neuralized Functions Cell-Autonomously to Regulate a Subset of Notch-Dependent Processes during Adult Drosophila Development

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neuralized (neu) represents one of the strong neurogenic mutants in Drosophila. Mutants of this class display, among other phenotypes, a strong overcommitment to neural fates at the expense of epidermal fates. We analyzed the role of neu during adult development by using mutant clonal analysis, misexpression of wild-type and truncated forms of Neu, and examination of genetic interactions with N-pathway mutations. We find that neu is required cell-autonomously for lateral inhibition during peripheral neurogenesis and for multiple asymmetric cell divisions in the sensory lineage. In contrast, neu is apparently dispensable for other N-mediated processes, including lateral inhibition during wing vein development and wing margin induction. Misexpression of wild-type Neu causes defects in both peripheral neurogenesis and wing vein development, while a truncated form lacking the RING finger is further capable of inhibiting formation of the wing margin. In addition, the phenotypes produced by misexpression of wild-type and truncated Neu proteins are sensitive to the dosage of several N-pathway components. Finally, using epitope-tagged Neu proteins, we localize Neu to the plasma membrane and reveal a novel morphology to the sensory organ precursor cells of wing imaginal discs. Collectively, these data indicate a key role for neu in the reception of the lateral inhibitory signal during peripheral neurogenesis.

Key Words: neuralized; Notch signaling; Drosophila; sensory organ development; wing vein development.

INTRODUCTION

The Notch pathway is a conserved signaling system that is essential for the proper determination of cell fates throughout development (Artavanis-Tsakonas et al., 1999). A major class of cell fate decisions commonly associated with the Notch pathway are so-called “lateral inhibitory” processes, whereby the adoption of a particular cell fate is restricted within a group of equipotent cells. Typically, loss of Notch-pathway activity during one of these cell fate decisions results in the overcommitment of one type of cell fate at the expense of another. Many of the known members of the Notch pathway were initially characterized as “neurogenic” genes because of the neural hypertrophy that occurs at the expense of epidermis (Lehmann et al., 1983). However, most of the neurogenic mutants display additional defects, including overcommitment to muscle in the mesoderm, overspecification of midgut precursors in the endoderm, misspecification of photoreceptor cell fates, thickening of wing veins, and so forth (Corbin et al., 1991; de Celis et al., 1996; Hartenstein et al., 1992; Ligoxygakis et al., 1998). These observations indicate that the neurogenic genes are typically utilized in concert.

The key signaling components of the Notch pathway, as it is most often used in lateral inhibitory processes in Drosophila, are currently believed to be as follows. Interaction of the transmembrane ligand Delta with the transmembrane receptor Notch results in the release of a proteolytic fragment including the intracellular domain of Notch (N	extsuperscript{IC}) (Lecourtois and Schweisguth, 1998; Rebay et al., 1991; Schroeter et al., 1998; Struhl and Adachi, 1998). N	extsuperscript{IC} then translocates to the nucleus and acts as a coactivator for the sequence-specific DNA-binding protein Suppressor of Hairless (Hsieh et al., 1996; Harriault et al., 1995; Schweisguth and Posakony, 1992). This complex activates transcription of various target genes, including multiple bHLH and Bearded family genes in the Enhancer of split Complex [E(spl)-C] (Bailey and Posakony, 1995; Furukawa et al., 1995; Lai et al., 2000; Lecourtois and Schweisguth, 1995;
FIG. 1. Phenotypes of adult neu clones. (A–C, H) neu<sup>A101</sup> clones, (D–F, I–K) neu<sup>IF65</sup> clones, (G) wild-type clones were generated with a heat shock-inducible source of FLP recombinase except for (C), which utilized ey-FLP. (A, B, D, E, H–K) Examples of neu clones marked with y (light yellow pigmentation of cuticle and bristles) and Sb<sup>+</sup> (Sb bristles are short and stout). (A–C) neu<sup>A101</sup> clones display an autonomous increase in microchaete density as well as tufting of microchaetes and macrochaetes. Some clones are marked by arrows in (A); the clone boundary in (B) is marked with a line. (D, E) neu<sup>IF65</sup> notum clones exhibit an autonomous balding phenotype and never differentiate external sensory structures; the clone boundary in (E) is outlined. (F) Generation of neu<sup>IF65</sup> clones within the sensory lineage can result in double-shaft structures where the socket cell (arrow) has been transformed into another shaft (arrowhead). (G) Wild-type wing margin includes rows of stout mechanosensory organs (arrow) and chemosensory organs (arrowhead). (H) neu<sup>A101</sup> clone on the anterior wing margin displays strong increase in density of chemosensory organs (arrowheads) and loss of most stout mechanosensory organs (arrow). (I) No external sensory structures differentiate within a large neu<sup>IF65</sup> clone, although the thickness of the associated vein and overall integrity of the wing margin are unaffected. (J) neu<sup>IF65</sup> mutant bristles can differentiate on the wing margin, and these are typically double-shafted (arrows). (K) A neu<sup>IF65</sup> clone on the posterior wing margin displays similar phenotypes, including loss of the nonsensory hairs (bracket) as well as occasional double-shaft hairs (arrow).
Nellesen et al., 1999). The E(spl)bHLH genes encode transcription factors that may directly repress genes that promote various cell fates antagonized by the Notch pathway (i.e., proneural genes) (Jimenez and Ish-Horowicz, 1997).

