The Drosophila EGF domain protein Uninflatable sets the switch between wrapping glia growth and axon wrapping instructed by Notch

Marie Baldenius[1], Steffen Kautzmann[1], Rita Kottmeier[1,2], Jaqueline Zipfel[1,3] and Christian Klämbt[1]

1)
Institut für Neuro- und Verhaltensbiologie
Röntgenstraße 16
D-48149 Münster
Germany
2)
Universität zu Köln
Dezernat Forschungsmanagement
Dezernat 7, Abt. 72
Albertus-Magnus-Platz
D-50923 Köln Germany
3)
LVR-Universitätsklinik Essen
Klinik für Psychiatrie und Psychotherapie
Virchowstraße 174
D-45147 Essen
Germany

Key words: Drosophila, wrapping glia, axon-wrapping, RTK signaling, Uninflatable, Notch, differentiation

Abstract

In the peripheral nervous system, sensory and motor axons are generally covered by wrapping glial cell processes. This neuron-glia interaction requires an intricate coordination of glial growth and differentiation. How this is controlled molecularly is largely unknown. At the example of Drosophila larval nerves, we show that glial growth, that occurs without any cell division, is initially triggered by the FGF-receptor tyrosine kinase Heartless (Htl). In a screen for genes acting downstream of activated FGF-receptor, we identified the large membrane protein Uninflatable (Uif), which supports the growth of excessive plasma membrane domains but does not support glial axon wrapping. Uif is also known to inhibit Notch. Surprisingly, we find that Notch signaling is required in postmitotic wrapping glia. While compromised *Notch* signaling results in a reduced wrapping efficiency, gain of *Notch* activity in wrapping glia leads to a hyperwrapping phenotype. Thus, Notch signaling is both necessary and sufficient for glial wrapping in Drosophila larvae. In addition, *Notch* suppresses both *uif* and *htl* function and thus stabilizes the switch between glial growth and glial axon wrapping. Given the general conservation of signaling mechanisms controlling glia development in mice and flies, similar mechanisms may act in the mammalian nervous system to control final glial differentiation.

Introduction

The generation of the many cell types during development is governed by both intrinsic as well as extrinsic regulatory mechanisms. Extrinsic information on the status of the immediate neighbors is often conveyed by the evolutionary well conserved transmembrane receptor Notch, that plays a decisive role in many developmental contexts (Henrique and Schweisguth, 2019; Ho et al., 2020; Sachan et al., 2023; Salazar et al., 2020). In addition, individual cell types often have to undergo discrete developmental steps once they have been specified. In the nervous system, this can be illustrated by the example of axon-wrapping glial cells. These cells must first grow to a certain size before they can begin to differentiate. How such a switch is made, is not yet well understood.

Axon wrapping glial cells are generally very large cells. In the peripheral nervous system (PNS), myelin forming Schwann cells cover large segments of a single axon. Myelin formation is directed by the axon and only large diameter axons are wrapped with myelin. Small caliber axons, in contrast, are covered by a simple glial wrap in so-called Remak fibers. Here, a single non-myelinating Schwann cell covers many axons. In the peripheral nervous system, myelin formation is initiated by a Neuregulin-dependent activation of the EGF-receptor (Michailov et al., 2004; Taveggia et al., 2005). Interestingly, *neuregulin* mutants become normally myelinated within the CNS, indicating that oligodendrocytes have evolved an independent mechanism of myelination control (Brinkmann et al., 2008). An alternative pathway that might act in oligodendrocytes is initiated by the Notch receptor, that can be activated by neuronally expressed F3/Contactin, a GPI-linked membrane protein of the Ig-domain family (Hu et al., 2003).

Within the fly nervous system two main glial cell types are associated with axons (Bittern et al., 2021; Yildirim et al., 2022). Within the CNS, the ensheathing glia establishes a barrier

around the CNS neuropil and also wraps axons that connect the neuropil with the periphery (Pogodalla et al., 2021). The ensheathing glial cells are formed during embryonic development through divisions of a single glioblast that also generates all astrocytes (Jacobs et al., 1989; Peco et al., 2016). The underlying asymmetric division of the glioblast is in part controlled by *Notch* (Peco *et al.*, 2016). Together with the transcription factor Pointed, Notch directs the formation of astrocytes at the expense of the ensheathing glial lineage (Gabay et al., 1996; Klämbt, 1993; Peco *et al.*, 2016). In glial cells, Pointed is a target of receptor tyrosine kinase (RTK) signaling (Klaes et al., 1994; Klämbt, 1993; Peco *et al.*, 2016).

Within the Drosophila PNS, axon wrapping is mediated by the so-called wrapping glia (Kottmeier et al., 2020; Matzat et al., 2015; Stork et al., 2008). It follows a similar strategy as described for vertebrate Remak fibers. Wrapping glial cells are similarly large as their vertebrate counterparts and a single cell covers more than a millimeter of axon length (Matzat *et al.*, 2015). In adult stages, excessive differentiation of glial processes can be observed around large caliber motor axons which eventually leads to the establishment of myelin-like structures (Rey et al., 2023).

The development of wrapping glial cells is in part controlled by RTK signaling, that is initiated by either the EGF-receptor, the FGF-receptor, or the Discoidin domain receptor (Corty et al., 2022; Franzdóttir et al., 2009; Kottmeier *et al.*, 2020; Matzat *et al.*, 2015; Shishido et al., 1997; Stork et al., 2014; Wu et al., 2017). In the developing adult visual system, the FGF-receptor Heartless initially controls proliferation and migration of the wrapping glial progenitor cells, which upon contact to axons stop their migration to then grow in size and differentiate (Franzdóttir *et al.*, 2009; Sieglitz et al., 2013).

Glial cell development does not only depend on RTK-signaling. In Drosophila embryos, proper migration of postmitotic, peripheral glial cells requires *Notch* activity (Edenfeld et al.,

2007). Additional post-mitotic *Notch* functions have been reported in the adult Drosophila CNS. *Notch* activity is needed in olfactory neurons innervating specific glomeruli for long-term memory formation, depending on presentation of Delta by projection neurons as well as on neuronal activity (Ge et al., 2004; Kidd et al., 2015; Lieber et al., 2011; Zhang et al., 2013; Zhang et al., 2015). Moreover, since *Notch* is prominently expressed by postmitotic glial cells in the adult Drosophila CNS, it may be involved in glial differentiation (Allen et al., 2020; Davie et al., 2018; Li et al., 2022; Seugnet et al., 2011).

We have previously shown that in peripheral larval nerves, the FGF-receptor Heartless controls glial growth (Kottmeier *et al.*, 2020). To gain a deeper understanding on how differentiation of wrapping glial cells is regulated, we initiated a genetic screen looking for genes that act downstream of activated *heartless*. In Drosophila, such suppressor screens have been used successfully many times (Macagno et al., 2014; Rebay et al., 2000; Therrien et al., 2000). Our screen led to the unexpected identification of the large transmembrane protein Uninflatable (Uif), which in epithelial cells localizes to the apical plasma membrane. Loss of *uninflatable* suppresses the phenotype caused by activated RTK signaling. In addition, we found that *uif* knockdown as well as *uif* knockout larvae show impaired glial growth. In contrast, an excess of Uninflatable leads to the formation of ectopic membrane processes that, however, fail to interact with axons. *uninflatable* is also known to inhibit *Notch* (Xie et al., 2012). Indeed, we could show that canonical Notch signaling is activated in wrapping glia by the unconventional ligand Contactin, where it is required and sufficient for axon wrapping. Moreover, *Notch* counteracts both *uninflatable* as well as *heartless* function. Thus, Uninflatable acts to switch the balance between glial growth induced by RTK signaling and wrapping of axons.

Results

The FGF-receptor tyrosine kinase Heartless triggers glial growth

The development of wrapping glial cells in Drosophila depends on the activity of several receptor tyrosine kinases (Corty *et al.*, 2022; Franzdóttir *et al.*, 2009; Kottmeier *et al.*, 2020; Matzat *et al.*, 2015; Wu *et al.*, 2017). Loss of the FGF-receptor Heartless specifically in wrapping glial cells causes a reduced complexity of wrapping glial cell processes in the peripheral nervous system of third instar larvae (Kottmeier *et al.*, 2020) (**Figure 1A,B**). This is reflected in a reduced wrapping index which indicates the percentage of individually wrapped axons or axon fascicles (Matzat *et al.*, 2015). While in control larvae the wrapping index is around 0.18, it drops to 0.07 when *heartless* is specifically silenced in wrapping glia (Kottmeier *et al.*, 2020).

In contrast, gain of *heartless* function that is caused by expression of *λhtl* (Michelson et al., 1998) specifically in the wrapping glia, results in exuberant glial growth but does not trigger cell division (**Figure 1C**). Segmental nerves are swollen in an area that is demarcated by the position of the wrapping glial cell nucleus. To determine axonal wrapping we prepared third instar larvae as open book filet preparations for electron microscopic analyses. This allowed to analyze the nerve ultrastructure at positions indicated (**Figure 1C**). Far from the nerve bulge, only little remnants of a poorly differentiated wrapping glial cell can be detected (**Figure 2A,B**). In positions closer to the bulged nerve area, the wrapping glia ramifies. However, in many cases, glial processes rather grow along each other than around axons, resulting in fascicles surrounded by extensive glial cell processes (**Figure 2C**). These glial membrane formations become more evident as the diameter of the nerve gets larger (**Figure 2D-G**). In the central area of the nerve bulge, liquid-filled areas are detected with only few

thin glial cell processes (**Figure 2H**). In conclusion, expression of activated FGF-receptor Heartless triggers glial growth but does not instruct increased wrapping of axons.

Identification of genes acting downstream of *heartless*

In order to identify genes that are responsible for this excess in glial growth, we performed a wrapping glia specific RNA interference (RNAi)-based suppressor screen in animals that concomitantly express activated Heartless and double stranded RNA directed against individual genes (see **Table S1** for all genes tested). For this we generated a stable stock that allows Gal4-based expression of activated *heartless* in wrapping glia and also allows easy scoring of the wrapping glial shape [*nrv2-Gal4/CyO*^{weep}*; UAS-λhtl, repo4.3-stinger::GFP/TM6*]. Virgins collected from this stock were crossed against males carrying the different *UAS-dsRNA* elements (**Table S1**). The offspring third instar larvae were assayed for the presence of nerve bulges under an UV-dissecting microscope (see Materials and Methods and **Figure S1** for classification scheme). *repo4.3-stinger::GFP* directs expression of nuclear GFP in all glial nuclei. Among those, the wrapping glial nucleus can be easily identified based on the large size and typical position (**Figure S1**).

To test the efficacy of the above screening settings, we first silenced genes known to be involved in FGF-receptor signaling (Ashton-Beaucage and Therrien, 2017; Brunner et al., 1994; Klämbt, 1993; Vincent et al., 1998). The activated Heartless receptor tyrosine kinase recruits the adaptor protein Stumps which then signals via Son of sevenless (Sos), Ras85D, members of the MAPK cascade, the MAPKKK raf, the MAPKK Downstream of raf1 (*Dsor1*), the MAPK rolled (rl), and the transcription factor Pointed that can be phosphorylated by Rl. Knockdown of all these genes, except *Raf*, either fully or almost fully rescued the nerve bulging phenotype (**Table S1**, **Figure S1**). Full rescue (classified as 4): *stumps*, *Ras85D, pnt*;

almost full rescue (classified as 3): *Dsor1*, *rl*, *sos*. These findings indicate that the screening works efficiently.

We next selected 2,679 genes which we tested for their ability to suppress the *λhtl*-induced glial phenotype (**Table S1**). The different genes were chosen based on RNAseq data, glial expression and their involvement in major signaling pathways (Avet-Rochex et al., 2014), Petri & Klämbt, unpublished). The knockdown of 2,106 genes did not modify the nerve bulging phenotype, while knockdown of 105 genes caused a mild rescue and the knockdown of 97 genes a moderate rescue (classified as 1 or 2, respectively, see **Figure S1**; **Table S1**). Knockdown of 318 genes caused an almost complete or full rescue of the phenotype (classified as 3 or 4, respectively, see **Figure S1**; **Table S1**). Knockdown of the remaining 33 genes caused variable phenotypes or early lethality (**Table S1**).

We then tested additional UAS-dsRNA transgenes for the 318 genes whose knockdown efficiently rescued the nerve bulge phenotype in the initial screen. For 62 of these genes, we identified at least two independent UAS-dsRNA transgenes targeting independent regions of the mRNA that rescue the *λhtl* induced nerve bulging phenotype (**Table S2**). For additional 57 genes, a second but overlapping UAS-dsRNA construct was able to rescue the *λhtl* induced nerve bulging phenotype (**Table S3**).

