

Requirement of Notch in adulthood for neurological function and longevity

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Although Notch proteins rely upon presenilins for activation and can modulate neuritic architecture, their role in aging adults and Alzheimer's disease is unknown. Here we examine *Drosophila* in which Notch function was selectively diminished in adulthood. An outcrossing strategy was employed to reduce the effect of recessive modifiers of lifespan, and a temperature-sensitive allele or inducible dominant-negative Notch transgenes were used to reduce Notch function. A progressive

neurological syndrome with loss of flight and shortened lifespan was observed in adults with compromised Notch function. Notch protein persists in aging adult *Drosophila* brains. However, no evidence of neurodegeneration in the central nervous system was detected. We conclude that Notch activity is constitutively required in the adult fly for neurological function. *NeuroReport* 12:3321–3325 © 2001 Lippincott Williams & Wilkins.

Key words: Aging; Alzheimer's disease; Lifespan; Notch; Presenilin

INTRODUCTION

Notch proteins are receptors for the Delta-Serrate-Lag2 (DSL) ligand family and serve essential roles in the differentiation of multicellular organisms [1]. Despite evidence that Notch proteins and their ligands are expressed in adult tissues of many species particularly in the brain [2,3], little is known about their function in mature organisms. Additionally, recent evidence that the Notch pathway relies upon the presenilins and studies showing Notch3 mutations in the neurodegenerative disorder, CADASIL [4], have focused interest in the role of Notch proteins in the adult brain. Recent studies have shown that young neurons in vitro utilize the Notch pathway to guide the branching and complexity of their neuritic processes [5–7]. Moreover, in embryonic *Drosophila*, a loss of Notch or Delta function during axon extension elicits marked structural and targeting abnormalities of extending neuronal processes [8,9]. In line with these observations, mature *C. elegans* with hypomorphic mutations in genes homologous to Notch or presenilin (*lin12* or *sel12*) show abnormal projections of sensory interneurons. Interestingly, learning or behavioral deficiencies were found in addition to the characteristic developmental anomalies of other tissues [10]. Although these studies indicate that certain neurons may utilize Notch signals to establish axonal or dendritic structures or targets, no study has yet examined the importance of the Notch pathway in neurological function in an adult organism which has completed its neuronal development. We therefore evaluated the function of the Notch pathway in adult *Drosophila* after all steps of neural development were complete.

MATERIALS AND METHODS

Strains: Flies were reared in standard cornmeal molasses medium at 18°C or 25°C as indicated. *DpN⁺::SM1* is a small translocation that contains the *Notch* locus recombined onto the *SM1* balancer (*SM1 Cy Dp(1;2)w⁺51b7N⁺/+*) [8]. The study of lifespan at 29°C was conducted on F1 animals from crosses of *w N^{ts1}* females with *w N^{ts1}* males, wildtype Canton S males [13], or *N^{ts1};DpN⁺::SM1/+* males yielding *w N^{ts1}/+*, *w N^{ts1}/N^{ts1}* or *w N^{ts1}/N^{ts1}; SM1::DpN⁺/+* progeny. Studies on lifespan at 18°C and flight were performed on F1 adults, *w N^{ts1} cv¹ v² f¹/w N^{ts1}* and *w N⁺ cv¹ v² f¹/w N^{ts1}*. These were derived from recombination with *w N^{ts1}* or *w* strains and *y² cv¹ v² f¹* [13] to increase isogeny between control and experimental strains. The *hs-Notch* rescue experiment tested F1 animals generated from crosses between homozygous *N^{ts1}* females and male parentals carrying a copy of an inducible *Notch* transgene *P{w⁺,hsEGF1-18}* bearing heterozygous transgenes (*hs-ΔEGF1-18*) [11] or *P{w⁺,hsN}(hs-N⁺)* [12] in an *N^{ts1}* background allowing for study of each transgene's action in comparison to a control sibling. Dominant negative experiments utilized F1 animals derived from crosses between *w* females [13] and male parentals carrying transgenes *hs-ΔEGF1-18*, *hs-ΔCDC10rpts* or *hs-ΔC1693> S::ΔCDC10rpts* [11] in a wildtype *N* background.

