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Requirement for Dynamin during Notch Signaling in *Drosophila* Neurogenesis

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Singling out of a unique neural precursor from a group of equivalent cells, during *Drosophila* neurogenesis, involves Notch-mediated lateral signaling. During this process, activation of the Notch signaling pathway leads to repression of neural development. Disruption of this signaling pathway results in the development of an excess of neural cells. The loss of activity of dynamin, which is encoded by the gene *shibire* and is required for endocytosis, results in a similar phenotype. Here we have investigated the requirement of *shibire* function for Notch signaling during the segregation of sensory bristles on the notum of the fly. Overexpression of different constitutively active forms of Notch in *shibire* mutant flies indicates that *shibire* function is not necessary for transduction of the signal downstream of Notch, even when the receptor is integrated in the plasma membrane. However, when wild-type Notch is activated by its ligand Delta, dynamin is required in both signaling and receiving cells for normal singling out of precursors. This suggests an active role of the signaling cell for ligand-mediated receptor endocytosis in the case of transmembrane ligands. We discuss the possible implications of these results for normal functioning of Notch-mediated lateral signaling.

INTRODUCTION

Membrane receptors, as well as most cell surface proteins, are passively recycled or actively eliminated by endocytosis (Watts and Marsh, 1992; Schmid, 1992; Robinson, 1994). This process plays an important role in the regulation of signal transduction in the course of time by modulating the number of receptors available for the reception of the signal and, consequently, the capacity of the cell to receive and transduce it. For example, the study of a noninternalizing epidermal growth factor receptor has shown that endocytosis could be required to abrogate the long-term actions of this receptor (Wells *et al.*, 1989; Vieira *et al.*, 1996). The importance of endocytosis has been intensively investigated for tyrosine kinase receptors, but little is known about the role it plays in other signal transduction pathways such as the Notch pathway (Baass *et al.*, 1995).

Unlike many other ligands which are proteolytically cleaved, Delta, the ligand for Notch, is a transmembrane protein. Nevertheless, it has been shown that Delta–Notch complexes are internalized: they can be found in multivesicular bodies, apparent endocytotic vesicles, inside the cells (Fehon *et al.*, 1991; Kooh *et al.*, 1993; Parks *et al.*, 1995). Endocytosis of the Notch receptor has been observed in cultured cells and *in vivo* during the period in which it is required for cell fate determinations in *Drosophila* (Kooh *et al.*, 1993; Fehon *et al.*, 1990, 1991). Endocytosis appears to be correlated with activation of the receptor for it seems to follow the binding of Delta to Notch, in cell cultures (Fehon *et al.*, 1990). Similarly, during cell–cell interactions mediated by GLP-1 (a Notch homologue) in the gonad of the nematode *Caenorhabditis elegans*, a chimeric LAG-2 protein, a *C. elegans* Delta homologue, is found to be internalized into receiving cells which express GLP-1 and not LAG-2, suggesting here too that activation of the receptor is followed by the internalization of the ligand–receptor complex (Henderson *et al.*, 1994). Endocytosis of ligand–receptor complexes involving membrane-bound ligands is not restricted to the Delta–Notch family: it has also been observed for another membrane-bound ligand, Bride of sevenless, that is also internalized along with its receptor Sevenless (Krämer *et al.*, 1991).

One way to study the importance of endocytosis for cell interactions during development *in vivo* is afforded by the *shibire* mutant of *Drosophila*. *shibire* encodes a *Drosophila* homologue of dynamin, a protein with a GTPase activity required to pinch off the endocytic vesicles (Van der Bliek and Meyerowitz, 1991; Chen *et al.*, 1991). Ultrastructural studies in *shibire* temperature-sensitive mutant tissues strongly indicate that dynamin is specifically required for

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the process of endocytosis. Indeed, the coated pits form normally in the mutant but the vesicles fail to pinch off from the cell surface (Poodry and Edgar, 1979; Kosaka and Ikeda, 1983). The specificity of dynamin action has also been confirmed in mammalian cells by overexpression of dominant negative forms of the molecule. In this case too, receptor-mediated endocytosis was disrupted, whereas all other vesicular traffic continued normally (Van der Bliek *et al.*, 1993; Herskovitz *et al.*, 1993).

Thus, the *shibire* mutant allows us to analyze *in vivo* the effect of a lack of endocytosis on Notch signaling. In *Drosophila*, the general inhibition of endocytosis caused by *shibire* mutants results in a phenocopy of the mutant phenotype in the nervous system observed when the Notch signaling pathway is disrupted (Poodry, 1990; Ramaswami *et al.*, 1993; Poodry *et al.*, 1973; Lujan, 1981). This is in contrast to other signaling pathways, such as that of Wingless, which are not disrupted in the *shibire* mutant (Bejsovec and Wieschaus, 1994).

Here we have investigated the basis of the apparent specific requirement for dynamin and endocytosis for Notch signaling. We studied the selection of the precursors of sensory bristles on the notum. In *Drosophila*, determination of the precursors occurs in two steps (Simpson, 1990). First, a group of cells acquires the potential to become neural by the expression of the proneural genes. These cells constitute an equivalence group and each of them expresses both the ligand, Delta, and the receptor, Notch. Within the group, neural potential is progressively restricted to a single cell by Notch-mediated cell–cell interactions. This cell continues to produce the signal and becomes the precursor. Activation of Notch in the other cells leads to a cessation of proneural expression and differentiation into epidermis. This process is called lateral inhibition (for review see Artavanis-Tsakonas *et al.*, 1995). In the absence of either Delta or Notch, signaling is abolished and all the cells of the group become bristle precursors; this is known as a ''neurogenic phenotype.'' Conversely, constitutive activation of the pathway by dominant activated forms of Notch causes all cells to develop as epidermis (Rebay *et al.*, 1993; Struhl *et al.*, 1993; Lieber *et al.*, 1993).

Here we show that dynamin is not required downstream of activated Notch receptors for signal transduction, even when the activated receptor is anchored in the plasma membrane. However, when the wild-type receptor is activated by binding to Delta, *shibire* is required autonomously in the receiving cells and also, to a lesser extent, in the signaling cells for precursor selection to take place normally. We suggest that ligand-mediated receptor endocytosis involves an active participation of the signaling cell when the ligand is membrane-bound. We further postulate that removal of the ligand–receptor complexes is necessary to allow signaling to continue in order to maintain cell fate choices for some time. This may be linked to particular features of Notch activation, such as a possible cleavage of the intracellular domain of Notch itself, which would render the receptors inactive. Endocytosis would then be required to remove inactive complexes in order to allow formation of new functional receptor complexes.

MATERIALS AND METHODS

Fly Strains and shibire Mutant Conditions

The temperature-sensitive alleles of *shibire*, shi^{ts1} and shi^{ts3}, were made by Grigliatti in a screen for immobile adults (Grigliatti *et al.*, 1973). shi^{ts1} and shi^{ts3} are EMS-induced point mutations, causing the replacement of glycine[268] by an aspartate residue in the GTPase domain of the protein (Van der Bliek and Meyerowitz, 1991; Garcia, 1994). *shibire* flies were raised at 22°C. White pupae were collected individually at 0 hr after puparium formation (APF) and shifted to 29°C at 14 hr APF for 6 hr. The animals were then shifted back to 22°C until the end of development or used to perform β-galactosidase enzyme staining. $Delta^{revF10}$ (Haenlin *et al.*, 1990) and $Delta^{RF}$ (Parody and Muskavitch, 1993) were used as a *Delta* temperature-sensitive allele combination (Dl^{ts}). The Dl^{ts} white pupae were treated as for *shibire* and shifted to 33°C instead of 29°C during the same time window.