The above 119 candidates are likely to act in the wrapping glia. We therefore performed wrapping glia-specific knockdown experiments using the following stock [*w*; *nrv2-Gal4/ nrv2-Gal4; nrv2-Gal4, UAS-CD8::mCherry/TM6*], where the presence of CD8::mCherry served as a proxy to determine the morphology of the wrapping glia. Indeed, knockdown of the majority of the 119 candidate genes caused wrapping glia differentiation defects (92 with phenotype, 7 no phenotype, 20 not tested, **Tables S2,S3**). In most cases, wrapping glial differentiation was impaired similar to what has been noted upon loss of *heartless* activity (Kottmeier *et al.*,

2020). Moreover, pan-glial knockdown of most of these genes caused lethality (**Tables S2, S3**), further supporting the notion that we identified genes relevant for glial development. Among the group of candidate genes, most encode proteins involved in translation and protein stability (48/119), transcription and splicing (23/119), lipid metabolism and membrane dynamics (20/119). This further supports the notion that a main function of FGF-receptor signaling is to promote cellular growth of the wrapping glia.

Uninflatable is required for wrapping glial cell growth

uninflatable (*uif*) is among the 119 candidate genes that when silenced, suppresses the nerve bulging phenotype induced by activated *heartless* (**Figure 3A-C**). It encodes a large, single-pass transmembrane adhesion protein with eighteen EGF-like repeats in its extracellular domain that specifically localizes to the apical membrane domain of epithelial cells (Zhang and Ward, 2009) (**Figure S2**). Since *uif* null mutants die at late embryonic stages due to their inability to inflate their trachea properly (Zhang and Ward, 2009), we utilized CRISPR/Cas9 to generate *uif* deficient wrapping glial cells to independently verify the RNAi-based phenotype. Four different sgRNAs were generated, targeting different regions of the *uif* locus (**Figure S2**; see materials and methods for details). Assuming that Cas9-induced double strand breaks will generate deletions or indels that will cause the formation of a correspondingly shortened open reading frame, we anticipate that the sgRNA targeting the second exon generates an almost null situation. In contrast, the sgRNA targeting Cas9 to the presumed cleavage site $uif^{sgRNA\ CS}$ or the one targeting a sequence 5' to transmembrane domain might result in the formation of a secreted and not membrane bound Uif protein. Finally, the sgRNA that targets sequences coding for the short cytoplasmic domain might allow the formation of an almost intact Uif protein.

When Cas9 is expressed ubiquitously together with any of these four *sgRNA* constructs in *trans* to an *uif* deficiency, development is arrested during early larval stages with defects in tracheal inflation indicating the functionality of the *sgRNA* constructs ([*w; UAS-Cas9/ Df(2L)ED438; da-Gal4 /UAS-uif*$^{sgRNA\ X}$] data not shown).

When Cas9 is expressed specifically in wrapping glial cells together with any of the four *sgRNA* constructs, wrapping glial cells appear thin and patchy, similar to what we noted following silencing of *uif* expression by RNA interference ([*+/+; UAS-Cas9/+; nrv2-Gal4,UAS-CD8::mCherry/UAS-uif*$^{sgRNA\ X}$], **Figure 3**). These data indicate that all parts of the protein are required in wrapping glial development, as even induction of a C-terminal mutation impairs morphology.

To further analyze the poorly differentiated wrapping glial cells we initiated an electron microscopic analysis of nerves in third instar larval filet preparations. We took ultrathin sections 200 µm distal from the ventral nerve cord. As a control genotype we utilized animals that expressed double stranded RNA targeting GFP encoding mRNA in all wrapping glial cells (*UAS-GFP*^{dsRNA}). In cross sections we found severely reduced glial cell processes (**Figure 4A,B**). Upon knockdown of *uif*, the wrapping index drastically decreases from 0.17 to 0.03 (**Figure S3**, n=3 larvae, 5-9 nerves per specimen, $p= 2.88x10^{-7}$).

To test how *uif* affects wrapping glial development we performed overexpression studies. Gain of *uif* function in wrapping glia caused bulge formation around the wrapping glial nucleus (**Figures 4C**). Similar to what we had noted following expression of activated Heartless, more distal and proximal parts of the wrapping glia cell remained thin and do not fully develop (**Figures 1C, 4C**). Subsequent ultrastructural analysis revealed a reduced wrapping index outside of the bulge region where only little glial wrapping is observed and most axons are devoid of any glial cell contact (**Figures 4D, S3A**). Within the nerve bulge, an

excess of wrapping glial membranes can be seen (**Figure 4E,F**). These processes fail to wrap individual axons which results in a significantly decreased wrapping index of 0.08 (**Figure S3A**, $p=2.56x10^{-9}$, n=3 larvae, 5-6 nerves per specimen). Interestingly, the excess glial cell processes form multilayered membrane stacks (**Figure 4F**). Thus, *heartless* appears to direct wrapping glial cell growth, while *uif* is needed for growth and stabilization of a specific, but still elusive, membrane compartment, possibly matching the apical domain of epithelial cells. To directly test this we stained for the distribution of PIP2 and PIP3 using specific PH-domain sensors, but in contrast to a differential distribution of PIP2 and PIP3 in ensheathing glia (Pogodalla *et al.*, 2021), we found no specific localization of these membrane lipids in wrapping glia.

Notch is required for wrapping glial cell differentiation

Importantly, although *uif* is needed for wrapping glial cell growth, it is not sufficient to instruct the wrapping of axons. Thus, Uif might define the wrapping glial cell interface required to wrap axons, and Uif interacting proteins organize subsequent wrapping. One of these interacting proteins is Notch. Uif can bind Notch and antagonizes the canonical Notch signaling pathway (Loubéry et al., 2014; Xie *et al.*, 2012). Likewise, it has been reported that *Notch* negatively regulates *uif* transcription (Djiane et al., 2013). Thus, the interaction of Uif and Notch might set a switch triggering glia-axon interaction leading to axon wrapping.

To determine a possible role of *Notch* signaling during wrapping glia differentiation, we first silenced *Notch* expression by RNAi in wrapping glia of otherwise normal animals. Knockdown of *Notch* expression using three different dsRNA constructs targeting different sequences of the *Notch* mRNA specifically in wrapping glia resulted in the appearance of thin wrapping glial cells in larval filet preparations (**Figure 5A-C**). A similar phenotype was also induced by

removing *Notch* expression using conditional CRISPR/Cas9-based knockout (**Figure 5D,E**) or in mutant $Notch^{ts1}$ animals (Shellenbarger and Mohler, 1975) that were kept at the restrictive temperature of 29°C during larval stages, only (**Figure 5F,G**). In contrast, upon expression of activated *Notch* (N^{ICD}) no significant changes of wrapping glial morphology could be detected using the confocal microscope (**Figure S4**).

Notch instructs axonal wrapping

To analyze the role of *Notch* signaling for wrapping glial differentiation in more detail, we performed an electron microscopic analysis. Upon expression of $Notch^{dsRNA}$ the wrapping index drops significantly to 0.12 (**Figure 6A,B,D**, p=0.00079; n=5, larvae with 7-9 nerves per specimen). In contrast, upon expression of the active form of *Notch*, $Notch^{ICD}$, we observed a dramatic and significant increase of the wrapping index to 0.28 (**Figure 6C,D**, p=0.014, n=5 larvae with 3-8 nerves per specimen). This indicates that the wrapping glia more efficiently enwraps axons compared to control larvae. Importantly, overexpression of *Notch* does not cause any bulge formation along the nerves as it is noted upon expression of the activated receptor tyrosine kinase Heartless (**Figure 1C**).

In summary, these data clearly demonstrate that *Notch* is not only required for development of wrapping glial cells but it is also sufficient to guide axon wrapping as gain of *Notch* function triggers extensive formation of glial processes resulting in a hyperwrapping phenotype.

Notch signaling is active in some adult wrapping glial cells

The above data suggest that *Notch* is expressed by differentiated wrapping glia in the PNS. To directly test this, we utilized a CRIMIC-based transposon insertion into the first coding intron

of the *Notch* locus (*Notch*$^{CR00429\text{-}TG4.1}$) that carries a Trojan Gal4 element, which allows GAL4 expression under control of the endogenous *Notch* promoter (Diao et al., 2015; Lee et al., 2018). However, no Gal4 activity is associated with the *Notch*$^{CR00429\text{-}TG4.1}$ insertion. To alternatively detect *Notch* activity we utilized the common *Notch* activity reporter, *GbeSu(H)-lacZ*, where 3 copies of the Grainy head (Grh) protein binding element (Gbe) and 2 Suppressor of Hairless (Su(H)) protein binding sites drive *lacZ* reporter expression (Furriols and Bray, 2001). In larvae, *Notch* reporter activity was detected in neuroblasts of the brain lobes and the thoracic neuromeres but not in peripheral wrapping glia (**Figure S5A-C**). In adults, however, *Notch* activity could be detected in ensheathing glial cells as well as in peripheral wrapping glial cells (**Figure S5D-F**).

The above-mentioned data do not demonstrate whether canonical *Notch* signaling acts in larval wrapping glia. We therefore suppressed expression of the gene *Su(H)*, which encodes a transcription factor critically required for *Notch* dependent gene expression, specifically in wrapping glia. In such knockdown larvae, overall wrapping glial morphology appeared impaired when analyzed using a confocal microscope (**Figure S6A,B**). In an electron microscopic analysis, we could observe a clear reduction in the wrapping index (**Figure 7A,B**, **Figure S3A**, WI=0.075, $p=4.21x10^{-11}$, n=4 larvae with 6-8 nerves per specimen). This finding indicates an involvement of *Su(H)* in wrapping glia development. Moreover, when we silenced the gene *mastermind* (*mam*), that encodes a further transcription factor involved in *Notch* signaling (Henrique and Schweisguth, 2019), we noted a severe impairment of glial morphology at the confocal microscope. Wrapping glial cells accompanying the abdominal nerves appeared thin and not well differentiated (**Figure S6C)**. Similarly, when looking at electron microscopic images, we noted a dramatic reduction in the wrapping index with a corresponding loss of glial complexity (**Figure 7C**, **Figure S3**, WI=0.07, $p=4.29x10^{-9}$,n=3 larvae

with 5-9 nerves per specimen). In conclusion, these data indicate that canonical Notch signaling acts within larval wrapping glial cells to guide engulfment of axons.

Notch **activation is not mediated by canonical ligands**

Notch is generally activated by the transmembrane EGF-domain proteins encoded by *Delta* or *Serrate*. Both ligands, like the Notch receptor, are evolutionarily well conserved. To determine the expression of both genes we again utilized insertion of Trojan Gal4 elements in either Delta or Serrate. While Serrate does not appear to be expressed in neurons, we noted some expression of Delta in peripheral sensory neurons and in very few CNS neurons (**Figure S7A,B**). We then performed RNAi based knockdown experiments specifically in neurons using the nSyb-Gal4 driver and found no effect on wrapping glial morphology at the light microscopic and the electron microscopic level (**Figure S7C-E**, data not shown). These results were confirmed by a second dsRNA construct as well as by sgRNA / Cas9 mediated, cell type specific knockout experiments (**Figure S8**). In summary, these data suggest that during larval development of the peripheral wrapping glia, *Notch* is neither activated by Delta nor by Serrate.

In mice, F3/Contactin, a GPI-linked member of the Ig-domain superfamily, activates Notch during oligodendrocyte differentiation (Hu *et al.*, 2003). F3/Contactin is well conserved during evolution and a homolog is encoded in the Drosophila genome. Drosophila Contactin binds Neuroglian and is an essential component of septate junctions which establish the occluding junctions in all epithelial cells and the glial blood-brain barrier (Faivre-Sarrailh et al., 2004; Izumi and Furuse, 2014; Peles and Salzer, 2000). In addition, single cell sequencing data indicate that *Contactin* is also expressed by neurons (Davie *et al.*, 2018). We therefore silenced *Contactin* expression in all neurons using the neuron specific driver *nSyb-Gal4*. Such

larvae survive and in third instar stage, a significant reduction in the wrapping index from 0.15 to 0.11 can be noted (**Figure 7D,E**, **Figure S3B**, p=0,006, n=5 larvae with 10 nerves per specimen for both RNAi lines). Wrapping glia morphology is impaired similar to what is noted upon knockdown of *Notch* and its downstream signaling components *Su(H)* and *mam* (**Figures 6,7**). This suggests that the non-canonical ligand Contactin acts in both flies and mammals to actively control wrapping glial differentiation and, moreover, indicates that additional ligands may exist to achieve full Notch activation.

