Development 136, 1147-1157 (2009) doi:10.1242/dev.030254

Neurexin IV and Wrapper interactions mediate *Drosophila* midline glial migration and axonal ensheathment

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Glia play crucial roles in ensheathing axons, a process that requires an intricate series of glia-neuron interactions. The membrane-anchored protein Wrapper is present in *Drosophila* midline glia and is required for ensheathment of commissural axons. By contrast, Neurexin IV is present on the membranes of neurons and commissural axons, and is highly concentrated at their interfaces with midline glia. Analysis of *Neurexin IV* and *wrapper* mutant embryos revealed identical defects in glial migration, ensheathment and glial subdivision of the commissures. Mutant and misexpression experiments indicated that Neurexin IV membrane localization is dependent on interactions with Wrapper. Cell culture aggregation assays and biochemical experiments demonstrated the ability of Neurexin IV to promote cell adhesion by binding to Wrapper. These results show that neuronal-expressed Neurexin IV and midline glial-expressed Wrapper act as heterophilic adhesion molecules that mediate multiple cellular events involved in glia-neuron interactions.

KEY WORDS: Axon, Cell adhesion, *Drosophila*, Midline glia, Neurexin IV, Wrapper

INTRODUCTION

During development, CNS axons undergo a series of pathway choices to find their synaptic targets, and in the vertebrate spinal cord most axons cross the midline in commissures before joining longitudinal axon tracts. The floorplate cells of the spinal cord reside along the ventral midline, where they closely associate with commissural axons (Campbell and Peterson, 1993) and extend processes that subdivide axon bundles (Yoshioka and Tanaka, 1989). Like the floorplate cells, the midline glia (MG) of the *Drosophila* CNS are centrally located and serve as a source of secreted factors required for attracting axons to the midline and ensuring that they do not recross (Garbe and Bashaw, 2004). In each segment, the MG ensheath the anterior (AC) and posterior (PC) commissures; each individual commissure is then further subdivided into axon-containing regions separated by MG projections (Noordermeer et al., 1998; Stollewerk and Klambt, 1997). The process of commissural axon ensheathment resembles vertebrate myelination, requiring that the glia migrate towards, recognize, adhere to and completely surround the axonal processes. These similarities make the *Drosophila* MG an attractive system for studying the development and function of neuron-glia interactions.

Initially, there are ten MG composed of two classes: anterior midline glia (AMG) and posterior midline glia (PMG) (Dong and Jacobs, 1997; Kearney et al., 2004; Wheeler et al., 2006). The AMG initially consist of six cells. These cells migrate towards, make contact with and ensheath the developing axon commissures through a series of stereotyped movements and process extensions. On average, only three AMG closely contact the commissural axons, resulting in their continued survival, while the remaining AMG undergo apoptosis (Bergmann et al., 2002). All PMG die via apoptosis, and do not contribute to the mature MG (Dong and Jacobs, 1997; Sonnenfeld and Jacobs, 1995). Genetic studies have shown that the EGF, FGF and PVF signaling pathways are involved in aspects of MG development (Jacobs, 2000; Learte et al., 2008). However, members of these pathways are unlikely to mediate the adhesive interactions that underlie MG-neuron interactions. Analysis of the immunoglobulin (Ig) superfamily member *wrapper* showed that it is highly expressed in MG and is required for MG-neuron adhesion, as well as for the proper ensheathment and subdivision of the axon commissures (Noordermeer et al., 1998). One key issue is the identity of the binding partner on neuronal membranes that functions with Wrapper in MG-neuron adhesion.

A strong candidate is *Neurexin IV* (*Nrx-IV*), which is expressed throughout the CNS and encodes a transmembrane protein containing four extracellular Laminin G domains and two EGF domains (Baumgartner et al., 1996). While distantly related to other vertebrate and invertebrate Neurexin proteins, *Drosophila* Nrx-IV is orthologous to vertebrate Caspr (paranodin; Cntnap1) (Banerjee et al., 2006b). Caspr is localized to paranodal axo-glial junctions of myelinated neurons, where it binds to the Ig superfamily proteins contactin and neurofascin (Bhat, 2003; Charles et al., 2002). *Drosophila* Nrx-IV is also localized to septate junctions at axo-glial interfaces, and interacts with Contactin and Neuroglian (Banerjee et al., 2006a), which is highly related to neurofascin.

Since *Nrx-IV* is expressed in neurons and binds to Ig superfamily members, we tested the hypothesis that Nrx-IV and Wrapper physically interact and mediate MG-neuron interactions. Using a *sim-Gal4 UAS-tau-GFP* midline cell marker strain (Wheeler et al., 2006) we were able to carefully examine midline cell morphology, movement and axonal ensheathment during embryonic development. Genetic analysis of *Nrx-IV* mutants revealed defects in MG-neuron and MG-axon interactions that are identical to those observed with *wrapper* mutants (Noordermeer et al., 1998). Nrx-IV protein was highly localized to the interface between MG and neuronal surfaces (both axon and cell body). The localization of Nrx-IV on neuronal membranes was dependent on the presence of Wrapper, and immunoprecipitation experiments demonstrated a physical interaction. Using cultured *Drosophila* S2 cells, we showed that mixing *wrapper*- and *Nrx-IV*-transfected cells resulted in cellular aggregation, and this effect was dependent upon the presence of both proteins. As in embryos, the Nrx-IV present in the aggregated cells was highly localized at sites of cell-cell contact.

