# Canoe functions at the CNS midline glia in a complex with Shotgun and Wrapper-Nrx-IV during neuron-glia interactions

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## SUMMARY

Vertebrates and insects alike use glial cells as intermediate targets to guide growing axons. Similar to vertebrate oligodendrocytes, *Drosophila* midline glia ensheath and separate axonal commissures. Neuron-glia interactions are crucial during these events, although the proteins involved remain largely unknown. Here, we show that Canoe (Cno), the *Drosophila* ortholog of AF-6, and the DE-cadherin Shotgun (Shg) are highly restricted to the interface between midline glia and commissural axons. *cno* mutant analysis, genetic interactions and co-immunoprecipitation assays unveil Cno function as a novel regulator of neuron-glia interactions, forming a complex with Shg, Wrapper and Neurexin IV, the homolog of vertebrate Caspr/paranodin. Our results also support additional functions of Cno, independent of adherens junctions, as a regulator of adhesion and signaling events in non-epithelial tissues.

KEY WORDS: Neuron-glia interactions, Canoe/AF-6, *Drosophila*, CNS, Wrapper, DE-cadherin, Nrx-IV, Midline

## INTRODUCTION

Intricate and reciprocal neuron-glia interactions are essential for proper nervous system development across species (Crews, 2010; Klambt, 2009; Lemke, 2001). Early on during neuronal development, glial cells act as guidepost cells, i.e. intermediate targets for growing pioneer axons on the way to their final destination (Bastiani and Goodman, 1986; Bentley and Caudy, 1983; Learte and Hidalgo, 2007). Neurons, in turn, are essential for glia migration, proliferation and survival (Birchmeier and Nave, 2008; Brinkmann et al., 2008; Klambt, 2009). Whereas vertebrate glial cells, oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS), generate myelin sheaths around nerves to isolate and protect axonal tracts, many invertebrates, such as *Drosophila melanogaster*, do not produce myelin. However, *Drosophila* CNS midline glial cells ensheath and separate axonal commissures in a process in which neuron-glia interactions are crucial. Hence, *Drosophila* midline glia constitute an amenable model system for untangling the complex and still largely unknown molecular mechanisms that underlie neuron-glia interactions. This, in turn, is essential for our in-depth understanding of the myelination process in development and disease (Bhat, 2003; Crews, 2010; Edenfeld et al., 2005; Jacobs, 2000; Sherman and Brophy, 2005).

*Drosophila* CNS midline cells are extremely well characterized in terms of both their location and molecular markers. In a mature CNS, ~22 midline cells are present per segment, three of which constitute the midline glia (MG) (Beckervordersandforth et al., 2008; Bossing and Technau, 1994; Jacobs, 2000; Klambt et al., 1991; Wheeler et al., 2006). At early stages, two types of MG have been molecularly characterized:

anterior (six cells) and posterior (four cells) (Kearney et al., 2004). At later stages, all posterior MG (PMG) die and only three of the six anterior MG (AMG) survive (Wheeler et al., 2009); these are the three AMG present per segment in a mature CNS (stage 17), as mentioned above. One of the main functions of the MG is to provide the guidance cues required for proper axon pathfinding at the midline (Harris et al., 1996; Kidd et al., 1999; Mitchell et al., 1996). Another crucial function of the *Drosophila* MG is to separate and ensheath the anterior and posterior commissures present per segment. Neuron-glia intercellular communication is essential during these processes, although the molecules involved remain largely uncharacterized (Edenfeld et al., 2005; Jacobs, 2000). Recently, the transmembrane protein Neurexin IV (Nrx-IV), which is the *Drosophila* ortholog of vertebrate Caspr/paranodin and is present in axonal membranes, has been shown to interact physically with the Immunoglobulin (Ig) superfamily member Wrapper, a glycophosphatidylinositol (GPI)-linked protein expressed on the surface of the MG. Moreover, this interaction is fundamental for neuron-glia adhesion and, consequently, for the correct ensheathment of commissural axon fascicles (Banerjee et al., 2006; Baumgartner et al., 1996; Noordermeer et al., 1998; Stork et al., 2009; Wheeler et al., 2009).

Here, we show that the PDZ (PSD-95, Discs large, ZO-1) domain-containing protein Canoe (Cno) (Miyamoto et al., 1995) and the DE-cadherin Shotgun (Shg) (Tepass et al., 1996) are expressed in the MG, where they are highly concentrated at the interface between MG and commissural axons. $cno^2$ loss-of-function mutant embryos showed clear defects in MG migration as well as in commissural axon ensheathment and subdivision. Indeed, Cno colocalized with Nrx-IV and Wrapper at the axon-glia interface and interacted genetically with them during this process. *shg* mutant embryos also displayed strong defects in MG enwrapping of commissural axons and a failure in Cno subcellular localization. Moreover, Wrapper formed a complex in vivo with both Shg and Cno. We propose that Cno and Shg in the MG link with the Wrapper-Nrx-IV complex and act as novel functional players that are crucial for regulating neuron-glia interactions at the midline.

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## MATERIALS AND METHODS

### *Drosophila* strains and genetics

The following mutant stocks were used (all from the Bloomington Stock Center unless stated otherwise): $cno^2$, $cno^{mis10}$ (Miyamoto et al., 1995), *Df(3R)ED5147* (*Drosophila* Genetic Resource Center, Kyoto, Japan), $wrapper^{175}$, $shg^2$, $Nrx-IV^{4304}$, *Nrx-IV-GFP* (CA06597) (Morin et al., 2001), *sim-Gal4*, *slit-Gal4* (Scholz et al., 1997), *elav-Gal4*, *maternal-Gal4* [*V32* (Speicher et al., 2008)], *eagle-Gal4*, *Df(3L) Exel 6116* and *UAS-Nrx-IV-Exon4* (Stork et al., 2009) and *UAS-mCD8::GFP*, *UAS-cno* (Carmena et al., 2006). The crosses GAL4×UAS were carried out at 25°C and 29°C. *yw* was used as the reference control wild-type strain. Balancer chromosomes containing different *lacZ* or *GFP* transgenes were used for identification of homozygous mutant embryos.

