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The glycosyltransferase Fringe promotes Delta-Notch signaling between neurons and glia, and is required for subtype-specific glial gene expression

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The development, organization and function of central nervous systems depend on interactions between neurons and glial cells. However, the molecular signals that regulate neuron-glial communication remain elusive. In the ventral nerve cord of *Drosophila*, the close association of the longitudinal glia (LG) with the neuropil provides an excellent opportunity to identify and characterize neuron-glial signals in vivo. We have found that the activity and restricted expression of the glycosyltransferase Fringe (Fng) renders a subset of LG sensitive to activation of signaling through the Notch (N) receptor. This is the first report showing that modulation of N signaling by Fng is important for central nervous system development in any organism. In each hemisegment of the nerve cord the transcription factor Prospero (Pros) is selectively expressed in the six most anterior LG. Pros expression is specifically reduced in *fng* mutants, and is blocked by antagonism of the N pathway. The N ligand Delta (Dl), which is expressed by a subset of neurons, cooperates with Fng for N signaling in the anterior LG, leading to subtype-specific expression of Pros. Furthermore, ectopic Pros expression in posterior LG can be triggered by Fng, and by Dl derived from neurons but not glia. This effect can be mimicked by direct activation of the N pathway within glia. Our genetic studies suggest that Fng sensitizes N on glia to axon-derived Dl and that enhanced neuron-glial communication through this ligand-receptor pair is required for the proper molecular diversity of glial cell subtypes in the developing nervous system.

KEY WORDS: ***Drosophila*, Glia, Neuron, Fringe, Notch, Delta, Prospero, Axon**

INTRODUCTION

Interactions between neurons and glial cells are vital for the development, function, plasticity and maintenance of nervous systems (Freeman and Doherty, 2006). During nervous system development in *Drosophila*, glial cells are important for neuron survival and for the guidance, pruning and ensheathment of axons; in return, neurons provide trophic support for glia, stimulate glial proliferation and guide glial migrations (Booth et al., 2000; Chotard and Salecker, 2004; Hidalgo and Griffiths, 2004; Klambt et al., 2001). Although progress has been made in understanding cellular aspects of neuron-glial interactions, the molecular signals that regulate communication between neurons and glial cells remain largely undetermined. In the ventral nerve cord (VNC) of *Drosophila*, several glial classes have been defined based on their morphology, position, function and additional embryologic and molecular characteristics (Ito et al., 1995). The longitudinal glia (LG) lie dorsal to the longitudinal axon tracts of the VNC (Jacobs et al., 1989). These axon tracts, called connectives, lie within a dense neuropil enwrapped by plasma membrane from LG. The close association of LG with the neuropil provides an excellent system to identify and characterize neuron-glial signals in vivo.

LG are derived from a glioblast, which gives rise to 10-12 glial progeny only (Griffiths and Hidalgo, 2004; Jacobs et al., 1989; Schmidt et al., 1997). Proliferation within this lineage is controlled in part by Epidermal growth factor receptor (EGFR) signaling in response to an axon-derived signal, Vein (Hidalgo et al., 2001). Mitosis occurs in LG precursors that express the transcription factor Prospero (Pros) at high levels (Griffiths and Hidalgo, 2004). Pros is thought to promote cell division through EGFR-dependent activation of the MAPK pathway. Of the 10-12 LG at late stages of embryogenesis, only six continue to express Pros.

Subclasses of glia such as LG are highly specialized in both form and function, and probably arise from intrinsic genetic programs as well as extrinsic cues experienced in part through contact with neurons. All glia in *Drosophila*, except midline glia, are intrinsically specified by a regulatory cascade of transcription factors including Glial cells missing (Gcm), Tramtrack, PointedP1 and Reversed polarity (Repo), which act in concert to promote glial-specific gene expression (Jones, 2005). Targets of this cascade encode the regulator of G-protein signaling Locomotion defects (Loco) and the transcription factor Retained (Retn) (Granderath et al., 1999; Shandala et al., 2002; Yuasa et al., 2003). However, Loco and Retn are expressed in only restricted subsets of glia, including LG, as are a number of other genes, including the Fibroblast growth factor receptor Heartless (Htl), the transcription factor Distal-less and Pros. The individual or combined activities of these and other factors are likely to endow LG with their specialized morphological and functional properties. Indeed, genetic mutants for *retn* exhibit defects of LG position and reduced Loco and Pros expression, while mutants of *loco* or *htl* each have defects in LG membrane morphology (Granderath et al., 1999; Shandala et al., 2003; Shishido et al., 1997).

The restriction of gene expression to specific subsets of glia suggests there may be context-dependent, locally derived regulators that direct aspects of glial differentiation, including extrinsic molecular signals provided by axons. The expression of Pros in only six LG, and its absence in the remainder of the LG with which they share a common lineage, provided an opportunity to identify and

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characterize local molecular signals that mediate the differentiation of glial subtypes. We have found that expression of *fringe* (*fng*) is restricted to a small population of cells in the VNC that include a subset of LG. Fng is a β-1,3-N-acetyl-glucosaminyl (GlcNac) transferase that catalyzes the addition of GlcNac to O-linked fucose monosaccharides on specific EGF repeats of the extracellular domain of the Notch (N) receptor (Blair, 2000; Haltiwanger and Stanley, 2002). N is a single-pass transmembrane receptor that has two known ligands in *Drosophila*, Serrate (Ser) and Delta (Dl). Fng modulates signaling through N by reducing the sensitivity of N for Ser and increasing its sensitivity for Dl (Bruckner et al., 2000; de Celis and Bray, 2000; Fleming et al., 1997; Hicks et al., 2000; Moloney et al., 2000; Okajima et al., 2003; Panin et al., 1997). Only some of the many developmental events controlled by N also involve Fng. In *Drosophila*, Fng is an important determinant of boundary formation in the wing, eye and leg imaginal discs, and for specifying polar cell fates during oogenesis (Cho and Choi, 1998; de Celis et al., 1998; Dominguez and de Celis, 1998; Grammont and Irvine, 2001; Irvine and Wieschaus, 1994; Klein and Arias, 1998; Rauskolb et al., 1999; Rauskolb and Irvine, 1999). To date, modulation of N by Fng has not been implicated in central nervous system (CNS) development in any organism, although Fng orthologs are expressed in the developing brains of mice and fish (Ishii et al., 2000; Moran et al., 1999; Qiu et al., 2004).

Here we show that Fng is required for subtype-specific Pros expression in LG. Pros expression can be triggered by N ligands derived from neurons but not glia, and this effect can be mimicked by direct activation of the N pathway within glia. N is expressed in LG while Ser and Dl are each restricted to unique subsets of neurons. Our genetic studies in vivo suggest that Fng sensitizes N on LG to axon-derived Dl and that neuron-glial communication through this ligand-receptor pair is required for the proper molecular diversity of glial cell subtypes in the developing nervous system.

