

# Different cofactor activities in $\gamma$ -secretase assembly: evidence for a nicastrin–Aph-1 subcomplex

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The  $\gamma$ -secretase complex is required for intramembrane cleavage of several integral membrane proteins, including the Notch receptor, where it generates an active signaling fragment. Four putative  $\gamma$ -secretase components have been identified—presenilin (Psn), nicastrin (Nct), Aph-1, and Pen-2. Here, we use a stepwise coexpression approach to investigate the role of each new component in  $\gamma$ -secretase assembly and activation. Coexpression of all four proteins leads to high level accumulation of mature Psn and increased proteolysis of Notch. Aph-1 and Nct

may form a subcomplex that stabilizes the Psn holoprotein at an early step in  $\gamma$ -secretase assembly. Subcomplex levels of Aph-1 are down-regulated by stepwise addition of Psn, suggesting that Aph-1 might not enter the mature complex. In contrast, Pen-2 accumulates proportionally with Psn, and is associated with Psn endoproteolysis during  $\gamma$ -secretase assembly. These results demonstrate that Aph-1 and Pen-2 are essential cofactors for Psn, but that they play different roles in  $\gamma$ -secretase assembly and activation.

## Introduction

Signaling by several integral membrane proteins involves the proteolytic release of an intracellular fragment mediated by the  $\gamma$ -secretase complex (for review see Kopan and Goate, 2000; Sisodia and St. George-Hyslop, 2002). Cell surface receptors cleaved by  $\gamma$ -secretase include amyloid precursor protein (APP),\* Notch, the ErbB4 receptor tyrosine kinase, CD44, and the low density lipoprotein receptor-like protein. An unusual feature of this signaling mechanism is that substrates are cleaved within their transmembrane domain in a hydrophobic environment that is thought to impede efficient hydrolysis of peptide bonds. Numerous papers have now implicated presenilin (Psn) as a central catalytic component of  $\gamma$ -secretase (for review see Kopan and Goate, 2000; Sisodia and St. George-Hyslop, 2002).

Psn itself is synthesized as an immature holoprotein that undergoes endoproteolysis to generate NH<sub>2</sub>- and COOH-terminal fragments, which are thought to associate to form the active enzyme (Thinakaran et al., 1996; Ratovitski et al., 1997; Levitan et al., 2001). The trafficking and processing

events associated with maturation of Psn and its substrates have complicated efforts to understand the assembly of  $\gamma$ -secretase and its proteolytic activation. Significantly, only a fraction of Psn is endoproteolyzed and assembled into these high mol wt complexes, with the majority of the Psn holoprotein instead being rapidly degraded (Ratovitski et al., 1997; Thinakaran et al., 1997).

Recent work has identified three additional putative  $\gamma$ -secretase components, termed nicastrin (Nct), Aph-1, and Pen-2. Nct is a type I integral membrane protein that associates with Psn and COOH-terminal fragments of APP and Notch (Yu et al., 2000; Chen et al., 2001). Aph-1 and Pen-2 are polytopic membrane proteins containing seven and two predicted transmembrane segments, respectively, and were identified through genetic screens in *Caenorhabditis elegans* (Francis et al., 2002; Goutte et al., 2002). Many questions remain about the roles of these components in  $\gamma$ -secretase assembly, trafficking, and enzymatic activation. The fact that only a fraction of Psn is endoproteolyzed has led to the proposal that limiting cellular factors regulate the amount of active  $\gamma$ -secretase that is produced (Ratovitski et al., 1997; Thinakaran et al., 1997). Are Nct, Aph-1, and Pen-2 these postulated limiting factors, and are they, together with Psn, sufficient for functional  $\gamma$ -secretase assembly? Which of these components are associated with mature  $\gamma$ -secretase, and do any display specific roles in  $\gamma$ -secretase complex assembly or function? Here, we investigate the activities of the four putative components of *Drosophila*  $\gamma$ -secretase in

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\*Abbreviations used in this paper:  $\Delta$ ECN, deletion of extracellular Notch; APP, amyloid precursor protein; dsRNA, double-stranded RNA; Nct, nicastrin; N(intra), intracellular Notch; Psn, presenilin; RNAi, RNA-mediated interference; S2, Schneider-2; SOP, sensory organ precursor.