### Immunohistochemistry, immunofluorescence and microscopy

Embryo fixation and antibody staining were carried out by standard protocols except as specified below. The following primary antibodies were used: rabbit anti-Cno 1/400 (Speicher et al., 2008); mouse BP102 1/100 [Developmental Studies Hybridoma Bank (DSHB)]; C555.6 mouse anti-Slit 1/100 (DSHB); 10D3 mouse anti-Wrapper 1/50-1/200 (DSHB); rat anti-DE-cadherin 1/20 (DSHB); rat anti-Elav 1/400 (DSHB); rabbit anti-HRP 1/5000 (Jackson); goat anti-HRP 1/100-1/500 (Jackson); rabbit anti-β-galactosidase 1/100,000 (Cappel); and mouse anti-β-galactosidase 1/8000 (Promega). Secondary antibodies coupled to biotin (Vector Labs), Alexa Fluor 488, 546 or 633 (Molecular Probes) were used. For immunostaining with the anti-Cno antibody, embryos were fixed using the heat-methanol method (Tepass, 1996). Fluorescent images were recorded using a Leica upright DM-SL microscope and assembled using Adobe Photoshop. All micrographs shown in figures represent single sections from confocal z-stacks (1 μm between each optical plane).

### Co-immunoprecipitations (Co-IPs)

For in vivo Co-IPs, lysates were prepared from ~500 μl of 16- to 18-hour *yw*, *V32-Gal4>>UAS-cno::GFP* or *Nrx-IV::GFP* embryos. Embryos were homogenized in lysis buffer [50 mM Tris pH 8, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% Triton X-100, 1 mM NaF, 100 μM Na$_3$VO$_4$, 50 μg/ml PMSF and Complete Protease Inhibitors (Roche)]. Extracts were centrifuged for 2 minutes at 14,000 rpm (18,700 *g*) at 4°C and passed through a filter disc (Whatman, 25 mm diameter). The extract was then pre-cleared with Protein A or Protein G beads for 2 hours at 4°C followed by incubation with the appropriate primary antibodies overnight at 4°C. The supernatant-antibody mix was incubated with 40-60 μl pre-washed Protein A/G beads for 2 hours at 4°C. The beads were then washed three times with lysis buffer without inhibitors and then heated at 95°C for 5 minutes. Precipitates were resolved by SDS-PAGE and immunoblotted with rabbit anti-GFP (Abcam), rabbit anti-Cno (affinity purified), mouse anti-Wrapper or rat anti-DE-cadherin. Each experiment was repeated at least two or three times.

## RESULTS

### Cno is localized at the neuron-glia interface in the midline

We had observed that Cno is expressed in the midline during the differentiation of the CNS (our unpublished observations). To investigate a potential function of Cno in this process, we analyzed its midline expression in detail. Cno was first detected at stage 12, at the sites of contact between the MG and the ectoderm and at some locations in the AMG (Fig. 1A-A‴). Later, during stage 12 [12/3; stage according to Klämbt et al. (Klämbt et al., 1991); see also Wheeler et al. (Wheeler et al., 2009)], Cno was mainly detected in the PMG (Fig. 1B-B‴). This expression persisted as far as stage 14. At this point, the AMG have already enwrapped the anterior commissure and Cno was present at the interface between the AMG and the anterior commissural axons (Fig. 1C-C‴). By stage 15 Cno was highly concentrated at the interface between the MG and the two commissures (Fig. 1D-D‴). At late stages (stage

17) only AMG are present and the three AMG extend processes that further subdivide the commissures into distinct axon bundles. Cno was highly restricted to these processes at this stage, colocalizing with the glial marker Slit, which itself is more widely distributed in the MG (Fig. 1E,E‴″). In a ventral view at stage 17, Cno was detected in the MG, along with Slit, in close contact with anterior and posterior commissural axons (Fig. 1F-F‴″). Therefore, Cno is present in the MG throughout the process of MG migration, commissural axon enwrapping and subdivision.

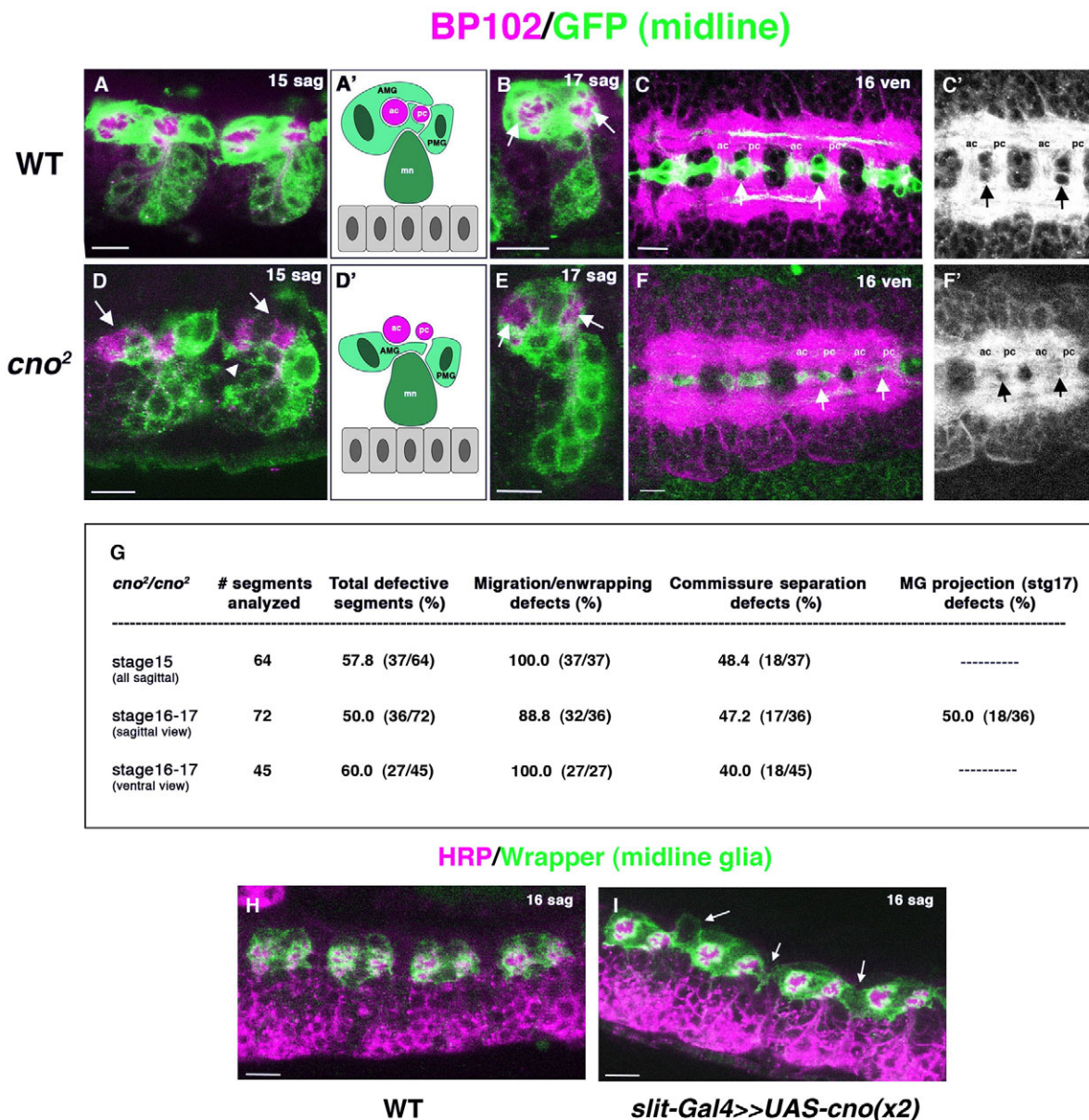### Cno is necessary for MG migration and for commissural axon ensheathment and subdivision

