# lin-12 Notch functions in the adult nervous system of C. elegans 

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# lin-I 2 Notch functions in the adult nervous system of C. elegans <br> Michael Y Chao ${ }^{\dagger 1,2}$, Jonah Larkins-Ford ${ }^{\dagger 1}$, Tim M Tucey ${ }^{1}$ and Anne C Hart ${ }^{* 1,2}$ 

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#### Abstract

Background: Notch signaling pathways are conserved across species and traditionally have been implicated in cell fate determination during embryonic development. Notch signaling components are also expressed postdevelopmentally in the brains of adult mice and Drosophila. Recent studies suggest that Notch signaling may play a role in the physiological, rather than developmental, regulation of neurons. Here, we investigate a new non-developmental role for Caenorhabditis elegans lin-I2 Notch signaling in neurons regulating the spontaneous reversal rate during locomotion. Results: The spontaneous reversal rate of $C$. elegans during normal locomotion is constant. Both lin- 12 gain and loss of function mutant animals had significantly increased reversal rates compared to wild type controls. These defects were caused by lin- 12 activity, because the loss of function defect could be rescued by a wild type lin-I2 transgene. Furthermore, overexpression of lin-I2 recapitulated the gain-of-function defect. Increasing or decreasing lin-/2 activity in the postdevelopmental adult animal was sufficient to rapidly and reversibly increase reversals, thereby excluding a developmental role for lin-I2. Although lin-/2 is expressed in the vulval and somatic gonad lineages, we find that these tissues play no role in regulating reversal rates. In contrast, altering lin- 12 activity specifically in the nervous system was sufficient to increase reversals. These behavioral changes require components of the canonical lin- 12 signaling cascade, including the ligand lag-2 and the transcriptional effector lag-I. Finally, the C. elegans AMPA/kainate glutamate receptor homolog glr-I shows strong genetic interactions with lin-I2, suggesting that glr-I and/or other glutamate gated channels may be targets of lin- 12 regulation.


Conclusion: Our results demonstrate a neuronal role for lin-l 2 Notch in C. elegans and suggest that lin- $/ 2$ acutely regulates neuronal physiology to modulate animal behavior, without altering neuronal cell fate specification or neurite outgrowth. This is consistent with a role for Notch signaling in neurological disease with late onset symptoms.

## Background

The conserved Notch signaling pathway has well established roles in cell fate determination during develop-
ment. Transmembrane Notch receptors are activated by transmembrane DSL (Delta/Serrate/LAG-2) family ligands [1-6]. The intracellular (IC) domain of Notch is
proteolytically released by presenilins and translocates to the nucleus [7-11], where it acts as a transcriptional activator abetted by CSL (ㄷBF1/ $\underline{\mathrm{S}} \mathrm{u}(\mathrm{H}) / \underline{\mathrm{LAG}}-1$ ) proteins [1215]. In C. elegans, the LIN-12 Notch receptor is activated by LAG-2 and related DSL ligands [16-19], and proteolytically processed by the presenilins SEL-12 and HOP-1 [2022]. The CSL protein LAG-1 interacts with LIN-12IC to activate transcription of target genes [23].

Notch receptors and ligands are expressed in adult vertebrate neurons [24,25]; recent studies in Drosophila and mice suggest that altering Notch signaling results in defective neuronal function [Costa, 2003 \#50; Ge, 2004 \#46; Presente, 2004 \#31; Saura, 2004 \#32; Wang, 2004 \#51;Yoon, 2005 \#69]. The importance of these findings is underscored by the fact that several genetic diseases associated with neuronal defects and/or late onset symptoms map to mutations in Notch pathway genes [32-36]. However, it remains unclear from these studies whether Notch signaling is acutely affecting neuronal physiology or if it is causing permanent changes in cell fate and/or structure due to developmental defects or aberrant growth.

Here, we report a new role for lin-12 signaling in the adult C. elegans nervous system, using behavior as an indicator of neuronal activity. C. elegans predominantly move forward, but they spontaneously initiate backward locomotion. Genetically modulating lin-12 activity alters the rate of initiation of spontaneous reversals. Using inducible RNAi and a conditional, gain-of-function allele of lin-12, we show that this behavioral change can occur within a few hours of altering lin-12 activity in post-developmental adults. We also show that these inducible behavioral changes are rapidly reversible, strongly suggesting that lin12 mediated behavioral changes are unlikely due to changes in cell fate. Altering lin-12 activity in a subset of interneurons is sufficient to alter behavior. $g l r-1$, an AMPA/kainate receptor homolog gene expressed in these interneurons, genetically interacts with lin-12. Our results demonstrate a novel, post-developmental role for lin-12 signaling that is clearly distinct from its role in cell fate determination.

## Results

## Altering lin-I 2 activity increases spontaneous reversals during locomotion

To assess a role for lin-12 Notch signaling in behavior, we first examined spontaneous reversal rates during locomotion in lin-12 mutant animals (Fig. 1). Normal animals moving forward consistently initiate backward locomotion approximately 10 times per 3 minutes. Reversal rates were significantly increased in lin-12(n941) loss of function (lf) animals, which completely lack lin-12 gene function. The behavioral defect of lin-12(lf) animals was rescued by a previously described transgene containing a


Figure I
Altering lin- 12 activity increases spontaneous reversal rates. Animals of the genotypes indicated were tested for mean number of reversals per 3 minutes on NGM agar plates (see Methods for details). lin-I2(If) is lin-I2(n94I), a complete loss of function allele. lin-I2(gfcs) is lin$12(n 137 n 460)$, a cold sensitive, gain of function allele. lin-I2p::lin-I2(+) and lin-I $2 p:: l i n-12(O E)$ are transgenic animals that have been injected (at different concentrations, see Methods) with plin-I2::gfp, a plasmid that expresses a functional lin- 12 cDNA fused to gfp under the control of the lin12 promoter 37. ${ }^{* * *} p<0.000$ I vs. wild type.
lin-12 cDNA driven by the lin-12 promoter [37]. Furthermore, lin-12(lf) behavioral defects could be recapitulated by RNAi (see below). These results indicated that loss of function in lin-12 caused increased reversal rates.