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Accepted 4 February 2009

Thus, Nrx-IV and Wrapper function as heterophilic adhesion molecules that mediate MG migration and the ensheathment and subdivision of commissural axons.

MATERIALS AND METHODS

Drosophila strains

Drosophila strains used were: *ap-Gal4* (Calleja et al., 1996), *arm-Gal4* (Sanson et al., 1996), *en-Gal4* (Ward et al., 1998a), *Nrx-IV*^{4304} (Baumgartner et al., 1996), *Nrx-IV-GFP* (CA06597) (Buszczak et al., 2007; Laval et al., 2008; Morin et al., 2001), *sim-Gal4* (Xiao et al., 1996), *slit-Gal4* (Scholz et al., 1997), *UAS-GFP-lacZ.nls* (Shiga et al., 1996), *UAS-tau-GFP* (Brand, 1995), *UAS-wrapper* (Noordermeer et al., 1998) and *wrapper*^{175} (Noordermeer et al., 1998).

In situ hybridization, immunostaining and immunoprecipitation

Embryo collection, in situ hybridization, immunostaining and immunoprecipitation were performed as previously described (Kearney et al., 2004; Banerjee et al., 2006a). Primary antibodies used were: mouse MAb BP102 (Developmental Studies Hybridoma Bank, DSHB), rat anti-Elav MAb 7E8A10 (DSHB), mouse anti-En MAb 4D9 (Patel et al., 1989), chicken anti-GFP (Upstate), rabbit anti-GFP Ab290 (Abcam), guinea pig anti-Lim3 (Broihier and Skeath, 2002), rabbit anti-Nrx-IV (Baumgartner et al., 1996), mouse anti-Nrt MAb BP106 (DSHB), guinea pig anti-Runt (East Asian Distribution Center) (Kosman et al., 1998), mouse anti-Wrapper MAb 10D3 (DSHB) (Noordermeer et al., 1998) and guinea pig anti-Wrapper. Generation of the guinea pig anti-Wrapper utilized a 6×His-tagged fusion protein containing amino acids 245-444 of Wrapper as immunogen. Midline cells were examined in abdominal segments A1-8. Owing to the three-dimensional structure of the midline cells, it was difficult to represent all relevant cells in a single focal plane; so, for clarity, irrelevant portions of single images within a stack of confocal images were subtracted before projections were generated.

Cell culture, RNAi and immunofluorescence

Cell culture and RNAi experiments were performed as described (Rogers and Rogers, 2008). *wrapper* (*pAc-wrapper*) and *Nrx-IV* (*pAc-Nrx-IV*) open reading frames were PCR amplified from full-length cDNA clones and cloned into the pAc-V5/His A vector (Invitrogen) providing expression induced by the constitutively active *Actin 5C* promoter (Han et al., 1989). For immunofluorescence experiments, cells were plated onto poly-lysine-coated coverslips 24 hours after transfection, fixed, and stained with antibodies against Nrx-IV and Wrapper. All experiments were performed in duplicate and more than 100 cells were analyzed for each sample. Control cells were transfected with *pMt-GFP* to assess background aggregation. For cell aggregation assays, S2 cells were transfected with either *pAc-wrapper* or *pAc-Nrx-IV* by electroporation (Amaxa Nucleofector, Lonza). After 24 hours, the cells were resuspended, mixed, gently rocked on a Nutator for 1 hour, and plated onto poly-lysine-treated coverslips. For RNAi experiments, cells were treated daily with 10 μg dsRNA for 6 days followed by transfection with *pAc-wrapper* for 1 day. Negative controls were conducted with dsRNA generated against pBluescript vector, whereas controls to ensure that cells were competent for RNAi treatment were conducted with dsRNA targeted to *Rho1* (Rogers and Rogers, 2008). Nrx1 and Nrx2 dsRNAs were generated by PCR of an *Nrx-IV* cDNA with two primer sets: set 1, 5′-TAATACGACTCACTATAGGGGTCAATAATGGCATTAAATCGGTG-CAAGCAGACG-3′ and 5′-TAATACGACTCACTATAGGCCCAGTTC-AGTGCTGTCTTTCATCACTCG-3′; set 2, 5′-TAATACGACTCACTA-TAGGGCACAGTACTATATTGCTGGTGGAAAGGACAAAAATGG-3′ and 5′-TAATACGACTCACTATAGGGCATCGGCAATGCGGAAGGTC-ACCACATTAC-3′.