Western blots: Immunoblots were performed on tissue extracts from heads, thoraces, and abdomens of 10 animals at the indicated ages. Animals were dissected, then dounce homogenized in 2× sample buffer: 0.125 M Tris pH 6.8, 20% glycerol, 4% SDS, 2% 2-mercaptoethanol, 20 μg/ml

PMSF, 10 µg/ml antipain, 10 µg/ml trypsin inhibitor, 10 µg/ml pepstatin A, 0.044% benzamidine, 0.02% bromphenol blue and an equal volume of water was added prior to boiling. Samples were run on 6% SDS/PAGE gel, transferred to nitrocellulose and blotted with 1:1000 mAb C17.9C6 (Developmental Studies Hybridoma Bank). Goat anti-mouse-peroxidase secondary 1:2000 (Boehringer-Mannheim) and ECL (Pierce) were used to detect Notch.

Lifespan assays: Animals of indicated genotypes were allowed to develop at 18°C or 25°C as indicated. Animals were collected on the day of eclosion, separated the following day, and at 3 days post-eclosion were placed at a new temperature (29°C) or into a heat-shock regimen (25°C with two one hour 37°C heat shocks per day). Fewer than 50 animals were kept together and vials were changed every 2–4 days as needed. Survival was scored either every day, or every other day for animals maintained at 29°C and 25°C or every fourth day for animals maintained at 18°C. Mean lifespans and 95% confidence intervals were determined by Kaplan-Meier Survival analysis using SPSS. Lost or crushed animals accounted for <10% of cohorts and were censored.

Flight testing: Flies were placed at room temperature ≥15 min prior to testing. At approximately the same time each afternoon, animals were tapped to the bottom of a vial and then emptied into a modified version of the Benzer flight testing tube [14] initially suggested previously [15]. Flies that adhered to the walls of the 60 × 6 cm diameter plastic tube were scored as able to fly. Groups of ~50 animals were tested, and the fraction flying was calculated. The mean time to loss of flight was calculated using Kaplan-Meier with dead animals censored.

RESULTS

To ascertain the role of Notch in adult animals, we first examined posteclosion flies for the persistence of the Notch protein as animals aged at 29°C (Fig. 1). Notch proteins

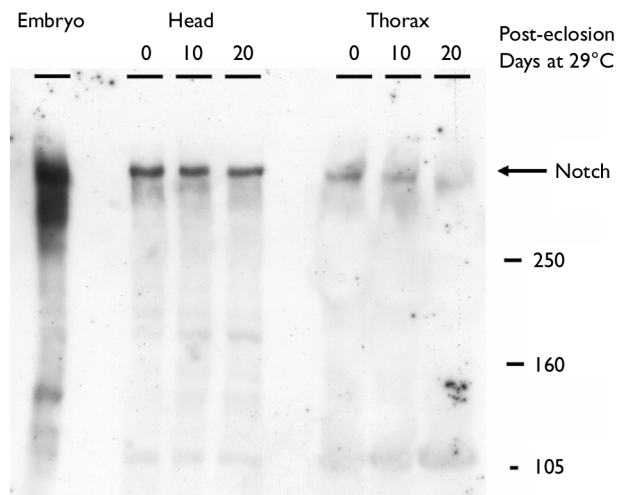


Fig. 1. Notch expression in aging adults. Tissue extracts from wildtype (Canton-S) *Drosophila* reared at 29°C blotted with MAb C17.9C6 (anti-Intracellular Notch). Sections from 10 animals are loaded in each lane as indicated.

were observed at high levels in extracts of adult heads and thorax while significantly less Notch was apparent in abdomen extracts (data not shown). Notch levels in the head and thorax from eclosion to 20 days post-eclosion were approximately steady, however, Notch protein in the abdomen declined. Thus Notch protein persists into late adulthood in the head and thorax.