The effect of increased lin-12 activity on reversal rates was then assessed using lin-12(n137n460) (Fig. 1), a gain of function, cold sensitive (gfcs) allele [38,39]. Reversals were not significantly increased in lin-12(gfcs) animals raised at the permissive temperature $\left(25^{\circ} \mathrm{C}\right)$, but were dramatically increased in animals raised at the restrictive temperature $\left(15^{\circ} \mathrm{C}\right)$. Cultivation temperature had no effect on reversal rate in wild type animals. The increased reversal rate of lin-12 (gfcs) animals was due to increased lin-12 activity, as transgenic animals that overexpress LIN12 (lin-12p::lin-12(OE)) also had increased reversals. Thus, both gain and loss of function in lin-12 causes increased reversal rates.

## An allelic series of lin-I 2 mutants reveals complex regulation of behavior

To further characterize the relationship between lin-12 activity and reversal rates, we assessed behavior across the lin-12 allelic series ordered based on the severity of previously determined vulval defects (Table 1). lin-12 alleles can be grouped into 4 classes: strong loss of function, weak gain of function, moderate gain of function, and strong gain of function. lin-12(n941) null animals are

Table I: Allelic series of lin-I 2 mutants. Vulval phenotype abbreviations are as follows: Pvl, protruding vulva; WT, wild type; Vul, vulvaless; and Muv, multiple pseudovulvae. All animals were tested at $25^{\circ} \mathrm{C}$ except animals carrying the cold-sensitive lin-I2( $n / 37 \mathrm{n} 460$ ) allele were raised at the non-permissive temperature $15^{\circ} \mathrm{C}$. Control animals raised at 15 or $25^{\circ} \mathrm{C}$ had wild type reversal rates (see Figs. $I$ and 2).

| strain | vulval phenotype | reversals/3 min. $\pm$ S.E.M. | n |
| :---: | :---: | :---: | :---: |
| lin-I2(n94I) (null) | Pvl | $17.3 \pm 1.7$ | 15 |
| lin-12(n941)/+ | WT | $10.8 \pm 1.5$ | 15 |
| lin-12(+) | WT | $10.5 \pm 0.7$ | 45 |
| lin-12(n302)/lin-12(n941) | Vul | $9.3 \pm 1.3$ | 15 |
| lin-12(n302) | Vul | $8.8 \pm 1.1$ | 13 |
| lin-12(n379) | Vul | $7.7 \pm 0.5$ | 15 |
| lin-12(n676) | Vul | $6.0 \pm 0.3$ | 5 |
| lin-I2(n137n460)/lin-12(n941) | WT | $22.9 \pm 1.5$ | 12 |
| lin-12(n137n460)/+ | WT | $20.1 \pm 1.8$ | 9 |
| lin-12(n137n460) | Muv | $23.7 \pm 1.8$ | 12 |
| lin-12(n137)/lin-12(n941) | Muv | $17.6 \pm 1.2$ | 5 |
| lin-12(n137)/+ | Muv | $6.0 \pm 1.6$ | 7 |
| lin-12(n427) | Muv | $3.9 \pm 0.2$ | 15 |
| lin-12(n137) | Muv | $3.6 \pm 1.1$ | 10 |

sterile and display protruding vulva that usually burst in adult animals; these animals had increased reversals. The vulval and reversal phenotypes of lin-12(n941)/+ animals were normal, indicating that the lin-12(n941) mutation is recessive. lin-12(n302), lin-12(n379), and lin-12(n676) are weak gain of function alleles [39]; these animals were fertile, vulvaless, and had slightly decreased reversal rates. lin-12(n137n460) acts as a moderate gain of function allele; these animals are cold-sensitive, display multiple pseudovulvae at the restrictive temperature, and had increased reversals. Finally, lin-12(n137) and lin-12(n427) are strong gain of function alleles that display multiple pseudovulvae and had strongly decreased reversals. We note that lin-12(n137)/lin-12(n941) hemizygote animals have multiple pseudovulvae and have high reversal rates consistent with lin-12(n137n460) phenotypes. Since lin$12 p::$ inn-12(OE) (Fig. 1) and other transgenic animals overexpressing lin-12 (see Fig. 5B) recapitulate the moderate lin-12 gain of function allele, we expected that injection of the lin-12p::lin-12 construct at a higher concentration would lead to transgenic animals that recapitulate the strong lin-12 gain of function alleles. However, we were unable to generate viable transgenic lines using higher concentrations of lin-12p::lin-12 (data not shown; see Methods for details). We conclude that altering lin-12 activity results in complex changes in the pattern of reversal behavior; the implications of this allelic series are discussed below.