β-Galactosidase Staining

The anterior and posterior extremities of pupae were cut in a 0.5% glutaraldehyde/0.1% Triton PBS solution. The tissues were then fixed for 15 min in this solution and washed in 0.1% Triton X-100/PBS. Thoraxes were then dissected, washed again for 15 min in 0.1% Triton X-100, and then stained overnight at 37°C in a 3 m*M* $K_4[FeII(CN)_6]3H_2O$, 3 m*M* $K_3[FeIII(CN)_6]$, 0.1% Triton X-100, 0.2% XGal PBS solution. They were then mounted between coverslips in 50% glycerol/PBS.

UAS Constructs

Three different UAS constructs were used in this study: UAS ΔNLS2, UAS $Torso^{4021}$–Notch, and UAS Notch. All coding sequences were inserted into the pUAST vector (Brand and Perrimon, 1993). The wild-type Notch sequence used in all constructs comes from an *Xba*I fragment containing the last three introns of the *Notch* gene and the 5′ and 3′ untranslated region of the mRNA (Kidd *et al.*, 1986). The ΔNLS2 construct results from a digest by *Aat*II and religation, which removes amino acids between Arg^{8} and Arg^{1812}. This is followed by a digest by *Sfi*I and *Sac*II, repair, and religation: a frameshift is induced which adds five new residues (Gly, Leu, Ser, Ser, Ileu) after Lys^{2199}. ΔNLS2 contains the cdc10/ANK repeats of the intracellular domain of Notch but lacks the C terminal region including the second nuclear localization signal and the PEST motif. Expression of this molecule results in gain of function phenotypes in the imaginal discs. It is less effective than the full-length intracellular domain (Struhl *et al.*, 1993; Rebay *et al.*, 1993; Lieber *et al.*, 1993), but permits embryonic survival. The $Torso^{4021}$–Notch construct contains the extracellular and transmembrane domain of the receptor encoded by the tor^{4021} mutant allele of *torso* (Sprenger and Nüsslein, 1992; Dickson *et al.*,1992) and the intracellular domain of Notch. The fusion between *N* and tor^{4021} was constructed by PCR, creating an *Eco*RI site between the two fragments. The fusion site is $Cys^{420(torso)}$ $Arg^{(new)}$ $Ileu^{(new)}$ $Gln^{1787(Notch)}$. The $Torso^{4021}$–Notch construct is inserted as an *Xba*I (3′ end of *N*) *Kpn*I (start of *tor* coding sequence) fragment in pUAST

and does not carry the 5′ untranslated region of the *N* mRNA. Constructs were transformed into flies by standard methods.

Generation of Mitotic Clones in the Adult Thorax

Mutant clones were produced by mitotic recombination induced by X-irradiation or by the FRT/FLP method (Golic and Lindquist, 1989; Golic, 1991; Xu and Rubin, 1993). We did not analyze a complete loss-of-function allele, because *shibire* also affects cell fate decisions later in the differentiation of the bristles and might affect the ability of cells to produce cuticular structures that can be analyzed (Hartenstein and Posakony, 1990; Poodry *et al.*, 1973; Lindsley and Zimm, 1992). Twenty-four-hour egg collections were made and heat-shocked (1 hr, 37°C) between 24 and 48 hr after egg laying. White pupae were then collected and shifted as previously described. As the neurogenic phenotype induced in the *shibire* mutant clones was as strong as in *shibire* homozygous flies, we concluded that the perdurance of the wild-type product in the mutant cells plays a negligible role at the time we performed the temperature shift. Clones were marked with *multiple wing hair (mwh)*, which labels epidermal cells, and *forked* (f^{36a}), which labels the bristles, and were induced in flies of the following genotypes:

(1) shi^{ts1} f^{36a} FRT^{19A}/Dp(3;Y;1)M2 FRT^{19A}, emc^{+}, mwh^{+}, y v; emc^{1} mwh/mwh;
(2) shi^{ts3} f^{36a} FRT^{19A}/Dp(3;Y;1)M2 FRT^{19A}, emc^{+}, mwh^{+}, y v; emc^{1} mwh/mwh;
(3) Ax^{59b} shi^{ts1} f^{36a} FRT^{19A}/Dp(3;Y;1)M2 FRT^{19A}, emc^{+}, mwh^{+}, y v; emc^{1} mwh/mwh;
(4) Ax^{59b} f^{36a} FRT^{19A}/Dp(3;Y;1)M2 FRT^{19A}, emc^{+}, mwh^{+}, y v; emc^{1} mwh/mwh;
(5) N^{ts1} shi^{ts1} f^{36a} FRT^{19A}/Dp(3;Y;1)M2 FRT^{19A}, emc^{+}, mwh^{+}, y v; emc^{1} mwh/mwh;
(6) N^{ts1} f^{36a}/Dp(3;Y;1)M2, mwh^{+}, y Co; mwh/mwh;
(7) f^{36a} FRT^{19A}/Dp(3;Y;1)M2 FRT^{19A}, emc^{+}, mwh^{+}, y v; emc^{1} mwh/mwh.

Thoraxes were mounted between coverslips in canada balsam.

RESULTS

The shibire Mutant Phenotype

The notum of *Drosophila* is covered with small mechanosensory bristles, called microchaetes, that are regularly spaced (Fig. 1, IA). Spacing of these bristles depends upon the Notch signaling pathway and in the absence of any component of this pathway a great number of additional, adjacent microchaetes are formed at the expense of epidermal tissue (Heitzler and Simpson, 1991; Hartenstein and Posakony, 1990; Parody and Muskavitch, 1993; Heitzler *et al.*, 1996). We analyzed the effects of the *shibire* mutant on the determination and spacing of the microchaete precursors. We employed two previously characterized temperature-sensitive alleles of *shibire*, shi^{ts1} and shi^{ts3}, that allow the study of *shibire* function at different developmental stages (Poodry *et al.*, 1973; Lindsley and Zimm, 1992; see Materials and Methods).