RESULTS

Imaging MG migration and axonal ensheathment in *sim-Gal4 UAS-tau-GFP* embryos

We have recently employed a *sim-Gal4 UAS-tau-GFP* transgenic strain and confocal microscopy to study the development of *Drosophila* CNS midline cells (Wheeler et al., 2006; Wheeler et al., 2008). In *sim-Gal4 UAS-tau-GFP* embryos, *GFP* is localized to the cytoplasm of all midline cells – both neurons and glia (Wheeler et al., 2006). When examined in sagittal views, this allows visualization of the morphology of each midline cell type during development. Our identification of specific midline cell types employed immunostaining or in situ hybridization with more than 90 validated cell type-specific reagents (Wheeler et al., 2006; Wheeler et al., 2008). In this paper, we use this system to investigate the dynamics of MG development during embryonic stages 12-17 in both wild-type and mutant embryos. MG were identified by their distinct shape, relatively dorsal position within the CNS and expression of *wrapper*, which is high in AMG, low in PMG, and absent from neurons (Noordermeer et al., 1998; Wheeler et al., 2006). PMG were additionally identified by expression of *engrailed* (*en*). Antibodies recognizing all neurons (anti-Elav), their axons (MAb BP102), and the midline precursor 1 (MP1) neurons (anti-Lim3) provided a comprehensive view of MG interactions with nearby neurons and axons. At the beginning of CNS axonogenesis [stages 12/3 to 12/0; subdivisions of stage 12 according to Klambt et al. (Klambt et al., 1991)], commissural axons initially converged into a single axon bundle (Fig. 1A,B). At stage 12/3, the AMG resided in the anterior of the segment and had an elongated morphology as they migrated toward the axon commissure (Fig. 1A). Approximately three AMG made contact with the anterior surface of the commissure while the remaining AMG underwent apoptosis, in keeping with published observations (Bergmann et al., 2002). At stage 12/0, the AMG sent processes across both the dorsal and ventral surfaces of the commissure (Fig. 1B). As the commissure separated into AC and PC, the AMG membranes completely ensheathed the AC (Fig. 1C). AC ensheathment concluded with the movement of an AMG cell body between the commissures (Fig. 1D). After the AC was completely ensheathed, a single, dorsally located AMG migrated across the dorsal surface of the PC during stages 15-16 (Fig. 1E). This AMG extended processes posteriorly to ensheath the PC (Fig. 1F). During stages 15-17, the AMG also sent cytoplasmic projections into both the AC and PC that became more elaborate as development proceeded (Fig. 1E,F). Using electron microscopy, Stollewerk and Klambt (Stollewerk and Klambt, 1997) showed that these cytoplasmic projections divide each commissure into distinct subdomains.

Both the MP1 neurons and PMG were also in proximity to AMG and the commissures. Their positions were constant relative to the migrating AMG. The MP1 neurons remained in close contact with the ventral-most AMG from stages 11-17 (Fig. 1B-F; see Fig. S1A-D in the supplementary material), and prior to commissure separation the MP1 neurons were closely associated with the commissure along its ventral side (Fig. 1B; see Fig. S1A in the supplementary material). The PMG migrated dorsally and in an anterior direction during stage 12, and at least one PMG abutted the posterior side of the PC from stages 12-16 (Fig. 1A-E; see Fig. S1E-H in the supplementary material) before undergoing apoptosis (Bergmann et al., 2002; Sonnenfeld and Jacobs, 1995). The cell bodies of non-midline-derived neurons also made extensive contacts with the AMG (see Fig. 4D) and, together with the PMG and MP1 neurons, they might play important roles in AMG development. Overall, the view of MG development described above is in general agreement with that described by others (Jacobs, 2000), validating the use of *sim-Gal4 UAS-tau-GFP* to study MG development. However, there are some important differences in nomenclature and PMG migration (see Discussion).

Nrx-IV mutants have disrupted MG-neuron interactions

During an earlier analysis of the role of *Nrx-IV* in septate junctions (Banerjee and Bhat, 2007; Baumgartner et al., 1996), a failure of commissure separation was observed in *Nrx-IV* mutants (Fig. 2A,B). Previously, disruption of the commissures was correlated with MG defects (Klambt et al., 1991). To investigate a potential *Nrx-IV* midline phenotype, $Nrx\text{-}IV^{4304}$, a null allele, was used in combination with *sim-Gal4 UAS-tau-GFP* to examine the dynamics of AMG migration, axonal ensheathment and commissure subdivision. During stage 12, the migration of the AMG and their juxtaposition to the unseparated commissure were normal (Fig. 2C). Once the AMG contacted the commissure, they sent processes across the AC as in the wild type (Fig. 2D). However, instead of ensheathing the AC, both the dorsally and ventrally located AMG migrated past the AC towards the PC (compare Fig. 2E,F with Fig. 1C,D). Strikingly, the AMG were frequently dissociated from the MP1 neurons residing at a more distant and dorsal location away from the midline neurons (Fig. 2H,I). This phenotype was the most common defect observed in stage 17 *Nrx-IV* mutant segments (Table