Key words: presenilin; Pen-2; Notch; *Drosophila*; amyloid

the context of Notch signaling and proteolysis. We identify *aph-1* mutant flies, which exhibit defects in Notch function that are indistinguishable from *Drosophila* *Psn* and *nct* mutants. In fly Schneider-2 (S2) cells, high level accumulation of mature Psn and Pen-2, as well as increased  $\gamma$ -secretase cleavage of Notch, are only observed if all four putative  $\gamma$ -secretase components are coexpressed in S2 cells. In contrast to other components, Aph-1 is stabilized by Nct expression, but destabilized by the additional coexpression of Psn. We propose an assembly pathway in which Aph-1 and Nct associate in a subcomplex that acts early in  $\gamma$ -secretase complex formation to stabilize Psn holoprotein, and in which Pen-2 is required for endoproteolysis of Psn holoprotein.

## Results and discussion

### Characterization of a *Drosophila* *aph-1* mutant

We identified alleles of *aph-1* among preexisting lethal mutations mapping to cytological region 23A6-B1 (DiAntonio et al., 1993; Littleton and Bellen, 1994). One mutant group of two alleles (*D35* and *AD25*) exhibits a lethal phenotype at the larval-pupal transition closely resembling *Psn* mutants (Struhl and Greenwald, 1999; Ye et al., 1999). Sequencing of *D35* revealed that it contains a mutation predicted to convert the Arg-99 codon of Aph-1 into a stop codon, which would truncate the protein between transmembrane domains 3 and 4.

The postembryonic lethality of *aph-1* mutants indicated that an embryonic requirement for Aph-1 in Notch signaling might be masked by maternally deposited protein. Therefore, we generated adult females bearing *aph-1* mutant germline clones, and found that embryos lacking both maternal and zygotic Aph-1 activity exhibit a *Notch*-like hyperplasia of the embryonic nervous system (Fig. 1, A and B). Similarly, *aph-1* mutant wing discs contain clusters of supernumerary sensory organ precursor (SOP) cells in addition to an overall developmental arrest phenotype, as seen in other neurogenic mutants (Fig. 1, I and J). To examine adult tissue phenotypes, we induced homozygous mutant *aph-1* somatic tissue clones in heterozygous hosts. Numerous phe-

notypes characteristic of impaired Notch function were observed, including wing notching and bristle loss (Fig. 1, C–H). Together, these results demonstrate that Aph-1 is required for Notch signaling throughout development, and that *aph-1* mutants show phenotypes indistinguishable from *Psn* and *nct* mutants.

### Aph-1 is required for signaling from membrane-anchored Notch

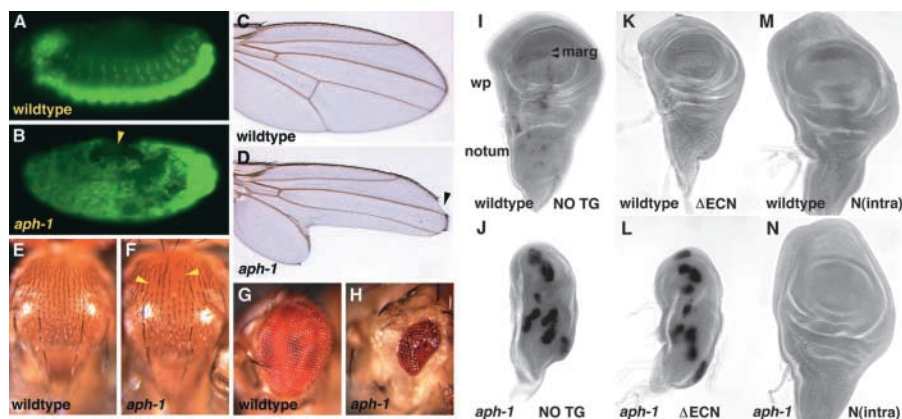
Next, we examined the ability of activated forms of Notch to signal in the absence of *aph-1* gene function. An intracellular COOH-terminal fragment of *Drosophila* Notch lacking extracellular and transmembrane sequences—termed intracellular Notch (N[intra])—suppresses neuronal specification independent of Psn and Nct, whereas a membrane-anchored COOH-terminal fragment (deletion of extracellular Notch [ $\Delta$ ECN]) also signals constitutively, but requires Psn and Nct (Struhl and Greenwald, 1999; Chung and Struhl, 2001; Hu et al., 2002; López-Schier and St. Johnston, 2002). N(intra) displays nearly complete SOP inhibition in *aph-1* mutant wing discs, whereas  $\Delta$ ECN has no discernible inhibitory effect on SOP formation in the *aph-1* mutants (Fig. 1, K–N). The equivalent genetic requirements for *aph-1*, *Psn*, and *nct* for signaling by  $\Delta$ ECN and N(intra) confirms that Aph-1 is required specifically for the  $\gamma$ -secretase-mediated intramembrane cleavage of Notch.

