A Genetic Analysis of the Role of *neuralized* in the Notch and EGFR pathways of *Drosophila melanogaster*.

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Zoology University of Toronto

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A Genetic Analysis of the Role of *neuralized* in the Notch and EGFR pathways of *Drosophila melanogaster*. Master of Science, 1999, Nicholas James Rudzik, Department of Zoology, University of Toronto.

The *neuralized* gene of *Drosophila melanogaster* is thought to be a member of the neurogenic signalling pathway involved in the development of the embryonic and adult nervous systems. Overexpressing *neuralized* during development causes dominant phenotypes in the eye, notum and wing of adults. These phenotypes include the production of extra and misplaced bristles in the eye, an increase in the number of notal microchaetae and decrease in macrochaetae, and deletion of portions of wing veins. These dominant phenotypes were used to screen a deficiency kit for modifiers of *neuralized*. The screen identified deficiencies which uncovered members of both the neurogenic and the EGFR signalling pathways. This interaction was confirmed by directly testing alleles of genes in both pathways for the ability to modify the *neuralized* overexpression phenotype. It appears that *neuralized* could play a role in both of these pathways. Biochemical tools to test these interactions have been produced.
Acknowledgements

I would like to acknowledge the hard work of the following people, without whom this thesis would not have been possible: Lin Naemsch, whose work led directly into this project; Edward Yeh, who produced the UAS-\textit{neuralized} transgenic flies used in Chapter 1; Kjerstin Gustafson, who produced the A78GAL4 line used here; Gianfranco Pellicori, who performed the deficiency screen outlined in Chapter 1; and Lily Zhou, who made the constructs and produced many of the lines described in Appendix 1. I wish to thank all of my colleagues in the Boulianne lab, past and present, for creating such a pleasant working environment. I would like to thank Steven Doyle and Battista Calvieri for instruction in and assistance with scanning electron microscopy. I would also like to thank Patrycja Zuk for her support and encouragement during my graduate work. Thanks to Thomas Nguyen for useful discussions and countless cups of coffee. I would like to thank my family for their support, and for putting up with me during both research and writing. Finally, I would like to thank my supervisor, Dr. Gabrielle Boulianne, for giving me the opportunity to pursue graduate work, and for her guidance throughout the course of my Masters. This work was supported in part by Ontario Graduate Scholarships from the Province of Ontario.

THIS THESIS IS DEDICATED TO MY PARENTS.
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**List of abbreviations**

acv: anterior cross vein

AS-C: *achaete-scute* gene complex

bp: base pairs (of DNA)

bHLH: basic Helix-Loop-Helix

bib: *big brain*

cDNA: complementary DNA

CNS: Central Nervous System

COIP: coimmunoprecipitation

CsCl₂: cesium chloride

CyO: *Curly of Oster*

DER: *Drosophila*-homologue of the EGF Receptor

Dfd: *Deformed*

dH₂O: distilled water

Dl: *Delta*

DNA: deoxyribonucleic acid

EDTA: ethylene diamine tetraacetic acid

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

Elp: *Ellipse* (gain-of-function DER allele)

EMS: ethyl methane sulphonate

E(spl): *Enhancer of split*

ESPL-C: *Enhancer of split complex*
F1: First-generation progeny in genetic crosses

flb: faint little ball (loss-of-function DER allele)

G0: Parental generation in genetic crosses; injected flies when making transgenics

kb: kilobases (of DNA)

KCl: potassium chloride

kDa: kilo Daltons

kV: kilovolts

M: molar

mam: mastermind

mM: millimolar

mRNA: messenger RNA

N: Notch

NaPO₄: sodium phosphate

neu: neuralized

PCR: polymerase chain-reaction

pcv: posterior cross vein

PNS: peripheral nervous system

pnt: pointed

rho: rhomboid

RNA: ribonucleic acid

S: Star

Sb: Stubble
Ser:  Serrate
spi:  spitz
SOP: Sensory Organ Precursor
Su(H): Suppressor of Hairless
TE: Tris-EDTA buffer
Tris: tris(hydroxymethyl)aminomethane
UAS: Upstream Activating Sequences
Ubx: Ultrabithorax
Introduction
One of the most common processes in the development of *Drosophila melanogaster* (and in development in general) is the production of multiple cell types from an originally homogeneous population of precursors. These cell fate decisions occur during the development of the embryonic nervous system and midgut, the development of the sensory organs of the adult fly, and the development of the venation pattern of the wing, to name a few cases. One of the best studied of these processes is the neural/epidermal cell fate decision of cells in the ventral neurogenic ectoderm during embryonic neurogenesis.

Neurogenesis in the *Drosophila* embryo is thought to occur in several steps (Jan and Jan, 1990). First, the anterior-posterior and dorsal-ventral patterns of the embryo are laid down by "prepattern" genes (gap, pair-rule and segment polarity genes). Second, so-called proneural genes are expressed in clusters of cells, giving these cells neural potential. Last, the expression of the proneural genes (and so the neural potential) is refined to a single cell by the action of the neurogenic genes, through a process of lateral and mutual inhibition (Hassan and Vaessin, 1996; Jan and Jan, 1990). Those cells that are not selected to become neuroblasts become epidermoblasts and give rise to the embryonic epidermis, while the selected neuroblasts delaminate from the epithelium and go on to form the central nervous system.

The neurogenic genes were first identified in a screen for embryonic lethal mutations that display a cuticular phenotype, and were so called based on the neural hyperplasia displayed in recessive homozygous mutant embryos (Lehmann et al., 1981; Lehmann et al., 1983). In addition to the neural hyperplasia, homozygous mutant embryos also lacked cuticle, suggesting that the epidermal cells that secreted the cuticle
were also affected. The neurogenic genes include Notch, Delta, mastermind, big brain, genes of the Enhancer of split complex, and neuralized; these are the genes originally identified as neurogenic by Lehmann et al. (1983). Additionally, complete loss of Suppressor of Hairless has been shown to cause a neurogenic phenotype (Schweisguth and Posakony, 1994).

Genetic analysis suggests that the neurogenic genes act in a common pathway. This is based on experiments in which duplications for one locus were tested for their ability to modify the homozygous null embryonic phenotypes of the other neurogenic loci. According to these studies, neuralized was placed upstream of Notch and Delta (de la Concha et al., 1988). mastermind was placed upstream and Enhancer of split downstream of Notch and Delta. big brain was tentatively placed in a parallel pathway to the neurogenics, based on the fact that duplications of the other neurogenic genes did not modify a big brain null phenotype, and big brain duplications did not modify the phenotype of mutations in the other neurogenics (de la Concha et al., 1988). Recent molecular characterizations of several of these molecules have caused aspects of this genetically described pathway to be questioned; mastermind has been shown to act downstream of Notch and Delta (Schuldt and Brand, 1999) and big brain seems to act within the neurogenic pathway (Doherty et al., 1997).

Evidence that the neurogenic genes interact in a pathway is further provided by the molecular characterization of these genes. The most studied of the neurogenic genes is Notch. Notch encodes a 300 kDa single-pass transmembrane protein that contains 36 EGF-like repeats and 3 so-called "Notch" repeats in the extracellular domain. Within its intracellular domain NOTCH also contains six ankyrin repeats, several opa repeats and a
PEST region, as well as two nuclear localization signals, one on either side of the ankyrin repeats. This protein functions as a receptor of intercellular signals. It has recently been shown that the Notch holoprotein is cleaved into extracellular and membrane bound fragments in the trans Golgi network, and that these fragments are attached by disulphide bonds to form heterodimers on the cell surface; these heterodimers are the active form of the protein (Blaumueller et al., 1997). It has also been shown that the membrane bound fragment can be cleaved upon ligand binding, producing a smaller fragment which may be translocated to the nucleus and directly activate the transcription of target genes (Struhl and Adachi, 1998); however, the relevance of this potential translocation to the biological role of Notch is still controversial, as endogenous NOTCH has never been found in the nucleus.

Delta encodes a 100 kDa transmembrane protein containing 9 EGF repeats and a DSL (Delta-Serrate Like) domain in the extracellular portion. This protein is the main ligand for NOTCH activation during embryonic neurogenesis. Serrate has also been shown to act as a ligand for Notch, and ectopic expression of Serrate can rescue the homozygous null Delta phenotype, but not the Notch phenotype (Gu et al., 1995). It has been suggested that Delta and Serrate may be involved in Notch activation in different developmental contexts. Serrate’s main function seems to be in the establishment of the wing margin during imaginal development.

It has been shown that Notch and Delta are the receptor and ligand responsible for lateral inhibition. Biochemically, DELTA can bind NOTCH in trans (Fehon et al., 1990), leading to the activation of NOTCH. This interaction is mediated through NOTCH EGF repeats 11 and 12 (Rebay et al., 1991). The NOTCH-SERRATE
interaction involves the same NOTCH EGF repeats as the NOTCH-DELTA interaction (Rebay et al., 1991). The intracellular portion of NOTCH is then cleaved, and together with SUPPRESSOR OF HAIRLESS [Su(H)] activates the transcription of genes of the Enhancer of split [E(spl)] complex. The E(spl) complex genes encode bHLH proteins that repress the transcription of the proneural genes of the achaete-scute complex (AS-C). This in turn leads to a reduction in the level of Delta in the receiving cell, forming a positive feedback loop that can rapidly transform minor stochastic differences in ligand levels into a situation where all cells but one lack ligand entirely (see Figure 1).

Interestingly, it has recently been shown that DELTA can also bind NOTCH in cis, in which case it acts as a repressor of Notch signalling (Jacobsen et al., 1998).

The neurogenic pathway functions in many other cell fate decisions, including the development of the wing vein territories in the adult (de Celis et al., 1997), the specification of adult sensory organs (e.g. the notal sensory bristles, and the photoreceptors of the compound eye (Cagan and Ready, 1989)), oogenesis (Ruohola et al., 1991), and the specification of endodermal cell types in the embryonic midgut (Tepass and Hartenstein, 1995). Based on the number and variety of processes affected by the neurogenic genes, it has been suggested that the neurogenic pathway may act as a signalling cassette wherever binary cell fate decisions occur. The role of the neurogenic genes in the determination and differentiation of the mechanosensory bristles of the adult notum provides a good example of neurogenic signalling functioning in adult development.

The sensory bristles of the notum are primarily mechanosensory, and comprise two types: 13 large macrochaetae (per heminotum), which develop at stereotyped
Figure 1: The Notch signalling pathway. Notch and Delta have been shown to encode the receptor and ligand of a commonly used developmental signalling pathway. A.

Wildtype NOTCH is cleaved into two fragments, an extracellular fragment that contains 36 EGF repeats (boxes) and 3 "Notch" repeats (not shown), and an intracellular fragment that contains 6 cdc10/ankyrin repeats (ovals). These fragments form active heterodimers at the cell surface. The DELTA ligand binds to NOTCH EGF repeats 11 and 12 via its own EGF repeats. Ligand binding causes cleavage of the intracellular portion of NOTCH, allowing SUPPRESSOR OF HAIRLESS (Su(H)) to enter the nucleus (either with or without the NOTCH fragment) and activate transcription of downstream targets, such as the Enhancer of split complex (E(spl)). DELTA can also interact with NOTCH in cis. B. Model for the involvement of the neurogenic genes in lateral inhibition.