## Altering lin- 12 activity in adult animals is sufficient to increase reversal rates

lin-12 Notch plays well established roles in development. Therefore, we asked whether increased reversals in lin-12
mutant animals depended on lin-12 activity during development or in adults. lin-12 loss of function was induced by expressing an inverted repeat of a lin-12 cDNA fragment under the control of a heat shock promoter to knock down lin-12 activity by RNAi (hsp::lin-12(RNAi)) in otherwise normal adult animals (Figure 2A). Uninduced $h s p:: l i n-12(\mathrm{RNAi})$ adult animals (filled triangles) raised at $25^{\circ} \mathrm{C}$ had normal reversal rates, while heat shock induction resulted in dramatically increased reversal rates within 4 hours. Reversals returned to near basal levels after overnight recovery (approx. 14 hours). Heat shock had no effect on wild type control animals (filled circles). As a control, we generated transgenic animals containing an inverted repeat of a cDNA fragment from the Gprotein coupled receptor kinase-2 grk-2) gene under control of the heat shock promoter (hsp::grk-2(RNAi)). grk-2 loss of function causes sensory defects [40], but had no effect on reversal rates (data not shown). Heat shock induction of $h s p:: g r k-2(\mathrm{RNAi})$ did not alter reversal rates (open squares), indicating that neither the presence of the heat shock vector nor overexpression of an unrelated dsRNA influenced reversal rates. We conclude that loss of function of lin-12 in adult animals is sufficient to alter behavior.

We examined lin-12(gfcs) animals in temperature shift experiments (Figure 2B). lin-12 (gfcs) adults raised at the restrictive temperature $15^{\circ} \mathrm{C}$ (filled square, $\mathrm{t}=0$ ) initially had increased reversal rates. When these animals were moved to the permissive temperature of $25^{\circ} \mathrm{C}$ (dotted line with filled squares), reversal rates gradually decreased, and after 3 hours reversals decreased to wild type levels. In reciprocal experiments, lin- 12 (gfcs) adults raised at $25^{\circ} \mathrm{C}$


Figure 2
Changing lin- 12 activity in adults alters spontaneous reversal rates. Changing lin- 12 activity in adults alters spontaneous reversal rates. A. Reversal rate changes in hsp::linI 2(RNAi) animals. Filled triangles indicate hsp::lin-I2(RNAi) animals; filled circles indicate wild type control animals; and open squares indicate $h s p:: g r k-2(R N A i)$ control animals. Animals were heat shocked at $33^{\circ} \mathrm{C}$ for 2 hours, allowed to recover at $25^{\circ} \mathrm{C}$ then tested 4 hours later. After recovery at $25^{\circ} \mathrm{C}$ overnight (approx. 16 hours), animals were tested again. ${ }^{* * *} p<0.001$ vs. wild type. B. Reversal rate changes in lin-I 2 (gfcs) animals. lin-12 (gfcs) animals raised at the permissive temperature $\left(25^{\circ} \mathrm{C}\right)$ are indicated by open squares, and those raised at the restrictive temperature $\left(15^{\circ} \mathrm{C}\right)$ by filled squares. Open and filled circles indicate wild type animals raised at 25 and $15^{\circ} \mathrm{C}$, respectively. Temperature shifts from 25 to $15^{\circ} \mathrm{C}$ are indicated by solid lines, and those from 15 to $25^{\circ} \mathrm{C}$ are indicated by dotted lines. $* p<0.0 \mathrm{I}$ vs. $\mathrm{t}=0 \mathrm{hrs} . ; * *$ $p<0.01$ vs. $\mathrm{t}=4 \mathrm{hrs} . ;{ }^{* * *} p<0.00 \mathrm{I}$ vs. $\mathrm{t}=0 \mathrm{hrs}$.
(open square, $\mathrm{t}=0$ ) initially had almost normal reversal rates. When they were moved to $15^{\circ} \mathrm{C}$ (solid line with open squares), reversal rates gradually increased until they reached levels comparable to those of lin-12 (gfcs) animals raised at $15^{\circ} \mathrm{C}$. When these animals were moved back to $25^{\circ} \mathrm{C}$ (dotted line with open square), reversal rates decreased to original levels within 2 hours. Temperature shifts and cultivation temperature had only minimal effects on control wild type animals (open and filled circles). Taken together, these data demonstrate that altering lin-12 Notch activity for a few hours in post-developmental adult animals is sufficient to change behavior and suggests that lin-12 activity is regulating a physiological, not a developmental, process.

## lin-I2 is not required in the vulval lineage to regulate reversals

Where does lin-12 function to regulate reversal rates? LIN12 is expressed in the somatic gonad and vulval lineages, based on previous studies using a functional lin-12::gfp transgene [37]. To test if lin-12 activity in these tissues regulated reversal rates, we eliminated the somatic gonad and the vulva by killing the progenitor cells of these lineages using a laser and then determining the reversal rates of the operated animals. Vulval development depends on cell-cell signaling from the anchor cell to the vulval precursor cells [41]. The anchor cell (and somatic gonad) is derived from one of two equipotent cells called Z1 and Z4 in L1 larvae; thus, killing Z1 and Z4 results in animals that lack gonads and vulvae.

Wild type Z1-Z4-killed adult animals had normal reversal rates, indicating that these tissues play no role in regulating the reversal rate in wild type animals (Fig. 3). We tested the role of the vulval and gonadal lineages in regulating reversal rates in lin-12(RNAi) (see Methods for details) and lin-12p::lin-12(OE) animals. Z1-Z4-killed lin12(RNAi) and lin-12p::lin-12(OE) animals maintained high reversal rates comparable to mock treated animals. These data indicate that lin-12 must function outside of the somatic gonad and vulva to regulate reversal rates.