To evaluate the role of Notch in the viability of the adult, we utilized methods to selectively reduce Notch function in adult flies. We first evaluated the survival of cohorts of flies carrying the N^{ts1} allele [16] reared at 18°C and then shifted to the non-permissive temperature of 29°C 3 days after eclosion. We chose to shift animals 3 days following eclosion to assure that all previously examined aspects of development were complete and because neuronal apoptosis continues in the ganglia for the first days following eclosion [17,18]. In initial experiments, several isolines of N^{ts1} and wildtype strains were evaluated for their mean lifespan and an inconsistent pattern was observed (data not shown). Interestingly, unselected cohorts are thought to acquire mutations at numerous loci affecting lifespan in as few as 10 generations [19] and inbred strains have been shown to carry homozygous alleles at numerous loci [20], resulting in differences in age-specific mortality, environmental susceptibility and lifespan among stocks of flies [19,20]. To reduce the effect of these background modifiers, all of the subsequent experiments were carried out on outcrossed progeny whose parents came from different stocks of the N^{ts1} allele. Utilizing F1 flies effectively renders most modifiers heterozygous.

After standardizing the experiments with F1 flies, a marked reduction in lifespan was observed in female N^{ts1} flies compared to half-sibs which were $N^{ts1}/+$ or those carrying a duplication of the N locus ($N^{ts1}/N^{ts1};Dp(N^+)$; Fig. 2a, Table 1a). The effect on lifespan is partially specific to females as male N^{ts1} flies did not show differences from controls at 29°C (not shown). However, in subsequent tests, adult males also showed a reliance on Notch signals (see below).

Although the previous experiments implicate the N locus in lifespan, only a single strong temperature sensitive allele of N was tested. A trivial explanation for our observation is that a linked modifier of lifespan could have been carried with the original N^{ts1} chromosome to all of its progeny. Although the partial rescue by the duplication of the N locus (DpN) argues against this possibility, to verify that the effect on lifespan was specifically due to a defect in the N gene, we assessed the ability of a N cDNA to rescue the early lethality observed at 29°C. We compared F1 strains homozygous for N^{ts1} carrying a heat inducible transgene driving either a wild type *Notch* cDNA, *hs-N* [21] or a negative control transgene, *hs-NΔEGF1-18* [12] which encodes a defective version of *Notch* that is unable to rescue N hypomorphs and lacks dominant negative activity. While the presence of *hs-NΔEGF1-18* had no effect on the shortened lifespan of N^{ts1} flies, animals carrying *hsN* showed a statistically significant suppression of the lifespan defect at 29°C (Table 1b). This result indicates that the N gene alone is sufficient to correct the deficiency of lifespan found in the N^{ts1} F1 cohort.

The previous experiments showing a requirement for Notch function in adult flies rely upon the assumption that

