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The regulation of apoptosis by Numb/Notch signaling in the serotonin lineage of *Drosophila*

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SUMMARY

Apoptosis is prevalent during development of the central nervous system (CNS), yet very little is known about the signals that specify an apoptotic cell fate. In this paper, we examine the role of Numb/Notch signaling in the development of the serotonin lineage of *Drosophila* and show that it is necessary for regulating apoptosis. Our results indicate that when Numb inhibits Notch signaling, cells undergo neuronal differentiation, whereas cells that maintain Notch signaling initiate apoptosis. The apoptosis inhibitor p35 can counteract Notch-mediated apoptosis and rescue cells within the serotonin lineage that normally undergo apoptosis. Furthermore, we observe tumor-like overproliferation of cells in the CNS when Notch signaling is reduced. These data suggest that the distribution of Numb during terminal mitotic divisions of the CNS can distinguish between a neuronal cell fate and programmed cell death.

Key words: Numb, Notch, Apoptosis, Serotonergic neurons, *Drosophila*

INTRODUCTION

The *Drosophila* central nervous system (CNS) develops from a small number of precursor cells that divide to produce a variety of neurons and glial cells. During the specification of cell fates, subsets of cells are induced to undergo programmed cell death or apoptosis (Abrams et al., 1993). Elucidating both the intrinsic and extrinsic signals that establish cell fates and trigger apoptosis is fundamental to understanding CNS development. This paper examines the regulation of apoptosis in the ventral nerve cord of *Drosophila*, specifically within in the cell lineage that produces the serotonergic neurons. The serotonin lineage is a relatively simple lineage, producing only four neurons. However, additional cells are observed when this lineage is examined in the apoptosis deficient line *H99* (Isshiki et al., 2001; Novotny et al., 2002). This suggests that the mitotic divisions of the serotonin lineage also produce several cells that undergo apoptosis.

The segmented *Drosophila* nerve cord develops from stereotyped division of 30 neuroblasts (NB) in each hemisegment (Doe, 1992; Goodman and Doe, 1993). A pair of serotonergic neurons in each hemisegment arise from NB7-3 (Lundell et al., 1996). NBs are stem cells that undergo several asymmetric divisions producing a specific number of ganglion mother cells (GMCs). Each GMC divides once to form two neuronal or glial progeny (Hartenstein et al., 1987). The divisions of the NB7-3 lineage have recently been determined using a combination of molecular markers and clonal analysis (Isshiki et al., 2001; Novotny et al., 2002). NB7-3 produces three GMCs. GMC-1 produces two neurons: GW, a motoneuron, and EW1, the more medial serotonergic neuron. GMC-2 produces EW2, the more lateral serotonergic neuron. GMC-3 produces EW3, a neuron that synthesizes the neuropeptide corazonin. The GW neuron projects an axon ipsilateral and posteriorly, and the three EW interneurons all project axons anteriorly to the posterior commissure (Fig. 1) (Bossing et al., 1996; Higashijima et al., 1996; Dittrich et al., 1997; Schmid et al., 1999).

Several genes have been shown to be essential in the differentiation of the NB7-3 lineage. Sequential expression of the segmentation transcription factors, Hunchback→Krüppel→Pdm1, within neuroblasts has been shown to be important in the development of several lineages, including NB7-3 (Brody and Odenwald, 2002). The subsequent GMCs and neuronal progeny maintain the expression of the transcription factors that are present in the NB at their birth. In the case of NB7-3, Hunchback (Hb) is expressed only in GMC-1 and its progeny, and is both necessary and sufficient to define the fates of these cells (Isshiki et al., 2001; Novotny et al., 2002). Krüpple (Kr) is expressed in both GMC-1 and GMC-2 and is necessary and sufficient to establish the fate of the EW2 serotonergic neuron (Isshiki et al., 2001). Pdm1 is expressed primarily in EW2 (Fig. 1) (Lundell and Hirsh, 1998). Differentiation of the NB7-3 lineage is also affected by mutations in *wingless* (*wg*) and other members of the Wingless signaling pathway such as, *engrailed* (*en*), *hedgehog* (*hh*) and *patched* (*ptc*) (Patel et al., 1989; Lundell et al., 1996). In addition, mutations in the transcription factors *eagle* (*eg*) (Dittrich et al., 1997; Lundell and Hirsh, 1998) and *huckebein* (*hkb*) (Lundell et al., 1996) also disrupt NB7-3 differentiation. *eg* has been shown to suppress a rough eye phenotype caused by the overexpression of *Ras1*, suggesting that *eg* may be

involved in Ras signaling (Hay et al., 1997). *hkb* is regulated by both *en* and *hh* in the NB7-3 lineage (Lundell et al., 1996; McDonald and Doe, 1997). The exact relationship between these genes and signal transduction pathways within the NB7-3 lineage remains to be determined.

In this study, we examine the role of Numb/Notch signaling, in the differentiation of the NB7-3 lineage. Numb is a membrane-associated protein that has been shown to partition asymmetrically with one daughter cell during binary mitotic divisions and to be responsible for establishing alternative cell fates in the *Drosophila* CNS, PNS (reviewed by Lu et al., 2000; Cayouette and Raff, 2002; Skeath and Thor, 2003) and myogenic precursor cells (Ward and Skeath, 2000). Numb has also been suggested to have a role in the differentiation of CNS lineages in vertebrates (reviewed by Lu et al., 2000; Cayouette and Raff, 2002; Shen et al., 2002; Sommer and Rao, 2002). Numb affects cell fate by inhibiting the intercellular Notch signaling pathway. During cell division, the cell that inherits Numb will lack Notch signaling, while the sibling cell that does not receive Numb will maintain Notch signaling (reviewed by Lu et al., 2000; Cayouette and Raff, 2002; Sommer and Rao, 2002; Skeath and Thor, 2003). The mechanism for how Numb inhibits Notch signaling is still under investigation, but Numb binds to the intercellular domain of Notch (Frise et al., 1996) and may regulate α-Adaptin, a protein involved in receptor-mediated endocytosis (Berdnik et al., 2002).

We present evidence that Numb/Notch signaling regulates apoptosis within the NB7-3 lineage. Notch signaling has previously been associated with apoptosis in an array of both negative and positive effects. Notch has been shown to inhibit apoptosis in T and B lymphocytes (Pear et al., 1996; Deftos et al., 1998; Jehn et al., 1999; Shelly et al., 1999; Morimura et al., 2000; Jundt et al., 2002). Constitutive Notch activity has been associated with cell transformation and cancer, presumably by inhibiting apoptosis (reviewed by Artavanis-Tsakonas et al., 1999; Miele and Osborne, 1999; Mumm and Kopan, 2000). Notch has also been implicated in inhibiting apoptosis in the brain. In the cerebellum of mice, Notch1 mediates the onset of neurogenesis and prevents apoptosis of neuroepithelium (Lütolf et al., 2002). A number of investigations have linked neurodegeneration in Alzheimer's disease to a reduction in Notch signaling (reviewed by Miele and Osborne, 1999). Presenilins, which are often mutated in individuals with Alzheimer's disease, are also necessary for Notch processing. It has been proposed that a loss in presenilin function may lead to a decrease in Notch signaling and a subsequent increase in cell death. Furthermore it has been shown in *Drosophila* that constitutive expression of Notch can inhibit presenilin-induced apoptosis (Ye and Fortini, 1999). Thus Notch signaling may protect neurons from apoptotic cell death.

By contrast, Notch has also been shown to induce apoptosis during development of the *Drosophila* retina (Cagan and Ready, 1989; Wolff and Ready, 1991; Rusconi et al., 2000; Yu et al., 2002), wing (Milan et al., 2002) and PNS (Orgogozo et al., 2002). Constitutive expression of Notch in zebrafish causes cells of the developing retina to enter apoptosis (Scheer et al., 2001). Notch has also been shown to induce apoptosis in vertebrate neural crest cells (Maynard et al., 2000). Recently, several studies have found a reduction in Notch activity to be coincident with cell proliferation and cancer (Verdi et al., 1999; Wakamatsu et al., 1999; Talora et al., 2002), suggesting that Notch-induced apoptosis may be an important mechanism in regulating cell growth. In this study, we demonstrate that during terminal mitotic divisions of the *Drosophila* CNS Notch signaling can induce an apoptotic cell fate.

MATERIALS AND METHODS

Drosophila stocks

eg^{289} and eg^{mz360} were both provided by G. Technau. eg^{mz360} contains a gal-4 P-element insertion 5′ of *eg*. eg^{289} contains a *lacZ* P-element insertion 5′ of *eg* and was generated by B. Genisch and G. Korge. $numb^{1}$, $spdo^{G104}$ and $numb^{1}$: $spdo^{G104}$ were provided by C. Doe. *UAS-Notch*^{ACT} was provided by Y. N. Jan. *Df(3L)H99*, *en-gal4* and *UAS-p35* were obtained from the Bloomington Stock Center.