Notch **suppresses** ***heartless*** **and** ***uninflatable*** **during glial development**

To further test whether Notch instructs glial axon wrapping downstream of *heartless* and *uninflatable*, we conducted epistasis experiments. Gain of *uif* function results in nerve bulges (**Figure 4C**). When we concomitantly silenced *Notch* using RNA interference, the nerve bulging phenotype is not changed (**Figure 8A,B**). However, when we co-expressed the intracellular domain of Notch, *UAS-N*^{ICD}, resembling the activated form of Notch, the bulging phenotype is rescued (**Figure 8A,C**). These data suggest that similar to the developing wing (Xie *et al.*, 2012), Uninflatable suppresses Notch function.

Expression of a constitutively active FGF-receptor, *λhtl*, in wrapping glial cells results in prominent nerve bulging (**Figure 8D**). Upon co-expression of *Notch* dsRNA, the size of the *λhtl*-induced nerve bulges increases (**Figure 8E**). A similar enhancement of the bulging phenotype was noted when using the temperature-sensitive *Notch*^{ts1} allele and an appropriate temperature regime (**Figure 8F**). In contrast when we co-expressed N^{ICD} we noted a suppression of the severity of the nerve bulging phenotype (**Figure 8G**). Thus, *Notch* function appears to counteract both *uif* and *htl* during glial development.

Model underlying wrapping glia development

We conclude the following model underlying wrapping glia development in the Drosophila PNS (**Figure 9**). Initially, the activity of the FGF-receptor Heartless triggers wrapping glial cell growth. The large transmembrane protein Uif is present in the wrapping glia and is needed to stabilize the formation of a specific membrane domain capable to interact with axons. In addition, Uif suppresses Notch to inhibit precocious axon wrapping. As Heartless negatively regulates expression of Uif (Avet-Rochex *et al.*, 2014), Notch becomes more and more active which also contributes to the silencing of *uif* and *htl*. This then sets the switch to initiate axon wrapping by the wrapping glia (**Figure 9**).

Discussion

Here we present a model underlying cell growth and subsequent wrapping of axons by the wrapping glia in the larval PNS of Drosophila. In a first step, wrapping glia shows an enormous increase in its size, which is regulated by the receptor tyrosine kinase Heartless. The large transmembrane protein Uninflatable, which harbors an array of EGF-domains in its extracellular domain, is needed to form excessive membrane domains capable of eventually wrapping axons. The last process is controlled by Notch whose activation then orchestrates axon-glia interaction.

Wrapping glial development is initiated by the activation of receptor tyrosine kinases (RTK) including the FGF-receptor Heartless (Htl). In perineurial glia, RTK activity will trigger cell division, whereas in the remaining glial cells RTK activity is used to support cell growth to subsequently allow differentiation (Avet-Rochex et al., 2012; Avet-Rochex *et al.*, 2014; Franzdóttir *et al.*, 2009; Read et al., 2009; Stork *et al.*, 2014; Witte et al., 2009; Wu *et al.*, 2017). This dual function of RTK signaling in controlling either proliferation, cell growth or differentiation is accompanied by a switch in activating ligands as well as the expression of additional regulator proteins that modulate RTK signaling strength (Franzdóttir *et al.*, 2009; Ohm et al., 2024; Sieglitz *et al.*, 2013).

Wrapping glial cells eventually have to cover all axons in the peripheral nerves to prevent degeneration of sensory axons (Kautzmann et al., 2025). In Drosophila larvae, wrapping glial cells can reach 2 mm in length and thus, have an enormous size (Matzat *et al.*, 2015). To achieve this, they block mitosis and instead undergo endoreplication (Unhavaithaya and Orr-Weaver, 2012; Von Stetina et al., 2018; Zülbahar et al., 2018). Here we found that in larval peripheral wrapping glia, Htl supports this cell growth. This is corroborated by our suppressor screen, which identified many genes affecting translation. Similarly, we identified several

genes that control metabolite transport across the wrapping glial membrane. Four of these genes are predicted to be involved in sugar/carbohydrate transport (*CG3409*, *CG4797*, *CG5078* and *CG6901*).

Initially, wrapping glial cells form only very thin processes that in peripheral nerves follow the axon bundles (Matzat *et al.*, 2015). Starting from second instar larval stages onwards, glial cells extend processes that wrap around axon fascicles or single axons (Kautzmann *et al.*, 2025; Matzat *et al.*, 2015). This process requires the formation of membranes dedicated to axon-glia contact. Possibly, this is mediated by the large transmembrane protein Uninflatable that in epithelial cells is found specifically at the apical plasma membrane and in tracheal cells also can affect polyploidy (Zhang and Ward, 2009; Zhou et al., 2020).

Using existing antibodies (Zhang and Ward, 2009), Uninflatable cannot be detected in third instar larval nerves. Moreover, recent microarray data indicate that activated Heartless suppresses *uif* transcription (Avet-Rochex *et al.*, 2014), which suggests that the amount of Uif present in wrapping glia decreases during larval development. Notably, Uif not only induces the generation of numerous membrane sheets, but it can also bind and inhibit Notch function (Loubéry *et al.*, 2014; Xie *et al.*, 2012). Thus, the inhibition of Notch by Uif is expected to gradually diminish during larval development. This antagonistic action of Uif will stabilize the switch from a glial cell growth phase to a subsequent phase with pronounced axon-glia interaction leading to axon wrapping.

During axon wrapping, Notch likely acts via its canonical signaling cascade. Although mutant analysis and RNAi-based knockdown studies clearly indicate a role of *Notch* in wrapping glia differentiation, we could detect Notch activity only in a subset of adult glial cells. This may be due to the fact that these glial cells form myelin-like structures (Rey *et al.*, 2023) and thus require a different level of Notch activity.

Postmitotic functions of Notch have been reported in several instances. In the Drosophila nervous system, *Notch* is expressed strongly in axon-associated, postmitotic glial cells (Allen *et al.*, 2020; Davie *et al.*, 2018; Li *et al.*, 2022; Seugnet *et al.*, 2011). During embryogenesis, Notch is needed for the exact positioning of glial cells as they migrate along peripheral nerves (Edenfeld *et al.*, 2007). Here we show that in larval wrapping glia *Notch* instructs axon wrapping. Thus, Notch promotes neuron-glia contact.

How could Notch signaling regulate axon-glia adhesion? The tight interaction of axons and glial cells calls for highly regulated adhesion of the two cell types. The transmembrane proteins of the Ig-domain superfamily Borderless and Turtle bind each other and mediate the differentiation of the wrapping glia in the developing eye (Cameron et al., 2013; Cameron et al., 2016; Chen et al., 2017). While Turtle is expressed on photoreceptor axons, Borderless is found on the ensheathing glia. Interestingly transcriptomic studies have indicated that in the developing eye, Borderless is downstream of Notch (Nfonsam et al., 2012).

Given our finding that Notch signaling is acting during axonal engulfment by the wrapping glia, we asked whether one of the canonical Notch ligands, Delta and Serrate (Henrique and Schweisguth, 2019), is responsible for Notch activation. However, neither Delta nor Serrate are broadly expressed in neurons, and moreover, neuronal knockdown of neither gene did result in a glial wrapping phenotype. In addition to these canonical ligands, the GPI-linked F3/Contactin protein has been suggested to activate Notch during myelin formation in oligodendrocytes (Hu *et al.*, 2003). Here, Notch activation leads to a gamma-secretase-dependent nuclear translocation of $Notch^{ICD}$ to upregulate expression of the myelin-related protein MAG and promote myelination. Quite similar, also in Drosophila, knockdown of *contactin* specifically in neurons causes a prominent defect in wrapping glial cell morphology. This surprising evolutionary conservation of the molecular control underlying the

differentiation of wrapping glia suggests that Drosophila models may be useful to gain a deeper understanding of myelin biology.

Acknowledgements:

We are thankful to Robert Ward for sending antibodies, Stefanie Schirmeier and Marcos Gonzalez Gaitan for flies and DNA. We are grateful to Elena Rinne for help in generating guide RNA constructs and all our lab colleagues for support and discussions. This work was supported by the Deutsche Forschungsgemeinschaft through funds to C.K. (SFB 1348 B5).

Figure legends

Figure 1 Heartless is required for wrapping glia development. Confocal images of third instar larval filet preparations, stained for CD8::mCherry expression. The segmental nerves posterior to the ventral nerve cord are shown. Wrapping glial morphology is (**A**) not changed in control animals expressing (*nrv2-Gal4*) mock GFP^{dsRNA}. (**B**) Differentiation of wrapping glial cells is affected following expression of a dominant negative form of Htl and thin wrapping glial cells are detected (arrowhead). (**C**) Expression of a constitutively active form of Htl [*nrv2-Gal4; UAS- λhtl*] leads to nerve bulges around the wrapping glial nuclei (C, arrowhead). The white dashed lines indicate the level of sections shown in Figure 2.

Figure 2 Heartless controls growth of wrapping glia cells. Electron microscopic images of segmental nerves from wandering third instar larvae. (**A**) Nerve of a control larva expressing GFP in wrapping glia sectioned 150 µm posterior to the ventral nerve cord. Normally differentiated wrapping glia can be seen. (**B-H**) Nerves of larvae expressing activated Heartless in wrapping glia [*nrv2-Gal4; UAS-λhtl*] sectioned at the positions indicated in Figure 1C. (**B**) Note the poorly differentiated wrapping glial cells distant from the nerve bulge. (**C,D**) At the beginning of the nerve bulge, excessive differentiation of wrapping glial cell processes start to be detected that not always grow around axons (magenta). (**E-G**) Higher magnifications of the boxed areas in (D). Note the formation of wrapping glial cell processes that do not contact axons (ax, magenta) but rather contact glial cell processes. (**H**) In the central area of the nerve bulge, liquid-filled vacuolar structures (asterisks) can be detected. Thin wrapping glial cell processes (arrows) span the bulged area.

Figure 3 *uninflatable* affects differentiation of wrapping glia. Confocal images of third instar larval filet preparations, stained for CD8::mCherry expression. The segmental nerves posterior to the ventral nerve cord are shown. (**A**) Filet preparation of a control third instar larva. (**B**) *uif* knockdown in wrapping glial cells [*nrv2-Gal4*; *UAS-uif*$^{dsRNA\text{-}HMS01822}$] impairs their development, which (**C**) cannot be rescued by co-expression of activated Htl. (**D-H**) Ubiquitous expression of (D) mock control *GFP*^{sgRNA}, (E) *uif*$^{sgRNA\ 2nd\ Exon}$, (F) *uif*$^{sgRNA\ CS}$, (G) *uif*$^{sgRNA\ TMD}$ or (H) *uif*$^{sgRNA\ CD}$ in *Cas9* expressing larvae. All *uif* sgRNAs disrupt wrapping glial cell development, wrapping glia appear thin and fragmented (arrowheads). n=5 larvae for all genotypes. Scale bars 100 µm.

Figure 4 *uif* affects axonal ensheathment of wrapping glia. (**A+B,D-F**) Electron microscopic cross sections of third instar larval abdominal peripheral nerves with wrapping glia specific expression [*nrv2-Gal4; R90C03-Gal80*] of (**A**) *GFP*^{dsRNA} mock control transgene, (**B**) *uif*$^{dsRNA\text{-}HMS01822}$. Upon knockdown of *uif,* wrapping glial cell complexity is reduced. (**C**) Single plane of a confocal image of a third instar larval nerve stained for CD8::mCherry expression. Expression of *uif::GFP* in wrapping glial cells causes bulge formation, while outside the bulge wrapping glia appear thin (arrowheads). The dashed lines indicate the plane of section in relation to the bulge of the images shown in (D,E). (**D**) Upon *uif::GFP* overexpression specifically in wrapping glia, glial morphology is reduced outside the bulge region. (**E**) Within the nerve bulge, wrapping glial membrane increases in size while most axons lack proper wrapping. (**F**) Close up of region indicated in (E). *GFP*^{dsRNA} n=4 larvae, 4-7 nerves per specimen; *uif*^{dsRNA} n=3 larvae, 5-9 nerves per specimen; *uif::GFP* n=3 larvae, 5-6 nerves per specimen. Scale bars 2 µm unless indicated otherwise.

Figure 5 *Notch* is required for wrapping glial development. Filet preparations of wandering third instar larvae stained for CD8::mCherry expression. The segmental nerves just posterior to the ventral nerve cord are shown. (**A**) Control larvae expressing the mock control GFP^{dsRNA} in wrapping glial cells specifically [*nrv2-Gal4; R90C03-Gal80*]. Upon expression of dsRNA targeting *Notch* mRNAs [(**B**) N^{GD144}, (**C**) N^{9G}. (**D**) Control larva expressing sgRNA directed against the GFP open reading frame in [*nrv2-Gal4; UAS-Cas9*]. (**E**) Conditional knockout of *Notch* leads to dramatically altered morphology of wrapping glial cells. (**F**) Control larva cultured at the same temperature regime as the N^{ts1} larva shown in (**G**). Wrapping glial cells appear smaller compared to the control. Scale bars are 100 µm.

