

Dlg5 and Cadherins Cooperate to Ensure Peripheral Glia and Septate Junction Integrity

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The formation of the glial sheath is essential in nervous system development to insulate and protect peripheral nerves and axons, yet the regulation of adhesion junctions in nonmyelinating glia has not been clearly established. Many components of adhering junctions contain PDZ domains or are recruited by PDZ binding motifs. To identify PDZ domain proteins with roles in peripheral glia, we carried out an RNAi screen using *Drosophila melanogaster* to knockdown each of the 66 predicted PDZ proteins in larvae of either sex. We identified six PDZ genes with potential roles in glial morphology, and we further characterized the role of Dlg5, a scaffolding protein with no previously known function in any glia. In our investigation of Dlg5, we focused on cadherins and found both N-cadherin and E-cadherin are expressed throughout peripheral glia. Knockdown of E-cadherin (ECad) phenocopied the loss of Dlg5 leading to gaps in the subperineurial glia and septate junctions (SJs), while only simultaneous loss of both N-cadherins (NCad and CadN2) had the same effect. The loss of all three cadherins enhanced these phenotypes as did loss of Dlg5 when paired with cadherin knockdown yet Dlg5 does not colocalize with cadherins. The Hippo signaling pathway represents a common convergence point for both Dlg5 and cadherins, and we found that expression of Yorkie is able to rescue the loss of Dlg5 and ECad. This leads to a model where Dlg5 and cadherins function in the same pathway and play a role in glial membrane stabilization and SJ formation.

Key words: adherens junctions; glia; hippo; peripheral nerve; Yorkie

Significance Statement

Glial ensheathment of peripheral nerves is critical to generate the insulating sheath and the blood–nerve barrier. We found that the PDZ domain protein Discs-large 5 (Dlg5) plays a key role in the developmental of the insulating glial sheath and does so in cooperation with cadherins, both E- and N-cadherins. Dlg5 and cadherins converge to regulate the Hippo pathway and thus glial growth and blood–brain barrier formation.

Introduction

A critical function of glia is to wrap and ensheath axons in both the central and peripheral nervous systems. In vertebrate peripheral nerves, myelinating Schwann cells enable rapid and efficient transduction of electrical signals by forming the myelin sheath to ensheath and insulate large caliber axons. While there are

extensive studies that have identified the protein complexes that mediate myelin sheath formation and stabilization, almost nothing is known about the adhesion junctions, the scaffolding proteins, and the signaling processes that mediate sheath formation in the nonmyelinating Schwann cells and other classes of peripheral glia.

PDZ proteins mediate the recruitment of protein complexes to create signaling centers and adherent junctions. PDZ domains are 80–90 amino acids and mediate protein–protein interactions by binding to short three amino acid PDZ binding motifs found at the C-terminus of many proteins (Harris and Lim, 2001; Subbaiah et al., 2011). Most PDZ domain proteins contain a range of protein domains, for example, the family of membrane-associated guanylate kinases (MAGUKs) contains PDZ domains, L27, SH3, and GUK domains. Through these different interactions, PDZ proteins regulate processes such as cell polarity, adhesion, migration, and signaling through their ability to scaffold proteins to coordinate and regulate protein function (Ye et al., 2018). It is likely that PDZ proteins play a

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role in nonmyelinating glia, yet the identity and role of these proteins have yet to be determined. Thus, we set out to identify PDZ proteins and their role in nonmyelinating glia.

The peripheral glia of *Drosophila melanogaster* are an excellent model to further our understanding of the mechanisms underlying nonmyelinating glia development and function. Each peripheral nerve is ensheathed by three glial layers (Yildirim et al., 2019). The innermost glia layer, the wrapping glia (WG), resembles nonmyelinating Schwann cells and extends their processes around individual or bundles of motor and sensory axons. The WG layer is surrounded by the subperineurial glia (SPG), which form septate junctions (SJs) and establish the blood–nerve barrier to protect peripheral axons from the high K⁺ hemolymph (Auld et al., 1995; Baumgartner et al., 1996). Lastly, the perineurial glia (PG), which are the equivalent to vertebrate perineurial cells, wrap the nerve and adhere to the extracellular matrix that surrounds and stabilizes the entire nerve. The mechanisms that drive glial sheath formation in the *Drosophila* peripheral nerve are highly conserved (Freeman and Doherty, 2006), yet the role of PDZ proteins has yet to be determined.

There are only 66 PDZ proteins in *Drosophila* (Aranjuez et al., 2012), compared with the 250 predicted PDZ proteins in humans (Tonikian et al., 2008), and we screened all PDZ-encoding proteins using RNAi knockdown to determine which play a role in peripheral glia. We identified seven PDZ domain proteins where loss of function lead to disruption of peripheral glia and focused our subsequent investigation on Discs-large 5 (Dlg5). Dlg5 is a MAGUK protein with a coiled-coil domain at the amino terminus, four PDZ domains, an SH3 domain, and a GUK domain, and has been implicated in cell polarity and cadherin complex formation (Nechiporuk et al., 2007; Wang et al., 2014; Reilly et al., 2015; Liu et al., 2017). The role of Dlg5 and those of the classical cadherins in nonmyelinating glia, including the peripheral glia of *Drosophila*, had not been determined. Therefore, we went on to investigate Dlg5 and the *Drosophila* cadherins: E-cadherin (ECad, *shotgun*), N-cadherin (NCad, *CadN*), and N-cadherin2 (NCad2, *CadN2*) in the peripheral glia. Knockdown of Dlg5 in the subperineurial and WG disrupted glial morphology and ensheathment. We found that ECad, NCad, and CadN2 perform redundant functions in the SPG and that loss of cadherins phenocopied and enhanced the loss of Dlg5 phenotypes. However, unlike in other tissues, Dlg5 and cadherins did not colocalize within the glia. Rather, Dlg5, similarly to E-cadherin, feeds into the Hippo pathway as expression of Yorkie was able to rescue the Dlg5 RNAi phenotypes. Overall, our results show that Dlg5 and cadherins function to mediate peripheral glia development and integrity through regulation of the Hippo/Yorkie pathway.

Materials and Methods

Fly strains and genetics. The following fly strains were used in this study: elav-Gal4 (Bloomington Stock Center); repo-Gal4 (Sepp et al., 2001); Nrv2-Gal4 (Sun et al., 1999); 46F-Gal4 (Xie and Auld, 2011); Gli-Gal4 (Sepp and Auld, 1999); SPG-Gal4 (aka moody-Gal4; Schwabe et al., 2005); UAS-mCD8::GFP (Lee and Luo, 1999); UAS-Dicer2 (Dietzl et al., 2007); UAS-Yorkie::GFP (Oh and Irvine, 2008); *Df(2L)BSC148*; and UAS-mCD8::RFP (Bloomington Stock Center). The following GFP and RFP protein-trap insertions were used: Dlg5::GFP (Saroy et al., 2016); Nrv2::GFP; NrXIV::GFP (Morin et al., 2001; Kelso et al., 2004; Buszczak et al., 2007); and NCad::cherry, ECad::cherry (Venken et al., 2011). The following RNAi lines were used with those lines used for the majority of experiments in bold: Dlg5-RNAi (VDRC: 46234, 101596; BDSC: 41832); ECad-RNAi (VDRC: 27802); NCad-RNAi (VDRC: 1092); and CadN2-RNAi (BDSC: 27508). All RNAi experiments

carried out after the initial screen were raised at 25°C. Those experiments with Dicer2 are indicated in the figure. For all experiments, controls were the driver line crossed with *w¹¹¹⁸* or Dicer2 where appropriate.

Screen of RNAi knockdown of PDZ genes. RNAi lines were obtained from TRiP and VDRC stock collections. Dataset S1 provides the list of RNAi lines used in the screen. *repo>mCD8::RFP/TM6,Tb* virgin flies were crossed with males from each UAS-RNAi line at 29°C. As a control, *repo>mCD8::RFP/TM6,Tb* virgin flies were crossed with *w¹¹¹⁸* males and raised at 29°C. A minimum of six larvae were dissected from each cross and stained with mAb 22C10 (anti-Futsch) to label the peripheral axons. The morphology of the glial membranes were visualized using UAS-mCD8::RFP.

Immunolabeling and image analysis. The peripheral nervous system of third instar wandering larvae were dissected and fixed for immunolabeling using previously described methods (Sepp et al., 2000). The following primary antibodies were used in this study: guinea pig anti-Inx2 (1:500; Smendziuk et al., 2015); mouse anti-Futsch/22C10 [1:1,000, Developmental Studies Hybridoma Bank (DSHB)]; rabbit anti-GFP (1:600, Life Technologies); chicken anti-GFP (1:1,000, Life Technologies); mouse anti-GFP (1:300, Novus Biologicals); rabbit anti-Cherry (1:300, Abcam); mouse anti-βPS (1:50, CF.6G11 DSHB; Brower, 1984); rabbit anti-HRP (1:500, Jackson ImmunoResearch Laboratories); mouse anti-Futsch (1:1,000, 22C10 DSHB); and rabbit anti-Nrv2.1 (1:1,000, Abcam). The following secondary antibodies were used at a 1:300 dilution: goat anti-mouse Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 647; goat anti-chicken Alexa Fluor 488; goat anti-rabbit Alexa Fluor 488 and Alexa Fluor 568; and goat anti-guinea pig Alexa Fluor 647 (Molecular Probes). DAPI (1:1,000, Invitrogen) was used to label nuclei. Images were acquired with a Delta Vision Spectris compound microscope (Applied Precision/GE Healthcare) using a 60× oil immersion objective (NA 1.4). An image was captured every 0.2 μm, and the resulting stacks were deconvolved (SoftWorx) using a point spread function measured with 0.2 μm beads conjugated to Alexa dyes (Molecular Probes) and mounted in Vectashield (Vector Laboratories). Cross sections were generated using SoftWorx. Images were imported into Fiji (ImageJ; Schindelin et al., 2012). Alternatively, images were acquired with an Evident IXplore SpinSR Spinning Disk Confocal Microscope (Evident/Olympus) using a 60× oil immersion objective (NA 1.35) and a z-step of 0.2 μm. A single z-slice was chosen from each z-stack, and images were compiled using Adobe Photoshop and Adobe Illustrator CC. For transmission electron microscopy analysis, larvae were dissected and prepared using previously described methods (Matzat et al., 2015) using a Tecnai Spirit 120 kV TEM at the UBC BioImaging Facility.

Dye penetration assay. Third instar larvae were washed using 1× PBS, then opened at the posterior end, and inverted in Schneider's insect medium (Millipore Sigma, S0146). The inverted larvae were incubated in Schneider's medium containing 2.5 mM of 10,000 MW Dextran Texas Red-conjugated dye (Invitrogen, D1863, Lysine Fixable) for 40 min at room temperature on an orbital shaker. The larvae were then fixed using 4% PFA with 1× PBS for 20 min. Following fixation, samples were washed with 1× PBS twice for 5 min each and then washed with 0.1% PBST with 1× PBS twice for 10 min each. Lastly, the brains were dissected and mounted in Vectashield. The samples were imaged using an Olympus FV1000 confocal microscope (BioImaging Facility UBC) using a 30× silicone oil immersion objective (NA 1.05) with 1 μm steps. Laser (559 nm) intensity was set at 7% intensity, 750 V digital gain, 1× master gain, and 6% offset for all genotypes except for NrXIV-RNAi, which was 5% intensity, 750 V digital gain, 1× master gain, and 6% offset. Images were imported into Fiji, fluorescence intensity was calculated, and images were displayed using the Fire lookup table.

Locomotion assay. Locomotion assays were performed as previously described (Brooks et al., 2016). Larvae of the desired genotype were gently placed onto a freshly made 2% agar plate with food-safe dye added to enhance contrast. Larval movements were recorded continuously for 60 s using a Canon VIXIA HF R800 video camera. The recordings were

then converted from *.MOV to *.AVI files using FFmpeg (ffmpeg.org) and analyzed using the wrMTrck plug-in (Nussbaum-Krammer et al., 2015; Brooks et al., 2016) in Fiji to calculate locomotion and movement trajectory. Larvae with noncontinuous paths throughout the entirety of the recording were discarded from the total travel distance quantification but were included in the average travel speed.

Quantification and statistics. Prism (GraphPad Software) was used for all statistical analyses. For all graphs, the mean and standard deviation are indicated. For fluorescence image analyses, 6–8 abdominal nerves were analyzed per larvae, the average nerves affected was calculated per animal, and each graph point represents a single animal. Pearson's coefficients were calculated using SoftWorx across the entire z-stack using a region of interest (ROI) limited to each peripheral nerve with each graph point representing a single nerve. For sAJ quantification, micrographs from at least three peripheral nerves (in the nerve extension region) per animal were scored for the presence of sAJ between the SPG and WG membrane. Each graph point represents the mean number of sAJ observed in an individual larva.