MATERIALS AND METHODS

Fly stocks

Fly stocks were obtained from the Bloomington Stock Center or alternative published sources. Gal4 lines used: *elav*^{C155}-Gal4 (Lin et al., 1994), *htl-Gal4* (Shishido et al., 1997), *repo-Gal4* (Sepp et al., 2001), *scrt*$^{11-6}$-Gal4 (Boyle et al., 2006). UAS lines: *UAS-fng* (Kim et al., 1995), *UAS-N*^{ICD} (Go et al., 1998), *UAS-H* (Maier et al., 1999), *UAS-Numb* (Yaich et al., 1998), *UAS-Ser* (Speicher et al., 1994), *UAS-Dl* (Fleming et al., 1997), *UAS-mCD8::GFP* (Lee and Luo, 1999), *UAS-Tom* (Lai et al., 2000) and *UAS-mycGFP*, which has a nuclear localization signal. Mutant alleles: Df(3L)riXT1 (*fng*^{Df}), *fng*^{L73}, *fng*^{13} and *fng*^{80} (Correia et al., 2003; Irvine and Wieschaus, 1994), *fng*^{RF584} (Grammont and Irvine, 2001), *ser*$^{+r83k}$ (Hukriede et al., 1997), *ser*^{Rx82} (Speicher et al., 1994), *ser*$^{Rev2-11}$ (Fleming et al., 1990), *loco*^{rC56} (Granderath et al., 1999) here called *loco-lacZ*, and *Dl*^{Rev10}(Heitzler and Simpson, 1991).

Immunohistochemistry

Embryos at stages 13-17 were collected at 25°C, dissected to reveal the VNC, fixed in 4% paraformaldehyde, and stained according to standard procedures. Antibodies from the Developmental Studies Hybridoma Bank: mouse anti-prospero (dilution 1:25), mouse anti-BP102 (1:50), mouse C17.9C6 (anti-N, 1:50), rat anti-elav (1:50) and mouse C594.9B (α-Dl, 1:100). Other antibodies used: rabbit anti-GFP (1:1000, Molecular Probes), rabbit anti-β-Gal (1:1000, MP Biomedicals), mouse anti-Gs2 (1:100, Chemicon), and Cy2-conjugated goat anti-horseradish peroxidase (1:100, Jackson ImmunoResearch). Rat anti-Ser polyclonal antiserum (1:1000) was a gift from K. Irvine, and rabbit anti-Dll (1:100) was a gift from S. Carroll.

In situ hybridization

In situ hybridization was performed in whole mount on embryos collected 4-16 hours after egg laying. A digoxigenin-labeled cRNA riboprobe (Roche) was made from the *fng* cDNA RE03010. Hybridization was done overnight at 55°C, and visualized using α-Dig-AP (1:1000; Roche). For fluorescence in situ hybridization, anti-Dig-POD (1:100; Roche) and Cy-3 conjugated tyramide reagent were used for probe detection (1:50; Perkin-Elmer).

Microscopy and imaging

Confocal microscopy was performed using a Yokogawa spinning disk confocal system (Perkin-Elmer) and an Eclipse TE2000-U microscope (Nikon). *Z*-series images were collected using Metamorph software (Molecular Devices). To better visualize anti-N in LG, the images in Fig. 3A-F were processed for 3D-deconvolution with Autodeblur software (Autoquant), and 3D-rendering of stacks was performed using Imaris software (Bitplane AG). Images were compiled with Adobe Photoshop.

RESULTS

Fng is expressed in LG of the *Drosophila* CNS

At late stages of *Drosophila* embryogenesis (stages 16-17), the cell bodies of neurons and glia within the VNC surround a dense neuropil comprised of axon projections, dendrites and synapses. Neurons can be immunostained with an antibody to horseradish peroxidase (HRP), highlighting their axons and revealing the ladder-like major axon tracts (Fig. 1A), called commissures and longitudinal connectives. The glial cell marker Repo highlights the nuclei of all lateral glia (Fig. 1B). LG are distinguished from other populations of lateral glia by their dorsal location, their proximity to the longitudinal connectives, and their expression of Htl. *htl-Gal4* drives expression of *UAS-nGFP* in most or all LG (Fig. 1D), and in the LG lineage (Griffiths and Hidalgo, 2004). There are 10-12 LG in each hemisegment (Griffiths and Hidalgo, 2004), 8-11 of which can be readily counted in Htl-nGFP-expressing embryos (Fig. 1D). The uncounted LG are either obscured by other Htl-nGFP cells or occasionally fail to express GFP. Interestingly, the LG can be further divided into an anterior and posterior subpopulation. The transcription factor Pros is expressed in the six anteriormost LG, which are positioned along the connectives, mostly in the interval between the anterior and posterior commissures (Fig. 1E,F). With confocal imaging we found that, in a compressed stack of *Z*-series images, some hemisegments appear to show only five Pros-positive LG, because the sixth is obscured by another.

We used in situ hybridization to determine the spatial and temporal expression pattern of *fng* transcripts in the VNC. Beginning at stage 14-15, *fng* mRNA was observed in two continuous stripes of cells that lay dorsal to the longitudinal connectives (not shown). This pattern was reminiscent of mature LG, although expression of *fng* transcript was not observed in the longitudinal glioblast at earlier stages of development, nor in migrating LG precursors. In older embryos, the two rows of *fng*-positive cells were somewhat discontinuous (Fig. 1G), and expression persisted to stage 17. To confirm whether *fng* mRNA is expressed in LG, fluorescence in situ hybridization (FISH) was performed (Fig. 1H), and embryos co-labeled with the markers Repo (Fig. 1I) and Pros (not shown). There was clear enrichment of *fng* labeling surrounding nuclei of the anterior LG. We cannot rule out the possibility that *fng* transcripts were expressed at very low levels by posterior LG and perhaps a few other cells near the neuropil, consistent with our observations of embryos carrying a *lacZ* P-element insertion in the *fng* locus (*fng*^{RF584}, not shown). Together, the results indicate that *fng* expression probably begins in all LG after their final division, and becomes progressively enriched in the anterior LG.

Fng is necessary for the correct expression of Pros in LG

Expression of *fng* in the Pros-expressing LG prompted us to test whether Pros expression was affected in LG of *fng* mutant embryos. Four different *fng* alleles were studied: *fng*^{L73} and *fng*^{13} each contain