Figure 6 *Notch* function affects axonal wrapping. Electron microscopic images of segmental nerves from wandering third instar larvae sectioned 100 µm posterior to the ventral nerve cord. (**A**) Control nerve of an animal expressing *dsRNA* directed against GFP [*nrv2-Gal4; R90C03-Gal80*]. Axons are wrapped by processes of the wrapping glia. The entire nerve is engulfed by the perineurial glia and the subperineurial glia. For quantification of glial wrapping see (D). (**B**) Upon expression of *dsRNA* targeting *Notch mRNA* (N^{GD144}) glial wrapping is reduced. (**C**) Upon expression of an activated form of Notch (N^{intra}) glial wrapping is increased. (**D**) Quantification of Wrapping Index (WI). While *Notch* knockdown significantly decreases the WI (from 0.17 to 0.12, $p = 0.00079$), activation of Notch signaling significantly increases glial wrapping and the WI (from 0.17 to 0.27, $p = 0.014$). For statistical analysis a t-test was performed for normally distributed data (Shapiro-test), Mann-Whitney-U test was performed for not normally distributed data. GFP^{dsRNA} n=4 larvae with 4-7 nerves each; N^{dsRNA} n=5, larvae with 7-9 nerves each; N^{ICD} n=5 larvae with 3-8 nerves each. α=0.05, * $p \leq 0.05$, *** $p \leq 0.001$. Scale bars 2 µm.

Figure 7 Knockdown of *mam*, *Su(H)* and *contactin* impairs axonal wrapping. Electron microscopic cross sections of third instar larval abdominal peripheral nerves. Glial cell morphology is indicated in orange color. (**A-C**) Wrapping glia specific [*nrv2-Gal4; R90C03-Gal80*] expression of GFP^{dsRNA} as mock control, (B) *Suppressor of Hairless* (*Su(H)*) $dsRNA^{HMS05748}$ or (C) *mastermind* (*mam*) $dsRNA^{KK110687}$. Note reduced complexity of glial cell processes. (**D**) Neuron specific [*nSyb-Gal4*] expression of $LacZ^{dsRNA}$ as mock control and (**E**) *Contactin* (*Cont*) $dsRNA^{GD12610}$ inserted on the third Chromosome. Upon neuronal knockdown of *Cont*, glial wrapping of peripheral axons is impaired. Scale bars 2 µm.

Figure 8 *Notch* counteracts *heartless* and *uninflatable* function. Squeeze preparations of wandering third instar larvae. The segmental nerves posterior to the ventral nerve cord are shown. (**A-C**) Larvae expressing *uninflatable* and CD8::mCherry in the wrapping glia. The nerve bulge shown in the white dashed box is enlarged. (A) Control larvae co-expressing *LacZ*. Uif expression results in nerve bulges. (B) Upon co-expression of $Notch^{dsRNA}$ (*GD144*) the nerve bulging phenotype is not suppressed. (C) Upon activation of Notch signaling by expression of N^{ICD}, the nerve bulging phenotype is suppressed. (**D-G**) Larvae expressing an activated Heartless receptor (*λhtl*) in the wrapping glia [*nrv2-Gal4*] carrying a *repo3.4-stinger::GFP* element leading to a panglial nuclear GFP expression. (D) Control larvae co-expressing *dsRNA* directed against *mCherry*. Note the prominent nerve bulges. (E) Upon co-expression of $Notch^{dsRNA}$ the nerve bulging phenotype is enhanced. (F) Likewise, the nerve bulging phenotype is enhanced in a $Notch^{ts1}$ mutant background when the larvae are kept at the restrictive temperature. (G) Upon activation of Notch signaling by expression of N^{ICD}, the nerve bulging phenotype is significantly rescued. n=7 larvae for all genotypes.

Figure 9 Model The interplay of FGF-receptor, Uninflatable and Notch signaling controls the switch between glial cell growth and glial cell differentiation leading to extensive neuron-glia contact. For details please see the text.

STAR Methods

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

All *Drosophila* work was conducted according to standard procedures. All fly stocks were kept at room temperature in plastic vials containing *Drosophila* standard food and dry yeast. Crosses were set up with male and virgin female flies in a ratio of 1:3 and kept at 25 °C. Induction of N^{ts1} allele was performed by placing Stage 16 embryos at 29°C.

METHOD DETAILS

sgRNA GENERATION

To generate flies carrying sgRNAs targeted to different regions of the *uif* gene, sgRNA sequences specifically designed for the target gene region of interest were integrated into the pUAST-dU63gRNA vector carrying a ubiquitous U6:3 promoter. To do so, sense and anti-sense oligonucleotides containing the respective sgRNA template sequence ($uif^{2ndExon}$: TTCAATATCAAGCACTCGT; uif^{CS}: TGTTCTGCGTACCTCGGTAG; uif^{TMD}: CGCTGTGTGGGCTCCTTTAC; uif^{CD}: CTACAATGAAACGTACATGA) were phosphorylated, annealed and ligated into the vector. Flies were tested via single-fly PCR. The position of the different guide RNAs is indicated (**Figure S2**).

HEARTLESS MODIFIER SCREEN

To test whether candidate genes can be linked to fibroblast growth factor (FGF) receptor signaling in wrapping glia, we utilized the nerve bulging phenotype caused by expression of a constitutively active Heartless (*UAS-λhtl*) in wrapping glia (*nrv2-Gal4*). The *repo4.3-stinger::GFP* reporter line labels all glial nuclei independent of *Gal4* while *nrv2-Gal4* was used to express *UAS-λhtl*. This strain was crossed against a collection of *UAS-dsRNA* lines. For screening, living larvae were mounted as live squeezed preparations, to ensure best signal to noise ratio (Kottmeier, 2013). Third instar wandering larvae were collected in ice-cold PBS. With their ventral side up, animals were transferred to a drop of silicon fat (KORALISON, medium viscosity, Kurt Obermeier GmbH) onto a microscope slide. To fix larvae in their position, they were covered with a cover slip and squeezed. Tracheae were oriented towards the microscope slide for visualization of the nervous system. About seven larvae per genotype were assessed using a Nikon fluorescence binocular (AZ-100).

IMMUNOHISTOCHEMISTRY

For confocal analyses, at least six to ten animals including an equal ratio of both female an male animals were dissected. For experiments using the N^{ts1} allele, only hemizygous males were examined. For larval filet preparations third instar wandering larvae were collected in ice-cold PBS. The larvae were placed on a silicon pad with their dorsal side facing up and secured at both ends using needles. They were then carefully opened along the dorsal midline using dissection scissors and stretched out with four needles. Gut, fat body, and trachea were removed. For adult brain preparations, adult flies were anesthetized with CO_2 and briefly dipped in 70% ethanol. The head capsule was cut open with dissection scissors, and the tissue surrounding the brain was removed using forceps. Legs and wings were

excised, and the thorax was opened dorsally. The ventral nerve cord was freed from the surrounding tissue to isolate the sample. After dissection, the samples were fixed by covering them with Bouin's solution for 3 min at room temperature. This was followed by three quick buffer exchanges and three additional washes with PBT lasting 20 min each. Following blocking in 10% goat serum/PBT for one hour at room temperature primary antibodies were applied and incubated overnight at 4°C. Then samples were washed three times with PBT for 20 min each and then incubated with secondary antibodies for 3 h at room temperature. The tissues were covered with Vectashield mounting solution (Vector Laboratories) and stored at 4°C until imaging using an Zeiss LSM880 Fast-Airyscan microscope. Confocal images were analyzed using Fiji.

ELECTRON MICROSCOPIC ANALYSIS

For electron microscopy analyses larvae were dissected in 4% PFA and fixed as filet preparations for 45 min at room temperature, which was followed by fixation in 4% paraformaldehyde (PFA) and 0.5% glutaraldehyde in 0.1 M P-buffer at 4°C overnight. The PFA was replaced by 2% OsO_4 in 0.1 M P-buffer for 1 hr on ice (dark). Uranyl acetate staining was performed en bloque using a 2% solution in H_2O for 30 min (dark). Following an EtOH series (50%, 70%, 80%, 90%, and 96%) on ice for 3 min each step, final dehydration was done at RT with 2×100% EtOH for 15 min and 2x propylene oxide for 15 min. Following slow epon infiltration specimens were embedded in flat molds and polymerized at 60°C for 2 days. After trimming, ultra-thin sections of segmental nerves about 150µm distant from the tip of the ventral nerve cord were obtained using a 35° ultra knife (Diatome). Sections were collected on formvar-coated copper grids on which they were left to dry for at least 1 h prior to

imaging, which was performed with a Zeiss TEM 900 at 80 kV in combination with a Morada camera (EMSIS).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical details of every experiment can be found in the figure legends, with n representing the number of examined animals. Normal distribution of values was performed using the Shapiro Wilk test. To determine the level of significance the t-test was applied for normally distributed data, while the Man Whitney U test was applied for not normally distributed. Python was also used to generate all statistics and boxplots. For statistical analyses of EM data, the wrapping index was obtained by putting the number of individual wrapped axons or axon fascicles into relation to the number of all axons. A wrapping index of 1 implies that every single axon of the nerve is individually wrapped. All nerves that contained less than 76 or more than 82 axons were not included in the statistical analysis.

Table S1: Summary of Heartless modifier screen, related to STAR Methods > METHOD DETAILS > Heartless modifier screen

Table S2: Rescue of the Htl gain of function wrapping glial phenotype by two independent dsRNA transgenes, related to STAR Methods > METHOD DETAILS > Heartless modifier screen, Figure 3

Table S3: Rescue of Htl gain of function wrapping glial phenotype by two overlapping dsRNA transgenes, related to STAR Methods > METHOD DETAILS > Heartless modifier screen

References

Allen, A.M., Neville, M.C., Birtles, S., Croset, V., Treiber, C.D., Waddell, S., and Goodwin, S.F. (2020). A single-cell transcriptomic atlas of the adult Drosophila ventral nerve cord. eLife *9*. 10.7554/eLife.54074.

Ashton-Beaucage, D., and Therrien, M. (2017). How Genetics Has Helped Piece Together the MAPK Signaling Pathway. Methods Mol Biol *1487*, 1-21. 10.1007/978-1-4939-6424-6_1.

Avet-Rochex, A., Kaul, A.K., Gatt, A.P., McNeill, H., and Bateman, J.M. (2012). Concerted control of gliogenesis by InR/TOR and FGF signalling in the Drosophila post-embryonic brain. Development *139*, 2763-2772. 10.1242/dev.074179.

Avet-Rochex, A., Maierbrugger, K.T., and Bateman, J.M. (2014). Glial enriched gene expression profiling identifies novel factors regulating the proliferation of specific glial subtypes in the Drosophila brain. Gene expression patterns : GEP *16*, 61-68. 10.1016/j.gep.2014.09.001.

Bittern, J., Pogodalla, N., Ohm, H., Bruser, L., Kottmeier, R., Schirmeier, S., and Klambt, C. (2021). Neuron-glia interaction in the Drosophila nervous system. Developmental Neurobiology *81*, 438-452. 10.1002/dneu.22737.

Brinkmann, B.G., Agarwal, A., Sereda, M.W., Garratt, A.N., Müller, T., Wende, H., Stassart, R.M., Nawaz, S., Humml, C., Velanac, V., et al. (2008). Neuregulin-1/ErbB signaling serves distinct functions in myelination of the peripheral and central nervous system. Neuron *59*, 581-595. 10.1016/j.neuron.2008.06.028.

Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H., and Klambt, C. (1994). The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. Nature *370*, 386-389. 10.1038/370386a0.

Cameron, S., Chang, W.T., Chen, Y., Zhou, Y., Taran, S., and Rao, Y. (2013). Visual circuit assembly requires fine tuning of the novel Ig transmembrane protein Borderless. J Neurosci *33*, 17413-17421. 10.1523/jneurosci.1878-13.2013.

Cameron, S., Chen, Y., and Rao, Y. (2016). Borderless Regulates Glial Extension and Axon Ensheathment. Developmental Biology. 10.1016/j.ydbio.2016.04.020.

Chen, Y., Cameron, S., Chang, W.-T., and Rao, Y. (2017). Turtle interacts with borderless in regulating glial extension and axon ensheathment. Molecular brain *10*, 17. 10.1186/s13041-017-0299-6.

Corty, M.M., Hulegaard, A.L., Hill, J.Q., Sheehan, A.E., Aicher, S.A., and Freeman, M.R. (2022). Discoidin domain receptor regulates ensheathment, survival and caliber of peripheral axons. Development *149*. 10.1242/dev.200636.