Immunohistochemistry

Dissected CNS were fixed in 4% paraformaldehyde and incubated with primary and secondary antisera as previously described (Lundell and Hirsh, 1994). Primary antibodies used were: rabbit anti-eg (1:2000, G. Technau), guinea pig anti-Hb (1:600, C. Doe), rabbit anti-Ddc (1:50, M. Lundell), rat anti-Ddc (1:30, J. Hirsh), rabbit anti-corazonin (1:200, C. Doe), rabbit anti-zfh-1 (1:400, R. Lehman), rat anti-zfh-2 (1:200, A. Tomlinson), rabbit anti-pdm1 (1:1000, T. Dick), guinea pig anti-*lacZ* (1:1000, M. Lundell) and rabbit anti-*lacZ* (1:1000, Cappel). All Texas Red, FITC and Cy5 secondary antibodies were from Jackson Laboratories and used at 1:200. Images were obtained using a BioRad 1024 laser-scanning microscope and processed with Adobe Photoshop.

TUNEL assay

Apoptosis was assayed by TUNEL using a TACS 2 TDT-Fluor In Situ Kit from Trevigen. To reduce background the Strep-Fluor solution was preabsorbed against embryonic tissue and used at a 1:400 dilution. After the TUNEL procedure, tissue was washed with an Avidin/Biotin blocking kit from Vector.

RESULTS

Molecular markers uniquely identify the progeny of NB7-3

All four neuronal progeny of the NB7-3 lineage can be uniquely identified with the specific molecular markers shown in Fig. 1. Several previous investigations have contributed to this knowledge (Lundell and Hirsh, 1992; Bossing et al., 1996; Higashijima et al., 1996; Dittrich et al., 1997; Lundell and Hirsh, 1998; Schmid et al., 1999; Isshiki et al., 2001; Novotny et al., 2002). NB7-3 begins division in late stage 11 and produces four progeny that are all positive for Eg expression (Fig. 1B,C). GW and EW1, the progeny of GMC-1, are distinguishable by their expression of Hb (purple cells in Fig. 1B). The GW motoneuron is usually the most posterior cell in the cluster, often reduced in size and shows expression of Zfh-1 (purple cells in Fig. 1C). EW2 and EW3, the progeny of GMC-2 and GMC-3, express Zfh-2 (orange cells in Fig. 1B,C). EW1 and EW2 produce serotonin and are detectable with antibodies against either Dopa decarboxylase (Ddc) (Fig. 1D-F) or serotonin (data not shown). The two serotonergic cells are distinguishable from each other owing to their birth order, EW1 is more medial and expresses Hb, whereas EW2 is more lateral and expresses Zfh-2 (Fig. 1D). EW3 is usually the most

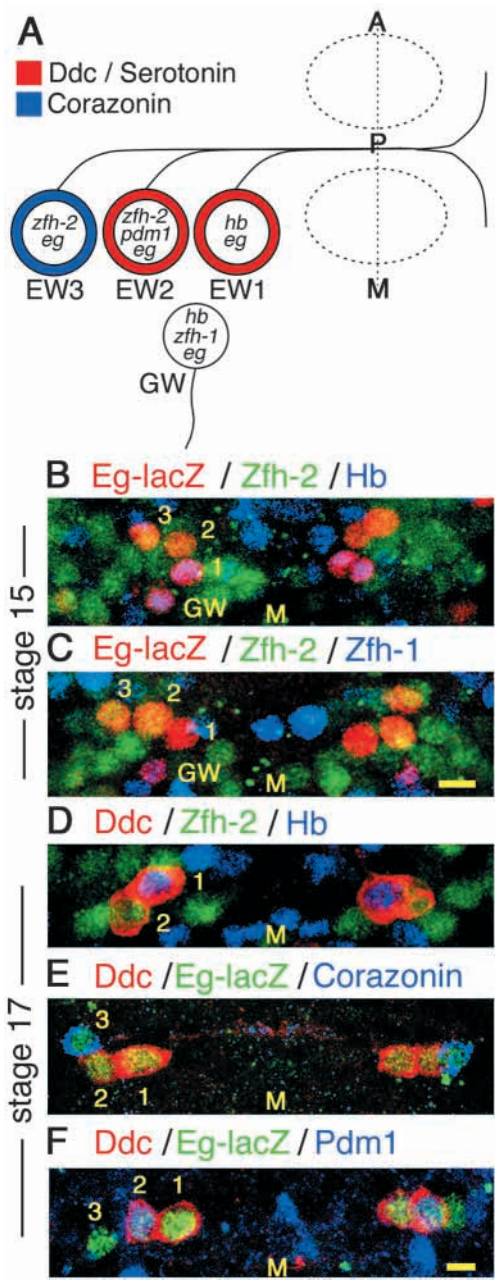

Fig. 1. Molecular markers uniquely identify all NB7-3 progeny neurons. (A) Schematic representation of the NB7-3 lineage showing molecular markers of the four neuronal progeny, axonal projections and positions relative to the midline (M), anterior commissure (A) and posterior commissure (P). Ddc and corazonin are cytoplasmic antigens; all others are nuclear. The yellow numbers 1, 2 and 3 label the EW1, EW2 and EW3 neurons, respectively. M indicates the midline. (B,C) One abdominal segment from the ventral cord of stage 15 eg^{289}/+ embryos, shows the four Eg-*lacZ* positive neurons of the NB7-3 lineage. The EW2 and EW3 neurons express Zfh-2 (orange cells). (B) The GW and EW1 neurons express Hb (purple cells). (C) The GW neuron expresses Zfh-1 (purple cell). (D-F) One abdominal segment from the ventral cord of stage 17 eg^{289}/+ embryos. (D) The two serotonergic neurons (1, 2) express Ddc and can be distinguished by their differential expression of Hb and Zfh-2. (E) The EW3 neuron expresses corazonin. (F) The EW2 neuron expresses Pdm1. The GW neuron is usually not detectable at stage 17. Scale bars: in C, 5 μm for B,C; in F, 5 μm for D-F.

lateral cell and is unique in its production of the neuropeptide corazonin (Fig. 1E). At stage 15, Pdm1 has been reported to be expressed in several different progeny (Isshiki et al., 2001; Novotny et al., 2002), but by stage 17, its expression is limited to EW2 (Fig. 1F) (Lundell and Hirsh, 1998). Not all of these markers are expressed simultaneously during development. Eg expression begins to decline just as Ddc and corazonin become detectable. Eg-*lacZ*, Hb, Zfh-2 and Pdm1 expression persists to stage 17 and into larval stages. By stage 17 the GW neuron becomes undetectable with Eg-*lacZ* (Fig. 1E,F).

Numb is necessary for development of GMC-2 and GMC-3 progeny

As the GMC divisions of NB7-3 appear to be asymmetric, we asked whether Numb might have a role in specifying cell fates within this lineage. We found that in the mutant allele $numb^1$, the development of EW2 and EW3 was dramatically altered, but there was only a mild effect on the development of EW1 (Fig. 2). Comparison of stage 17 CNS immunoassayed for Ddc/Zfh-2/Hb from wild-type (Fig. 2A,A′) and $numb^1$ (Fig. 2B,B′) embryos showed that the number of immunoreactive Ddc cells decreased from two per hemisegment in a wild-type CNS to one per hemisegment in a $numb^1$ CNS (Table 1C). The Ddc cells that remained in $numb^1$ mutants were immunoreactive for Hb (95%, n=65), indicating they were EW1 cells (Fig. 2B). The EW1 cell was detectable in most hemisegments, but in 7% of the $numb^1$ mutant hemisegments there were no detectable Ddc cells (Fig. 2D, Fig. 4D and Table 1C). Comparison of stage 17 CNS immunoassayed for Ddc/Eg-*lacZ*/corazonin from wild-type (Fig. 2C) and $numb^1$ (Fig. 2D) embryos showed that no corazonin-positive EW3 cells were detectable in the $numb^1$ mutant (Table 1D). Not only were EW2 and EW3 undetectable with Ddc and corazonin in a $numb^1$ mutant, but they also failed to express Eg-*lacZ* at stage 17. In a wild-type CNS three cells per hemisegment were detected with Eg-*lacZ*, whereas in a $numb^1$ mutant CNS only one cell per hemisegment was detected with Eg-*lacZ* (Fig. 2C,D and Table 1E). These data indicate that in a $numb^1$ mutant development of the EW1 neuron proceeds normally, but the development of the EW2 and EW3 neurons is significantly altered.

Previous studies have shown that *numb* mutants alter cell fates but do not usually affect cell divisions. To examine whether NB7-3 divides normally in a $numb^1$ mutant we examined the lineage in stage 15 embryos (Fig. 3). In a wild-type stage 15 hemisegment there were four Eg-positive cells (Fig. 3A). In a $numb^1$ mutant 66% of the hemisegments had only two Eg-positive progeny that were both Hb positive, the EW1 and GW neurons (Fig. 3B and Table 1A). In 22% of the hemisegments there was a third Eg positive cell that expressed reduced levels of Zfh-2 (Fig. 3C and Table 1A). This cell must be a derivative of GMC-2 or GMC-3, however, by stage 17 only 3% of hemisegments had an EW2 cell (Table 1C) and 0% of hemisegments had an EW3 cell (Table 1D). These results suggest that in the $numb^1$ mutant at least some or possibly all divisions of GMC-2 and GMC-3 do occur, but the progeny of these divisions quickly take an alternate fate that renders them undetectable by several different molecular markers.