The precise molecular functions of several cloned neurogenic genes with respect to the core N pathway remain unclear, including those of neuralized, mastermind, and big brain (Boulianne et al., 1991; Price et al., 1993; Rao et al., 1990; Smoller et al., 1990). Of these, the extreme neurogenic phenotype of neuralized (neu), coupled with its specific expression pattern at many sites of Notch-pathway activity, make it of particular interest (Boulianne et al., 1991; Lehmann et al., 1983). In this report we analyzed the requirement of neu for adult development. We find that neu is essential for multiple stages of adult peripheral neurogenesis, but not for other N-regulated processes such as wing vein differentiation or wing margin development. In spite of the apparent restriction of both neu transcripts and enhancer trap activity to sensory organ precursors (SOPs) (Boulianne et al., 1991; Huang et al., 1991), we find that neu is required cell-autonomously throughout cells of the proneural cluster. This suggests that very low levels of Neu may be sufficient for lateral inhibition. Using a misexpression assay, we find that wild-type Neu mimics gain-of-N receptor activity when expressed at lower levels, but phenocopies loss-of-N receptor activity at higher levels. These effects of Neu misexpression are observed in both peripheral neurogenesis and vein development. A form of Neu lacking the C-terminal RING finger strongly antagonizes the N pathway during neurogenesis, vein development, and wing margin development. This suggests that the RING finger is required not only for negative regulation of the Neu protein but also for controlling the specificity of its interaction with the N pathway. We further demonstrate that phenotypes produced by overexpression of Neu and NeuΔRING are sensitive to the dosage of many components of the N pathway. Finally, although Neu was previously hypothesized to be a nuclear protein (Boulianne et al., 1991), we instead observe that tagged Neu proteins are localized to the plasma membrane. This raises the possibility that Neu may somehow be involved in N-receptor function. Taken together, these results strongly indicate a key role for neuralized in the operation of the N pathway.

MATERIALS AND METHODS

Fly Stocks

The following GAL4 driver lines were used for over-/ misexpression studies by the GAL4/UAS method (Brand and Perrimon, 1993): sca-GAL4 (Hinz et al., 1994); GMR-GAL4 (Freeman, 1996); bx(MS1096) (Capdevila and Guerrero, 1994; Milan et al., 1998; Zeng et al., 1998a); neu-Gal4 (Reddy et al., 1999); VMQ-Gal4 (gift of Michael Brodsky, unpublished). UAS-eGFPF was obtained from Michael Mckewon (Finley et al., 1998). All of the mutant alleles used in clonal analysis and genetic interaction tests were previously described (Lindsley and Zimm, 1992) and were obtained from Jose de Celis, James Posakony, or the Bloomington Stock Center. We used the following chromosomes in clonal analysis:

FIG. 2. Cellular basis of neu adult clonal phenotypes. (A–F) Third instar wing imaginal discs or (G–J) pupal nota at 32 h after puparium formation (APF) containing neu<sup>iso5</sup> clones (A–D, G–J) or neu<sup>19166</sup> clones (E, F) were stained for Achaete (A, B), Asense (C, D), β-galactosidase (E, F), Prospero (G, H), or Elav (I, J); all of these are detected in the red channel. Clone boundaries are marked by the absence of GFP detected in the green channel and are outlined in white. (A, B) Ac protein is present throughout proneural clusters but is upregulated in the SOP. Most proneural cluster cells that fall in neu clones at the anterior wing margin express a high level of Ac (arrowhead). (C, D) Ase protein is present in SOPs. Single Ase-positive cells normally develop (arrow); supernumerary Ase-positive cells are found within a clone that overlaps a portion of the anterior wing margin (arrowhead). (E, F) A101 activity is restricted to SOPs; in an neu<sup>iso5</sup> clone, supernumerary cells autonomously express β-galactosidase (arrowhead), while A101-positive cells bordering neu clones are normally patterned (arrow). (G, H) At 32 h APF, Prospero is present in nuclei of sheath cells in the pupal notum; no Prospero staining is observed within the neu<sup>iso5</sup> clone that covers a portion of the microchaete field. (I, J) At 32 h APF, Elav is expressed by neurons. Within a neu<sup>iso5</sup> clone that overlaps the position of the anterior post-alar macrochaete, a large cluster of Elav-positive nuclei is seen (arrowhead); compare with a single Elav-positive cell outside of the clone (arrow).
RESULTS

An Autonomous Function of neutralized Is Required for Multiple N-Regulated Cell Fate Decisions during Adult Peripheral Neurogenesis