Levels of Notch and Delta are initially the same in all cells of a proneural cluster (1). A stochastic increase in AS-C activity in one cell leads to increased DELTA production, causing increased NOTCH activation in its neighbours (2). This leads to increased E(SPL) activity, down regulating Delta levels and driving all of the neighbouring cells into the epidermal fate. (Adapted from Hassan and Vaessin, 1996).
positions on the notum, and numerous smaller microchaetae, which form in similar areas in all adults, but without the precise localization seen with macrochaetae. It is thought that bristle precursors are determined in a manner analogous to the selection of neuroblasts in the ventral neurogenic region; intercellular communication allows the selection of one bristle precursor from amongst a group of equipotent cells. Macrochaete precursors develop from proneural clusters similar to those seen inneuroblast development, and lateral inhibition is believed to be involved in their specification (Simpson, 1990). However, microchaete precursors have been shown to develop from larger proneural regions, or stripes of neural potential, in which mutual inhibition between cells leads to the specification of precursor cells which “win out” in the competition (Usui and Kimura, 1993). Mutual inhibition based upon stochastic fluctuations in ligand levels would explain the less rigid patterning of the microchaetae versus the invariant patterning of the macrochaetae.

After determination each SOP undergoes mitosis, producing two daughter cells (termed pIIa and pIIb). Each daughter cell then divides to produce either a tormogen (socket) and trichogen (shaft) cell (pIIa progeny) or a thecogen (sheath) and neuron (pIIb progeny) (Hartenstein and Posakony, 1989). Notch and Delta are intimately involved in the production of these cells, acting in each cell fate decision (SOP vs. epidermis, pIIa vs. pIIb, tormogen vs. trichogen and thecogen vs. neuron). Macrochaete and microchaete precursors are determined at different times; macrochaete SOPs are determined during the late third instar larval stage, while microchaete SOPs are specified between 10 and 12 hours after pupariation formation (APF). During early pupariation, Notch, Delta and
achaete are expressed in a dynamic pattern, which eventually resolves into the microchaete pattern.

**The other neurogenic genes**

The functions of *Delta*, *Notch*, and several of the signal transducing molecules that modulate intercellular interactions are well defined. Ligand binding (*Delta*) causes *Notch* receptor activation, which, through action of the major signal transduction molecule *Su(H)* leads to transcription of target genes (such as *E(sp1)*). Unfortunately, the roles of other neurogenic genes are not so clear. The process of cell fate specification by the neurogenic signalling pathway cannot be fully understood without knowing more about the functions of these molecules. *big brain* encodes a putative pore protein, and appears to modulate the levels of *Notch-Delta* signalling (Doherty et al., 1997); however, the mechanism of this modulation is still unknown. It has also been shown that *mastermind* encodes a putative transcription factor, as does *neuralized*, but what they control (or, indeed, whether they are in fact transcription factors) is still unknown. To help clarify aspects of neurogenic signalling I have focussed on *neuralized*. I have attempted to determine its role in the *Notch* signalling pathway (or, alternatively, whether it functions in a different pathway and simply phenocopies the neurogenic phenotype), and have also investigated its role in later development.

*neuralized* was first identified by Lehman *et al.* in 1981, as having a classic (and severe) neurogenic phenotype (Lehmann *et al.*, 1981; Lehmann *et al.*, 1983). It has a widespread and dynamic expression pattern during both embryonic and imaginal development (as seen by *in situ* hybridization) (Boulianne *et al.*, 1991). Embryonic
expression of *neuralized* mRNA is first detectable around cellularization, in a ventral stripe. It is then expressed throughout the ectoderm during germ band extension, primarily in delaminating and delaminated neuroblasts. RNA levels then decrease, until there is only low level expression in parts of the brain, musculature and ectoderm.

*neuralized* is also expressed during imaginal development, within wing, leg and eye-antenna discs, and the larval CNS (Boulianne et al., 1991). *neuralized* mRNA is found in all sensory organ precursors, including those that will give rise to the notal macrochaetae and the bristles along the anterior wing margin. *neuralized* is also expressed in cells posterior to the morphogenetic furrow in the eye disc, in chordinotonal organ (stretch receptor) precursors in the leg discs, and in regions of cell proliferation in the CNS. Finally, *neuralized* RNA has been found in delaminating follicle cells of the germarium, and in the polar follicle cells in the vitellarium during oogenesis (Ruohola et al., 1991).

There is currently no *neuralized* antibody, so protein expression data is unavailable; however, the A101 *lacZ* enhancer trap line is inserted into the *neuralized* locus (Boulianne et al., 1991), and is believed to report the NEURALIZED expression pattern reliably. A101 expresses in the sensory organ precursors (SOPs) of the larval and pupal notum (Huang et al., 1991), and is often used as a marker of SOP fate. The widespread expression of *neuralized*, particularly during imaginal development, suggests that it may function in several developmental contexts, and may have a role in the development of the adult, as well as the embryonic, peripheral nervous system.

*neuralized* encodes a novel 753 amino acid protein with a putative C3HC4 zinc finger motif at the carboxy terminus and a putative nuclear localization signal near the
amino terminus (Price et al., 1993). These motifs suggest that neuralized may encode a transcription factor, but its subcellular localization is still unknown. neuralized is highly conserved among the Drosophilidae, with a Drosophila virilis homologue having been found (Zhou and Boulianne, 1994). A putative human homologue has also been cloned recently from a chromosomal region associated with astrocytoma (Nakamura et al., 1998). Analysis of protein homologues from different species can indicate regions that may have functional importance, these regions tending to be more conserved than others. The D. virilis NEURALIZED has 84.4% identity overall (at the amino acid level) to D. melanogaster NEURALIZED, while the putative human homologue has 30% identity. However, some regions are more highly conserved; the putative nuclear localization signal and zinc finger are 100% identical between D. virilis and D. melanogaster, while the zinc finger of the human NEURALIZED has 41% identity. There are also two other regions of high conservation, termed the Neuralized Homology Regions by Nakamura et al. (1998) (see Figure 2). Interestingly, the putative human homologue lacks a nuclear localization signal. These observations suggest that the zinc finger and the Neuralized Homology Regions may be important for NEURALIZED function, while the nuclear localization signal may be dispensable.

**Hypothesis/Goals**

The process of Notch signalling is a very important one in development. However, the functions of many of the genes in the pathway, including neuralized, are still unknown. To this end, I have investigated the role of neuralized in the development of adult sensory structures using two different but complementary approaches. The first, an F1 modifier screen, identifies genetic modifiers of neuralized function; the
Figure 2: Comparison of neuralized homologues. Putative homologues of Drosophila melanogaster neuralized have been identified in Drosophila virilis and in humans. Comparisons of conserved regions of protein homologues can indicate areas that may be required for the function of the protein. D. mel neu, D. vir neu and H neu represent NEURALIZED and its D. virilis and human homologues, respectively. Percentages on the left of the diagram indicate overall identity between the homologue and D. melanogaster NEURALIZED, at the amino acid level; percentages below the figures indicate identity for the indicated region. Numbers above the figures indicate amino acid position for each region. NLS = putative nuclear localization signal. NHR1 and 2 = neuralized homology region 1 and 2 (see Nakamura et al., 1998). C3HC4 = zinc-finger motif. The NLS is 100% conserved between D. melanogaster and D. virilis, but is not found in the human homologue.
Modified from Nakamura et al. (1998) and Zhou and Boulianne (1994).
methodology and results of this screen are described in Chapter 1. The second approach is more biochemical, and involves the creation of transgenic flies expressing epitope-tagged NEURALIZED. These constructs should assist in determining the subcellular localization of NEURALIZED, and so shed light on possible functions. Epitope-tagged NEURALIZED will also make it possible to use biochemical approaches to study neuralized function. These experiments are described in Appendix 1. Taken together, the series of experiments outlined in this thesis should assist in elucidating both the function of NEURALIZED and its role (or otherwise) in Notch signalling.
Chapter 1:

A genetic approach to finding modifiers of neuralized.
Summary

The Notch signalling pathway is known to regulate numerous cell fate decisions during Drosophila development. Although neuralized is thought to function within this pathway, its precise role is presently unknown. To gain insight into its function and to determine its role in Notch signalling, we generated dominant adult phenotypes in the wing, eye and notum by overexpressing neuralized. These phenotypes were used to perform genetic screens to identify neuralized interacting genes. It was found that several deficiencies uncovering neurogenic genes, as well as several uncovering genes of the EGFR pathway, interacted with neuralized. To confirm the interaction of neuralized with both the neurogenic genes and the EGFR pathway genes, the ability of specific alleles of the various genes to modify the neuralized overexpression phenotype was tested directly. The results of these tests suggest that neuralized can genetically interact with both the neurogenic and the EGFR pathways, indicating that it may have a role in both pathways.
**Introduction**

Genetic screens have provided a powerful way of studying gene function and identifying components of various signalling pathways. There are two basic kinds of screen, phenotypic screens and modifier screens.

Phenotypic screens involve creating mutant animals (using EMS, P-element or X-ray mutagenesis) and then looking for effects in specific tissues or on specific developmental processes. The neurogenic genes were first identified in this type of screen; a large collection of embryonic lethal mutations was screened for cuticular phenotypes, and those mutants selected were examined further for neural hyperplasia (Lehmann et al., 1981; Lehmann et al., 1983).

Modifier screens identify second-site mutations that can suppress or enhance the phenotype generated by mutations in a gene. This can involve looking at modification of gain-of-function or loss-of-function phenotypes. To investigate modification of loss-of-function phenotypes, lines that have "threshold" phenotypes are created. These lines have just sufficient gene activity to develop normally, but often display a phenotype. If a mutation in a different gene reduces the function of the gene of interest, it results in a more severe loss-of-function phenotype, while mutations that increase the function of the gene of interest can ameliorate the phenotype displayed. The easiest form of threshold screen to perform is one in which further reduction of gene function causes lethality. To investigate modification of gain-of-function phenotypes, dominant phenotypes (either spontaneous or artificial, for example, caused by deliberate overexpression of the gene) are crossed to mutations, and effects on the phenotype are assessed. Both approaches can identify both enhancers and suppressors of the gene activity.
A recent development is the misexpression screen. This involves the use of randomly inserted UAS promoter units to misexpress genes at random (Rorth, 1996). The misexpressed genes are then characterized based on their ability to modify a dominant GAL4-generated phenotype.

Modifier screens have been used successfully to investigate and expand the Notch pathway. The results of these screens indicated that Notch interacts with many genes in addition to the original neurogenic mutations identified by Lehmann et al. (1983). For example, one screen for suppressors of Abruptex (gain-of-function alleles of Notch, which are lethal in some combinations) lethality identified an unknown modifier of Notch on the X chromosome (Xu et al., 1990). Further characterization identified the modifier to be the already discovered deltex gene (Gorman and Girton, 1992; Xu and Artavanis-Tsakonas, 1990), which had not previously been known to interact with Notch. This is the strength of the genetic modifier screen; unlike a candidate gene approach, in which specific mutations affecting specific genes are examined for their effects on the phenotype of another gene (such as was used to generate the model of the neurogenic pathway (de la Concha et al., 1988)), modifier screens are unbiased, and so can uncover interactions which have never before been reported.

To gain insight into the role of neuralized in adult development, dominant adult phenotypes were generated by overexpression of neuralized in imaginal discs, using the GAL4-UAS gene expression system. Briefly, genes under control of GAL4-responsive promoters (Upstream Activating Sequences, UAS) are driven by the yeast transcriptional activator GAL4, which is regulated by endogenous promoter and enhancer sequences in the fly (Brand and Perrimon, 1993). The dominant phenotypes produced by neuralized
overexpression allowed a genetic modifier screen to isolate modifiers of *neuralized* to be performed. The observed interactions were then confirmed using a candidate gene approach.