Results

RNAi induced knockdown of PDZ proteins in glial cells

To understand the role of PDZ proteins in mediating glia interactions, we utilized the peripheral nerve of the third instar *Drosophila* larva to investigate the consequences on glial morphology of knockdown of PDZ domain proteins. There are 66 genes predicted to encode PDZ domain proteins in the *Drosophila* genome (Aranjuez et al., 2012). Using the pan-glial driver *repo-Gal4*, we expressed each UAS-PDZ-RNAi line while coexpressing *UAS-mCD8::RFP* to label the glial membranes. We scored morphological defects in the labeled peripheral glial membranes, and axons were labeled using anti-Futsch to reveal any axonal defects. When available, multiple RNAi lines were used to target each PDZ gene. For the 66 genes, 150 individual UAS-RNAi lines were tested for abnormal glial phenotypes in the PNS (Fig. 1A; Dataset S1). As a control, *repo>mCD8::RFP* flies were crossed to *w¹¹¹⁸* flies (Fig. 1B,B'). We classified PDZ genes as positive hits (Fig. 1A; Dataset S1) if knockdown using multiple RNAi lines resulted in lethality or glial phenotypes. Seven PDZ proteins, Discs Large 1 (*dlg1*), Scribbled (*scrib*), Varicose (*vari*), Stardust (*sdt*), Discs Large 5 (*Dlg5*), Locomotion defects (*loco*), and a Rho-type guanine nucleotide exchange factor (*RhoGEF2*), were identified as positive hits with potential functions in glial cells (Fig. 1A,C–F; Dataset S1). The screen resulted in a range of phenotypes such as glial swelling observed with the knockdown of *RhoGEF2* and *Loco* (Fig. 1C–C',F–F'). Knockdown of *Dlg1* resulted in abnormal axonal wrapping (Fig. 1D–D'), whereas *Vari* knockdown in all glia was lethal (Dataset S1). Of note is that loss of known apical polarity proteins including *Bazooka* (*baz*, *Par-3*), *Par-6*, and *Patj* had no effect (Fig. 1A; Dataset S1). Conversely, the basolateral polarity proteins such as *Dlg1* and *Scrib* had strong effects as did *Dlg5*. Abnormalities in the glial membranes and in particular axonal wrapping by the inner wrapping glial membrane were observed with *Dlg5* knockdown (Fig. 1F,F'). Overall, we identified a group of PDZ proteins that may play necessary roles in peripheral glia development.

Dlg5 is expressed and is required in glial cells of the larval peripheral nerve

Given the role of *Dlg5* in cell polarity and cell–cell adhesion and the fact that *Dlg5* has not been studied in glia, we chose to investigate *Dlg5* further. *Dlg5* is a MAGUK protein with a CARD (Caspase Activation and Recruitment Domain) domain at the

amino terminus, a coiled-coil (CC) domain, four PDZ domains, an SH3 domain, and a GUK domain (Fig. 2G). We examined the distribution of *Dlg5* in the glial layers of the peripheral nerve in third instar larvae using *Dlg5* endogenously tagged with GFP (*Dlg5::GFP*; Sarov et al., 2016). *Dlg5* puncta were observed throughout the peripheral nerve, with puncta present within the glial cell membranes (identified with *mCD8::RFP*; Fig. 2A–A'). Some *Dlg5* puncta might also be present in the axons (immunolabeled with anti-Futsch; Fig. 2B–B'). This pattern mirrored similar “dotty” or punctate patterns of *Dlg5* distribution observed previously in epithelia (Venugopal et al., 2020).

To assess *Dlg5* function in the peripheral nerves, we tested verified *Dlg5* RNAi lines (Aranjuez et al., 2012; Reilly et al., 2015; Luo et al., 2019; Venugopal et al., 2020; Kaya-Copur et al., 2021) that target different regions of the *Dlg5* transcripts (Fig. 2G). When driven with a pan-glial driver (*repo-Gal4*), all three lines tested disrupted peripheral glial morphology with a range of phenotypes that included membrane accumulation (Fig. 2D, arrowheads), regions with vacuole or hole-like structures (Fig. 2E,F, arrowheads) and overall disorganized membranes. The glial defects observed with each RNAi were significantly greater than in controls (Fig. 2H). When tested, RNAi-mediated knockdown (*repo>Dlg5::RNAi 46234*, *mCD8::RFP*) resulted in the loss of *Dlg5::GFP* from the peripheral nerves (Supplemental Fig. S1) compared with control (*repo>mCD8::RFP*). Knockdown of *Dlg5* using the pan-neuronal driver *elav-Gal4* (*elav>Dlg5-RNAi 101596*) did not affect either peripheral glial or axon morphology (*n* = 60 nerves in 8 larvae).

To further characterize the effects of *Dlg5* loss in the peripheral glia, we knocked down *Dlg5* in individual glial layers using *Gal4* drivers specific to each of the three glial layers [PG, 46F-*Gal4* (Xie and Auld, 2011); SPG, *Gli-Gal4* (Sepp and Auld, 1999), SPG-*Gal4* (Bainton et al., 2005); WG, *Nrv2-Gal4* (Sun and Salvaterra, 1995; Sun et al., 1999)] driving a membrane marker, *mCD8::RFP* (Fig. 3). Knockdown of *Dlg5* (RNAi lines 41832, 101596) in the PG using 46F-*Gal4* (*46F>mCD8::RFP*, *Dlg5-RNAi*; *n* = 6 larvae, 36–40 nerves per RNAi) did not affect PG membrane morphology or ensheathment of the peripheral nerves (Fig. 3B–B') compared with control (*46F>mCD8::RFP*; Fig. 3A–A'). Knockdown of *Dlg5* in the WG using *Nrv2-Gal4* (*Nrv2>mCD8::RFP*, *Dlg5-RNAi*) resulted in reduced and discontinuous WG strands (Fig. 3D,D', arrowheads, E) compared with controls where multiple, continuous WG strands ensheath the axons (*Nrv2>mCD8::RFP*; Fig. 3C,C'). We also noted an increase in WG processes extending through the nerve and the presence of gaps or holes flanking these processes (Fig. 3F,G, arrowheads), which became more apparent when the image stack was rotated along the longitudinal axis 10 and 65° (Fig. 3G,G'). These defects were significantly higher compared with controls for both RNAi lines tested (Fig. 3H).

We next tested the effects of knockdown of *Dlg5* using *Gli-Gal4* to drive expression in the intermediate SPG layer. Knockdown of *Dlg5* (*Gli>mCD8::RFP*, *Dlg5-RNAi*) disrupted SPG morphology (Fig. 4B,B') compared with controls (*Gli>mCD8::RFP*; Fig. 4A,A'). SPG membrane morphology was disrupted with breaks/gaps in the membrane (yellow arrows) and occasionally accompanied by swellings (asterisks). We next tested if the defects in the SPG extended to the SJ protein complex, which in *Drosophila* forms the blood–nerve and blood–brain barriers (BBB). We observed nerves with gaps in the normally uniform SJ strands when labeled with *NrxIV::GFP* (Fig. 4D', yellow arrows), and these corresponded to thinning or gaps in the *mCD8::RFP*-labeled membrane (Fig. 4B',G').

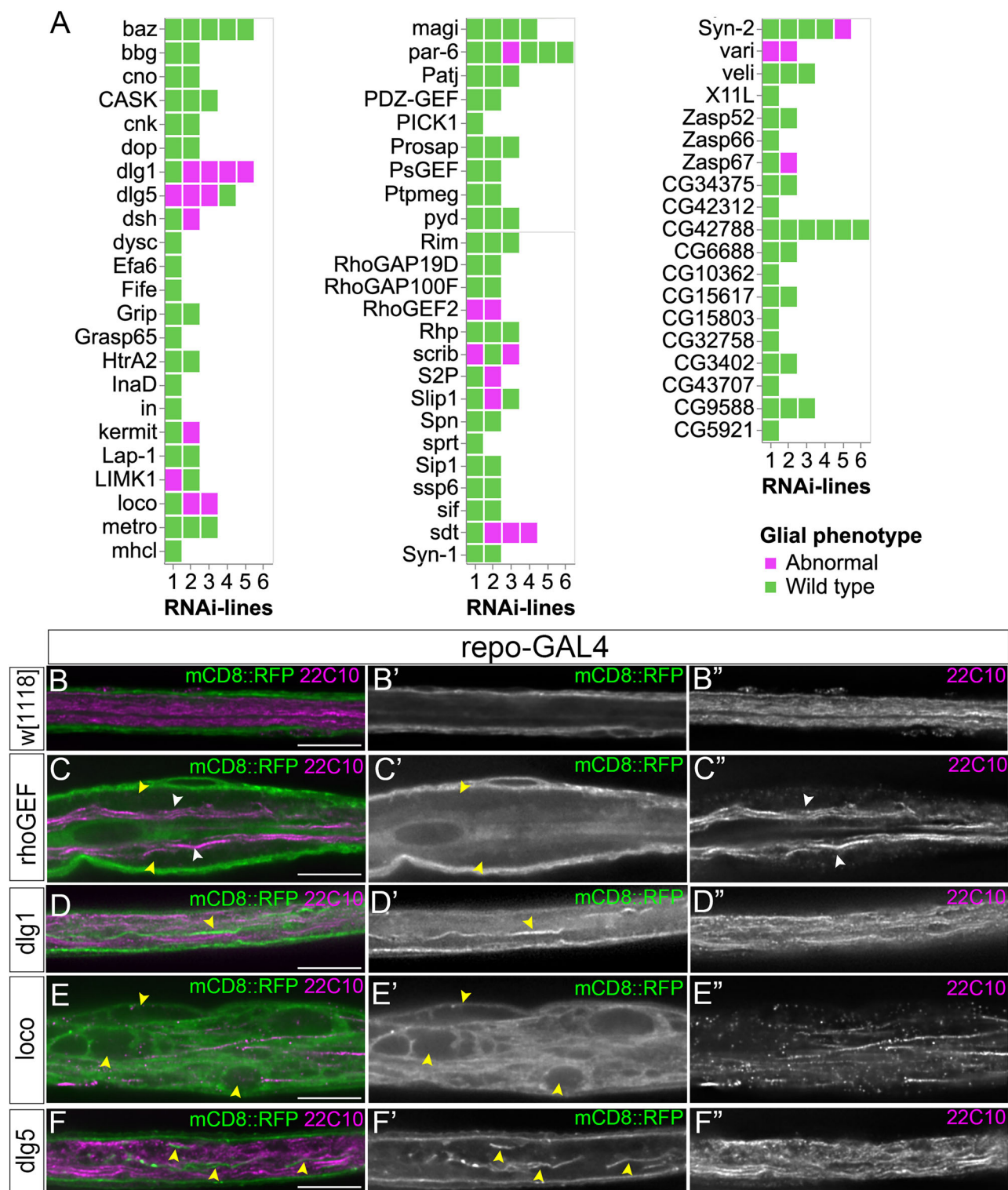


Figure 1. Identification of PDZ proteins required in glial cells of the peripheral nerve. **A**, Summary of PDZ protein RNAi screen. Details of RNAi lines used to target each candidate gene can be found in [Dataset S1](#). Each box represents an individual RNAi line with green meaning wild-type appearance and magenta representing abnormal glial phenotypes. **B–F**, Longitudinal sections of peripheral nerves from control and selections of positive candidate identified in the RNAi screen. All glial membranes labeled with mCD8::RFP (green) and axons immunolabeled with anti-Futsch (22C10; magenta). The pan-glial driver repo-Gal4 was used and crossed to: (**B–B''**) control (*w¹¹¹⁸*), normal peripheral nerve. **C–C''**, Rho-GEF2-RNAi. Swollen regions are indicated (**C'**, yellow arrowheads), and these swellings were accompanied by defasciculation of the axons in that region (white arrowheads, **C''**). **D–D''**, Dlg1-RNAi. Glial membranes were disrupted (**D'**, yellow arrowhead). **E–E''**, Loco-RNAi. Peripheral nerves were swollen and vacuole-like structures were observed within the glial membranes (**F**, yellow arrowheads). **F–F''**, Dlg5-RNAi. Peripheral nerves had disrupted inner glial membranes (**F'**, yellow arrowheads). The morphology of axons in these nerves was unaffected (**F''**). Scale bars, 15 μ m.