We considered the possibility that the gross morphological vulval defects in lin-12 mutant animals, but not lin-12 signaling per se, might account for changes in reversal rates. lin-12(lf) animals have a large protruding vulva, while lin-12 (gfcs) animals raised at the restrictive temperature $15^{\circ} \mathrm{C}$ have multiple pseudovulvae. We measured basal locomotion rates in lin-12(lf) and lin-12(gfcs) animals and found that they had slight but significant decreases in basal movement rate (Table 2). However, lin12(RNAi) and lin-12p::lin-12(OE) animals, which phenocopy the reversal phenotypes but not the vulval phenotypes of the mutant animals, had normal basal movement rates. These data indicate that morphological defects of


Figure 3
lin-I2 is not required in the gonadal or vulval lineages to regulate reversal rates. The somatic gonads and vulval tissues were eliminated by killing the progenitor ZI and Z4 cells in LI larvae with a laser microbeam (see text for details). Successful ablation of ZI and Z4 was scored visually as follows: animals in which both ZI and Z4 were ablated lacked gonads and vulvae; animals in which only one of the two cells were killed results in a protruding vulva; and animals in which neither cell was killed resulted in fertile animals with normal vulvae. Only animals in which both ZI and Z4 were killed were scored for behavior. The reversal rates of mock treated lin-I 2(RNAi) and lin-I $2 p:: l i n-12(O E)$ animals were slightly lower compared to untreated animals, but they were still significantly higher than that of wild type. ${ }^{* *} p<0.01$, *** $p<0.00$ I. Statistical comparisons are to wild type.

Table 2: Basal locomotion rates of animals with altered lin-I2 activity. Animals were tested under identical conditions as reversal assays in 10 second bins. A single body bend was scored as a complete dorsal to ventral oscillation. Only forward moving animals were scored; if an animal reversed direction during the assay, that data point was discarded.

| strain | body bends/ $10 \mathrm{sec} \pm$ S.E.M. | n | $p$ value |
| :---: | :---: | :---: | :---: |
| wild type | $5.2 \pm 0.2$ | 26 |  |
| lin-12(If) | $3.9 \pm 0.2$ | 43 | $<10^{-5} \mathrm{vs}$. wild type |
| lin- 12 (gfcs) $25^{\circ} \mathrm{C}$ | $5.1 \pm 0.2$ | 20 |  |
| lin-12(gfcs) $15^{\circ} \mathrm{C}$ | $3.8 \pm 0.2$ | 24 | $<10^{-4} \mathrm{vs}$. wild type |
| lin-12(RNAi) | $5.3 \pm 0.3$ | 24 |  |
| lin- I 2p::lin-I2(OE) | $5.2 \pm 0.2$ | 24 |  |

the vulva in lin-12 mutant animals cannot account for the altered behavior. Taken together, we conclude that lin-12 expressed in the vulva and somatic gonad does not contribute to the regulation of reversal rates during locomotion.


Figure 4
LIN-I 2::GFP is expressed in RIG neurons. RIG neurons are indicated by arrowheads. Expression of LIN-I2::GFP was detected in approximately $25 \%$ of $\mathrm{LI} / \mathrm{L} 2$ animals. The identity of RIG neurons was confirmed by using a nmr- $1::$ dsRed reporter gene that labels the AVG neuron, which is located in between the RIG neurons (data not shown). Scale bar = 10 $\mu \mathrm{m}$.

## lin- 12 acts in a subset of glr-I expressing neurons to regulate reversals

lin-12 might act in or upon neurons to control C. elegans behavior. Previous studies have implicated the command interneurons AVA, AVB, AVD, AVE and PVC in regulating normal forward and backward locomotion [42]. The intrinsic activation of these interneurons affects reversal rates [43]. Other neurons presynaptic to the command interneurons, such as ASH [43] and AIY [44-46], can affect reversal rates as well. We did not detect any overt cell fate changes or morphological defects in any of these or other neurons in lin-12(lf), lin-12(gfcs) or lin-12(n137gf) mutant animals (data not shown), consistent with a previous report [47] and supporting our conclusion that lin12 mediated behavioral changes were not due to developmental defects. LIN-12 expression was not detected in any of these neurons either by immunohistochemical analysis or by GFP fluorescence (data not shown). However, occasional LIN-12::GFP expression was observed in the RIG neurons of young larvae (Fig. 4). These observations and the preceding temperature shift experiments suggested that LIN-12 may be expressed in adult neurons at levels too low to detect. Increasing LIN-12::GFP levels further caused lethality (data not shown); therefore, a