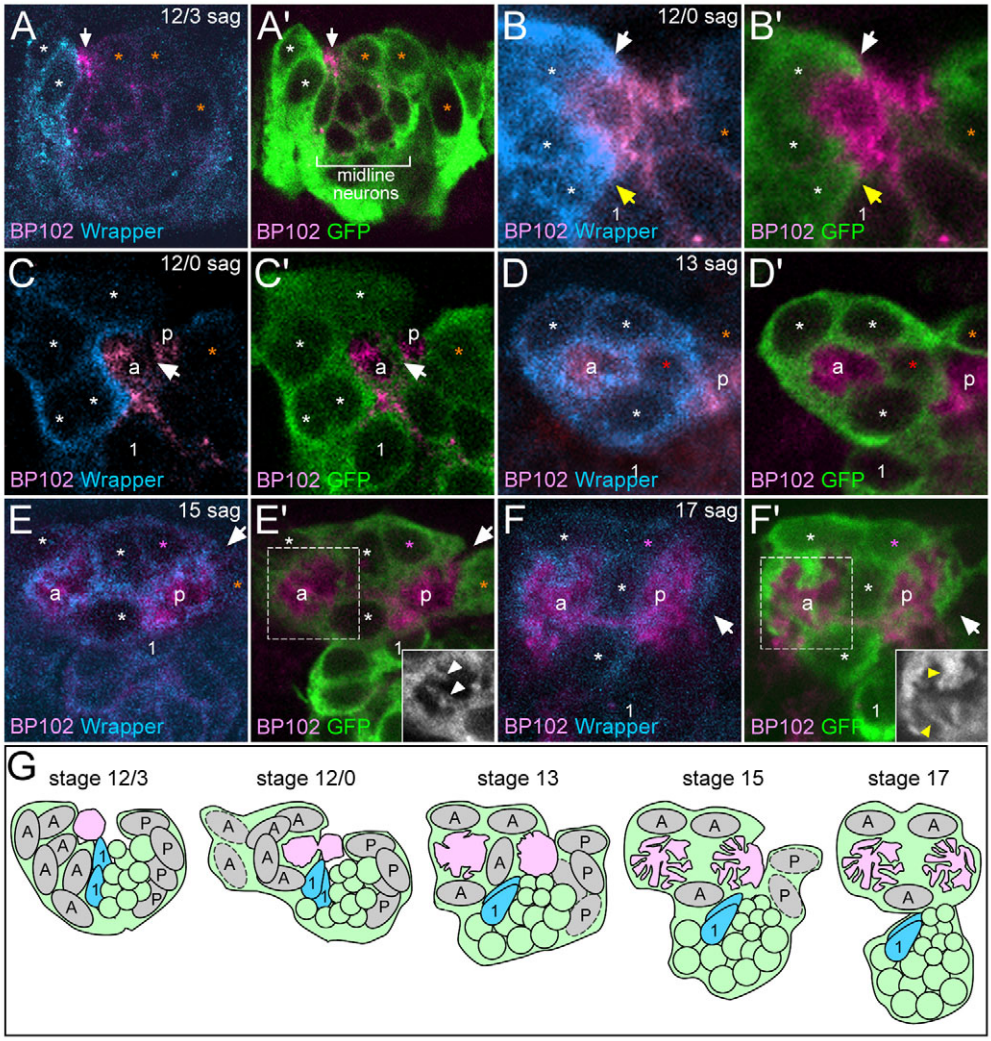

Fig. 1. MG migration and commissural axon ensheathment and subdivision. (**A-F'**) Composite confocal images of *sim-Gal4 UAS-tau-GFP* *Drosophila* embryos in sagittal views. (A,A') Single segment and (B-F') high-magnification views focused primarily on MG and axon commissures. Anterior is left and dorsal (internal) is up. Developmental stage and orientations are shown in the upper right corner. Sag, sagittal; ven, ventral. White asterisks, anterior midline glia (AMG); orange asterisks, posterior midline glia (PMG); 1, MP1 neurons; a, anterior commissure (AC); p, posterior commissure (PC). MP1 neurons were identified by their characteristic shape, position and axonal trajectories. (A,A') At stage 12/3, the AMG and PMG moved internally, contacting the single commissure (arrow). Only two AMG and three PMG are shown in this focal plane. The position of midline neurons is shown for reference (bracket). (B,B') At stage 12/0, the AMG began a posterior migration along the dorsal (white arrow) and ventral (yellow arrow) aspects of the commissure. (C,C') During stage 12/0, the AMG extended a cytoplasmic process between the AC and PC (arrow). (D,D') Following the process, an AMG (red asterisk) migrated between the AC and PC. (E,E') A single, dorsally located AMG (magenta asterisk) began to migrate over the PC during stage 15. A small gap (arrow) was still present between AMG and PMG. Inset shows GFP staining in the boxed region, showing that the AMG extended projections (arrowheads) into the AC. (F,F') During stage 17, the more posterior of the dorsal AMG (magenta asterisk) sent out a thin process (arrow) that surrounded the PC. Glial membranes continued to project into the commissures (arrowheads in inset). (**G**) Schematic summary of MG migration. The schematic depicts an idealized view; actual segments vary in MG number and position. Sagittal views. Axon commissures, pink; circles indicate midline cell nuclei surrounded by cytoplasmic Tau-GFP, green; MP1s, blue (1); AMG, gray (A); PMG, gray (P); midline neurons, green. Dashed lines indicate nuclei of cells undergoing apoptosis. Individual cell borders are not shown.