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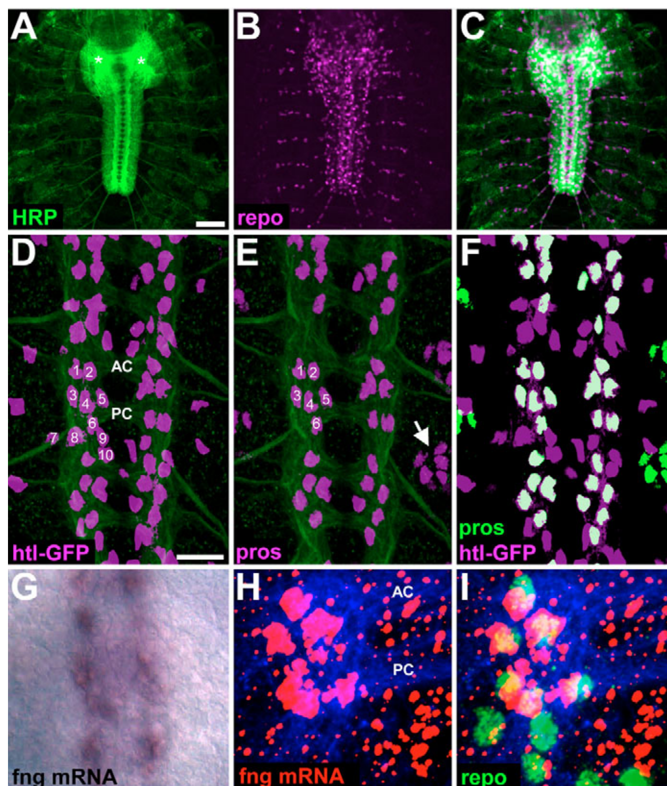

Fig. 1. Fng expression in a subset of longitudinal glia (LG). (**A-C**) Dorsal view of the nervous system of a filleted wild-type embryo at stage 16-17. (A) Anti-HRP (green) reveals the brain lobes (asterisks) and the major axon tracts of the VNC. (B) Repo-positive lateral glia (magenta). (C) Overlay of A and B. (**D**) Three segments of the VNC of a *htl-Gal4,UAS-nGFP* embryo. Anti-HRP (green) shows the anterior and posterior commissures in each segment, and the two longitudinal connectives over which the LG (anti-GFP, magenta) are positioned. (**E**) The six most anterior LG in each hemisegment express Pros (magenta), as do a small number of neurons (arrow). (**F**) Overlay of D and E without anti-HRP. Note that the Pros channel in D has been converted to green in F, clarifying the distinction between the anterior LG, which express Pros (white), and posterior LG, which do not (magenta). (**G**) Whole-mount *fng* in situ hybridization of a stage 15 embryo. Transcripts for *fng* are observed in a two rows of cells at the dorsal surface of the VNC. (**H**) Fluorescence in situ hybridization for *fng* (red), showing a single hemisegment with axons (anti-HRP, blue) and the commissures labeled. (**I**) The six anterior LG coexpress *fng* mRNA (red) and Repo (green). Anterior is at the top in all panels of all figures. Scale bars: 50 μm in A for A-C; 10 μm in D for D-F. AC, anterior commissure; PC, posterior commissure.

distinct single base-pair mutations resulting in a premature stop codon, while fng^{80} and fng^{Df} are small and large deletions of the *fng* locus, respectively (Correia et al., 2003). In wild-type animals, *fng* heterozygotes (Fig. 2A) and *fng* hemizygotes (Fig. 2G,J), 5-6 of the 10-12 LG express Pros in nearly 100% of hemisegments. We tested the *fng* alleles in various combinations and found significant losses of Pros-positive LG (Fig. 2D,H,J), indicating a recessive defect. Depending on the allelic combination, between 38 and 62% of hemisegments in *fng* mutants had fewer than five Pros-positive LG, with an average of 50% (299/597) for all combinations (Fig. 2J). Of the hemisegments that exhibited a defect, 53% were reduced to three Pros-positive LG or fewer. Among the LG that remained Pros-positive, the intensity of Pros immunoreactivity was often reduced in *fng* mutants relative to wild type (Fig. 2D,H). In each hemisegment, Pros is also expressed by a small cluster of neurons (Doe et al., 1991; Vaessin et al., 1991). Pros expression in these neurons was unaffected in *fng* mutants, indicating that the effect was specific for LG (Fig. 2D,H).

The loss of Pros immunoreactivity could indicate a reduction in the total number of LG or, alternatively, that *fng* is necessary for Pros expression. To determine whether there was a normal number of LG in the absence of Fng, we crossed the LG-specific *lacZ* reporter $loco^{rc56}$ (*loco-lacZ*) into *fng* mutants. There was no difference in the number of cells expressing *loco-lacZ* between *fng* mutants (fng^{13}/fng^{L73}, Fig. 2E) and heterozygous controls (Fig. 2B), nor was the positioning of LG within the VNC affected. Similar results were observed using Htl-nGFP to mark LG, although *loco-lacZ* reported on LG more faithfully. These results demonstrate that in *fng* mutants the LG are intact, yet Pros expression is significantly reduced.

The evidence is consistent with the idea that *fng* is necessary for maintenance, not initiation, of Pros expression in LG. First, *fng* expression begins at stages 14-15 of embryonic development, whereas Pros expression in LG precursors begins at the two-cell stage of the lineage, at stage 11-12 (Griffiths and Hidalgo, 2004). Second, although Pros expression was markedly reduced in *fng* mutants, low levels could still be observed in five to six cells in many hemisegments, suggesting Pros expression could be initiated in many instances, but not maintained.

To demonstrate the specificity of the *fng* mutant phenotype for glia, *repo-Gal4* was used to drive the expression of a *UAS-fng* transgene in *fng* mutants (fng^{13}/fng^{Df}). Pros expression was restored to levels comparable to wild type in the anterior LG (Fig. 2I). The rescue was nearly complete; 89% of hemisegments had five to six Pros-positive anterior LG, and the remaining 11% had four (Fig. 2J). In rescued *fng* mutants, ectopic Pros-positive cells were rarely observed. However, in a wild-type background, they did appear occasionally (not shown), suggesting that *fng* is both necessary and sufficient for Pros expression in LG. The sufficiency of Fng for Pros expression will be addressed again below.

The expression of Notch and its ligands at late stages of embryonic VNC development

Previous studies have demonstrated that Fng acts cell-autonomously on nascent N polypeptides within the Golgi (Bruckner et al., 2000; Munro and Freeman, 2000). Therefore, we surmised that Notch should be expressed on LG. Upon immunostaining embryos at stages 15-17, N was found to be expressed on the surface of most cell bodies in the VNC and was broadly distributed throughout the neuropil (Fig. 3A), consistent with previous studies (Fehon et al., 1991). Due to this robust expression, and the close apposition of LG with axons, it was difficult to assess whether N was expressed in LG. To address this, N expression was examined in embryos that expressed a membrane-targeted GFP reporter in LG (*Repo-Gal4, UAS-mCD8GFP*), and co-stained with antibodies to detect GFP and HRP to reveal glia and axons, respectively. In optical sections dorsal to the longitudinal connectives, N co-localized with LG membrane (Fig. 3B,C). In cross-section, N was localized primarily to the ventral side of LG (closest to the neuropil), and to contact sites between adjacent glia (Fig. 3B′,C′). In addition to LG, N was also found in other glia, and N overlapped with HRP in the dorsal neuropil (Fig. 3F). Our results show that N is indeed expressed in LG, where its sensitivity to ligand could be modulated by Fng.