Figure 5
lin-I2 likely acts in a subset of glr-I expressing neurons to regulate reversals. A. RNAi driven by the glr-l promoter expressing dsRNA does not spread to nearby tissues. The glr-I promoter drives expression in the command interneurons AVA, AVB, AVD, AVE, and PVC, as well as AIB, AVG, AVJ, DVC, PVQ, RIG, RIM, RIS, RMD, RMEL/R, SMD, and URY, all of which (except DVC, PVQ, and PVC) are located in the head 48, 49. The lin- 12 cDNA fragment used to express lin-/2 dsRNA also contains GFP sequences in cis. The dsRNA expressing construct was introduced into strains expressing osm-IOp::gfp in ASH neurons or elt- $2 p:: g f p$ in intestinal cells, and compared to control strains for GFP expression levels. Adult animals from multiple transgenic lines were scored; representative images are shown. B. Effect of altering lin- $/ 2$ activity in glr-l expressing neurons. $g l r-l p$ indicates the $g \mid r-l$ promoter used to drive expression of various transgenes, and $g \mid r-l p::(0)$ indicates the promoter only control. lin- $12(R N A i)$ and $g f(R N A i)$ indicate lin- 12 and $g f p d s R N A$, respectively. lin- $12(\mathrm{OE})$ indicates transgenic animals injected with a rescuing lin-I2 cDNA construct at a high concentration (see Methods). lin-I 2IC indicates a truncated, activated lin- $/ 2$ allele that lacks the extracellular domain. $* * p<0.01$ and $* * * p<0.00 \mathrm{I}$ vs. wild type, respectively. C. Expressing lin12 cDNA in glr-I expressing neurons rescues the lin-I2(If) reversal defect. ${ }^{* * *} p<0.00$ I.
functional approach was taken to determine whether lin12 acts in the nervous system.
lin-12 activity was knocked down by RNAi in a subset of neurons by expressing lin-12 dsRNA under the control of the $g l r-1$ promoter, which drives expression in the aforementioned command interneurons and twelve other classes of neurons including RIG $[48,49]$ ( $g l r-1 p::: / i n-$ 12(RNAi)). Because RNAi effects can spread systemically [50], we first validated the cellular specificity of this approach. The lin-12 cDNA fragment used to generate the $g l r-1:: l i n-12(\mathrm{RNAi})$ constructs was derived from a lin$12:: g f p$ fusion; thus, the dsRNA expressed in these transgenic animals contains both lin-12 and gfp sequences. When $g l r-1 p:: l i n-12(\mathrm{RNAi})$ constructs were injected into strains that express GFP in the intestine or in ASH sensory neurons (which are physically close to glr-1 expressing neurons), no decreases in GFP fluorescence were observed (Figure 5A). Furthermore, these transgenic animals had grossly normal fertility and vulval morphology (data not shown). Thus, RNAi effects did not appear to spread from glr-1 expressing neurons to nearby neurons, the intestine, or to the vulva. Also, we found that transgenic animals injected with the $g l r-1$ promoter fragment alone ( $g l r$ $1 p::(0))$ or constructs expressing $g f p$ only dsRNA under control of the glr-1 promoter had no effect on reversal rates (Fig. 5B). When we examined the behavior of glr$1 p::$ in-12(RNAi) animals, we found that reversal rates increased significantly. Thus, knocking down lin-12 activity in $g l r-1$ expressing neurons was sufficient to recapitulate lin-12(lf) behavioral defects.

The requirement for lin-12 activity in the nervous system was also tested by driving lin-12 cDNA expression using the glr-1 promoter (Fig. 5B). Increasing lin-12 activity by overexpressing either a full length lin-12 cDNA or a truncated, activated form of lin-12 under the control of the glr1 promoter ( $g l r-1 p:: l i n-12(\mathrm{OE})$ and $g l r-1 p:: l i n-12 I C$, respectively) also increased reversal rates. Expression of GFP using the $g l r-1$ promoter ( $g l r-1 p:: g f p$ ) as a control had no effect (Fig. 5B). Finally and most significantly, expression of the lin- 12 cDNA under the control of the glr-1 promoter ( $\mathrm{glr}-1 \mathrm{p}:$ :lin-12(+)) rescued the behavioral defects of lin-12(lf) animals, restoring reversal rates to wild type levels (Fig. 5C). These results demonstrate that lin-12 activity in $g l r-1$ expressing neurons is sufficient to regulate reversal rates.

## Increased lin-I 2 activity affects reversal rates via RIG neurons

The RIG neurons, in which we observed weak LIN12::GFP expression, express $g l r-1$. Interestingly, the RIG neurons are presynaptic to command interneurons. The role of RIG neurons in spontaneous reversal rates was tested by laser ablation. Laser ablation of the RIG neurons


Figure 6
The RIG neurons are a likely site of lin- 12 gain of function action. To facilitate RIG neuron identification, these experiments were carried out in a $f 1 p-18 p:: g f p$ background (see Methods). lin-/2(gfcs) animals were raised at $25^{\circ} \mathrm{C}$, then were shifted as young adults to $15^{\circ} \mathrm{C} 4$ hours prior to behavioral assays. ${ }^{*} p<0.05$, $* * * p<0.000$ I.
did not dramatically affect reversal rates in wild type or lin-12(RNAi) animals. However, eliminating the RIG neurons of lin-12p::lin-12(OE) animals ameliorated reversal rate increases (Fig. 6), Thus, increased lin-12 function in the RIG neurons is likely responsible for increased reversal rates in lin-12p::lin-12(OE) animals.

Despite the fact that lin-12 overexpression recapitulated lin-12(gfcs) behavioral defects, we considered the possibility that the lin-12p::lin-12(OE) transgene might act ectopically or during development to alter reversal rates. Increasing lin-12 activity in adult lin-12(gfcs) animals in temperature shift experiments was sufficient to increase reversal rates (Fig. 2B). Therefore, we carried out RIG laser ablations in lin-12 (gfcs) animals, using the same temperature shift paradigm described above (Fig. 2B). RIG killed, temperature shifted lin-12(gfcs) animals had normal reversal rates, while mock treated lin-12(gfcs) control animals retained high reversal rates. We conclude that increased lin-12 activity in the RIG neurons of adult animals increases reversal rates.