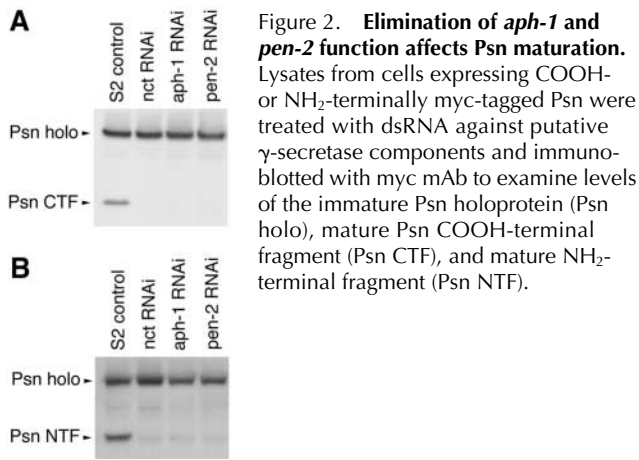
### Aph-1 and Pen-2 are required for Psn maturation in *Drosophila* cells

Using a form of Psn with a myc epitope inserted in the hydrophilic loop to detect the COOH-terminal subunit, or a form with an NH<sub>2</sub>-terminal myc tag, we observed loss of both the mature NH<sub>2</sub>- and COOH-terminal fragments, but no effect on the immature Psn holoprotein when cells were treated with double-stranded RNA (dsRNA) directed against either *aph-1* or *pen-2* (Fig. 2). These results are similar to those reported for *aph-1* and *pen-2* in *C. elegans*, *Drosophila*, and mammalian cells (Francis et al., 2002; Lee et al., 2002; Steiner et al., 2002; Gu et al., 2003; Luo et al., 2003). Our result with *pen-2* RNA-

Figure 1. Notch signaling in *Drosophila* *aph-1* mutants.

(A and B) Neurogenic phenotype of embryos lacking maternal and zygotic *aph-1* function (B) compared with wild type (A), using immunostaining for ELAV to visualize neuronal nuclei. Arrowhead in B indicates a small remaining patch of dorsal epidermis. (C–H) Clones of homozygous *aph-1* mutant tissue exhibit *Notch*-like phenotypes, including notches and missing margin structures (arrowhead) in the wing (D), thoracic cuticle regions lacking microchaetae (F, arrowheads), and small eyes with disordered ommatidial packing (H). (I–N) SOP cell differentiation in larval imaginal wing discs from wild-type flies expressing no transgene (I), the membrane-tethered Notch fragment  $\Delta$ ECN (K), or the intracellular Notch fragment N(intra) (M), and from homozygous *aph-1* mutant flies expressing no transgene (J),  $\Delta$ ECN (L), or N(intra) (N); *aph-1* genotype indicated at bottom left, transgene listed at bottom right. Abbreviations: wp, wing pouch; marg, margin SOP cells; notum, region that develops into adult thorax; NO TG, no transgene.