Given the characteristic expression of Cno in the MG from stage 12, we analyzed a potential requirement of Cno during MG migration and commissural axon enwrapping. In $cno^2$ null mutant embryos, striking defects in these processes were observed in 55.2% of the segments analyzed throughout stages 15-17 (*n*=181) (Fig. 2A-G). Indeed, anterior and posterior commissures were hardly separated from each other (Fig. 2C,C′,F-G) or they displayed an abnormal, wider morphology (not shown). Likewise, the projections that the AMG normally send into the commissures at stage 17 to enwrap individual axons were not formed in 50% of $cno^2$ mutant defective segments analyzed at this stage (*n*=72) (Fig. 2B,E,G). All these phenotypes were also observed with a similar expressivity and penetrance in $cno^2$ over the *Df(3R)ED5147*, a deficiency that eliminates *cno*, and over $cno^{mis10}$, another *cno* null allele (Miyamoto et al., 1995) (data not shown). *cno* gain-of-function at the MG (*slit-Gal4>>UAS-cno*) also caused defects (10.3%, *n*=87 segments at 25°C; 11.1%, *n*=81 at 29°C), as revealed by Wrapper expression as a MG marker. Specifically, the MG of adjacent segments were very frequently in contact as if cell adhesion were enhanced. The same phenotype was observed at slightly higher penetrance when two copies of *cno* were used (17.5%, *n*=126 segments at 25°C) (Fig. 2H,I). The overexpression of Cno using the neural-specific driver *elav-Gal4* did not cause any apparent phenotype in the MG (1.4%, *n*=70 segments at 25°C). Intriguingly, the $cno^2/cno^2$ mutant phenotype was partially rescued when Cno was specifically overexpressed in the MG under the *slit-Gal4* line (11% of defective segments at stage 16-17, *n*=109, compared with 50.0% defective segments, *n*=75, in $cno^2/cno^2$ mutant embryos at this stage; see also above). These results strongly support a function of Cno during MG migration, commissural axon ensheathment and subdivision.