It was found that mutations in *Notch, Delta, big brain, mastermind* and Enhancer of split modified the dominant *neuralized* phenotype, providing additional evidence that *neuralized* plays a role in the Notch pathway. Additionally, *neuralized* interacts with several members of the EGFR pathway. This suggests that *neuralized* may play a role in multiple pathways that affect cell fate decisions during development.

**Materials and Methods**

*Fly lines*

Flies were raised on standard yeast agar medium at room temperature (20 ± 2°C).

The following fly stocks were used:

A78GAL4-UASneu/TM3, Sb. This line carries a recombinant chromosome on which the A78GAL4 enhancer trap line (which expresses ubiquitously throughout third larval instar imaginal discs) is combined with a strongly-expressing UAS-*neu* line. Flies carrying this chromosome display a dominant adult phenotype, that was used as the basis of interaction tests with neurogenic and EGFR pathway genes.

**Neurogenic alleles:** N*pl*/*FM7; N*axl*/*FM7; N*md*/*FM7; Dl*'/TM2, Ubx; Dl*'/In[3R] p, Dfd, ca; mam*'/CyO; bib'/CyO; E(spl)D; Su(H)'/CyO. N*pl* is a hypomorphic allele caused by a mutation in EGF repeat 14 of the extracellular domain of *Notch* (Xu et al., 1990). N*md* is a hypomorphic allele caused by a missense mutation in the intracellular domain of *Notch* (Hing et al., 1994). N*axl* is a gain-of-function *Notch* allele that causes deletion of the L2,
and partial deletions of the L4 and L5 wing veins; it is thought that the gain-of-function aspect is caused by an enhanced affinity for Delta (Heitzler and Simpson, 1993). \(Di^{l}\) is a strong allele of Delta (Mortin et al., 1988). \(Di^{l+}\) is a moderate hypomorphic allele of Delta (Alton et al., 1989). \(mam^{x}\) is a strong hypomorphic allele of mastermind (Lehman et al., 1983). \(bib^{'}\) is an amorphic allele of big brain (de la Concha et al., 1988). \(E(spl)^{p}\) is a dominant allele of the Enhancer of split locus, comprising a 700 bp deletion and a 5 kb insertion in the gene complex (Oellers et al., 1993). \(Su(H)^{'}\) is a loss of function mutation in Suppressor of Hairless (de Celis et al., 1996).

EGFR pathway genes: \(DER^{Ep}/CyO; DER^{fibled7}/CyO; argos^{A7}/TM3, Sb; pnt^{A88}/TM3, Sb; rho^{18}/TM3, Sb; S^{l}/Cy; S^{H23}/CyO; spi^{l}/CyO. DER^{Ep}\) is a gain-of-function mutation in the EGF receptor (Baker and Rubin, 1989). \(DER^{fibled7}\) is a loss-of-function mutation in the EGF receptor (Clifford and Schupbach, 1989). \(argos^{A7}\) is an amorphic allele of argos (Freeman et al., 1992). \(pnt^{A88}\) is an amorphic allele of pointed (Scholz et al., 1993). \(rho^{18}\) is a loss-of-function allele of rhomboid. \(S^{H23}\) and \(S^{l}\) are amorphic alleles of Star (Kerber et al., 1998). \(spi^{l}\) is a loss-of-function allele of spitz (Freeman, 1994).

Ectopic expression: A78GAL4/TM3, Ser; UAS-\(DER^{DN}\); 2x UAS-\(DER^{DN}\); UAS-\(Di^{l78}\); UAS-activated \(dRas2\); UAS-\(DER\). A78GAL4 is a GAL4 insertion line which expresses ubiquitously throughout imaginal discs. UAS-\(DER^{DN}\) and 2x UAS-\(DER^{DN}\) express one and two copies respectively of a dominant negative EGFR construct under UAS control (Freeman, 1996). UAS-\(DER\) expresses wildtype EGFR under UAS control. UAS-activated \(dRas2\) expresses an activated form of Drosophila Ras under UAS control.
Viability studies

Crosses were set up between virgin female neu^{If65}/TM3 and males of the following genotypes: DER^{flb1e07}/CyO; argos^{A7}/TM3, Sb; pnt^{A88}/TM3, Sb; rho^{38}/TM3, Sb; S'/Cy; spi^{1}/CyO. The progeny were examined for the presence or absence of transheterozygotes (non-balancer flies).

Interaction studies

Virgin female A78GAL4-UASneu/TM3, Sb flies were mated to male flies carrying the neurogenic and EGFR pathway alleles listed above, except in the case of Notch crosses, in which male A78GAL4-UASneu/TM3, Sb flies were mated to virgin female Notch mutants. All crosses were carried out at room temperature. Progeny of these crosses were then observed for modification of the A78GAL4-UASneu phenotype under a dissecting microscope; possible modifications were confirmed using electron microscopy (for modifications of the eye and notal phenotypes). Female experimental and sibling (internal) controls were used, except for the following crosses: A78GAL4-UASneu/TM3, Sb x rho^{38}/TM3, Sb, where male rho^{38}/TM3, Sb controls were used due to a lack of females; A78GAL4-UASneu/TM3, Sb x DER^{flb1e07}/CyO, where male experimental and controls were used; and in all crosses involving Notch alleles, where w/Y; A78GAL4-UASneu/+ animals were used as the ectopic neuralized controls.

Phenocopy/overexpression studies

A78GAL4/TM3, Ser virgin females were crossed with males carrying the UAS constructs listed above. All crosses were maintained at room temperature. The crosses
were scored for production of F1 progeny, and flies carrying both GAL4 and UAS insertions (that is, non-balancer flies) were examined for phenotypic effects, as described above.

Scanning electron microscopy
Fly specimens were dissected, and the eyes and nota fixed overnight at 4°C in 4% formaldehyde, 2% glutaraldehyde, 0.1 M NaPO₄ (pH 7.2). The samples were then washed 3 x 15 minutes in 0.1 M NaPO₄ (pH 7.2), put through an ethanol dehydration series (10 minutes 25% ethanol, 10 minutes 50% ethanol, 15 minutes 70% ethanol, 30 minutes 80% ethanol, 2x20 minutes 90% ethanol, 3x20 minutes 100% ethanol) and stored at 4°C. Specimens were then rinsed with 100% ethanol and dried at the critical point using CO₂ as the drying medium. They were then mounted on aluminum stubs, spatter coated with gold, and viewed using a Hitachi S-570 scanning electron microscope at an acceleration voltage of 15 kV. Images were captured using Quartz PCI, and brightness and contrast were optimized using Adobe Photoshop.

Observations of wings
Wings were dissected and mounted in Gary’s Magic Mountant (2 parts Canada Balsam : 1 part methylsalicilate); the mountant was cured at 60°C overnight. The wings were then photographed under a dissecting microscope; photos were scanned using Fotolook SA 2.08, and made into montages using Adobe Photoshop.
**Results**

*Ectopic expression of neuralized causes dominant adult phenotypes*

To investigate the role of neuralized in postembryonic development, a number of UAS-*neuralized* constructs (UAS-*neu*) were overexpressed previously in the lab using a battery of GAL4 lines (the GAL4-UAS system (Brand and Perrimon, 1993)), and adult phenotypes were closely examined (Naemsch, 1996). It was found that overexpression of UAS-*neu* by the A78GAL4 line (which is expressed ubiquitously in third instar larval imaginal discs (Gustafson and Boulianne, 1996)) caused three reproducible phenotypes, in the eye, notum and wing of adult flies (Naemsch, 1996) (see Figure 3).

In the eye, overexpression of *neuralized* caused defects in both the number and positioning of the interommatidial bristles. Multiple bristles were frequently seen at normal bristle positions, and bristles were also often located at adjacent (as opposed to alternate) vertices. No ommatidial defects were seen in these flies (Figure 3, A, C).

Overexpression of *neuralized* in the notum (by A78GAL4) caused loss of landmark macrochaetae and an increase in the number of microchaetae. However, despite the increased numbers of microchaetae, there was no clustering or tufting of bristles on the notum. Each microchaete remained separated from all others by epidermal cells, although the distance between microchaetae was somewhat reduced relative to wildtype (Figure 3, B, D).

*neuralized* overexpression in the wing pouch (again using A78GAL4) caused the loss of distal portions of wing veins L4 and L5 (see Figure 3, E-F). This phenotype was variable, with some animals displaying extensive deletions of each vein, while others had much smaller deletions. Also, L4 was sometimes unaffected in these flies; L5 was
Figure 3: Over-expression phenotypes generated by *neuralized*. Over expression of a wildtype *neuralized* construct generates phenotypes in the eye (A,C), notum (B, D) and wing (E, F). A. Wildtype eye. Note the stereotypical array of interommatidial bristles at alternating vertices. B. Wildtype notum. C. A78GAL4/UASneuG2 eye. Note the clusters of bristles in both normal and ectopic locations (arrows). D. A78GAL4/UASneuG2 notum. Note the loss of landmark macrochaetae (arrows) and the increased number of microchaetae, compared to wildtype. E. Wildtype wing. F. A78GAL4/UASneuG2 wing. The distal portions of longitudinal veins L4 and L5 are deleted. A, C. 200X magnification. B, D. 110X magnification.
always partially deleted.

A similar eye phenotype was seen when UAS-neu was driven specifically in the eye using sevenlessGAL4 (Naemsch, 1996). Interestingly, the sevGAL4-UASneu phenotype was completely different from that resulting from the ectopic expression of either Notch or Delta, which caused ommatidial fusion and rough eyes in addition to mild bristle patterning effects. This suggests that neuralized may have a novel function in the neurogenic pathway, or may affect parallel pathways.

Deficiencies unmasking neurogenic and EGFR pathway genes modify these phenotypes

To identify candidate modifiers of neuralized an F1 deficiency screen was performed by Gianfranco Pellicori, looking for enhancers and suppressors of the neuralized overexpression phenotype (see Figure 4). This type of screen involves crossing flies expressing the dominant neuralized overexpression phenotype to flies heterozygous for deficiency chromosomes and looking for enhancement or suppression of the phenotype by the deficiency. The principle behind this screen is that reduction in gene dosage by half (through total loss of genes present in the deficiency) is sufficient to modify the phenotype. Therefore, it identifies dosage-sensitive interactions, as there is always a wild type copy of the candidate modifiers present in experimental flies. Previous studies have shown that the neurogenic pathway is dosage sensitive, so a 50% reduction in gene product should be sufficient to produce an effect. Deficiency screens are also fast and relatively easy. The deficiency kit used uncovered approximately 80% of the Drosophila genome. Only deficiencies that modified the A78GAL4-UASneu eye, wing and notal phenotype in a consistent manner (or two of the three at least) were
Figure 4: Phenotypes caused by enhancers and suppressors of ectopic neuralized.

The phenotypes generated by ectopic expression of *neu* (C, D; see also Figure 3) allowed an F1 deficiency screen for modifiers of *neu* to be performed. This screen uncovered several deficiencies that either enhanced (E, F) or suppressed (G, H) the phenotypes. A, B. Wild-type eye and notum. C, D. A78GAL4-UASneu G2 eye and notum. Note the increased number of interommatidial bristles, and the increased density of microchaetae on the notum. E, F. Enhancement of the phenotype. Ommatidial bristle clusters are larger and denser, and there appear to be more microchaetae present. G, H. Suppression of the phenotype. There are fewer interommatidial bristles present, and the microchaete density is reduced to almost wild-type levels. A, C. 200X magnification. B, D, F, H. 110X magnification. E, G. 150X magnification.
regarded as true modifiers of *neuralized* and investigated further.