As mentioned previously, 7% of $numb^1$ hemisegments had no detectable Ddc cells at stage 17 (Table 1C). Interestingly, at stage 15, 10% of $numb^1$ hemisegments show two Eg cells that

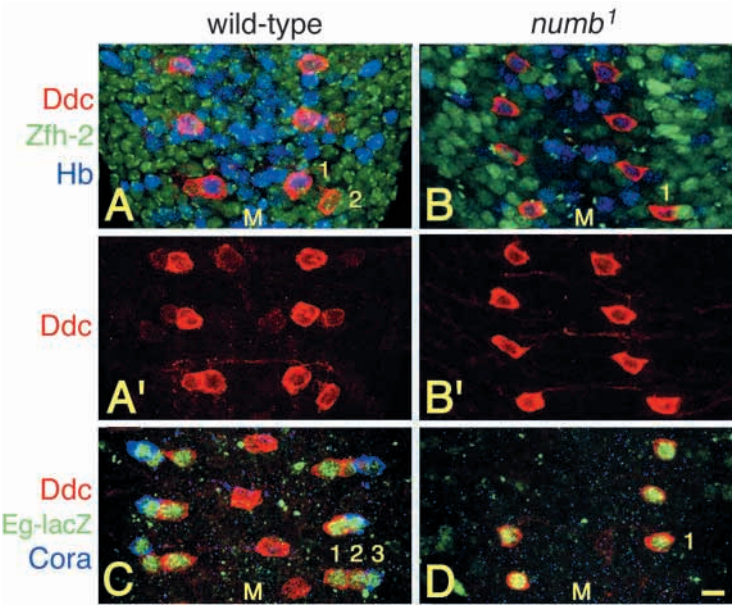

Fig. 2. Numb is required for development of EW2 and EW3. All images are ventral cord abdominal segments from stage 17 embryos. The yellow numbers 1, 2 and 3 label the EW1, EW2 and EW3 neurons, respectively. M indicates the midline. (A,B) Immunostaining with Ddc (red), Zfh-2 (green) and Hb (blue) shows a preferential loss of EW2 cells in embryos homozygous for *numb*^{1}. The remaining Ddc-positive cells in *numb*^{1} are Hb positive, indicating they are EW1 cells. (A′-B′) Same images as (A,B), but showing only Ddc immunoreactivity. (C,D) Immunostaining with Ddc (red), Eg-*lacZ* (green) and corazonin (blue), showing a reduction of Eg-*lacZ* in *numb*^{1} relative to wild type. Corazonin expression is completely absent in *numb*^{1}. Midline dopaminergic cells (M) are visible in wild type but are undetectable in *numb*^{1}. Scale bar in D: 5 μm for A-D.

both express Zfh-1 (Fig. 3J and Table 1B). Wild-type hemisegments and most *numb*^{1} hemisegments only have one Zfh-1-positive cell, the GW neuron (Fig. 3H,I and Table 1B). This suggests that in a *numb*^{1} mutant, division of GMC-1 may sometimes produce two cells with the GW fate, rather than a single GW and EW1 cell. This 10% transformation in cell fate to two GW cells, may account for the 7% of hemisegments that showed no detectable EW1 cell with Ddc immunoreactivity. Taken together, these results indicate that the differentiation of GMC-1 progeny is mostly independent of Numb function, whereas Numb is essential for the differentiation of GMC-2 and GMC-3 progeny.

Table 1. Statistical analysis of molecular marker expression in the NB 7-3 lineage

		Number of of immunoreactive cells/hemisegment								
Antibody/stage	Genotype	0	1	2	3	4	5	6	*n*	Mean+s.e.m.
A										
Eagle/stage 15	Wild type	–	–	2.6%	18.5%	77.5%	1.3%	–	151	3.77+0.04
	numb^{1}/*numb*^{1}	1.8%	9.9%	66.1%	22.2%	–	–	–	171	2.09+0.05
	UAS-Notch^{ACT}	–	10.9%	69.6%	15.2%	4.3%	–	–	92	2.13+0.07
	spdo^{G104}/*spdo*^{G104}	–	–	–	8.8%	41.2%	38.2%	11.8%	34	4.53+0.14
	UAS-P35	–	–	–	1.9%	41.5%	46.2%	10.3%	106	4.65+0.07
	UAS-numb	–	–	–	2.1%	56.3%	38.5%	3.1%	96	4.43+0.06
B										
Zfh-1/stage 15	Wild type	20.3%	79.7%	–	–	–	–	–	79	0.80+0.05
	numb^{1}/*numb*^{1}	–	90.5%	9.5%	–	–	–	–	84	1.10+0.03
	UAS-Notch^{ACT}	–	10.8%	89.2%	–	–	–	–	83	1.89+0.03
C										
Ddc/stage 17 or third instar	Wild type	–	19.0%	81.0%	–	–	–	–	290	1.81+0.02
	numb^{1}/*numb*^{1}	7.1%	89.5%	3.4%	–	–	–	–	294	0.96+0.02
	UAS-Notch^{ACT}	90.3%	9.0%	0.7%	–	–	–	–	996	0.10+0.01
	spdo^{G104}/*spdo*^{G104}	–	–	5.2%	42.1%	47.4%	5.3%	–	19	3.52+0.16
	UAS-p35	1.9%	2.9%	89.4%	5.8%	–	–	–	104	1.99+0.04
D										
Corazonin/stage 17 or third instar	Wild type	2.8%	97.2%	–	–	–	–	–	72	0.97+0.02
	numb^{1}/*numb*^{1}	100%	–	–	–	–	–	–	54	0.00+0.00
	UAS-Notch^{ACT}	99.7%	0.3%	–	–	–	–	–	624	0.00+0.00
	spdo^{G104}/*spdo*^{G104}	46.7%	53.3%	–	–	–	–	–	15	0.53+0.52
	UAS-p35	5.0%	86.0%	9.0%	–	–	–	–	100	1.04+0.04
E										
Eg-*lacZ*/stage 17	Wild type	–	1.3%	36.7%	55.1%	5.7%	1.3%	–	158	2.69+0.05
	numb^{1}/*numb*^{1}	8.5%	82.3%	7.1%	2.1%	–	–	–	141	1.03+0.04

For each genotype, hemisegments were counted for the number of NB7-3 cells that expressed the specific antigen. The results are presented as a percentage of total number of hemisegments counted and as an average number of cells per hemisegments. Abdominal segments 1-7 were counted for all antigens except corazonin, where wild-type expression is only found in segments 1-6. *UAS-Notch*^{ACT} and *UAS-p35* were activated by *eg-gal4*.

(A,B) Eg and Zfh-1 expression was assayed in stage 15 embryonic CNS.

(C,D) Ddc and corazonin expression was assayed in stage 17 embryonic CNS for the *numb*^{1} and *spdo*^{G104} genotypes and in third instar larval CNS for the other three genotypes.

(E) Eg-*lacZ* expression was assayed in stage 17 embryonic CNS.

numb^{1} alters the identity of the serotonergic cell in segment A8

In a wild-type CNS, there is a pair of serotonergic neurons within each hemisegment, with two exceptions: in A8, the most posterior abdominal segment, there is a single serotonergic cell; and in T1, the most anterior thoracic segment, there is a triplet of serotonergic cells (Fig. 4A) (Valles and White, 1988). We found that in a *numb*^{1} mutant there is a change in the identity of the A8 serotonergic cell. In a wild-type animal the single cell in A8 expressed Zfh-2, indicating that it has characteristics similar to EW2 cells (Fig. 4B). In a *numb*^{1} mutant, there was also a single cell in A8, but it expressed Hb similar to an EW1 cell (Fig. 4E) and similar to the more anterior segments of a *numb*^{1} mutant (Fig. 2B).

Examination of A8 in a wild-type embryo, at stage 15, showed three Eg immunoreactive cells. Two of the cells expressed Hb and one expressed Zfh-2 (Fig. 4C). The Zfh-2 expressing cell presumably develops into the Ddc-expressing neuron. EW3 cells were not detected with corazonin in either segments 7 or 8 (Fig. 5A), suggesting that GMC-3 does not form in these segments. These results suggest that although GMC-1 and GMC-2 both produce progeny in A8, only EW2 develops into a serotonergic neuron and the development of EW1 into a serotonergic neuron is suppressed.

Examination of A8 in a *numb*^{1} embryo, at stage 15, also had three Eg immunoreactive cells. Hb expression was unaltered in two of the cells, but Zfh-2 expression was dramatically reduced in the third (Fig. 4F). These results suggest that the loss of Numb function in A8 prevents EW2 from developing into a mature serotonergic cell, just as it does in the more anterior segments. Furthermore, the loss of Numb function apparently induces GMC-1 to produce a Ddc expressing cell in A8 when it normally would not. This suggests that the EW1 fate is a default state in all hemisegments of a *numb*^{1} mutant.