### Cno colocalizes and genetically interacts with Wrapper and Nrx-IV

The Ig superfamily protein Wrapper is expressed in the MG and is crucial to properly ensheath commissural axons (Noordermeer et al., 1998). Very recently, two studies have shown how Wrapper acts through the transmembrane protein Nrx-IV, which is expressed mainly on neuronal membranes and it is highly enriched at the interface between neuronal surfaces and the MG. This interaction is fundamental for neuron-glia adhesion and, consequently, for the correct ensheathment of commissural axon fascicles (Banerjee et al., 2006; Baumgartner et al., 1996; Stork et al., 2009; Wheeler et al., 2009). Given that the midline phenotype of the $cno^2$ mutants that we observed was very similar to that described for *Nrx-IV* and *wrapper* mutants (Stork et al., 2009; Wheeler et al., 2009), we analyzed functional relationships between *cno*, *wrapper* and *Nrx-IV*. First, double immunostaining showed that Cno colocalizes with Wrapper (Fig. 3A-B″) and Nrx-IV (Fig. 3C,C′) at the MG. Then, we studied the phenotype of double heterozygotes to detect
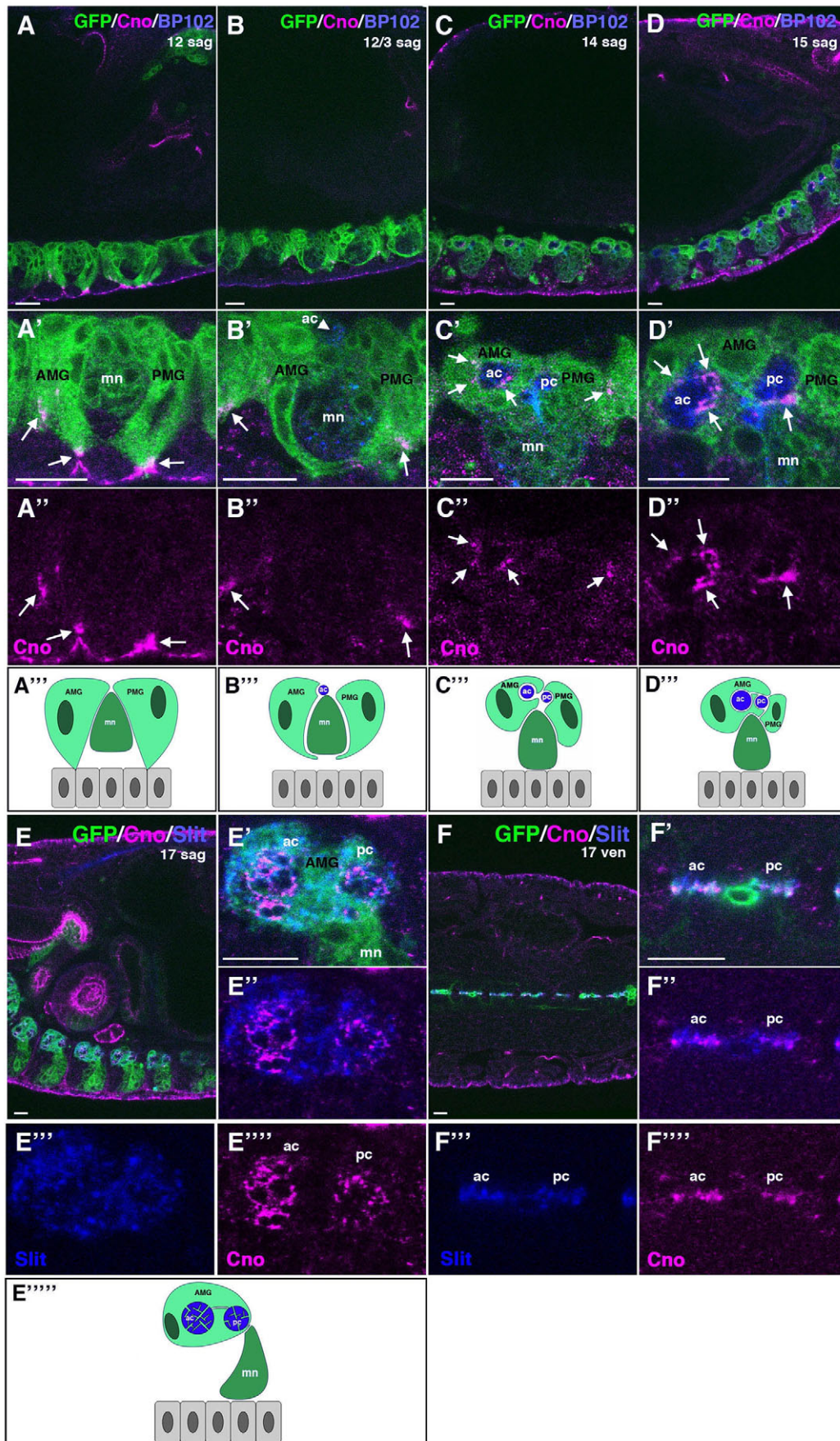
**Fig. 1. Cno is expressed in the midline glia.** (**A-F''''**) Cno expression in the midline glia (MG) throughout embryogenesis of *sim-Gal4 UAS-CD8::GFP Drosophila* embryos. Composite confocal images of single CNS segments in sagittal (sag; A'-D'',E'-E'''') or ventral (ven; F'-F'''') views. A lower magnification overview of the CNS, including several segments, is shown for each stage (A-D,E,F). Anterior is left. Stage and orientation are shown in the upper right corner. mn, midline neurons; ac, anterior commissure; pc, posterior commissure; AMG, anterior midline glia; PMG, posterior midline glia. (A-B'') Cno (magenta) is first detected in the MG (arrows) at stage 12 (BP102, a general axonal marker, is in blue). (C,C'') At stage 14, when AMG has already enwrapped the anterior commissure, Cno is detected at the interface between AMG and this commissure (arrows in the AMG). Cno is also detected at the PMG (arrow). (D,D'') By stage 15, when both commissures have clearly separated, Cno is concentrated at the sites of close contact between MG and commissures (arrows). (E-F'''') At stage 17, Cno is highly restricted to the cytoplasmic projections that MG extend to further subdivide commissural axons into distinct axon bundles (E-E''''). A ventral view of a stage 17 embryo is shown (F-F''''). Slit (labeling the MG) is in blue. (A''',B''',C''',D''',E''''') Schematics representing midline cells (including MG and mn) in a sagittal single segment illustrating the changes that occur at the stages shown in the corresponding confocal micrographs. For clarity, only one cell of the AMG and PMG is shown (see text for details). Scale bars: 10 μm.

## BP102/GFP (midline)



## HRP/Wrapper (midline glia)



WT                    slit-Gal4>>UAS-cno(x2)

**Fig. 2. Cno is necessary for MG migration and for commissural axon ensheathment and subdivision.** (A-F′) *sim-Gal4 UAS-CD8::GFP Drosophila* embryos stained with BP102 (magenta) in a wild-type (A-C′) or *cno²* mutant (D-F′) background. Stage and orientation are shown in the upper right corner. (A,A′,D,D′) At stage 15, the MG of wild-type embryos completely enwrap and separate both commissures (A). At this stage, *cno²* mutant embryos show failures in MG migration (arrowhead) and commissure ensheathment (arrows) (D). Schematics of wild-type (A′) and *cno²* mutant (D′) segments at stage 15. (B,E) At stage 17, wild-type MG extend projections into the commissures, further subdividing individual axon bundles (arrows in B). In *cno²* mutants this process fails (arrows in E). (C,C′,F,F′) Ventral views of stage 16 embryos. In wild-type embryos, both commissures appear well separated at this stage (arrows in C,C′). In *cno²* mutants, anterior and posterior commissures are hardly separated, almost forming a continuous commissure (arrows in F,F′). (G) Quantitation of *cno²/cno²* mutant phenotypes at the midline. (H,I) Sagittal views of wild-type (H) and *slit-Gal4>>UAS-cno (x2)* (I) embryos stained for HRP (a general neuronal marker) and Wrapper (as a MG marker). MG are very frequently detected in between segments (arrows in I; compare with H). Scale bars: 10 µm.