1). In stage 17 wild-type embryos, 3.1±0.4 AMG (n=33 segments) were present, whereas in embryos with dissociated AMG this was reduced to 2.1±0.5 AMG (n=28 segments). The reduction was likely to be due to the lack of AMG ensheathment of the PC, and the corresponding inability of the AMG to receive a sufficient axon-derived survival signal (Bergmann et al., 2002). Additional *Nrx-IV* mutant phenotypes were: (1) dissociation with incomplete migration and ensheathment of the AC and PC (Fig. 2J; Table 1), and (2) a complete absence of AMG (Fig. 2K; Table 1). In all mutant segments, the AMG failed to extend glial projections to subdivide either the AC or PC (Fig. 2A,B; and compare Fig. 2I with Fig. 1F). The inability of the AMG to properly migrate along the AC coupled with their inability to send projections into the commissures indicated that *Nrx-IV* was required for interactions between AMG and commissural axons. Furthermore, the dissociation of AMG from the MP1 neurons indicated that interactions between these two cell types are important for AMG positioning.

Nrx-IV protein is present in neurons and localizes to MG-neuron interfaces

To understand how mutations in *Nrx-IV* lead to defects in AMG development, the CNS expression and protein localization of *Nrx-IV* was analyzed in wild-type embryos by in situ hybridization and immunostaining. Transcripts were detected at a low level throughout the CNS (Fig. 3A; see Fig. S1I-K in the supplementary material), with no increase in expression levels at or near the midline. This was in sharp contrast to Nrx-IV immunostaining, which showed high levels of protein at the midline (Fig. 3B,C) (Baumgartner et al., 1996). We argue that the Nrx-IV midline staining is due to *Nrx-IV* neuronal expression and protein localization at MG-neuron interfaces, and is absent from MG (or present at insignificant levels).

There were two major locations at which Nrx-IV was concentrated (Fig. 3C): (1) along the axon commissures, and (2) at the boundaries between MG and neurons. At stages 12/0 to 13, Nrx-IV was found at the interface where the migrating AMG contacted the single, unseparated commissure (Fig. 3D). After commissure separation and ensheathment, Nrx-IV localized to the boundaries where commissures and AMG were juxtaposed and along the AMG projections that subdivided the commissures (Fig. 3E).

Nrx-IV was localized at detectable, but low, levels around the membranes of neurons (Fig. 3B), and at high levels at sites of contact between lateral CNS neurons and AMG (Fig. 3F). Neurons also showed Nrx-IV localization where they contacted PMG, but the accumulation was weaker than with AMG (Fig. 3C). Nrx-IV was highly localized to the contacts between AMG and the MP1 neurons (Fig. 3G); this localization was prominent from stage 12/3 until the MP1 neurons underwent apoptosis during stage 17 (Miguel-Aliaga and Thor, 2004). To further investigate the subcellular localization