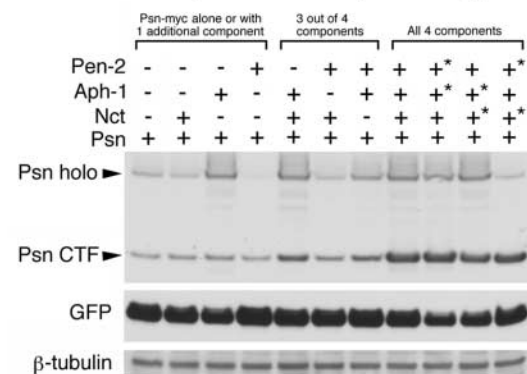


**Figure 2. Elimination of *aph-1* and *pen-2* function affects Psn maturation.** Lysates from cells expressing COOH- or NH<sub>2</sub>-terminally myc-tagged Psn were treated with dsRNA against putative  $\gamma$ -secretase components and immunoblotted with myc mAb to examine levels of the immature Psn holoprotein (Psn holo), mature Psn COOH-terminal fragment (Psn CTF), and mature NH<sub>2</sub>-terminal fragment (Psn NTF).

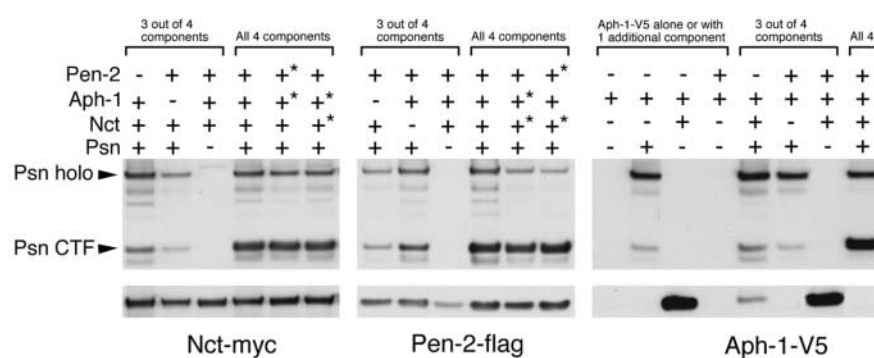
mediated interference (RNAi) was also similar to that observed by Takasugi et al. (2003) in *Drosophila* cells, but their work found that *aph-1* RNAi leads to loss of both

Psn holoprotein and cleaved fragments. This discrepancy is likely to reflect the fact that their approach used a relatively weak promoter to drive Psn expression, such that all newly synthesized Psn holoprotein was able to enter the Aph-1-dependent early steps of  $\gamma$ -secretase assembly. Under these conditions, depletion of Aph-1 would eliminate all the stabilized Psn holoprotein and its derivative fragments. Our use of a stronger promoter presumably results in an excess of Psn holoprotein that is unable to enter this stabilization pathway, and which, therefore, is insensitive to removal of Aph-1 or Nct. Interestingly, although Takasugi et al. (2003) found that *nct* RNAi also abolished all Psn holoprotein in their cell culture assay, a previous in vivo genetic analysis detected Psn immunoreactivity in early compartments of the secretory pathway of *nct* mutant flies (Chung and Struhl, 2001), suggesting that physiological levels of Psn expression might be high enough to allow some Psn holoprotein to accumulate in the absence of these  $\gamma$ -secretase cofactors.

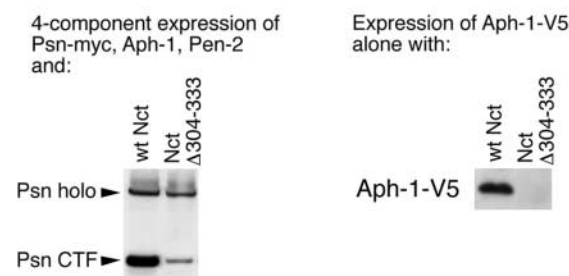
**A. Combinatorial expression of Psn-myc with untagged Nct, Aph-1, and/or Pen-2**



