Neuralized functions cell autonomously to regulate Drosophila sense organ development

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Neurogenic genes, including Notch and Delta, are thought to play important roles in regulating cell–cell interactions required for Drosophila sense organ development. To define the requirement of the neurogenic gene neuralized (neu) in this process, two independent neu alleles were used to generate mutant clones. We find that neu is required for determination of cell fates within the proneural cluster and that cells mutant for neu autonomously adopt neural fates when adjacent to wild-type cells. Furthermore, neu is required within the sense organ lineage to determine the fates of daughter cells and accessory cells. To gain insight into the mechanism by which neu functions, we used the GAL4/UAS system to express wild-type and epitope-tagged neu constructs. We show that Neu protein is localized primarily at the plasma membrane. We propose that the function of neu in sense organ development is to affect the ability of cells to receive Notch-Delta signals and thus modulate neurogenic activity that allows for the specification of non-neuronal cell fates in the sense organ.

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Introduction

The process of sense organ (SO) formation in Drosophila is well characterized at the genetic and cellular levels and provides an ideal model to study the role of cell lineage and cell–cell interactions during development (Hartenstein and Posakony, 1989; Huang et al., 1991; Posakony, 1994). Bristle SOs are comprised of four cell types—tormogen (socket), tricogen (shaft), thecogen (sheath) and neuron—that arise from a single sense organ precursor (SOP). A fifth cell, the soma sheath cell or glia cell, is associated with each SO. SOP determination occurs within an equivalence group called a proneural cluster and requires the action of proneural group genes (Garcia-Bellido, 1979; Ghysen and O’Kane, 1989; Simpson, 1990). After primary SOP determination, all other cells within the proneural cluster are prevented from becoming SOPs through a process of lateral or mutual inhibition, and these other cells adopt an epidermal cell fate (Ghysen et al., 1993). The primary SOP divides asymmetrically to produce two secondary SOPs called pIIa and pIIb. pIIa will divide to produce the shaft and socket cell (Hartenstein and Posakony, 1989), while pIIb founds a lineage that produces the neuron and sheath cell. Recently, it has been demonstrated that the pIIb cell divides first to produce a glial cell and a daughter cell named pIIIb. The pIIIb cell divides to produce the neuron and sheath, indicating that the SOP lineage yields all five cells associated with a mature SO (Gho et al., 1999). Although the SOP undergoes a stereotypical pattern of cell division and cell lineage is important in cell fate determination, cell–cell interactions have also been shown to play an important role in the determination of SOP and daughter cell fates (Hartenstein and Posakony, 1990).

Many of the cell–cell interactions that are important in SO development are thought to be regulated by neurogenic genes. Neurogenic genes, including Notch (N), Delta (Dl), big brain (bib), mastermind (mam) and neuralized (neu), were first identified as embryonic recessive lethal mutations that cause hyperplasia of the embryonic nervous system at the expense of epidermal tissue (Lehmann et al., 1983). The best characterized members of the group, N and Dl, function as a receptor and ligand, respectively. Other members of the group are believed to play a role in generating the signal or propagating the signal. Besides providing the signalling pathway that is believed to be responsible for lateral or mutual inhibition within proneural fields, it is thought that neurogenic genes function together as a genetic cassette to regulate cell–cell interactions important for cell fate decisions in a variety of tissues during development (Ruohola-Baker et al., 1994). The role of neurogenic genes in SO development was demonstrated by generating mutant clones of N or Dl cells during development (Dietrich and Campos-Ortega, 1984). These studies revealed that mutant clones of N or Dl exhibit specific defects in bristle development. In Dl clones, these defects included tufting (supernumerary SO bristles), whereas in N clones both tufting and balding (absence of bristles) were observed. The variability observed in N clones appeared to be due to spatial differences within the notum; some regions yielded the tufted phenotype while other regions produced only the balding phenotype.

A detailed analysis of mutant clones of N, Dl and shaggy (sgg), another neurogenic gene, using adult epidermal markers confirmed the involvement of these genes in SO development (Heitzler and Simpson, 1991). Analysis of the genotypes of bristles found at the boundaries between mutant and wild-type cells revealed that N is required autonomously for receiving the neurogenic signal that prevents cells within the proneural cluster from adopting the SOP fate. The same type of analysis revealed that Dl is
required non-autonomously to produce the signal that allows epidermal cell specification. shaggy was found to be required cell autonomously to send and receive the neurogenic signal. Taken together, these results provided evidence that N and DI function in signalling as a receptor and ligand and that sgg probably plays a role in a feedback-based regulatory mechanism (Heitzler and Simpson, 1991).