In comparison, intact and continuous SJs were observed along the length of control nerves (*Gli>mCD8::RFP*; [Fig. 4C](#), yellow arrows). The discontinuities in the SJ and SPG membrane appear

to correspond to thinning or uneven distribution of SJ proteins or the membrane marker ([Fig. 4F,G](#)). We observed regions of “holes” or swellings along the SPG membrane ([Fig. 4G](#)”, yellow

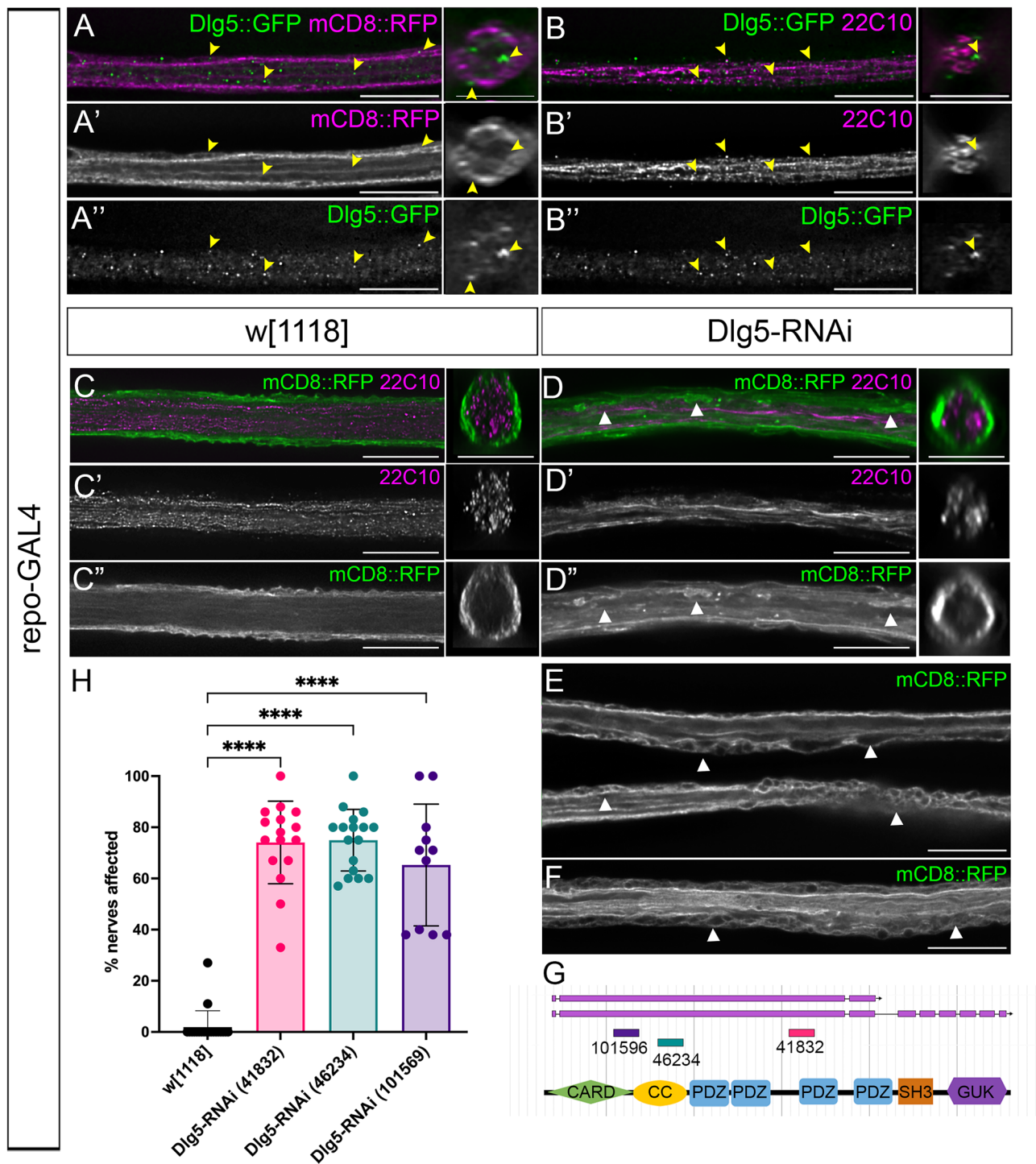


Figure 2. Knockdown of Dlg5 in glia leads to peripheral glial disruptions. **A, B**, Endogenously tagged Dlg5 (Dlg5::GFP, green) reveals punctate expression (yellow arrowheads) within glia and axons in the PNS. **A**, Glial membranes labeled with mCD8::RFP (repo-Gal4, magenta) or **B** axons immunolabeled with anti-Futsch (22C10, magenta). Longitudinal sections with cross sections of third instar peripheral nerves shown to the right. Cross sections reveal puncta of Dlg5::GFP throughout the glial layers (yellow arrowheads). **C–F**, The pan-glial driver repo-Gal4 crossed to *w[1118]* (**C**) or Dlg5-RNAi (**D–F**). Glial layers labeled with mCD8::RFP (green) and axons with anti-Futsch (22C10, magenta). Longitudinal sections and cross sections of third instar peripheral nerves. Disruptions in the glial membranes are indicated (white arrowheads). **G**, The *Dlg5* gene with the targeted region of each RNAi line and the conserved Dlg5 domains indicated (CARD, CC, PDZ, SH3, and GUK). **H**, Quantification of glial membrane disruptions. The average number of affected nerves per animal with repo-Gal4 crossed to *w[1118]* (control) and three independent Dlg5-RNAi lines (41832, 46234, 101569). The mean and standard deviation are indicated. Statistical significance was determined by one-way ANOVA with Dunnett's post hoc multiple-comparison test for each group (*****p* < 0.0001). Scale bars: 15 μ m in longitudinal sections, 10 μ m in cross sections.

arrow) where the corresponding SJ marker (NrXIV) was still present but at much lower levels than flanking regions (Fig. 4G', yellow arrow) suggesting uneven deposition of SJ proteins or

stretching of the membranes in these regions. These defects were significantly higher with Dlg5 knockdown compared with controls (Fig. 4E).

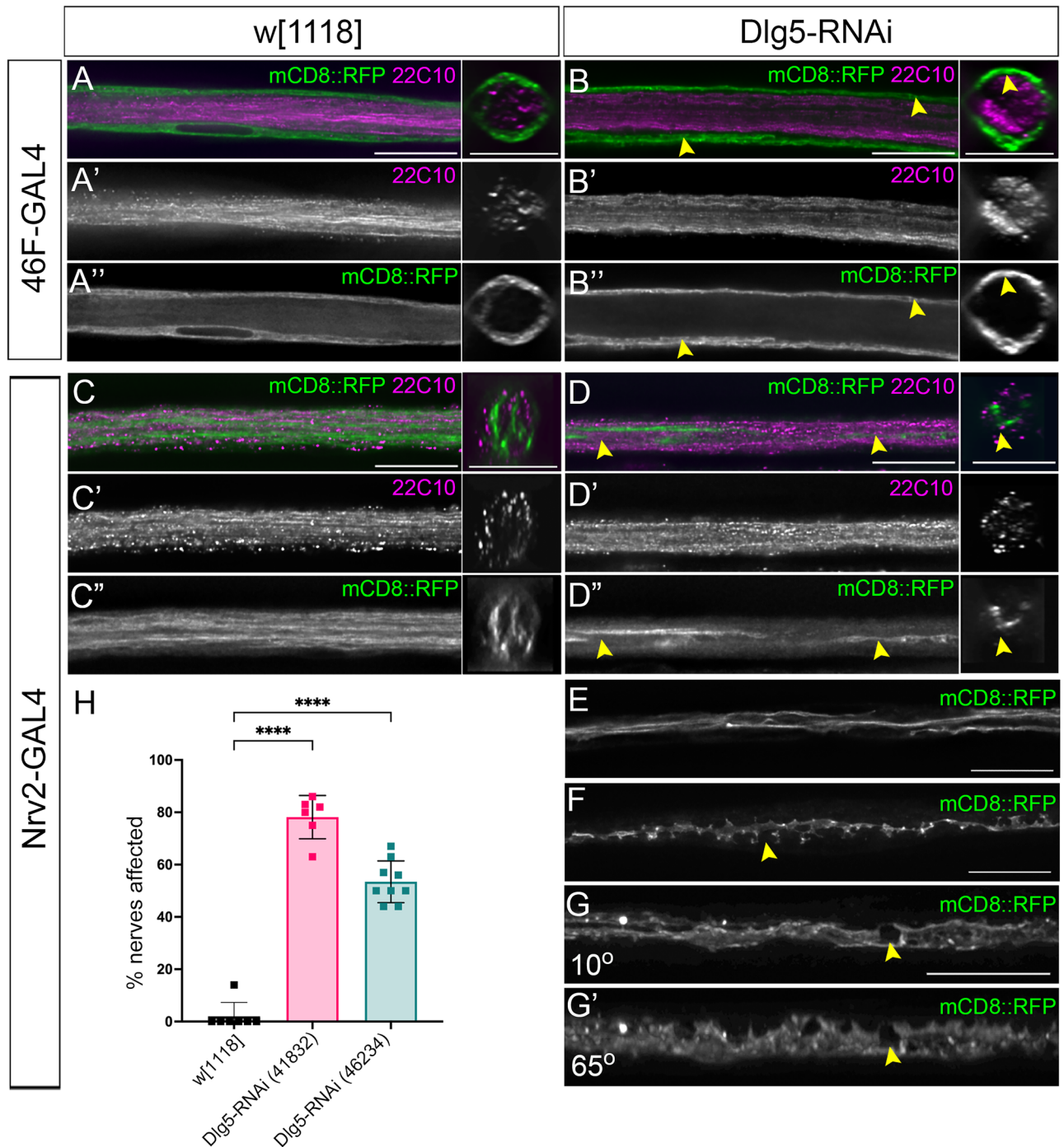


Figure 3. Loss of Dlg5 affects WG but not PG. **A, B**, PG. The PG driver 46F-Gal4 crossed to *w[1118]* (**A**) or Dlg5-RNAi (**B**). The PG membranes (mCD8::RFP, green) and axons immunolabeled with anti-Futsch (22C10, magenta) appeared normal in longitudinal and cross sections in both groups. **C–G**, WG. The WG driver Nrv2-Gal4 crossed to *w[1118]* (**C**) and Dlg5-RNAi (**D–G**). The WG membranes were labeled with mCD8::RFP (green), and axons were labeled with anti-Futsch (22C10, magenta). WG in control nerves (**C**) extended normal processes along the length of the nerve and in cross sections surrounded the axons (magenta). WG in Dlg5-RNAi nerves had fewer and collapsed processes (**D**), yellow arrowheads; **E**). In cross sections the WG processes did not extend around the axons (yellow arrowhead). Glial membranes appeared to surround bubbles or swellings (**F, G**; yellow arrowheads) that can be observed with rotation (**G** 10° to **G'** 65°). **H**, Quantification of WG disruptions. The average number of affected nerves per animal with Nrv2-Gal4 crossed to *w[1118]* (control) and two independent Dlg5-RNAi lines (41832, 46234). Statistical significance was determined by one-way ANOVA with Dunnett's post hoc test (**** $p < 0.0001$). Scale bars: 15 μ m in longitudinal sections, 10 μ m in cross sections.

Dlg5 distribution within the peripheral glia

Dlg5 has been found associated with SJ proteins such as Dlg1 and Neurexin-IV (Nrx-IV) in *Drosophila* border cells (Liu et al., 2014), yet we found that Dlg5 did not localize with any of the SJ markers tested such as Nrx-IV, Nervana2.1, or Dlg1 (Supplemental Fig. S1F). Prior studies have suggested that Dlg5 can interact with β -catenin and cadherin family members in a

number of cell types. Specifically, Dlg5 is colocalized with cadherins (Liu et al., 2017) and is involved in trafficking of cadherin–catenin adhesion complexes (Nechiporuk et al., 2007; Wang et al., 2014; Reilly et al., 2015). We tested the possibility that Dlg5 also interacts with cadherins in peripheral glia. The presence and distribution of cadherins in *Drosophila* peripheral glia had not been established, so we began by looking for the