Adult clones of neu tissue have not been previously examined in detail, although they were reported to cause a balding phenotype in the notum and scars in the eye (Dietrich and Campos-Ortega, 1984). We used the FRT-FLP system (Xu and Rubin, 1993) to generate mutant clones of the null allele neu and the lacZ enhancer trap neu (Zeng et al., 1998b), a neu hypomorph; mutant clones are yellow (y) and Stubble positive (Sb). Notum clones of neu displayed an autonomous increase in microchaete density as well as tufts of microchaetes and macrochaetes, phenotypes that are typical of a reduction in N-pathway activity (Figs. 1A and 1B); similar effects were seen at a variety of other locations (Fig. 1C and data not shown). In contrast, notum clones of neu failed to differentiate the external components of sensory organs (shafts and sockets), resulting in bald cuticle (Figs. 1D and 1E). Again, the effect was cell-autonomous, as mutant cells at the clone border never differentiated bristles, while the patterning of wild-type cells adjacent to neu clones was not affected. When mitotic recombination of neu was induced within the sensory lineage (Zeng et al., 1998b), we observed a low frequency of double-shaft structures, where the socket cell has apparently adopted the fate of its sister cell, the shaft cell (Fig. 1F). Double shafts are indicative of a loss of N-pathway activity at this cell division (Hartenstein and Posakony, 1999). In experiments in which the genotype of the shaft cell was genetically marked with forked (f’), we found that double shafts could not be generated when the recombination event occurred at the cell division that produces the shaft and socket cells (which would have resulted in one f’ and one f shaft). Only double-f shafts were ever observed, indicating that the recombination event occurred at the first division of the sensory organ precursor (SOP) (Posakony, 1994). This result, along with the overall low frequency of double-shaft structures that could be produced, suggests that Neu protein inherited from the SOP may be sufficient for subsequent asymmetric divisions in the sensory lineage.

In the adult wing, neu clones displayed an increase in chemosensory bristles and lacked most stout mechanosen-
as well as a balding phenotype (Fig. 1K), indicating that multiple stages in the development of these noninnervated bristles are also regulated by neu.

We next analyzed the cellular bases of the neu phenotypes in adult PNS development. We generated mutant clones that were marked by the absence of nuclearly localized GFP and examined the expression of several sensory cell markers. First, we stained for Achaete (Ac) protein, which is present in all cells of proneural clusters but becomes upregulated in the SOP (Skeath and Carroll, 1991). Neu clones did not affect the distribution of Ac, indicating that it does not have a proneural function. However, cells within neu

expression becomes restricted to the sheath cell, while Elav is a neural-specific RNA-binding protein. At 32 h after expression is limited to SOPs (Brand et al., 1993); ectopic Ase-positive cells were present within mutant clones (Figs. 2C and 2D). The cell-autonomy of the neu mutant phenotype was clear in these experiments: excess upregulation of Ac and ectopic Ase expression was limited to the clone and not observed in neighboring wild-type cells. Conversely, neighboring wild-type cells did not prevent mutant cells on the clone borders from adopting the SOP fate.

We also examined the behavior of the neu

enhancer trap in neu

homozygous tissue. A101 appears to faithfully report the SOP fate even in neu mutant tissue: we observed an autonomous increase in cells expressing β-galactosidase within A101 mutant clones (Figs. 2E and 2F). Thus, neu is formally required to negatively regulate its own transcription. To assess the differentiation of internal sensory cell fates in neu

clones, which lack external sensory organ structures, we examined expression of Prospero (Pros) and Elav. Pros is a homeodomain protein whose expression becomes restricted to the sheath cell, while Elav is a neural-specific RNA-binding protein. At 32 h after puparium formation, neu

clones in the pupal notum failed to express Pros (Figs. 2G and 2H) and instead differentiated large numbers of Elav-positive cells (Figs. 2I and 2J).

Taken together, these results indicate that the basis of the balding phenotype in neu adult clones is apparently a successive transformation of pIIA cells into pIIb cells, and transformation of sheath cells into neurons (Posakony, 1994). Since we have also shown that neu is involved in lateral inhibition within proneural clusters and in the socket/shaft cell fate decision, neu is therefore required for all cell fate decisions in adult peripheral neurogenesis that are known to depend on N and Dl function (Hartenstein and Posakony, 1990; Parks and Muskavitch, 1993). We did not investigate the fate of the recently described glial cell, the fifth cell in the sensory organ lineage, as its migratory nature precludes conventional clonal analysis (Gho et al., 1999; Reddy and Rodrigues, 1999). The relationship of the glial cell fate with respect to the N pathway is currently unknown.

Miseexpression of Wild-Type and Mutant Neu Proteins Affects Lateral Inhibition during Peripheral Neurogenesis

To date all Neuralized orthologues identified, from multiple invertebrate and vertebrate species, share the following structure: two copies of a novel domain termed the neuralized homology repeat (NHR) followed by a C-terminal RING finger (Nakamura et al., 1998). We used the Gal4/UAS system (Brand and Perrimon, 1993) to misexpress various forms of Neuralized during larval development. Constructs tested include the wild-type protein, epitope-tagged Neu (myc-Neu), each of the individual NHR domains alone (Neu-NHR1 and Neu-NHR2), Neu deleted for NHR1 (NeuΔNHR1), Neu deleted for the RING finger (NeuΔRING), and finally the RING finger alone (Neu RING) (Fig. 3).

Animals carrying a single copy of UAS-neu and sca-Gal4 displayed mild notum and head sensory organ loss (Figs. 4A–4C and data not shown). Since this phenotype (as well as other Neu misexpression phenotypes) was not strongly modified by the neu

background, we made use of A101 as an SOP marker in our misexpression studies; sca-Gal4/UAS-neu; A101/+ discs showed loss of SOPs (not shown). These effects are thus the opposite of the neu loss-of-function phenotype. Interestingly, higher levels of Neu (2x UAS-neu) provoked an oppositely directed phenotype: although many bristles remained absent, many macrochaete positions were, in fact, now tufted (Fig. 4D). Strong increases in microchaete density could also be generated when expression of 2x UAS-neu was driven with bx(MS1096) (Fig. 4E). Wing discs from sca-Gal4/2x UAS-neu; A101/+ animals display missing SOPs in some regions as well as supernumerary SOPs in other regions (Figs. 4F and 4K). Thus, Neu appears to exhibit some dominant negative behavior when present at higher levels.

