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Numb Antagonizes Notch Signaling to Specify Sibling Neuron Cell Fates

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Summary

Asymmetric cell divisions play a key role in establishing neuronal diversity in the mammalian and Drosophila CNS, but the mechanisms involved are mostly unknown. The Drosophila MP2 precursor divides asymmetrically to generate the dMP2/vMP2 interneurons. Delta–Notch signaling is required to specify vMP2 fate, whereas the localized determinant Numb is segregated into dMP2 and is required to specify dMP2 fate. *Notch*; *numb* double mutants have two dMP2 neurons; hence, Numb is not required for dMP2 fate, but antagonizes the Delta–Notch "vMP2" signal. In vivo Delta expression and in vitro culture experiments show that vMP2 fate is specified by an "inductive" signal from outside the MP2 lineage. Thus, intrinsic and extrinsic cues converge to specify binary cell fates in the MP2 cell lineage.

Introduction

Development involves generating diverse cell fates from a single cell. Two general mechanisms are known to specify sibling cell fates: intrinsic "determinants" segregated to one daughter cell at mitosis, and diffusible or contact-mediated extrinsic cues. Good examples of intrinsic determinants include PAR1 and PAR3 proteins, which are involved in specifying cell fates in the early embryo of Caenorhabditis elegans (Guo and Kemphues, 1995; Etemad-Moghadam et al., 1995). In Drosophila, the Numb and Prospero proteins are intrinsic determinants of sibling cell fate (Rhyu et al., 1994; Spana and Doe, 1995; Spana et al., 1995). Much more is known about the role of extrinsic cues in specifying cell fate, in both vertebrates and Drosophila. For example, secreted WNT and Hedgehog proteins (Perrimon, 1994) and contact-mediated signals transduced by the Notch transmembrane receptor (Artavanis-Tsakonas et al., 1995) control cell fate in a diverse array of tissues.

Both intrinsic and extrinsic factors are required for CNS development in Drosophila. Proneural genes are expressed in clusters of ectodermal cells and promote neuroblast formation (Skeath and Carroll, 1994); within each cluster, cell interactions mediated by Delta (ligand) and Notch (receptor) result in only one cell becoming the neuroblast (reviewed by Campos-Ortega, 1993). Intrinsic factors are involved in later steps of neurogenesis. Neuroblasts divide asymmetrically to produce a series of ganglion mother cells (GMCs), each of which produces two postmitotic neurons or glia. The intrinsic factors Prospero and Numb are segregated into the smaller GMC at each neuroblast division (Rhyu et al., 1994; Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995); Prospero is required for normal GMC development (Spana and Doe, 1995).

The MP2 precursor develops from a proneural cluster and is morphologically identical to a neuroblast, but it has a much simpler cell lineage (Bate and Grunewald, 1981; Spana et al., 1995). MP2 divides asymmetrically to produce a small ventral cell (vMP2) and a larger dorsal cell (dMP2). Subsequently, the neurons migrate to the same dorsoventral plane, with vMP2 anterior to dMP2, and extend axons in opposite directions (Spana et al., 1995). vMP2 is an interneuron with an anterior axon projection, and dMP2 is an interneuron with posterior axon projection. During MP2 mitosis, Numb is asymmetrically localized to the dorsal cortex of the MP2 (similar to its dorsal localization in dividing neuroblasts) and selectively partitioned into the dMP2 neuron. In the absence of Numb, the dMP2 is transformed into the vMP2 fate; conversely, ectopic Numb can transform vMP2 into dMP2. Thus, Numb is an intrinsic determinant of dMP2 fate (Spana et al., 1995).