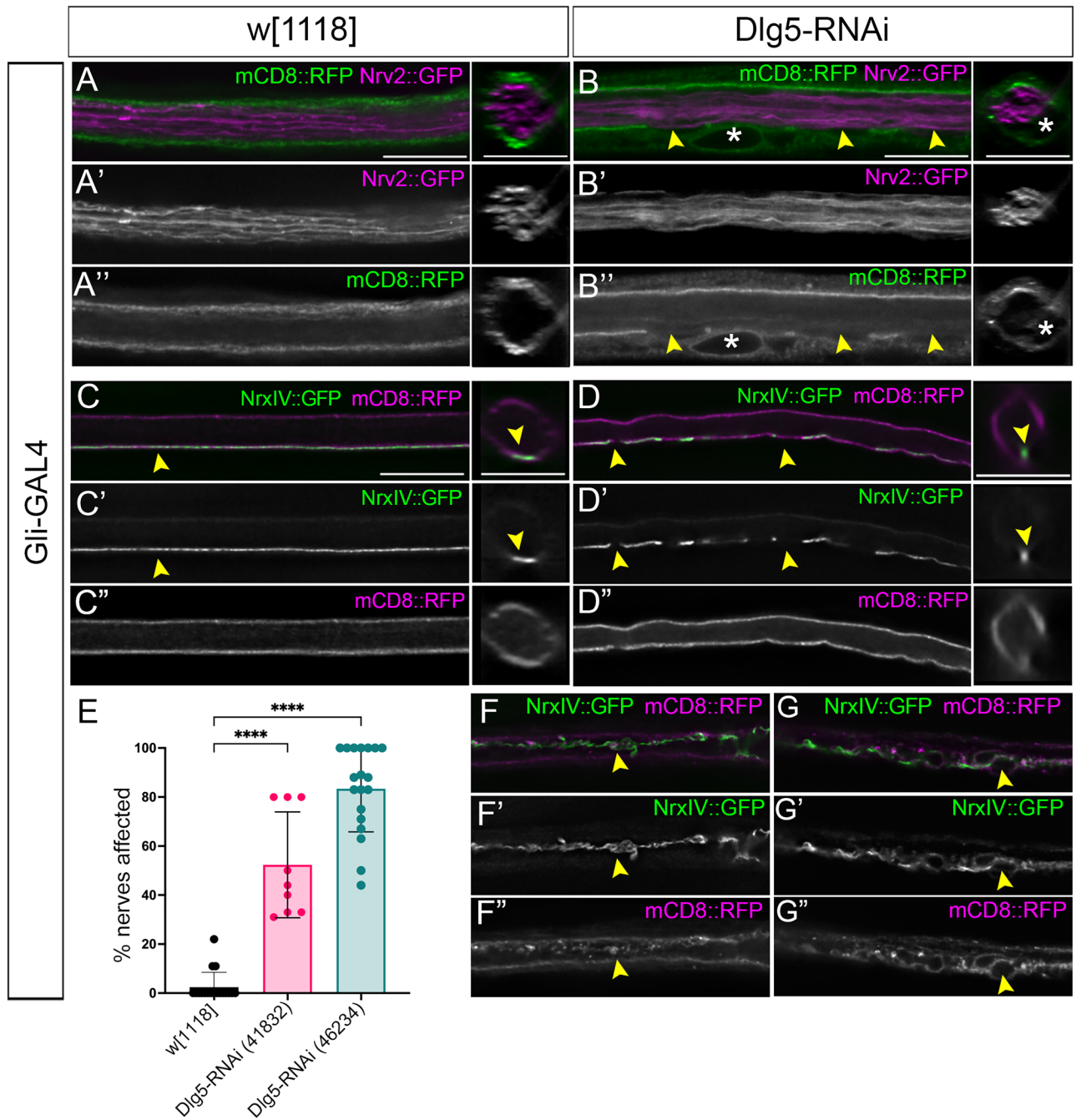


Figure 4. Knockdown of Dlg5 in the SPG disrupts SJ and membrane morphology. **A, B**, Dlg5-RNAi in the SPG disrupts SPG membranes but not the WG. Control (**A**) and Dlg5-RNAi (**B**) peripheral nerves with the SPG membrane labeled with mCD8::RFP (green) and the WG labeled with Nrv2::GFP (magenta). In controls, *w[1118]* SPG membranes (**A**) were continuous, while in *Dlg5-RNAi* nerves SPG membranes are disrupted (**B**) with thinning or breaks (**B'**, arrowheads) or swellings (**B'** asterisk). **C–G**, Dlg5-RNAi in the SPG disrupts the SJ. Control (**C**) and Dlg5-RNAi (**D, F, G**) peripheral nerves with the SPG membrane labeled with mCD8::RFP (magenta) and SJs labeled with NrxIV::GFP (green). In control *w[1118]*, the SJs are continuous along the length of the glia. In Dlg5-RNAi nerves, SJ strands were disrupted (**D'**) or form whirls (**F**) or swellings (**G**) that corresponded to changes in the SPG membrane (**F'**, **G'**). **E**, Quantification of the SJ disruptions. The number of nerves per animal with disrupted SJs was quantified for SPG-Gal4 crossed to *w[1118]* (control) and two independent lines of Dlg5-RNAi (41832, 46234). The mean and standard deviation are indicated. Statistical significance was determined by one-way ANOVA with Dunnett's post hoc multiple-comparison test (*****p* < 0.0001). Scale bars: 15 μm in longitudinal sections, 10 μm in cross sections.

presence of the two classical *Drosophila* cadherins; E-cadherin (ECad; *shotgun*) and N-cadherin (NCad; *CadN*) using genes endogenously tagged with mCherry or GFP (ECad::mCherry, ECad::GFP, and NCad::mCherry; Venken et al., 2011). Both NCad and ECad puncta were observed throughout the peripheral nerve (Fig. 5*A,B*) with both cadherins forming occasional short belt-like patterns in some parts of the nerve. More frequently,

NCad and ECad were observed in puncta, and we observed cadherin puncta throughout all three glial layers (Fig. 5*A,B*, yellow arrows). The majority of the NCad (Fig. 5*C'*) and ECad (Fig. 5*C''*) puncta were distinct with minimal colocalization (Fig. 5*C*, yellow arrows; Fig. 5*I*). The individual NCad and ECad puncta likely represent glial spot adherens junctions (sAJs; Matzat et al., 2015), and using TEM, sAJs were observed

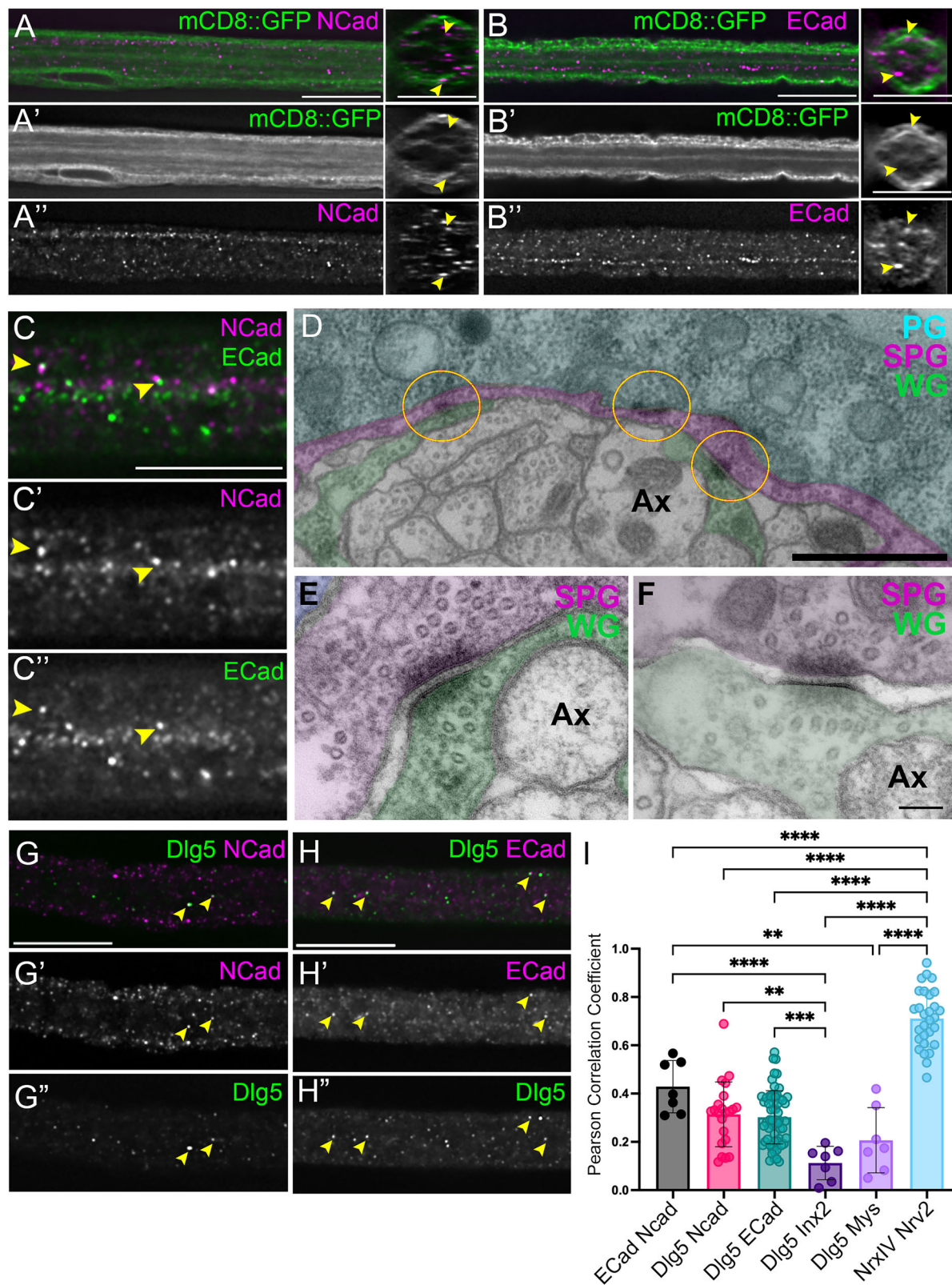


Figure 5. N-cadherin and E-cadherin create sAJs in the larval peripheral nerve. **A, B**, Control third instar peripheral nerves with NCad::mCherry (magenta, **A, A'**) and ECad::mCherry (magenta, **B, B'**). The glial membranes were labeled repo-Gal4 driving mCD8::GFP (green). Both cadherins were distributed throughout the nerve indicated in the cross sections of each peripheral nerve (yellow arrowheads). **C**, Longitudinal sections of peripheral nerves with NCad::mCherry (magenta, **C, C'**) and ECad::GFP (green, **C, C'**). Some but not all NCad overlaps with ECad puncta (yellow arrowheads). **D–F**, TEM analysis of a control (repo-Gal4) nerve with the three glial layers false colored: WG (green), SPG (magenta), PG (cyan), and Axons (Ax). sAJs are indicated (yellow circles). Scale bars: **D**, 0.5 μ m; **E, F**, 0.1 μ m. **G, H**, Longitudinal sections of peripheral nerves with Dlg5::GFP (green) and NCad::mCherry (magenta, **G**) and ECad::mCherry (magenta, **H**). Occasional Dlg5 puncta overlap with NCad and ECad puncta (yellow arrowheads). **I**, Pearson's correlation coefficient of Dlg5 and cadherin localization. Dlg5::GFP, ECad::mCherry, and Ncad::mCherry puncta do not consistently colocalize. Dlg5 is not localized to gap junctions or focal adhesions (Supplemental Fig. S1). All combinations were significantly less compared with the positive control of SJ markers NrxIV + Nrv2.1. Statistical significance was determined by one-way ANOVA with Tukey's post hoc multiple-comparison test for each RNAi compared across multiple genotypes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bars, 15 μ m for confocal images.

between the three different glial layers with the majority occurring between the PG–SPG and SPG–WG membranes (Fig. 5D–F).

Next, we tested for Dlg5 and cadherin colocalization using Dlg5::GFP crossed with NCad::cherry (Fig. 5G) or ECad::cherry (Fig. 5H). We only observed occasional overlap between NCad (Fig. 5G',G'') and ECad (Fig. 5H',H'') puncta with Dlg5 in the peripheral nerve (arrows). Analysis of the degree of overlap found no strong correlation between the distribution of NCad, ECad, and Dlg5 in the peripheral nerve (Fig. 5I). We also did not observe any colocalization between Dlg5::GFP and other junctional complexes that form puncta such as gap junctions immunolabeled with Innexin 2 (Innx2) or focal adhesions, immunolabeled for the integrin beta-subunit Myospheroid (Mys; β PS; Fig. 5I; Supplemental Fig. 1G,H). Overall, there was a higher correlation of Dlg5 with sAJs compared with gap junctions or focal adhesions, but the Pearson's coefficient for each combination was significantly lower than the benchmark SJ proteins NrX-IV and Nrv2.1, which are known to colocalize and as expected had a high positive coefficient (Fig. 5I).

Loss of Dlg5 disrupts glial adhesion and the formation of sAJs

In some tissues, Dlg5 is involved in trafficking of cadherin–catenin adhesion complexes (Nechiporuk et al., 2007; Wang et al., 2014; Reilly et al., 2015) suggesting that Dlg5 may not colocalize but may be necessary for sAJ formation. We attempted to test the effects of Dlg5 knockdown on the formation of sAJ using immunolabeling with ECad::cherry and NCad::cherry; however, this analysis was difficult due to the distribution of cadherin puncta within all three glial layers. To better assess the effects on sAJ, we used TEM analysis of Dlg5-RNAi knockdown using the pan-glial driver (*repo>Dlg5-RNAi, Dicer2*) and assessed for the presence of sAJ (Fig. 6). In controls (*repo>Dicer2*), we observed sAJ between multiple glial layers, specifically between the PG and SPG (magenta) and between the SPG and WG (green) with an average of 1.2 sAJ and 1.9 sAJ per section, respectively (Fig. 6A,B, yellow circles). Given the strong phenotypes observed with Dlg5-RNAi in the SPG and WG, we then quantified the effect of loss of Dlg5 on the sAJ found between these two glial layers. For this analysis, as a positive control we used a previously verified ECad-RNAi line (27082; Schwabe et al., 2013; Cai et al., 2014; Sarpal et al., 2019). Knockdown of Dlg5 (*repo>Dlg5-RNAi*) and ECad (*repo>ECad-RNAi*) led to a significant reduction in the sAJ between the SPG–WG membranes (Fig. 6D–I, yellow circles; Fig. 6C). Our data suggest that knockdown of Dlg5 resulted in a reduction but not the complete loss of sAJ junctions between the glial layers.