Temperature-sensitive alleles have been used to elaborate further the role of N and DI in SO development (Hartenstein and Posakony, 1990; Parks and Muskavitch, 1993). These studies have shown that loss of N function prior to or during the determination of the primary SOP causes supernumerary primary SOPs to form. These extra SOPs develop normally and produce bristle tufts. When N loss-of-function is induced, subsequent to primary SOP determination and during the division and differentiation of the accessory cells, all the cells in the SOP lineage are transformed into neurons, resulting in a bald phenotype. Further analysis revealed that N is required at every step of the SOP lineage; proper determination of the pIIa and pIIb fates, as well as the accessory cell fates, requires N signalling. These results explain the apparent spatially dependent phenotypes caused by N mutant clones and suggest that chaetae do not develop synchronously. This approach also revealed similar requirements for DI during SOP development.

The neurogenic gene neu has also been implicated in SO development. neu, like N and DI, was first identified by means of loss-of-function mutations that cause hyperplasia of the central and peripheral nervous system at the expense of the epidermis (Lehmann et al., 1983). Although neu interacts genetically with other neurogenic genes, its role within this pathway remains unclear. The function of Neu protein is also unknown. The amino acid sequence of Neu suggests that it might encode a nuclear protein with a putative nuclear localization signal, helix–turn–helix domain and a C3H2 Zn-finger ("RING") domain at the C-terminus (Boulatane et al., 1991; Price et al., 1993). However, the subcellular distribution of Neu has yet to be determined. Homologues of neu have been identified in other species including human (Nakamura et al., 1998), mouse (Moschonas, 1998) and Caenorhabditis elegans (Wilson et al., 1994) suggesting that the function of neu in N–DI signalling has been conserved. Comparison of these sequences reveals that the RING finger domain is present within all homologues. However, the putative nuclear localization signal and helix–turn–helix domains have not been well conserved.

In situ hybridization studies have shown that neu is broadly expressed during early embryogenesis, but becomes restricted to the ventral neurogenic region and eventually to neuroblasts during neuroblast determination. During the third larval instar stage, neu is expressed in SOP cells that will give rise to macrochaetae on the adult notum (Boulatane et al., 1991). Dietrich and Campos-Ortega (1984) carried out mosaic analysis to determine the role of neu in SO development and found that neu mutant clones gave rise to a balding phenotype, which, in contrast to N, is spatially independent. However, these studies did not reveal the cellular nature of these defects. Furthermore, in the absence of appropriate markers, these studies could not establish whether neu was required autonomously or non-autonomously during SO development.

To characterize further the role of neu in the neurogenic signalling pathway we have studied its function during SO development by generating mutant clones using the FLP-FRT system (Golic and Lindquist, 1989). Using two independent neu alleles we find that neu is required for epidermal cell fate determination within the proneural cluster. neu mutant clones that overlap proneural regions exhibit supernumerary determination of SOPs. Analysis of the bristle genotypes found at clonal boundaries reveals that neu functions cell autonomously in receiving the signal that prevents SOP determination. Also, loss of neu function produces phenotypes similar to those seen in loss-of-function mutants for N and DI, indicating that it is required for proper determination of cell fates in the SO lineage. To gain insight into where neu functions in the neurogenic pathway, we examined the localization of Neu within the cell. Using the GAL4/UAS system (Brand and Perrimon, 1993) to express wild-type and epitope-tagged neu constructs during development, we show that both constructs are able to partially rescue the embryonic neurogenic phenotype caused by a mutation in neu and that Neu localizes to the plasma membrane. We propose that the function of neu during SO development is to modulate the efficacy of neurogenic signalling within the proneural cluster by affecting the ability of cells to receive or propagate signals through N and DI.

Results

Loss of neu function affects cell fate decisions during sense organ development

To assay the effects of loss of neu during SO development we generated mutant clones using FLP/FRT-mediated somatic recombination (Golic and Lindquist, 1989; Xu and Harrison, 1994). For these purposes, two alleles of neu were recombinated onto third chromosome arms containing FRT sequences at 82B. neuAl01 is a hypomorphic allele resulting from the insertion of a lacZ enhancer trap into the upstream regulatory region of the neu locus (Bellen et al., 1989), while neuAl05 is an amorphic ethylmethane sulfonate-induced allele (Brand and Campos-Ortega, 1988; de la Concha et al., 1988). Both alleles produce severe hyperplasia of the embryonic nervous system leading to lethality and both fail to complement any other known neu allele.