numb was originally identified as a gene controlling cell fate specification in the PNS. Sense organ precursors (SOPs) produce daughter cells called IIa and IIb: the IIa cell divides to make the bristle and socket cells and the IIb cell divides to make a neuron and a glial cell (reviewed by Jan and Jan, 1995). Numb is asymmetrically segregated into the IIb cell during SOP division (Knoblich et al., 1995; Spana and Doe, 1995), and it is necessary and sufficient to specify IIb cell fate (Uemura et al., 1989; Rhyu et al., 1994). Interestingly, extrinsic signals are also required to produce a normal SOP lineage. Loss of either *Delta*, *Notch*, or *Suppressor of Hairless* (*Su(H)*) function results in two IIb cells, the opposite of the *numb* loss of function phenotype (Parks and Muskavitch, 1993; Hartenstein and Posakony, 1990; Schweisguth and Posakony, 1992). This has led to the prediction that Numb might confer resistance to the Notch-mediated signal in the IIb cell (Posakony, 1994). Thus, both intrinsic and extrinsic cues are involved in specifying cell fate within the SOP lineage.

To understand the mechanism by which the intrinsic Numb protein and extrinsic Delta–Notch signaling control sibling cell fate, we focus on the simple cell lineage of the MP2 precursor. Loss of either *Delta* or *Notch* function in the MP2 lineage results in two dMP2 neurons, the opposite of the *numb* phenotype. Double mutants (*Notch*; *numb* or *numb*; *Delta*) have the same phenotype as the *Delta* or *Notch* single mutants (two dMP2 neurons). Thus, Numb is not required for dMP2 fate, but acts by antagonizing the Delta–Notch-mediated specification of vMP2 fate. Delta protein is detected in adjacent tissues, not in the MP2 neurons, suggesting that vMP2 fate is "induced" by adjacent tissues rather than by "lateral" signaling between the sibling neurons. We confirm this using in vitro culture of MP2 precursors: when MP2 develops in isolation, two dMP2 neurons develop; when MP2 develops in cell aggregates, the normal vMP2/dMP2 neurons develop.

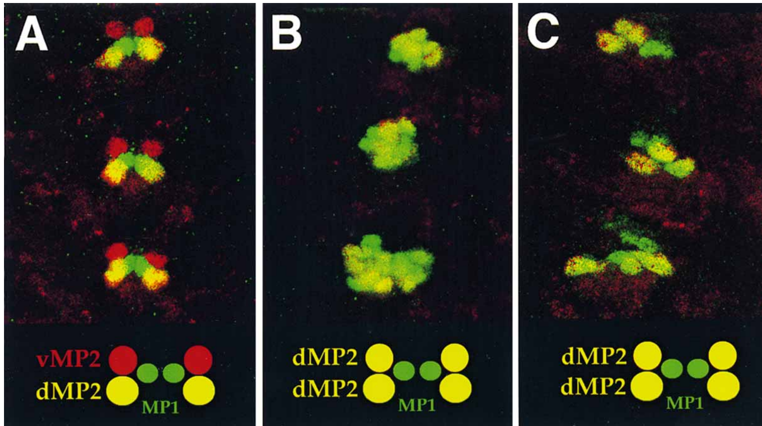

Figure 1. *Delta* or *Notch* Mutations Transform vMP2 into dMP2

vMP2, dMP2, and the lineally unrelated MP1 neurons can be uniquely identified using double label confocal imaging to detect the AJ96 (β-gal, red) and Odd (green) markers. Both dMP2 and vMP2 express AJ96 (red), but only dMP2 expresses Odd (green + red = yellow); the lineally unrelated MP1 neurons also express Odd (green). In all panels, three segments of a stage 15 CNS is shown, with anterior up. The schematic below each panel summarizes the cell fate changes.

(A) In wild-type embryos, there is one vMP2 neuron (red), one dMP2 neuron (yellow), and one MP1 neuron (green) per hemisegment.

(B) In *Delta* mutant embryos, there are supernumerary MP2 precursors due to failure of lateral inhibition in the ectoderm (see text). There are no vMP2 neurons (red), about 20 dMP2 neurons (yellow; not all are in this focal plane), and about 12 MP1 neurons (green) per segment.

(C) In *Notch* mutant embryos, there is no vMP2 neuron (red), two dMP2 neurons (yellow), and one MP1 neuron (green) per hemisegment. Maternal Notch results in normal MP2 formation, but vMP2 specification is defective.