However, our TEM analysis of both the Dlg5 and ECad-RNAi knockdowns also highlighted other changes to the SPG and WG. Qualitative assessments for both RNAi treatments revealed an increased number of gaps between the SPG and WG membranes and between the PG and SPG membranes (Fig. 6D–I, red asterisks). These gaps may correspond to the swellings or discontinuities we observed within the glial membranes in the light microscopy analyses of Dlg5-RNAi. We also observed increased layers present in the SPG membranes with ECad-RNAi that generated a fenestrated appearance (Fig. 6E,F, black dashed circles). Of interest, we found that, in both Dlg5- and ECad-RNAi, the SJs were present with electron dense septae present in all samples analyzed (Fig. 6D–I, white dashed circles) but often appeared shorter with more discontinuous sections (Fig. 6L–O, arrows) compared with control (Fig. 6J,K). Overall, our combined light microscopy and TEM data suggest that the SPG phenotypes we

observed with Dlg5-RNAi were due to the presence of increased gaps between the glial membranes possibly due to spreading and thinning of the glial membrane and the SJ domain.

E-cadherin is required in glial cells of larval peripheral nerve

To better quantify the abnormalities in glial morphology seen with ECad knock down, *repo-Gal4* was crossed to the established ECad-RNAi line (27082; Schwabe et al., 2013; Cai et al., 2014; Sarpal et al., 2019). We assessed membrane morphology with mCD8::RFP and SJ morphology using the SJ markers, Nrv2.1 or NrX-IV, which were normal in all control nerves (*repo>mCD8::RFP, Dicer2*; Fig. 7A–A'', yellow arrowheads). Pan-glial knockdown of ECad (*repo>ECad-RNAi, Dicer2*) resulted in disrupted glial membranes and the appearance of “hole-like” structures within the glial membrane (Fig. 7B', white arrows) and changes to the SJs (Fig. 7B'', yellow arrows) with a significant increase in affected nerves compared with control (Fig. 7C). We then tested for changes to the SPG and WG. ECad knockdown in the WG using Nrv2-Gal4 resulted in fewer and discontinuous WG strands (*Nrv2>ECad-RNAi, Dicer2*; Fig. 7E,E'', yellow arrowheads). Control nerves (*Nrv2>Dicer2*) had the expected multiple, continuous wrapping glial strands ensheathing the peripheral axons (Fig. 7D,D'', asterisk). When quantified, the frequency of glial defects was significantly higher with ECad-RNAi compared with the control (Fig. 7F). Therefore, ECad is required in the WG and is necessary for axon ensheathment. We next tested the function of ECad in the SPG layer using Gli-Gal4 to drive the RNAi and mCD8::RFP to label the SPG membranes. In all control nerves, the SPG membranes (*Gli>mCD8::RFP, Dicer2*) were continuous, and the SJ labeled with NrX-IV formed a normal continuous line (Fig. 7G–G'', yellow arrowheads). Knockdown of ECad (*Gli>ECad-RNAi, Dicer2*) resulted in nerves with disorganized SPG membranes (Fig. 7H''), along with disruption to the SJ and the presence of NrXIV aggregates (Fig. 7H,H'', yellow arrowheads). The presence of gaps or disruption to the SJ was quantified, and the percentage of nerves with disrupted SJ was significantly higher compared with controls (Fig. 7I). Overall, the phenotypes we observed within the SPG were similar to that of loss of Dlg5; however, on average, only 53% of nerves were affected with ECad-RNAi even with the presence of Dicer2 compared with 83% with the Dlg5-RNAi (without Dicer2).

N-cadherins play redundant roles in the larval peripheral nerve

We observed a lower penetrance of peripheral nerve defects with ECad-RNAi when expressed in the SPG compared with Dlg5-RNAi, and, given the presence of NCad in the peripheral glial layers, this suggested NCad may play a function. Thus, we next tested to see if loss of NCad also disrupted glial morphology. Surprisingly, pan-glial knockdown of NCad (*repo>NCad-RNAi, Dicer2*) using an established RNAi line (1092; Schwabe et al., 2013; Cai et al., 2014; Trush et al., 2019) did not affect glial morphology (*n* = 6 larvae, 48 nerves). Similarly, knockdown of NCad in the SPG (*SPG>NCad-RNAi, Dicer2*) did not disrupt SPG or SJ morphology (Fig. 8E). There are two NCad genes in *Drosophila*, CadN (NCad) and a duplication CadN2 (NCad2; Prakash et al., 2005). CadN2 shares 73% amino acid sequence identity and is partially redundant to NCad (Yonekura et al., 2007), and it is possible that CadN2 is compensating for the loss of NCad. We knocked down CadN2 in the SPG (*SPG>CadN2-RNAi, Dicer2*) using an established RNAi line (27508; Harrison et al., 2021), and similar to NCad knockdown, CadN2 knockdown did not

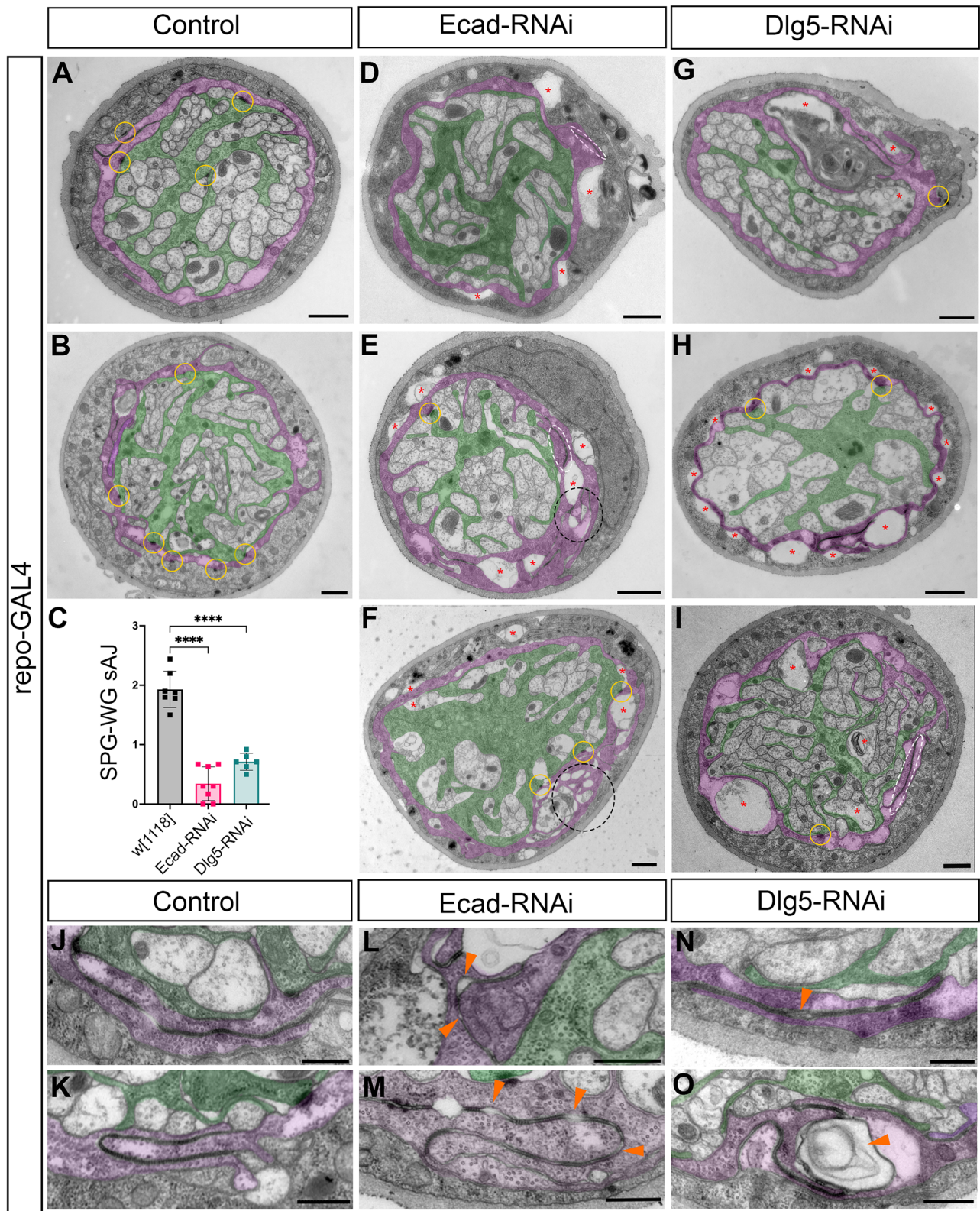


Figure 6. Knockdown of Dlg5 and Ecad leads to loss of sAJs. **A–I**, Transmission electron microscope images of repo-GAL4 crossed to *w1118* (**A, B**), *Ecad-RNAi* (**D–F**), or *Dlg5-RNAi* (**G–I**) with the SPG and WG layers false colored (SPG, magenta; WG, green). The outer PG layer and the inner axons are not colored. Yellow circles, sAJs. White dashed circles, SJs. Black dashed circles, areas of increased gaps or fenestration. Red asterisks, the unusual gaps within the SPG or between the SPG and WG. **C**, The number of sAJ observed between the SPG and WG membranes. Each point represents the mean number of sAJ observed in an individual larva across multiple nerves. The mean and standard deviation are indicated. Statistical significance was determined by one-way ANOVA with Dunnett's post hoc test compared with control ($****p < 0.0001$). **J–O**, SJs in control (**J, K**), *Ecad-RNAi* (**L, M**), and *Dlg5-RNAi* (**N–O**). Septae were clearly observed in all nerves with some SJ having clear gaps between the ladders of septae (orange arrowheads). Scale bars: **A–C**, 1 μ m; **J–O**, 0.1 μ m.