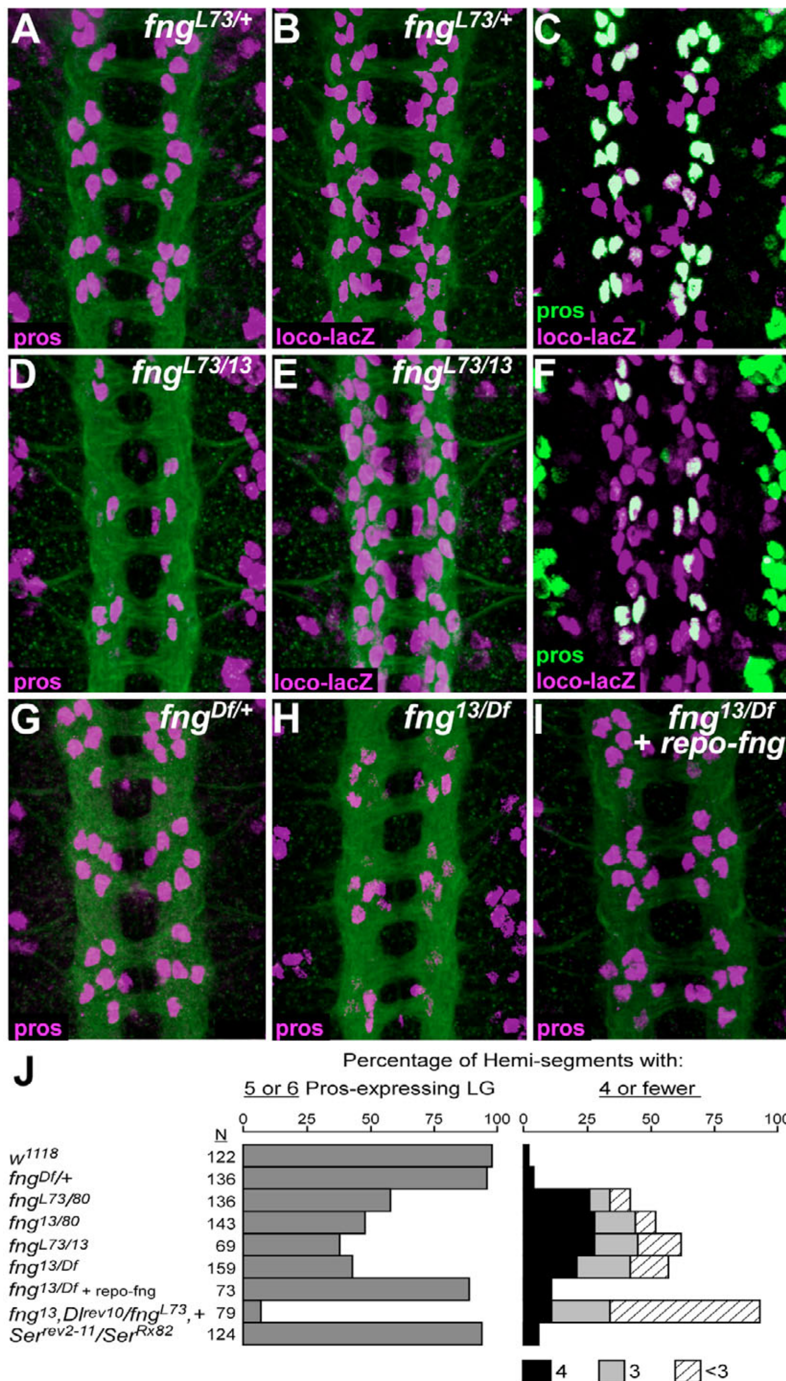

Fig. 2. Mutations in fng cause defects of Pros expression in LG. All panels except C and F show three segments of stage 15-16 VNC stained with anti-HRP (green) and co-stained for either Pros or β-galactosidase, as indicated (magenta). (**A**) *fng*^{L73} heterozygotes, like wild type, have six Pros-positive LG per hemisegment. (**B**) The reporter *loco-lacZ* reveals the number and position of LG in *fng* heterozygotes. (**C**) Overlay of D and E without anti-HRP, and with Pros converted to green. (**D**) *fng* mutants have fewer Pros-positive LG, and the level of Pros expression is often reduced. (**E**) The number and position of LG in *fng* mutants appears normal. (**F**) Overlay of D and E. (**G**) *fng*$^{Df/+}$ hemizygotes also have six Pros-positive LG per hemisegment as normal. (**H**) Reduced Pros expression in an *fng*$^{13/Df}$ embryo (D). (**I**) Pros expression is rescued in *fng*$^{13/Df}$ mutants using *repo-Gal4* to express *UAS-fng*. (**J**) Quantification of Pros-positive LG per hemisegment. Every Pros-expressing cell in the vicinity of the neuropil was counted as positive, regardless of staining intensity. The number of abdominal hemisegments counted for each genotype is indicated (N). Because one of the six Pros-expressing LG per hemisegment is often obscured in the compiled images, the results were binned as five or six Pros-expressing LG, versus four or fewer.

Dl/Ser ligands are transmembrane proteins that activate N in a contact-dependent manner. If these ligands are required for N activation in LG, one would expect them to be expressed on cells that neighbor the LG or on neurons that project axons that contact LG. Immunochemistry for Dl revealed that at stage 15-17, Dl expression was restricted to a cluster of roughly 20-30 cells with somata that were located at the lateral edge of each hemisegment (Fig. 3G,H), approximately in the middle of the dorsoventral axis. All these cells appeared to be neurons projecting axons in two fascicles, one through each of the two commissures (Fig. 3H). Interestingly, we observed that Dl immunoreactivity was dramatically reduced where these axons met the longitudinal axon tracts and LG (Fig. 3H).

Ser expression in the VNC has been noted previously (Thomas et al., 1991). We found that Ser expression was restricted to two or three commissural interneurons per hemisegment. Each cell extended an axon through either the anterior or posterior commissure (Fig. 3I), which then projected anteriorly within the longitudinal connectives.

In summary, both Dl and Ser were expressed in restricted populations of neurons in the embryonic VNC but were not observed in LG. By contrast, N was more broadly distributed and exhibited polarized subcellular localization within LG. These findings are consistent with the possibility that expression of Dl or Ser ligands on axons could activate N signaling in LG. Expression of Fng in a subset of glia might confer differential

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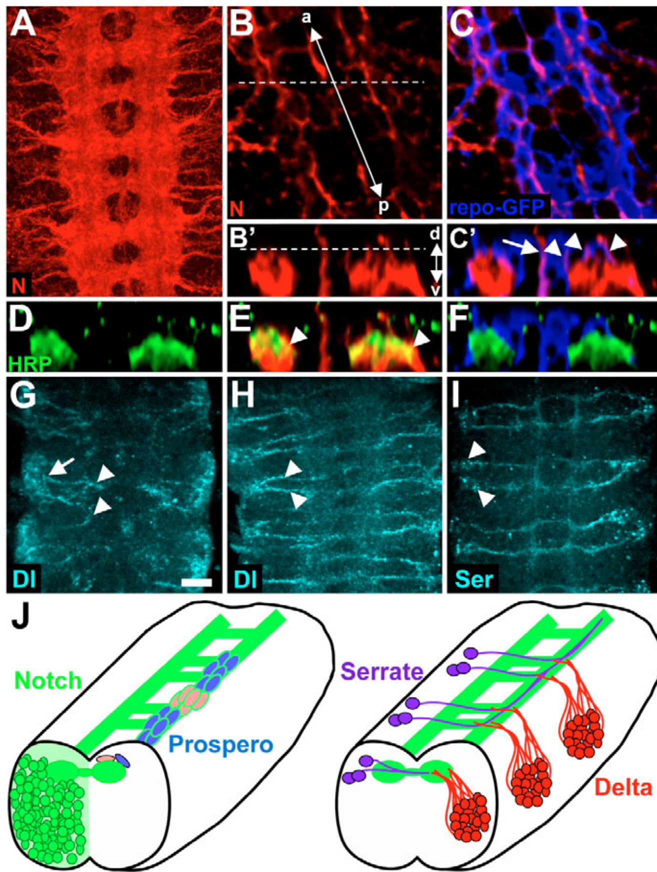