Results

Loss of *Delta* or *Notch* Transforms vMP2 into dMP2

The MP2 precursor divides asymmetrically to produce a larger dorsal neuron (dMP2) and a smaller ventral neuron (vMP2). Cell interactions mediate binary sibling neuron fates in the grasshopper embryo (Kuwada and Goodman, 1985), and the Delta (ligand) and Notch (receptor) proteins are known to mediate a wide variety of "lateral" and "inductive" cell interactions in Drosophila (Artavanis-Tsakonas et al., 1995). Here, we test whether cell–cell interactions mediated by *Delta* and *Notch* play a role in specifying MP2 sibling neuron identity.

Expression of β-galactosidase (β-gal) in the AJ96 enhancer–trap line can be used to identify the dMP2/vMP2 neurons, and the Odd-skipped (Odd) protein can be used to uniquely identify the dMP2 neuron (Spana et al., 1995). In wild-type embryos, all segments show a bilateral pair of dMP2/vMP2 neurons (Figure 1A). In embryos lacking zygotic *Delta* function, about 10–12 MP2 precursors are formed per segment due to failure of lateral inhibition in the proneural cluster producing MP2 (Skeath and Carroll, 1992). Nevertheless, it is still possible to score dMP2 and vMP2 fates using AJ96 and Odd. We find that all AJ96-positive neurons maintain Odd expression, characteristic of the dMP2 cell fate (Figure 1B). We observe approximately 10 small and 10 large cells expressing AJ96 and Odd per segment, suggesting that the asymmetric division of all the MP2 precursors formed is not altered, but the smaller "vMP2" has been transformed into the dMP2 fate.

A similar cell fate change is observed in embryos lacking zygotic *Notch* function. In these embryos, only one MP2 precursor is formed per hemisegment (probably due to the maternal contribution of Notch; Menne and Klambt, 1994), yet all MP2 neurons maintain Odd expression, characteristic of the dMP2 cell fate (Figure 1C). Thus, loss of either *Delta* or *Notch* function transforms the vMP2 neuron into the sibling dMP2 neuron, showing that Delta–Notch mediated cell interactions are required to specify vMP2 identity.

Numb Autonomously Inhibits *Delta–Notch* Specification of vMP2 Identity

The membrane-associated Numb protein is asymmetrically segregated at MP2 mitosis into the dMP2 neuron (Spana et al., 1995), and loss of *numb* leads to two vMP2 neurons (Figure 2A). Loss of *Delta* or *Notch* results in two dMP2 neurons, the opposite of the *numb* mutant phenotype (Figure 1). One model accounting for these opposite phenotypes is that Numb specifies the dMP2 fate and that *Delta* and *Notch* are responsible for keeping *numb* out of vMP2. In this case, the double mutants would show the *numb* phenotype (two vMP2s). Alternatively, Delta–Notch signaling could induce vMP2 fate and localization of Numb into dMP2 could inhibit this signal. In this case, the double mutants would show the *Delta* or *Notch* phenotype (two dMP2s).

To test these models, embryos mutant for both *numb* and *Delta* (Figure 2B) or both *numb* and *Notch* (Figure 2C) were stained for Odd and AJ96. In both double mutants, all of the AJ96-positive cells express Odd, indicating the dMP2 fate. This shows that *numb* is not required to specify dMP2 fate, but that the dMP2 fate is due to lack of productive *Delta–Notch* signaling. The double mutant result, in combination with the asymmetric localization of Numb to the dMP2 neuron, shows that the function of Numb is to antagonize the Delta–Notch signal specifying vMP2 fate.

Delta Is Detected Adjacent to, but Not within, the dMP2 and vMP2 Neurons

The Delta signal could be produced by the dMP2 and vMP2 neurons, consistent with a "lateral inhibition" model for specifying sibling cell fates (Greenwald, 1989). Alternatively, Delta might be produced by cells outside the MP2 lineage and act as an "inductive" signal specifying vMP2 fate. These alternatives can be tested by assaying the expression of Delta and Notch in the MP2