Deletion of the Neu RING finger (NeuΔRING) resulted in a protein that acted as a potent antagonist of lateral inhibition, especially during macrochaete development. Both the size of macrochaete tufts and the extent of A101-positive territories obtained following misexpression of two copies of this construct with sca-Gal4 are consistent with adoption of the SOP fate by all or nearly all cells of these proneural clusters (Figs. 4F and 4L). Miseexpression of high levels of NeuΔRING in the SOP and sensory lineage using neu-Gal4 also affected the differentiation of lineage fates. Notably, we observed a low but reproducible frequency of partial shaft-to-socket transformations, as well as fully double-socket structures (Fig. 4G). Curiously, this phenotype is correlated with an increase in N-pathway activity (Bang et al., 1991) and is the opposite of double-shaft phenotypes observed with neu loss-of-function. Thus, although ectopic NeuΔRING has primarily dominant-negative effects, it provides apparent gain-of-neu function in some circumstances.

Miseexpression of the RING finger alone (Neu RING) in single copy was highly toxic, as evidenced by poor viability
FIG. 3. Summary of phenotypes induced by misexpression of full-length and truncated Neu proteins. All Neu homologues have the same overall structure as Drosophila Neu, including two copies of a novel domain (neuralized homology domain, NHR, blue boxes) and a C-terminal RING finger (red box). Misexpression in the PNS was achieved using sca-Gal4, except for (*) which utilized neu-Gal4; misexpression in the wing was performed with VMQ-Gal4 or bx(MS1096). (lo) and (hi) refer, respectively, to lower or higher UAS-transgene number and/or Gal4 activity. (*) Bristle and SOP loss resulting from Neu-RING misexpression may not reflect changes in cell fate, but may be the result of an effect on cell viability.
when transheterozygous with sca-Gal4. In spite of this, the effect on patterning of the PNS was minimal, typically leading to loss of only one or two dorsocentral macrochaetes (data not shown). However, misexpression of multiple copies of UAS-neu RING yielded a dramatic balding phenotype in the rare flies that survived to the late pharate adult stage; this was correlated with loss and overall diminution of A101 activity (Figs. 4H and 4M). Although this phenotype appears to be consistent with a failure to specify SOPs, we do not interpret this as resulting from specific modulation of the N pathway. Misexpression of Neu RING with bx(MS1096) led to an extreme disruption in the development and patterning of all cell types in the notum (Fig. 4I). Many sensory organs were missing and overall polarity was strongly affected. In addition, development of the epidermal cells was also clearly disrupted, as large regions were devoid of the hairs normally produced by the epidermis. As these phenotypes do not appear to be obviously related to the Notch pathway, the phenotype caused by expression of the RING finger may not be the result of any specific effect on Notch-controlled pattern formation, as is apparently the case for the other Neu constructs.

Finally, misexpression of the single NHR domain proteins Neu-NHR1, Neu-NHR2, and Neu1NHR1 did not affect PNS development (data not shown). Although we did not assay the stability of these truncated versions, these results suggest that both NHR domains are necessary for Neu function.

**Misexpression of Wild-Type and Mutant Neu Proteins Affects Wing Development**

The same Neu constructs were tested for their ability to perturb wing development. As is the case with PNS development, misexpression of full-length Neuronal caused oppositely directed phenotypes on wing vein development. In females heterozygous for bx(MS1096) and UAS-neu we observed vein truncation, a phenotype often seen under conditions of Notch-pathway hyperactivity (i.e., Abruptex and Hairless mutant backgrounds, Fig. 5D; also see below at Figs. 7M and 7P). However, at higher levels of misexpression, we found a recovery of vein length concomitant with a strong thickening of veins (Figs. 5E and 5F). The vein thickening phenotype is comparable to that induced by raising Nts animals at the restrictive temperature during the first 2 days of puparium development (Shellenberger and Mohler, 1978). Although misexpression of Neu is able to affect at least one process that does not require endogenous Neu (vein differentiation), it was incapable of affecting the integrity of the wing margin when expressed at any level.

The NeuRING construct acted as a Notch-pathway antagonist in the wing, as it did in the notum PNS. Surprisingly, this form was able to strongly antagonize wing margin development, resulting in a severe loss of wing margin and a strong serrated phenotype (Figs. 5G-5I). A similar phenotype is generated Nts animals raised at the restrictive temperature during the second and third instars (Sturtevant and Bier, 1995). A single copy of this construct driven by bx(MS1096) in males resulted in nearly wingless flies. Nevertheless, in the struts and stumps of wings left in severely affected flies, the remaining venation was only mildly thickened. Therefore, deletion of the RING finger seems to have had the dual effect of allowing NeuRING to interfere with Notch-controlled processes that its wild-type counterpart could not affect (wing margin specification), while also apparently making NeuRING less effective in some processes relative to wild-type Neu (wing vein thickness). It is important to note, however, that all mutant phenotypes caused by Neu and NeuRING expression can be interpreted as resulting from specific alterations in Notch-pathway activity.