Davie, K., Janssens, J., Koldere, D., De Waegeneer, M., Pech, U., Kreft, L., Aibar, S., Makhzami, S., Christiaens, V., Bravo Gonzalez-Blas, C., et al. (2018). A Single-Cell Transcriptome Atlas of the Aging Drosophila Brain. Cell *174*, 982-998 e920. 10.1016/j.cell.2018.05.057.

Diao, F., Ironfield, H., Luan, H., Diao, F., Shropshire, W.C., Ewer, J., Marr, E., Potter, C.J., Landgraf, M., and White, B.H. (2015). Plug-and-play genetic access to drosophila cell types using exchangeable exon cassettes. Cell reports *10*, 1410-1421. 10.1016/j.celrep.2015.01.059.

Djiane, A., Krejci, A., Bernard, F., Fexova, S., Millen, K., and Bray, S.J. (2013). Dissecting the mechanisms of Notch induced hyperplasia. Embo j *32*, 60-71. 10.1038/emboj.2012.326.

Edenfeld, G., Altenhein, B., Zierau, A., Cleppien, D., Krukkert, K., Technau, G., and Klämbt, C. (2007). Notch and Numb are required for normal migration of peripheral glia in Drosophila. Dev Biol *301*, 27-37. 10.1016/j.ydbio.2006.11.013.

Faivre-Sarrailh, C., Banerjee, S., Li, J., Hortsch, M., Laval, M., and Bhat, M.A. (2004). Drosophila contactin, a homolog of vertebrate contactin, is required for septate junction

organization and paracellular barrier function. Development *131*, 4931-4942. 10.1242/dev.01372.

Franzdóttir, S.R., Engelen, D., Yuva-Aydemir, Y., Schmidt, I., Aho, A., and Klämbt, C. (2009). Switch in FGF signalling initiates glial differentiation in the Drosophila eye. Nature *460*, 758-761. 10.1038/nature08167.

Furriols, M., and Bray, S. (2001). A model Notch response element detects Suppressor of Hairless-dependent molecular switch. Curr Biol *11*, 60-64. 10.1016/s0960-9822(00)00044-0.

Gabay, L., Scholz, H., Golembo, M., Klaes, A., Shilo, B.Z., and Klämbt, C. (1996). EGF receptor signaling induces pointed P1 transcription and inactivates Yan protein in the Drosophila embryonic ventral ectoderm. Development *122*, 3355-3362.

Ge, X., Hannan, F., Xie, Z., Feng, C., Tully, T., Zhou, H., Xie, Z., and Zhong, Y. (2004). Notch signaling in Drosophila long-term memory formation. Proc Natl Acad Sci U S A *101*, 10172-10176. 10.1073/pnas.0403497101.

Henrique, D., and Schweisguth, F. (2019). Mechanisms of Notch signaling: a simple logic deployed in time and space. Development *146*, dev172148. 10.1242/dev.172148.

Ho, D.M., Artavanis-Tsakonas, S., and Louvi, A. (2020). The Notch pathway in CNS homeostasis and neurodegeneration. Wiley Interdiscip Rev Dev Biol *9*, e358. 10.1002/wdev.358.

Hu, Q.D., Ang, B.T., Karsak, M., Hu, W.P., Cui, X.Y., Duka, T., Takeda, Y., Chia, W., Sankar, N., Ng, Y.K., et al. (2003). F3/contactin acts as a functional ligand for Notch during oligodendrocyte maturation. Cell *115*, 163-175. 10.1016/s0092-8674(03)00810-9.

Izumi, Y., and Furuse, M. (2014). Molecular organization and function of invertebrate occluding junctions. Seminars in cell & developmental biology *36*, 186-193. 10.1016/j.semcdb.2014.09.009.

Jacobs, J.R., Hiromi, Y., Patel, N.H., and Goodman, C.S. (1989). Lineage, migration, and morphogenesis of longitudinal glia in the Drosophila CNS as revealed by a molecular lineage marker. Neuron *2*, 1625-1631. 10.1016/0896-6273(89)90051-2.

Kautzmann, S., Rey, S., Krebs, A., and Klämbt, C. (2025). Cholinergic and glutamatergic axons differentially require glial support in the Drosophila PNS. Glia. 10.1002/glia.70011.

Kidd, S., Struhl, G., and Lieber, T. (2015). Notch is required in adult Drosophila sensory neurons for morphological and functional plasticity of the olfactory circuit. PLoS Genet *11*, e1005244. 10.1371/journal.pgen.1005244.

Klaes, A., Menne, T., Stollewerk, A., Scholz, H., and Klämbt, C. (1994). The Ets transcription factors encoded by the Drosophila gene pointed direct glial cell differentiation in the embryonic CNS. Cell *78*, 149-160.

Klämbt, C. (1993). The Drosophila gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. Development *117*, 163-176.

Kottmeier, R., Bittern, J., Schoofs, A., Scheiwe, F., Matzat, T., Pankratz, M., and Klämbt, C. (2020). Wrapping glia regulates neuronal signaling speed and precision in the peripheral nervous system of Drosophila. Nature communications *11*, 4491-4417. 10.1038/s41467-020-18291-1.

Lee, P.-T., Zirin, J., Kanca, O., Lin, W.-W., Schulze, K.L., Li-Kroeger, D., Tao, R., Devereaux, C., Hu, Y., Chung, V., et al. (2018). A gene-specific T2A-GAL4 library for Drosophila. eLife *7*. 10.7554/eLife.35574.

Li, H., Janssens, J., De Waegeneer, M., Kolluru, S.S., Davie, K., Gardeux, V., Saelens, W., David, F.P.A., Brbić, M., Spanier, K., et al. (2022). Fly Cell Atlas: A single-nucleus transcriptomic atlas of the adult fruit fly. Science *375*, eabk2432. 10.1126/science.abk2432.

Lieber, T., Kidd, S., and Struhl, G. (2011). DSL-Notch signaling in the Drosophila brain in response to olfactory stimulation. Neuron *69*, 468-481. 10.1016/j.neuron.2010.12.015.

Loubéry, S., Seum, C., Moraleda, A., Daeden, A., Fürthauer, M., and González-Gaitán, M. (2014). Uninflatable and Notch control the targeting of Sara endosomes during asymmetric division. Current biology : CB *24*, 2142-2148. 10.1016/j.cub.2014.07.054.

Macagno, J.P., Diaz Vera, J., Yu, Y., MacPherson, I., Sandilands, E., Palmer, R., Norman, J.C., Frame, M., and Vidal, M. (2014). FAK acts as a suppressor of RTK-MAP kinase signalling in Drosophila melanogaster epithelia and human cancer cells. PLoS Genet *10*, e1004262. 10.1371/journal.pgen.1004262.

Matzat, T., Sieglitz, F., Kottmeier, R., Babatz, F., Engelen, D., and Klämbt, C. (2015). Axonal wrapping in the Drosophila PNS is controlled by glia-derived neuregulin homolog Vein. Development *142*, 1336-1345. 10.1242/dev.116616.

Michailov, G.V., Sereda, M.W., Brinkmann, B.G., Fischer, T.M., Haug, B., Birchmeier, C., Role, L., Lai, C., Schwab, M.H., and Nave, K.-A. (2004). Axonal neuregulin-1 regulates myelin sheath thickness. Science *304*, 700-703. 10.1126/science.1095862.

Michelson, A.M., Gisselbrecht, S., Buff, E., and Skeath, J.B. (1998). Heartbroken is a specific downstream mediator of FGF receptor signalling in Drosophila. Development *125*, 4379-4389.

Nfonsam, L.E., Cano, C., Mudge, J., Schilkey, F.D., and Curtiss, J. (2012). Analysis of the transcriptomes downstream of Eyeless and the Hedgehog, Decapentaplegic and Notch signaling pathways in Drosophila melanogaster. PLoS One *7*, e44583. 10.1371/journal.pone.0044583.

Ohm, H., Rey, S., and Klämbt, C. (2024). Clustering of voltage-gated ion channels as an evolutionary trigger of myelin formation. Neural Regen Res *19*, 1631-1632. 10.4103/1673-5374.389636.

Peco, E., Davla, S., Camp, D., M Stacey, S., Landgraf, M., and van Meyel, D.J. (2016). Drosophila astrocytes cover specific territories of the CNS neuropil and are instructed to differentiate by Prospero, a key effector of Notch. Development *143*, 1170-1181. 10.1242/dev.133165.

Peles, E., and Salzer, J.L. (2000). Molecular domains of myelinated axons. Current Opinion in Neurobiology *10*, 558-565.

Pogodalla, N., Kranenburg, H., Rey, S., Rodrigues, S., Cardona, A., and Klämbt, C. (2021). Drosophila ßHeavy-Spectrin is required in polarized ensheathing glia that form a diffusion-barrier around the neuropil. Nature communications *12*, 6357-6318. 10.1038/s41467-021-26462-x.

Read, R.D., Cavenee, W.K., Furnari, F.B., Thomas, J.B., and Rulifson, E. (2009). A Drosophila Model for EGFR-Ras and PI3K-Dependent Human Glioma. PLoS Genetics *5*, e1000374. 10.1371/journal.pgen.1000374.

Rebay, I., Chen, F., Hsiao, F., Kolodziej, P.A., Kuang, B.H., Laverty, T., Suh, C., Voas, M., Williams, A., and Rubin, G.M. (2000). A genetic screen for novel components of the Ras/Mitogen-activated protein kinase signaling pathway that interact with the yan gene of Drosophila identifies split ends, a new RNA recognition motif-containing protein. Genetics *154*, 695-712. 10.1093/genetics/154.2.695.

Rey, S., Ohm, H., Moschref, F., Zeuschner, D., Praetz, M., and Klämbt, C. (2023). Glial-dependent clustering of voltage-gated ion channels in Drosophila precedes myelin formation. eLife *12*, e85752. 10.7554/eLife.85752.

Sachan, N., Sharma, V., Mutsuddi, M., and Mukherjee, A. (2023). Notch signalling: multifaceted role in development and disease. Febs j. 10.1111/febs.16815.

Salazar, J.L., Yang, S.A., and Yamamoto, S. (2020). Post-Developmental Roles of Notch Signaling in the Nervous System. Biomolecules *10*. 10.3390/biom10070985.

Seugnet, L., Suzuki, Y., Merlin, G., Gottschalk, L., Duntley, S.P., and Shaw, P.J. (2011). Notch signaling modulates sleep homeostasis and learning after sleep deprivation in Drosophila. Curr Biol *21*, 835-840. 10.1016/j.cub.2011.04.001.

Shellenbarger, D.L., and Mohler, J.D. (1975). Temperature-sensitive mutations of the notch locus in Drosophila melanogaster. Genetics *81*, 143-162. 10.1093/genetics/81.1.143.

Shishido, E., Ono, N., Kojima, T., and Saigo, K. (1997). Requirements of DFR1/Heartless, a mesoderm-specific Drosophila FGF-receptor, for the formation of heart, visceral and somatic muscles, and ensheathing of longitudinal axon tracts in CNS. Development *124*, 2119-2128.

Sieglitz, F., Matzat, T., Yuva-Adyemir, Y., Neuert, H., Altenhein, B., and Klämbt, C. (2013). Antagonistic Feedback Loops Involving Rau and Sprouty in the Drosophila Eye Control Neuronal and Glial Differentiation. Science signaling *6*, ra96. 10.1126/scisignal.2004651.

Stork, T., Engelen, D., Krudewig, A., Silies, M., Bainton, R.J., and Klämbt, C. (2008). Organization and function of the blood-brain barrier in Drosophila. Journal of Neuroscience *28*, 587-597. 10.1523/JNEUROSCI.4367-07.2008.

Stork, T., Sheehan, A., Tasdemir-Yilmaz, O.E., and Freeman, M.R. (2014). Neuron-glia interactions through the Heartless FGF receptor signaling pathway mediate morphogenesis of Drosophila astrocytes. Neuron *83*, 388-403. 10.1016/j.neuron.2014.06.026.

Taveggia, C., Zanazzi, G., Petrylak, A., Yano, H., Rosenbluth, J., Einheber, S., Xu, X., Esper, R.M., Loeb, J.A., Shrager, P., et al. (2005). Neuregulin-1 type III determines the ensheathment fate of axons. Neuron *47*, 681-694. 10.1016/j.neuron.2005.08.017.

Therrien, M., Morrison, D.K., Wong, A.M., and Rubin, G.M. (2000). A genetic screen for modifiers of a kinase suppressor of Ras-dependent rough eye phenotype in Drosophila. Genetics *156*, 1231-1242.