**B. Levels of tagged Nct-myc, Pen-2-flag, and Aph-1-V5 in combinatorial expression with Psn-myc**



**C. Psn CTF hyperaccumulation and Aph-1-V5 stabilization require coexpression of functional Nct**



**Figure 3. Effects of combinatorial expression of putative  $\gamma$ -secretase components on Psn, Nct, Aph-1, and Pen-2 levels.**

(A) Different combinations of putative  $\gamma$ -secretase components were expressed in S2 cells, as indicated above each blot (+, present; -, absent), and cells were harvested and examined for levels of the immature Psn holoprotein (Psn holo) and mature Psn COOH-terminal fragment (Psn CTF), using a myc-tagged form of Psn. Samples were examined for endogenous  $\beta$ -tubulin and a cotransfected GFP plasmid as loading/transfection controls. In this experiment, all other transfected components (Nct, Aph-1, and/or Pen-2) were not epitope-tagged. Asterisks (rightmost lanes) denote pairs of components that were expressed together from two different cotransfected plasmids. (B) To monitor levels of each of the other coexpressed  $\gamma$ -secretase components, the experiment in A was repeated, using Psn-myc together with one other tagged component and two untagged components for each replicate (left, Nct-myc with untagged Aph-1 and Pen-2; middle, Pen-2-flag with untagged Nct and Aph-1; right, Aph-1-V5 with untagged Nct and Pen-2; asterisks as in A). For clarity, uninformative 1- and 2-component lanes of the Nct-myc and Pen-2-flag blots are omitted. Informative 1- and 2-component samples are included for the Aph-1-V5 immunoblot; the two-plasmid expression lanes of the Aph-1-V5 blot are omitted because this approach always produces a low level of residual Aph-1-V5 that is not seen when all four components are expressed from a single plasmid, and which we ascribe to a small percentage of cells that are not transfected by both plasmids. (C) Coexpression performed with a nonfunctional form of Nct with an internal deletion of residues 304–333.



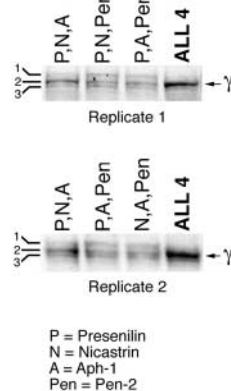
## Coexpression of putative $\gamma$ -secretase components in *Drosophila* cells

Because *Drosophila* S2 cells faithfully replicate the stringently regulated, low level accumulation of mature Psn seen in mammalian cells (Hu et al., 2002), we used them to test whether coexpression of all four putative  $\gamma$ -secretase components promotes the assembly of mature  $\gamma$ -secretase. However, we found that certain combinations of epitope-tagged components were unstable, particularly those involving coexpression of tagged Nct and tagged Aph-1 (see Fig. 5 A). Tagged Pen-2 was relatively well tolerated in most cases (see Fig. 5 A). Therefore, we initially expressed Psn-myc with untagged Nct, Aph-1, and/or Pen-2, monitoring only the levels of Psn holoprotein and COOH-terminal fragment in each combination. To monitor the levels of the other components, these works were repeated using Psn-myc with just one other epitope-tagged component (e.g., Aph-1-V5, Nct-myc, or Pen-2-flag). Levels of Psn-myc were monitored in these samples to verify that each component combination gave reproducible effects on Psn, whether other components were epitope-tagged or not. Using this strategy, we obtained consistent results for Psn levels without apparent complications due to epitope tags.

S2 cells were transfected with constructs encoding stepwise combination of the four  $\gamma$ -secretase components. No massive overproduction of Psn holoprotein or COOH-terminal fragments was observed in samples involving up to three coexpressed proteins. However, coexpression of all four proteins together results in high levels of mature  $\gamma$ -secretase, as indicated by dramatically elevated levels of cleaved Psn COOH-terminal fragments and tagged Pen-2 (Fig. 3, A and B). These effects were paralleled by a significant increase in the specific Notch p120 cleavage attributed to  $\gamma$ -secretase, implying an elevated production of functional  $\gamma$ -secretase (Fig. 4). Nct levels remain relatively constant in all samples, including those involving coexpression of all four proteins (Fig. 3 B). However, Aph-1 accumulates to high levels only when coexpressed with Nct or Nct with Pen-2, as described later (Fig. 3 B).