A number of interesting interactions were seen (see Table 1). Deficiencies uncovering several neurogenic genes (*mam, bib, and Delta*) modified the ectopic *neuralized* phenotype. The deficiency uncovering *Delta* suppressed both the eye and notum phenotypes caused by A78GAL4-UASneu, while the deficiency uncovering *big brain* suppressed the wing phenotype as well. The *mastermind* deficiency enhanced all phenotypes examined. Interactions were also seen with deficiencies uncovering *argos, Star, spitz,* and the *Drosophila* homologue of the *Epidermal Growth Factor Receptor* (EGFR), which are all members of the EGFR signalling pathway. The *argos* deficiency suppressed the eye and wing phenotypes, as did the deficiency uncovering *Star*. The *spitz* and *EGFR* deficiencies seemed to enhance the *sevGAL4-UASneu* eye phenotype. In addition to these, interactions were seen with regions which do not contain any obvious candidate genes. These results were confirmed using a different GAL4 driver, one under the control of the *sevenless* promoter. This experiment was necessary to show that the observed interactions were caused by effects on *neuralized* and were not due to secondary effects on the A78GAL4 promoter.

The observed interactions with EGFR pathway genes are intriguing, as they suggest a possible alternative pathway in which *neuralized* could act. However, a deficiency can remove more than one gene, and the possibility exists that the observed interactions could be due to deletion of genes other than those of the EGFR pathway. Therefore, the interactions seen between *neuralized* and the deficiencies must be confirmed by directly testing alleles of different neurogenic and EGFR pathway genes uncovered by the deficiencies.
Table 1: Results of the F1 deficiency screen for modifiers of an ectopic neuralized phenotype. Consistent interactions were seen with 30 deficiencies, including many which uncovered members of the EGFR pathway, and three which uncovered neurogenic genes.

* = deficiency uncovering a member of the EGFR pathway.

# = deficiency uncovering a neurogenic gene. 0 = no effect seen on the phenotype.

sup = phenotype was suppressed by this deficiency. enh = phenotype was enhanced.

? = interaction was difficult to determine. ND = this interaction not examined.
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2993 uncovers argos
3011 uncovers Delta
442 uncovers mam
368 uncovers bib
2606 uncovers EGFR
3084 uncovers Star
3346 uncovers spi
Viability studies

To examine the possibility of interactions between *neuralized* and the EGFR pathway genes further, flies transheterozygous for mutations in *neuralized* and EGFR pathway genes were generated. If two genes function in the same signalling pathway, flies that are heterozygous for mutations in both those genes may not be viable. For example, flies heterozygous for mutations in either *E(spl)* or *Delta* are viable, but transheterozygotes for these mutations are not (Vassin et al., 1985). Flies carrying the *IF65* null mutation in *neuralized* were crossed to loss-of-function mutations in EGFR pathway genes. All crosses between *neu*<sup>IF65</sup>/TM3 and the genes of the EGFR pathway were viable, yielding the expected percentages of transheterozygous progeny. These experiments have been performed previously with the neurogenic mutations, and no effect on viability was seen in transheterozygotes (Vassin et al., 1985).

**Interactions observed between neurogenic mutations and the ectopic neuralized phenotype**

To confirm a role for *neuralized* in neurogenic signalling, the ability of mutations in neurogenic genes to modify the *neuralized* overexpression phenotype was examined. This involved crossing flies carrying the recombinant A78GAL4-UASneu chromosome to flies heterozygous for alleles of the different neurogenic genes. These observations confirmed the interactions with neurogenic genes suggested by the deficiency screen. The effects of transheterozygosity for neurogenic mutations on the ectopic neuralized phenotype are summarized in Table 2. Briefly, reduction of neurogenic signalling enhanced the extra bristle phenotype in the eye, while generally suppressing the notal
Table 2: Crosses testing interactions directly reveal interactions between neuralized and both EGF receptor pathway and neurogenic genes. Modifications were observed either under a dissecting scope (for wings) or using EM (for eyes and nota).

sup = phenotype was suppressed by this allele. ench = phenotype was enhanced.

? = interaction was difficult to determine. NE = there was no interaction between this allele and the ectopic neuralized phenotype. ND = this interaction not examined.

*: In addition to suppressing the neuralized phenotype, the Delta wing vein and eye phenotypes were enhanced by ectopically expressing neuralized.

***: rho\textsuperscript{54} had an eye phenotype indistinguishable from that seen with ectopic neuralized.

#: Star alleles have an inherent eye phenotype; however, this interaction was clearly not simply additive.
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phenotype (see Figures 5 and 6). The exception to this statement is the X-ray induced
$Delta$ hypomorph $Dl^\prime$, which enhanced both the eye and notal phenotypes (see Figures 7
and 8). Effects on the wing vein phenotype were variable, ranging from no effect ($N^{p2l}$)
to complete suppression ($Dl^\prime$) (see Table 2 and Figures 9 and 10). The $Delta$ alleles
tested had the opposite effect to that seen with the deficiency uncovering $Delta$, which
suppressed the phenotype. This discrepancy could be due to the deficiency removing
more than one gene in the region, causing multiple effects on the $neuralized$
overexpression phenotype, and shows the necessity of checking alleles of the uncovered
genes for effects rather than relying on the data from the deficiency screen for
interactions.

The ectopic interommatidial bristle phenotype was chosen for quantitative
analysis, to allow the use of statistical significance tests. The results of this analysis are
summarized in Table 3. The wildtype number of interommatidial bristles in females is
approximately 550. Ectopically driving $neuralized$ using A78GAL4 led to the
production of $876\pm13$ bristles per eye in females, and $734\pm16$ bristles per eye in males.
Mutations in all of the neurogenic genes except $mastermind$ were seen to significantly
enhance this effect, although not all alleles tested had a significant effect (see Table 3).

Interestingly, $neuralized$ overexpression appeared to modify dominant $Notch$ and
$Delta$ phenotypes. The different $Notch$ alleles tested had no effect on the ectopic
$neuralized$ wing vein phenotype; however, the $Abruptex$ phenotype (deletion of
longitudinal vein L2) was suppressed by $neuralized$ overexpression (see Figure 9). The
wing notching caused by hypomorphic alleles of $Notch$ was not affected by ectopic
$neuralized$ expression. The $Delta$ alleles suppressed the A78-neu vein loss phenotype

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Figure 5: Reduction in big brain level has opposite effects on the ectopic neu bristle phenotypes. Animals containing both the A78GAL4-UASneu recombinant chromosome and a loss of function allele of big brain have an enhanced interommatidial bristle phenotype (compare A with B), while the microchaete phenotype is reduced in severity (compare C and D). A. A78GAL4-UASneu eye. B. bib/+; A78GAL4-UASneu/+ eye. C. A78GAL4-UASneu notum. D. bib/+; A78GAL4-UASneu/+ notum. Note the obvious reduction in microchaete number compared to the recombinant alone.

Figure 6: Reduction of *Suppressor of Hairless* causes a marked reduction in microchaete number. Transheterozygous animals having both A78GAL4-UASneu and a *Su(H)* allele have a notal phenotype of reduced severity, with both fewer numbers and a reduced density of microchaetae. A, C. A78GAL4-UASneu notum. B, D. *Su(H)/+; A78GAL4-UASneu/+* notum. Note the obviously reduced numbers of microchaetae, and apparent increase in bristle spacing. A, B. 110X magnification. C, D. 700X magnification.
Figure 7: Reduction in Delta level enhances the ectopic neu eye phenotype.

Transheterozygous A78GAL4-UASneuG2/Dl7 animals display an enhanced interommatidial bristle phenotype, while having increased ommatidial fusion (compare B and D). A, B. Dl7/TM3 eye. Note the mild roughening effect caused by minor ommatidial fusion, and the occasional bristle duplication. C, D. A78GAL4-UASneu G2/Dl7 eye. The ectopic neu ommatidial bristle phenotype is enhanced (compare to Figure 3C), and there is also a much greater degree of ommatidial fusion. A, C. 200X magnification. B, D. 1200X magnification.
Figure 8: Reduction of Delta function strongly enhances the ectopic neu notal phenotype. Animals transheterozygous for the ectopic neu recombinant and $Dl^7$ have a strongly enhanced microchaete phenotype. A, B. A78GAL4-UASneuG2 notum. Note that while there is an increase in the number of microchaetae, proper spacing is maintained and the microchaetae do not form clusters. C, D. A78GAL4-UASneuG2/$Dl^7$ notum. Note that the number of microchaetae is increased compared to A, but the spacing is also affected, with the formation of clusters. Also, there appears to be an increased incidence in bristle twinning (see D.), which occurs only rarely in A78GAL4-UASneuG2 animals. A, C. 110X magnification. B, D. 700X magnification.
Figure 9: Interaction between Notch and neuralized in the wing.

Notch alleles appear to have no effect on the A78GAL4-UASneu wing vein phenotype. In contrast, ectopic neuralized suppresses activated-Notch phenotypes. A. Wild type wing, with 5 longitudinal veins (L1-L5) and two cross veins (acv and pcv). B. A78GAL4-UASneu wing. Note the deletion of distal portions of L4 and L5 (see also Figure 3F). C. N^std/Y wing. D. N^std/X;A78GAL4-UASneu/+ wing. The vein deletion phenotype is unaffected by partial loss of Notch. The lack of a Notch phenotype in this wing is due to the presence of a wild-type X chromosome. E. N^axl/Y wing. L2 is fully deleted, while L5 is partially deleted. F. N^axl/X; A78GAL4-UASneu/+ wing. The neu phenotype is unaffected, while the dominant Abruptex phenotype of deletion of L2 is abolished.
(see Table 2), while at the same time the thick-vein wing phenotype typical of Delta heterozygotes was worsened by overexpression of neuralized (see Figure 10). In addition to effects on the Delta vein phenotype, neuralized overexpression also appeared to enhance a rough eye phenotype seen in Dl heterozygotes (see Figure 7).

In order to see if ectopic expression of neurogenic genes could mimic the dominant phenotype caused by overexpression of neuralized, UAS-Dl was overexpressed using A78GAL4. No A78GAL4/UAS-Dl progeny were seen, suggesting that the combination was lethal.

**Effects of mutations in the EGFR pathway on the ectopic neuralized phenotype**

To confirm that the observed interactions between ectopic neuralized and the various deficiencies uncovering EGFR pathway genes were specific for the lack of those genes, alleles of spitz (the major EGFR ligand), Star, rhomboid (involved in processing the SPITZ protein into an active form), argos (an inhibitor of EGFR activation) (Golembo et al., 1996), pointed (a transcriptional activator and downstream target of EGFR signalling) and the EGFR itself were tested for their ability to modify the ectopic neuralized phenotype. The effects caused by different EGFR pathway mutations on the A78GAL4-UASneu phenotype are summarized in Tables 2 and 3, qualitative observations in Table 2 and quantitative results in Table 3.