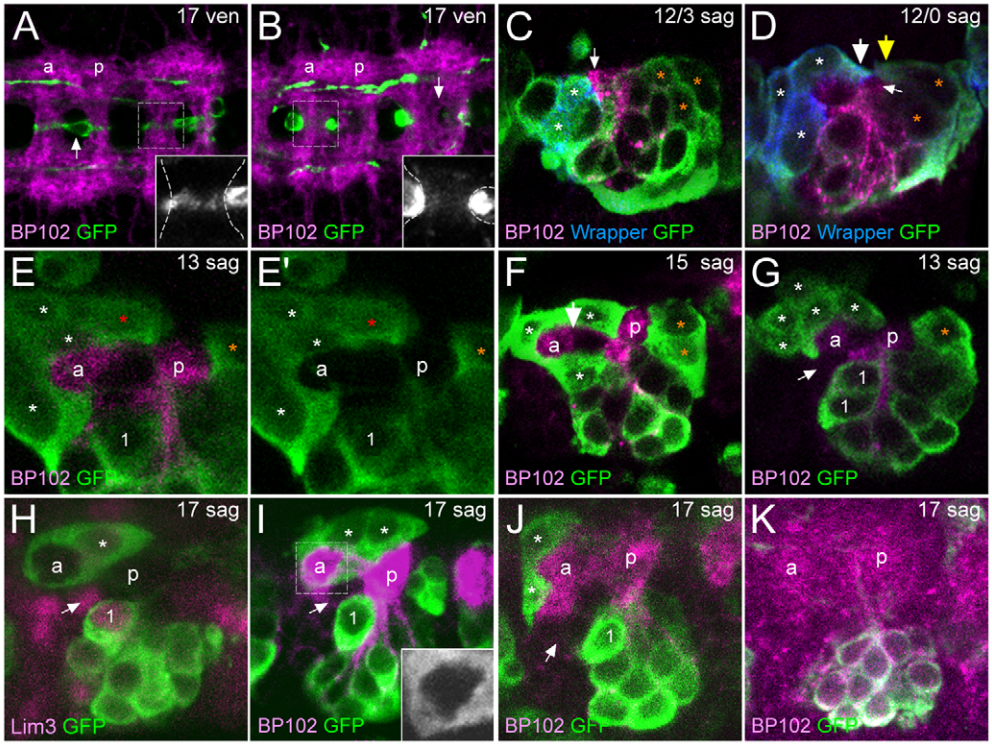

Fig. 2. *Nrx-IV* is required for MG migration and commissural axon ensheathment and subdivision. Wild-type (A) and *Nrx-IV* mutant *sim-Gal4 UAS-tau-GFP* (B-K) *Drosophila* embryos. (**A**) In wild type, AMG were between the AC and PC (arrow). Inset illustrates that AMG projections were present within the boundaries of the commissure (dashed lines). (**B**) In *Nrx-IV* mutants, the commissures were wider and poorly separated; AMG were not present between the commissures (arrow) and did not extend projections (inset). (**C**) *Nrx-IV* mutant AMG and PMG migrated dorsally toward the commissure (arrow). (**D**) AMG contacted the commissure (thin arrow) along its anterior side (white arrow), and the PMG contacted the commissure along its posterior side (yellow arrow). (**E**,**E′**) During stage 13, an AMG (red asterisk) migrated posteriorly across the AC, failed to ensheath the AC, and instead moved directly toward the PC. The PMG and MP1 neurons were positioned normally. (**F**) By stage 15, the AC was often surrounded by AMG, but AMG membranes were not closely associated with the AC along its posterior side (arrow). (**G**) At stage 13, AMG were often not in contact with the MP1 neurons (arrow indicates the gap) and the AC was not completely surrounded by AMG processes. (**H**) At stage 17 in *Nrx-IV* mutants, AMG were dissociated from the MP1 neurons (anti-Lim3); arrow indicates the gap. (**I**) AMG surrounded the AC, but not the PC, and AMG failed to send projections into the AC (inset) or PC. (**J**) Failure of AMG to surround either commissure. (**K**) AMG were completely absent; two commissures were apparent, but they were diffuse.

DEVELOPMENT

Table 1. *Nrx-IV* and *wrapper* MG phenotypes

Genotype	% Defective segments	Phenotypic classes		
		% Dissociated	% Incomplete	% Absent
Single mutant				
Nrx-IV^{4304}/TM3	3 (5/147)	40 (2/5)	0 (0/5)	60 (3/5)
Nrx-IV^{4304}/Nrx-IV^{4304}	95 (168/177)	68 (114/168)	23 (39/168)	9 (15/168)
wrapper^{175}/+	2 (4/185)	50 (2/4)	50 (2/4)	0 (0/4)
wrapper^{175}/wrapper^{175}	99 (137/139)	76 (104/137)	24 (33/137)	0 (0/137)
Double mutant				
wrapper^{175}/+; Nrx-IV^{4304}/+	2 (1/52)	0 (0/1)	100 (1/1)	0 (0/1)
wrapper^{175}/wrapper^{175}; Nrx-IV^{4304}/Nrx-IV^{4304}	96 (71/74)	30 (21/71)	68 (48/71)	3 (2/71)

Values in parentheses indicate the number of affected segments/total number of segments examined. For single mutants, segments A1-A7 were examined at stage 17 for defects in AMG migration using *sim-Gal4 UAS-tau-GFP* and anti-GFP to visualize all midline cells. AMG were identified based on morphology and position and the presence of Runt or Wrapper immunostaining. At stage 17, three phenotypes were observed: (1) Dissociated – dissociation of AMG from MP1 neurons and the failure of AMG to ensheath the PC; (2) Incomplete – dissociation coupled with a failure of AMG to ensheath the AC or PC (more severe than Dissociated alone); (3) Absent – complete absence of AMG. For double mutants, AMG were identified based on morphology, position and the presence of staining for the glial markers *argos* or *Ect3*. Homozygous *Nrx-IV* mutants were identified by the absence of Nrx-IV staining from the epidermis.

of Nrx-IV, we examined a protein-trap GFP fusion of Nrx-IV (Nrx-IV-GFP). The localization of Nrx-IV-GFP strongly resembled endogenous Nrx-IV protein with respect to cell type localization: it was present in neurons but absent or at low levels in MG (Fig. 3H,I). Although Nrx-IV-GFP localized at MG-neuron interfaces, it was less pronounced than endogenous Nrx-IV. Conversely, the Nrx-IV-GFP was more cytoplasmic than Nrx-IV and its localization was enhanced in the axon scaffold (Fig. 3I). Neither Nrx-IV immunostaining nor Nrx-IV-GFP was observed at appreciable levels at the membranes of AMG or PMG that were not contacting neurons (Fig. 3C,H). These data suggest that Nrx-IV is expressed in neurons and not MG.