### Figure 4. Activity of overproduced $\gamma$ -secretase assessed by Notch processing.

Coexpression of all four putative  $\gamma$ -secretase components is required for enhanced production of the Psn-dependent p120 fragment of the Notch receptor. Two replicated S2 cell transfection experiments are shown (total,  $n = 14$ ), in which combinations of three different components or all four were coexpressed with intact Notch. Lysates were resolved by SDS-PAGE and immunoprobed using antibodies that detect the cleaved COOH-terminal fragments of Notch in the 120-kD range. For samples involving only three components, similar amounts of the three Notch COOH-terminal fragments are detected (bands 1–3, left), as in cells expressing 0–2 components (not depicted). Coexpression of all four components leads to significant overproduction of the second 120-kD species ( $\gamma$ , right), corresponding to the  $\gamma$ -secretase-dependent 120-kD cleaved fragment of Notch.



These experiments were performed using single plasmids engineered to express 0–4 components, to avoid potential mixed-plasmid transfection artifacts. Two additional experiments were performed to verify the results. In the first, high level  $\gamma$ -secretase accumulation was confirmed in cells cotransfected with two separate plasmids, one expressing a given pair of proteins and the other expressing the remaining pair (Fig. 3, A and B). In the second, the *nct* gene of the four-component plasmid was altered by introducing the Nct  $\Delta 304$ – $333$  mutation that renders Nct nonfunctional in *Drosophila* cells (Hu et al., 2002). This construct does not promote high level accumulation of Psn, consistent with our conclusion that efficient  $\gamma$ -secretase assembly requires coexpression of functional component proteins (Fig. 3 C).

Given the technical difficulties associated with producing purified multipass membrane proteins such as Psn, Pen-2, and Aph-1 without relying on cells or microsomes, our coexpression strategy approximates a cellular “reconstitution” of purified  $\gamma$ -secretase components, with the caveat that this approach cannot rigorously exclude the potential involvement of unknown cellular factors. Using a similar stepwise coexpression approach, Takasugi et al. (2003) have recently reported similar results that coexpression of *Drosophila* Aph-1, Pen-2, and Nct increases levels of Psn fragments and increases APP  $\gamma$ -secretase cleavage. Similarly, Edbauer et al. (2003) have recently reported equivalent results by coexpressing mammalian  $\gamma$ -secretase components in yeast.

### Effects of Aph-1 and Pen-2 indicate different roles in $\gamma$ -secretase complex assembly

Aph-1 does not accumulate proportionally when all four putative  $\gamma$ -secretase components are coexpressed, suggesting that Aph-1 might be absent from the mature complex. Interestingly, coexpression of Aph-1 with Psn and Nct (without Pen-2) results in a moderate increase in both the Psn holoprotein as well as its processed fragments (Fig. 3, A and B). Conversely, coexpression of Pen-2 with Psn and Nct (without Aph-1) causes no discernible effect relative to coexpression of just Psn and Nct (Fig. 3, A and B). Because addition of Pen-2 to the Aph-1/Psn/Nct combination results in strong accumulation of the Psn COOH-terminal fragment with no apparent increase in Psn holoprotein, the simplest interpretation of these effects is that Aph-1 is important for determining the amount of Psn holoprotein that is stabilized and enters the maturation pathway, whereas Pen-2 is involved in subsequent Psn endoproteolysis. Our results are consistent with recent works showing that Aph-1 acts early during  $\gamma$ -secretase assembly in the initial stabilization of Psn holoprotein, and that Pen-2 is required for holoprotein endoproteolysis (Gu et al., 2003; Luo et al., 2003; Takasugi et al., 2003).