possible functional interactions between these proteins. *Nrx-IV⁴³⁰⁴, +/+, cno²* transheterozygotes showed specific defects in commissural axon ensheathment and separation in a significant number of segments (35.6%, *n*=177) (Fig. 4A,A′,C). *wrapper¹⁷⁵/+; cno²/+* embryos showed 10.5% defective segments (*n*=95). In addition, the penetrance of the *cno²/cno²* phenotype was significantly enhanced (*P*=0.004) in a sensitized background in which the dose of *wrapper* was reduced (i.e. in *wrapper¹⁷⁵/+;*
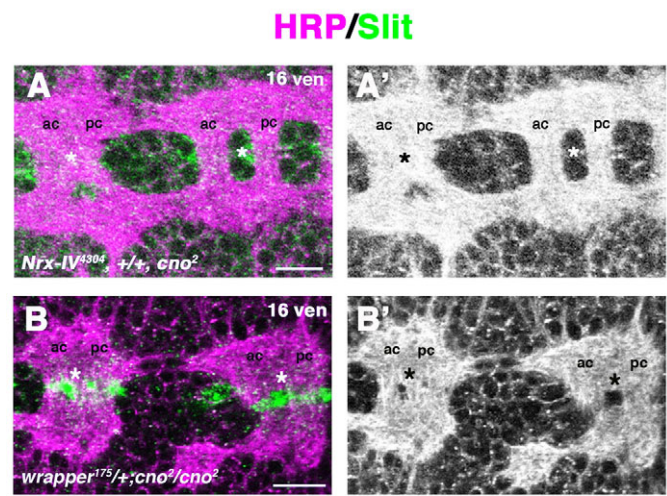
*cno²/cno²* mutant embryos) (Fig. 4C). The expressivity of the *wrapper¹⁷⁵/+; cno²/cno²* phenotype was also much higher than that of the *cno²/cno²* phenotype (in 71% of the *wrapper¹⁷⁵/+; cno²/cno²* segments with a phenotype, this was stronger than the phenotype found in *cno²/cno²* mutants; Fig. 4B,B′). Taking all these data into account, our results support a functional relationship between Cno and Nrx-IV/Wrapper at the midline of the CNS during neuron-glia interactions.
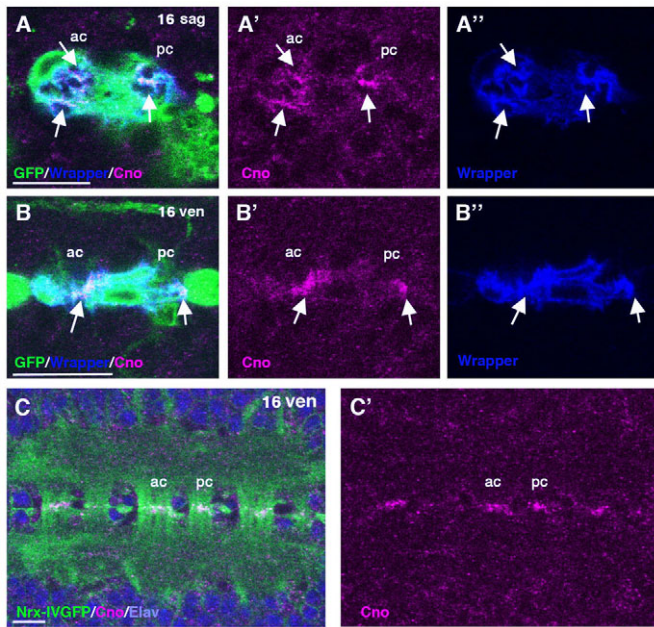
**Fig. 3. Cno colocalizes with Wrapper and Nrx-IV.** (**A-B″**) *sim-Gal4 UAS-CD8::GFP Drosophila* stage 16 embryos. Confocal micrographs of one CNS segment in a sagittal (A-A″) or ventral (B-B″) view. Cno (magenta) colocalizes with Wrapper (blue) (arrows in A-B″). In a sagittal view, the colocalization of the two proteins in the MG projections that subdivide the commissural axons is apparent (arrows in A-A″). (**C,C′**) Ventral cord of an *Nrx-IV-GFP* stage 16 embryo showing colocalization of Cno (magenta) and Nrx-IV (GFP) at the midline, where the MG are in close contact with both commissures (Elav, in blue, labels all neuronal cell bodies). Scale bars: 10 μm.

## Cno forms a complex in vivo with Wrapper and Nrx-IV

The functional relationships between Cno, Wrapper and Nrx-IV, as well as their colocalization, being highly restricted to the MG/commissural axon contacts, prompted us to investigate whether Cno forms a complex with Wrapper and Nrx-IV. Co-IP experiments from embryo extracts confirmed the presence of Cno-Wrapper and Cno-Nrx-IV aggregates in vivo (Fig. 5). We then investigated whether the localization of Cno was altered in *wrapper* and *Nrx-IV* mutant embryos. In both *Nrx-IV^{4304}* and *wrapper^{175}* mutants, the localization of Cno was affected, being completely or partially missing from the commissures and frequently located between them (Fig. 6A-C′). When Nrx-IV was expressed in a subset of commissural neurons (i.e. *eagle-Gal4>>UAS-Nrx-IV*) in an *Nrx-IV^{4304}* mutant background, the localization of Cno, along with that of Wrapper (MG), was partially rescued (see Fig. S1 in the supplementary material) (Stork et al., 2009). We next examined whether the defects in Cno localization found in *Nrx-IV^{4304}* and *wrapper^{175}* mutants were a secondary effect of the failure in MG migration and enwrapping of commissural axons found in these mutants. We used Slit as a MG marker along with Cno and analyzed higher magnifications of *Nrx-IV^{4304}* and *wrapper^{175}* mutants in sagittal views (Fig. 6D-F′). We found that failure in Cno localization (including the absence of Cno at MG projections) perfectly correlated with the loss of MG in these mutants (Fig. 6D-F′). These results further support a requirement for Cno at the MG, not at the commissural axons. However, the subcellular localization of Cno in the remaining MG found in *Nrx-IV^{4304}* and *wrapper^{175}*