Fig. 3. Expression of N and its ligands in the VNC. (**A-F**) Stage 15 embryo (genotype *repo-Gal4;UAS-mCD8GFP*) co-labeled for N (red), GFP (blue) and HRP (green). (A) N is found on most neurons and is highly expressed in the neuropil. (B,C) Single optical section dorsal to the neuropil reveals N expression (red) along the perimeter of many LG marked by the membrane-targeted GFP. (B′,C′) In a cross-section through LG and the longitudinal connectives, N co-localizes with the membrane of LG (arrowheads) and channel glia (arrow). (D,E) N expression overlaps with anti-HRP in the dorsal neuropil (arrowheads in E), while HRP is excluded from glia (F). The dotted line in B indicates the anteroposterior (a-p) level of the cross-section in B′,C′, and D-F. The dotted line in B′ indicates the dorsoventral (d-v) level of section for B and C. (**G**) Dl expression in a cluster of cells (arrow) near the lateral edge of the VNC of a stage 15-16 embryo. (**H**) Dl is expressed on proximal axons (arrowheads in G,H) although it is largely excluded from the distal axons in the region of the longitudinal connectives. (**I**) Ser is expressed on the cell bodies (arrowheads) and axons of two or three neurons in each hemisegment. Scale bar: 10 μm. (**J**) Cartoon depicting a summary of the expression of N, Dl and Ser relative to expression of Pros in the anterior LG.

sensitivity to N ligands, and could underlie the acquisition of distinct properties in response to different levels of N signaling.

N activity and Fng are each necessary and sufficient for Pros expression

Signaling through the N receptor has been well studied, and recently reviewed (Lai, 2004; Schweisguth, 2004). In the canonical signaling pathway, the N receptor is cleaved upon ligand-binding, releasing the intracellular domain, N^{ICD}. N^{ICD} is transported to the nucleus, where it binds Suppressor of Hairless [Su(H)]. In the absence of N^{ICD}, Su(H) is part of a repressor complex that includes Hairless (H). N^{ICD} replaces H, binds Su(H) and recruits another co-activator, mastermind, leading to the activation of target gene transcription.

Fng can either increase or decrease activity through the Notch pathway by modulating the sensitivity of the N receptor to ligand. Fng renders N more sensitive to activation by Delta, and less sensitive to activation by Serrate. Therefore, it is possible that the loss of Pros seen in *fng* mutants is due either to *hypo*-activity or *hyper*-activity of the N signaling pathway, depending upon whether the relevant ligand is Dl or Ser, respectively. To test these alternatives, we examined the effects of either inhibiting or activating the Notch pathway specifically in LG.

To inhibit the N pathway, *htl-Gal4* was used to express two different N antagonists: the Hairless (H) transcriptional repressor (Morel et al., 2001) and Numb, which promotes endocytosis of N and limits exposure to ligand (Berdnik et al., 2002; Jafar-Nejad et al., 2002). Expression of *UAS-H* in LG resulted in a near complete loss of Pros expression (Fig. 4A). Seventy-eight percent of hemisegments failed to express Pros at all, and none expressed the normal number of five to six (Fig. 4J). The overall number or position of LG was unaltered (Fig. 4B). This result suggests that Pros expression in LG is regulated, either directly or indirectly, through canonical Notch signaling involving transcription activation. Expression of *UAS-Numb* was also a potent inhibitor of Pros expression (Fig. 4C,J) (Griffiths and Hidalgo, 2004).

To determine whether the effect of N inhibition was specific for Pros expression, we sought markers other than Pros that distinguish the anterior LG from posterior LG. Glutamine synthetase 2 (Gs2) has been shown previously to be expressed in LG (Freeman et al., 2003). With in situ hybridization, we found Gs2 transcript limited to the anterior six LG in each hemisegment in stage 16-17 embryos (not shown). Immunochemistry with an anti-Gs2 antibody confirmed this specific pattern of expression (Fig. 4D,E). Glutamine synthetase is expressed in glia and converts the neurotransmitter glutamate into glutamine, which is then transported back into neurons. The limited expression of Gs2 in the Pros-positive anterior LG indicates that they are indeed functionally distinct from the Pros-negative posterior LG. Inhibition of N signaling by misexpression of Numb with *htl-Gal4* caused no changes in Gs2 expression (Fig. 4F), consistent with the idea that Pros expression is specifically regulated by N signaling, that Gs2 is regulated independently, and that inhibition of N in this context does not simply convert anterior LG to posterior LG.

Is N activity sufficient to induce Pros expression in LG that do not normally express it? To test this, *htl-Gal4* was used to express a constitutively active form of N (UAS-N^{ICD}). N^{ICD} was indeed sufficient to drive Pros in most of the posterior LG, and sometimes all of them, without altering their number or their general positioning (Fig. 4G,H), confirming the results of others (Griffiths and Hidalgo, 2004). We confirmed that the ectopic Pros-positive cells were indeed LG, as they coexpressed Distal-less, a transcription factor expressed exclusively in LG and only one additional cell (not shown). Together with the results of N inhibition, these data indicate that activation of Notch signaling promotes the maintenance of Pros expression in the anterior LG. As the *fng* mutant phenotype resembled that caused by N inhibition, it is likely that Fng increases N activity in the anterior LG. As *fng* transcripts were enriched in the anterior LG, but not in posterior LG, we reasoned that perhaps Pros is not expressed in posterior LG because Fng is not there to heighten the sensitivity of these cells to ligand. Consistent with this idea, expression of *UAS-fng* with *htl-Gal4* was sufficient to promote ectopic expression in posterior LG (Fig. 4J, Fig. 5A).