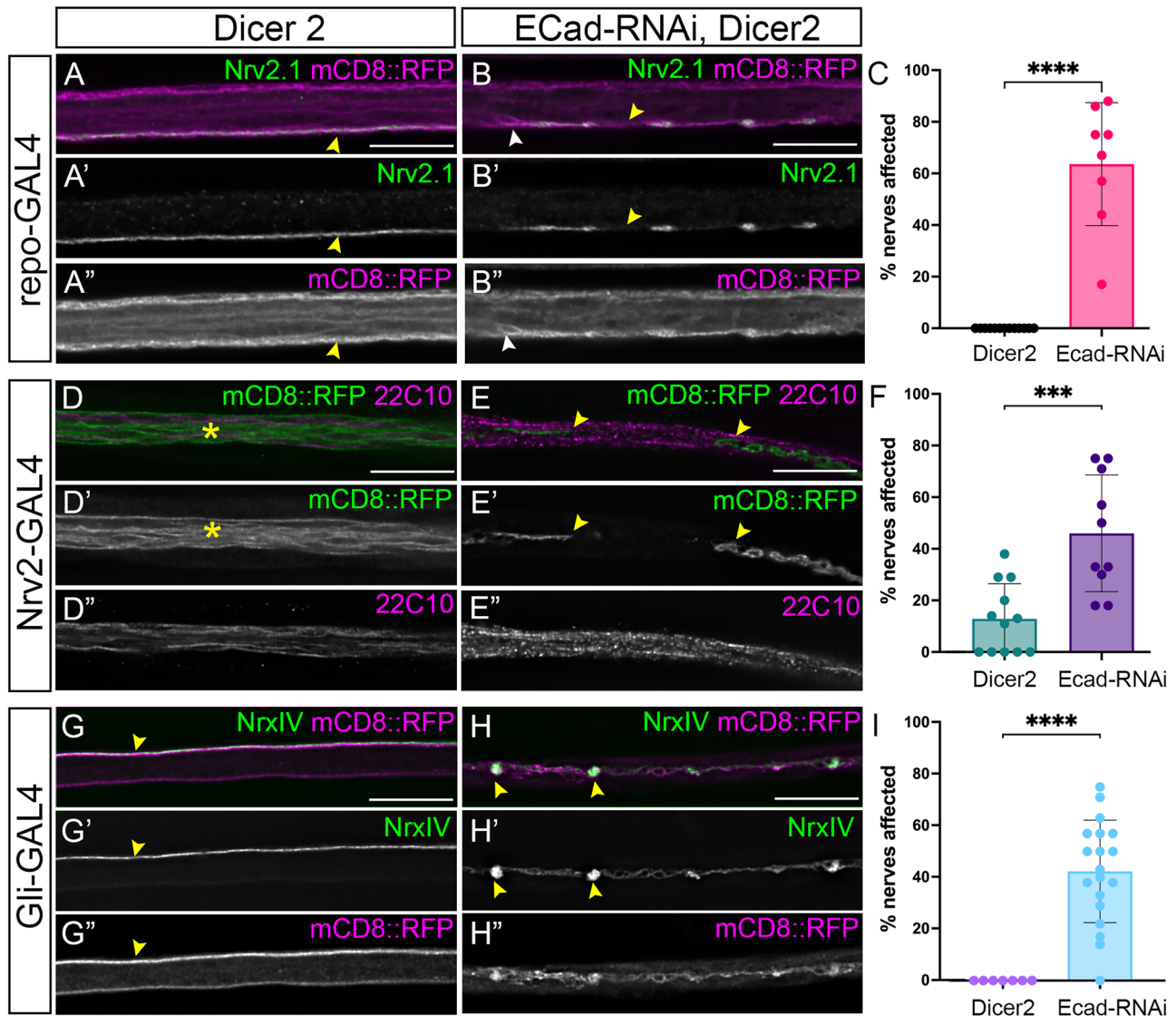


Figure 7. Knockdown of Ecad disrupts SPG and WG. Ecad-RNAi driven with a pan-glia driver (repo-Gal4, **A–C**), a WG driver (Nrv2-Gal4, **D–F**), and a SPG driver (Gli-Gal4, **G–I**). Dicer2 was expressed in all experiments. Scale bars, 15 μ m. **A–C**, Pan-glia, repo-GAL4: control (**A**) and Ecad-RNAi (**B**). Glial membranes marked with mCD8::RFP (magenta) and SJs immunolabeled with anti-Nrv2.1 (green). The SJs are intact in control (**A'**, yellow arrowhead) and had breaks with Ecad-RNAi (**B'**, yellow arrowheads). Glial membranes displayed swells or holes (**B'**, white arrowheads). **C**, Quantification of the phenotypes, two-tailed *T* test with mean and SD indicated. (*****p* < 0.0001). **D–F**, WG, Nrv2-Gal4: control (**D**) and Ecad-RNAi (**E**). Glial membranes marked with mCD8::RFP (green) and axons immunolabeled with anti-Futsch (22C10, magenta). Arrows indicate breaks in the wrapping glial processes (**E'**). **F**, Quantification of the phenotypes, two-tailed *T* test with mean and SD indicated (****p* < 0.001). **G–I**, SPG, Gli-Gal4: control (**G**) and Ecad-RNAi (**H**). Glial membranes marked with mCD8::RFP (magenta) and SJs with NrxiV::GFP (green). Arrows indicate the normal SJ (**G**) and accumulation of SJ proteins with Ecad-RNAi (**H**). **I**, Quantification of the phenotypes, two-tailed *T* test with mean and SD indicated (*****p* < 0.0001).

affect SPG or SJ morphology (Fig. 8E). To test for redundancy between these genes, we knocked down both NCad and CadN2 in the SPG (*SPG>NCad-RNAi*, *CadN2-RNAi*). Reducing the levels of both resulted in a slight but significant increase in SPG membrane and SJ defects (Fig. 8B–B'') compared with control (Fig. 8A–A'') and each RNAi alone (Fig. 8E). The SPG defects were similar to those we observed with the knockdown of Ecad suggesting that NCad and CadN2 are not only redundant with each other but also perform similar functions to Ecad. To test if Ecad and NCad are redundant, we performed a double knockdown of NCad and Ecad in the SPG (*SPG>NCad-RNAi*, *Ecad-RNAi*). Knockdown of NCad significantly enhanced the Ecad-RNAi phenotypes compared with either RNAi line alone (Fig. 8E). To further test if all three cadherins perform similar functions in the peripheral glia, we knocked down Ecad in

conjunction with a heterozygous deficiency [*Df(2L)BSC148*] that deletes both NCad and CadN2 (*CadN[Df]/+*; *SPG>Ecad-RNAi*). The reduction of both NCad genes strongly enhanced the Ecad-RNAi phenotypes (Fig. 8C–C'') with gaps or breaks within the SJ and glial membranes (yellow arrows) and aggregates of NrxiV::GFP and glial membranes (white arrowheads). The penetrance of these defects was significantly greater compared with control, Ecad-RNAi alone, or the Ecad + NCad double RNAi knockdown (Fig. 8E). In summary, these results suggest that Ecad, NCad, and CadN2 are partially redundant in the peripheral glia.

The knockdown of Ecad alone or in combination with loss of NCad specifically in the SPG phenocopied the SJ phenotypes observed with the knockdown of Dlg5, and loss of Dlg5 affected the number of sAJs within the peripheral glial layers. This led to

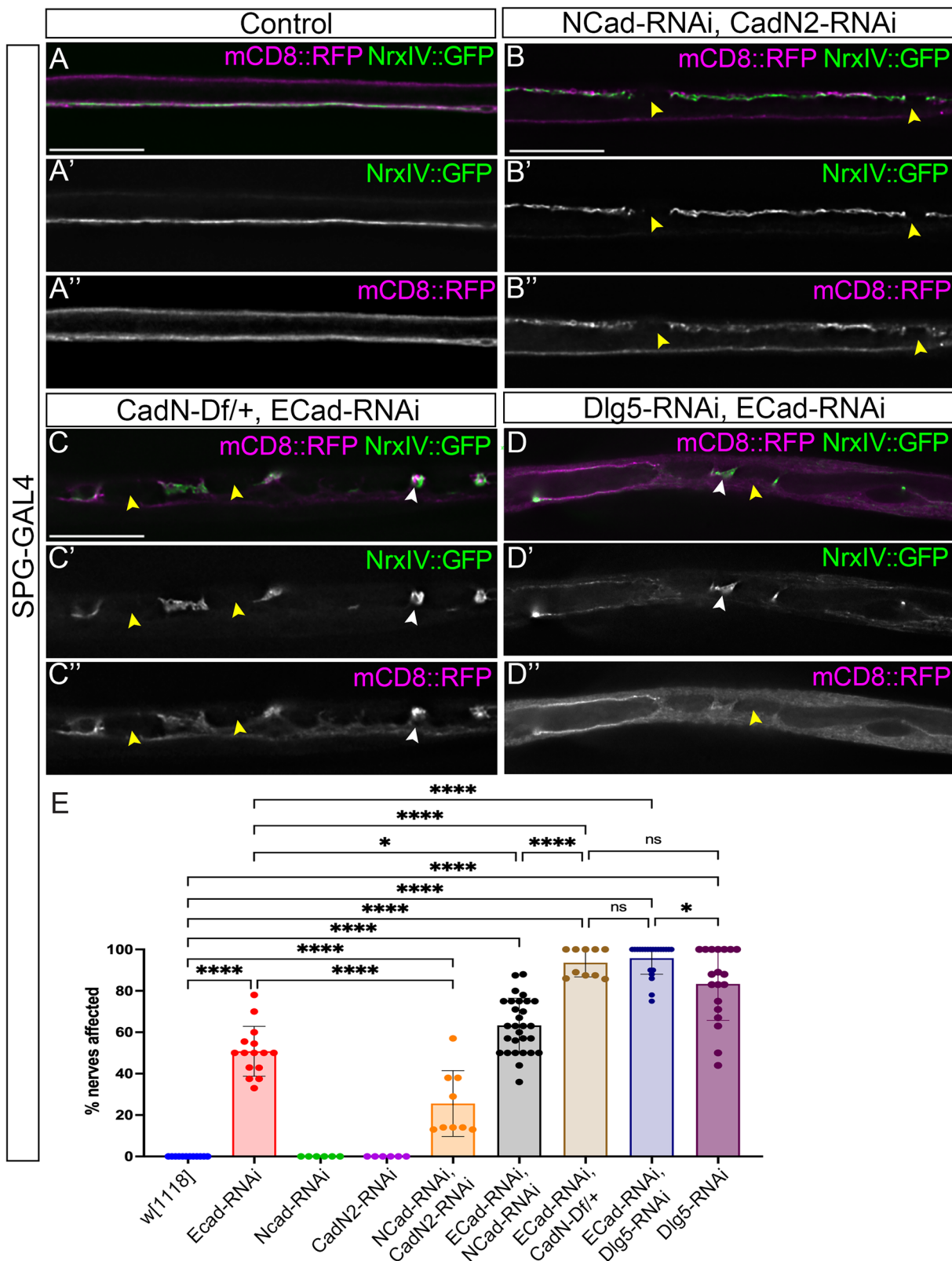


Figure 8. Ecad, NCad, and Dlg5 RNAi combinations enhance the SJ phenotypes. **A–D**, Longitudinal sections of third instar peripheral nerves with SPG-Gal4 driving mCD8::RFP (magenta) to label the glial membranes and with Nr4V::GFP (green) to mark the SJ. Scale bars, 15 μ m. **A**, Control. SPG-Gal4 crossed to *w¹¹¹⁸*. Glial membranes (**A'**) and a linear SJ (**A'**) extends along the length of the nerve in control. **B**, NCad-RNAi, CadN2-RNAi. SPG-Gal4 crossed with NCad-RNAi, CadN2-RNAi. The SJ (**B'**) and glial membranes (**B'**) contained thin areas with breaks or holes (yellow arrowheads). **C**, Ecad-RNAi, CadN-Df/+. SPG-Gal4 crossed with Ecad-RNAi and a deficiency that deletes both NCad and CadN2 (CadN-Df/+). Nr4V formed aggregates (**C'**) and those corresponded to membrane concentrations (white arrowhead). Disruptions of the SJ occurred in the same places as loss of the glial membrane (yellow arrowheads). **D**, Dlg5-RNAi, Ecad-RNAi. SPG-Gal4 crossed with Dlg5-RNAi and Ecad-RNAi. Nr4V formed aggregates (**D'**) and those corresponded to membrane concentrations (white arrowhead). SJ were reduced and the glial membranes were reduced or lost (yellow arrowheads). **E**, Quantification of the RNAi effects on the SJ. The percentage of nerves affected per larva was determined for each genotype. Statistical significance was determined by one-way ANOVA with Tukey's post hoc multiple-comparison test for each RNAi compared across multiple genotypes. *p* values compared with control *w¹¹¹⁸* are not significant (ns) for NCad-RNAi or CadN2-RNAi. (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

the hypothesis that these proteins might function in the same or parallel pathways. To test this hypothesis, we combined the Dlg5 and ECad RNAi lines. We observed a strong enhancement of the ECad-RNAi phenotypes by Dlg5-RNAi (Fig. 8D), where the percentage of nerves affected with Dlg5 + ECad-RNAi were significantly greater than ECad-RNAi alone and on par with the severity and penetrance observed with ECad-RNAi plus the heterozygous NCad + Cadn2 deficiency (Fig. 8E). The combination of Dlg5 + ECad-RNAi was slightly but significantly stronger than Dlg5-RNAi alone (Fig. 8E). These results suggest that Dlg5 and cadherin function synergistically in the SPG and may function together to regulate SPG morphology and SJ integrity.

Loss of Dlg5 and ECad disrupts the BBB and larval locomotion

Glial SJs in the SPG form the BBB in the *Drosophila* peripheral nerves and around the brain lobes and ventral nerve cord in the CNS. Given the effects of loss of both Dlg5 and the cadherins on SJ morphology, we next tested for changes to the BBB around the CNS using a dextran dye assay (Paluri and Auld, 2025; Fig. 9). As expected, over the time course of 40 min, dye was blocked

from entry into the brain lobes and ventral nerve cord of the CNS in controls (SPG-Gal4; Fig. 9A,F). Knockdown of NrX-IV as a positive control (SPG>NrX-IV-RNAi) disrupted the SJ and led to significant dye penetration (Fig. 9B,F). Dye penetration with NrX-IV knockdown was quantified at a lower laser intensity than the other genotypes and is graphed separately. Knockdown of ECad but not Dlg5 lead to a mild but statistically significant increase in the penetration of dye into the CNS compared with the control (Fig. 9C,D,F). The double knockdown of Dlg5 + ECad (SPG>Dlg5-RNAi, ECad-RNAi) had significantly more dye penetration compared with either RNAi alone and to the control (Fig. 9E,F). Thus, the strength of the disruption of the BBB was correlated with the degree of disruption of the glial membranes and SJs, where loss of Dlg5 or ECad alone had much weaker effects compared with when both were knocked down. These results suggest that Dlg5 and cadherin are components of a common pathway that functions to regulate SPG morphology and SJ integrity.