**Fig. 4. *cno* genetically interacts with *wrapper* and *Nrx-IV*.** (**A-B′**) Two segments of *Nrx-IV^{4304}, +/+, cno2 Drosophila* transheterozygotes (A,A′) or *wrapper^{175}/+; cno2/cno2* embryos (B,B′) are shown in a ventral view. HRP is in magenta (A,B) or gray (A′,B′) and Slit (MG) is in green (A,B). The asterisks indicate the space between the anterior commissure (ac) and the posterior commissure (pc). Strong defects in MG enwrapping (A,B) and commissure separation (A′,B′) are shown. (**C**) Quantitation of *cno-Nrx-IV* and *cno-wrapper* genetic interactions. Scale bars: 10 μm.

mutants was unaltered: Cno was still highly restricted to the interface between MG and commissural axons (Fig. 6E-F′). This suggests that other protein(s) stabilize Cno at this location (see below).

## Shotgun localizes and functions at the MG in a complex with Wrapper

Our results indicate that Cno at the MG forms a complex in vivo with Wrapper and Nrx-IV. But, how could the cytoplasmic protein Cno be interacting at the MG with Wrapper, a GPI-linked protein that lacks a cytosolic domain? Additionally, why is Cno still highly localized at the interface between MG and commissural axons in *Nrx-IV^{4304}* and *wrapper^{175}* mutants? The adhesion and transmembrane protein Shg, a DE-cadherin, provided a potential link between Cno and Wrapper at the MG. Shg has a PDZ-binding motif at its C-terminus that interacts with the PDZ domain of Cno (Sawyer et al., 2009). Interestingly, we found that Shg was present at the MG and was highly enriched, as is Cno, at the interface between the MG and commissural axons (Fig. 7A-C′). Moreover, *shg^2* mutant embryos showed strong defects in the MG in 46.5% of the segments analyzed (*n*=88), with commissural axons frequently fused (61%; Fig. 7D,E), MG missing (14.6%; Fig. 7D,E) or MG misplaced (85.4%; Fig. 7E,G, compare with 7D,F).
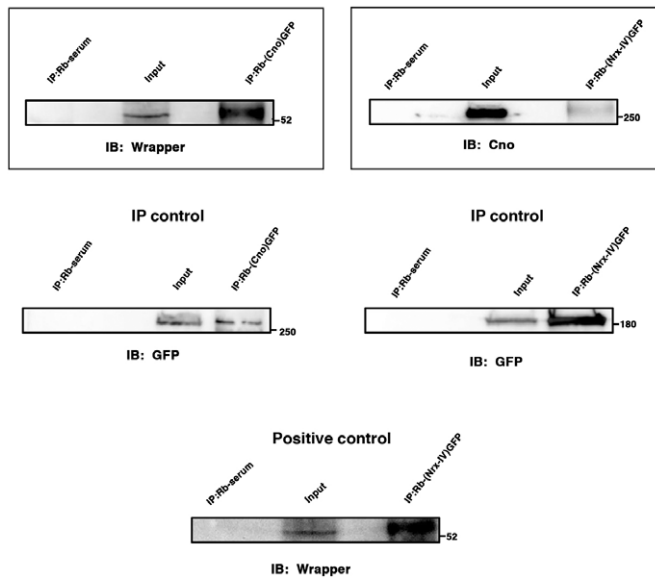
**Fig. 5. Cno forms a complex in vivo with Wrapper and Nrx-IV.** *UAS-cno-GFP Drosophila* embryo lysates were subject to immunoprecipitation (IP) with anti-GFP antibody and probed on immunoblots (IB) with anti-Wrapper and with anti-GFP (as an IP control). *Nrx-IV-GFP* embryo lysates were subject to IP with anti-GFP and probed on immunoblots with anti-Cno, anti-Wrapper (as a positive control) and with anti-GFP (as an IP control).

Intriguingly, the subcellular localization of Cno at the MG was altered in *shg²* mutants (Fig. 7H-I'). Moreover, Shg formed a complex in vivo with Wrapper (Fig. 7J). Taking all these data into account, we propose that Shg functions at the MG, where it acts as a linker between Cno and the Wrapper-Nrx-IV complex (Fig. 7K).

## DISCUSSION

The midline constitutes a key boundary of bilateral organisms. In vertebrates, it is the floorplate and the functionally equivalent structure in *Drosophila* is the mesectoderm, which gives rise to all midline cells, neurons and glia, in the most ventral part of the embryo. MG are of great relevance at the midline as an intermediate target during axonal pathfinding, providing both attractive and repulsive guidance cues. These signals allow contralateral axons to cross the midline but never to recross, and they also keep ipsilateral axons away from the midline. In addition to this early function in guiding commissural axons towards the midline, MG are also fundamental later on to separate the commissures by enwrapping and subdividing them. Here, we show that the PDZ protein Cno and the DE-cadherin Shg participate in, and contribute to, the regulation of these later stage neural differentiation events, in which neuron-glia interactions play a central role.