Ectopic expression of *Notch* disrupts the differentiation of NB7-3 progeny

As Numb has previously been shown to inhibit Notch signaling, we next asked whether ectopic expression of Notch within the NB7-3 lineage would produce a similar phenotype to the *numb*^{1} mutant. Ectopic expression of Notch was achieved using the UAS/Gal4 system (Brand and Perrimon, 1993), where the expression of a *UAS-Notch*^{ACT} transgene was driven by an *eg-gal4* transgene. The extracellular domain of Notch is absent in the *Notch*^{ACT} allele, thus this allele provides constitutive Notch activity (Doherty et al., 1996). In *UAS-Notch*^{ACT} embryos and larvae, very few Ddc- and corazonin-containing cells were detected (Fig. 5C and Table 1C,D). This phenotype is extremely penetrant, most CNS show no serotonergic or corazonin-containing cells, the example presented in Fig. 5C is an exception. Some of these flies actually eclosed as adults, but died within several days. This phenotype is similar to *numb*^{1} in that EW2 and EW3 are undetectable, but is different in that EW1 is also undetectable.

The number of Eg immunoreactive cells detectable in the *UAS-Notch*^{ACT} allele at stage 15 was variable (Fig. 3D,K; Table 1A). Four percent of hemisegments showed four Eg-positive cells, with the normal distribution of Hb and Zfh-2 expression (Fig. 3D). Fifteen percent of hemisegments showed three Eg-positive cells, but most hemisegments showed two Eg-positive cells (Fig. 3D). This variable number of Eg immunoreactive cells suggests that the NB7-3 lineage divisions occur when Notch is ectopically expressed, but that the cells quickly follow an alternative fate that makes them undetectable, similar to the

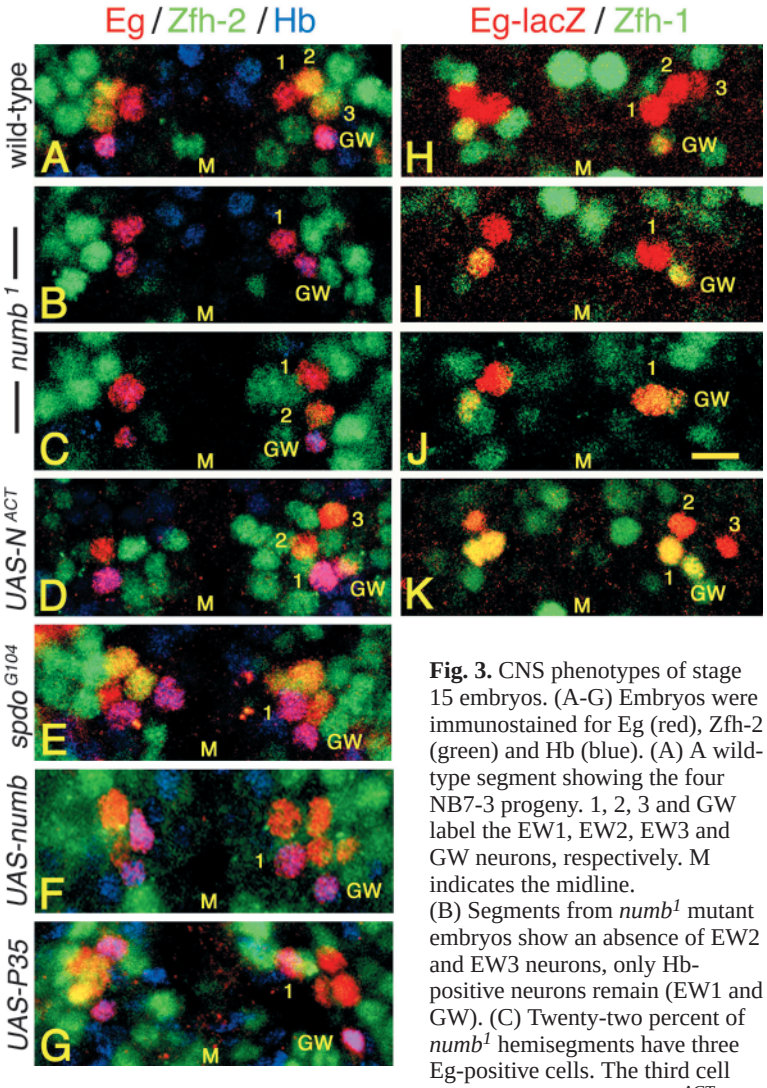

Fig. 3. CNS phenotypes of stage 15 embryos. (A-G) Embryos were immunostained for Eg (red), Zfh-2 (green) and Hb (blue). (A) A wild-type segment showing the four NB7-3 progeny. 1, 2, 3 and GW label the EW1, EW2, EW3 and GW neurons, respectively. M indicates the midline. (B) Segments from *numb*^{1} mutant embryos show an absence of EW2 and EW3 neurons, only Hb-positive neurons remain (EW1 and GW). (C) Twenty-two percent of *numb*^{1} hemisegments have three Eg-positive cells. The third cell expresses Zfh-2 at reduced levels relative to wild type. (D) *UAS-Notch*^{ACT}*: eg-gal4* embryos shows a variable pattern, some hemisegments are identical to the wild-type pattern and some hemisegments having a reduced number of Eg-positive cells. (E-G) Hemisegments from *spdo*^{G104}*, UAS-numb:eg-gal4 and eg-gal4/UAS-p35* embryos show a similar phenotype of five to six Eg-positive cells. The ectopic cells express Zfh-2. (H-K) Embryos were immunostained with Eg (red) and Zfh-1 (green). (H) A wild-type segment showing the four NB7-3 progeny and expression of Zfh-1 in the GW neuron. (I,J) Hemisegments from *numb*^{1} mutant embryos show that the expression of Zfh-1 in GW is unaltered. J shows an example of a *numb*^{1} hemisegment were both remaining cells express Zfh-1, indicating a transformation of EW1 to a GW cell fate. (K) *UAS-Notch: eg-gal4* embryos show a variable number of cells similar to D and two Zfh-1 staining cells indicating a transformation of EW1 to a GW cell fate. Scale bar in J: 5 μm.

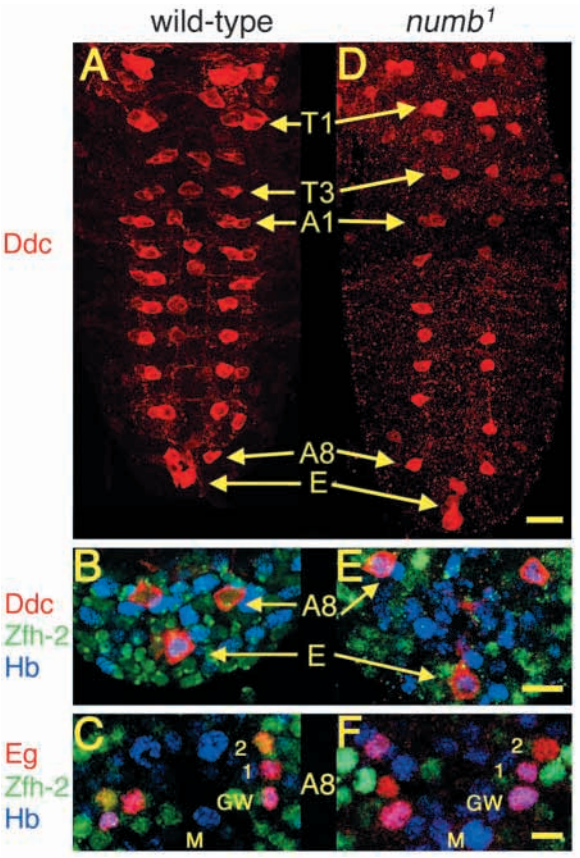

Fig. 4. Comparison of serotonergic cells in segment A8 from wild-type and *numb*^{1} mutant embryos. (A,D) Ddc immunoreactivity of stage 17 embryos showing the entire ventral cord and demonstrating that A8 cells are not absent in *numb*^{1} embryos. T1-T3 are thoracic segments. A1-A8 are abdominal segments. E is the embryonic posterior Ddc cluster that is only visible during embryogenesis. (B,E) A magnification of A8 from the same CNS as in A,D, showing immunoreactivity of Ddc (red), Zfh-2 (green) and Hb (blue). The single Ddc-expressing cell in A8 shows Zfh-2 expression in wild-type and Hb expression in *numb*^{1} embryos. (C,F) Stage 15 embryos immunostained with Eg (red), Zfh-2 (green) and Hb (blue). (C) A wild-type A8 hemisegment shows three Eg cells, two are Hb positive and one is Zfh-2 positive. (D) A *numb*^{1} A8 hemisegment shows that Eg and Hb expression are unaltered, but Zfh-2 expression is reduced. Scale bars: in D, 15μm for A,D; in E, 10 μm for B,E; in F, 5 μm for C,F.

numb^{1} phenotype. Interestingly, 89% of the *UAS-Notch*^{ACT} hemisegments had two cells that were immunoreactive for Zfh-1 (Fig. 3K and Table 1B). This suggests that most EW1 cells were converted to a GW cell fate in the *UAS-Notch*^{ACT} genotype. This would explain the inability to detect EW1 cells with Ddc immunoreactivity (Fig. 5C). This transformation in cell fate is similar to the 10% of hemisegments in the *numb*^{1} mutant that had two Zfh-1-positive cells. Therefore in both the *numb*^{1} and *UAS-Notch*^{ACT} genotypes, the number of hemisegments that have no detectable Ddc immunoreactivity is proportional to the number of hemisegments that show two Zfh-1 immunoreactive cells (Table 1B-C).