Misexpression of Neu-NHR1 or Neu-NHR2 had minimal effects on wing development (Figs. 5B and 5C and data not shown), while misexpression of NeuNHR1 weakly antagonized lateral inhibition during vein development, resulting in distal vein thickening (Figs. 5J and 5K, arrows).
addition, the wings were mildly spoon-shaped, suggesting a possible proliferation or viability defect. Because bx(MS1096) is predominantly active on the dorsal surface of the wing, expression of molecules that inhibit growth or viability would be expected to result in curvature of the wing. Misexpression of Neu RING caused a more severe decrease in wing size, as well as resulting in strongly curved wings (Fig. 5L).

We also examined the behavior of L3 campaniform sensilla, which provides a secondary assay of PNS development. Overall, we find that their behavior paralleled that of the notum mechanosensory organs. Lower levels of ectopic Neu induced loss of campaniform sensilla, while higher levels caused occasional clustered sensilla (Figs. 5M–5O). Overexpression of the NeuΔRING construct caused a strong increase in the density and clustering of these sensilla (Fig. 5P). Finally, in the disorganized wings that result from Neu RING expression, we did find certain pattern elements present, including the appearance of singularized, morphologically normal campaniform sensilla (Fig. 5Q).

Consistent with the loss of wing margin in adult wings, we found that misexpression of NeuΔRING resulted in a strong reduction of both Cut and Wingless (Wg) protein at the wing margin (Figs. 6A, 6B, 6E, and 6F). At the same time, ectopic Cut-positive cells are found in sensory positions, in accord with the effect of NeuΔRING on PNS development. In contrast, misexpression of either Neu or Neu RING had no significant effect on margin Cut expression (Figs. 6C and 6D). As well, mutant clones of neuIF65 that overlapped the prospective wing margin also showed normal levels of Cut and Wg (Figs. 6G–6J), in agreement with the failure of neu clones to affect the integrity of the wing margin in adult wings. NeuΔRING thus exhibits a switch in the specificity of N-regulated processes that Neu (both endogenous as well as ectopically produced) can normally affect.

Phenotypes Produced by Misexpression of Neu Proteins Are Sensitive to the Dosage of N-Pathway Components

Many components of the N pathway display dosage-sensitive interactions with each other. Indeed, many of the relatively few Drosophila genes that exhibit a haploinsufficient phenotype (including Delta (Dl), N, and Hairless (H)) function in the N pathway (Lindsley et al., 1972). However, neu does not generally display dosage-sensitive interactions with other neurogenic genes, in spite of its extreme neurogenic mutant phenotype. Consistent with this, neu was never isolated in a series of genetic modifier screens for components of the N pathway (Brand and Campos-Ortega, 1990; Go and Artavanis-Tsakonas, 1998; Verheyen et al., 1996). In fact, the general failure of neu to modify most other mutant phenotypes has made A101 a convenient SOP marker in a large number of studies conducted over the past decade, even though it is a lethal allele of neu. All of these observations suggest that neu levels are rarely limiting, even under genetic conditions that are compromised for N-pathway function. We therefore sought to obtain further evidence for genetic interactions between neu and members of the N pathway by testing for modification of neu misexpression phenotypes in various N-pathway mutant backgrounds.

We constructed recombinant chromosomes containing VMQ-Gal4 and UAS-neu, UAS-neuΔRING, or UAS-neu RING. In contrast to its behavior when misexpressed with bx(MS1096), we observed that VMQ-neuΔRING wings displayed a mild truncation of wing vein L5, similar to the phenotype produced with VMQ-neu (Figs. 7B and 7C). This suggests that, even though NeuΔRING typically acts as a neu antimorph, in certain circumstances it may exhibit gain-of-neu activity. Flies homozygous for this recombinant chromosome showed strong loss of wing margin and vein thickening (Fig. 7C, inset).

VMQ-neu RING displayed either no genetic interactions or only additive effects in combination with all N-pathway mutants tested, supporting the hypothesis that misexpression of this domain does not specifically affect N-pathway activity (data not shown). On the other hand, VMQ-neu and VMQ-neuΔRING displayed complex genetic interactions with many N-pathway mutants, with the two displaying similar effects in some cases and opposite effects in other cases. For example, VMQ-neu strongly enhanced the excess vein phenotype seen in heterozygotes of the amorphic allele D19P39 and of DpN (Figs. 7E and 7H, compare with Figs. 7D and 7G), whereas VMQ-neuΔRING displayed either no interaction or even mildly suppressed the phenotype of Dl and DpN (Figs. 7F and 7I). On the other hand, only VMQ-neuΔRING strongly enhanced the loss of wing margin in females heterozygous for the null allele N81K1 (Figs. 7J–7L). In other instances, the two transgenes acted similarly. For example, both enhance the vein loss seen with Ax1 (encoding a gain-of-function N receptor) or H431 (a mutated negative regulator of the N pathway) heterozygous flies (Figs. 7M–7R). That VMQ-neuΔRING behaved similarly to VMQ-neu by itself and in some genetic interaction tests supports the idea that the RING finger is not necessarily essential for Neu activity, even though this deleted form typically acts as an antimorph. These interaction data are in agreement with our proposition that Neu misexpression specifically modifies N-pathway signaling; however, the inconsistent direction of various interactions does not lead to any simple conclusions regarding epistasis.