The wing vein phenotype was suppressed in the presence of one copy of the gain-of-function Ellipse allele of DER, and also by amorphic alleles of argos and pointed. An amorphic rhomboid allele enhanced the phenotype, while loss-of-function mutations in DER (flb) and Star had no effect in the wing.
Figure 10: The *Delta* vein phenotype is epistatic to the ectopic *neu* wing vein phenotype. Transheterozygous A78GAL4-UAS*neu G2/Dl* animals display a worsened *Delta* vein phenotype, rather than the A78GAL4-UAS*neu G2* vein phenotype (compare B and D). A. Wild-type wing, with five longitudinal veins of normal thickness. B. A78GAL4-UAS*neu G2* wing, showing the typical deletion of veins L4 and L5. While this phenotype is variably penetrant, there is always some degree of vein deletion observed. C. *Dl*'/+ wing. Note the thickening at the junction of L2 and the margin, and the vein delta formed between L5 and the posterior cross vein (pcv). D. A78GAL4-UAS*neu G2/Dl* wing. Note that there is no deletion of L4 or L5, while the thickening of L2 and the delta between the pcv and L5 are increased.
Table 3: The observed interactions between *neuralized* and other genes are statistically significant. Images of eyes from transheterozygotes were obtained using EM, and the bristle numbers counted. S.E.M. = standard error of the mean (sd/n\(^{1/2}\))

* = The experimental animals in this case were males. n.s. = not significant

*r*-values were obtained using Student's t-test with n-1 degrees of freedom; one-tailed for the neurogenic genes, two-tailed for the EGFR interactions.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average</th>
<th>S.E.M.</th>
<th>n</th>
<th>t</th>
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<tr>
<td>A78-neu</td>
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Most mutations of the EGFR pathway studied had no effect on the notal phenotype. *Ellipse* and *Star* appeared to increase the number of bristles on the notum (see Figure 11), while an *argos* mutation reduced the number.

Quantitative analysis of the effect of spitz-group genes on the ectopic neuralized eye bristle phenotype revealed that amorphic mutations in *argos* and *pointed* significantly increased the number of bristles in the eye, as did *flb* (see Figure 12). Amorphic alleles of *Star* significantly reduced this number (see Figure 11). Mutations in *spitz*, as well as the *Elp* allele of *DER* had no significant effect on bristle number when combined with A78GAL4-UASneu.

**Phenocopy/overexpression studies**

The observed modifications of the dominant neuralized overexpression phenotypes by mutations in the EGFR pathway genes suggested that these phenotypes could be due to an effect of NEURALIZED on EGF receptor expression or activation. To investigate this possibility, various forms of the EGFR were expressed using A78GAL4, including dominant negative forms. A constitutively activated RAS line was also used; this line gives rise to similar effects to a constitutively activated EGFR line, as EGFR acts through the Ras pathway. The rationale for these experiments is that overexpression of an EGFR line that produces a similar phenotype to neuralized overexpression could be informative about the effect of neuralized on the EGFR. For example, were a dominant negative form of the EGFR found to phenocopy the neuralized overexpression phenotype, NEURALIZED might play a role in repressing EGFR activation or expression. If overexpression of the dominant negative EGFR had the
Figure 11: Mutations in members of the EGFR pathway modify the ectopic neuralized phenotype. Mutations in Star enhanced the notal phenotype while suppressing the interommatidial bristle phenotype. A. A78GAL4-UASneu eye. B. S/+;A78GAL4-UASneu eye. The Star mutation reduces the overall number of bristles in the eye. The eye roughness is due to a Star dominant phenotype (see C). C. Star control eye. D. A78GAL4-UASneu notum. E. S/+;A78GAL4-UASneu notum. Microchaete numbers are increased. F. The microchaete density is also increased, and twinned bristles are seen; twinned bristles are rarely seen in A78GAL4-UASneu nota. Star alone has no notal phenotype. A-C. 200X magnification. D, E. 110X magnification. F. 700X magnification.
Figure 12: Reduction in EGFR level causes an enhancement of the A78GAL4-UASneu eye phenotype. Reducing EGFR levels by one half (using the faint little ball IIE07 allele) caused a significant enhancement of the eye phenotype.

A, C. A78GAL4-UASneu eye. B, D. flbIIE07/+;A78GAL4-UASneu/+ eye. The size of bristle clumps are increased at almost every position. A, B. 200X magnification. C, D. 1200X magnification.
opposite effect to neuralized overexpression, NEURALIZED might instead be involved in enhancing EGFR activation.

The following EGFR constructs were found to be lethal when overexpressed using A78GAL4: UAS-\textit{DER}^{DN}, (2x UAS-\textit{DER}^{DN}), and UAS-\textit{activated dRas2}. The first two lines carry one and two copies respectively of a dominant negative EGFR, while the UAS-\textit{activated dRas2} line functions as a constitutively active EGFR. None of these crosses produced non-balancer flies, while progeny carrying one or other balancer were produced, indicating that the overexpression of these genes in the A78 pattern was lethal. However, overexpression of UAS-\textit{DER} in the A78GAL4 pattern produced a very similar phenotype to that seen with neuralized overexpression, in the wing, eye and notum (see Figure 13). A78GAL4;UAS\textit{DER} eyes appeared rough and had increased numbers of bristles. The rough eye phenotype is not seen with the overexpression of neuralized. The notum had a marked increase in bristle number compared to wild type controls, and the distal portions of L4 and L5 were deleted.

**Discussion**

A genetic interaction screen to identify genes that interact with neuralized was performed to further investigate the role of neuralized in development. This screen made use of dominant adult phenotypes generated by overexpression of neuralized in imaginal discs. A deficiency kit uncovering approximately 80\% of the genome was used in an F1 deficiency screen looking for enhancement or suppression of the dominant neuralized overexpression phenotypes by deletions of chromosomal regions. This screen identified thirty deficiencies that modified the neuralized eye, wing and notal phenotypes in a
Figure 13: Ectopic expression of DER using A78 generates a similar phenotype to that seen with *neuralized*. A, C, E. A78GAL4-UASneu. B, D, F. A78GAL4-UASDER. Overexpression of EGFR in the same pattern as *neuralized* produces similar phenotypes in the eye (A, B), notum (C, D) and wing (E, F). The roughness seen in B is similar to that seen with the hyperactive EGFR allele *Ellipse*, and so is most likely due to inappropriate activation of EGFR during eye development.

consistent manner. These interactions were confirmed using a different GAL4-UASneu recombinant line. Among these deficiencies were several uncovering neurogenic genes, and also several uncovering EGFR pathway genes. The observed interactions were then tested for specificity to loss of the neurogenic and EGFR pathway genes by directly testing the ability of mutations in these genes to modify the neuralized overexpression phenotype.

These results are very informative. First, they confirm that neuralized interacts genetically with the neurogenic genes, especially Notch and Delta. Although this interaction has been shown previously (de la Concha et al., 1988), these additional data strengthen the case for neuralized acting in the neurogenic pathway. Second, they validate the results of the deficiency screen by confirming that neuralized can also interact with the EGFR pathway. This possibility has never before been reported for neuralized.

neuralized was first identified (along with Notch and Delta) in a screen for embryonic lethal mutations that caused cuticular loss (Lehmann et al., 1981); these embryos were then seen to also have hyperplastic neural tissue. As a result of genetic experiments that investigated the ability of triploidy for one neurogenic gene to modify the homozygous null cuticular phenotype of the others, it was placed upstream of both Notch and Delta, and downstream of mastermind (de la Concha et al., 1988). However, mastermind has since been reported to act downstream of Notch (Schuldt and Brand, 1999), based on experiments in which homozygous mastermind mutations blocked the ability of activated Notch constructs to induce cell fate changes. This means that neuralized's position in the pathway is only tentative. No other genetic interactions
between neuralized and the other neurogenics have been reported, so the role of neuralized in the neurogenic pathway remains unclear.

The results of the experiments performed in the course of this work indicate that mutations in the neurogenic genes can modify the phenotype caused by overexpressing neuralized. The neuralized overexpression phenotype is a neurogenic one, producing more sensory organs in the eye and notum. Reductions in neurogenic gene levels had the expected neurogenic effect in the eye; the number of bristles was increased with mutations in all genes except mastermind (see Figures 5 and 7, and Table 3). Unexpectedly, the notal microchaete phenotype was suppressed by all neurogenic mutations tested (with the exception of Delta mutations), rather than enhanced in a neurogenic manner. This observation suggests that the interaction between neuralized and the neurogenic genes is not a simple one.

In the wing, Delta and Enhancer of split mutations caused a reduction in the extent of the vein deletion; the Delta mutations completely suppressed the ectopic neuralized vein phenotype (see Figure 10). Suppressor of Hairless and mastermind mutations enhanced the phenotype. In contrast, neither gain-of-function nor loss-of-function Notch mutations had any effect on the A78GAL4-UASneu phenotype.

neuralized overexpression was also seen to modify dominant phenotypes produced by Delta and Notch alleles, enhancing Delta vein and eye phenotypes seen with heterozygous Delta loss-of-function alleles (see Figures 7 and 10), while suppressing the gain-of-function Abruptex phenotype of Notch (see Figure 9). The Abruptex phenotype is thought to be due to an enhanced affinity of the NOTCH receptor for DELTA (Heitzler and Simpson, 1993), and so functions in a ligand dependent manner.
Members of the Enhancer of split gene complex, which are downstream targets of Notch activation, have been ectopically expressed in larval wing discs, resulting in loss of sensory microchaetae (Ligoxygakis et al., 1998). This antineurogenic phenotype is the opposite of the effect we see with neuralized. As such, it suggests that neuralized may be having its effect by reducing Notch signalling levels.

The effects of neuralized on the Delta and Abruptex phenotypes also suggest that neuralized is reducing Notch signalling, by reducing functional levels of either Delta or Notch. Similar phenotypes of increased numbers of microchaetae and increased numbers of interommatidial bristles in the anterior portion of the eye are produced by raising flies carrying conditional alleles of Delta at the restrictive temperature during early pupariation (Parody and Muskavitch, 1993); this effect is also seen with conditional alleles of Notch (Cagan and Ready, 1989; Shellenbarger and Mohler, 1978). It is therefore possible that the neuralized overexpression phenotype is due to reduction of Notch signalling by the excess NEURALIZED present.

The enhancement of the eye phenotype caused by reduction in neurogenic gene levels supports the conclusion that the neuralized overexpression phenotypes are due to a reduction in lateral inhibition. However, mutations in these same genes (with the exception of Delta) suppress the notal phenotype. What causes these different effects?

First, the nature of the proneural regions for the two bristle types differs. The eye bristles are selected from small clusters of cells with neural potential, in a similar manner to the embryonic neuroblasts. On the other hand, the notal microchaetae are selected from stripes of cells with neural potential (Usui and Kimura, 1993), making the bristle/epidermis decision slightly more graded. That is to say, slight reduction in
neurogenic signalling should have the effect of increasing microchaete density, while still allowing bristle SOPs to inhibit development of immediately neighbouring cells. This is in fact what is seen with ectopic neuralized; there are more microchaetae present, but bristle spacing is maintained to a certain extent, and there is no clustering of bristles (see Figure 3).

Second, it is possible that the strong Delta7 allele used may have an increased effect on Notch signalling levels relative to the weaker (hypomorphic) Notch alleles used. It has been shown previously that affecting Notch signalling levels by different degrees can have different phenotypic effects (most notably when comparing mosaic clones for null or hypomorphic Notch alleles). Also, mastermind and big brain generally have weaker effects than mutations in the other neurogenic genes, so it is possible they do not have the same strength of effect as do the stronger Delta alleles used here.