These results suggest that Notch signaling must be inactivated within the NB7-3 lineage for normal development of EW1, EW2 and EW3. As the previous results show that Numb is required for the development of EW2 and EW3, Numb is most probably responsible for repressing Notch signaling in EW2 and EW3. However, as the effect of a *numb*^{1} mutation on EW1 development is minor, some additional mechanism must be primarily responsible for repressing Notch signaling in EW1.

sanpodo^{G104} produces ectopic Ddc cells and can rescue the *numb*^{1} mutant phenotype

Because the ectopic expression of Notch produced a phenotype that inhibited development of the NB7-3 lineage, we next investigated the phenotype associated with a loss of *Notch* function in the NB7-3 lineage. To produce a loss of *Notch* function we used a mutant allele of *sanpodo* (*spdo*). *spdo* has been reported to be a tropomodulin-like molecule (Dye et al., 1998) but its functional capacity has not yet been shown. Previous experiments indicate that *spdo* mimics loss-of-function *Notch* mutations (Buescher et al., 1998; Dye et al., 1998; Park et al., 1998; Skeath and Doe, 1998; Ward and Skeath, 2000). It is advantageous to use a *spdo* mutation, instead of a *Notch* mutation, because *spdo* is not involved in Notch-mediated lateral inhibition during early neurogenesis and thus does not have the severe morphological defects of a *Notch* embryo (Salzberg et al., 1997).

In a homozygous *spdo*^{G104} mutant embryo at stage 15, 50% of the hemisegments had one or two ectopic cells immunoreactive for Eg (Fig. 3E and Table 1A). The additional cells were also immunoreactive for Zfh-2 suggesting that they are derivatives of GMC-2 or GMC-3. After stage 16 the homozygous *spdo*^{G104} mutant CNS became quite fragile and difficult to dissect, nevertheless we were able to isolate fragments of stage 17 CNS from *spdo*^{G104} mutants. These CNS showed random ectopic expression of Ddc cells and altered morphology. In the position of the NB7-3 lineage the thoracic clusters showed numerous Ddc immunoreactive cells (Fig. 5D), and there were three to five Ddc immunoreactive cells in each abdominal cluster (Fig. 5E and Table 1C). These clusters of Ddc-expressing cells showed two cells that were immunoreactive for Hb, with the remaining cells expressing Zfh-2 (Fig. 5F). A wild-type CNS has only one Ddc cell that expresses Hb (Fig. 1D), suggesting that in the *spdo*^{G104} mutant there is a transformation in cell fate such that, GMC-1 produces two EW1 cells, rather than a single EW1 cell and the GW cell. Corazonin immunoreactivity was poorly developed in *spdo*^{G104} mutants, only 50% of the hemisegments had a corazonin-immunoreactive cell (Table 1D) and there was no evidence of ectopic corazonin-containing cells. This phenotype of ectopic Ddc cells in *spdo*^{G104} is opposite to the absence of Ddc cells observed in *numb*^{1} and *UAS-Notch*^{ACT} phenotypes.

We then asked whether the *spdo*^{G104} allele could rescue the *numb*^{1} mutant phenotype. Because the *spdo*^{G104} homozygous phenotype is so severe, we attempted the rescue with just one copy of the *spdo*^{G104} allele. The *spdo*^{G104} heterozygote alone showed a phenotype that was similar to wild-type at stage 17 (Fig. 5G). When the *spdo*^{G104} allele was combined with the homozygous *numb*^{1} mutation we found evidence that EW2 and EW3 were partially rescued. 23% of hemisegments showed both EW1 and EW2 Ddc neurons and 7% of hemisegments showed an EW3 corazonin-containing neuron (Fig. 5H; Table 2). This is

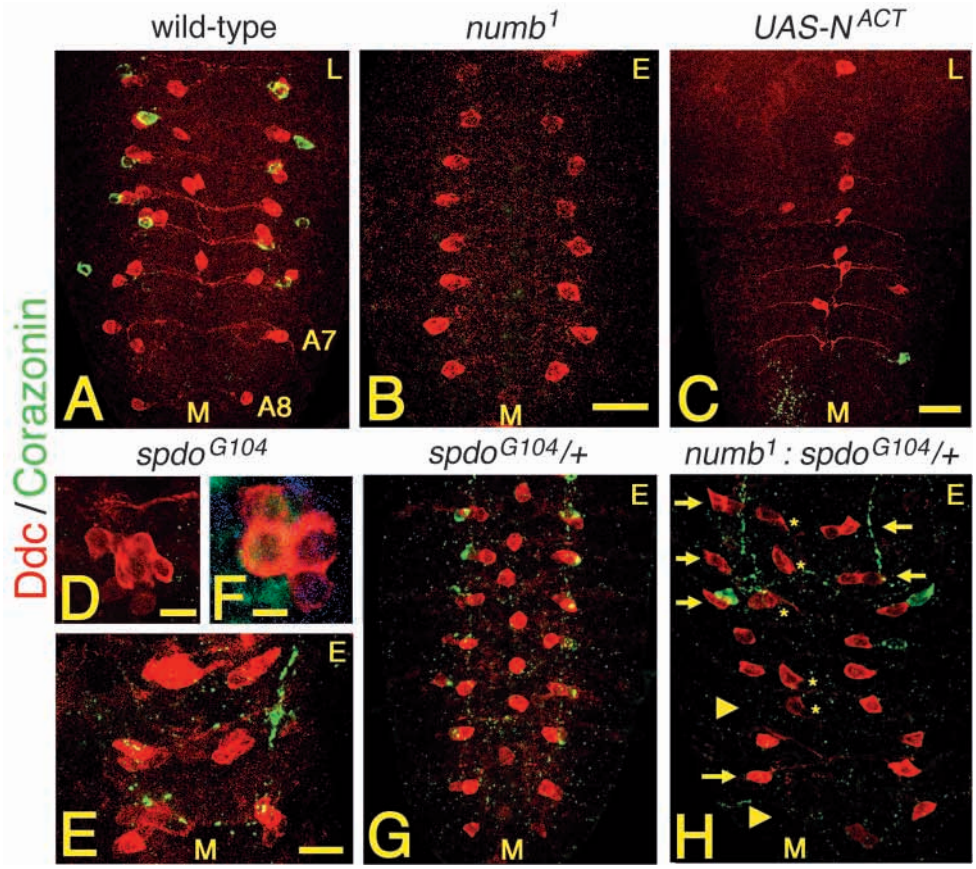

Fig. 5. Numb/Notch regulation of NB7-3 development. All panels with the exception of F show segments of the ventral cord immunostained for Ddc (red) and corazonin (green). (A) The wild-type larval pattern shows that corazonin is not expressed in segments A7 and A8. (B) *numb*^{1} mutant embryos show the absence of EW2 and EW3. (C) The *UAS-Notch: eg-gal4* larval pattern shows the absence of EW1, EW2 and EW3. (D-F) *spdo*^{G104} mutant embryos show ectopic Ddc-expressing cells, D is a thoracic hemisegment and E shows abdominal segments. (F) A *spdo*^{G104} mutant embryo immunostained for Ddc (red), Zfh-2 (green) and Hb (blue), shows two Ddc cells immunoreactive for Hb and three Ddc cells immunoreactive for Zfh-2. (G) A *spdo*^{G104} heterozygous embryo shows a pattern that is wild type but expression of markers in EW2 and EW3 is incomplete at this stage. (H) *numb*^{1}/*numb*^{1}:*spdo*^{G104}/+ embryos show rescue of the *numb*^{1} mutant phenotype. Arrows indicate hemisegments that have two Ddc-expressing cells, arrowheads indicate hemisegments with no Ddc-expressing cells (indicative of the *numb* phenotype) and asterisks mark the midline dopamine cells. E and L indicate whether the image is from a stage 17 embryo or third instar larva, respectively. M indicates the midline. Scale bar: in B, 15 μm for B,G,H; in C, 15 μm for A,C; in D, 10 μm for D; in F is 3 μm for F; in E, 10 μm for E.

a substantial increase over the number of hemisegments in the *numb*^{1} mutant that have a wild-type pattern of EW neurons. The ability of *spdo* to rescue the *numb*^{1} mutant phenotype demonstrates that *spdo* is epistatic to *numb*, which has been demonstrated in other lineages (Dye et al., 1998; Skeath and Doe, 1998; Ward and Skeath, 2000). These results imply that the function of Numb in the NB7-3 lineage is to inactivate Notch signaling during differentiation of EW2 and EW3 neurons.

We next asked whether ectopic expression of Numb would be sufficient to inhibit Notch signaling and produce a phenotype similar to the *spdo*^{G104} mutation. Ectopic expression of Numb was achieved using a *UAS-Numb* transgene driven by an *eg-gal4* transgene. At stage 15, *UAS-Numb*:*eg-gal4* embryos had one or two ectopic cells immunoreactive for Eg in 42% of the hemisegments, similar to the *spdo*^{G104} phenotype (Fig. 3F and Table 1A). However, at stage 17, we did not detect any ectopic Ddc or corazonin-containing cells using several different gal4 drivers to activate *UAS-Numb* (data not shown). Although *UAS-Numb* is sufficient to produce ectopic NB7-3 cells at stage 15, it is apparently insufficient to induce terminal differentiation of Ddc and corazonin-containing neurons. Taken together, the results presented thus far show that inhibiting Notch signaling with either *spdo*^{G104} or *UAS-Numb* produces ectopic cells in the NB7-3 lineage, whereas enhancing Notch signaling with either *numb*^{1} or *UAS-Notch*^{ACT} alters the fate of NB7-3 progeny so that they become undetectable with several different molecular markers.