Flies heterozygous for neuAl01 and carrying a source of FLP (e22cGAL4, UAS-FLP; FRT82B, pM/FRT82B, neuAl01) displayed a bristle tufting phenotype affecting both macrochaetae and microchaetae. The severity of the phenotype ranged from duplicated bristles to tufts containing several bristles (Figure 1). Supernumerary macrochaetae and microchaetae were always found in characteristically normal positions. With the exception of extreme cases of microchaetae tufting observed only at the anterior-most part of the notum (Figure 1C), regions between bristles and bristle tufts did not appear to be affected. This suggests that neu functions to prevent cells from adopting SOP fates within the proneural cluster.

The effects of neuAl01 clones were not limited to bristles on the notum. Tufting could also be observed with adult head sensilla surrounding the eye and ocelli. In addition,
bristle sensilla throughout the body, including the dorsal and ventral abdomen, also appeared to form tufts. As was observed for macrochaetae, these tufts always occurred in the location where normal bristles are formed. \textit{neuA}\textsuperscript{101} clones also gave rise to defects in the adult eye. The severity of the phenotype ranged from ectopic inter-ommatidial bristles and aberrant ommatidial size to scarring (Figure 1H and I) and defective photoreceptor development (data not shown). In addition, defects in wing development, including irregular wing margin sensory bristle formation and ectopic wing vein formation, were observed (data not shown).
Tufting caused by neuA101 mutant clones is the result of supernumerary SOP determination

To determine whether the supernumerary bristles (i.e., tufting) that we observe in mutant clones are due to commitment to the SOP fate, we took advantage of the fact that the neu mutant allele neuA101 is a lacZ enhancer trap line in the neu locus that can be used as a marker of SOP determination. Previous studies have demonstrated that neuA101 expression can be detected within third larval instar wing imaginal discs in primary SOPs that give rise to macrochaetae on the notum and sensory bristles along the wing margin (Huang et al., 1991). As development proceeds, expression of neuA101 can also be detected in secondary SOPs as well as the accessory cells that are associated with each primary SOP. neuA101 is similarly expressed in SOPs on the pupal notum that will give rise to microchaetae. Since the appearance and differentiation of each macrochaeta SOP is well documented, it is possible to examine the fate of each SOP at particular developmental time points. For example, the primary SOPs that will give rise to bristles along the adult wing margin are determined during late third larval instar, but do not divide until 5–10 h after puparium formation (APF) (Huang et al., 1991). Therefore, any supernumerary β-gal positive cells along the wing margin that are observed during third larval instar development are most likely primary SOPs rather than secondary SOPs. Using the pMyc marker to identify neuA101 clones, we found that supernumerary SOPs arose from neuA101 cells (Figure 2). Since supernumerary SOPs are not observed in ectopic locations in the wing disc this suggests that neu functions normally in the proneural cluster to determine epidermal cell fates.

To ask whether the supernumerary primary SOPs arose from determination of excess primary SOPs within the proneural cluster or from abnormal proliferation of the primary SOPs, BrdU labelling experiments were performed. As described earlier, wild-type primary SOPs at the wing margin remain mitotically quiescent until after pupariation. Thus, they do not incorporate BrdU during third instar larval development (Figure 3A). Similarly, the supernumerary SOPs that developed along the wing margin in neuA101 mutant clones did not incorporate BrdU (Figure 3B, inset). Using an antibody against a phosphorylated form of histone H3 as a marker of mitosis (Hendzel et al., 1997) further reveals that supernumerary SOPs are not actively dividing (Figure 3D). These two results demonstrate that the increase in primary SOPs was not a result of aberrant mitotic events.

neu is also required for plla/pllb and accessory cell fate determination

neu mutant clones were also generated using neuF65. In this case, we found that mutant clones gave rise to a balding phenotype characterized by the absence of chaetae on the adult notum (Figure 4B). This is consistent with a role for neu in the determination of both plla/pllb and accessory cell fates. In N and Dl mutant clones, loss-of-function during secondary SOP and accessory cell fate determination causes cells to assume a neuronal cell fate (Hartenstein and Posakony, 1990). To determine whether neuF65 clones give rise to similar alterations in secondary SOP and accessory cell fates, pupal nota (24 h APF) were dissected and stained with the neuronal marker 22C10. In wild-type notum at this stage, 22C10 expression is detected in a single neuron comprising each individual sense organ (as identified by the double axon processes;
Fig. 4. The amorphic allele, neu<sup>F65</sup>, causes a bald cuticle phenotype. (A) Notum of a wild-type fly. (B) When clones of neu<sup>F65</sup> are generated, bristles do not form causing a bald cuticle (arrow). Epidermal cells appear to form normally. The bald phenotype of neu<sup>F65</sup> clones is caused by transformation of the SOP accessory cell fates into neuronal fates. (C) Expression of a neuronal marker 22C10 in wild-type pupal nota 24 APF. Neurons can also be unambiguously identified by their double axon projections. (D) Clusters of cells expressing 22C10 (some containing more than four positive staining cells) can be detected in nota (arrowheads) in which clones have been generated, indicating that multiple primary SOPs are determined and that each SOP divides and differentiates into the neuronal cell fate.