To test the requirement for *Nrx-IV* in neurons or glia, we used a neural driver (*elav-Gal4*) and an MG driver (*slit-Gal4*) to express *UAS-Nrx-IV* in a protein-negative *Nrx^{4304}*-null mutant and assayed Nrx-IV protein localization and rescue of the *Nrx-IV* MG migration defects. In *elav-Gal4* rescue embryos, Nrx-IV was localized at the boundaries between neurons and both AMG and PMG (compare Fig. 3J with 3B), similar to wild type. Unlike in *Nrx-IV* mutants, in these rescue embryos AMG ensheathed the AC by stage 15 and migrated between the AC and PC (Fig. 3K-M; Table 2). However, by late stage 17, the AMG failed to ensheath the PC or to send projections into either commissure (data not shown). Thus, misexpression of *Nrx-IV* using *elav-Gal4* was able to rescue the initial steps of MG migration, but not the later aspects. We noted that Nrx-IV protein levels were lower in the rescue embryos from stages 15 to 17 as compared with wild-type embryos (data not shown), a result consistent with the decrease in *elav-Gal4* expression reported previously (Lin and Goodman, 1994). This might explain the lack of full rescue late in development. *slit-Gal4* rescue embryos did not show localization of Nrx-IV at MG-neuron boundaries (see Fig. S1L,N in the supplementary material) and the *Nrx-IV* mutant MG migration defects were not rescued either at stage 15 or 17 (Table 2; see Fig. S1L-O in the supplementary material). In summary, only when expressed in neurons did Nrx-IV accumulate at MG-neuron interfaces and partially rescue the *Nrx-IV* mutant phenotype.

Wrapper localizes to MG membranes and *wrapper* MG mutant phenotypes are identical to those of *Nrx-IV*

From stages 11-17, *wrapper* is expressed at high levels in AMG and at low levels in PMG (see Fig. S1E-H in the supplementary material) (Noordermeer et al., 1998; Wheeler et al., 2006). Unlike *Nrx-IV*, *wrapper* in situ hybridization showed strong MG expression (Fig. 4A). Wrapper protein was first observed at stage 12/3 and was present throughout AMG migration and ensheathment. The protein was uniformly localized on MG membranes (Fig. 4B), including where they contacted MP1 neurons (Fig. 4C), commissural axons (Fig. 4C,E), lateral CNS neuronal cell bodies (Fig. 4D) and other MG (Fig. 4B). At later stages, Wrapper was present on AMG projections during commissure subdivision (Fig. 4E).

Genetic analysis of *wrapper^{175}*-null mutant embryos revealed MG phenotypes that were identical to *Nrx-IV* mutant embryos, and to the *wrapper* phenotypes reported previously using electron microscopy (Noordermeer et al., 1998). At stage 12, the AMG and PMG of *wrapper* mutants migrated dorsally and contacted the commissures normally. Defects appeared after the AMG contacted the AC. By stage 15, AMG failed to ensheath the AC, and an AMG cell body was not interposed between the AC and PC (Fig. 4F). The *wrapper* mutant phenotype was highly penetrant: 99% of stage 17 segments possessed defects (n=139) (Table 1). In 76% of mutant segments, AMG surrounded the AC but failed to surround the PC (Fig. 4G), and in the other 24% of mutant segments, the AMG failed to surround either the AC or PC (Fig. 4H). In all segments examined, the AMG were dissociated from the MP1s (Fig. 4I) and failed to extend projections to subdivide the commissures (Fig. 4G). As in *Nrx-IV* mutants, the number of AMG present during stage 17 was reduced, from 3.1±0.4 (n=33) in wild type to 2.3±0.7 (n=14). Thus, *wrapper* is required for AMG migration, commissural ensheathment and commissure subdivision. Phenotypes of *Nrx-IV wrapper* double mutants were similar to, but more severe than, either single mutant: the same percentage of segments showed defects as in the single mutants, but more segments showed incomplete ensheathment of either the AC or PC (Table 1). One interpretation of the more severe double-mutant phenotype is that both Nrx-IV and Wrapper might be interacting with additional proteins on the apposing cell.

Nrx-IV membrane localization is dependent on Wrapper

In wild-type embryos, Nrx-IV protein was present at low levels throughout the membranes of neuronal cell bodies and axons, but was highly concentrated where they contacted AMG (Fig. 3B; Fig. 5A). The similarity of the *Nrx-IV* and *wrapper* mutant phenotypes coupled with the concentration of Nrx-IV opposite Wrapper^{+} MG membranes led us to hypothesize that the high-level membrane localization of Nrx-IV was dependent on the presence of Wrapper on the apposing membranes. To investigate this possibility, Nrx-IV localization was examined in *wrapper* mutant and misexpression