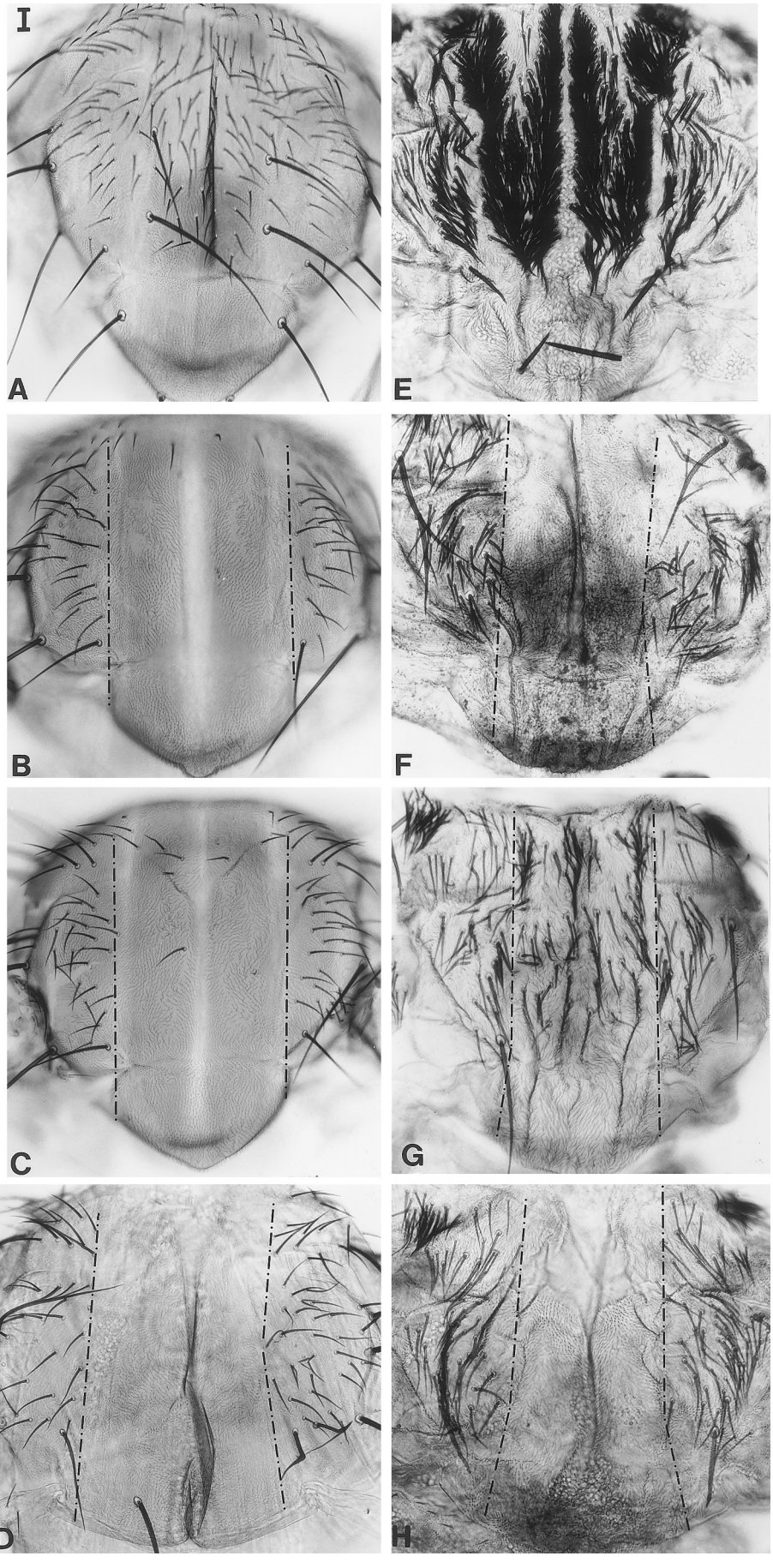
Mutant flies were shifted to the nonpermissive temperature (29°C) at the time of precursor determination: 14–20 hr APF. The mutants differentiated a large number of extra microchaetes, most of which could be seen to be adjacent to one another (Fig. 1, IE). This phenotype is indistinguishable from that of hypomorphic, temperature-sensitive alleles of *Notch* and *Delta*, N^{ts1} or Dl^{RF}/Dl^{revF10} (Hartenstein and Posakony, 1990; Lujan, 1981; Seugnet, 1997). In all three cases, the phenotype is more severe in medial than in lateral areas of the thorax.

We also examined the development of the microchaete precursors in *shibire* mutants using the A1O1 marker, which is specifically expressed in all bristle precursors (Huang *et al.*, 1991). In the most medial (dorsal) part of the thorax, a great number of supernumerary, adjacent neural precursors can be seen (Fig. 1, IIE). The phenotype is less severe in the lateral parts of the notum. Therefore, the *shibire* mutant phenotype results from the development of an excess of neural precursors at the expense of epidermal cells. The same alleles and temperature regime were used for all of our experiments and the phenotype will be referred to as the ''*shibire* neurogenic phenotype.''

Activated Notch Receptor Is Epistatic over shibire

In principle a neurogenic phenotype can result from a failure of Notch signaling at almost any step in the pathway: upstream of the receptor, ligand–receptor interactions, or at any step in signal transduction. Is the neurogenic phenotype of *shibire* due to a blockage of the intracellular events that occur after activation of the receptor? To test this, we generated cells doubly mutant for *shibire* and constitutively active Notch receptors. The latter display a phenotype opposite to that of the loss of *Notch* function, which is a loss of sensory bristles. We looked at the consequences of overexpression of three different forms of Notch, two activated

FIG. 1. Imaginal phenotypes resulting from expression of Notch constructs during determination of thoracic microchaete precursors in wild-type and in shi^{ts1} mutant backgrounds. Expression of the UAS constructs in the central domain of the thorax (outlined by a black line on the photographs) was obtained using the Gal^{md237} driver. See Materials and Methods for complete genotypes. Thoraces are shown of adult (I) and pupal flies at 20 hr APF when neural precursors have just appeared (II: A101 staining; note that at this stage some of the neural precursors have already divided). IA and IIA are wild type. IE and IIE are *shibire* (shi^{ts1}); note the excess of precursors and bristles. IB, IF, IIB, and IIF show the effects of expression of ΔNLS2. Note that this construct causes a similar loss of precursors and bristles in the wild-type and the mutant backgrounds. IC, IG, IIC, and IIG show expression of $Torso^{4021}$–Notch. This strongly reduces the bristle hyperplasia seen in the *shibire* mutant. However, the phenotype obtained in the mutant background (IG and IIG) is less extreme than that seen in the wild type where some bristles still form (IC and IIC, see text). ID, IH, IID, and IIH show the effects of overexpression of wild-type Notch molecules: a complete loss of precursors and bristles in both the wild-type (ID and IID) and mutant backgrounds (IH and IIH).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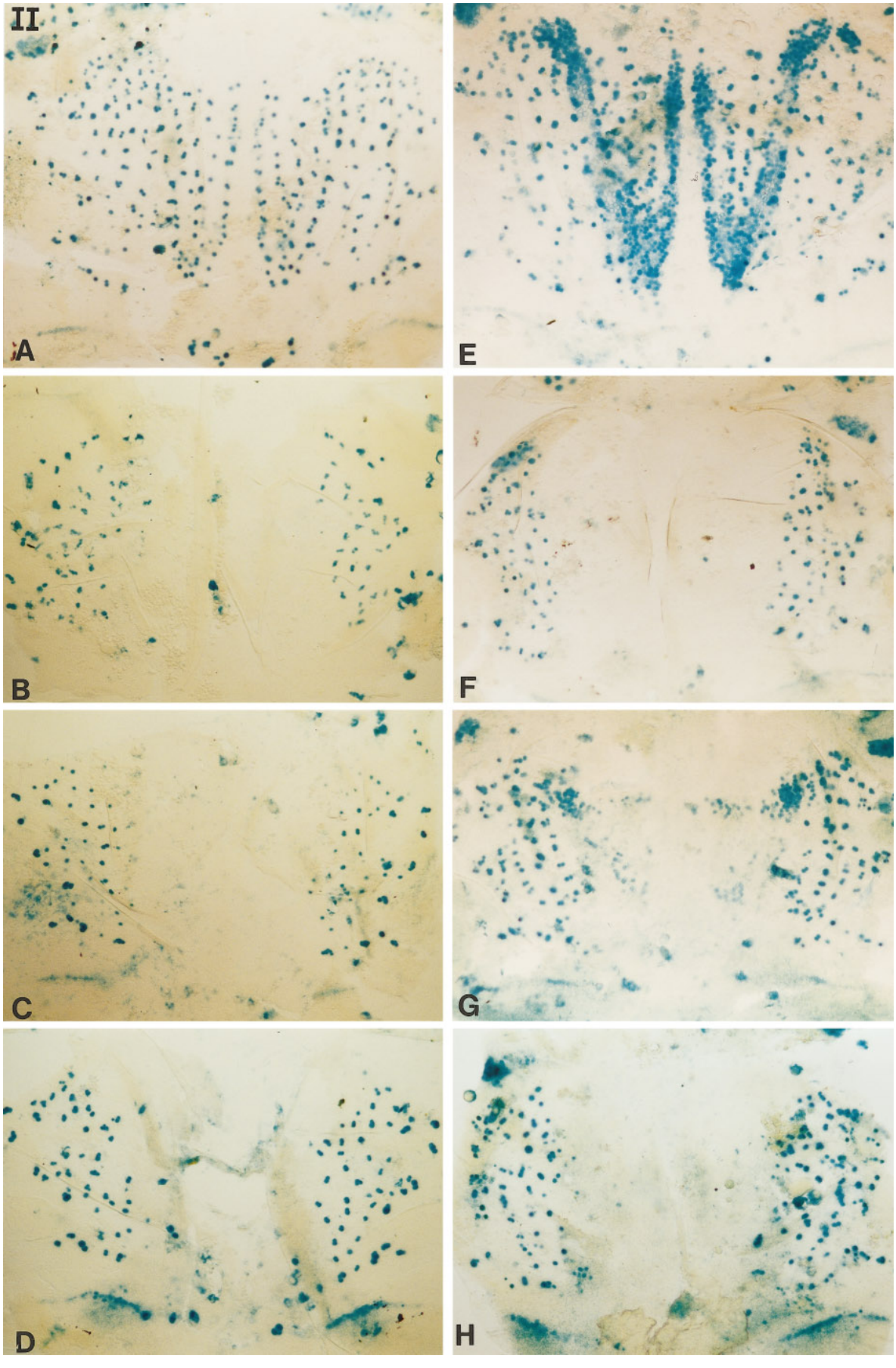
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forms, ΔNLS2 and Torso^{4021}–Notch, and a wild-type form (see Materials and Methods). ΔNLS2 is a truncated form carrying only part of the intracellular domain of Notch, but its expression causes a loss of bristles similar to that seen with the previously characterized full-length intracellular domain (Struhl *et al.*, 1993; Seugnet, 1997). The Torso^{4021}–Notch chimeric molecule behaves as a constitutively active, ligand-independent receptor presumably due to dimerization of the mutant extracellular domain of Torso (Sprenger and Nüsslein, 1992; Dickson *et al.*, 1992; Seugnet, 1997). Unlike ΔNLS2, but like the wild-type full-length receptor, this protein is probably inserted in the plasma membrane and thus might behave differently from ΔNLS2 with respect to the *shibire* mutant background.