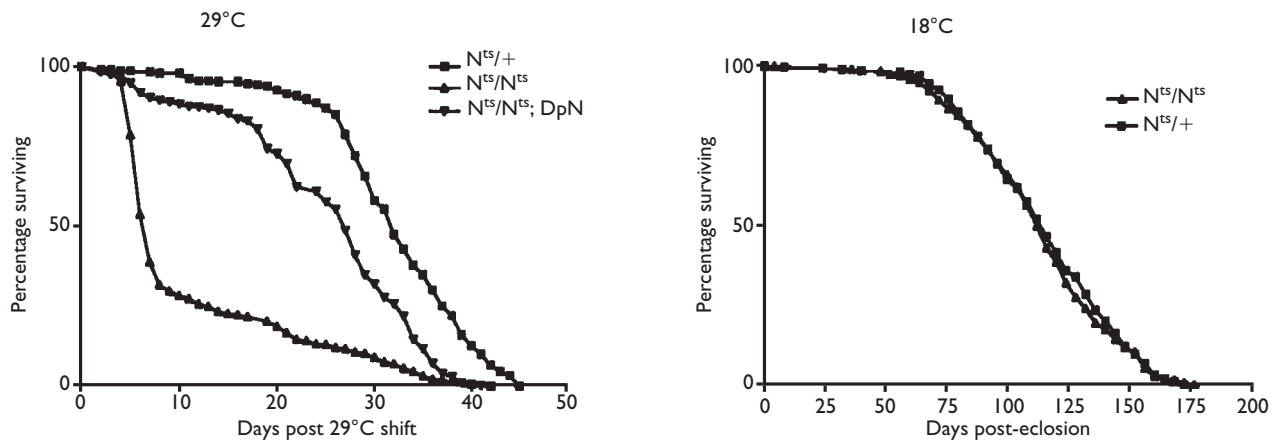


Fig. 2. Effect of Notch on survival of adults. (a) Survival at 29°C post shift. Animals of genotypes $N^{ts1}/+$, N^{ts1}/N^{ts1} , $N^{ts1}/N^{ts1}; DpN+/+$ were reared at 18°C (permissive temperature) and then shifted to 29°C (non-permissive) 3 days after eclosion. (b) Survival at 18°C. Animals of genotypes $N^{ts1}/+$, N^{ts1}/N^{ts1} were reared and maintained at 18°C (permissive temperature).

Table 1. Survival and Adult Notch Function.

Genotype	mean life span	(95% CI)	n
A. Decreased life span and small duplication rescue (females)			
$wN^{ts1}/+$	31.86	(31.22,32.51)	607
wN^{ts1}/N^{ts1}	11.46	(10.68,12.25)	573
$wN^{ts1}/N^{ts1}; SMI::DpN+/+$	24.93	(24.02,25.84)	397
B. Rescue with heat-shock induced Notch (females)			
$wN^{ts1}; hs-\Delta EGF1-18/+$	29.02	(27.62,30.42)	239
wN^{ts1} (siblings)	27.24	(25.53,28.94)	196
$wN^{ts1}; hs-N+/+$	35.00	(33.43,36.57)	206
wN^{ts1} (siblings)	25.54	(23.13,27.96)	151
$wN^{ts1}/+$	43.81	(43.12,44.51)	384
C. Adult induced dominant negative notch (males)			
$w; hs-\Delta EGF1-18/+$	35.27	(33.77,36.76)	193
$w; hs-\Delta EGF1-18/+$ heat shock	34.69	(33.49,41.00)	111
$w; hs-\Delta CDC10/+$	39.70	(38.40,41.00)	111
$w; hs-\Delta CDC10/+$ heat shock	27.47	(25.35,29.58)	112
$w; hs-cl69/+$	33.05	(29.99,36.10)	44
$w; hs-cl69/+$ heat shock	25.27	(22.58,27.96)	65
D. Normal lifespan with maintenance at permissive temperature			
$w N^{ts1}/w N^{ts1}$	114.32	(111.29,117.36)	383
$w N^{ts1}/w$	112.60	(109.64,115.57)	403

Genotypes, mean life spans, 95% confidence intervals, and number of animals tested are shown. (A) and (B) Animals were reared at 18°C (permissive temperature) until 3 days following eclosion and then they were shifted to 29°C (non-permissive temperature). Sibling controls which did not inherit the transgene (listed above) were used in (B). (C) Animals were reared at 25°C then subjected to two 1 h heat pulses per day (as indicated) beginning 3 days post-eclosion. (D) Animals were reared and maintained at 18°C.

flies carrying the N^{ts1} allele are essentially normal at the permissive temperature. Since the N^{ts1} allele produces pleiotropic developmental effects at non-permissive temperatures [16], N^{ts1} flies raised at 18°C could possibly have been subtly enfeebled and thus susceptible to early death because of their developmental disability. In theory, enfeeblement could occur despite a lack of obvious morphological malformations, leading to a shortened lifespan for N^{ts1} flies regardless of a requirement in adults for Notch function. Therefore, we subjected N^{ts1} F1 cohorts to a test of their lifespan at 18°C. We observed no differences in the mean lifespan when compared to half-sibs carrying the

wildtype N locus on chromosome 1 (Fig. 1b, Table 1d). These results indicate that the N^{ts1} allele at 18°C does not produce a developmental susceptibility to early death, and strengthens the conclusion that Notch function is continuously required in adults for survival.