## Genes that interact with lin- 12 to regulate reversals

To determine if the canonical lin-12 signaling pathway regulates spontaneous reversal rates, we examined the reversal rates of animals that are defective in genes of the lin-12 pathway, specifically lag-1 (which encodes a transcriptional cofactor that is the major effector for lin-12 signaling) and lag-2 (which codes for a Notch ligand) (Fig. 7A). The reversal rates of partial loss-of-function lag-1 and


Figure 7
Genes that interact genetically with lin-I 2 to regulate reversals. A. lag-I and lag-2 likely function with lin-I 2 to regulate reversals. Complete loss of function in lag-I and lag2 cause lethality; therefore, partial loss of function alleles were used (oml3 and q420, respectively). lag-2(sd) is lag2(sa37), a semidominant suppressor of lin- $/ 2$ gain of function. B. The AMPA/kainate glutamate receptor glr-l genetically interacts with lin-I 2 . glr-l (If) is glr-I (n246I), a genetic null allele. glr-l $p:: g / r-1$ (OE) is nuls25 (see Methods for details). *** $p<0.001$ vs. wild type.
lag-2 mutant animals were relatively normal. However, partial loss of lag-1 function suppressed increased reversal rates in $g l r-1 p:: l i n-12 I C$ animals that had constitutively activated lin-12 signaling, consistent with lag-1 functioning downstream of lin-12 regulating reversal rates. Also, a semidominant allele of lag-2 that suppresses the lin-12 gain-of-function multivulval phenotype caused increased reversal rates. Although strong loss of function alleles could not be tested due to embryonic lethality, our results suggest that lin-12, lag-2, and lag-1 likely act together in the nervous system to regulate reversals.

Finally, given the previously described role of the glr-1 AMPA receptor in the command interneurons [43,48,49,51], we examined more closely the role of glr-1 in spontaneous reversals and lin-12 mediated behavioral changes (Fig. 7B). Consistent with a previous report, complete loss of $g l r-1$ function ( $g l r-1$ (lf)) alone had no effect
on reversal rates [43]. However, we found that overexpression of $g l r-1$ ( $g l r-1 p:: g l r-1(\mathrm{OE})$ ) increased spontaneous reversal rates. We note that different constructs are used here than previous studies [43] (see Methods for details) and that reversal rates can be dependent on assay conditions. Both $g l r-1(\mathrm{lf}) ; l i n-12 p:: l i n-12(\mathrm{OE})$ and $g l r-1(\mathrm{lf}) ; g l r-$ $1 p:: l i n-12(\mathrm{RNAi})$ animals had dramatically decreased reversal rates (below wild type levels). Yet, there were no dramatic changes in the expression of a $g l r-1 p:: g f p$ transcriptional reporter in lin-12 (gfcs) or (lf) animals (data not shown). Our results suggest that glr-1 AMPA receptor activity, but not levels, are modulated by lin-12 signaling to regulate reversals.

## Discussion

In this study we demonstrate a non-developmental role for lin-12 Notch in the adult nervous system regulating C. elegans behavior. lin-12 mediated behavioral changes can be rapidly induced within a few hours in adult animals and are reversible. Knocking down lin-12 activity by RNAi or by activating lin-12 in glr-1 expressing neurons is sufficient to reproduce the behavioral defects of lin-12 mutant animals. The rapidity with which behavioral changes can be induced in post-developmental adult animals argues that neither lin-12 mediated cell fate changes nor de novo neurite outgrowth are the likely mechanisms for altering behavior. Rather, our results are consistent with a novel role for lin-12 signaling acutely regulating neuronal physiology via transcriptional activation, clearly distinct from previously described roles in cell fate specification.

Signaling pathways used to pattern the developing nervous system can also play important roles in the adult nervous system. For example, ephrins and Eph receptors function both in nervous system patterning during development and in synaptic plasticity in the adult nervous system (reviewed in [52]). Recent studies suggest that Notch signaling may also play a role in adult neurons. In Drosophila, adult animals harboring temperature sensitive, loss-of-function Notch alleles are defective for long term memory formation after one to two days at the restrictive temperature $[27,28]$. In mice, Notch1 and CBF1 heterozygous adult animals have specific defects in spatial learning and memory [26]. Similarly, adult mice in which Notch protein levels have been partially depleted by antisense RNA are defective in long term potentiation (LTP) [30]. Conditional knockout of both presenilin genes in the postnatal forebrain in mice results in defects in long-term contextual memory and LTP, when assayed in two month old animals [29]. Our heat shock and temperature shift experiments indicate that behavioral defects appear within hours, suggesting that Notch mediated alterations in neuronal function can occur on a much shorter timescale than days $[27,28]$ or months [29] as previously reported.

The lin-12 allelic series for reversal rates is complex. In particular, lin-12(n137n460) gain-of-function hemizygotes, heterozygotes, and homozygotes all have high reversal rates, while stronger gain-of-function alleles ( $n 427$ and $n 137$ ) have decreased reversals, raising the possibility that lin-12(n137n460) could be a neomorphic allele. Several lines of evidence argue against this hypothesis. First, based on vulval phenotypes, there is no evidence of any neomorphic activity. lin-12(n137n460), which is a recessive hypermorphic allele, is a revertant of $\operatorname{lin}-12(n 137)$, a dominant hypermorphic allele; the $n 460$ mutation confers a temperature sensitive, partial loss of function onto $n 137$ [38,39]. Both the $n 137$ and the $n 137 n 460$ alleles cause multiple pseudovulvae, indicating that lin$12(n 137 n 460)$ is simply a weaker hypermorph than lin12(n137). Second, modestly increasing lin-12 activity through several other independent means also caused increased reversals. These include moderate overexpression of lin-12 (lin-12p::lin-12(OE)) at levels that do not affect fertility and vulval development, and placing the strong hypermorphic allele lin-12(n137) over the null allele (i.e., lin-12(n137/lin-12(n941) animals).