### Aph-1 and Nct might associate in a subcomplex

Another reliably observed effect involves the dramatic overaccumulation of Aph-1 when coexpressed together with Nct in the absence of Psn (Fig. 3, B and C). All other pairwise coexpression combinations involving Aph-1 fail to show significantly elevated levels of Aph-1. We observed strong up-regulation of Aph-1 by Nct using two different tagged ver-

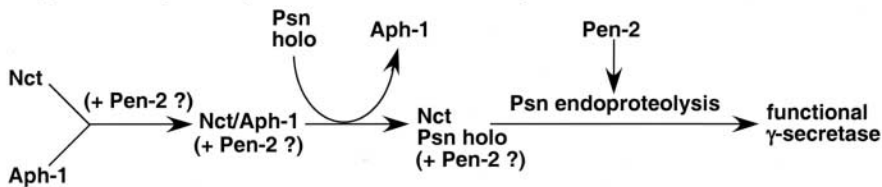
### A. Summary chart of coexpression experiments

Coexpression studies involving all four components					Replicates	Increase in Psn CTF levels
tagged Psn only	Psn-myc	Nct	Aph-1	Pen-2	4	++++
tagged Psn and one other tagged component	Psn-myc	<u>Nct-myc</u>	Aph-1	Pen-2	3	++++
	Psn-myc	Nct	<u>Aph-1-V5</u>	Pen-2	3	++++
	Psn-myc	Nct	<u>Aph-1-flag</u>	Pen-2	4	++++
	Psn-myc	Nct	Aph-1	<u>Pen-2-flag</u>	3	++++
tagged Psn and two other tagged components	Psn-myc	<u>Nct-myc</u>	<u>Aph-1-V5</u>	Pen-2	2	++
	Psn-myc	Nct	<u>Aph-1-V5</u>	<u>Pen-2-flag</u>	2	++++
	Psn-myc	<u>Nct-myc</u>	Aph-1	<u>Pen-2-flag</u>	2	++++
all four tagged	Psn-myc	<u>Nct-myc</u>	<u>Aph-1-V5</u>	<u>Pen-2-flag</u>	2	+

Coexpression studies on Nct::Aph-1 subcomplex					Replicates	Increase in Aph-1 levels
wildtype Nct	-	Nct	<u>Aph-1-V5</u>	-	5	++++
	-	<u>Nct-myc</u>	<u>Aph-1-V5</u>	-	3	+
	-	Nct	<u>Aph-1-flag</u>	-	1	++++
Nct $\Delta$ 304-333 deletion mutant	-	Nct $\Delta$	<u>Aph-1-V5</u>	-	5	-

### B. Hypothetical pathway for $\gamma$ -secretase assembly and activation



sions of Aph-1 (Fig. 3 B and Fig. 5 A), and we also determined that it does not occur with Nct $\Delta$ 304–333 and thus requires functional Nct (Fig. 3 C). Although Nct strongly enhances Aph-1 accumulation, this effect disappears if Psn is also present, but it is unaffected by Pen-2. It should be noted that these effects were not observed in a related paper by Takasugi et al. (2003). This discrepancy might be due to the fact that their work relied on coexpression of tagged Nct-V5 and tagged Aph-1-flag, similar to coexpression conditions under which we failed to detect strong Aph-1–Nct stabilizing interactions due to epitope tag interference problems (Fig. 5 A).

Together, our biochemical results suggest that Aph-1 and Nct interact early in  $\gamma$ -secretase assembly, perhaps forming a stable subcomplex. This subcomplex might regulate subsequent maturation of  $\gamma$ -secretase by facilitating productive associations between Nct, Psn, and Pen-2 during endoproteolytic maturation of the complex. Because Psn prevents the over-accumulation of Aph-1 by Nct, it is possible that stabilization of the Psn holoprotein by Aph-1 leads ultimately to the removal and degradation of Aph-1 from the maturing complex.

