G-protein stimulated adenylate cyclase activity is lower in NF1/- mouse brains (Tong et al. 2002). Williams and colleagues were the first to show that Drosophila NF1 acts through Ras/MAPK to maintain normal circadian locomotor activity (Williams et al. 2001). I have shown that the perineurial glial phenotype exhibited by ine'; NF1p2 mutants is rescued by reducing Ras activity, and that increased Ras activity in the peripheral glia does not synergize with the NF1p2 mutation to promote perineurial glial growth. These results demonstrate that Drosophila NF1 acts to down-regulate Ras activity in the control of perineurial glial growth.

I have also identified five other genes, push, amn, Axs, ine and eag that play a role in the control of perineurial glial growth in Drosophila. Several of these genes bear similarity to human genes. For example, the push gene bears 45% identity with a human cDNA (GenBank accession no. AB007931) (Stephen Richards Personal communication). The amn gene encodes a putative neuropeptide bearing similarity to the pituitary adenyl cyclase activator peptide (PACAP) in humans (Feany and Quinn 1995). Perhaps the identification of these genes in the control of perineurial glial
growth in *Drosophila* will suggest target genes to investigate that may interact with NF1 and play a role in neurofibroma formation in humans.

### 4.5 Neuronal hyperexcitability/ Perineurial glial growth

Three genes involved in the control of perineurial glial thickness (*eag*, *ine* and *push*) also exhibit neuronal hyper-excitability, this observation raises the possibility that the perineurial glial thickness phenotypes may be a result of increased neuronal hyper excitability or that increased perineurial glial thickness causes neuronal hyper-excitability. It is unclear if there is a cause and effect relationship between perineurial glial thickness and motor neuron function. M. Stern was unable to detect any increase in neuronal excitability in either the *amn* or *ine*; *NF1* mutant larvae (greater that that exhibited by the *ine* mutation alone) suggesting that perineurial glial growth does not affect neuronal hyper-excitability (M. Stern, personal communication). It is possible that effects on neuronal excitability in these mutants are too subtle for our assays to detect. M. Stern has also visually inspected *eag Sh* double mutant larvae, which exhibit an extreme neuronal hyper-excitability phenotype, and noted that they do not possess thick peripheral nerves (M. Stern, personal communication). A more detailed analysis of these mutants will be performed. These data suggest that perineurial glial growth and neuronal hyper-excitability phenotypes are not directly related. However,
mutations that confer neuronal hyper-excitability may promote the release of neurotransmitters that act to initiate signaling pathways that control perineurial glial growth and a thickened perineurial glial phenotype may be exhibited only when neuronal hyper-excitability is paired with the elimination of another pathway that controls perineurial glial growth. The correlation between neuronal hyper-excitability and perineurial glial growth will be investigated further. To determine if the role of push in control of perineurial glial growth is related to its role in normal motor neuron function, RNAi elimination of push specifically in the neurons or the peripheral glia in the ine^{-} background will be performed to see if the two phenotypes are separable. If knocking out push in the neurons but not the peripheral glia confers neuronal hyperexcitability, but not perineurial glial growth, then perineurial glial growth is not the result of increased neuronal hyperexcitability. If knocking out push in the peripheral glia in the ine^{-} background confers the perineurial glial phenotype but not neuronal hyperexcitability, then neuronal hyperexcitability is not the result of increased perineurial glial thickness.
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