Notch-mediated apoptosis in the NB7-3 lineage

Previous studies with the apoptosis deficient line *H99* have shown that supernumerary Eg cells were produced within the NB7-3 lineage (Isshiki et al., 2001; Novotny et al., 2002). This result suggests that there are cells within the NB7-3 lineage that undergo apoptosis. The ectopic NB7-3 progeny we observe by inhibiting Notch signaling with *spdo*^{G104} and *UAS-Numb* might be due to an inhibition of apoptosis and the rescue of cells in the NB7-3 lineage that normally undergo apoptosis. Conversely, the loss of NB7-3 progeny we observe by enhancing Notch signaling with *numb*^{1} or *UAS-Notch*^{ACT}, might be due to Notch-mediated apoptosis.

To test this hypothesis, we first examined the NB7-3 lineage for apoptosis using TUNEL analysis, which measures DNA fragmentation, a hallmark of programmed cell death. In wild-type embryos we examined the occasional hemisegments that had five immunoreactive Eg cells rather than the standard four Eg cells. In these hemisegments we found that the extra Eg-positive cell undergoes apoptosis (Fig. 6A,A′). This indicates that during the mitotic divisions of the NB7-3 lineage some cells are eliminated by apoptosis. In *numb*^{1} mutant embryos, we examined the hemisegments that had three immunoreactive Eg cells and found that the cell, which we previously showed to be Zfh-2 positive (Fig. 3C), underwent apoptosis (Fig. 6B). We also found a small fraction of *numb*^{1} hemisegments where the EW1 and GW neurons were apoptotic (Fig. 6C). It is likely that these hemisegments correspond to the 7% of *numb*^{1}

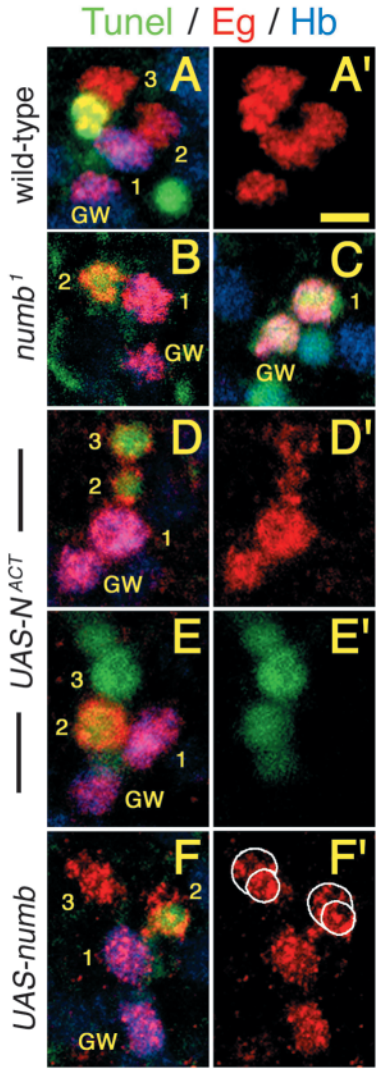

Fig. 6. TUNEL analysis of apoptosis in the NB7-3 lineage. Stage 15 embryos were immunostained for TUNEL (green), Eg (red) and Hb (blue). All panels are arranged with anterior at the top and the midline towards the right. (A-A′) A wild-type hemisegment showing an extra NB7-3 Eg cell that is apoptotic. (B) A *numb*^{1} hemisegment showing three Eg cells; the two GMC-1 progeny and an apoptotic cell. (C) A *numb*^{1} hemisegment showing apoptosis of the two GMC-1 progeny, these cells are immunostained for all three labels. (D-D′) A *UAS-Notch*^{ACT}*:eg-gal4* hemisegment showing four Eg cells; the two GMC-1 progeny and two apoptotic cells. (E-E′) A *UAS-Notch*^{ACT}*:eg-gal4* hemisegment showing six cells; the two GMC-1 progeny and four closely associated cells that are apoptotic and appear as mitotic pairs. Only one of the apoptotic cells maintains Eg expression. (F-F′) A *UAS-numb:eg-gal4* hemisegment showing six Eg cells; the two GMC-1 progeny and paired GMC-2 and GMC-3 progeny (outlined). Only one cell in this hemisegment is apoptotic. Scale bar: 5 μm.

hemisegments that have no detectable Ddc neurons at stage 17 (Table 1C and Fig. 2D, Fig. 4D). Thus, a loss of *numb* function initiates novel apoptosis within the NB7-3 lineage.

The *UAS-Notch*^{ACT}*:eg-gal4* mutant also induced apoptosis within the NB7-3 lineage. Fig. 6D,D′ show a hemisegment in which two Eg cells, that were not GMC-1 progeny, were TUNEL positive. Fig. 6E,E′ shows a hemisegment that had the two GMC-1 progeny and four closely associated TUNEL-positive cells. The four TUNEL cells are arranged in distinct pairs, suggesting they are mitotic siblings. These cells are most likely the GMC-2 and GMC-3 progeny. One of the four TUNEL cells is marked with Eg immunoreactivity, presumably apoptosis in the other three cells has either prevented or eliminated Eg expression. We did not find evidence of apoptosis in GMC-1 progeny of *UAS-Notch*^{ACT} embryos. Interestingly, we also found apoptosis in *UAS-Numb:eg-gal4* embryos. Fig. 6F,F′ show a *UAS-Numb* hemisegment that had six cells immunoreactive for Eg, where one of the GMC-2/3 progeny is apoptotic. Apparently, expression of *UAS-Numb* initially allows the recovery of ectopic Eg cells, but these cells eventually undergo apoptosis. This would explain the lack of ectopic Ddc and corazonin-containing cells in stage 17 *UAS-Numb* embryos. Taken together, the results of our TUNEL analysis shows that apoptosis occurs in the wild-type NB7-3 lineage and that novel apoptosis can be induced by either a loss of *numb* function or the ectopic expression of *Notch*.

We next addressed whether inhibition of apoptosis could produce ectopic Ddc and corazonin-containing neurons. We first examined the apoptosis deficient line *H99* (White et al., 1994), which had previously been shown to produce ectopic NB7-3 cells at stage 15 (Isshiki et al., 2001; Novotny et al., 2002). We found that *H99* mutant embryos showed a wild-type pattern of expression for Ddc and corazonin at stage 17 (data not shown). Apparently, the extra cells produced at stage 15 in *H99* mutants are unable to mature into Ddc- and corazonin-containing neurons. Therefore, we tried another approach and examined the effect of ectopic expression of the apoptosis inhibitor, p35. Ectopic expression of p35 was achieved using a *UAS-p35* transgene driven by an *eg-gal4* transgene. At stage 15, *UAS-p35*/*eg-gal4* embryos produced ectopic Eg cells in 56% of all hemisegments (Fig. 3G; Table 1A). This phenotype is similar to *H99* (Isshiki et al., 2001; Novotny et al., 2002), *spdo*^{G104} and *UAS-Numb* embryos (Fig. 3E-G; Table 1A). At stage 17, *UAS-p35/eg-gal4* embryos produced a pattern of Ddc and corazonin immunoreactivity that was mostly wild-type with occasional ectopic cells (Fig. 7A,D; Table 1C,D). Two corazonin-containing cells were found in 9% of the hemisegments instead of the normal one per hemisegment (Fig. 7A). Corazonin-containing cells were also found in A7, where corazonin is normally not expressed (data not shown). Triplets of Ddc-expressing cells were observed in 6% of the hemisegments (Fig. 7D). These ectopic cells suggest that p35 can, to a limited degree, rescue cells of the NB7-3 lineage that would normally undergo apoptosis.

We next examined whether ectopic expression of p35 could rescue the *numb*^{1} mutant phenotype. We found partial rescue of the *numb*^{1} phenotype (Fig. 7B); 28% of hemisegments showed at least two Ddc neurons and 34% of hemisegments showed at least one corazonin-containing neuron (Table 2). Interestingly, A8 showed two Ddc cells (Fig. 7B), presumably the EW1 cell was present due to the *numb*^{1} mutation and the EW2 cell was rescued with p35. As the *numb*^{1} phenotype can be rescued both by the inhibition of apoptosis with p35 and by the inhibition of Notch signaling with *spdo*^{G104}, we hypothesize that in a wild-type animal Numb inhibits both Notch signaling and apoptosis in the NB7-3 lineage.