Fig. 5. neu functions cell autonomously in cell fate decisions. (A and B) Supernumerary bristles are genetically homozygous mutant as γ<sup>-</sup> bristles are never seen within tufts. Arrowhead indicates supernumerary macrochaetae, arrow indicates supernumerary microchaetae. Adult neu clones identified using pwn confirm the cell autonomous nature of neu function. (C) The majority of bristles observed at clonal boundaries are mutant (arrow) for neu<sup>A101</sup>. (D) A smaller fraction of bristles at boundaries are wild type (arrowhead). Also, single bristles that are neu<sup>A101</sup> (arrow) can exist at clonal boundaries (D).

neu functions cell autonomously in sense organ development

Using mosaic analysis, we have demonstrated a role for neu in sense organ development. To determine whether neu is required autonomously in this process, we generated neu mutant clones that were genetically marked with y in a y<sup>-</sup> background. Somatic recombination was induced by heat-shocking flies of the genotype y w hsflp; FRT 82B, neu<sup>A101</sup>/FRT 82B, γ<sup>-</sup>Sb pM during late embryogenesis. We found that the supernumerary bristles were non-Sb and γ<sup>-</sup> (Figure 5A and B) demonstrating that they arose from neu<sup>A101</sup> cells; mixtures of wild-type and mutant bristles were never observed.

To ascertain the ability of mutant cells to send or receive the signal that prevents neural determination, and thus delineate autonomous versus non-autonomous neu function, adult clones were examined using the epidermal hair marker pawn (pwn), which can be used to identify clonal boundaries on the adult cuticle. Since pwn affects bristle morphology (producing truncated bristles), mutant cells can be identified as well. If mutant bristles can be found at the clonal boundary, and these are unaffected by neighbouring wild-type cells, then neu must be required autonomously to inhibit neuronal cell fates. In contrast, if wild-type bristles are found at the boundary next to neu mutant cells, then neu must act non-autonomously within cells since they fail to suppress neighbouring cells from becoming SOs. We found that mutant bristles exist at clonal boundaries next to wild-type cells more frequently than wild-type bristles next to mutant cells (81 versus 19%, respectively, n = 382). Furthermore, mutant bristles at the clonal boundary were observed as either single bristles or tufts (Figure 5C and D). Thus, neu mutant cells are affected in their ability to receive the signal that prevents neural determination and they form SOs at clonal borders despite the presence of wild-type cells. The ability of mutant cells to send a signal does not appear to be affected as mixtures of wild-type and mutant bristles were not observed. Taken together, these results clearly demonstrate that neu functions cell autonomously during SOP determination to specify epidermal fates in Drosophila.

Neu is associated with the plasma membrane

To understand how neu could function in the signalling process that allows for epidermal cell fate determination, we examined the expression pattern of neu during SOP determination using in situ hybridization techniques on
Fig. 6. Expression of neu during SOP development. Detection of neu using in situ hybridization reveals expression within SOPs in the third instar imaginal disc. Early (A) and mid (B)-larval wing discs reveal no expression of neu in proneural regions, and weak expression in emerging SOPs. Late (C) third instar wing discs show expression of neu within emerging SOPs. (D) In situ analysis of pupal nota (24 APF) reveals neu expression within the neuron (characterized by dual processes of the axons) of each future SO.

Fig. 7. Wild-type and myc-tagged neu constructs can rescue the neu<sup>1065</sup> neurogenic phenotype. (A) A wild-type embryo stained with anti-HRP at late stages of embryogenesis displays an organized central nervous system characterized by its fasicles. (B) A neurogenic neu<sup>1065</sup> embryo displays severe hyperplasia of the central nervous system. Fasicles do not form and the central nervous system is greatly expanded. A neurogenic neu<sup>1065</sup> embryo that contains ptc-GAL4-driven expression of wild-type neu (C) or myc-tagged neu (D) has a partially rescued nervous system. The central nervous system is not as expanded and is more organized, with partial fasicles forming.

staged third larval instar wing imaginal discs. neu was undetectable in proneural clusters prior to SOP determination (Figure 6A and B). The first detectable neu expression occurs within SOPs in wing discs of late third larval instars as previously described (Figure 6C; Boulianne et al., 1991). neu expression was also examined within the notum at 24 h APF where its expression was found to be associated with the neuron of each SO cluster (Figure 6D). At this stage, all the accessory cells of each SO have been determined and the neuron can be identified based on its shape.