We have used the Gal-4 UAS system (Brand and Perrimon, 1993) to express the two mutant constructs, as well as the wild-type protein, in a large area of the thorax. The *Gal*^{md237} driver is expressed specifically in a broad central band along the dorsal midline of the notum; there is no expression on the lateral half of the notum (Calleja *et al.*, 1996; Fig. 1). Expression of ΔNLS2 (Figs. 1, IB; 1, IIB; 1, IF; and 1, IIF) or Torso^{4021}–Notch (Figs. 1, IC; 1, IIC; 1, IG; and 1, IIG) suppresses formation of bristle precursors in both wild-type and *shibire* mutant backgrounds. The phenotype due to expression of ΔNLS2 is the same in *shibire* and *shi*$^{+}$ flies. The bristle loss due to Torso^{4021}–Notch is not as strong in the *shibire* background as in the wild-type background, but Torso^{4021}–Notch does not completely suppress the mutant phenotype of *Notch* loss of function mutants either, unlike ΔNLS2. Nevertheless, Torso^{4021}–Notch does very significantly reduce the neurogenic phenotype of *shibire* and so it can therefore activate the Notch pathway in the *shibire* mutant background. Similar results have been obtained with *Gal*$^{410.2}$ (Hinz and Campos-Ortega, unpublished) which is ubiquitously expressed at the time of precursor formation (not shown).

The full-length wild-type Notch receptor can also cause a loss of bristles when it is overexpressed to sufficiently high levels during determination of the bristle precursors. This effect is dominant and is completely epistatic over the loss of function of *Delta* (Seugnet, 1977). When overexpressed with *Gal*^{md237} or *Gal*$^{410.2}$, wild-type Notch suppresses development of the neural precursors to the same extent as ΔNLS2 in the *shibire* mutant background (Figs. 1, ID; 1, IH; 1, IID; and 1, IIH).

Together these results suggest that *shibire* is not required downstream of Notch for signal transduction, even when receptor activation occurs at the cell surface, as with overexpressed wild-type protein or as expected with Torso^{4021}–Notch. It may therefore be required upstream of the receptor or at the time of receptor activation by Delta.

shibire Is Epistatic over the Abruptex Gain of Function Alleles of Notch

Abruptex^{59b} (N^{Ax}) encodes a dominant gain of function form of Notch resulting from a point mutation in the extracellular domain; it prevents the development of most bristle precursors (Kelley *et al.*, 1987; Heitzler and Simpson, 1993). However, unlike the dominant activated forms of Notch, N^{Ax} remains dependent on activation by Delta (Heitzler and Simpson, 1993). Thus, in double-mutant flies loss of function alleles of *Delta* are epistatic over N^{Ax}, and, furthermore, the phenotype of N^{Ax} is modulated by the dosage of Dl^{+}. In contrast, the phenotype of bristle loss due to overexpression of wild-type Notch is completely epistatic over *Delta* mutants (Seugnet, 1997). Therefore, unlike ΔNLS2, Torso^{4021}–Notch, and full-length wild-type Notch, the hyperactive N^{Ax} molecules are both membrane bound and ligand dependent. We tested whether *shibire* is required for receptor activation in the case of N^{Ax} by making clones of cells doubly mutant for N^{Ax} and shi^{ts1}. At restrictive temperature such clones present a strong neurogenic phenotype similar to *shibire* itself (Fig. 2 and Table 1). At permissive temperature they are completely devoid of bristles. Thus, shi^{ts1} is epistatic over N^{Ax} and shi^{+} is therefore required for Notch signaling in N^{Ax} mutants.

Together these results indicate that *shibire* is required either at the cell surface at the time of activation of the receptor by its ligand or for the presentation of the ligand.

shibire Mutant Cells Are Defective in Both Sending and Receiving the Lateral Signal

The selection of sensory organ precursors relies on Notch-mediated lateral signaling. Initially all cells have the potential to become the precursor and express both Notch and Delta (Fehon *et al.*, 1991; Kooh *et al.*, 1993). With time this resolves to a single signaling cell which will become the precursor (Ghysen *et al.*, 1993). Mosaic analysis allows the assignment of gene function to either the signaling cell or the receiving cells (Heitzler and Simpson, 1991). Earlier results showed that *Delta* mutant cells adopt the epidermal fate when juxtaposed to wild-type cells. *Delta* is therefore not required for signal reception. In contrast, *Notch* is required for signal reception, and the mutant cells always become bristle precursors even when adjacent to wild-type signaling cells. In double-mutant *Notch Delta* clones, lacking both signal and receptor, both mutant and wild-type bristles form along the mosaic border and can be adjacent to one another (Heitzler and Simpson, 1993).