To test directly if Notch signaling induces apoptosis within the NB7-3 lineage, we examined whether inhibiting apoptosis with ectopic expression of p35 would rescue the phenotype produced by the ectopic expression of *Notch*. When both *UAS-p35* and *UAS-Notch*^{ACT} were expressed simultaneously with *eg-gal4*, p35 was able to protect cells from Notch-induced apoptosis (Fig. 7C). However, only 18% (n=146) of larval CNS showed the rescued phenotype depicted in Fig. 7C, the majority of CNS appeared identical to the *UAS-Notch*^{ACT} phenotype alone (Fig. 5C). Of the larval CNS that showed rescue there were no patterns of partial expression (Table 2), all hemisegments showed the wild-type number of Ddc- and corazonin-expressing cells with occasional ectopic cells. This all-or-nothing phenotype suggests that there is some global trigger that determines whether *UAS-Notch*^{ACT} or *UAS-p35* will

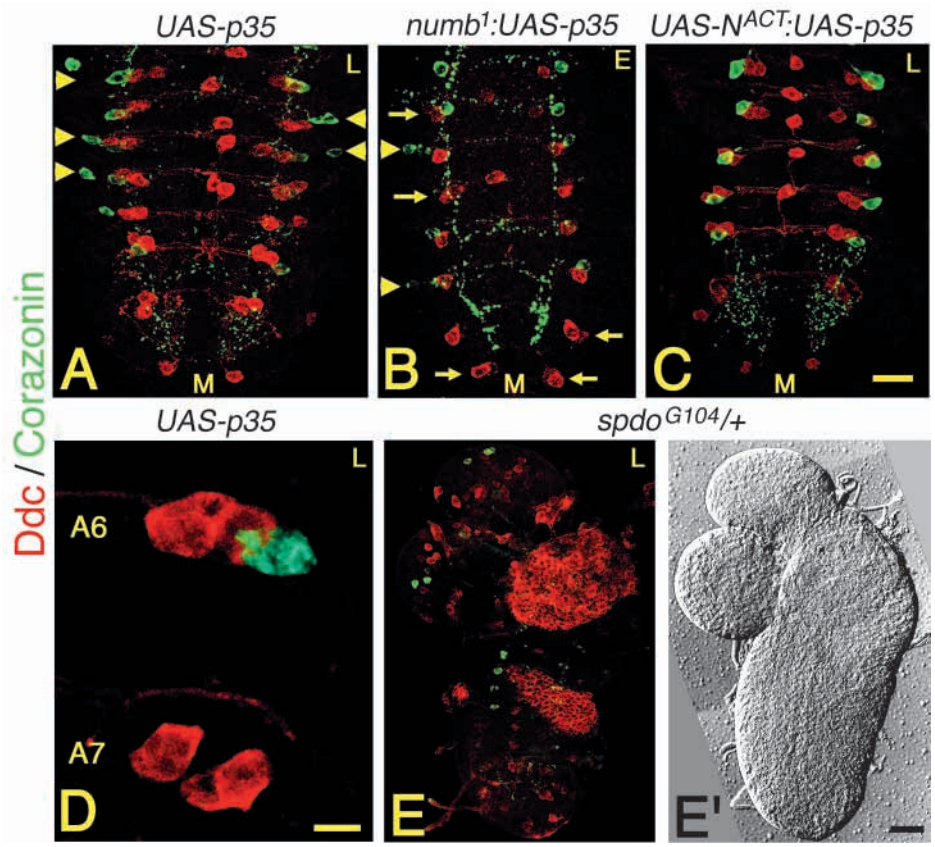

Fig. 7. *Notch*-mediated apoptosis in the NB7-3 lineage. (A-D) Abdominal segments of the ventral cord showing immunoreactivity of Ddc (red) and corazonin (green). E and L indicate whether the image is from a stage 17 embryo or a larva, respectively. M indicates the midline. (A) A *eg-gal4/UAS-p35* third instar larval nerve cord shows a wild-type pattern of cells plus ectopic corazonin-containing cells marked with arrowheads. (B) A *numb*^{1}*/numb*^{1}*:eg-gal4/UAS-p35* embryos show evidence that p35 can rescue the *numb*^{1} mutant phenotype. Arrows indicate hemisegments that have two Ddc-expressing cells, and the arrowheads marks a hemisegment with ectopic corazonin-containing cells. A8 shows two Ddc-expressing cells. (C) A *UAS-Notch*^{ACT}*/+:eg-gal4/UAS-p35* third instar larval nerve cord has a pattern identical to wild type. (D) A *en-gal4/+:UAS-p35/+* third instar larval CNS shows two abdominal hemisegments with triplets of Ddc-expressing cells. (E-E′) An entire *spdo*/+ first instar larval CNS showing overproliferation of Ddc expressing cells. E′ is a DIC image of the same CNS as in E, showing the top surface and a protrusion that matches the largest area of cell proliferation in E. Scale bars: in C, 15 μm for A-C; in D, 5 μm for D; in E′, 30 μm for E,E′.

Table 2. Quantification of rescue experiments

Genotype	% of hemisegments (actual number) Ddc (EW1 + EW2)	Corazonin (EW3)
numb^{1}*/numb*^{1}	3.4 (294)	0 (54)
numb^{1}*/numb*^{1} : *spdo*^{G104}*/+*	23 (244)	7 (244)
numb^{1}*/numb*^{1} : *UASp35/eg-gal4*	28 (64)	34 (64)
UAS-N^{ACT}*/+* : *eg-gal4/+*	0.7 (996)	0.3 (624)
UAS-N^{ACT}*/+* : *eg-gal4/UASp35*	97 (64)	98 (54)

For each genotype, abdominal hemisegments were counted for the number of NB7-3 cells that had at least two Ddc-expressing cells and at least one corazonin cell. The results are presented as a percentage of total number of hemisegments counted. Stage 17 embryos were analyzed in the rescue of the *numb*^{1} phenotype. Third instar larvae were analyzed in the rescue of the *UAS-Notch*^{ACT} phenotype. The *UAS-Notch*^{ACT}*/+:eg-gal4/UASp35* genotype produces two distinct CNS phenotypes, those that show rescue and those that look like *UAS-Notch*^{ACT} phenotype. Only the fraction of CNS that showed evidence of rescue were counted.

have the dominant effect on development of the NB7-3 lineage. When *en-gal4* was used to drive expression of both transgenes, 100% (*n*=14) of larval CNS showed the rescued wild-type pattern. *eg* expression fades during late embryogenesis, whereas *en* expression persists into larval stages. Therefore the extended expression of *en-gal4* may maintain sufficient levels of p35 to prevent apoptosis, whereas declining expression of *eg-gal4* may cause levels of p35 to drop such that Notch-induced apoptosis predominates.

Our results suggest that Notch-induced apoptosis is an essential mechanism for regulating cell fates within the CNS. In support of this hypothesis, we observed in heterozygous *spdo*^{G104} larval CNS, tumor-like overproliferation of Ddc-expressing cells that protrude from the surface (Fig. 7E,E′). These areas of cell growth appeared in random positions throughout the CNS and varied in size and frequency. Ten percent of the CNS showed regions of cell proliferation as large as those in Fig. 7F and another 30% of the CNS showed smaller foci of cell proliferation. We do not know the origin of these cells, but because we have seen overproliferation in the brain lobes they cannot originate exclusively from the NB7-3 lineages. They may not even originate from Ddc-expressing lineages, as induction of Ddc expression is a characteristic of human small-cell lung carcinomas (Bepler et al., 1987). This observation that a reduction in Notch signaling can promote abnormal cell growth, suggests that Notch-induced apoptosis may have an important role in preventing overproliferation of cells during CNS development.

DISCUSSION

The results of this study demonstrate that the intercellular Notch signaling pathway can be modulated during terminal divisions of the CNS to direct a choice between neuronal development and programmed cell death. Fig. 8 summarizes our results and presents a model of how Numb/Notch signaling may be used to specify cell fates in the NB7-3 lineage.

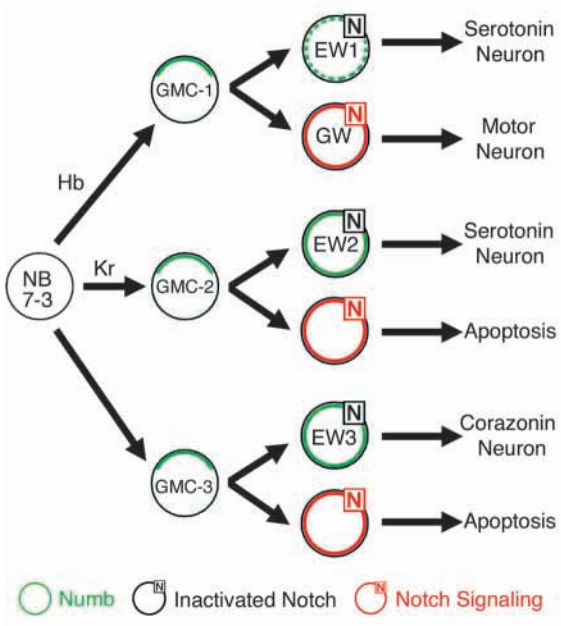

Fig. 8. Specification of NB7-3 cell fates by Numb/Notch signaling. GMC terminal divisions in the NB7-3 lineage are asymmetric, with only one daughter cell receiving Numb protein. Daughter cells that receive Numb inactivate Notch signaling, which leads to a neuronal cell fate. The broken green line in EW1 indicates that Numb is not an absolute requirement for this cell fate. In the progeny that do not receive Numb, Notch signaling is maintained. In GMC-1 progeny, Notch signaling induces the differentiation of the GW motoneuron. In GMC-2 and GMC-3 progeny, Notch signaling activates apoptosis. Previous work has shown that Hb is necessary for development of the GMC-1 lineage and that Kr is necessary for development of the GMC-2 lineage (Isshiki et al., 2001; Novotny et al., 2002).