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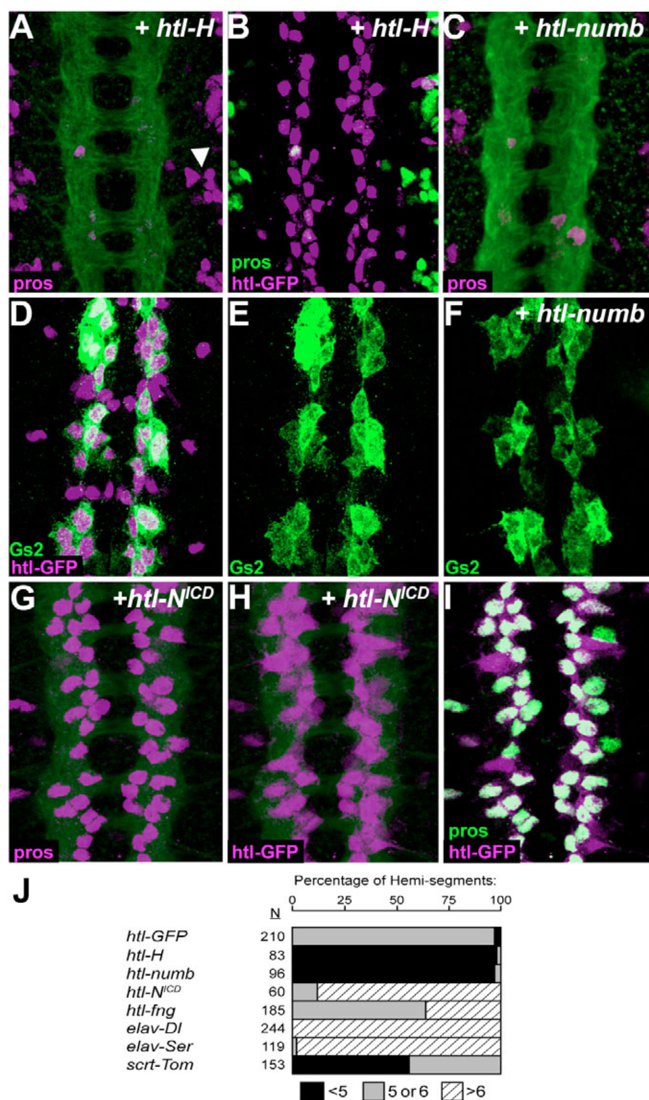

Fig. 4. N activity specifically influences Pros expression in LG. All panels show three segments of a stage 15-16 VNC with axons (anti-HRP) shown in green in A,C,G,H. All other antibodies are as labeled. *htl-Gal4* was used to drive expression of UAS transgenes. (**A**) Misexpression of Hairless (H) abolishes nearly all Pros expression in LG, while Pros in neurons is unaffected (arrowhead). (**B**) Overlay of Pros in A with anti-GFP reveals that H does not alter the number or positioning of LG. (**C**) Misexpression of Numb also abolishes Pros expression. (**D**,**E**) In wild type, the enzyme Gs2 is restricted to the six anterior LG. (**F**) In contrast to Pros, Gs2 expression in the anterior LG is unaffected by misexpression of Numb. (**G**) Misexpression of N^{ICD} causes Pros expression in most or all LG, but does not alter the number or position of LG (**H**). (**I**) Overlay of G and H without anti-HRP and with Pros converted to green. (**J**) Quantification of Pros-positive LG per hemisegment: misexpression studies. Quantification performed as in Fig. 2J, with results binned as shown in the legend.

Pros expression is upregulated in glia upon pan-neuronal misexpression of Dl or Ser

What factors in addition to Fng might limit N-dependent expression of Pros to the anterior LG? If the restricted expression of Dl on subsets of axons were to limit N activation, we predicted that misexpression of Dl in all axons would lead to excessive N activation and ectopic Pros expression. To test this, a *UAS-Dl* transgene was misexpressed using *elav^{C155}-Gal4*, which drives expression in all postmitotic neurons but is not expressed earlier in neuronal lineages and is not expressed in glia (Lin and Goodman, 1994). Misexpression of Dl did indeed lead to ectopic expression of Pros (Fig. 5B). Interestingly, co-labeling for Distal-less revealed that the effect was specific for the posterior LG. In posterior LG, one to three extra Pros-positive cells were evident in all hemisegments examined (Fig. 4J, Fig. 5B,C).

As Fng is not enriched in the posterior LG, and therefore cannot render the posterior LG less sensitive to N activation by misexpression of Ser, we predicted that Ser too might give rise to ectopic Pros expression in the posterior LG, and we found that this indeed was the case (Fig. 4J). Despite broad endogenous N expression in the VNC (Fig. 3A), misexpression of Dl (Fig. 5B) or Ser (not shown) with *elav^{C155}-Gal4* caused no obvious defects in the pattern of the major axon tracts, as determined by α-HRP (Fig. 5B) and α-BP102 immunostaining (not shown).

As a control, we tested the effects of misexpression of Dl in LG rather than neurons. By contrast to neuron-derived Dl, which increased Pros expression, glial-derived Dl suppressed Pros expression and mimicked N inhibition (Fig. 5D). This result could be a consequence of cis-inhibition of N signaling in LG by coexpression of Dl. Cis-inhibition of N is a phenomenon that has been observed previously, but the mechanism by which it operates remains poorly understood (de Celis and Bray, 2000; Jacobsen et al., 1998; Ladi et al., 2005; Sakamoto et al., 2002).

These results confirm that Notch ligands derived from neurons, but not glia, are capable of driving ectopic Pros expression specifically in posterior LG. We next asked whether Dl or Ser, like Fng, were required for the maintenance of endogenous Pros expression in the anterior LG. As Fng is known to sensitize N to the ligand Dl, and as Fng function in the anterior LG correlates with N activation and not N inhibition, we hypothesized that Dl, and not Ser, was the relevant ligand. We first tested trans-heterozygotes for *fng* and *Dl* and found no reduction of Pros expression (not shown). Homozygous Dl mutants could not be studied because of the earlier roles Dl and N have in neurogenesis, whereby certain cells of the developing neurectoderm adopt neural fate while inhibiting others from doing the same. Instead, we tested whether the loss of Pros expression observed in *fng* mutants could be further reduced by simultaneous reduction of Dl. Indeed, *fng* mutants heterozygous for *Dl^{rev10}* had dramatically lower levels of Pros than did *fng* mutants alone (Fig. 2J, Fig. 5E), with 93% of hemisegments failing to express the correct number of five to six Pros-positive LG, and 82% with three or fewer. To test whether Ser was required, Pros expression was examined in *Ser* mutants (*Ser$^{rev2-11}$*/*Ser^{Rx82}*), which was feasible because *Ser* is not required for neurogenesis. Pros expression was unaffected in *Ser* mutants (Fig. 2J, Fig. 5F), suggesting either that Ser has no function in Pros expression or that it is redundant with Dl. However, given that Fng renders cells less sensitive to N activation by Ser and more sensitive to Dl, we believe that Ser is unlikely to play a role and that Dl is the N ligand important for the maintenance of Pros in anterior LG.