**Neu Is Localized to the Plasma Membrane**

To assess the subcellular localization of Neu, we generated a version of Neu fused to five myc tags (myc-Neu). Tagged Neu proteins are biologically active and cause similar phenotypes to wild-type Neu when misexpressed during development of the PNS and wing veins (data not shown). This suggests that their localization is likely to reflect that of the native protein. When activated with...
FIG. 5. Ectopic Neu and truncated Neu proteins disrupt adult wing development. Genotypes of wings are indicated on the panels; the Gal4 driver is bx(MSI96). (A–C) Misexpression of Neu NHR2 has minimal effects on wing development. (D–F) Expression of Neu at lower levels leads to truncation of veins (D); vein length is restored by expression of higher levels of Neu, but veins are thickened (E, F). Inset in (F) shows a tuft of nonsensory bristles on the alula, indicating that development of nonsensory bristles on the posterior wing margin is regulated by lateral inhibition (see also Fig. 1K). (G–I) Misexpression of NeuΔRING leads to a strong serrated phenotype; wings in (G) and (H) show examples of weak and strong insertions of this construct. Note that even in severely affected wings (H, I), the thickness of the veins is not strongly affected (compare with E, F). (J, K) Misexpression of NeuΔNHR1 leads to a mild delta at the distal tips of veins (J, arrow); higher levels of this construct lead to stronger vein thickening (K, arrow) as well as smaller, spoon-shaped wings. (L) Misexpression of NeuΔRING leads to small, spoon-shaped wings in which the dorsal and ventral surfaces often fail to be opposed. (M–O) Closeups of a portion of the L3 wing vein that normally contains campaniform sensilla (M, arrowheads). Campaniform sensilla are often missing after misexpression of lower levels of Neu (N), but "tufts" of these sensilla are often present after misexpression of higher levels of Neu (O, arrowheads). (P) Misexpression of NeuΔRING leads to large numbers of campaniform sensilla all over L3 (arrowheads). (Q) Even in severely disrupted wings caused by Neu RING misexpression, relatively normal numbers of campaniform sensilla are present (arrowheads).
FIG. 6. Effect of neu gain- and loss-of-function on the expression of Cut (A–D, G, H) and Wingless (Wg; E, F, I, J) in third instar wing imaginal discs. (A–F) bx(MSL096)Y discs expressing the following transgenes: (A, E) none, (B, F) UAS-neuRING, (C) UAS-neu, (D) UAS-neu RING. (A) Aspects of Cut expression include wing margin (arrow) and sensory organ lineage (dorsal radius, arrowhead). (B) Misexpression of NeuRING abolishes margin Cut staining (arrow) but leads to ectopic sensory organ lineage staining (arrowhead). (C) Misexpression of wild-type Neuralized has little effect on margin Cut staining, although it may be somewhat increased; note decrease in the number of Cut-positive cells in the dorsal radius (arrowhead). (D) Cut expression is normal following misexpression of Neu RING. (E, F) Misexpression of NeuRING also leads to strong reduction of Wg expression at the wing margin (arrows), as well as in the analogous region of the haltere disc (insets). (G–J) neu<sup>res5</sup> clones (marked by the absence of GFP; outlined) that overlap the wing margin maintain normal levels of Cut (G, H, red) and Wg (I, J, red).
FIG. 7. Wing phenotypes produced by misexpression of Neu or NeuΔRING are modified by mutations in the N pathway. Recombinant flies carrying both vestigial margin quadrant enhancer-Gal4 (VMQ-Gal4) and either UAS-neu or UAS-neuΔRING were constructed and tested in the heterozygous backgrounds listed to the left of the figure. Wings from females are shown in all panels. (A) Wild-type wing. (B, C) Both VMQ-neu and VMQ-neuΔRING heterozygotes display some wing vein truncation (arrowheads), while VMQ-neu also displays a small amount of vein thickening (B, arrow). (C, inset) Flies homozygous for VMQ-neuΔRING display thickened veins and strong loss of wing margin. (D) Dl^{9P39} and (G) DpN heterozygous flies display extra vein material, particularly at the distal tips of veins; this phenotype is enhanced by VMQ-neu (E, H) but not by VMQ-neuΔRING (F, I). A large majority of VMQ-neu/+; Dl/+ wings carry a large blister. (J) N^{81K4} heterozygous flies have small notches at the distal portion of the wing; this phenotype is made more variable by VMQ-neu (K), with different individuals having more or less notching. (L) A strong increase in wing serration is observed in N/+; VMQ-neuΔRING flies. Both Ax^{M1} (M) and H^{E31} (P) heterozygous flies show a small amount of wing vein truncation, mostly at the tip of L5; this wing vein loss is enhanced by both VMQ-neu (N, Q) as well as VMQ-neuΔRING (O, R).
FIG. 8. Tagged Neu proteins are localized to the plasma membrane. (A–E) Expression of myc-Neu driven by neu-Gal4 or (G) sca-Gal4 and detected with α-myc antibody; (F) expression of membrane-localized GFP (eGFPF) driven by neu-Gal4. Approximate magnifications: (A) ×160, (B) ×1000, (C) ×2200, (D) ×800, (E) ×1600, (F) ×800, (G) ×600. (A) Expression of myc-Neu in wing (W), leg (L), and haltere (H) discs sensory organ precursors (SOPs). (B) Confocal optical cross section through the double row of SOPs at the wing margin; myc-Neu accumulates largely at the plasma membrane. (C) Confocal optical cross section through a single notum SOP shows myc-Neu in punctate structures at the plasma membrane. (D–F) Morphological features of SOPs. (D) Notum SOPs are roughly apically–basally oriented; focal plane is focused on the long arms that are extended toward the apical surface. These arms are oriented in a parallel manner and appear to end in a bulb. Maculae abbreviations: pDC, posterior dorsocentral; tr1, trichodeum sensilla1; pSA, posterior supra-alar; pNP, posterior notoplural; aNP, anterior notoplural. (E) Close view of an SOP in the dorsal radius, whose orientation is more parallel to the plane of the disc. Fine processes are associated with the long arm (arrowheads) and a short arm is seen at the opposite side of the cell body. (F) SOPs at the wing margin expressing eGFPF; cellular extensions and fine processes are observed (arrowhead). (F) Expression of myc-Neu with sca-Gal4 is also largely membrane-associated, but most cells do not appear to share the morphology of SOPs.
neu-Gal4, which drives gene expression predominantly in SOPs (Reddy et al., 1999), we found that myc-Neu was largely associated with the cell membrane in various locations of endogenous Neu function in imaginal discs (Figs. 8A–8C). Confocal microscopy further indicated that accumulation of myc-Neu is not uniform, but appears to localize to punctate structures (Fig. 8C). These experiments also revealed the unusual morphology to SOPs. Although imaginal disc cells are generally thought to have a roughly columnar morphology, it was previously reported that SOPs are widened basally and constricted apically (“bottle cells”) (Hartenstein and Hartenstein, 1997; Hartenstein et al., 1994). We find that notum SOPs labeled with myc-Neu have an elongated cell body with bipolar extensions, with a short arm projected basally and a much longer arm projected apically (Figs. 8D and 8E). Close examination of these arms revealed that they are associated with fine processes (Fig. 8E, arrowheads). We also examined the expression of membrane-localized GFP [eGFPF (Finley et al., 1998)] under control of neu-Gal4 and confirmed that the cellular extensions are not a consequence of Neu overexpression (Fig. 8F, arrowhead). The cellular projections and processes of SOPs we observe appear distinct from both cytonemes and peripodial cell projections based on their sizes (Cho et al., 2000; Gibson and Schubiger, 2000; Ramirez-Weber and Kornberg, 1999). We were unable at present to definitively assess whether these characteristics are specific to SOPs, since they may have been obscured when more general Gal4 drivers were used to express reporter genes. However, when sca-Gal4 was used to express myc-Neu, we did not readily detect the unique morphological features of SOPs in most stained cells, although localization to the plasma membrane remained evident (Fig. 8G). The biological significance of these unusual features of SOP morphology is under investigation.