### Cno forms a complex with Shg, Wrapper and Nrx-IV at the MG

In *Drosophila*, Wrapper and Nrx-IV physically interact to promote glia-neuron intercellular adhesion at the MG (Stork et al., 2009; Wheeler et al., 2009). We propose that Cno and Shg are important components of this adhesion complex and key to its function. We found that both Cno and Shg are present at the MG, being highly restricted to the interface between MG and commissural axons. Cno and Shg were detected in a complex in vivo with Wrapper at the CNS MG. Nrx-IV, which is located on the surface of commissural
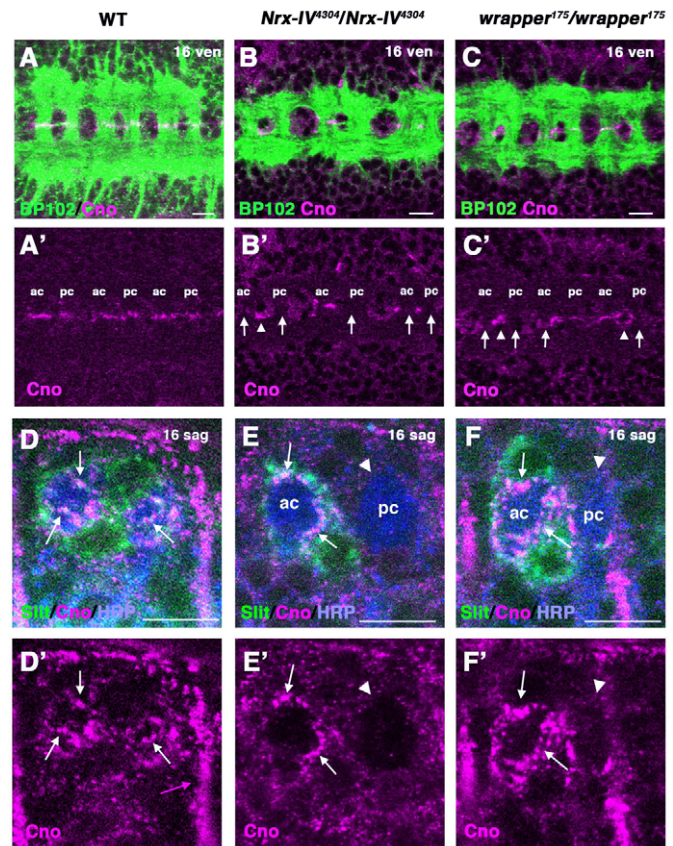


**Fig. 6. Cno subcellular localization at MG is not altered in *Nrx-IV* and *wrapper* mutants.** (**A-C'**) Ventral views of three segments are displayed. In wild-type *Drosophila* embryos (A,A'), Cno (magenta) is detected at the anterior (ac) and posterior (pc) commissures. BP102 is shown in green. In *Nrx-IV⁴³⁰⁴* (B,B') and in *wrapper¹⁷⁵* (C,C') mutant embryos, Cno was frequently missing from commissures (arrows) or detected between them (arrowheads). (**D-F'**) High magnifications of one segment in a sagittal view. Slit (as a marker of MG) is in green, HRP (neurons) is in blue and Cno is in magenta. In wild-type embryos (D,D'), Cno is highly concentrated at the interface between MG and both ac and pc. Notice also the presence of Cno at the MG projections within the commissures (arrows). The magenta arrow points to strong Cno expression independent of the MG. In *Nrx-IV⁴³⁰⁴* (E,E') and *wrapper¹⁷⁵* (F,F') mutants, MG fail to completely enwrap commissures (arrowheads). Cno at the remaining MG still accumulates at contacts between MG and commissural axons (arrows in E-F'). Scale bars: 10 μm.

axons, was also consistently found in a complex with Cno, although the amount of Cno protein that we were able to co-immunoprecipitate was much lower than that present in Cno-Wrapper complexes. One plausible explanation is that whereas Cno and Wrapper are present in the same cell (MG), Cno and Nrx-IV are in different cell types (MG and neurons, respectively) and, in addition, Cno is a cytoplasmic protein that is indirectly linked to Nrx-IV through other proteins in the same complex (i.e. Shg and Wrapper). Intriguingly, we found stronger genetic interactions between Cno and Nrx-IV than between Cno and Wrapper (double heterozygote analysis). A possible explanation for this is that Nrx-IV is not only acting through Wrapper-Shg-Cno in the MG but also through other partners, as previously proposed (Wheeler et al., 2009). In this way, when the dose of Cno and Wrapper was halved, Nrx-IV could still function fully through these other, putative partners.
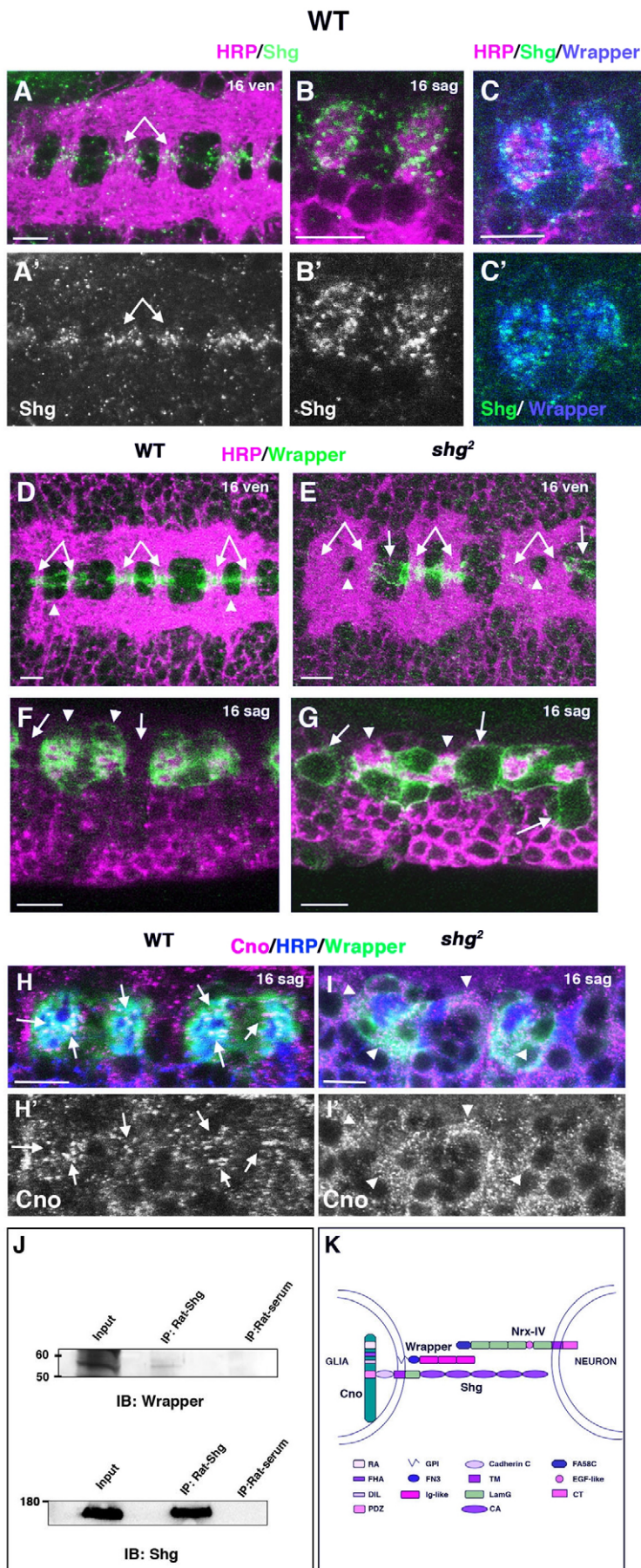
**Fig. 7. Shg localizes and functions at the MG in a complex with Wrapper.** (**A-C′**) Expression of Shg (green) at the MG. HRP is in magenta, Wrapper is in blue. Ventral view of three segments (A,A′) showing Shg accumulation in a punctuate pattern around anterior and posterior commissures (arrows). In a sagittal view, Shg is also detected in close contact with commissures (B,B′). Shg colocalizes with Wrapper in the MG (C,C′). (**D-G**) Ventral views of stage 16 wild-type (D) and *shg²* mutant (E) *Drosophila* embryos stained for HRP (magenta) and Wrapper (green). Commissures are poorly separated in *shg²* mutants (arrowheads) and MG fail to enwrap the commissures (double arrows, compare with wild type). MG are also detected between commissures (single arrows). In sagittal views (F,G), defects in commissure ensheathment are apparent in *shg²* mutants (arrowheads), as well as the presence of MG between commissures (arrows in G, compare with F). (**H-I′**) The subcellular localization of Cno is altered in *shg²* mutant embryos. Cno is in magenta, HRP in blue and Wrapper in green. Sagittal views of wild-type embryos show the localization of Cno within the MG at the contacts with the axonal commissures (arrows in H,H′). In *shg²* mutants, Cno is delocalized throughout the cytoplasm of MG (arrowheads in I,I′). (**J**) Co-IPs from wild-type embryo extracts show that Wrapper and Shg form a complex in vivo. (**K**) Model of neuron-glia interactions at the MG. The transmembrane protein Shg links the cytoplasmic protein Cno and the GPI-anchored protein Wrapper at the MG. Wrapper, in turn, binds to the transmembrane protein Nrx-IV present at commissural axons. The modular structure of all these proteins is shown (from NCBI 'conserved domains'). RA, Ras-association domain; FHA, Forkhead; DIL, Dilute; PDZ (PSD-95, Discs large, ZO-1); GPI, glycophosphatidylinositol; FN3, Fibronectin type 3; Ig-like, Immunoglobulin-like; TM, transmembrane; LamG, Laminin G; CA, Cadherin; FA58C, Coagulation factor 5 or 8 C-terminal domain; EGF-like, Epidermal growth factor-like; CT, C-terminal. Scale bars: 10 μm.