N signaling in LG through neuron-glia interactions

The data suggested that Dl-bearing axons could activate N signaling in LG. In addition, Dl immunoreactivity was dramatically reduced at the position where these axons intersect with the longitudinal axon tracts and LG. It is possible that this reduction in Dl immunoreactivity resulted from increased endocytosis of Dl in the vicinity of the neuropil. Endocytosis of Dl into ligand-bearing cells is a crucial step in reception of the N signal by N-expressing cells

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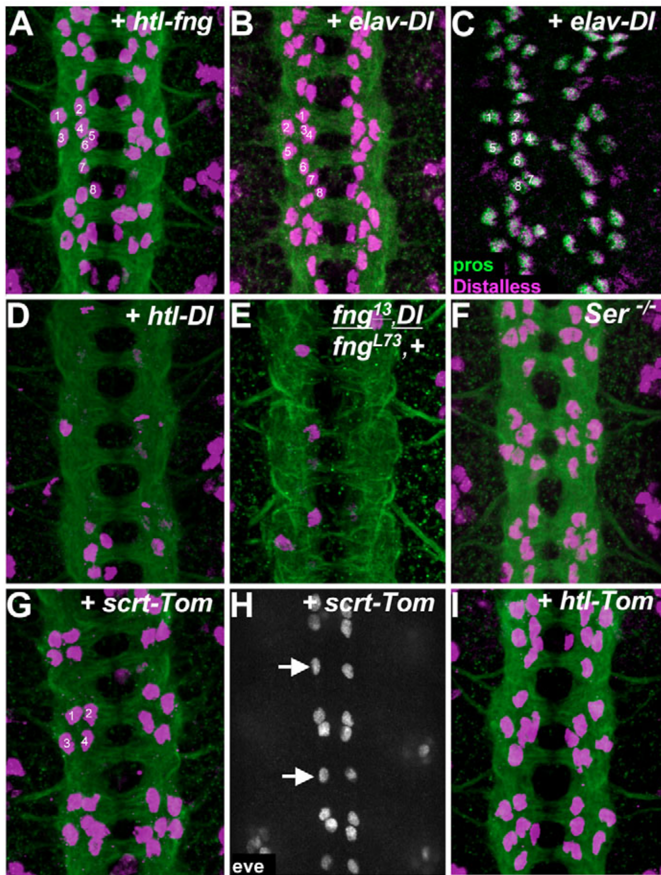

Fig. 5. Pros expression in LG depends on Dl-N signaling through neuron-glial interactions. All panels (except C and H) show three segments of a stage 15-16 VNC with the axon tracts shown in green (anti-HRP) and Pros in magenta. (**A**) Misexpression of *UAS-fng* with *htl-Gal4* leads to ectopic expression of Pros in posterior LG, labeled 7 and 8. (**B**,**C**) Ectopic Pros is also observed upon misexpression of either Dl (B) or Ser (not shown) in postmitotic neurons using *elav^{C155}-Gal4.* (C) Co-labeling for Pros (green) and the LG marker Distal-less (magenta) shows that the ectopic Pros- positive cells are indeed posterior LG. (**D**) By contrast, Pros is inhibited upon misexpression of Dl in LG with *htl-Gal4*. (**E**) Pros expression in *fng* mutants is further reduced in embryos also heterozygous for *Dl^{rev10}* (compare with Fig. 2D). (**F**) Pros expression is unaffected in *Ser$^{rev2-11}$/Ser^{Rx82}* mutants. (**G**) Fewer cells express Pros in embryos in which misexpression of *UAS-Tom* is driven by *scrt$^{11-6}$-Gal4* in neuronal lineages. (**H**) Three segments of a stage 16-17 embryo with *scrt$^{11-6}$-Gal4* driving *UAS-Tom*. Anti-Eve immunochemistry revealed no transformations of cell fate of RP2 (arrows) or other Eve-positive neurons. (**I**) Pros expression is unaffected by Tom misexpression in LG.

(Le Borgne et al., 2005a). Dl endocytosis is promoted by the activity of the E3 ubiquitin ligases Neuralized and Mind bomb (Lai et al., 2001; Lai et al., 2005; Le Borgne et al., 2005b; Pavlopoulos et al., 2001; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005). Neuralized is inhibited by interactions with members of the Brd class family of proteins (Bardin and Schweisguth, 2006; De Renzis et al., 2006). Brd proteins block Neuralized from interactions with Dl and prevent Dl endocytosis, thereby inhibiting N signaling in a non-cell-autonomous manner. To examine whether N signaling in LG occurred through neuron-glia interactions, we used the Brd protein Twin of m4 (Tom) to block Dl endocytosis in neurons, and examined the effects on Pros expression in LG. As the population of Dl-expressing neurons remains poorly defined, and there are no Gal4 lines specific for these cells, we used two alternatives. The first, *elav^{C155}-Gal4*, expresses in all postmitotic neurons, whereas the second, *scrt$^{11-6}$-Gal4*, expresses at moderate levels in most, perhaps all, neuroblasts, ganglion mother cells and postmitotic neurons. Misexpression of Tom with *elav^{C155}-Gal4* had a mild effect on Pros, with 13% of hemisegments showing fewer than five to six Pros-positive cells (not shown). Due to the inherent delay of the GAL4-UAS system, perhaps *elav^{C155}-Gal4* activated the expression of *UAS-Tom* too late to strongly inhibit N activation upon axon-glial contact. By contrast, misexpression of *UAS-Tom* with *scrt$^{11-6}$-Gal4* caused 55% of hemisegments to exhibit fewer than five to six Pros-positive cells (Fig. 4J, Fig. 5G). There was increased expression of Dl on neuron cell bodies and axons (not shown), consistent with inhibition of Dl endocytosis. Immunoreactivity was still reduced on distal portions of axons, perhaps reflecting incomplete inhibition of Dl endocytosis. This may account for milder effects caused by Tom misexpression in neurons than those caused by direct blockage of N signaling in LG using H or Numb (Fig. 4A,C,J). Nevertheless, our finding that inhibition of Dl endocytosis in neurons reduced Pros expression in LG provides strong evidence in vivo for Dl-N signaling through neuron-glial interactions.

To control for the possibility that *scrt$^{11-6}$-Gal4* might also be expressed in the LG lineage, and therefore affect Pros directly within LG rather than through Dl expressed on axons, *UAS-Tom* was also driven by *htl-Gal4*. However, Pros expression was unaffected (Fig. 5I).

During differentiation of neuronal lineages, N acts to distinguish asymmetric and unique fates of sibling cells. As *scrt$^{11-6}$-Gal4* was used to express Tom in neuronal lineages, it had the potential to inhibit Dl endocytosis, influence N signaling and cause defects of sibling cell fate determination. If axon guidance defects arose as a consequence of altered cell fates, they could interrupt neuron-glial interactions and thereby indirectly influence Dl-N signaling. Although the cell bodies and axon projections of the Dl neurons appeared normal (not shown), as did axon patterning in general (Fig. 5G), we looked for additional evidence that N-dependent cell fate decisions were unaffected. We tested whether overexpression of Tom with *scrt$^{11-6}$-Gal4* caused defects in cell fate by studying the expression of Even skipped (Eve). The cell fates of the RP2 motoneuron and its sibling (RP2sib), in addition to other neurons that are also distinguishable by anti-Eve immunochemistry, are dependent on N signaling (Doe et al., 1988; Frasch et al., 1988; O'Connor-Giles and Skeath, 2003), and can be used to indicate whether N-dependent cell fate decisions in neuronal lineages are intact. For example, when the strongly expressing *scabrous-Gal4* was used to misexpress Tom in neural lineages, losses of Eve expression in RP2 neurons were observed (not shown). By contrast, overexpression of Tom with *scrt$^{11-6}$-Gal4* caused no changes in Eve expression: RP2 and other Eve-positive neurons such as the U neurons were all present in normal numbers and positions (Fig. 5H). Together, the data suggest that the timely inhibition of Dl endocytosis in neurons can specifically block N signaling and Pros expression in LG.