Unhavaithaya, Y., and Orr-Weaver, T.L. (2012). Polyploidization of glia in neural development links tissue growth to blood-brain barrier integrity. Genes Dev *26*, 31-36. 10.1101/gad.177436.111.

Vincent, S., Wilson, R., Coelho, C., Affolter, M., and Leptin, M. (1998). The Drosophila protein Dof is specifically required for FGF signaling. Mol Cell *2*, 515-525. 10.1016/s1097-2765(00)80151-3.

Von Stetina, J.R., Frawley, L.E., Unhavaithaya, Y., and Orr-Weaver, T.L. (2018). Variant cell cycles regulated by Notch signaling control cell size and ensure a functional blood-brain barrier. Development *145*, dev157115. 10.1242/dev.157115.

Witte, H.T., Jeibmann, A., Klämbt, C., and Paulus, W. (2009). Modeling glioma growth and invasion in Drosophila melanogaster. Neoplasia (New York, N.Y.) *11*, 882-888.

Wu, B., Li, J., Chou, Y.-H., Luginbuhl, D., and Luo, L. (2017). Fibroblast growth factor signaling instructs ensheathing glia wrapping of Drosophila olfactory glomeruli. Proceedings of the National Academy of Sciences of the United States of America *18*, 201706533.

Xie, G., Zhang, H., Du, G., Huang, Q., Liang, X., Ma, J., and Jiao, R. (2012). Uif, a large transmembrane protein with EGF-like repeats, can antagonize Notch signaling in Drosophila. PLoS ONE *7*, e36362. 10.1371/journal.pone.0036362.

Yildirim, K., Winkler, B., Pogodalla, N., Mackensen, S., Baldenius, M., Garcia, L., Naffin, E., Rodrigues, S., and Klambt, C. (2022). Redundant functions of the SLC5A transporters Rumpel, Bumpel, and Kumpel in ensheathing glial cells. Biol Open *11*. 10.1242/bio.059128.

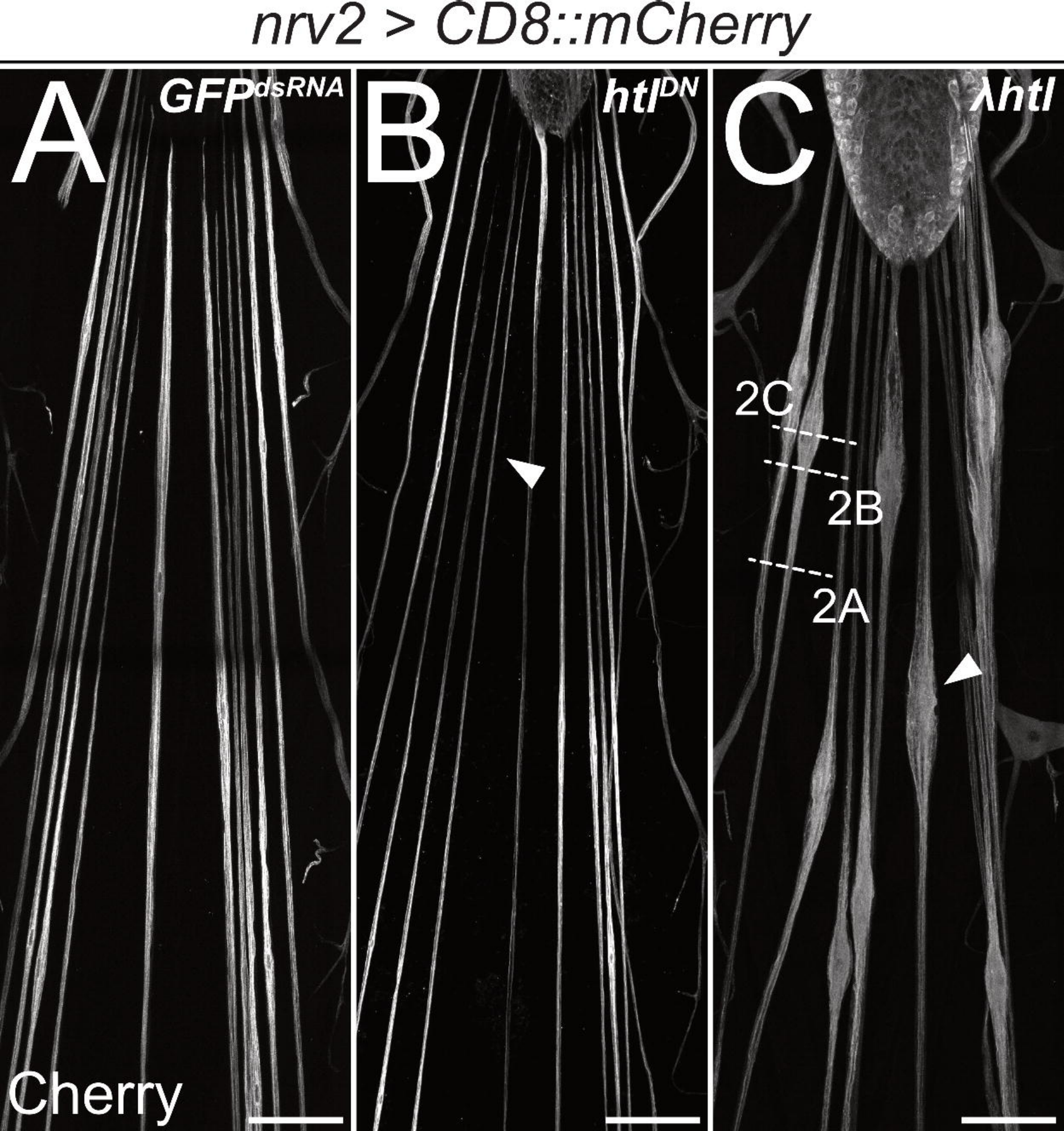
Zhang, J., Little, C.J., Tremmel, D.M., Yin, J.C., and Wesley, C.S. (2013). Notch-inducible hyperphosphorylated CREB and its ultradian oscillation in long-term memory formation. J Neurosci *33*, 12825-12834. 10.1523/jneurosci.0783-13.2013.

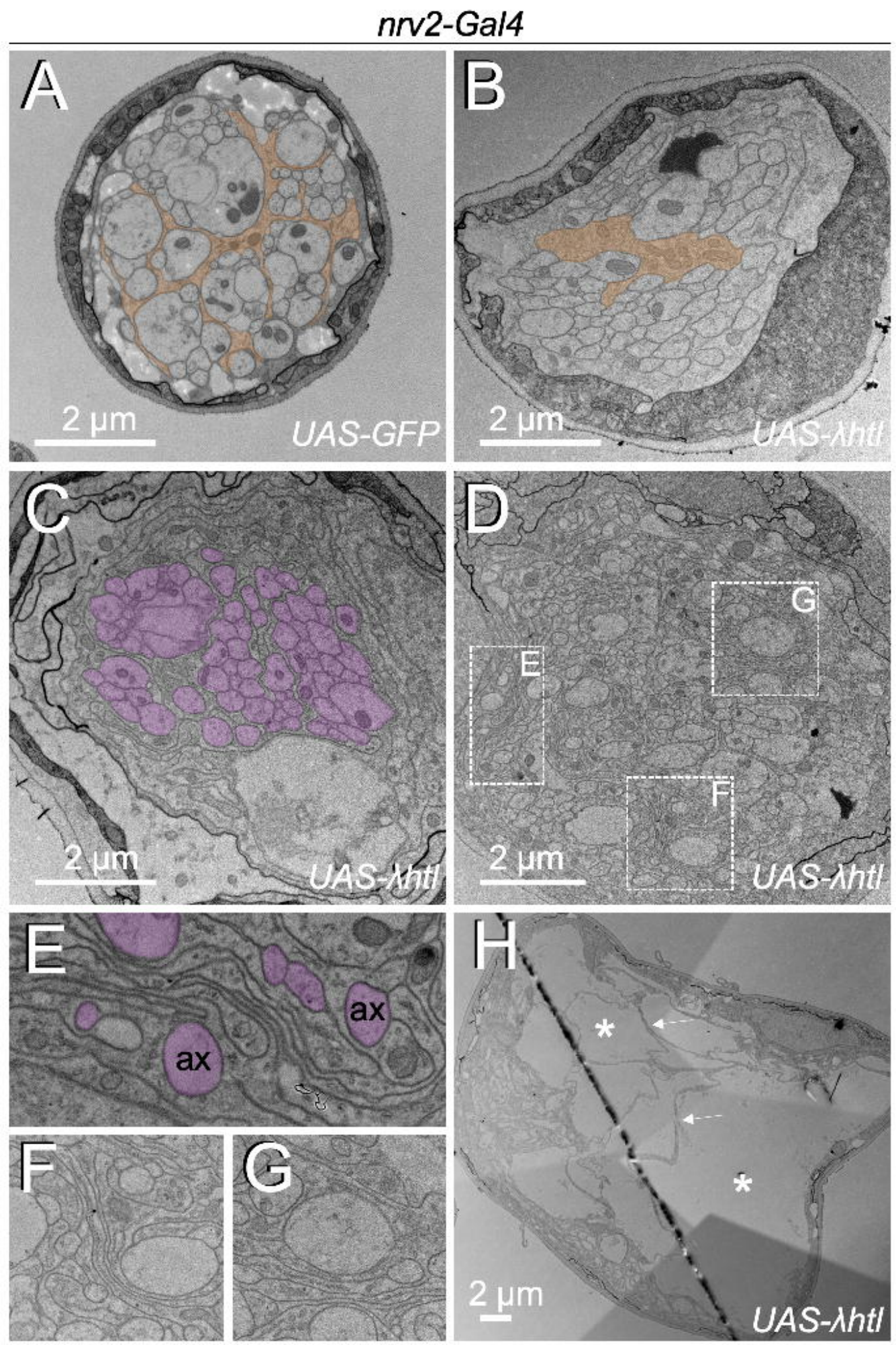
Zhang, J., Yin, J.C., and Wesley, C.S. (2015). Notch Intracellular Domain (NICD) Suppresses Long-Term Memory Formation in Adult Drosophila Flies. Cell Mol Neurobiol *35*, 763-768. 10.1007/s10571-015-0183-9.

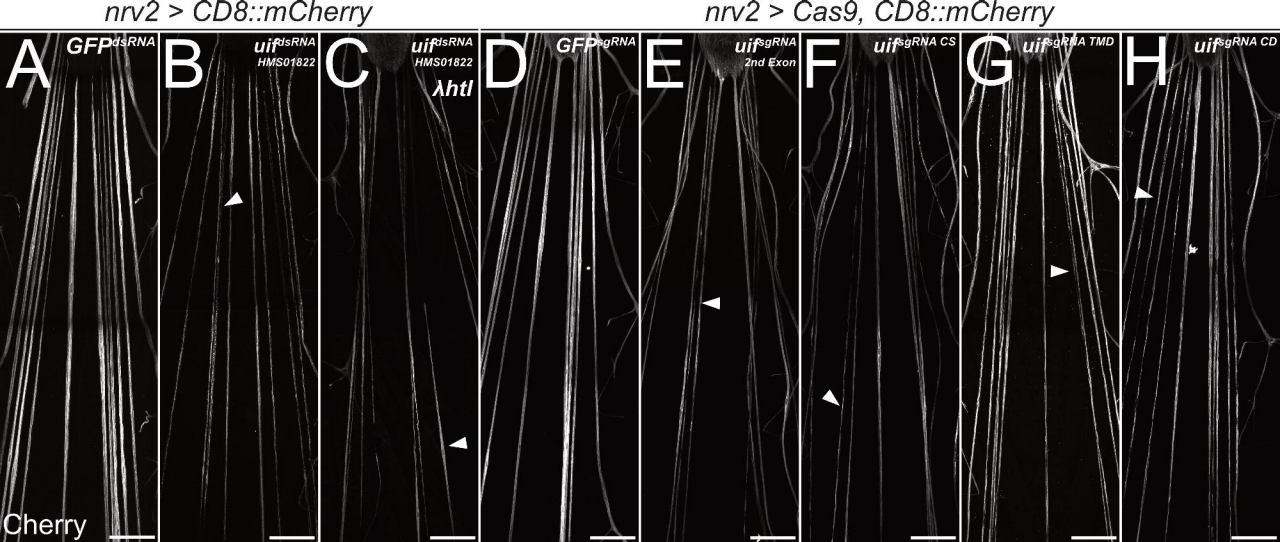
Zhang, L., and Ward, R.E. (2009). uninflatable encodes a novel ectodermal apical surface protein required for tracheal inflation in Drosophila. Developmental Biology *336*, 201-212. 10.1016/j.ydbio.2009.09.040.