DISCUSSION

neu Is Essential for a Subset of N-Dependent Processes during Larval Development

neu mutants have been studied extensively with respect to the patterning of many embryonic tissues and cell types, and neu has been found to be essential for virtually all N-dependent processes in the embryo. For example, it is required for lateral inhibition in all three germ layers and for inductive processes such as formation of the mesectoderm (Corbin et al., 1991; Hartenstein et al., 1992; Lehmann et al., 1983; Martin-Bermudo et al., 1995). We examined the role of neu in detail with respect to adult peripheral neurogenesis. During the preparatory stages of this study, similar results concerning the phenotypes of adult neu clones were reported (Yeh et al., 2000), including the tufting phenotype of neu^{A101} clones and a balding phenotype of neu^{IF65} clones. Our results agree with those of Yeh et al. (2000) and extend their observations in demonstrating a requirement for neu in both socket-shaft and in sheath-neuron cell fate choices. In fact, we showed neu to be required for all steps during PNS development that were previously shown to depend on N activity, including lateral inhibition in proneural clusters and three asymmetric cell divisions in the sensory lineage. Recently, we have observed a strict requirement for neu also during lateral inhibition of the R8 photoreceptor fate (in preparation).

In contrast, we find that neu is not required for certain other N-dependent processes in larval development, including formation of the wing margin and lateral inhibition during vein development. Since the neu enhancer trap A101 is active in vein cells, analogous to its expression in SOPs, one might have expected neu to function in both settings (Blair et al., 1992). However, there is precedent for such a discrepancy in the expression and apparent function of neurogenic genes. For example, deletions of the E(spl)-C fail to induce defects in wing margin integrity, despite the observations that multiple E(spl) genes are active along the wing margin by reporter or in situ analysis, and mutant clones of the E(spl)-C fail to activate Cut at the wing margin (de Celis et al., 1996; Lecourtois and Schweiguth, 1995; Ligoxygakis et al., 1999). It may be that there is an overlapping or redundant function of neu that operates in the restriction of vein fates. Although there is a single Neu orthologue in Drosophila, we identified two other Drosophila genes encoding NHR-domain proteins (E. C. Lai, unpublished observations). The functional relationship of these proteins to Neu, if any, remains to be determined.