DISCUSSION

We have identified the glycosyltransferase Fng as a means by which a specific subtype of glia, the anterior LG, are made sensitive to N activation, and we have provided evidence that Dl, expressed on axons, activates N signaling in these glia leading to subtype-specific gene expression. We have shown that Fng is required for maintenance of Pros expression in the anterior LG, which can also

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be blocked by antagonism of the N pathway with no effect on their survival or positioning. This is in contrast with studies of *pros* mutants, which found a role for Pros earlier in CNS development in establishing glial cell number (Griffiths and Hidalgo, 2004). The role of Pros in mature LG is poorly understood, but it has been proposed to retain mitotic potential in these cells for use in repair or remodeling of the nervous system in subsequent larval or adult stages (Griffiths and Hidalgo, 2004; Hidalgo and Griffiths, 2004). It will be important to determine the consequences of lost Pros expression from mature anterior LG, and whether additional features and functions of the anterior LG are controlled by N signaling from axons.

The importance of glycosylation for N function has been demonstrated in vivo. The addition of O-linked fucose to EGF repeats in the N extracellular domain is essential for all N activities and is mediated by O-fucosyltransferase-1 (O-fut1) (Okajima and Irvine, 2002; Sasamura et al., 2003; Shi and Stanley, 2003). By contrast, Fng is selectively used in specific developmental contexts, and has been best studied in the formation of borders among cells in developing imaginal tissues (Cho and Choi, 1998; de Celis et al., 1998; Dominguez and de Celis, 1998; Irvine and Wieschaus, 1994; Klein and Arias, 1998; Panin et al., 1997; Rauskolb et al., 1999; Rauskolb and Irvine, 1999). Fng catalyzes the addition of GlcNac to O-linked fucose, to which galactose is then added. The resulting trisaccharide is the minimal O-fucose glycan to support Fng modulation of Notch signaling (Haltiwanger and Stanley, 2002). Fng activity reduces the sensitivity of N for the ligand Ser but increases its sensitivity for Dl. By contrast with imaginal discs, in which modulation of N sensitivity to both ligands appears to be important, loss of Fng in LG resulted in reduced N activation only, consistent with reduced response to Dl. Expression of Pros in LG can be triggered by Dl derived from neurons but not glia, and this effect can be mimicked by direct activation of the N pathway within glia. Our genetic experiments implicate neuron-derived Dl as the relevant N ligand for Pros expression in anterior LG, consistent with the ability of Fng to sensitize N to signaling by Dl. Enriched Fng expression in the anterior LG probably renders them differentially sensitive to sustained N signaling from Dl-expressing axons.

The final divisions of the six LG precursors that give rise to 12 LG are thought to be symmetric, with low levels of Pros first distributed evenly between sibling cells after division. However, Pros is maintained and in fact upregulated in the anterior LG, and downregulated in sibling LG that migrate posteriorly (Griffiths and Hidalgo, 2004). We observed that *fng* transcripts first appear to be expressed in all LG, then become enriched in the anterior LG and reduced in the posterior LG. We speculate that refinement of *fng* expression may involve a positive feedback mechanism to consolidate and enhance N signaling in the anterior LG, as we have preliminary evidence to suggest that N signaling can positively influence *fng* expression in the LG (G.B.T., D.J.vM. and Jennie Yang, unpublished).

Like Pros, Gs2 is specifically expressed in the anterior LG but not posterior LG, indicating that these are functionally distinct glial subtypes with respect to their ability to recycle the neurotransmitter glutamate. The specificity of N signaling for Pros but not Gs2 indicates that N signaling is unlikely to influence cell fate decisions in the LG lineage and that Fng is unlikely to be the primary determinant of anterior versus posterior LG identity. Rather, Fng probably serves to consolidate this distinction through sustained N signaling.

N^{ICD} was a potent activator of Pros expression in the posterior LG. This has led us to consider what factors limit Pros expression to the anterior LG in wild-type animals, as posterior LG are indeed capable of expressing Pros in response to constitutive N activity. First, based on our analysis of *fng* mutants and Fng misexpression, we propose that Fng is a major determinant. Our finding that misexpression of Fng causes ectopic Pros in posterior LG supports the argument that Dl-expressing axons do not contact the anterior LG only. It is likely that they make contact with at least some of the posterior LG. Therefore, in wild-type animals, in which Fng is reduced on posterior LG, contact from the subset of Dl axons is alone not sufficient to drive Pros expression. Second, misexpression of Dl in all postmitotic neurons led to ectopic expression of Pros in posterior LG, indicating that the restricted expression of Dl on a subset of neurons also limits N activation. Third, N appears to be expressed in most or all LG, though we have also found that overexpression of full-length N caused ectopic expression of Pros (not shown). From these data we propose a threshold model for N activation in LG that invokes a combination of factors, including Fng-regulated N sensitivity, exposure of N to ligand, N expression levels, and perhaps others. Increasing any of these factors can provide sufficient signaling for ectopic Pros induction in posterior LG. In wild-type embryos, these factors are also likely to combine with one another in the anterior LG to achieve supra-threshold N signaling and sustained Pros expression during normal development.

Signaling through N is important for glial cell development in *Drosophila*, although it is context-dependent. Both an embryonic sensory lineage and the subperineurial CNS glial lineage utilize N activation to promote Gcm expression and glial fate (Udolph et al., 2001; Umesono et al., 2002). By contrast, in the sensory organ of adult flies, antagonism of N leads to Gcm expression in the glial precursor cell (Van De Bor and Giangrande, 2001). In vertebrates, signaling through Notch receptors promotes the differentiation of peripheral glia (Morrison et al., 2000), astrocytes (Ge et al., 2002; Grandbarbe et al., 2003; Tanigaki et al., 2001), Müller glia (Bernardos et al., 2005; Furukawa et al., 2000), Bergmann glia (Eiraku et al., 2005; Lutolf et al., 2002; Weller et al., 2006), radial glia (Dang et al., 2006; Gaiano et al., 2000; Yoon et al., 2004), oligodendrocyte precursors (Grandbarbe et al., 2003; Park and Appel, 2003) and mature oligodendrocytes (Hu et al., 2003). An Fng ortholog, lunatic fringe, is expressed in the developing mouse brain in a pattern consistent with glial progenitors (Ishii et al., 2000). It will be interesting to determine whether Fng-related proteins in vertebrates have a role in glial cell differentiation, and whether they too can modulate N sensitivity and the context of N signaling between neurons and glia.

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