Zhou, F., Green, S.R., Tsay, M., Hsu, S., Dibbs, R., and Beckingham, K.M. (2020). The roles of jim lovell and uninflatable in different endopolyploid larval tissues of Drosophila melanogaster. PLoS ONE *15*, e0237662. 10.1371/journal.pone.0237662.

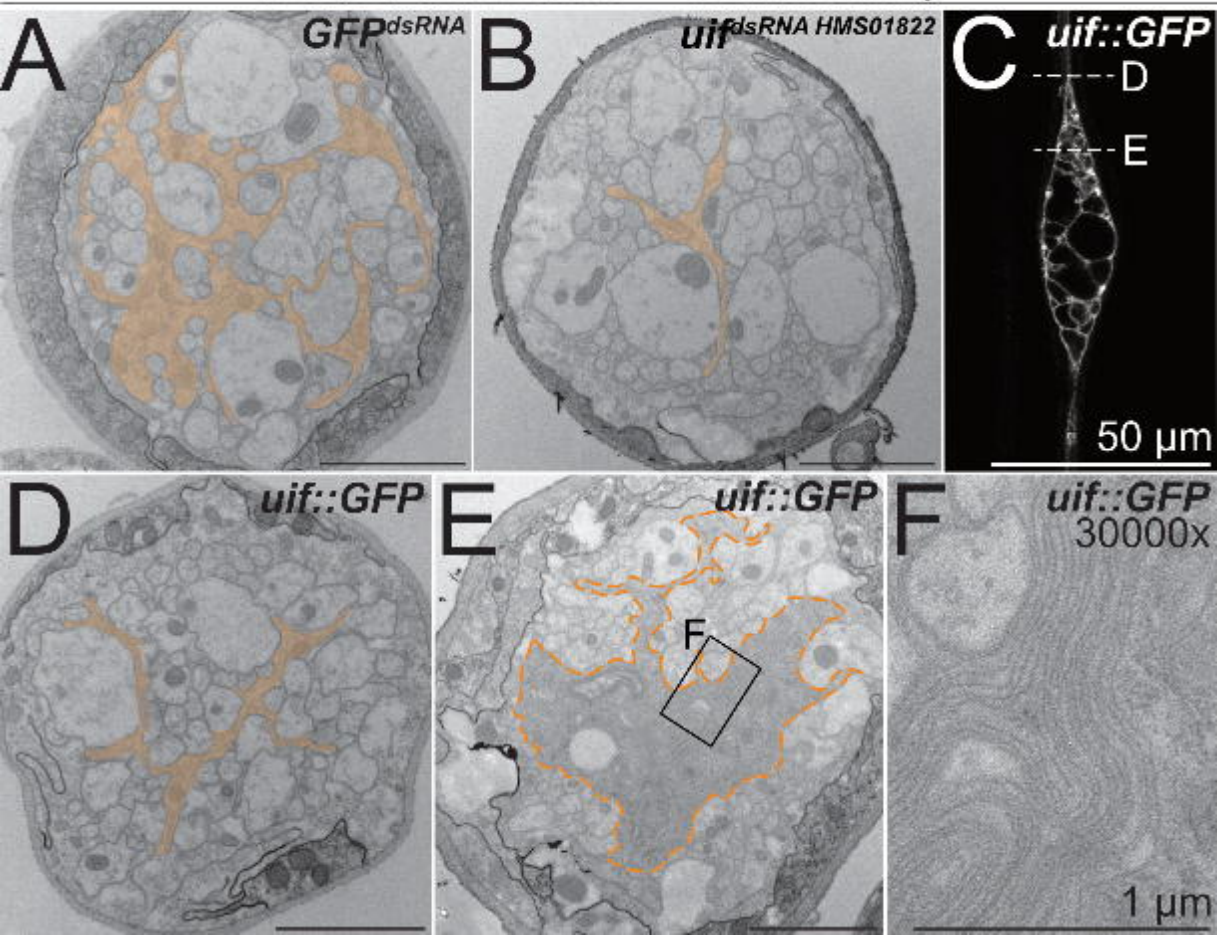
Zülbahar, S., Sieglitz, F., Kottmeier, R., Altenhein, B., Rumpf, S., and Klämbt, C. (2018). Differential expression of Öbek controls ploidy in the Drosophila blood-brain barrier. Development *145*, dev164111. 10.1242/dev.164111.

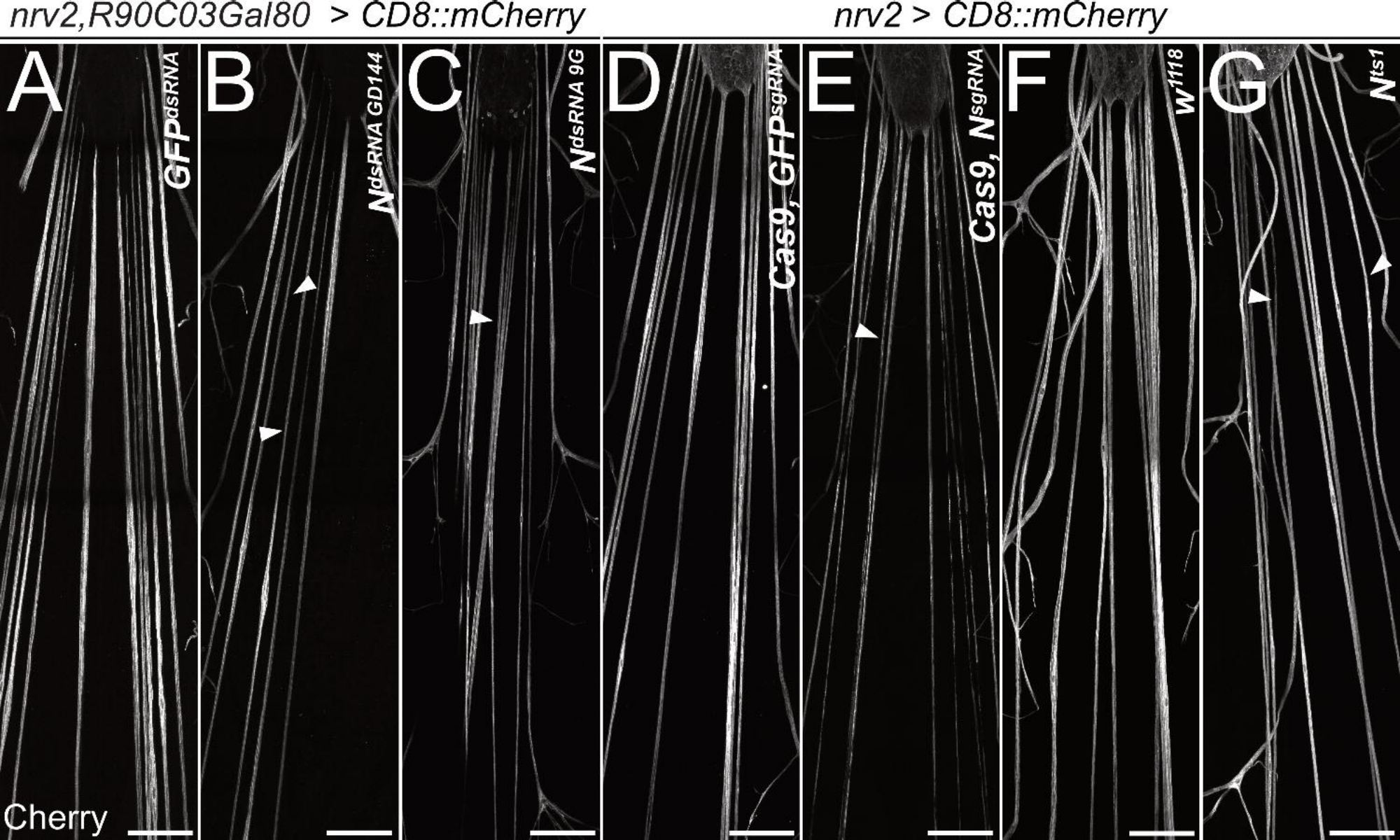

nrv2 > CD8::mCherry
A
GFPdsRNA
B
htlDN
C
λhtl
2C
2B
2A
Cherry

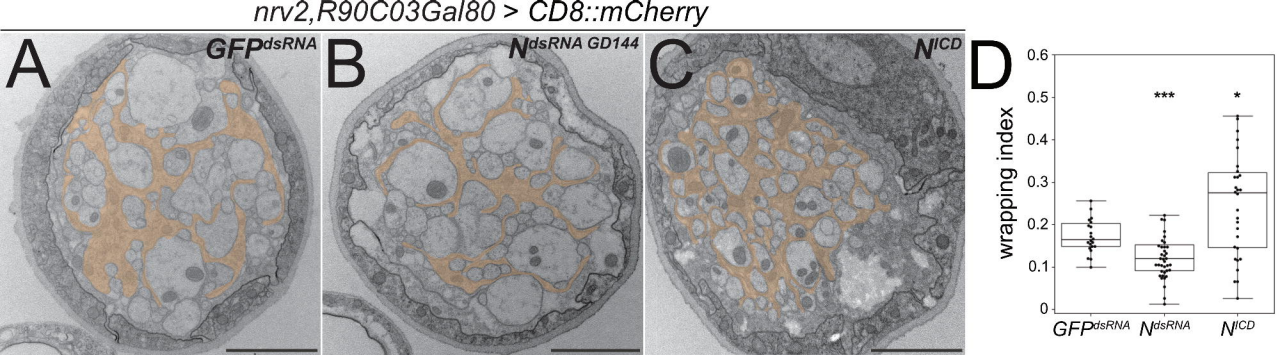

nrv2-Gal4
A
2 µm
UAS-GFP
B
2 µm
UAS-λhtl
C
2 µm
UAS-λhtl
D
G
E
F
2 µm
UAS-λhtl
E
ax
ax
F
G
H
*
*
2 µm
UAS-λhtl


nrv2 > CD8::mCherry
nrv2 > Cas9, CD8::mCherry
A
GFP dsRNA
B
uif dsRNA
HMS01822
C
uif dsRNA
HMS01822
λhtl
D
GFP sgRNA
E
uif sgRNA
2nd Exon
F
uif sgRNA CS
G
uif sgRNA TMD
H
uif sgRNA CD
Cherry

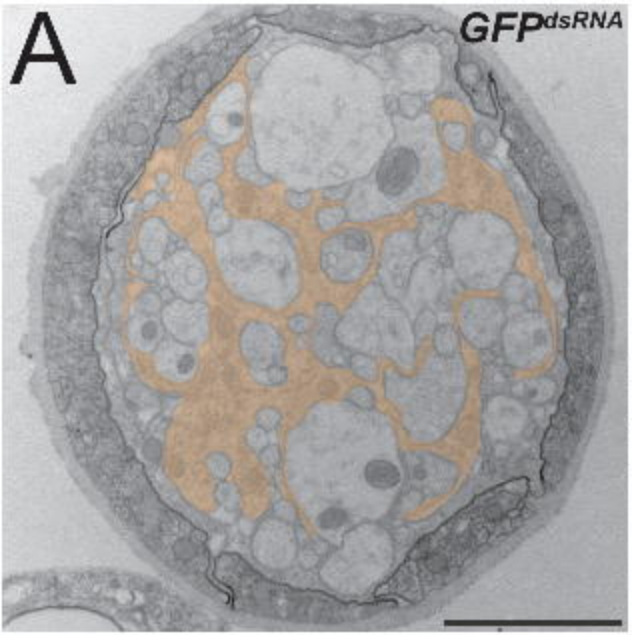
nrv2,R90C03Gal80 > CD8::mCherry

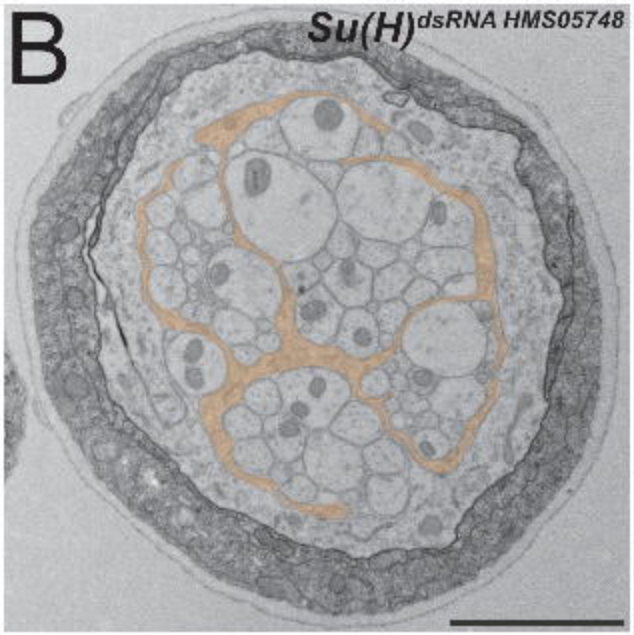


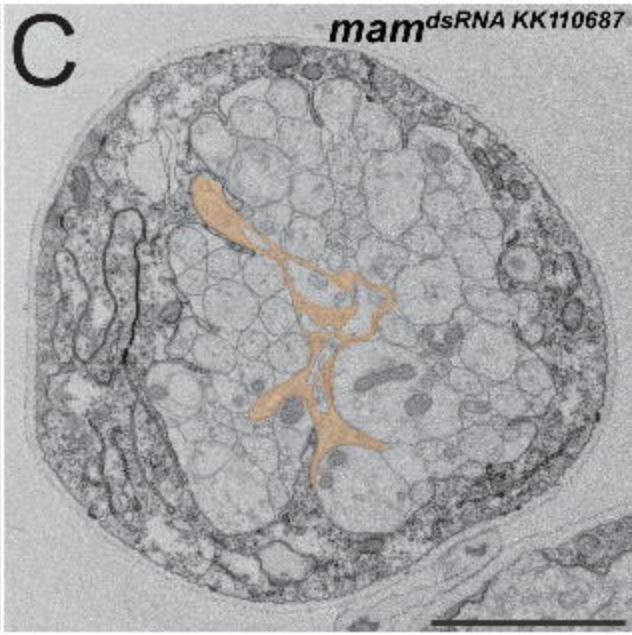

nrv2,R90C03Gal80 > CD8::mCherry
nrv2 > CD8::mCherry
A
GFPdsRNA
B
NdsRNA GD144
C
NdsRNA 9G
D
Cas9, GFPsgRNA
E
Cas9, NsgRNA
F
W1118
G
Nts1
Cherry