Another means to reduce Notch function selectively in adult flies is to utilize dominant negative versions of N under hs control. Although the exact mechanism for their dominant effect is not known, Notch proteins missing their intracellular domains ($N\Delta cdc10$) are thought to bind the available ligand and thus prevent wildtype Notch proteins from signal transduction [11,12]. A protein with an altera-

tion in a conserved cysteine (N-C169) may produce inactive heterodimers with wildtype proteins. We therefore compared F1 flies grown at 25°C with wildtype *N* alleles carrying one copy of a control transgene (*hs-NΔEGF1-18*) to the dominant negative *hs-NΔcdc10* and *hs-N-C169* strains. These strains were given two daily 37°C heat pulses to activate the *hs* promoter beginning 3 days after eclosion. The dominant negative *N* alleles had no effect upon the lifespan of female flies (data not shown); however, F1 males showed a significant decline in lifespan when the dominant negative alleles were induced (Table 1c). At 25°C there was no effect upon the lifespan of flies carrying transgenes but receiving no 37°C heat stress. Thus an independent means to reduce Notch function selectively during the adult phase of the life cycle reveals a requirement for constitutive Notch activity for a normal lifespan.

Although the observation that Notch is required for longevity has not previously been made, it has been noted [22] that N^{ts1} flies lost their ability to fly after a shift to 29°C. We confirmed and extended these observations in F1 females homozygous for the N^{ts1} allele. Using a test for the ability to fly to the walls of a tube [14], cohorts were examined and a significantly shorter mean interval to loss of flight was observed for homozygous N^{ts1} flies at 29°C (21.4 ± 0.53 days, $n = 451$) compared with sibs carrying one wildtype *N* allele (27.1 ± 0.54 days, $n = 415$; Fig. 3a) with dead flies censored. A greater difference was observed when dead flies were included in the non-flying category (not shown). This implies that loss of flight represents an earlier stage in the progression of a lethal neurological impairment, since N^{ts1} flies typically lost their ability to fly prior to their death. Additionally, no significant differences in the fraction flying were observed in cohorts maintained at the permissive temperature (18°C) at 80 days post-eclosion (Fig. 3b). This observation is consistent with the contention that the loss of flight was a consequence of the temperature sensitive Notch allele and not a developmental defect.

We also observed an impairment in negative geotaxis and righting reflexes in flies of both genders carrying the N^{ts1} allele within a few days of a shift to 29°C (Shaw and Nye, unpublished). These data indicate that an impairment of gross neurological function initiates after a reduction in Notch function in adults. Although these experiments point to a defect in nervous system function we have observed no evidence of neurodegeneration including apoptosis or vacuolization in silver stained sections of adult N^{ts1} brains from flies shifted to 29°C post-eclosion (not shown).

DISCUSSION

In this study, we demonstrate that a continuous Notch signal is required for neurological function and survival in adult flies. This requirement for Notch in adults was masked by its importance during pre-adult stages. The argument that Notch signals are involved is verified by two independent means to reduce Notch function, a temperature sensitive allele and dominant negative transgenes. Importantly, the reduction of Notch function initiated only after development was complete in contrast to most prior studies of the Notch pathway and its related genes. We provide direct evidence that *Notch* and not a linked