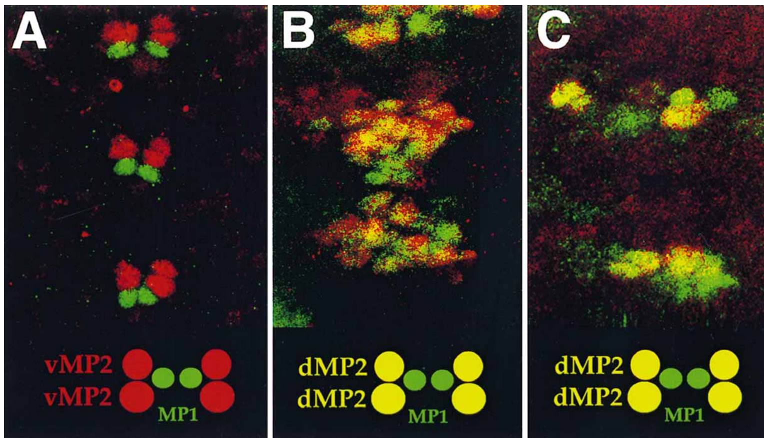

Figure 2. *Delta* and *Notch* Are Epistatic to *numb* in the MP2 Lineage

Loss of *Delta* or *Notch* transforms vMP2 into dMP2, even in the absence of the *numb*. MP2 cell fates are assayed as described in Figure 1 and summarized below each panel.

(A) In *numb* mutants, there two vMP2 neurons (red); dMP2 has been transformed into vMP2.

(B) In *numb*; *Delta* double mutants, all MP2 neurons develop as dMP2 (yellow). There are extra MP2 precursors due to failure of lateral inhibition in the neuroectoderm (see text).

(C) In *Notch*; *numb* double mutants both MP2 neurons develop as dMP2 (yellow).

lineage. We stained AJ96 embryos with antibodies against Delta, Notch, and β-gal (to identify the MP2 neurons). Delta cannot be detected in the newborn dMP2 and vMP2 neurons (Figure 3A) at the time their cell fates are specified (Spana et al., 1995). However, Delta is expressed at high levels in the mesoderm adjacent to dMP2 and at lower levels in the neuroectoderm and neuroblasts adjacent to vMP2 (Figure 3A). In contrast, Notch is uniformly distributed throughout all cell types, including the dMP2 and vMP2 neurons (Figure 3B). There is no sign of asymmetric Notch localization in MP2 or the adjacent neuroblasts (as observed for Notch1 immunoreactivity in dividing mammalian CNS precursors; Chenn and McConnell, 1995).

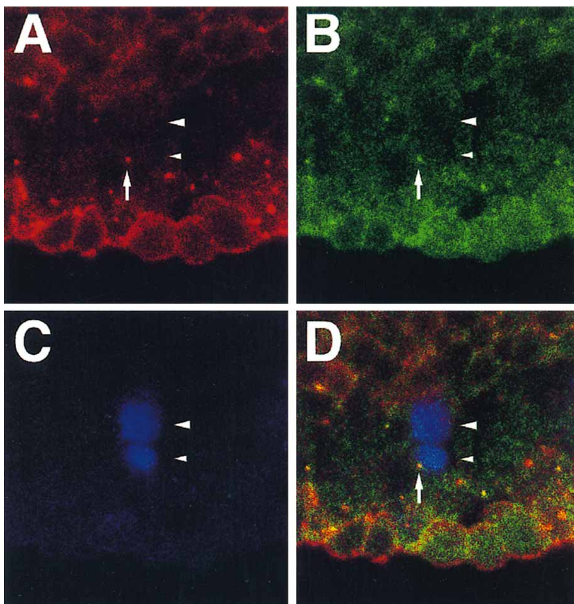

Figure 3. Delta Is Not Expressed in the MP2 Neurons

Expression of Delta and Notch in the MP2 neurons just after division. AJ96 embryos were labeled for Delta (red), Notch (green), and β-gal (blue). Lateral view of a stage 11 embryo; anterior to the left and dorsal up. dMP2, large arrowhead; vMP2, small arrowhead.

(A) Delta is not detectable on the membranes of the MP2 neurons, but is expressed at high levels on ectoderm and mesoderm cell membranes; in addition, Delta is observed in punctate "dots." Some of these dots colocalize Notch protein, including one in vMP2 (arrow).