To see whether *shibire* is required in signaling or receiving cells, or both, the borders of clones of cells mutant for *shibire* were analyzed. Clones of shi^{ts1} and shi^{ts3} generated during the first and second larval stages present a strong neurogenic phenotype and bristles along the border can be either wild type or mutant (Fig. 2). Occasionally, a mutant bristle can be found adjacent to a wild-type one (Table 1, Fig. 3B) a situation that is not observed in clones that are mutant for either N^{ts} or *Dl* alone (Heitzler and Simpson, 1991). These results suggest that in the absence of *shibire* both the sending of the signal and its reception are disrupted.

The Effects of shibire Are Autonomous to the Signaling Cell

Mutant *Notch* cells, devoid of receptor, can influence the fate of adjacent wild-type cells, causing them to consis-

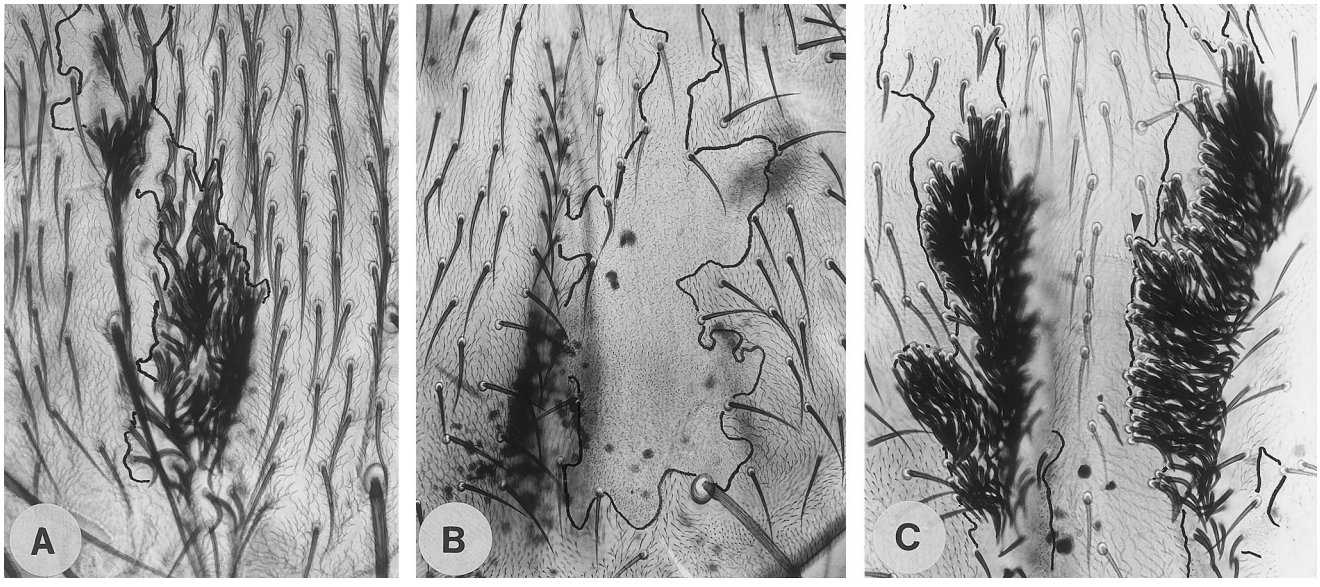

FIG. 2. Phenotypes of double-mutant N^{Ax} shi^{ts1} flies. Clones of mutant cells were generated during the larval stages. They are marked with f^{36A} for the bristles and *mwh* for the epidermal trichomes (see Materials and Methods for details and complete genotypes). The clones shown are located in the central, dorsal-most part of the thorax. The mosaic border is indicated by a black line. shi^{ts1} clones (A) show a strong neurogenic phenotype of an excess of bristles, whereas N^{Ax} clones (B) are devoid of bristles. The double-mutant N^{Ax} shi^{ts1} clones (C) display a neurogenic phenotype similar to that of shi^{ts1} alone. Occasionally, a wild-type bristle is found adjacent to a mutant one (arrowhead).

tently take up the epidermal fate. This suggests that *Notch* mutant cells constitutively send the inhibitory signal Delta (Heitzler and Simpson, 1991, 1993). Thus, along the borders of *Notch* mutant clones no wild-type bristles form. Signaling is therefore unidirectional in this special experimental situation which is therefore formally the equivalent of inductive signaling where one cell produces the ligand and the other the receptor (Greenwald and Rubin, 1991; Fig. 4B). To test whether *shibire* is autonomously required in the signaling cell we made clones of cells mutant for both *Notch* and *shibire*. N^{ts1} encodes a *Notch* protein containing a missense mutation in the extracellular domain which reduces

TABLE 1

Frequency with Which Mutant Bristles Are Found to Be Adjacent to Wild-Type Epidermal Hairs along the Borders of Mutant Clones

Mutant[a]	Phenotype	Mutant bristle adjacent to wild-type hairs (%)	Wild-type bristle adjacent to mutant hairs (%)	Number of mutant bristles adjacent to a wild-type bristle	Number of bristles scored
(1) shi^{ts1} (29°C)	Neurogenic	80	20	3	188
(1) shi^{ts1} (22°C)	Wild-type	54	46	0	166
(2) shi^{ts3} (29°C)	Neurogenic	81	19	1	152
(2) shi^{ts3} (22°C)	Wild-type	65	35	0	148
(3) Ax^{59b} shi^{ts1} (29°C)	Neurogenic	86	14	7	243
(3) Ax^{59b} shi^{ts1} (22°C)	Antineurogenic[b]	0	100	0	98
(4) Ax^{59b} (29°C)	Antineurogenic[b]	0	100	0	38
(5) N^{ts1} shi^{ts1} (29°C)	Neurogenic	93	7	2	118
(5) N^{ts1} shi^{ts1} (22°C)	Wild-type	77	23	0	111
(6) N^{ts1} (29°C)	Neurogenic	100	0[c]	0	159
(7) Wild-type (29°C)	Wild-type	53	47	0	169

[a] The flies were shifted on 29°C at 14–20 hr APF.

[b] Naked clones, with one or two microchaetes in some rare cases.

[c] Two wild-type bristles were present on the border but both were macrochaetes. The 2% wild-type bristles found at the border by Heitzler and Simpson (1991) were exclusively macrochaetes, the precise positioning of which depends on other factors.