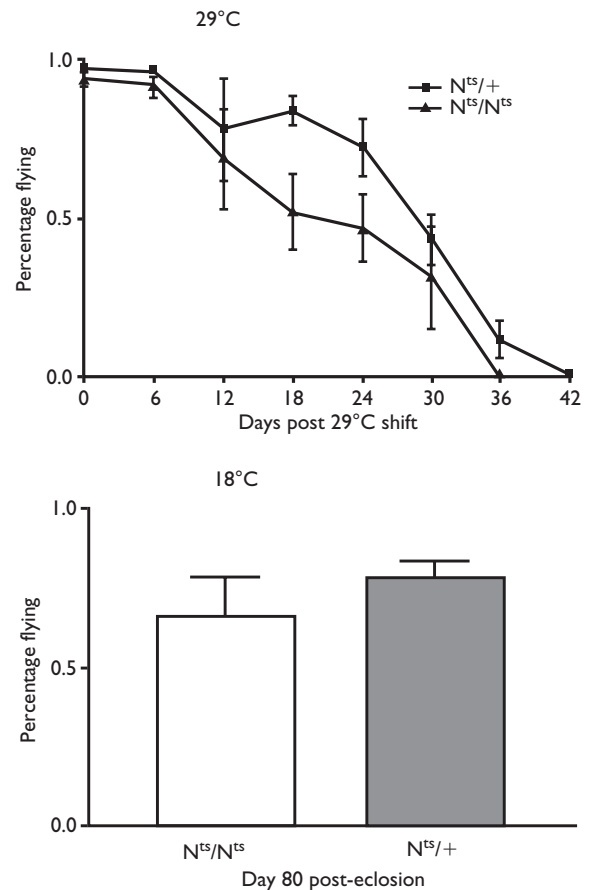


Fig. 3. Effect of Notch on flight. (a) Animals of genotypes $N^{ts1}/+$ and N^{ts1}/N^{ts1} were reared at 18°C, shifted to 29°C 3 days post-eclosion and then subsequently tested for flight. (b) Animals of genotypes N^{ts1}/N^{ts1} and $N^{ts1}/+$ reared and maintained at 18°C tested for flight on day 80.

modifier gene contributes to survival using a rescue with a duplication and a Notch cDNA. Thus we conclude that Notch dysfunction leads to functional neurological impairment and death in adult flies.

Interestingly, the effect of Notch on lifespan was revealed only when we took steps to reduce the effect of modifiers in the genetic background. This implies that its effects on lifespan were somewhat weaker than other genes that influence longevity, such as methuselah [23]. The lifespan effect was also gender specific. The effect of N^{ts1} was seen only in females and the dominant negative effects were seen only in males using the induction regimens that we attempted. Although the gender specificity may have been due to the control of gene dosage for chromosome 1, other techniques to reduce Notch signaling more completely in adulthood might possibly amplify the effects we have seen or reveal an effect in both genders. Consistent with our observations, gender-specific modifiers of lifespan have been mapped by QTL analysis and candidate genes have been identified in some cases [24,25]. In fact, one QTL showing female-specific sensitivity to temperature stress was mapped to the polytene segment 1B-3E that contains the Notch locus (3C7-9) [25]. Whether or not variations in the Notch locus play a significant role in this QTL remains

to be determined. Flies with a selective loss of Notch function in adulthood show a syndrome that includes loss of flight and premature death from unknown causes. We note that defects in other tissues could possibly underlie the syndrome seen here in the absence of pathological changes in the CNS. However, we propose that functional or subtle alterations in neurons or other cells of the CNS lead to impairment and death when Notch function is lost in adults. Further studies are clearly required to identify the biological basis of these functional deficits. Nonetheless, the identification of a role for Notch in adult neurological function paves the way for screens to illuminate the genetic pathway of Notch function in adulthood. The present study also raises the question as to what other developmentally important genes are similarly required in adulthood, but whose functions have been masked by their developmental roles.

Finally, this study raises the possibility that Notch may also have a direct role in adult-onset neurological disease. Presenilins mediate the cleavage and activation of Notch proteins. Recent studies in *Drosophila* and *C. elegans* [26–28] have indicated that all developmental abnormalities seen in PS mutants are a result of Notch dysfunction [29–31]. Since Notch signals control neuritic architecture *in vitro* [5–7] and in embryonic flies [8,9], and since neuritic branching has been shown to underlie neuroplasticity and learning, the present studies raise the possibility that a dysfunction of Notch may be an important factor in the loss of plasticity or learning seen in several adult-onset neurological disorders, including Alzheimer's disease. These studies also suggest that inhibitors of presenilins may cause neurological impairments by preventing Notch function in adults.

CONCLUSION

A deficiency in Notch function in adulthood produces a progressive neurological syndrome that includes loss of flight and premature death. These studies indicate that Notch signaling is constitutively required in the adult fly for neurological function. The lack of gross histological

abnormality in the brain of flies with Notch function reduced in adulthood suggests that a reduction in Notch activity produces a subtle or functional abnormality in the CNS.

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