We wanted to determine if changes to the glial layers, the SJs, and the disruption of the BBB affected nervous system function. To this end, we measured various parameters of larval locomotor

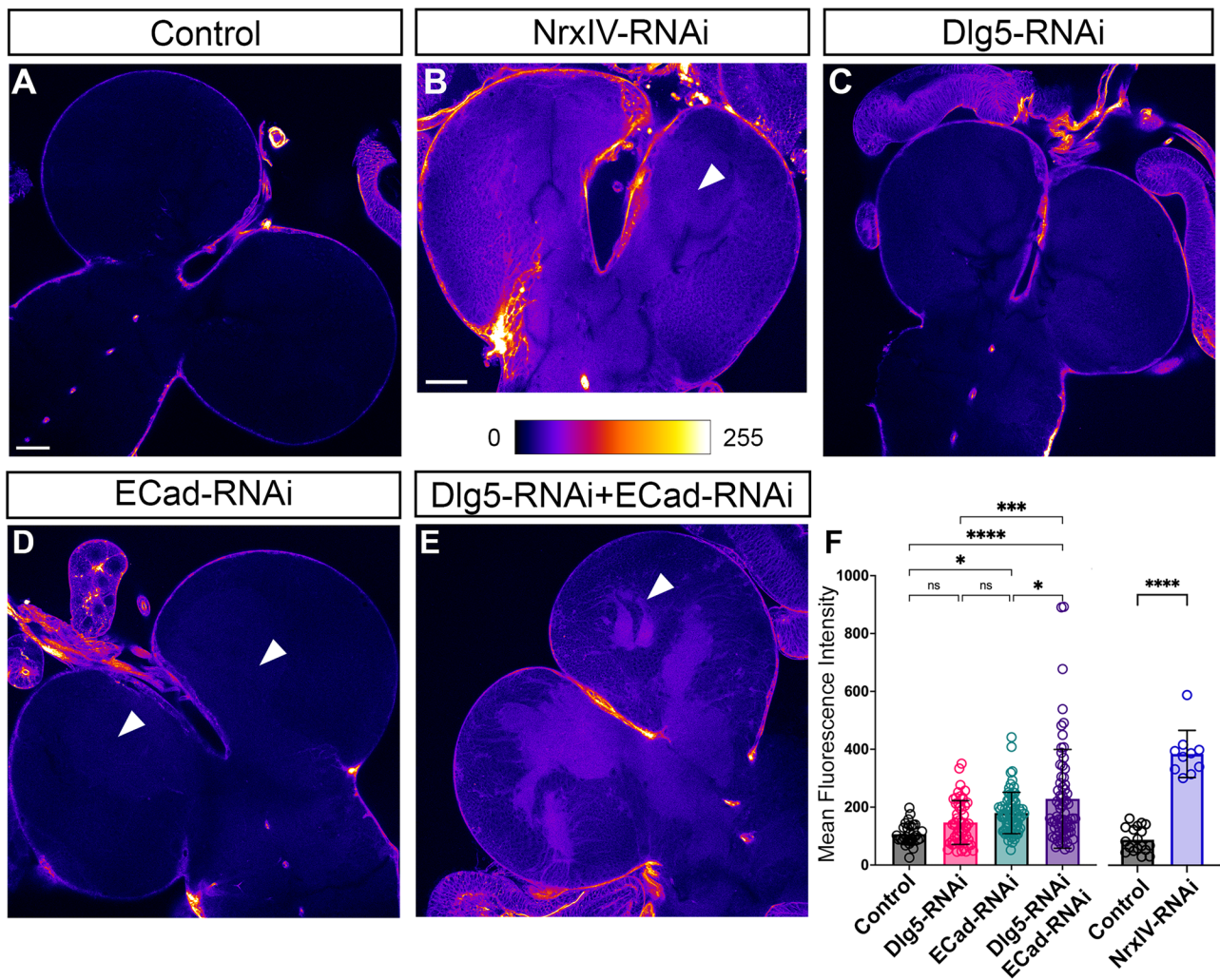


Figure 9. Knockdown of Dlg5 and ECad together impairs BBB integrity. **A–D**, Fire LUT showing blood–brain permeability assay using SPG>mCD8::GFP crossed to w[1118] (control; **A**), NrX-IV-RNAi (**B**), Dlg5-RNAi (**C**), ECad-RNAi (**D**), or Dlg5-RNAi+ECad-RNAi (**E**). White arrowheads highlight regions of significant dye labeling of the neuropile. Scale bars, 150 μm. **F**, Quantification of mean fluorescent intensity inside the brain lobe. The mean and standard deviation are indicated. Dye penetration for the NrX-IV-RNAi experiment and the corresponding control was determined at a lower laser intensity than the other genotypes and these were graphed separately. Statistical significance was determined by one-way ANOVA with Tukey's post hoc multiple-comparison test for each group (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

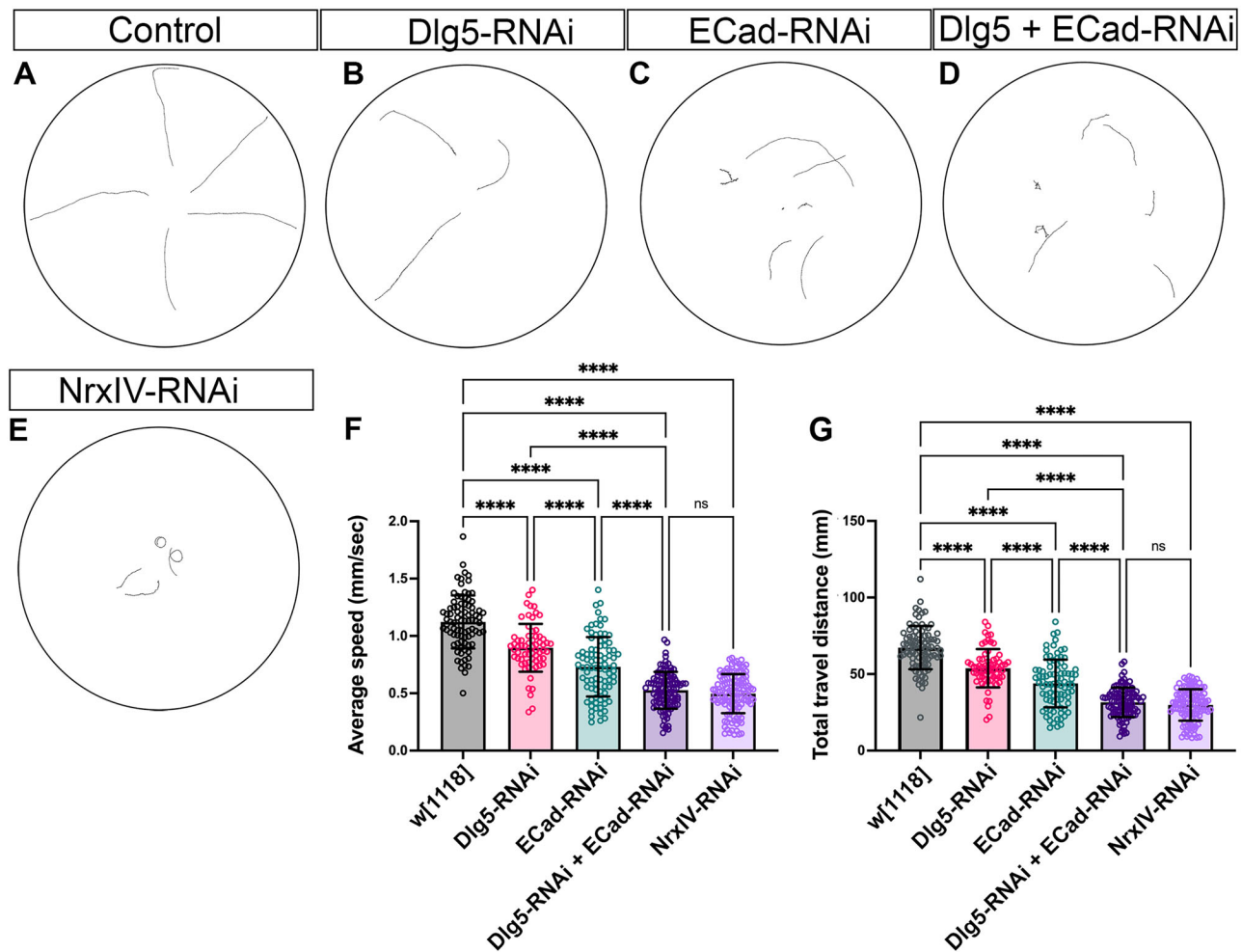


Figure 10. Loss of Dlg5 and Ecad in the SPG impairs animal locomotion. **A–E**, Movement trajectories of third instar larvae from SPG-Gal4 flies crossed with *w[1118]* (**A**), *Dlg5-RNAi* (**B**), *ECad-RNAi* (**C**), *Dlg5-RNAi*, *ECad-RNAi* (**D**), and *NrxIV-RNAi* (**E**). **F–G**, Comparison of average speed (**F**) and total travel distance (**G**) of SPG-Gal4 driving *Dlg5-RNAi*, *ECad-RNAi*, *Dlg5 + ECad-RNAi*, *NrxIV-RNAi*, and control, *w[1118]*. The mean and standard deviation are indicated. Statistical significance was determined by one-way ANOVA with Tukey's post hoc multiple-comparison test for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

behavior (Fig. 10), including average speed and total distance traveled. *Nrx-IV* knockdown (*SPG>Nrx-IV-RNAi*) was a positive control, and as expected larval motion was significantly compromised (Fig. 10E–G). When compared with control larvae (*w[1118]*), both locomotion parameters were mildly but significantly reduced after *ECad* knockdown (*SPG>ECad-RNAi*; Fig. 10C,F,G) and *Dlg5* knockdown (*SPG>Dlg5-RNAi*; Fig. 10B,F,G). The combined knockdown of *Dlg5* and *ECad* (*SPG>ECad-RNAi*, *Dlg5-RNAi*) was much stronger and significantly reduced locomotion (Fig. 10D,F,G) compared with *ECad-RNAi* or *Dlg5-RNAi* alone and matched the disruption in locomotion observed with the positive control, *Nrx-IV-RNAi*. Our results suggest that the disruption of the SPG and changes to SJs lead to changes in the integrity of the BBB with physiological effects leading to changes in animal locomotion, and these effects are much stronger when *Dlg5* and *ECad* are reduced simultaneously.

Dlg5 defects are rescued by increased Yorkie activity

Overall, our results suggested that *Dlg5* and cadherin function synergistically in the SPG. However, *Dlg5* and cadherins did not consistently colocalize within the peripheral glia, suggesting that they may function independently within the same pathway.

A signaling cascade that involves both *Dlg5* and *sAJ* is the Hippo pathway (Fig. 11F). The Hippo pathway regulates the transcriptional regulator Yorkie, specifically by inactivating Yorkie through phosphorylation and degradation (Comoletti et al., 2003). *Dlg5* regulates Hippo signaling in multiple *Drosophila* tissues, where *Dlg5* binds to Hippo (MST1/2) along with Par-1 to sequester and inhibit Hippo function, and where loss of *Dlg5* leads to activation of Hippo signaling and the repression of Yorkie (Fig. 11F; Kwan et al., 2016; Venugopal et al., 2020; Kaya-Copur et al., 2021). Similarly, basal *sAJ*s in the *Drosophila* imaginal wing disc regulate Hippo signaling through sequestration of Warts (Wts; LATS1/2; Kroeger et al., 2024), a key kinase in the Hippo pathway. Loss of *ECad* results in the release of Wts, activation of Hippo signaling, and repression of Yorkie (Fig. 11F; Kroeger et al., 2024). In *Drosophila*, Yorkie is necessary for normal glial cell numbers and expansion (Reddy and Irvine, 2011) and for the extensive growth of SPGs (Li et al., 2017). Loss of Yorkie activity in the SPG causes abnormal endoreplication, defective SJs, and BBB disruption (Li et al., 2017). Thus, we hypothesized that *Dlg5* and cadherins might regulate components of the Hippo pathway but likely through independent processes. To test the link between *Dlg5*, *ECad*, and Yorkie, we utilized a Yorkie transgene marked with GFP (*Yki*:

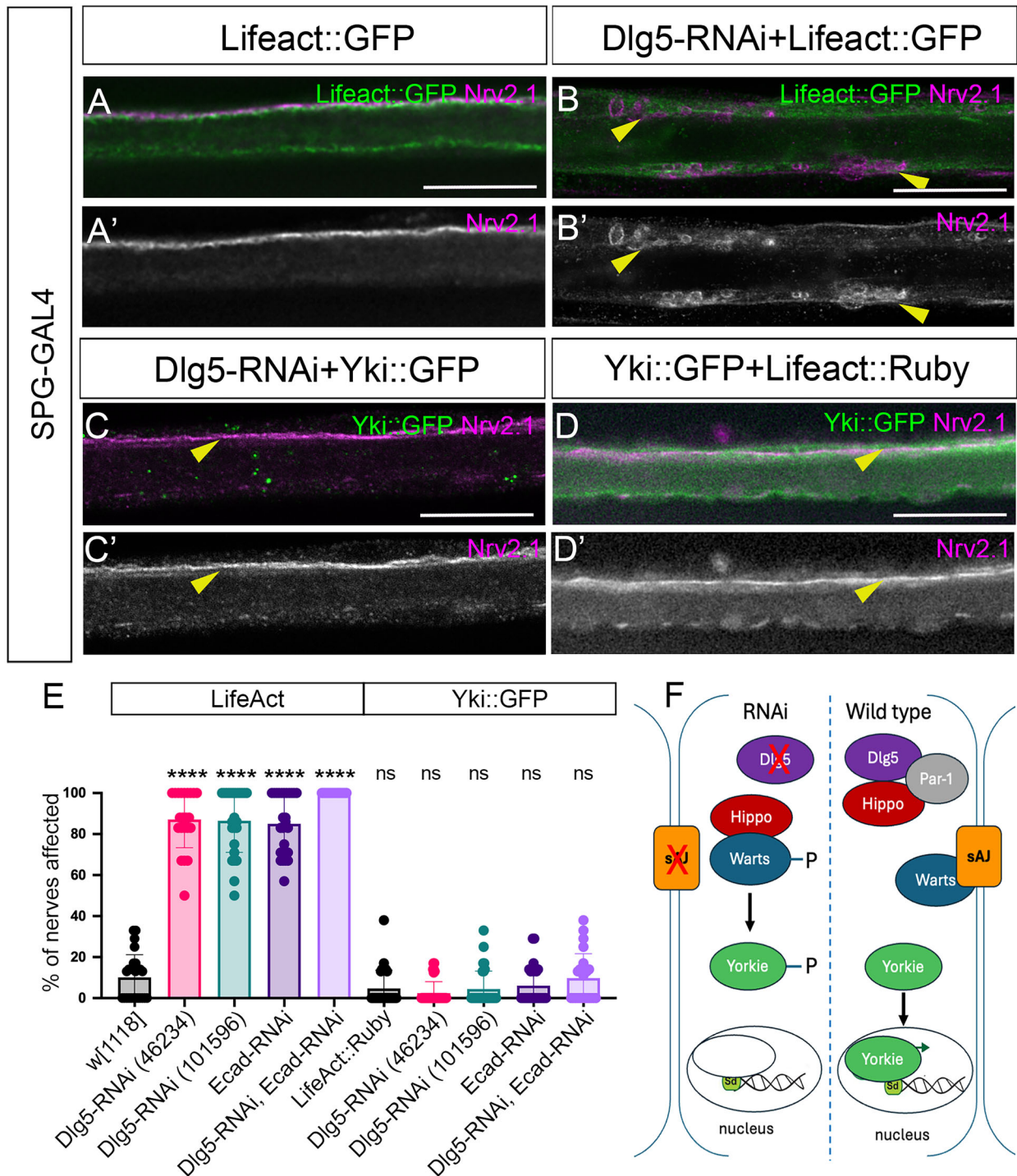


Figure 11. Yorkie rescues Dlg5-RNAi and Ecad-RNAi SJ defects. Longitudinal sections of third instar peripheral nerves with SJs immunolabeled for Nrv2.1 (magenta) to mark the SJ. Scale bars, 15 μ m. **A**, Control. SPG-Gal4 crossed with UAS-LifeAct::GFP. Glial processes (green) and a linear SJ (**A'**) extends along the length of the nerve in control. **B**, Dlg5-RNAi+LifeAct::GFP. SPG-Gal4 crossed with Dlg5-RNAi and LifeAct::GFP (green) as a control for the number of UAS transgenes. The SJ (**B'**, magenta) are disrupted and are no longer linear (yellow arrowheads). **C**, Dlg5-RNAi+Yki::GFP. SPG-Gal4 crossed with Dlg5-RNAi and wild-type Yorkie tagged with GFP (green). The SJ (magenta) are linear and extend uniformly along the nerve (**C'**, yellow arrowheads). **D**, Yki::GFP+LifeAct::Ruby. SPG-Gal4 crossed with wild-type Yorkie tagged with GFP (green) and LifeAct::Ruby. The expression of Yki does not disrupt the SJ (magenta), which are linear and extend uniformly along the nerve (**D'**, yellow arrowheads). **E**, Quantification of the Yorkie rescue of the Dlg5-RNAi and Ecad-RNAi defects. LifeAct::Ruby was used to maintain the same number of UAS lines across all experiments. The percentage of nerves affected per larva was determined for each genotype. The mean and standard deviation are indicated. Statistical significance was determined by one-way ANOVA with Dunnett's post hoc multiple-comparison test (**** $p < 0.0001$). There were no significant differences between the control, *w¹¹¹⁸*, and the lines expressing Yki::GFP. **F**, The model of Dlg5 and cadherin control of the Hippo to Yorkie signaling pathway. Dlg5 and sAJ containing cadherins control different aspects of this pathway and regulate the kinases Hippo and Warts, respectively. Both work upstream of Yorkie to inhibit its phosphorylation and subsequent degradation. When Dlg5 and cadherins are present (wild type), Yorkie enters the nucleus and turns on gene transcription. In the absence of Dlg5 and cadherins (RNAi), Hippo activity is present, and Yorkie is degraded leading to a lack of gene transcription.

GFP; Oh and Irvine, 2008) to determine if expression of Yorkie could rescue the Dlg5-RNAi and ECad-RNAi phenotypes. SJs were marked with immunolabeling for Nrv2.1. Controls were the SPG driver with Dlg5-RNAi coexpressing LifeAct::GFP or LifeAct::Ruby (*SPG>Dlg5-RNAi, LifeAct*) to balance the number of UAS-lines. Expression of wild-type Yorkie (Fig. 11C) was able to completely rescue the Dlg5-RNAi phenotypes (*SPG>Dlg5-RNAi, YkiWT::GFP*; Fig. 11C,E) and the ECad-RNAi phenotypes (*SPG>ECad-RNAi, YkiWT::GFP*; Fig. 11E). Yorkie expression was also sufficient to rescue the combined knockdown of both Dlg5 and ECad (*SPG>Dlg5-RNAi, ECad-RNAi, YkiWT::GFP*). Expression of wild-type Yorkie itself did not lead to deleterious effects (*SPG>YkiWT::GFP, Lifeact::Ruby*; Fig. 11D,E). Our results suggest that the effects of Dlg5 and ECad knockdown are caused by hyperactivation of the Hippo pathway leading to reduced Yorkie activity and disruption of the SPG, suggesting that Dlg5 and ECad synergistically interact in the SPG to regulate Hippo signaling (Fig. 11F).

Discussion

Our aim was to discover which PDZ domain scaffolding proteins play a role in glial ensheathment of peripheral axons. We identified that the MAGUK protein Discs-large 5 (Dlg5) has a role in SPG and SJ morphology. To investigate the role of Dlg5 further, we focused on cadherins and found that loss of N-cadherin and E-cadherin phenocopied the loss of Dlg5, leading to disruption of the SPG membranes and abnormal SJs. This leads to a model where Dlg5 plays a role in conjunction with cadherins in the SPG.

Dlg5 performs multiple independent functions in both mammals and *Drosophila* including regulation of cell proliferation, signaling complexes, polarity, growth, and trafficking of junctional complexes, and these functions are highly context specific (Kwan et al., 2016; Liu et al., 2017; Venugopal et al., 2020). In *Drosophila*, Dlg5 plays a role in establishing apical-basal polarity in follicular epithelia, where Dlg5 localizes at the apical membrane and adherens junctions in early egg chambers, with loss of Dlg5 generating epithelia polarity defects (Luo et al., 2016). In follicular epithelia, Dlg5 promotes the apical membrane localization of Crumbs to establish polarity. However, Dlg5 does not appear to play a similar role in glia as peripheral glia do not express Crumbs and knockdown of Crumbs had no effect. We also failed to detect Par-3 or Par-6 in the peripheral glia, and RNAi knockdown of these apical polarity proteins had no effect. We did find that Sdt, a component of the Crumbs complex, is required in the peripheral glia, which raises the question as to whether Sdt interacts with noncanonical partners to perform similar functions as seen in epithelial cells or whether Sdt might have an entirely different and novel function in glial cells.

Another potential role of Dlg5 could be through the interface between cadherin function and the glial cytoskeleton or in the assembly of sAJs. Dlg5 links the Vinculin-Vinculin complex and beta-catenin at sites of cell-cell contact in mammalian fibroblasts and epithelial cells (Wakabayashi et al., 2003). Loss of Dlg5 in the SPG resulted in reduction in the number of sAJs and enhanced the SPG defects generated by loss of cadherins. However, the degree of overlap between Dlg5 and cadherins in the third instar larvae was not extensive, suggesting an indirect role of Dlg5 rather than the maintenance and ongoing stability of these junctions. It is possible that Dlg5 function with regard to junctional complexes is highly context specific. In the *Drosophila* ovary, Dlg5 associates with basolateral SJ proteins

(including Dlg1, Neuroglian, ATPa, and Lachesin) and to a smaller extent the AJ in border cells (Luo et al., 2019). Dlg5 localizes with Dlg1 in stage 10 follicular epithelia (Luo et al., 2016). The loss of Dlg5 disrupts the distribution of Armadillo (beta-catenin) in border cells (Luo et al., 2019) and E-cadherin in follicular cells (Luo et al., 2016) yet has no effect on the distribution of Dlg1 or the SJ protein Neuroglian (Luo et al., 2019). In wing discs, Dlg5 mutants have a reduction of apical domain determinants but not a loss of cell polarity and a very specific defect in the recruitment of N-cadherin to adherens junctions but not E-cadherin (Venugopal et al., 2020); however, the effect on the basal sAJs was not tested. Our results show that in the SPG, Dlg5 was not found associated with any junctional complexes except occasionally with puncta of NCad and ECad. As well, loss of Dlg5 resulted in the disruption of both sAJ and SJ morphology suggesting a global defect rather than a specific disruption of a single junctional domain.

A third potential function for Dlg5 is in controlling cell proliferation (Nakamura et al., 1998; Liu et al., 2017) where Dlg5 knockdown cells grow faster. Dlg5 function appears to be highly cell specific as Dlg5 can function either as a tumor suppressor or tumor enhancer in different cancer cell lines (Liu et al., 2014; Andolfi et al., 2022). As SPG are polyploid cells and do not undergo mitosis, we do not think the role of Dlg5 is to control SPG proliferation.

However, the SPG are very large cells, and polyploidy (endoreplication) is necessary for glial growth to match the expanding CNS and required for a functional BBB (Unhavaithaya and Orr-Weaver, 2012). Thus, another point of potential Dlg5 function is through the regulation of glial growth acting through the Hippo pathway. Hippo is a key regulator of organ size and tissue homeostasis in many tissues (Limmer et al., 2014; Yu et al., 2015; Li and Guan, 2022), and the downstream target, Yorkie, is an important component of the SPG size increase during larvae growth (Liu et al., 2017). Yorkie is necessary for normal glial cell numbers and their expansion (Reddy and Irvine, 2011), and loss of Yorkie activity in the SPG causes abnormal endoreplication, defective SJs, and BBB disruption (Li et al., 2017). Dlg5 negatively regulates Hippo signaling where loss of Dlg5 leads to activation of Hippo signaling and the repression of Yorkie (Kwan et al., 2016; Venugopal et al., 2020; Kaya-Copur et al., 2021). Dlg5 binds Hippo (mammalian MST1/2) and recruits MARK3 (microtubule affinity-regulating kinases 3; *Drosophila* Par-1) leading to hyperphosphorylation of Hippo (MST1/2) and inhibition of kinase activity. Hippo normally phosphorylates and activates the downstream kinase Wrtts (LATs1/2, large tumor suppressor homologs 1/2). Dlg5 also inhibits the association between MST1/2 and LATs1/2 (Song et al., 2023). Overall, Hippo signaling is very active in both *dlg5*^{-/-} mice and *Drosophila* leading to increased degradation of Yorkie/Yap.

Similarly, ECad acting at basal sAJs also regulates Yorkie activity by sequestration of Warts, a key kinase in the Hippo pathway, where loss of ECad results in the release of Warts and activation of Hippo signaling (Kroeger et al., 2024).

Overall, our results found that loss of Dlg5 and cadherins leads to disruption of the SPG, SJs, and the BBB suggesting that loss of Dlg5 and cadherins leads to increased Hippo signaling and a decrease in Yorkie activity. We were able to confirm our hypothesis that Dlg5 and ECad functions converge into the Hippo signaling pathway by rescuing of the RNAi phenotypes with the coexpression of Yorkie. This leads to a model (Fig. 11F), where both Dlg5 and ECad regulate SPG and SJ development through the Hippo pathway but regulate different

aspects of the pathway with the same downstream effect, regulation of Yorkie levels.

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