To determine where Neu protein functions within the cell, we then generated transgenic lines that expressed wild-type or myc epitope-tagged neu constructs in the
vector pUAST. To ensure that the myc tag did not disrupt Neu function, the ability to rescue the neu^{P05} mutant allele with both the wild-type and myc-tagged construct was compared. The neu^{P05} allele produces a severe neurogenic

![Image](image_url)

**Fig. 8.** Ectopic expression of neu causes adult phenotypes. (A) Wild-type macrochaetae assume stereotyped locations on the notum (white arrows). (B) Ectopic expression of neu using A78 causes missing macrochaetae (white arrows) as well as incomplete wing vein formation (D, black arrows). A wild-type wing is shown in (C).

phenotype characterized by hyperplasia of the central and peripheral nervous system (Figure 7B), and complete lack of ventral cuticle. Using a ptc-GAL4 line to drive expression, we found that both constructs were equally able to partially rescue the neurogenic phenotype (Figure 7C and D) and restore ventral cuticle (data not shown). This indicates that the myc epitope does not disrupt the Neu protein and that the fusion protein is functionally wild-type. In addition to being able to rescue neu^{P05} embryonic phenotypes, we found that ectopic expression of either tagged or untagged neu constructs yielded identical adult phenotypes characterized by missing macrochaetae and incomplete wing vein formation (Figure 8).

The myc-tagged UAS-neu lines were then crossed to a sca-GAL4 line that drives expression of the transgene in proneural clusters in third larval instar wing imaginal discs. We found that myc-tagged Neu was primarily localized at the plasma membrane (Figure 9). Double labelling with an antibody to α-spectrin, a structural protein found associated with the plasma membrane (Dubreuil et al., 1997; Lee et al., 1997) confirmed this localization. Myc-tagged Neu protein expressed in third larval instar salivary glands was also found localized at the plasma membrane. Since the N signalling pathway is not active during this stage of salivary gland development, whereas N signalling is active in the proneural cluster, Neu

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**Fig. 9.** sca-GAL4 driven expression of a C-terminal myc-tagged neu construct reveals Neu localization at the plasma membrane. Localization of α-spectrin, shown in (A) (green) reveals that the protein is associated with the plasma membrane. (B) Expression of myc-tagged Neu (red) in a wing disc proneural cluster reveals localization at the plasma membrane. The superimposed image shown in (C) shows they are co-localized. Myc-tagged Neu protein expressed in salivary glands exhibits the same plasma membrane localization. (D) α-spectrin localization (green); (E) myc-tagged Neu localization (red); (F) superimposed images.
localization does not appear to be affected by N signalling. This suggests that Neu functions at the plasma membrane to affect neurogenic signalling.

Discussion

**neu is required cell autonomously for specification of non-neuronal cell fates within the proneural cluster**

We have examined the role of the neurogenic gene *neu* in sense organ development in *Drosophila* to gain insight into its function within the neurogenic signalling pathway. To observe the effects of removing *neu* during SOP determination, we generated mutant clones using two *neu* alleles. We find that *neu* is required within the proneural cluster to determine epidermal cell fates; mutant *neu* clones give rise to supernumerary SOPs within proneural clusters. This phenotype is very similar to the loss-of-function N phenotype. However, while *neu* appears to be needed for receiving the signal that allows epidermal cell fate determination, it is not essential for epidermal development. Support for this conclusion comes primarily from the observation that the majority of *neu* 

Using a number of genetic markers, we show that *neu* functions cell autonomously during the process of SOP determination. Mutant bristles were found next to wild-type cells more frequently than wild-type bristles next to mutant cells. As well, tufts that were generated in *neu* clones never contained mixtures of wild-type and mutant bristles. Taken together, these results indicate that *neu* mutant cells have a reduced ability to receive or propagate the signal that prevents SOP specification. *neu* mutant cells, however, appear to be unaffected in their ability to send this signal.