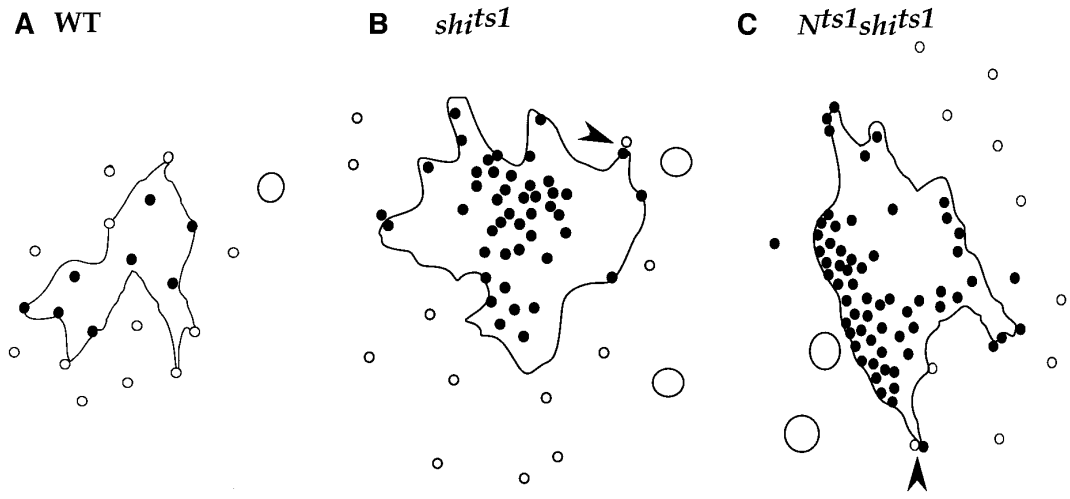

FIG. 3. Drawings of clones mutant for shi^{ts1} and N^{ts1} shi^{ts1}. Camera lucida drawings of wild-type (A), shi^{ts1} (B), and double-mutant N^{ts1} shi^{ts1} (C) clones. See Materials and Methods for complete genotypes. Black circles, mutant bristles; open circles, wild-type bristles. (A) In wild-type clones bristles are regularly spaced and at the border of the clone, while marked as well as nonmarked bristles form with equal frequency. In both shi^{ts1} (B) and N^{ts1} shi^{ts1} (C) wild-type as well as mutant bristles differentiate at the mosaic border and occasionally wild-type and mutant bristles are found adjacent to one another (arrowheads), a phenotype similar to that of N^{ts1} $Delta^{9P39}$ clones (Heitzler and Simpson, 1993).

its function at nonpermissive temperatures (Xu *et al.*, 1992). N^{ts1} clones present a strong neurogenic phenotype and, when only microchaetes are taken into consideration, all the bristles at the border are mutant (Table 1). N^{ts1} shi^{ts1} clones also present a strong neurogenic phenotype but, in contrast to N^{ts1} clones, in this case 7% of the bristles along the mosaic border are wild type. Thus, although it is not abolished, the inhibitory signal from the mutant *Notch* cells is impaired by the loss of shi^+ activity. Furthermore, like shi^{ts} clones, occasional cases of adjacent mutant and wild-type bristles are seen. Although this is rare, it should be noted that such cases have never been observed in the case of N^{ts} clones alone (Table 1, Fig. 3C).

Taken together, these observations show that the one-way constitutive inhibitory signal attributable to cells with little or no receptor is impaired in the absence of *shibire* and suggest that *shibire* is active autonomously in the signaling cell.

The Effects of shibire Are Autonomous to the Receiving Cell

Clones of cells mutant for N^{Ax} always differentiate as epidermis when adjacent to wild-type cells. Thus, all bristles along the border are wild type and no mutant bristles form (Table 1). This is therefore opposite to the effects of loss of function of *Notch*. In the N^{Ax} case too, signaling is unidirectional, only here the wild-type cells send the signal and the mutant cells receive it (Fig. 4C). Consequently, this is also formally equivalent to inductive signaling where one cell produces the ligand and the other the receptor. To test whether *shibire* is autonomously required in the receiving cell we made clones of cells mutant for both N^{Ax} and *shibire*. The borders of N^{Ax} shi^{ts1} clones bear 86% mutant bristles, in contrast to N^{Ax} clones which have none (Fig. 2C, Table 1). Therefore, the mutant N^{Ax} cells, which are always inhibited by their wild-type neighbors, lose the ability to receive the inhibitory signal when *shibire* is absent. In this case too, mutant and wild-type bristles can be adjacent (Table 1). We conclude that *shibire* is required autonomously in the receiving cell.

DISCUSSION

Endocytosis Is Not Required Downstream of Activated Notch for Signal Transduction in the Receiving Cell

We have used mutants of the gene *shibire* that encodes *Drosophila* dynamin in order to study the requirement for endocytosis during Notch-mediated cell signaling. *shibire* mutants display a neurogenic phenotype of the imaginal peripheral nervous system that is indistinguishable from that of *Notch* or *Delta* mutants. Thus, endocytosis is somehow required for the cell–cell interactions needed for the selection of spaced neural precursors. Dominant activated, truncated forms of Notch bearing only the intracellular domain are epistatic over *shibire*, however, showing that endocytosis is not required for signal transduction downstream of activated Notch. This conclusion is not unexpected since the truncated protein is not expected to be inserted in the cell membrane (Lieber *et al.* 1993; Rebay *et al.*, 1993; Struhl *et al.*, 1993). A presumed membrane-bound

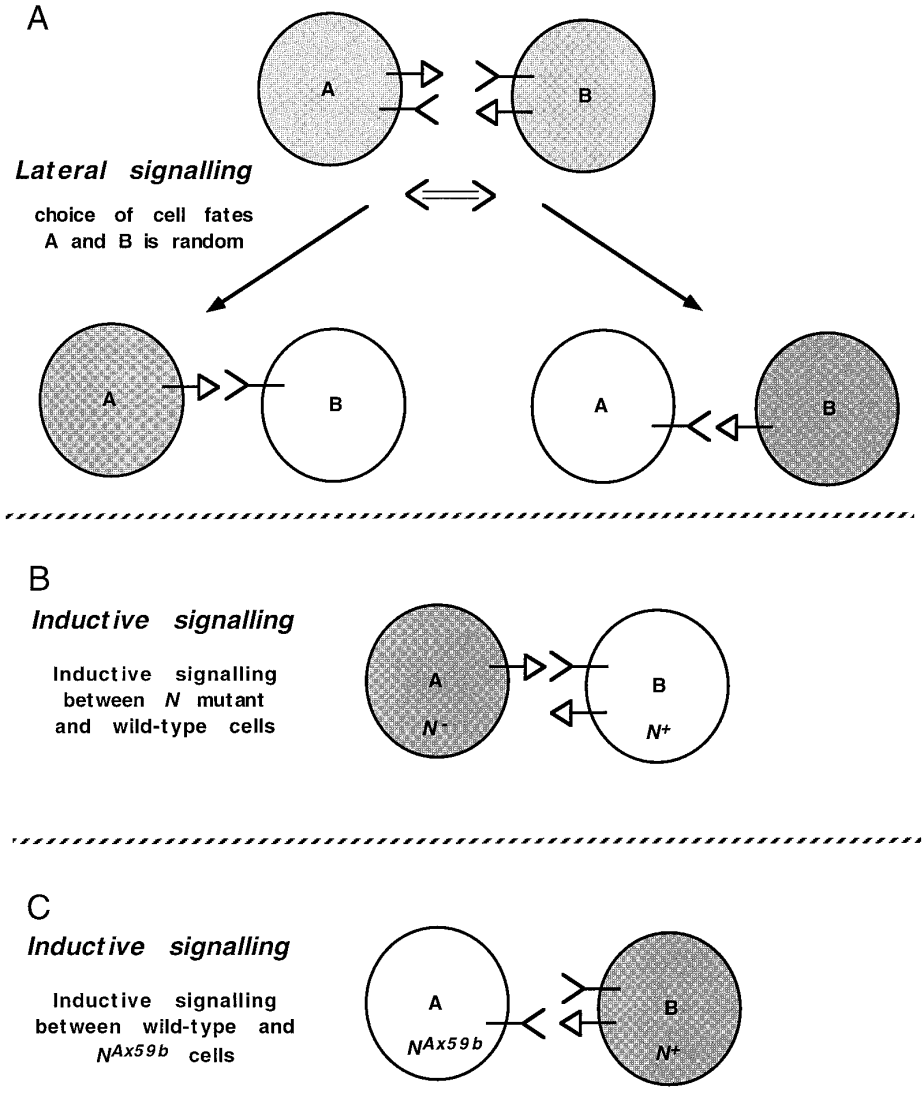

FIG. 4. Inductive and lateral signaling. Diagrams indicating lateral and inductive signals involving *Notch* mutants are shown. (A) Lateral signaling involves equipotential cells that express both ligand and receptor. Any small difference in the level of ligand or receptor between the cells can be amplified by means of a feedback loop linking receptor activation of a cell with its production of ligand. With time the situation resolves to one in which a single cell signals strongly and the others only receive. The final outcome may be random. (B) Signaling between mutant *Notch* cells and wild-type cells is inductive. The mutant cell is devoid of functional receptor and produces a strong signal, Delta. In the case of bristle precursor determination this cell always takes up the neural fate. The wild-type cell is always the receiving cell and it consistently takes up the epidermal fate. (C) Signaling between gain of function N^{Ax} cells and wild-type cells is inductive. The mutant cells bear hyperactive receptors and produce very little inhibitory signal. They are always the receiving cells and in the case of bristle precursor determination they always take up the epidermal fate. The wild-type cells are always the signaling cells and they consistently adopt the neural fate.