(B) Notch is detected in every cell in the developing CNS and mesoderm, but at highest levels in the ectoderm. It is also found in punctate dots that can colocalize with Delta.

(C) AJ96 is specifically expressed in the larger dMP2 (large arrowhead) and smaller vMP2 (small arrowhead).

(D) The merged image of (A)–(C) shows Delta and Notch colocalization at a point of contact between the future vMP2 and an adjacent neuroblast.

We observe colocalization of Notch and Delta in punctate "dots" in ectoderm, mesoderm, neuroblasts, and both dMP2 and vMP2 neurons. This may represent internalization, capping, or "clearing" of the Delta–Notch protein complex (Fehon et al., 1990; Kooh et al., 1993). In any case, it is not correlated with productive Notch signaling, since Delta–Notch dots are observed in dMP2. Thus, both dMP2 and vMP2 neurons express the Notch receptor and adjacent cells express the Delta ligand, consistent with a model in which Numb acts in the dMP2 cell to antagonize the function of Notch or downstream genes required for vMP2 determination. In addition, it suggests that vMP2 fate is induced by an extrinsic Delta–Notch signal, not by lateral signaling between sibling cells as observed in other systems (Kuwada and Goodman, 1985; Greenwald, 1989).

Specification of vMP2 Requires Contact with Cells Outside the MP2 Lineage

To determine unambiguously whether vMP2 can be specified by interactions with its sibling, or whether it requires a signal from outside the MP2 lineage, we assayed the development of MP2 precursors isolated in vitro. In vitro cultures were made from AJ96 embryos, in which the MP2 neurons can be uniquely identified by their β-gal expression and unequal cell size. Sibling fates can be assayed by Odd expression: vMP2 is Odd-negative; dMP2 is Odd-positive. When the MP2 lineage develops in isolation, lacking any detectable contact to other cells in the culture, both neurons preferentially differentiate as dMP2 (Table 1; Figures 4C–4D). This shows that contact between sibling neurons is insufficient to induce vMP2 fate. In contrast, when the MP2 neurons develop fully surrounded by other cells, the normal vMP2 and

Table 1. In Vitro Development of the MP2 Lineage: vMP2 Specification Requires Contact with Cells Outside the MP2 Lineage

Position of MP2 Neurons Relative to Other Cells[a]	Identity of MP2 neurons[b]		
	vMP2/dMP2	dMP2/dMP2	vMP2/vMP2
MP2 neurons isolated	2	21	0
MP2 neurons surrounded	38	4	0

[a] Isolated indicates no physical contact between MP2 neurons and other cells; surrounded indicates the MP2 neurons lie within a cluster of cells.
[b] The dMP2 and vMP2 neurons were identified as AJ96-positive unequal-sized pairs of cells; vMP2 is Odd-negative and dMP2 is Odd-positive (see Experimental Procedures).

dMP2 lineage is frequently observed (Table 1; Figures 4A–4B). These results show that vMP2 is specified by an inductive signal from outside the MP2 lineage, rather than by lateral signaling between the sibling neurons.