We favor the hypothesis that the unconventional lin-12 allelic series for reversal rates reflects the underlying complexity of Notch signaling and the neuronal signaling pathways that regulate behavior. lin-12 acts at multiple places during vulval cell fate specification, specifically the AC/VU decision and VPC lateral inhibition, resulting in a complex allelic series for vulval phenotypes. Similarly, lin12 gain and loss of function may have different cellular foci for action in the nervous system, making it difficult to predict the behavioral output based on simple genetic rules. This is partially supported by the RIG ablation studies, wherein killing RIG neurons in lin-12 gain of function animals ameliorated reversal increases, but had no effect in lin-12 loss of function animals. Alternatively, lin-12 may act coordinately with other genes to regulate reversals. Further genetic studies may lead to a clearer picture. Consistent with this hypothesis, we have found that $g l p-1$, another C. elegans Notch homolog, modulates reversal rates (in preparation). Our data suggest that lin-12 regulates reversal rates in a complex fashion.

The behavioral changes observed in lin-12 animals are dramatically dependent on GLR-1 AMPA receptor function. Taken together with our finding that lin-12 acts in $g l r-1$ expressing neurons to regulate reversals, it suggests a possible relationship between AMPA receptors and Notch receptors in post-developmental synaptic plasticity. This is consistent with a recent study that demonstrated that altering Notch signaling caused defects in LTP in mice [29,30]. Based on our genetic analysis, glr-1 may be a target of lin-12 signaling or lin-12 signaling may act in parallel with glr-1. For example, lin-12 signaling may modu-
late other glutamate-gated currents to influence membrane excitability. Consistent with this hypothesis, loss of function in avr-15, one of several semi-redundant C. elegans genes encoding conserved glutamate-gated chloride channel subunits [53], results in increased reversals. avr-15 is expressed in the AVA command interneurons (data not shown) and chloride currents have been observed in these interneurons [51], making AVR-15 a candidate target for regulation by LIN-12 signaling. Similarly, loss of function of $n m r-1$, which encodes an NMDA glutamate receptor subunit, results in decreased spontaneous reversals [54], suggesting that $n m r-1$ activity could be influenced by lin-12. Additional behavioral and genetic analysis will be required to further delineate the targets of lin-12 signaling in adult neurons.

It should be noted that defects in Notch signaling can result in pleiotropic developmental disorders and nervous system dysfunction. CADASIL syndrome is associated with mutations in human Notch3 and is characterized by seizures, late onset neurodegeneration and vascular defects [32]. Mutations in Jagged1 (a DSL protein family member) are implicated in Alagille syndrome, which is characterized by defects in liver, cardiac, and skeletal tissues, and less frequently, neurovascular defects and mental retardation [34,35]. Familial, early onset Alzheimer's disease is often caused by mutations in presenilin 1 or presenilin $2[33,36]$. The developmental defects associated with CADASIL and Alagille syndromes make it difficult to establish a role for Notch signaling in neurons, but it may play a role in the defects observed in some of the late-onset symptoms. Given the emerging role for Notch signaling in the adult nervous system, a role for defective Notch signaling in these and other neurological disorders warrants further investigation.

## Conclusion

We have demonstrated a novel role for lin-12 Notch in Caenorhabditis elegans in the adult nervous system. Changing lin-12 activity postdevelopmentally in adult animals alters the spontaneous reversal rates during locomotion. lin-12 activity in the vulva and somatic gonad, where lin12 expression was previously reported, is not required to control reversal rates. In contrast, altering lin-12 activity in specific neurons is sufficient to alter behavior. lin-12 likely acts through the canonical Notch signaling pathway that includes the ligand lag-2 and the downstream effector lag1. The neuronal function of lin-12 is clearly independent from cell fate specification during development.

## Methods

## Behavioral assays

Spontaneous reversals are modulated by sensory input, environmental conditions and feeding status $[43,55]$. To control these variables, animals were cultured on NGM
agar plates containing OP50 E. coli at $25^{\circ} \mathrm{C}$, except in temperature shift experiments, in which animals were cultured at $15^{\circ} \mathrm{C}$ and moved to room temperature 30 minutes prior to assays. Young adults (containing at least 4 eggs) were moved from the bacterial lawn of an uncrowded plate to an NGM plate lacking food, allowed to crawl around briefly to remove bacterial residue, then quickly transferred to another NGM plate lacking food for assays. Spontaneous initiation of backward locomotion was recorded over three minutes during the next 1.5 to 10.5 minutes with the lid on. Up to three animals per assay plate per trial were used; no effect on reversal rates was observed for up to three animals per plate. Freshly poured NGM agar plates were dried in a laminar flow hood for approx. 2 hours, sealed with Parafilm, then stored at $4^{\circ} \mathrm{C}$ at least overnight. Plates were allowed to warm up to room temperature for at least 30 minutes prior to use. Several assay plates were tested until a plate that resulted in an average of 10 reversals in 3 minutes was observed for N2 control animals; this plate was then used for all subsequent assays on that day. Each initiation of backward locomotion was scored as one reversal; omega turns without reversals were not scored. A subset of animals was scored blind as to genotype and/or transgene to confirm results. lin-12 mutants have defective vulvae, which are visually obvious; therefore, lin-12 mutant animals were scored independently by two observers. Statistical analysis was performed using the two tailed Student's t test.