However, halving the dose of Cno and Nrx-IV would impair not only the Nrx-IV-Wrapper-Cno signal but also the other potential pathways. In vertebrates, the ortholog of Nrx-IV, termed contactin-associated protein (Caspr or Cntnap) or paranodin, is located at the septate-like junctions of the axonal paranodes, where it interacts in cis with contactin (at neurons) and in trans with neurofascin (at the glia) (Poliak and Peles, 2003). The *Drosophila* homologs of these Ig superfamily proteins, Contactin and Neuroglian, interact in the same way with Nrx-IV at the septate junctions. However, there are no septate junctions at the neuron-MG interface (Jacobs and Goodman, 1989; Stollewerk et al., 1996) (see also below). Hence, other, as yet unknown partners of Nrx-IV might exist at this location.

## Cno: more than an adherens junction protein

Cno and its vertebrate orthologs afadin/AF-6/Mllt4 have been shown to localize at epithelial adherens junctions (AJs), where they regulate the linkage of AJs to the actin cytoskeleton by binding both actin and Nectin family proteins (Lorger and Moelling, 2006; Mandai et al., 1997; Matsuo et al., 1999; Sawyer et al., 2009; Takahashi et al., 1998). However, Cno is not exclusively present at the AJs of epithelial tissues. Indeed, we previously found that Cno is also expressed in mesenchymal tissues, where it dynamically regulates three different signaling pathways required for muscle/heart progenitor specification (Carmena et al., 2006). The asymmetric division of these muscle/heart progenitors and of CNS progenitors also requires an AJ-independent function of Cno to asymmetrically locate cell fate determinants and properly orientate the mitotic spindle (Speicher et al., 2008). Therefore, Cno seems to act through different mechanisms depending on the cell type. Here, we describe a novel function of Cno during neural differentiation. In the MG, Cno, through Shg, contributes to the tight adhesion between the MG and the commissural axons and perhaps even to the regulation of some intracellular signaling within the MG. Indeed, Cno has been shown to regulate different signaling cascades during development (Carmena et al., 2006). To the best of our knowledge, no AJs or septate junctions (SJs) have been described at the MG-commissural axon interface (Jacobs and Goodman, 1989; Stollewerk et al., 1996). This suggests that the function of Cno in the midline is independent of AJs. In fact, the partner of Cno at this location, the *Drosophila* Nectin ortholog Echinoid (Wei et al., 2005), is not detected at the midline (our unpublished observations). In this context, it is worth pointing out that Shg is an epithelial cadherin key at AJs. Here, we have shown that Shg can also be found in non-epithelial tissues with an important function independent of AJs. A similar situation occurs with Nrx-IV. Despite Nrx-IV being a very well established component of SJs (Baumgartner et al., 1996; Faivre-Sarrailh et al., 2004; Schulte et al., 2003), no SJs are formed in the midline and no other known components of SJs are expressed there (Stork et al., 2009). Thus, different modes of Cno action, either as an AJ protein or as a signaling pathway regulator, are possible and they are not mutually exclusive: it all depends on the cell type and context.

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