nrv2,R90C03Gal80 > CD8::mCherry
A
GFPdsRNA
B
NdsRNA GD144
C
NICD
D
wrapping index
0.6
0.5
0.4
0.3
0.2
0.1
0
***
*
GFPdsRNA
NdsRNA
NICD

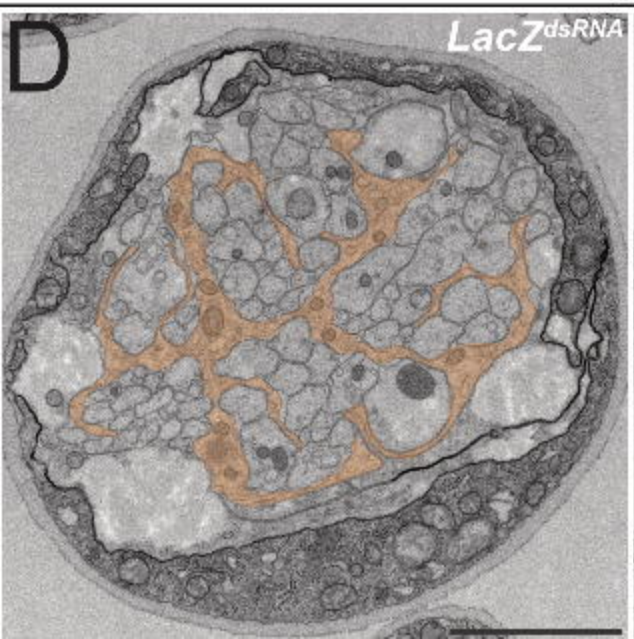
nrv2,R90C03Gal80 > CD8::mCherry

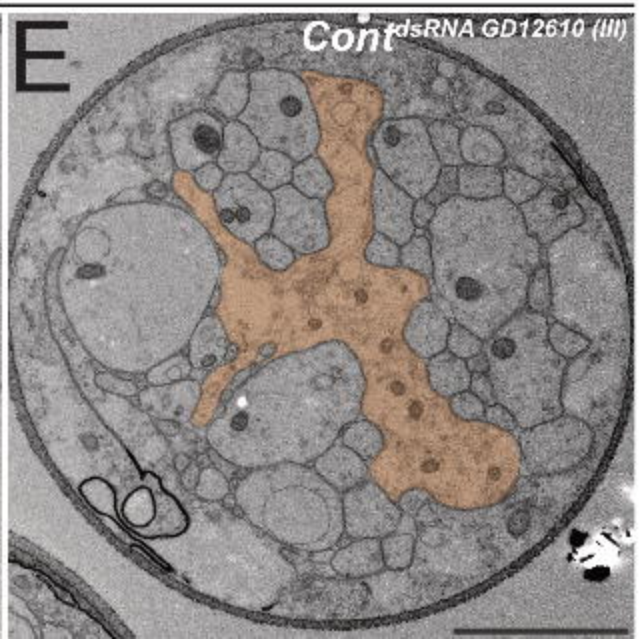


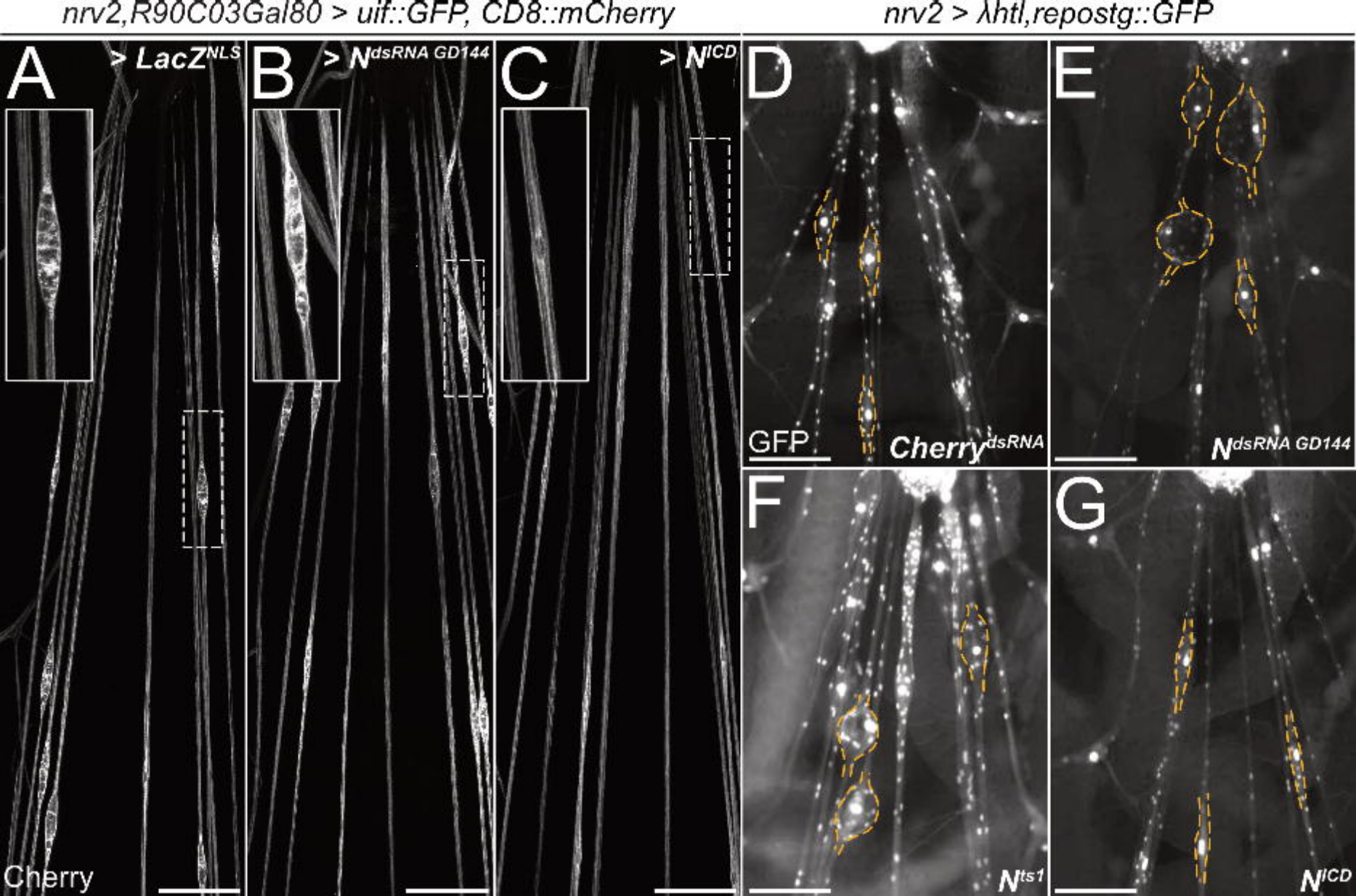


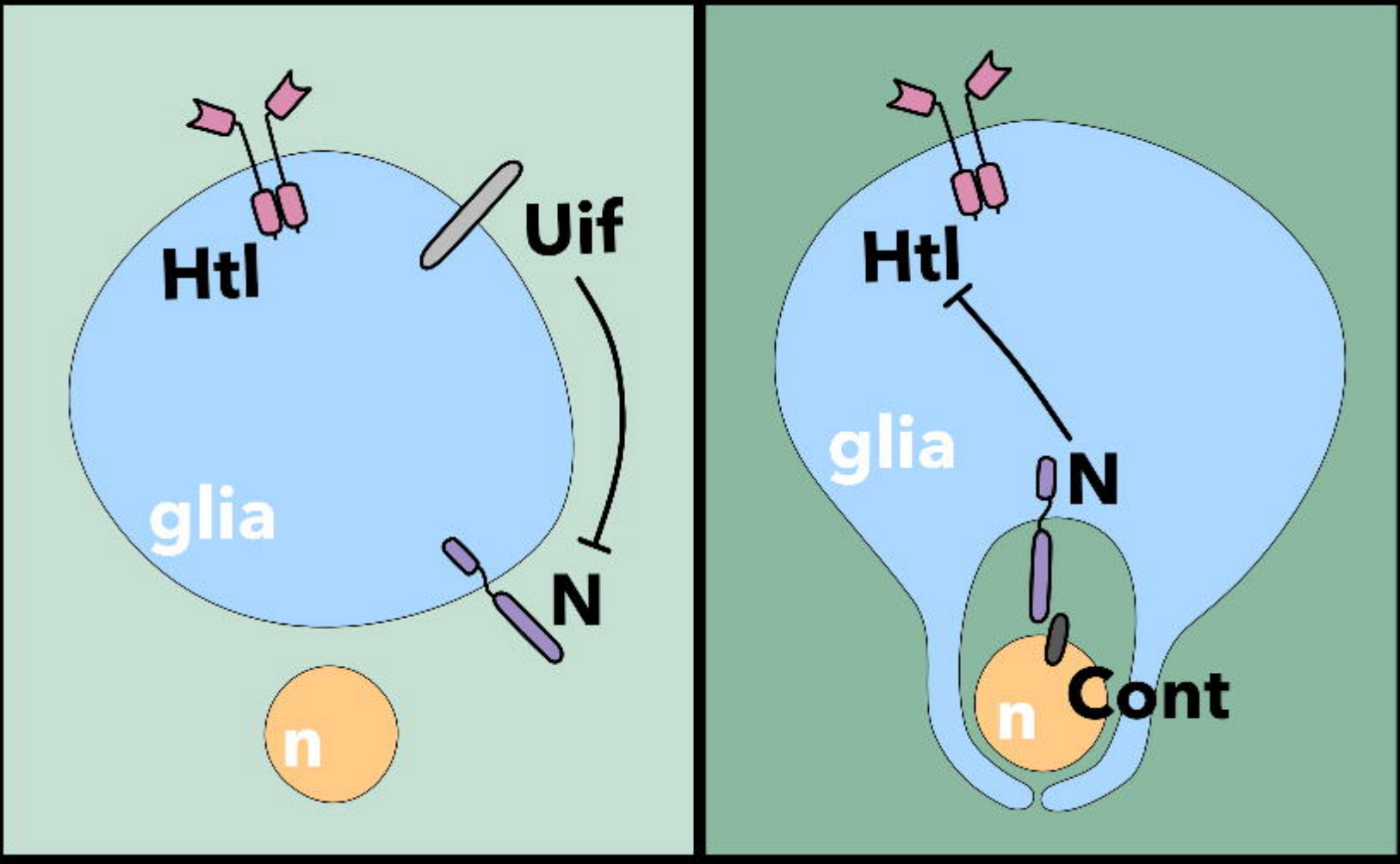


nSyb >






nrv2,R90C03Gal80 > uif::GFP, CD8::mCherry
nrv2 > λhtl,repostg::GFP
A
> LacZNLS
B
> NdsRNA GD144
C
> NICD
D
GFP
CherrydsRNA
E
NdsRNA GD144
F
Nts1
G
NICD
Cherry



growth	differentiation
cytoplasm/membrane	neuron/glia contact