Third, it is possible that reductions in neurogenic gene level may have different effects on the neuralized overexpression phenotype in different genetic backgrounds. Interactions have already been seen between the neurogenic genes and the EGFR pathway genes (de Celis et al., 1997), and their transcription patterns appear to be interdependent. It is therefore possible that reducing Notch levels in the eye (where EGFR pathway genes are highly active) has different effects (for example, through increasing rhomboid transcription) from reduction by the same allele in the notum.

The data appear to suggest that ectopic neuralized has its effect through reducing Notch signalling levels when expressed, possibly during early pupariation (0-24 hours after puparium formation [APF]). It could have this effect either by reducing Delta levels or by reducing Notch activation. The observed interactions in the wing support either
case; reduction of *Delta* would enhance the *Delta* phenotype and reduce *Delta* levels to
abolish the *Delta*-dependent *Abruptex* phenotype, while reduction of *Notch* could make
provein cells refractile to *Delta* signalling and broaden veins, while also reducing overall
*Notch* signalling in the wing and preventing establishment of the *Abruptex* phenotype.

In addition to the expected modification of the *neuralized* dominant phenotypes
by neurogenic mutations, mutations in EGFR pathway genes were also shown to modify
these phenotypes. This result is quite exciting, since interactions between *neuralized* and
this pathway have not been reported previously. The EGFR pathway is another important
signalling pathway during development; it would be very interesting if *neuralized* proved
to act as a linking molecule between this pathway and the neurogenic genes.

EGFR signalling is involved in many stages of *Drosophila* development, from the
earliest steps (determination of the embryonic axes) to late events (pupal eye and wing
development). Three activating ligands for EGFR have been found in *Drosophila*:
gurken, which is active only during oogenesis, *vein*, which has been mainly implicated in
wing vein development but may have additional functions (Simcox et al., 1996), and
*spitz*, which appears to be involved in all other areas of development. In addition to these
activators, there is also the inhibitory ligand *argos*, which is transcribed in response to
EGFR activation, forming a regulatory feedback loop which is thought to refine EGFR
activation patterns during development. The best characterized ligand is *spitz*, which
encodes a membrane-bound protein requiring cleavage for activation.

The model for *spitz*-mediated EGFR signalling involves the processing of
membrane-bound SPITZ into secreted SPITZ by two other membrane-associated
proteins, STAR and RHOMBOID; the cleaved SPITZ is then free to diffuse and bind to
EGFR (see Figure 14). This in turn activates the Ras/MAPK cascade and leads to activation and repression of transcription through downstream targets (such as activation of the transcriptional activator pointed and repression of the transcriptional repressor yan). Target genes include argos, which is translated and secreted, and which interferes with EGFR activation by SPITZ through an as yet unknown mechanism (Golembo et al., 1996). The genes involved in this process are also known as the spitz-group, on the basis of their phenotypic similarity to spitz mutants.

Interestingly, many of the genes of the spitz group appear to be expressed in the same areas as Notch, Delta, and neuralized, suggesting that there may be functional connections between the two pathways. Interactions between the neurogenic genes and the EGFR pathway have been reported previously (Verheyen et al., 1996). EGFR alleles have been identified in several screens for modifiers of Notch phenotypes, and an interaction between EGFR and groucho (one of the E(spl) complex genes) has been recently reported (Price et al., 1997). Furthermore, signalling between Notch and Delta has been shown to be important for rhomboid localization to the developing wing vein (de Celis et al., 1997). rhomboid has also been shown to be involved in inducing chordotonal organ fates in embryos, through its activating effects on EGFR (Okabe and Okano, 1997). These results make it likely that these two important developmental pathways intersect at some point, forming a regulatory network of intercellular signalling. Here, it has been demonstrated that mutations in genes of the EGFR pathway can affect dominant phenotypes produced by overexpression of UASneuralized using A78GAL4. In the wing, Ellipse (a gain-of-function EGFR allele), argos and pointed all suppressed the vein deletion phenotype, suggesting that enhanced EGFR signalling can block the
**Figure 14: Model of EGFR activation by spitz.** Membrane bound SPITZ is processed by STAR and RHOMBOID to produce a secreted product which diffuses out and activates EGFR. This leads to activation of POINTED and repression of YAN (not shown), which in turn causes the production of ARGOS in the receiving cell. ARGOS is then secreted and prevents EGFR activation by SPITZ in neighbouring cells. (Adapted from Schweitzer and Shilo, 1997).
SPI

MIDLINE

secreted spitz

secreted argos

VENTRAL ECTODERM

EGFR

EGFR
effect of ectopic neuralized. pointed is a transcriptional activator, which activates downstream targets of EGFR, and so mutations in this gene would normally lead to reduced EGFR signalling; however, argos is one of the targets of pointed, and so in this case reduction in pointed levels probably enhances the domain of EGFR activation, by reducing ARGOS-mediated suppression of activation. Both rhomboid and Star mutations enhanced the vein loss phenotype - this result is expected, as both these genes have been shown to be important for the specification of vein territories (Guichard et al., 1999). However, neuralized overexpression phenocopies a rhomboid null phenotype in the wing, and rhomboid heterozygotes have a similar eye phenotype to A78GAL4-UASneu (see Table 2; data not shown), so it is possible that there is more to this effect than a simple reduction in vein-competent area.

In the eye, faint little ball (a loss-of-function EGFR allele), argos and pointed mutations enhanced the interommatidial bristle phenotype. As with the wing effect, this is most likely due to the effect these mutations have on ARGOS levels; the effect of all three is to reduce ARGOS levels through reduced transcription of argos. ARGOS has previously been shown to be a repressor of inductive events in eye development (Freeman et al., 1992); since the eye bristle precursors are recruited to the ommatidium as a result of induction from previously differentiated ommatidial cells, it is possible that the increase seen in the bristle number is due to increased induction of bristle SOPs. Loss of ARGOS has also been seen to cause extra neural differentiation (Freeman et al., 1992). Loss of Star caused a suppression of the eye phenotype, suggesting that reduction in EGFR activation levels may reduce the effect of neuralized. It is possible that this apparent suppression may be due to an effect of Star on the size of the eye, which would
reduce the number of bristles as a secondary effect, and so this observation may not be informative. As mentioned above, *rhomboid* heterozygotes displayed an eye phenotype similar to that seen with ectopic *neuralized*, so the effect of the *rhomboid* mutation could not be determined.

*Ellipse* and *Star* enhance the notal microchaete phenotype, while *argos* suppresses it. This result is somewhat surprising, as a role in notal SOP formation has not been previously assigned to *spitz*-group genes. *Star* would tend to reduce EGFR activation, while *argos* should enhance it; *Ellipse* is a *rhomboid*-independent gain-of-function EGFR allele, which has previously been reported to drive excess ARGOS production. It appears from the interactions between the EGFR pathway genes and ectopic *neuralized* in the wing that *neuralized* may be having its effect by reducing levels of EGFR activation. It may be doing this by interfering with RHOMBOID. In the wing, ectopically expressed *neuralized* produces a similar phenotype to a dominant negative EGFR construct, and also to homozygous loss-of-function *rhomboid* mutants (Garcia-Bellido and de Celis, 1992). Mutations which would tend to increase EGFR activation (*Ellipse, argos*) suppressed the phenotype, while *rhomboid* enhanced it. Also, in the notum, *Star* mutations (which interact synergistically with *rhomboid* in EGFR activation (Guichard et al., 1999)) enhance the phenotype, while *argos* suppresses it; reduced ARGOS levels would have the effect of increasing EGFR activation. In this case, the *Ellipse* allele may actually reduce EGFR activation levels by promoting ARGOS production. Similar effects were seen in the eye, with mutations causing a reduction in EGFR activation enhancing the phenotype.
Interestingly, ectopically expressing EGFR gave a similar phenotype to that produced by A78GAL4-UASneu (see Figure 13), with some differences. Interommatidial bristle number was not increased as much by A78GAL4-UASDER as by A78GAL4-UASneu (~630 vs. 876; the wildtype number is ~550), and the eye was roughened slightly (most likely caused by inappropriate EGFR activation). The notal microchaetae increased in number, but appeared to be more dense between the dorsocentral macrochaetae compared to the uniform distribution of microchaetae on the A78GAL4-UASneu notum. Also, overexpression of EGFR had no obvious effect on the notal macrochaetae. Similar effects were also seen in the wing, with distal portions of L4 and L5 being deleted in A78GAL4-UASDER flies. Surprisingly, an identical vein deletion phenotype has been produced by overexpression of a dominant negative EGFR construct (UASDER<sup>DN</sup>) in the dorsal wing blade (Guichard et al., 1999). These contradictory results are difficult to interpret; it is possible that widespread overexpression of EGFR using A78GAL4 (which appears to express ubiquitously) may have negative effects on vein development (perhaps by increasing ARGOS production), or possibly the distal portions of L4 and L5 are more sensitive to relative levels of EGFR signalling in vein and intervein cells.

Taken together, the results of the interaction tests confirm many of the interactions revealed by the deficiency screen. Mutations in both neurogenic and EGFR pathway genes were seen to modify the neuralized overexpression phenotype. neuralized appears to have its effect in imaginal discs by reducing levels of Notch signalling, and also by reducing EGFR activation; mutations reducing either Notch signalling or EGFR signalling levels were seen to be able to enhance the neuralized overexpression
phenotype, in some contexts. These observations suggest that *neuralized* may function in both signalling pathways.

Alternatively, *neuralized* may act in only one of these pathways, and have secondary effects on the other. The neurogenic and EGFR pathways have been shown to interact in many developmental processes. As an example, both the *Notch* and EGFR pathways have been shown to be involved in the development of the wing veins. Veins are first seen in the third instar wing disc as a series of five stripes of *Delta* expression. *rhomboid* coexpresses with *Delta* in these areas, while *Notch* is expressed in the intervein regions. During pupal development, *Delta* expression is progressively restricted to the putative vein regions, while *Notch* expression becomes highest at the vein-intervein boundary. *rhomboid* expression is also restricted to the vein territory, through a process involving *Notch* signalling (de Celis et al., 1997). Provein regions must be specified by *rhomboid* before *Delta* and *Notch* can have effects on vein width, while *rhomboid* expression is regulated by *Notch* signalling. A similar interaction between these two pathways has also been shown to occur in the specification of ommatidial cell types and positions in the eye. One pathway depends on the other for its effect.

Given the intersection of the two pathways under discussion, it is possible that *neuralized* is a member of only one of them, but has secondary effects on the other pathway. For example, *rhomboid* is regulated by *Notch* signalling in the wing; if *neuralized* acted only in the neurogenic pathway, it could have secondary effects on *rhomboid* expression by affecting *Notch*.

The results of this genetic screen are therefore suggestive, but not conclusive. The two pathways that appear to interact with *neuralized* have internal feedback loops,
and also affect each other's expression. This makes the interpretation of genetic data, based on the modification of adult phenotypes, quite difficult. Also, it is not possible to determine conclusively whether *neuralized* acts in both pathways or only one, because of the high amount of feedback within and between the two pathways. Genetics is not informative about the function of the molecule encoded by a gene, but can only reveal interactions between genes. These interactions are not necessarily direct; some modifications seen genetically could be due to secondary effects on the later development of the bristle cells (as an example). Any investigation of the functional or molecular characteristics of the protein encoded by a gene requires a biochemical approach. For example, the neurogenic gene pathway, which was first discovered using genetics (de la Concha et al., 1988), has undergone considerable modification with the availability of biochemical evidence. *mastermind* was placed upstream of *Notch* and *Delta* genetically, but has recently been shown to be downstream of *Notch* (Schuldt and Brand, 1999). As there is currently no *neuralized* antibody, biochemistry cannot be done, and so the investigation of the nature of the interactions discovered here is not yet possible. To allow the biochemical investigation of *neuralized* function, I have produced epitope-tagged *neuralized* constructs. These constructs are described in Appendix 1.
General conclusions
The neuralized gene is one of the original neurogenic loci first reported by Lehmann et al. in 1981. The neurogenic genes, which include Notch and Delta, participate in many developmental decisions. Notch and Delta are well studied, but little is known about the other members of the pathway, such as mastermind, big brain and neuralized. This work concentrated on investigating the function of neuralized in development.