We also found that *neu* is involved in the specification of accessory cells. Clones generated by Dietrich and Campos-Ortega (1984) and in our experiments using *neu* produced a bald phenotype. Loss-of-function during the division of the SOP leads to a transformation of the pIIa daughter cell to the pIIb fate because bald regions contain no external SOP structures. This suggests that the pIIa cell fails to receive the signal from the pIIb cell that normally prevents it from adopting the same fate. Also, 22C10 staining reveals that supernumerary neurons develop in *neu* clones, suggesting that the lineage derived from the pIIb cell is affected. Specifically, the pIIb cell produces two neurons, indicating a failure of the sheath cell to receive a signal from the neuron. These phenotypes are much like the phenotypes seen when *N* is removed during development of the SOP lineage (Hartenstein and Posakony, 1990).

**Cell autonomous function and neu expression**

Careful examination of the expression pattern of *neu*, by in situ hybridization, suggests that expression of *neu* is restricted to the primary SOP. It is currently difficult to explain our expression data given the cell autonomous requirement for *neu* in inhibiting neuronal cell fate specification. Clonal analysis reveals that *neu* is required for receiving the signal that allows for suppression of SOP specification yet *neu* expression is undetectable using in situ hybridization in those cells that are prevented from becoming SOPs. While it is possible that the supernumerary SOPs that arise in *neu* mutant clones are a result of abnormal rounds of primary SOP cell division prior to SO differentiation, BrdU-labelling experiments and anti-phospho-histone H3 staining clearly show that supernumerary SOPs do not result from extra rounds of mitosis. An alternative explanation is that wild-type SOPs at the clonal boundary function normally to inhibit neighbouring cells from becoming neuronal. Thus, tufts could only arise when mutant SOPs were located away from the clone border. However, our analysis clearly shows that the majority of bristles at the boundary are mutant and can exist either as single bristles or tufts.

This leaves the possibility that in situ hybridization is not sensitive enough to detect low levels of *neu* transcripts within the proneural cluster before primary SOP determination occurs and that this low level of *neu* is required for epidermal cell specification. The high levels of *neu* observed in SOPs might then be attributable to elevated levels of proneural gene expression observed subsequent to SOP determination. This would be consistent with previous studies demonstrating that *neu* is a target of proneural gene expression (Hinz et al., 1994; Singh et al., 1994). The ability to generate two phenotypes with different alleles suggests that the determination of primary SOPs is more sensitive to levels of *neu* activity than is development of the SO lineage. In *neu* clones, in which Neu function is reduced, elevated levels of *neu* expression in the primary SOP might compensate for decreased Neu activity during pIIa/pIIb and accessory cell determination. Then, supernumerary SOPs essentially develop normally and give rise to tufts. However, low levels of *neu* expression during primary SOP determination might be insufficient to compensate for reduced Neu activity. In *neu* clones, lack of Neu function cannot be rescued by elevated levels of expression. The SOP lineage is affected—no pIIa cells are determined and excess neurons are produced.

**Neu is associated with the plasma membrane**

To gain further insight into the role of Neu in the neurogenic pathway, the subcellular localization of Neu was determined. The amino acid sequence of Neu (Boulmiane et al., 1991; Price et al., 1993) predicts a protein containing a C-terminal RING finger domain that is often found in DNA binding proteins. However, there has been no evidence to demonstrate that Neu functions in the nucleus. Also, it has been demonstrated that some RING fingers have functions outside the nucleus (Joazeiro et al., 1999). Using the GAL4/UAS system to express *neu* during embryogenesis, we are able to partially rescue *neu* phenotypes. *ptc*--GAL4 driven expression of either wild-type *neu* or a myc-tagged *neu* construct reduces the...
hyperplasia of the nervous system seen in neuF65 mutant embryos. The failure to completely rescue embryos is most likely due to spatial and temporal differences in ptc-GAL4 expression and normal neu expression. The ability of myc-tagged Neu to rescue the neuF65 mutation as well as wild-type Neu indicates that the epitope does not disrupt the function of the protein. Also, ectopic expression of either construct during early development yields similar adult phenotypes. Myc-tagged Neu was found to be closely associated with the plasma membrane. While this does not exclude the possibility that endogenous Neu, like Notch, may exist at low levels within the nucleus or elsewhere in the cell, it suggests that neurogenic signalling does not require nuclear Neu.