## Laser ablations

Laser ablations were performed as previously described [56] using a Micropoint ablation system (Photonic Instruments, St. Charles, IL). RIG ablations were undertaken in $n y I s 60$ animals expressing $f l p-18 p:: G F P$ [57]. These animals are uncoordinated but have normal spontaneous reversal rates. lin-12(lf) mutant animals were not subjected to laser microsurgery because they rarely survived the procedure. lin-12 (gfcs) mutant animals did not survive laser microsurgery as L1 larvae, but most survived when operated on as L2-L3 larvae. After laser microsurgery, lin-12 (gfcs) animals were allowed to recover at the permissive temperature $25^{\circ} \mathrm{C}$ for $1-2$ days, then were shifted to the restrictive temperature $15^{\circ} \mathrm{C}$ for 4 hours prior to behavioral assays. The $f l p-18 p:: g f p$ transgene did not affect the temperature dependence of lin-12 (gfcs) phenotypes (data not shown). After behavioral assays were completed, successful ablation of the RIG neurons was scored by the lack of GFP labeled neuronal cell bodies in the retrovesicular ganglion. In nearly all laser ablation experiments, mock treated animals with altered lin-12 activity had slightly lower reversal rates than untreated animals. However, they still had significantly higher reversal rates than wild type mock treated animals.

## Molecular biology

Plasmids used for transgenes are as follows: lin-12p:::in12(OE), plin-12::gfp; hsp::lin-12(RNAi) and lin-12(RNAi), pHA\#394; $h s p:: g r k-2(R N A i), \quad \mathrm{pHA} 327 ; ~ g l r-1 p::(0)$, pHA\#421; $g l r-1 p:: g l r-1(\mathrm{OE}), \quad \mathrm{pCR} \# 3 ; ~ g l r-1 p:: g f p(\mathrm{RNAi})$, pKP\#6 and pHA\#424; glr-1p::lin-12(OE) and glr-1p::lin12(+), pHA\#444; glr-1p::lin-12IC, pHA\#382; glr-1p::lin12(RNAi), pHA\#380 and pHA\#381. Plasmid details are available upon request.

## Genetics and strains

Strains used in this study: N2 Bristol wild type isolate, lin12(n137n460gfcs), lin-12(n941lf)/unc-32, lin-12(n941lf)/ eT1, lin-12(n941lf)/qC1, lin-12(n137)/unc-32, lin12(n302), lin-12(n379), lin-12(n427), lin-12(n676), lag1(om13), lag-2(sa37), lag-2(q420), glr-1(n2461) ncl1(e1865), pha-1(e2123ts), nyIs60 [lin-15(+) flp-18p::gfp], $m g I s 18$ [lin-15(+) ttx-3p::gfp], nuIs25 [lin-15(+) glr-1p::glr$1:: g f p]$, nuIs1 [lin-15(+) glr-1p:::gfp], rtIs11 [osm-10p::gfp], and rtIs18 [elt-2p::gfp]. Transgenes were co-injected using pha-1 (+) ( $\mathrm{pBX1}$ ), myo-2p::gfp (pPD48.33), and/or elt$2 p:: g f p$ (рJM67) as markers; details upon request. Heat shock induction occurred at $33^{\circ} \mathrm{C}$ for 2 hours. hsp::lin12(RNAi) introduced at $8 \mathrm{ng} / \mu \mathrm{l}$ yielded inducible transgenic lines (hsp::lin-12(RNAi)); introduction at $50 \mathrm{ng} / \mu \mathrm{l}$ resulted in lines with increased reversal rates even in the absence of heat shock ( $26.8 \pm 1.9$ reversals/ $3 \mathrm{~min} ., \mathrm{n}=11$; see also Fig. 3); these lines are designated lin-12(RNAi) in the text to distinguish them from the inducible hsp::lin12(RNAi) lines. Transgenic lines overexpressing lin$12 p:: l i n-12$ at very high levels ( $100 \mathrm{ng} / \mu \mathrm{l}$ ) often had extra vulvae and were difficult to generate and maintain; these animals were used only for expression analysis. Moderate overexpression ( $50 \mathrm{ng} / \mu \mathrm{l}$ ) of lin-12p::lin-12 was not overtly deleterious and vulval perturbations were infrequent; these animals were used for behavioral analysis. The integrated transgene nuIs 25 that overexpresses a GFP tagged glr-1 rescue construct [58] increased reversals (shown in Fig. 7B). We also generated extrachromosomal arrays marked by pha-1 that overexpress a glr-1 rescue construct lacking GFP; animals carrying these arrays also had increased reversals $(15.7 \pm 0.8$ reversals $/ 3 \mathrm{~min} ., \mathrm{n}=26$, $p<10^{-4}$ vs. wild type).

## Authors' contributions

M.Y.C, J.L.-F., T.T., and A.C.H. all contributed to the genetic, molecular, and behavioral experiments. M.Y.C. and A.C.H. drafted the manuscript. All authors read and approved the final manuscript.

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