### Concluding remarks

Our finding that coexpression of Psn, Nct, Aph-1, and Pen-2 leads to high levels of mature, functional  $\gamma$ -secretase, together with similar recent papers (Edbauer et al., 2003; Takasugi et al., 2003), suggests that these four proteins are likely to be the major limiting factors for  $\gamma$ -secretase assembly, and that true biochemical reconstitution of  $\gamma$ -secretase using purified proteins and membranes is feasible. A new result from our analysis is that Aph-1 and Nct appear to associate in a subcomplex that functions early in  $\gamma$ -secretase assembly. We have incorporated our results with those of

### Figure 5. Summary chart and hypothetical $\gamma$ -secretase assembly pathway.

(A) Chart summarizing the design and major outcomes of the coexpression experiments. Epitope tags on Nct, Aph-1, and Pen-2 are indicated by underlining. (B) Hypothetical model of  $\gamma$ -secretase assembly and activation. Nct and Aph-1 associate in an early subcomplex, leading to stabilization of Aph-1. The subcomplex regulates stabilization of Psn holoprotein and its incorporation into a complex with Nct and possibly Pen-2. Pen-2 is required for endoproteolysis of Psn holoprotein, although the point at which Pen-2 enters the pathway is unknown.

recent papers into a hypothetical model for  $\gamma$ -secretase assembly and activation (Fig. 5 B). Further analysis will be required to test this model and to define more precisely the biochemical activities of each  $\gamma$ -secretase component.

## Materials and methods

### Drosophila genetics

Candidate *aph-1* mutants were crossed to 23A/B deficiencies and progeny were evaluated for neurogenic phenotypes. Germline clones were made by FLP-mediated recombination using females heterozygous for a recombinant *P{neoFRT}40A aph-1<sup>D35</sup>* chromosome and *P{w[+mC]=ovoD1-18}2La P{w[+mC]ovoD1-18}2Lb P{ry[+7.2]=neoFRT}40A* fertilized by *Df(2L)N6/CyO* males. Adult clones were generated using *P{neoFRT}40A aph-1<sup>D35</sup>* together with *w hsFLP1; arm-lacZ FRT40A/CyO*. Eye-specific clones were produced using *P{ry[+7.2]=ey-FLP.N}2 P{GMR-lacZ.C(38.1)}TPN1*. For the  $\Delta$ ECN and N(intra) works, second-chromosome inserts of the transgenes *N<sup>ΔECN.hs</sup>* and *N<sup>int.hs</sup>* were recombined with *aph-1<sup>D35</sup>* and assayed as described previously (Hu et al., 2002).

### Immunohistology

Immunohistology of *Drosophila* tissues was performed as described previously (Ye and Fortini, 1998; Hu et al., 2002) using antibodies as follows: 1:200 rat anti-ELAV mAb 7E8A10, 1:30 mouse anti-Scabrous mAb sca1 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA).

### S2 cell experiments

The *aph-1* and *pen-2* genes were obtained by PCR from cDNA libraries and used for dsRNA preparation and protein expression. Epitope-tagged forms were constructed in which V5 and flag tags were inserted at the COOH termini of Aph-1 and Pen-2. The *Psn* cDNA (Ye and Fortini, 1998) was modified to insert myc tags after Psn residues I26 or S412. The *nct* cDNA (Hu et al., 2002) was used to express untagged Nct, and a form was made with two myc tags at the COOH terminus. All cDNAs were subcloned into the pIZ-V5/His vector (Invitrogen). S2 transfections and RNAi were performed as described previously (Hu et al., 2002), using dsRNA for *nct* (+674–1448), *aph-1* (+71–412) and *pen-2* (+96–400).

Western immunoblot analyses of S2 cells were performed as described previously (Ye et al., 1999; Hu et al., 2002). Gel loading was normalized according to total protein concentrations; some blots were also examined for levels of  $\beta$ -tubulin or expression of cotransfected pIZ-GFP for loading

and transfection controls. Western blots were probed with 1:1,000 mouse anti-Notch mAb C17.9C6, and anti-myc mAb (Sigma-Aldrich), anti-flag mAb (Upstate Biotechnology), anti-V5 mAb (Invitrogen), anti-GFP mAb (CLONTECH Laboratories, Inc.), or anti- $\beta$ -tubulin mAb (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) at recommended dilutions.

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