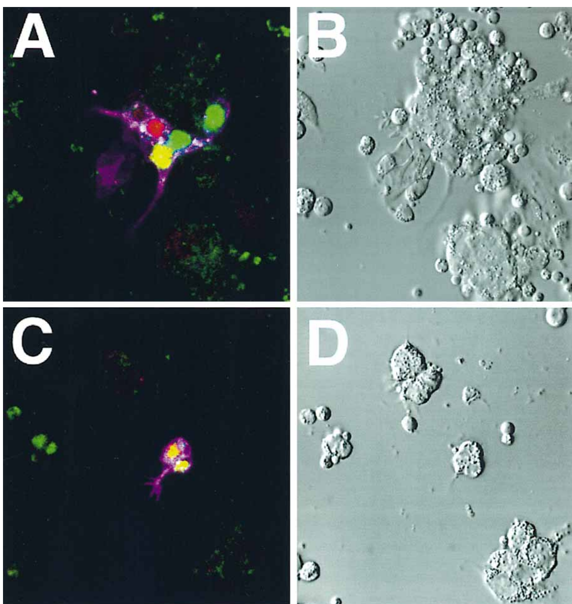

Figure 4. Extrinsic Signals Are Required for vMP2 Specification

dMP2/vMP2 identity was assayed following in vitro culture of MP2 either in isolation or in contact with other cells. Cell fates were assayed by four-channel imaging with AJ96 (red) to score MP2 neurons, Odd (green) to score dMP2, the axon marker α-HRP (purple) to verify that the neurons are old enough to extend axons and thus down-regulate Odd in vMP2, and Nomarski optics to assay the cell contacts between MP2 neurons and adjacent cells.

(A and B) Confocal triple label (A) and Nomarski (B) images showing normal specification of vMP2 (red; AJ96-positive) and dMP2 (yellow; AJ96- and Odd-positive) when surrounded by cells in culture. Divergent axons extend from the MP2 neurons (purple).

(C and D) Confocal triple label (C) and Nomarski (D) images showing transformation of vMP2 into dMP2 (yellow; AJ96- and Odd-positive) when MP2 neurons develop in isolation. A single axon fascicle extends from the duplicated dMP2 neurons (purple; the growth cone and axon images were taken from different focal planes and merged using Adobe Photoshop).

Discussion

Both intrinsic and extrinsic cues converge to specify binary cell fates in the simple lineage of the CNS precursor MP2. The intrinsic determinant Numb is asymmetrically localized into the dMP2 neuron; loss of *numb* transforms dMP2 to vMP2, whereas ectopic Numb transforms vMP2 to dMP2 (Spana et al., 1995). Extrinsic signals mediated by Delta and Notch are also necessary for normal dMP2/vMP2 development. The Notch receptor is expressed in the MP2 lineage; the Delta ligand is expressed adjacent to the MP2 lineage, but not within it. Loss of *Delta* or *Notch* produces two dMP2 neurons, even in the absence of *numb*. Thus, Numb is not required absolutely to specify dMP2 fate, but rather to antagonize the Delta–Notch-mediated induction of vMP2 fate (Figure 5).

In addition to the MP2 lineage, *numb* and *Delta–Notch* have opposing phenotypes in the adult external sense organ precursor (SOP) lineage. The SOP divides asymmetrically to produce the IIa and IIb cells; IIa produces the hair and socket cells, while the IIb produces the neuron and glia (reviewed by Jan and Jan, 1995). Numb is asymmetrically localized into IIb during SOP mitosis (Knoblich et al., 1995; Spana and Doe, 1995), and *numb* mutants produce two IIa cells (Uemura et al., 1989). Conversely, loss of *Delta* or *Notch* results in two IIb cells (Hartenstein and Posakony, 1990; Parks and Muskavitch, 1993). Similar to the MP2 lineage, Numb antagonizes the Notch-mediated specification of IIa cell fate in the SOP lineage (Guo et al., 1996, this issue of *Neuron*). Thus, in both MP2 and SOP lineages, asymmetric localization of Numb results in one sibling with functional Notch signaling (vMP2 and IIa) and one sibling containing Numb, which blocks Notch signaling (dMP2 and IIb).

The striking parallels between the SOP and MP2 lineage suggest that Numb affects a component of the Notch pathway common to both IIa and vMP2 specification. In the SOP lineage, IIa development is promoted by Delta, Notch, and the nuclear proteins Su(H) and Tramtrack (Ttk) (Parks and Muskavitch, 1993; Hartenstein and Posakony, 1990; Schweisguth and Posakony, 1992; Guo et al., 1995). In the MP2 lineage, vMP2 fate is promoted by Delta, Notch, and Mastermind (Mam) (this paper; E. P. S., unpublished data). The role of Su(H) has not been tested in the MP2 lineage, but *ttk* mutants do not alter vMP2/dMP2 cell fate (E. P. S., unpublished data). The SOP and MP2 pathways share some components (Delta and Notch) but not others (Ttk), and others have yet to be assayed in both lineages (Mam and Su(H)). It is likely that Numb antagonizes the function of a common component.