activated form of Notch, Torso^{4021}–Notch, is however also able to activate signal transduction downstream of Notch in the *shibire* mutant background. Furthermore, overexpression of the membrane-bound wild-type Notch, which causes dominant, ligand-independent receptor activation, is also completely epistatic over *shibire*. These arguments suggest that, even when the receptor is membrane-bound, endocytosis is not needed for signal transduction in the receiving cell. It has been shown that overexpression of a mutant form of dynamin prevents endocytosis of ligand–EGF receptor complexes in cultured cells. Here, too, transduction of the signal, in this case kinase activity, is not impaired (Vieira *et al.*, 1996).

The N^{Ax} alleles also lead to hyperactivation of the pathway but the receptors encoded by these alleles remain dependent upon the ligand, Delta (Heitzler and Simpson,

1993). In this case *shibire* is required and in N^{Ax} shi^{ts} animals a neurogenic phenotype, opposite to that of N^{Ax} itself, ensues. This suggests that *shibire* is needed for interactions between the ligand and its receptor. This places the requirement for dynamin at the cell surface, in agreement with the previously identified role for this protein in endocytosis (Liu and Robinson, 1995).

Dynamin Is Required in Both Signaling and Receiving Cells for Notch-Mediated Cell Interactions

The selection of sensory organ precursors in *Drosophila* relies on a particular type of lateral signaling during which initially all cells produce both ligand and receptor and are capable of receiving and transducing the signal (Simpson, 1994). With time this resolves to a situation in which a single signaling cell produces the ligand and the others the receptor (Ghysen *et al.*, 1993; see also Wilkinson *et al.*, 1994, for similar data in *C. elegans*). Experiments designed to assign the role of molecules such as dynamin to signaling or receiving cells are thus made difficult. To clearly distinguish between signaling and receiving cells, we used two experimental situations in which the signaling becomes inductive with one cell(s) producing and the other(s) receiving the signal. In mosaics where mutant N^{Ax} cells (bearing hyperactive receptors) are juxtaposed to wild-type cells, the signal is transmitted in one direction only from the wild-type to the mutant cells (Heitzler and Simpson, 1993). Mosaics of double-mutant N^{Ax} *shi* clones show that dynamin is required autonomously in the receiving cell. This is not surprising since the receiving cell is the one in which ligand-induced receptor endocytosis is taking place.

In mosaics where mutant *Notch* cells (deficient in reception) are juxtaposed to wild-type cells, the signal is transmitted in one direction only, this time from the mutant to the wild-type cells (Heitzler and Simpson, 1991). In this instance a study of flies mosaic for double-mutant *Notch shibire* clones reveals that dynamin is also required in the signaling cell. Indeed the inhibitory effect of the mutant *Notch* cells is somewhat impaired in the absence of *shibire:* a small percentage of wild-type bristles escape inhibition and also adjacent mutant and wild-type bristles can occasionally be found, a situation that has only been observed previously for clones mutant for both the signal and the receptor. We propose two possible explanations for this rather unexpected observation. First, endocytosis may be used for removal and downregulation of the ligand itself from the signaling cell. In the neural epithelium vesicles containing either Delta by itself or Delta plus Notch have been described by Kooh *et al.* (1993). These authors suggested that clearance of Delta from the cell surface involves both removal of ligand–receptor complexes by receiving cells and generalized clearance of Delta from expressing cells. However, this study suffered from the drawback that signaling and receiving cells could not be clearly distinguished. Another study in the *Drosophila* eye demonstrated the presence of Delta in subcellular vesicles in expressing cells and its accumulation in *shibire* mutants (Parks *et al.*, 1995).

A second explanation for the function for dynamin in the signaling cell is linked to the fact that Delta is a membrane-bound ligand. It is not proteolytically cleaved as in many other cases such as EGF. Furthermore, it is likely that the entire protein, including the intracellular domain, is taken up during endocytosis by the receiving cell (Henderson *et al.*, 1994; Cagan *et al.*, 1992). The removal of the ligand–receptor complex in these cases is likely to involve the transfer of plasma membrane from the signaling to the receiving cell. We suggest that a microvillar outgrowth containing the ligand is enveloped by the receiving cell and pinched off together with the endocytotic vesicle of the receiving cell (Fig. 5). Such a mechanism was suggested by Cagan *et al.* (1992) from their studies of the Bride of sevenless–Sevenless interaction. If such a mechanism prevails, dynamin could function in both the sending (Delta-expressing) cells to pinch off the microvillar outgrowth and the receiving (Notch-expressing) cells to pinch off the endocytotic vesicle.

If Signal Transduction Occurs in the Absence of Endocytosis Why Does a Failure of This Process Cause a Neurogenic Phenotype?

It has been proposed that ligand-induced endocytosis of EGF receptor is necessary to downregulate the cellular response when EGF signaling stops (Wells *et al.*, 1989; Vieira *et al.*, 1996). Addition of EGF to cultured cells expressing EGF receptor leads to a response, cell proliferation, which lasts for a short time and then stops. Overexpression of a mutant form of dynamin causes the cells to continue dividing as if the signal were continuous (Vieira *et al.*, 1996). Similarly, genetic interactions in *C. elegans* suggest that an impairment of endocytosis increases the level of activity of the EGF receptor (Lee *et al.*, 1994). In the case of Notch signaling, however, the failure of endocytosis in *shibire* mutants mimics phenotypes associated with a loss, rather than an increase, of receptor activity. The requirement for endocytosis in Notch-mediated lateral signaling may be linked to (1) the necessity to remove possibly inactive ligand–receptor complexes in order to maintain signaling for a considerable time and (2) the particular nature of signal transduction in the Notch pathway.