The division of GMC-1 produces two distinct neuronal cell fates: the EW1 interneuron and the GW motoneuron. In this division, genetic alteration in the expression of *Notch* leads to switching between these two cell fates. A loss of Notch activity in *spdo* mutants leads to two Ddc/Hb-expressing EW1 cells and the overexpression of Notch leads to two Zfh-1 expressing GW cells. Therefore, Notch signaling must be inactivated during development of the EW1 neuron. Numb appears to have a minor role in this inactivation. In a $numb^{1}$ mutant, 7% of the hemisegments do not develop an EW1 neuron, and a similar number of $numb^{1}$ hemisegments show two Zfh-1-expressing GW cells. This transformation from an EW1 cell fate to a GW cell fate is what one would expect if Numb were inhibiting Notch. However, most EW1 neurons develop normally in a $numb^{1}$ mutant and do not convert to the GW cell fate. Therefore, inactivation of Notch signaling in EW1 is mostly independent of Numb function. One possible explanation is that EW1 has a factor that is redundant for Numb function, which can inhibit Notch signaling and is capable of masking the effect of a $numb^{1}$ mutation in most hemisegments. The unique expression of Hb in GMC-1 progeny could be responsible for establishing this redundancy. However, if a redundant Numb-like factor does exist, it is insufficient to protect EW1 during expression of the $UAS\text{-}Notch^{ACT}$ transgene.

The A8 segment is unique in that it only has a single serotonergic neuron instead of the pair of serotonergic neurons found in the more anterior segments. In a wild-type fly, this cell appears to be a derivative of GMC-2, because it expresses Zfh-2, but in a $numb^{1}$ mutant, this cell appears to be a derivative of GMC-1, because it expresses Hb. A single Hb/Ddc-expressing cell in A8 is identical to the phenotype in the more anterior segments of a $numb^{1}$ mutant. This suggests that in a $numb^{1}$ mutant an EW1 cell is the default developmental pathway for this lineage. One possibility is that the redundant Notch-inactivating mechanism we proposed for EW1 is only induced in the presence of a *numb* mutation. This would explain why the A8 EW1 cell is only seen in the *numb* mutant and not in a wild-type animal. If this were true, then the preservation of EW1 cells in a *numb* mutant would be due to the mutation itself. Until a putative redundant factor is identified it is impossible to determine whether it is expressed normally in wild-type animals or is expressed only in *numb* mutant animals.

Like GMC-1, most GMCs divide producing two progeny cells. However, GMC-2 and GMC-3 of the NB7-3 lineage only produce one neuron. It has previously been suggested that the mitotic sisters of EW2 and EW3 may undergo apoptosis (Isshiki et al., 2001; Novotny et al., 2002). This idea is supported by our detection of apoptotic cells with TUNEL in the wild-type NB7-3 lineage and our experiments with the apoptosis inhibitor p35, which can produce ectopic Ddc and corazonin-containing cells. The origin of the ectopic cells within NB 7-3 has not been formally determined by lineage tracing; however, the hypothesis that they are mitotic sisters of EW2 and EW3 is supported by the observations that GMC-2 and GMC-3 progeny often appear as mitotic pairs (Fig. 6E,F) and that ectopic NB7-3 cells are immunoreactive for Zfh-2 (Fig. 3E-G).

During the divisions of GMC-2 and GMC-3, genetic alterations in the expression of Notch leads to a switching between a neuronal cell fate and apoptosis. A reduction of Notch signaling with either $spdo^{G104}$ or *UAS-Numb* embryos produces ectopic NB7-3 cells that express Zfh-2. Conversely, the overexpression of Notch in either $UAS\text{-}Notch^{ACT}$ or $numb^{1}$ embryos led to an increase in TUNEL labeling of GMC-2 and GMC-3 progeny. Additionally, inhibiting apoptosis with *UAS-p35* or reducing Notch activity with $spdo^{G104}$ can rescue the $numb^{1}$ phenotype. We hypothesize that during the divisions of GMC-2 and GMC-3, Numb partitions asymmetrically into EW2 and EW3 where it inactivates Notch signaling and leads to neuronal development. The mitotic sisters of EW2 and EW3 do not receive Numb, maintain Notch signaling and undergo apoptosis. The difficulty in detecting wild-type hemisegments that have more than four immunoreactive Eg cells, suggests that any other cells produced during divisions of the NB7-3 lineage quickly undergo apoptosis.

Ectopic Eg cells in the NB7-3 lineage can be induced at stage 15 by *H99* (Isshiki et al., 2001; Novotny et al., 2002), *UAS-Numb*, $spdo^{G104}$ and *UAS-p35* (Fig. 3E-G). However, the ability of these alleles to produce ectopic Ddc and corazonin-containing neurons at later stages is variable. We were unable to detect significant ectopic Ddc or corazonin-containing cells in either *H99* or *UAS-Numb* CNS. We have shown for *UAS-Numb* that the ectopic Eg cells detected at stage 15 can undergo apoptosis. $spdo^{G104}$ mutants produce only ectopic Ddc cells, but the reduction in the number of corazonin-containing cells in general suggests that either GMC-3 does not consistently

form in these mutants or that GMC-3 progeny may convert from a corazonin-containing cell fate to a serotonergic cell fate. *UAS-p35* mutants produce both ectopic Ddc and corazonin-containing cells at low frequency, but the allele is much more efficient at rescuing the EW neurons in *numb*^{1} and *UAS-Notch* mutants. Therefore, apoptosis is harder to reverse in cells that normally undergo apoptosis, than in the cells genetically induced to undergo apoptosis. The ability of these various alleles to produce ectopic Ddc- and corazonin-containing cells could be influenced by mutant affects they cause outside the NB7-3 lineage or may reflect different roles they have in the apoptotic pathway. The mechanism by which Notch induces apoptosis in the NB7-3 lineage remains to be determined, but the apoptotic genes *reaper*, *grim* and *hid* may be involved because all three of these genes are deleted in the *H99* allele (White et al., 1994).

Notch-induced apoptosis in the NB7-3 lineage will probably be regulated by other factors in addition to Numb. The Ras signaling pathway has been shown to inhibit Notch-induced apoptosis in the *Drosophila* pupal retina (Cagan and Ready, 1989). Wingless has been shown to mediate Notch signaling (Axelrod et al., 1996; Brennan et al., 1999; Wesley, 1999; Lawrence et al., 2001; Ramain et al., 2001) and mutations in the Wingless pathway can lead to ectopic serotonergic cells (Patel et al., 1989). It will be a challenge to determine how these different signaling pathways interact to specify apoptosis within the NB7-3 lineage.

The tumor-like expansion of Ddc-expressing cells we observe in heterozygous *spdo*^{G104} larvae suggests that Notch-induced apoptosis may be essential for regulating cell proliferation. This *spdo* phenotype is reminiscent of three tumor-suppressor genes; *discs large* (*dlg*), *lethal giant larvae* (*lgl*) and *scribble* (*scrib*), which produce tumors in the CNS and imaginal disks (Gateff, 1978; Manfruelli et al., 1996; Woods et al., 1996; Bilder et al., 2000). Interestingly, these three genes work in a common pathway that regulates cell polarity, and *lgl* and *dlg* have been shown to be essential for the distribution of Numb and other asymmetric determinants (Ohshiro et al., 2000; Peng et al., 2000). Further investigation will be necessary to determine if *spdo* is part of this same mechanism and exactly how *spdo* mutants inhibit Notch signaling. Spdo expression is ubiquitous throughout embryogenesis and persists through the larval stages and into adults (Dye et al., 1998). If a *spdo* mutation can alter the response of the Notch receptor to environmental cues that induce apoptosis, one would expect to see overproliferation in additional tissues.

Finally, although not discussed explicitly, our figures demonstrate that Numb/Notch signaling also affects development of the midline dopaminergic cells. The expression of Ddc is essential to the biosynthesis of both serotonin and dopamine. In the images presented, anti-Ddc antibody detects not only the serotonergic neurons, but also midline dopamine neurons. As a consequence of using Ddc as a marker for the serotonin lineage, a number of observations could be made about the development of midline dopamine cells. We found that in a *numb*^{1} mutant very few midline dopamine cells were detectable with Ddc (Fig. 2, Fig. 4D, Fig. 5B). *spdo*^{G104} mutants produce ectopic dopamine cells (data not shown) and can rescue dopamine cells in the *numb*^{1} mutant phenotype (Fig. 5H). Thus, Numb/Notch signaling also has a role in the development of midline dopamine cells, but further investigation into the significance and whether apoptosis is involved in this lineage will require lineage analysis to determine the origin of the midline dopamine cells. Neither *eg* (Fig. 2C) nor *en* (Lundell et al., 1996) is expressed in midline dopamine cells. As the UAS alleles in this paper were induced with either *eg-gal4* or *en-gal4*, the phenotype of dopamine cells in these experiments was not affected by these genetic backgrounds.

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