Currently, Notch is the only protein known to function autonomously to specify both IIa and vMP2 identity. Both Numb and Notch are membrane associated, raising the possibility that Numb physically interacts with Notch to block signal transduction. Numb has a myristoylation motif, which may lead to direct membrane association, but this has not been tested. In addition, Numb has a phosphotyrosine interaction domain (Bork and Margolis, 1995), but it is unknown if Notch is tyrosine

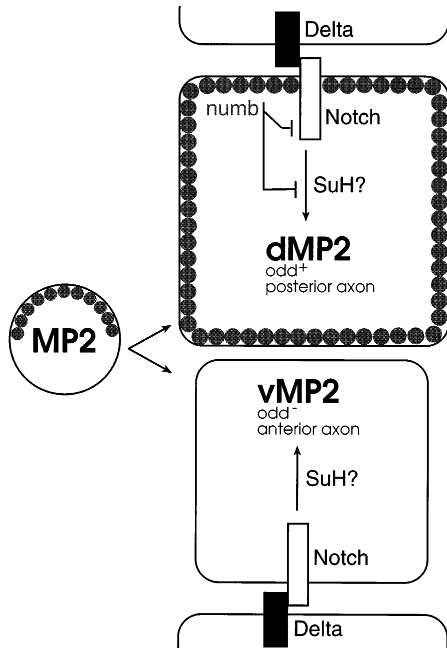

Figure 5. Intrinsic Numb and Extrinsic Delta–Notch Specify Sibling Neuron Fates

MP2 divides asymmetrically to produce the larger dMP2 and smaller vMP2; Numb (gray dots) is segregated into dMP2 at mitosis. The ligand Delta is expressed by cells adjacent to the MP2 neurons, but not in them, whereas the transmembrane receptor Notch is expressed by both dMP2 and vMP2. In vMP2, the Delta–Notch signal down-regulates Odd expression and promotes an anterior axon projection; the Su(H) protein may be a component of the signaling pathway (as it is in many Notch signaling pathways). In dMP2, the Delta–Notch interaction can occur, but signal transduction is blocked by the membrane-associated Numb protein. Numb may physically interact with the intracellular domain of Notch, or with downstream components of the pathway. In the absence of Notch-mediated signaling, Odd expression is maintained and the neuron extends a posterior axon projection.

phosphorylated. Three proteins are known to interact physically with the intracellular domain of Notch: Dishevelled, Deltex, and Su(H) (Axelrod et al., 1996; Matsuno et al., 1995; Fortini and Artavanis-Tsakonas, 1994; Tamura et al., 1995). Numb could interact with any of these proteins to block Notch signaling, but currently none is known to be involved in vMP2 specification.

If Numb antagonizes Notch signaling in the MP2 and SOP lineage, does it antagonize Notch function in other tissues? Delta–Notch signaling is usually associated with a lateral inhibition mode of signaling. Lateral inhibition is used to specify different cell fates within a group of two or more equivalent cells in which all cells of the group express both Delta and Notch (reviewed by Artavanis-Tsakonas et al., 1995). For example, Delta–Notch mediated lateral inhibition is used to "single out" neuroblasts and SOPs from a group of equivalent ectodermal cells in the embryo and adult (Campos-Ortega, 1993) or to specify AC/VU fate in C. elegans (Greenwald, 1989). Both Numb and Notch are expressed at high levels in ectoderm from which neuroblasts and SOPs develop. Loss of *Notch* or *Delta* function results in supernumerary neuroblasts and SOPs, but loss or overexpression of *numb* does not affect this process in the embryo (Spana et al., 1995) or in the adult (Rhyu et al., 1994). Thus, Numb does not appear to inhibit Notch lateral inhibition signaling in the ectoderm.