An important conclusion of this work concerns the autonomy of neu function, at least with respect to lateral inhibition within proneural clusters. The report of Yeh et al. (2000) similarly concludes that neu functions autonomously; however, their work was based primarily on characterization of the hypomorphic allele neu^{A101}. We showed that the null allele neu^{IF65} similarly behaves autonomously both in adult phenotype as well as with respect to cell fate choices assayed during imaginal disc and pupal development. The autonomy of neu appears to be contradictory to the reported localization of neu transcript and enhancer trap activity to the SOP, a cell fate inhibited by N signaling (Boulianne et al., 1991; Huang et al., 1991; Yeh et al., 2000). A possible reconciliation is that very low levels of neu, below that of conventional means of detection, may be sufficient for lateral inhibition. A parallel situation may be found for N itself, as subdetectable levels of nuclear N^{IC} are sufficient for target gene activation (Schroeter et al., 1998). Upregulation of neu in the SOP might then be a consequence of its particular transcriptional regulation that might not actually reflect a function with respect to the SOP-epidermal fate decision. Alternatively, upregulation of neu in the SOP might be required for successive alternative cell fate decisions in the sensory lineage, which we showed to also depend on neu.

Models for Neu Function

The first point to consider is the role of the only previously identified protein domain in Neu, the RING finger.
Diverse functions have been ascribed to various RING fingers, including as DNA-, RNA-, and protein-interaction domains. However, recent reports concluded that RING fingers may function generally as E3 ubiquitin ligases (Joazeiro et al., 1999; Lorick et al., 1999). The effect of deleting the Neu RING finger may have ubiquitin ligase activity, since much higher levels of wild-type Neu are required to generate antimorphic phenotypes seen with low levels of NeuΔRING. A similar effect was observed when the C-terminal RING finger was deleted from the Drosophila inhibitor of apoptosis-1 (DIAP-1); the ability of truncated DIAP-1 to block cell death was strongly increased relative to the full-length protein (Hay et al., 1995). We hypothesize that the C-terminal RING finger of Neu may possess ubiquitin ligase activity that negatively regulates Neu by recruitment of the ubiquitination machinery. Biochemical experiments are under way to test this hypothesis, and to address whether Neu functions in the ubiquitination of other proteins as well.

We next consider the unusual behavior of the full-length Neu protein in overexpression assays. In contrast to the recent report of Yeh et al. (2000), we find that Neu overexpression produces both gain- and loss-of-function phenotypes. Lower levels of ectopic Neu result in loss of sensory organs and truncation of wing veins, while higher levels of Neu expression result in tufted sensory organs and wing vein thickening. The former phenotypes are caused by over-activation of the Notch pathway and are opposite to the phenotype of neu clones in PNS development, while the latter phenotypes resemble a failure of Notch-pathway activity and are similar to the phenotype of neu clones in PNS development.

The ability of Neu to induce both gain- and loss-of-function phenotypes when overexpressed is most consistent with a model in which Neu functions as part of a multiprotein complex. Under conditions of elevated expression, the formation of the active complex may be encouraged, resulting in a gain-of-function phenotype. However, under conditions of highly elevated expression, components of the complex are titrated into inactive minicomplexes, causing a loss-of-function phenotype. This progression of causing gain-of-function phenotypes at lower levels and loss-of-function phenotypes at higher levels is indeed what we observed with Neu misexpression.

Although we favor a model in which the Neu RING finger may have ubiquitin ligase activity, there is ample precedent for RING fingers to function as protein–protein interaction domains (Meza et al., 1999; Oeda et al., 1998; Tanimura et al., 1999); indeed, the two functions need not necessarily be exclusive. In addition, both the complexity of the NHR domain and its rarity in the Drosophila proteome also make it an excellent candidate to mediate specific protein–protein interactions. Thus, the domain structure of Neu gives us further reason to hypothesize that, in accord with its behavior in overexpression assays in vivo, Neu may function as part of a multiprotein complex. Indeed, deletion of the RING finger domain resulted in a protein (NeuΔRING) with potent dominant-negative activity. We note that misexpression of the single-NHR derivatives Neu-NHR1, Neu-NHR2, and NeuΔNHR1, caused either a mild or no phenotype with respect to N-regulated cell fate decisions, though our model might predict they should have dominant negative activity similar to that of NeuΔRING. It is possible that two NHR domains are required to interact with the appropriate target in the N pathway, although it is also possible that these single-NHR proteins are either unstable or inappropriately localized in vivo.

The final points to consider in models of Neu function are its apparent localization to the plasma membrane and its cell-autonomous function, at least with respect to the adoption of the SOP fate; these conclusions are in general agreement with the report by Yeh et al. (2000). Both of these are characteristics of the N receptor as well. N is epistatic to neu, as a duplication of the N locus alleviates the neu null phenotype and overexpression of constitutively activated N bypasses the requirement of neu (de la Concha et al., 1988; Lieber et al., 1993). We note that our placement of Neu at the plasma membrane is based on misexpression of tagged proteins, and thus awaits verification with antibodies specific to Neu. However, a reasonable model that incorporates these observations is that Neu functions in a multiprotein complex that is somehow involved in the activation of the N receptor at the cell membrane. Recent observations that cis-interactions between DI and N may be important in regulating the ability of a cell to send and respond to DI signals suggest a further possibility that Neu may modulate DI–N interactions within the same cell (Jacobsen et al., 1998). Current efforts are aimed at identifying Neu-interacting proteins, which we hope may make evident the molecular function of Neu in the N pathway.

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REFERENCES


genes in response to Notch receptor activity. Genes Dev. 9, 2609–2622.


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