The first approach taken was to use a dominant phenotype generated by ectopic expression of neuralized to screen for interacting genes; the results of this screen and subsequent experiments are given in Chapter 1. Members of both the Epidermal Growth Factor Receptor and the neurogenic pathways were found to modify the ectopic neuralized phenotype. These observations indicate either that neuralized interacts with both pathways, or it acts in one pathway and has secondary effects on the other.

Several different kinds of approach can be taken to investigate further which of these possibilities is correct. First, further genetic experiments can be done. Second, molecular markers can be used to investigate changes in gene expression in particular genetic backgrounds. Third, a biochemical approach can be taken to determine which of the observed genetic interactions are direct and which are indirect.

It would be useful to repeat the interaction experiments using stronger neurogenic alleles. The two Notch loss of function alleles used here are hypomorphic; it would be interesting to see the effect of the Notch8 deficiency on the ectopic neuralized phenotype, and neuralized effects on the dominant wing notching phenotype of strong Notch alleles could be observed (spII and nd3 wing notching phenotypes are only present in Notch hemizygotes).
Epistasis experiments can also be performed, in which *neuralized* is coexpressed with a dominant negative EGFR construct; if coexpression results in an increased phenotype, it is likely that *neuralized* may be reducing EGFR signalling. *Notch, Delta* and cleavage independent forms of *Notch* can also be coexpressed with *neuralized*, and the phenotypes compared to those resulting from expression of any single construct. If *neuralized* acts by reducing *Notch* signalling, it would be expected that ectopic *neuralized* would block the *Notch* and *Delta* overexpression phenotypes, while being unable to reduce the effects of ectopically expressed “cleavage independent” *Notch*. *Notch, Delta* and *neuralized* could also be expressed all together, and the results compared to overexpression of *Notch* and *Delta* without *neuralized*; again, the expectation would be that *neuralized* ameliorates any phenotype produced by coexpression of *Notch* and *Delta*. Experiments of this kind have been attempted, but the effects were generally lethal (see Chapter 1, Results). This lethality is probably due to the widespread expression of A78GAL4, so a more defined driver, such as *sevenlessGAL4* should be used for these experiments.

The advantages of the genetic approach are that it is relatively fast and the progeny are easily scored. The disadvantage is that genetic results only indicate potential interactions between genes, and cannot discriminate between direct interactions, indirect interactions and effects on the underlying phenotype. This means that the genetic approach alone is insufficient to investigate interactions between genes fully.

Molecular markers can be used to follow changes in gene expression caused by the overexpression of *neuralized*. To determine if the *neuralized* phenotype in the wing is due to repression of *rhomboid*, discs from A78GAL4-UASneu flies which also carry an
enhancer trap in rhomboid could be stained to see if RHOMBOID levels are reduced.
ARGOS expression can be assayed in the same way, using the argos'' enhancer trap line; since ARGOS is a direct target of EGFR activation, this experiment will show whether or not neuralized affects EGFR activation. EGFR activation states can also be assayed directly using an antibody to activated MAPK. These three experiments will help to show the effects (if any) of increased levels of neuralized on the EGFR pathway.
Enhancer trap lines are available in many other genes, allowing this sort of analysis to be extended.

The expression pattern of A78GAL4 must also be better defined; if it is found to express during early pupariation, the case for neuralized mimicking the effects of reducing Notch or Delta levels at that time is strengthened, while if it is expressed at other times (for example, during the time sensitive period for Notch to cause bristle loss) the model will have to be rethought.

These kinds of experiment allow potential interactions to be confirmed at the molecular level. For example, overexpression of neuralized could lead to reduction in RHOMBOID levels, suggesting that it may help to regulate EGFR activation.
Information of this kind could eliminate the possibility that observed phenotypic effects are due to effects on the tissues underlying the phenotype. However, this approach cannot determine whether the effects of neuralized on gene expression are direct or indirect.

The biochemical approach is the only one that can answer questions about the nature of genetic interactions (direct versus indirect). However, there is currently no antibody to NEURALIZED, so a way of generating immunoreactive NEURALIZED was
required, to allow biochemical techniques to be used. This was done by creating constructs in which neuralized was fused to sequences for non-endogenous protein epitopes, and then using these constructs to make transgenic flies. This procedure is outlined in Appendix 1.

These constructs were shown to have neuralized activity by comparison of phenotypes generated by ectopically expressed native and tagged neuralized constructs. Staining against the tag indicates that NEURALIZED is localized to the plasma membrane (Yeh et al., submitted), and so is not a nuclear factor as has always been assumed. The membrane localization of NEURALIZED makes it possible that the protein does interact with either NOTCH or DELTA during neurogenic signalling. It also suggests that the putative Nuclear Localization Signal is not a functional part of the protein. While this was suggested from interspecific homologue comparison (human NEURALIZED lacks an NLS (Nakamura et al., 1998)), the staining result confirms this assumption. The neuralized zinc finger motif is thus not involved in DNA binding. It may instead be used as a protein interaction domain. It is possible that NEURALIZED interacts with NOTCH or DELTA through this domain.

In addition to localizing NEURALIZED, these constructs will also allow coimmunoprecipitation experiments to test interactions between RHOMBOID, DELTA and NEURALIZED directly. If neuralized acts through rhomboid repression, RHOMBOID would be expected to coimmunoprecipitate with tagged NEURALIZED. However, if it interacts with NOTCH or DELTA, then these proteins would be expected to be identified by COIP. If NEURALIZED binds to members of both pathways, then it most likely functions in both pathways.
Epitope-tagged *neuralized* deletion constructs were also made. These constructs will allow the performance of structure/function analysis of NEURALIZED domains. Taken together, the biochemical experiments made possible by the *neuralized* epitopes will enable the role of NEURALIZED in development to be determined, and will allow the testing of the genetic interactions for directness. The pathway (or pathways) in which *neuralized* acts can also be identified.

The results of directly testing neurogenic and EGFR pathway alleles for interactions suggested by an F1 deficiency screen confirm the interactions reported by this screen. Both neurogenic and EGFR mutations modified the A78GAL4-UASneu phenotype, suggesting that these genes can interact with *neuralized*. It appears *neuralized* may act by reducing levels of the EGFR ligand processing protein *rhomboid*, and so reducing EGFR signalling levels; alternatively, this may be a secondary effect caused by interactions with *Notch* or *Delta*. The genetic interactions found in Chapter 1, combined with the biochemical tools created in Appendix 1, will allow directed testing of potential NEURALIZED interactions. These two approaches have helped to clarify the role of *neuralized* in development, and future experiments using a combination of these approaches will further clarify this role. Finally, it appears that the deficiency screen identified real modifiers of *neuralized*, and further study of genes uncovered by those deficiencies which were shown to interact will yield useful information about genes which can interact with *neuralized*. 
Appendix 1:

Generation and preliminary characterization of epitope tagged neuralized constructs
**Introduction**

*neuralized* has always been considered to be a member of the neurogenic gene pathway. These genes have been shown to be active in cell fate decisions in many developmental processes. However, genetic experiments indicate that it interacts with genes of the Epidermal Growth Factor Receptor (EGFR) pathway as well (see Chapter 1). *neuralized* may thus have a role in both of these pathways, or may function in one and interact indirectly with the other. *neuralized* encodes a novel 753 amino acid protein with a C3HC4 zinc finger at the carboxy terminus (Price et al., 1993) and a putative nuclear localization signal near the amino terminus. These motifs suggest that the protein may act as a transcription factor, but this possibility has not been proven.

There are no antibodies to NEURALIZED currently available. As a result, the expression data available for *neuralized* is at the RNA level, or has been determined using the A101 lacZ enhancer trap insertion in the *neuralized* locus (Boulianne et al., 1991; Huang et al., 1991). This means that nothing is known about the subcellular localization of NEURALIZED; RNA localization patterns do not address this issue, and enhancer trap lines express a different protein, and so do not reflect the localization of their host protein.

Knowledge of the subcellular localization of NEURALIZED is essential to the clarification of its biochemical function. As an example, a protein which is found in the nucleus could act as a transcription factor, while a cytoplasmic localization increases the likelihood of the protein functioning in signal transduction.

To determine the subcellular distribution of NEURALIZED, I generated epitope-tagged *neuralized* constructs. These constructs were then used to create transgenic fly
lines. Fortunately, it is relatively simple to generate transgenic flies expressing chimaeric and tagged proteins, using P-element mutagenesis. This technique, together with the GAL4-UAS expression system (Brand and Perrimon, 1993) for driving genes in defined regions, allows us to determine the expression pattern of the tagged NEURALIZED protein. The subcellular localization of tagged NEURALIZED protein can be determined by expressing the tagged neuralized construct in a defined pattern and immunostaining against the epitope. While this method does not allow the investigation of wild type neuralized expression during development, it does permit NEURALIZED to be localized within a cell. In addition to revealing the subcellular distribution of NEURALIZED, these lines could also be useful for biochemical investigation of NEURALIZED interactions.

In order to produce a tool to resolve the subcellular localization of NEURALIZED, and to allow interesting interactions discovered genetically to be investigated more fully, neuralized constructs tagged with various epitopes were made, and transgenic lines containing these constructs were produced. These chimaeric proteins were then tested for neuralized function. Tagged neuralized deletion constructs were also made, to create tools to allow structure/function analysis to be performed.

Materials and Methods

Fly lines

All stocks were maintained on standard yeast agar medium at room temperature.

The following lines were used in this work:

\( w' \);
w, FM7/w, LETHAL; IF2, L/SM5, Cy;Ly/TM3, Sb. These stocks are X, second and third chromosome balancer lines, respectively.

A78GAL4/TM3, Ser. This is a GAL4 line with broad expression in third larval instar imaginal discs.

Constructs

myc and FLAG epitopes were added to neu cDNA using a PCR-based strategy.

To add epitope tags to the N-terminus:

A PstI site was added to the N-terminal portion of neuralized cDNA by performing PCR with the following primers:

5'GTGGGATCCGGTCTATCGGATATACCA3'
5'GGCCCTGCAGAAGCTCAAAGCAACG3'

SK neu was used as the template. The ~400 bp PCR product was ligated into SK neuRO as a PstI - BamHI fragment, producing SK neu nn. A myc oligonucleotide produced using the following primers

5'GGCCGCGCTGAAATGGAGCAGAAGCTGATCAGCGAGGACCTGAACG3'
5'GATCCGTTCAGGTCCTCCTCGCTGATCAGCTTCTGCTCCATTTCCAAGCGC3'

was then ligated to the SK neu nn as a NotI - BamHI fragment. Correct splicing and preservation of reading frame were confirmed by sequencing. The myc-neu fragment was then isolated from SK neu and subcloned into either pUAST as a NotI - KpnI fragment (producing UAS-mycneu) or pCaSpeR-hs as an XbaI - NotI fragment (producing hs-mycneu).