The finding that Neu protein associates with the plasma membrane suggests a possible role in promoting or modulating neurogenic signalling at the receptor/ligand level. One possible model is that neu affects the ability of the cell to receive or propagate signals by affecting N, and that the function of neu in the proneural cluster is to promote differences in the level of N-DI signalling activity required for mutual inhibition. According to this model, initial low levels of neu expression within the proneural cluster would be required to promote differences in neurogenic activity. Through mutual inhibition mechanisms that involve feedback between the proneural and neurogenic genes, these differences would then be amplified leading to selection of a single SOP. In the absence of neu function, the formation of multiple SOPs would be the result of loss in the ability to receive a N-DI signal. Expression of neu would then be upregulated in the SOP, and neu would function during the SO lineage in a similar manner to allow cells to respond to N-DI signalling. Ectopic expression of neu allows all cells within the field to signal equally, effectively causing gain-of-function N phenotypes. Interestingly, the RING finger in Neu shares high homology to the RING finger found in the oncogene c-cbl (Joaozeiro et al., 1999). c-cbl has been shown to have ubiquitin ligase activity and affects the strength of receptor tyrosine kinase (RTK) signalling activity by targeting RTKs for degradation. The ubiquitin ligase activity has been shown to be conferred by a domain encompassing the RING finger domain (Joaozeiro et al., 1999). Whether the RING finger in Neu regulates N-DI signalling by targeting either N or DI for ubiquitylation, remains to be determined.

Materials and methods

Fly stocks and transgenics
Fly stocks were kindly provided by the following individuals: sca-GAL4 (Y.N.Jan, University of California, San Francisco); c22c-GAL4 UAS-FLP/CyO; FRT82B, pM (D.Harrison, University of Kentucky); y w hsff; FRT82B, y+ mSp SB626 (T.Xu, Yale); pr pwn FLP[38]CyO; Ki kar2y506 and pr pwn; FRT82B, kar2y506/FRT82B, kar2y506/y506Dp(2;3)P32 (P.Simpson, IGBMC). FRT82Bm and ptc-GAL4 were obtained from the Bloomington Stock Center (B2004 and B2017). The allele neu1011 is a hypomorphic allele caused by a lacI enhancer trap insertion into the neu gene (Bellen et al., 1989) and is available from the Bloomington Stock Center (B4369). neu65 is an amorphic allele (Brand and Campos-Ortega, 1988; de la Concha et al., 1988) and was provided by Y.N.Jan. A78 is a GAL4 line generated from an enhancer trap screen (Gustafson and Boulianne, 1996), which expresses ubiquitously during embryonic and larval development. All fly stocks were maintained at either room temperature or 18°C on standard corn meal agar media.

A 3.2 kb full length neu cdNA was subcloned as a KpnI fragment into the vector pUAST (Brand and Perrimon, 1993) and introduced into flies by standard P-element-mediated transformation (Spradling, 1986). Thirteen independent insertions were generated and balanced over FM7B, SM5 or TM3. Seven lines expressing a C-terminal myc-tagged neu construct (generated by PCR using specific primers) were also created. Expression of wild-type and epitope-tagged neu during development was achieved by crossing lines carrying these constructs to various GAL4 lines including ptc-GAL4 and sca-GAL4.

Immunocytochemistry and in situ hybridization
Imaginal discs and pupal tissues were dissected in phosphate-buffered saline (PBS) (1× PBS recipe in Verheyen and Cooley (1994)). Immunocytochemistry was performed essentially as described previously (Patel, 1994). Primary antibodies were used at the following dilutions: mouse anti-22C10 (C.Goodman, University of California, Berkeley), 1:10; rabbit anti-β-gal (Cappel), 1:2000; mouse anti-bromodeoxyuridine (BrdU) (Sigma), 1:1000; rabbit anti-phospho-histone H3 (Upstate Biotechnology), 1:250; mouse anti-myc, undiluted, and rabbit anti-spectrin (kindly provided by D.Branton, Harvard), 1:250. HRP-conjugated anti-mouse and anti-rabbit secondarys (Bio-Rad) were used at a dilution of 1:200. FITC-conjugated donkey anti-rabbit and Cy3-conjugated donkey anti-mouse (Jackson Laboratories) were used at a dilution of 1:200. FITC conjugated sheep anti-HRP (ICN) was used at a dilution of 1:200. Samples stained with GCAP were treated as previously described (Verheyen and Cooley, 1994).

In situ hybridization was performed as previously described (Boulianne et al., 1991). Third instar larvae were staged based on feeding behaviour and the absence or presence of bromophenol blue dyed food in the gut.