How might Numb inhibit Notch function in some cells but not others? Asymmetric localization of Numb could increase its level in one cell enough to block Notch activity. Alternatively, Numb could directly interact with Notch or another component of the signaling pathway common to the MP2/SOP lineages; in the neuroectoderm, this inhibitory interaction would be blocked by a cell type–specific protein. Conversely, the MP2/SOP lineages may contain a cell type–specific cofactor necessary for Numb to block Notch signaling; in this case, Numb would not directly interact with a common component of the Notch pathway and would not inhibit Notch signaling in the neuroectoderm. Finally, cell type–specific forms of Numb protein may have different ability to inhibit Notch function; two forms of Numb are detectable on Western blots (Rhyu et al., 1994), but it is unknown if each has a different function. In the MP2 lineage, Delta–Notch acts as a polarized inducer of vMP2 fate. In the ectoderm, Delta–Notch is involved in lateral inhibition signaling between equivalent cells. Numb might block Delta–Notch inductive signaling, but not lateral inhibition signaling. It would be interesting to determine whether specification of IIa in the SOP lineage is due to an inductive Delta signal from adjacent tissues, similar to the role of Delta in the MP2 lineage.

Experimental Procedures

Fly Strains

We used the enhancer–trap line AJ96w^+ to identify the MP2 neurons. This line was crossed into the stocks *numb[1] pr cn Bc*/CyO *ftz–lacZ* and *l(1)N[81K1] w[a] rb*/FM7 *ftz–lacZ* and recombined onto *Dl[β] e*/TM3 *ftz–lacZ* to identify the MP2 neurons in mutants. *l(1)N[81K1]* is a small deficiency covering the *N* locus (Grimwade et al., 1985).

Antibody Production

Purified β-gal was purchased from Sigma and injected into rats until the diluted raw sera showed a staining pattern on Drosophila embryos containing a *lacZ* transgene. Monoclonal antibodies generated from the rat were first screened by ELISA. Twenty four ELISA-positive monoclonal lines were then screened on Drosophila embryos containing *lacZ* transgenes: both *ftz–lacZ* and *hkb–lacZ* (5953 w^+; Doe, 1992). Most ELISA-positive lines tested positive on embryos. Monoclonal line P_2D_{II}-1-B_{12} showed a slightly better signal to noise than the others and was used for all experiments.

Immunostaining

Embryos were stained using standard methods (Doe, 1992). Primary antibodies used were rabbit anti-β-gal (Cappel) at 1:2000 and rat anti-β-gal P_2D_{II}-1-B_{12} at 1:10. Antibodies against Notch (mouse monoclonal C.17.9C6 and rat polyclonal Rat-1) and against Delta (mouse monoclonal 9B) were used at 1:1000. Rabbit anti-Odd was used at 1:2000. Goat anti-HRP conjugated to DTAF (Jackson Immunoresearch) was used at 1:1000. Detection of primary antibodies was done using species-specific secondary antibodies conjugated to DTAF, LRSC, or Cy5 (Jackson Immunoresearch) at a concentration of 1:400. Embryos were mounted in 70% glycerol with 4% n-propyl gallate (Sigma), and immunofluorescent images were collected on a Bio-Rad MRC 1000 or 1024 confocal microscope.

In Vitro Embryonic Cell Culture

The dissociation procedure was done at 4°C using a minimally modified standard procedure (Seecof et al., 1971). AJ96 enhancer–trap

line embryos 3.5–4.5 hr old were dechorionated in 50% bleach and rinsed in tap water; they were washed with 90% ethanol for 5 min, washed twice with Schnieder's medium supplemented with 2% fetal bovine serum (SM-FBS; both GIBCO), homogenized in 5 ml of SM-FBS using a loose-fitting Dounce, and the dissociated cells were passed through a fine Nitex mesh to remove large debris. The cells were washed by pelleting/resuspension (setting 5, IEC tabletop centrifuge) three times in 5 ml of SM-FBS and plated in 0.5 ml aliquots of SM-FBS on clean glass coverslips. After 30 min, 1.5 ml of SM-FBS was added and the cultures were incubated at 25°C for 6 hr, and then fixed and stained according to standard procedures (Doe, 1992) except the primary and secondary antibody incubations were for 1 hr at room temperature. The MP2 neurons can be uniquely identified by their high level of β-gal expression and unequal cell size.

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