Internal deletions in the neuralized transcript were produced by digesting SK neu with StyI (which removes ~1.6 kb from the middle of the cDNA), religated and then
cloned into pUAST as a NotI - KpnI fragment, producing a tagged construct containing only the NLS and zinc finger of NEURALIZED.

A construct was made in which the zinc finger was deleted by digesting UAS-myc<em>neu</em> with BstEII and KpnI, filling the ends and religating into pUAST.

Conservation of reading frame for all constructs was confirmed by sequencing.

To add epitope tags to the C-terminus:

A BamHI site was added to the C-terminal portion of <em>neuralized</em> cDNA by performing PCR using the following primers:

5’GC<sub>GCGGCAGGCCAACGGCCACGGTAAACCTCC</sub>3’

5’CC<sub>GGATCCCCGTTGGTGGGTGCGGAT</sub>3’

SK<sup>−</sup> neu was used as the template. The ~400 bp PCR product was then subcloned into SK<sup>−</sup> neuWO as a BamHI - BstEII fragment, to produce SK<sup>−</sup> neu cc. myc and FLAG oligonucleotides were produced by PCR using the following primer sets:

To add <em>myc</em> to the C-terminus

5’GATCCGAGCAGAAGCTGATCAGCGAGGAGGACCTGAACTAGT3’

5’CTAGACCTAGTCAGGTCCTCCTCGCTGATCAGCTTCTGCTG3’

To add FLAG to the C-terminus

5’GATCCCTAATATTCCTGCTTACTACTCTTTTA3’

5’CTAGACTAAAACAGTAGTAGCAGGAATATTAG3’

These oligonucleotides were then ligated into SK<sup>−</sup> neu cc as BamHI - XbaI fragments. The <em>neumyc</em> or <em>neuFLAG</em> constructs were then isolated from SK<sup>−</sup> and ligated into either pUAST as XbaI - KpnI fragments (producing UAS-neumyc and UAS-neuFLAG constructs) or pCaSpeR-hs as XbaI - NotI fragments (producing hs-neumyc and hs-neuFLAG constructs). Conservation of reading frame was confirmed by sequencing.
Plasmid DNA was then isolated from a lysed bacterial culture by ultracentrifugation of a CsCl₂ gradient, precipitated with isopropanol, resuspended in TE and stored at -20°C until needed for microinjection.

**DNA microinjection**

DNA microinjection and isolation of transformants was performed using standard protocols. Briefly, w' embryos were collected for 30 minutes, dechorionated with 50% bleach for 45±15 seconds and rinsed with dH₂O. Embryos were then mounted on tape and air dried 15 minutes. After covering with halocarbon oil (Halocarbon Products Corporation), the embryos were injected with a cocktail consisting of 400 µg/ml construct plasmid with 100 µg/ml “wings-clipped” helper plasmid in injection buffer (5 mM KCl, 0.1 mM PO₄, pH 7.8); embryos which had already cellularized were destroyed. Hatched larvae were transferred to vials containing standard yeast agar medium with some moist instant fly food added. G0 adults were collected and mated in single crosses to w' flies. Red eyed F1s were then mated to balancer flies, and transgenic lines were maintained as a balanced stock.

**Assay of neuralized function**

To confirm that the epitope-tagged neuralized was functional, male transgenics were crossed to virgin female A78GAL4/TM3, Ser flies. Progeny were assayed for the ectopic neuralized phenotype as described above (Chapter 1, Materials and Methods).
Results

Constructs and transgenic lines

To allow the subcellular localization of NEURALIZED to be determined, the following epitope-tagged neuralized constructs were made (see also Figure 15): N-terminal myc-tagged neuralized, a line expressing a myc-NEURALIZED fusion protein, carrying the MYC epitope at the N-terminus of full length NEURALIZED; C-terminal myc-tagged neuralized, which expresses a NEURALIZED-myc fusion, consisting of full-length NEURALIZED with a MYC epitope attached to the C-terminus; C-terminal FLAG-tagged neuralized, similar to C-terminal myc-tagged neuralized except having a FLAG epitope instead of MYC; Zinc finger deleted neuralized, in which the carboxy terminal portion of NEURALIZED was deleted - this deletion removes the zinc finger motif from the protein. This fusion protein was tagged with myc at the N-terminus; Nuclear Localization Signal and zinc finger portions alone, in which the middle of the protein was deleted, leaving only the putative zinc finger and Nuclear Localization Signal motifs. This protein was also tagged with myc at the N-terminus. These constructs were all placed in pUAST plasmid; pUAST contains GAL4 responsive Upstream Activating Sequences (Brand and Perrimon, 1993) and permits defined expression of the fusion proteins under GAL4 control. The full length tagged NEURALIZED constructs were also subcloned into pCaSpeR-hs plasmid; this plasmid contains hsp70 stress-response control elements and so allows the tagged NEURALIZED to be expressed upon heat shock. Multiple transgenic lines were created for each construct.
Figure 15: Schematic of neuralized constructs. The following constructs were made:

A. Full-length wildtype neu, containing the putative Nuclear Localization Sequence and the C-terminal Zn finger motif. B. Full-length neu tagged at the C-terminus with either myc or FLAG. C. Full-length neu tagged at the N terminus with myc.

D. Zn finger deleted neu, tagged at the N-terminus with myc. E. The neu NLS and Zn finger motifs only, with the intervening region of the protein deleted, tagged with myc at the N-terminus.
Ectopic expression assay

To confirm that the epitope tag does not interfere with the normal function or binding properties, or cause abnormal localization, of the protein, tests of the function of the protein were carried out, by crossing UAS-tagged neu lines to the A78GAL4 line. The ectopic expression phenotype produced by A78GAL4 driving UASneu is an appropriate test of NEURALIZED function, because overexpressing non-functional protein should not cause the same effect as overexpressing native protein.

Overexpression of various epitope-tagged wild-type neuralized constructs produced a qualitatively similar phenotype to overexpression of untagged neuralized (see Figure 16). That is, overexpression of the tagged lines caused an increase in the numbers of microchaetae and interommatidial bristles as well as mispositioning of the interommatidial bristles. Quantitatively, the phenotypes generated varied between lines; this is most likely due to position effects on the expressivity of the UAS transgene.

Discussion

Numerous lines of transgenic flies carrying epitope-tagged neuralized constructs have been generated. The neuralized constructs have been placed under both heat-shock and UAS control, allowing either ubiquitous or restricted expression of the construct. Both wild-type neuralized cDNA and internally deleted neuralized constructs have been made (see Figure 15).

One concern with using epitope tags is that the attached peptide may disrupt the structure of the protein sufficiently to abolish its function; for this reason the tags were attached to the termini of the protein, in an effort to reduce effects on NEURALIZED
Figure 16: Epitope-tagged *neuralized* retains *neuralized* function. Ectopically expressed FLAG-tagged *neuralized* produces an ectopic sensory bristle phenotype in the eye which is qualitatively similar to that seen with wild-type *neuralized*. A, D. A wild-type eye, with three bristles per ommatidium, at alternating vertices. B, E. Ectopically expressed UAS-*neu*\textsuperscript{CFLAG 3}. C, F. Ectopically expressed UAS-*neu*\textsuperscript{CFLAG 1}. G, H. Ectopically expressed UAS-*neu*\textsuperscript{G2}. Note that while the degree of bristle duplication varies among the lines, the phenotype itself is constant; the variation is most likely due to position effects on the expression of the UAS-*neu* transgene. The UAS-*neu* lines were driven with the A78GAL4 line. A-C, G. 200X magnification. D-F, H. 1200X magnification.
structure. To test the possibility of the constructs having been rendered non-functional, tagged neuralized constructs under UAS control were expressed using A78GAL4, and the results compared to those seen with expression of an untagged neuralized.

Overexpression of the epitope tagged constructs using the A78GAL4 line produced a phenotype qualitatively similar to that produced by the overexpression of untagged neuralized constructs (see Figure 16). This observation suggests that the tags do not disrupt functional domains of NEURALIZED, as a non-functional protein would not be expected to cause a phenotype. Slight variations in the strength of the phenotype induced by ectopic expression of tagged NEURALIZED are most likely caused by position effects on the UAS promoters of the different lines. Similarity between ectopic tagged and native NEURALIZED phenotypes has been seen by others using other GAL4 lines; the same relative strengths of expression were also seen (data not shown). Therefore, the epitope tagged NEURALIZED retains NEURALIZED function and, presumably, localization.

Several of the generated tagged constructs have been overexpressed in a defined pattern using the GAL4-UAS system (Brand and Perrimon, 1993). Immunostaining using antibodies against the tag reveals that NEURALIZED is primarily localized to the plasma membrane (Yeh et al., submitted); this subcellular localization is seen consistently using both N-terminal and C-terminal tags (Yeh et al., submitted). This is an interesting result, as it suggests that NEURALIZED is not acting as a transcription factor as has always been assumed (Price et al., 1993), and may instead be involved in somehow regulating the sending or receiving of the signal responsible for lateral inhibition.
The creation of these tagged constructs permits the use of many biochemical techniques which were previously impossible. As an example, coimmunoprecipitation experiments, which allow the investigation of proteins which either directly bind to or form complexes with NEURALIZED, can now be done using antibodies against the epitope tags. This information is useful for discriminating between modes of NEURALIZED action suggested by genetic interactions. Knowledge of the proteins with which NEURALIZED interacts is invaluable to identifying possible functions of NEURALIZED. Genetic tests have suggested that NEURALIZED interacts with both the neurogenic gene pathway and the Epidermal Growth Factor Receptor pathway (see Chapter 1). Coimmunoprecipitation experiments using tagged NEURALIZED will resolve which of the interactions observed genetically are primary (direct) and which are due to secondary effects on the development of the tissue under investigation. Should NOTCH or DELTA be identified as interactors with NEURALIZED, and no EGFR pathway proteins are pulled out, the suggestion would be that neuralized functions primarily in the neurogenic pathway, and any interactions caused by EGFR pathway mutations are due to secondary (indirect) effects. On the other hand, if members of both pathways are identified as being capable of binding to NEURALIZED, neuralized most likely plays a role in both pathways. The use of different epitopes allows different kinds of experiment to be done; for example, myc tags are good for investigating the immunolocalization of proteins, while FLAG tags are good for biochemical techniques.

The creation of deletion constructs of neuralized also permits structure-function analysis of the protein. This analysis should assist in defining regions of NEURALIZED which are critical to its function. Epitope tagged constructs were made in which putative
functional domains (such as the C3HC4 zinc finger) were deleted; the tag allows any change in distribution or protein-protein interaction to be followed. As an example, if the zinc finger deleted constructs (Figure 15, D) fail to localize properly (as compared to wild-type constructs) and fail to produce the neuralized overexpression phenotype seen for full-length constructs, the zinc finger could be considered important to NEURALIZED function. Since it appears that NEURALIZED does not function as a transcription factor, the identification of potential protein interaction domains (such as the zinc finger) and other functional domains will also indicate a possible function for NEURALIZED. While some functional domains have already been suggested by comparison of NEURALIZED homologues from other species with *Drosophila melanogaster* NEURALIZED (see Figure 2), experiments of this kind will test these assumptions directly.

The tagged constructs generated in this work are not informative about the expression pattern of wild type NEURALIZED, as their expression is not under the control of the neuralized promoter. However, the subcellular localization data and the biochemical results they may yield will aid greatly in determining the function of neuralized in development, by suggesting pathways that may contain neuralized as an integral member, and by identifying regions of the protein that are essential for its function. These kinds of experiment have been impossible to perform until this time.
References


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