Rescue of neuF65
To assay the ability of wild-type neu or myc-tagged neu to rescue the neuF65 mutation, embryos from the cross ptc-GAL4/GFP, Cy; neuF65/TM3 and UAS-neu/UAS-neu, neuF65/TM6 or ptc-GAL4/GFP, Cy; neuF65/TM3 and UAS-neu/UAS-neuamy neuF65/TM6 were collected overnight and allowed to develop for an additional 24 h at room temperature. Embryos were then processed for in situ hybridization with antisense neu probe and immunocytochemistry with FITC-conjugated anti-HRP antibody. Rescue of the neurogenic phenotype was then determined in embryos displaying ptc-GAL4 driven expression.

Ectopic expression of neu during third larval instar imaginal disk development was achieved by crossing UAS-neu lines to the A78 GAL4 enhancer trap line.

Mosaic analysis
The alleles neu4101 and neuF65 were recombined onto third chromosome arms containing FRT sequences at 82B as outlined by Xu and Harrison (1994) and balanced over TM3. Mosaic clones of neu cells were generated using the FLP-FRT system (Golic and Lindquist, 1989; Xu and Rubin, 1993). The source of FLP-recombinase was either hsff, which consists of the fly gene under hsp70 control, or c22c-GAL4 UAS-flp, which consists of a GAL4 enhancer trap that is expressed ubiquitously at low levels during early Drosophila development when flies are reared at 29°C (data not shown; D.Harrison, personal communication).

To generate clones using the heat shock-FLP construct, y w hsff/y w hsff; FRT82B y+ mSp (w+) SB626/FRT82B y+ mSp (w+) SB626 females were crossed to FRT82B neu4101 or F65/TM3 males and y w hsff; FRT82B neu4101 or F65/FRT82B y+ mSp (w+) SB626 male progeny were then crossed back to y w hsff/y w hsff; FRT82B y+ mSp (w+) SB626/FRT82B y+ mSp (w+) SB626 females. Embryos from this cross were collected in vials for 48 h at 18°C, subjected to a 1 h heat shock at 37°C and allowed to complete development at room temperature. To mark adult clones with the epidermal hair marker pwn, FRT82B neu4101/TM3 were crossed to pr pwn FLP[38]CyO; Ki kar2y506 and Ki, Cy+ male progeny were mated to pr pw; FRT82B, kar2y506/FRT82B, kar2y506/y506Dp(2;3)P32. Progeny from this cross were collected at room temperature and heat shocked for 1 h at 37°C ~24 h after egg laying. GAL4-driven expression of FLP during early development was obtained by crossing c22c-GAL4, UAS-FLP/CyO; FRT82B, mCherry FRT82B, mCherry FRT82B, mCherry FRT82B, neu4101/TM3. Embryos from this cross were collected in vials for 24 h at room temperature, and then allowed to develop at 29°C.

BrdU labelling
Late larval third instar imaginal wing discs were dissected in Schneider’s medium (Schneider, 1964), transferred to fresh medium containing 0.01 mM BrdU and incubated at room temperature with slight rotation.
for 2 h. After three washes in PBS, the discs were fixed for 20 min with 4% formaldehyde in PBS, washed three times with PBT (1× PBS, 0.1% Triton-X) and blocked for 30 min with blocking solution (PBT, 2% bovine serum albumin, 1% normal goat serum). Detection of both β-gal and BrdU was as follows. Discs were first incubated with rabbit anti-β-gal overnight at 4°C, washed three times for 10 min with PBT, incubated for 30 min in blocking solution and incubated with FITC-conjugated donkey anti-rabbit antibody for 2 h at room temperature. After three washes of 10 min with PBT, a second fixation of 15 min with 4% formaldehyde in PBT was performed. Discs were then washed three times for 10 min with PBT and hydrolyzed with 2 N HCl (4 N HCl diluted 1:1 with PBT) for 35 min. Samples were washed three times for 10 min with PBT and incubated overnight at 4°C with anti-BrdU. Discs were then washed three times for 10 min with PBT and incubated with Cy3-conjugated donkey anti-mouse for 2 h at room temperature. After three washes of 10 min with PBT, discs were further dissected and mounted in 70% glycerol containing 2% DABCO (in PBS).

**Scanning and transmission electron microscopy**

Adult heads were fixed and embedded for scanning electron microscopy as described by Carthew and Rubin (1990). Sectioned adult eyes were fixed and embedded as described by Basler and Hafen (1988).

**Imaging and microscopy**

Adult structures and dissected tissue samples were examined using a Nikon Optiphot 2 equipped with Nomarski optics. Fluorescent and light images were captured using a Sony digital camera and analysed using Northern Exposure software. Confocal images were obtained using a Leica confocal microscope and PC computer running Scanware (Leica). Pseudo colour was added to confocal images using Adobe Photoshop.

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**References**


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