The kinetics of Notch-mediated lateral signaling during sensory organ precursor selection are rather different from those observed for EGF signaling. First, the cell–cell interactions must be maintained for a considerable time in order to select a single cell and for that cell to become the only signaling cell (Seugnet *et al.*, 1997). The neurogenic phenotype resulting from a failure of endocytosis could result from a failure to maintain the signal for a sufficient length of time. This would mean that, in contrast to EGF receptor signaling, clearance of ligand–receptor complexes may be necessary in this case to allow signaling to continue. It is not known how signal transduction takes place after activation of Notch. One hypothesis is that part of the intracellu-

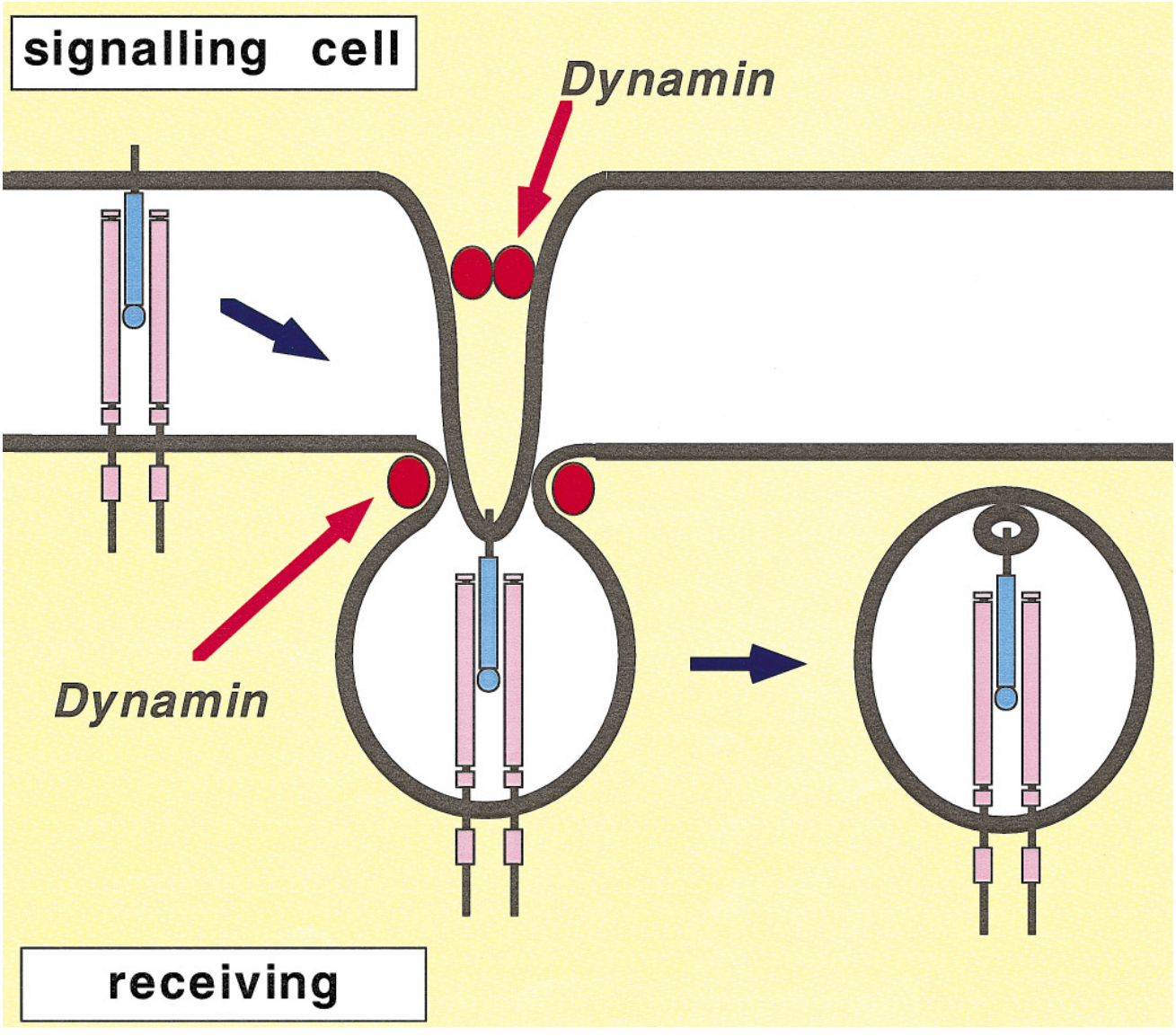

FIG. 5. Model for the internalization of the membrane-bound ligand, Delta, by the receiving cell after activation of the receptor Notch. After binding of Delta to Notch, internalization of the ligand–receptor complex is postulated to involve the plasma membrane of the signaling cell. A microvillar outgrowth of plasma membrane from the signaling cell into which Delta is inserted is visualized as being taken up within the endocytotic vesicle of the receiving cell. Dynamin would be required for pinching off the microvillar process of the signaling cell, as well as the endocytotic vesicle of the receiving cell.

lar domain of Notch may be cleaved and enter the nucleus (Jarriault *et al.*, 1995; Goodbourn, 1995; Hseih *et al.*, 1996; Kopan *et al.*, 1996). If this were to be the case, then after cleavage the ligand–receptor complexes would become inactive. Indeed, a phenotype similar to that of *shibire* is seen after overexpression of truncated Notch molecules in which the intracellular domain is lacking. These have been shown to act as dominant negatives probably because they result in the accumulation of too many inactive ligand–receptor complexes (Lieber *et al.*, 1993; Rebay *et al.*, 1993). Endocytosis may be necessary to remove inactive complexes and failure of this would not result in an enhanced response, but rather the opposite: their accumulation at the cell surface might interfere with the formation of new functional receptor complexes necessary for the continuation of the signal. Cellular trafficking in this case then may be needed for cell–cell signaling to be maintained.

Similarly, accumulation of Delta at the surface of the signaling cell, in the absence of endocytosis in *shibire* mutants, might also be deleterious. A recent study of Delta function at the dorsoventral wing boundary showed that when Delta is in great excess relative to Notch it does not

allow Notch signaling (Doherty *et al.*, 1995). Overexpression of *Delta* in imaginal discs can cause a neurogenic phenotype like that of *shibire* perhaps due to the formation of ligand–receptor complexes bearing only one Notch molecule instead of the two required for activation (Seugnet, 1997).

Perhaps surprisingly, endocytosis does not appear to be necessary for normal signal transduction from constitutively activated membrane-bound receptors as in the case of excess wild-type Notch or to a lesser extent the activated receptor Torso^{4021}–Notch. In addition to ligand-induced receptor internalization (Damke, 1996), internalization of receptors can take place independently of occupancy by a passive, constitutive mechanism (Wiley *et al.*, 1991). There is, furthermore, increasing evidence for an alternative, constitutively active pinocytic pathway that does not require either clathrin or dynamin (Damke *et al.*, 1995; Lamaze and Schmid, 1995). This pathway may allow downregulation of Torso^{4021}–Notch molecules or excess wild-type Notch molecules in the absence of dynamin. It is noteworthy that, unlike N^{Ax}, the constitutively active Torso^{4021}–Notch receptors or an excess of wild-type Notch does not require the ligand. We suggest that it is this characteristic that enables them to be internalized by a passive clathrin-independent pathway. It is likely that such constitutive internalization is only possible for Notch receptors that are not bound to Delta. N^{Ax} molecules, in contrast, require Delta for activation and do not allow precursor selection in the absence of dynamin. Delta is bound to the membrane of the signaling cell and it may sequester N^{Ax} so that it cannot be easily dislodged from the membrane. Our results have shown that, in the case of dynamin-dependent ligand-induced Notch receptor internalization, the signaling cell also actively participates.

In conclusion, since our experiments reveal a requirement for dynamin in both signaling and receiving cells to allow endocytosis of ligand–receptor complexes in the receiving cell, the resulting neurogenic phenotype may result from accumulation of Delta in the signaling cell or accumulation of inactive ligand–receptor complexes in the receiving cell, or both. Such a situation might be rather special to the Notch signaling pathway which combines several particular features. First, the ligand is membrane-bound; second, signal transduction is initiated in a manner different from that of other receptors; and third, lateral cell–cell signaling requires maintenance of the signal for some considerable time. Indeed the Notch signaling pathway appears so far to be the only one which is disrupted in the *shibire* mutant. Transmission of the signal in other cases such as that of Wingless, for example, does not require *shibire* (Bejsovec and Wieschaus, 1994).

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