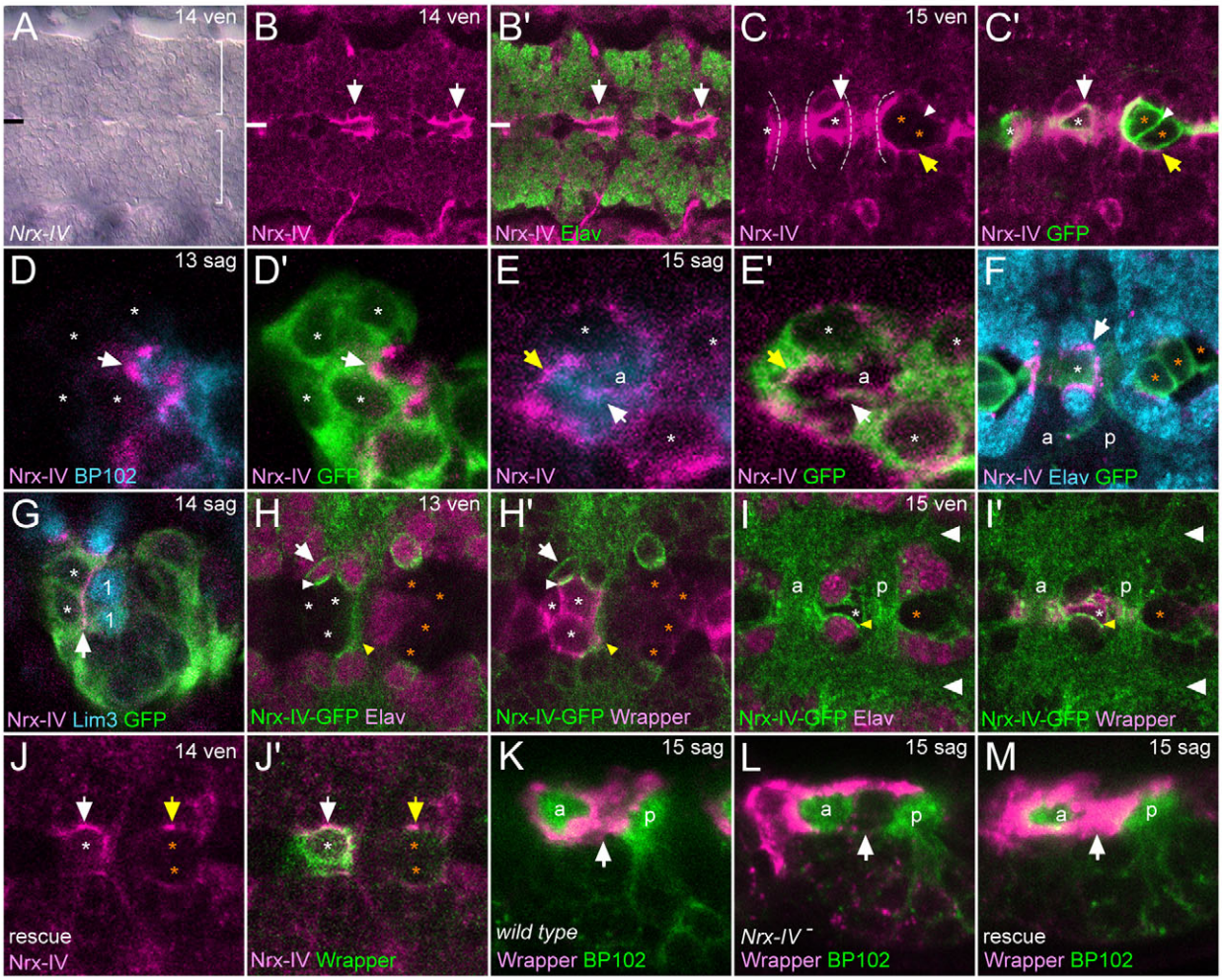

Fig. 3. Nrx-IV localizes to MG-neuron interfaces. (**A**) In situ hybridization for *Nrx-IV* RNA in wild-type *Drosophila* embryos showed relatively uniform low levels of expression throughout the CNS. Brackets, lateral CNS; horizontal bar, midline. (**B**,**B′**) Anti-Nrx-IV showed high protein concentrations (arrows) at the midline, and much lower levels in lateral CNS neurons (B′, anti-Elav). (**C**,**C′**) *sim-Gal4 UAS-tau-GFP* embryos were stained with anti-Nrx-IV and anti-GFP. Nrx-IV was mainly localized: (1) inside the commissures (between the dashed lines), and (2) along the borders of AMG (white arrow) and PMG (yellow arrow) with neurons. Staining was not observed at MG-MG boundaries (arrowhead). (**D**,**D′**) At stage 13, Nrx-IV was localized to the contact points (arrow) between AMG and the BP102$^+$ unseparated commissure. (**E**,**E′**) During stage 15, Nrx-IV was localized at the boundary of the BP102$^+$ AC with AMG (yellow arrow) and on AMG processes infiltrating the commissure (white arrow). (**F**) Nrx-IV was highly localized (arrow) to the membranes of Elav$^+$ lateral CNS neurons, where they contacted AMG. (**G**) At stage 14, Nrx-IV was highly concentrated at the long interface (arrow) between AMG and the Lim3$^+$ MP1 neurons. The Lim3$^+$ cells above the MG are non-midline cells. (**H**,**H′**) During stage 13, Nrx-IV-GFP localized to the membranes of Elav$^+$ neurons (arrow) and was enriched at the boundaries where neurons (white arrowhead) and commissural axons (yellow arrowhead) were adjacent to Wrapper$^+$ AMG. (**I**,**I′**) At stage 15, Nrx-IV-GFP was localized to axons in the commissures (a, p) and longitudinal connectives (white arrowheads), and to the contact points (yellow arrowhead) between neurons and Wrapper$^+$ AMG. (**J**,**J′**) At stage 14, in *Nrx-IV$^-$ elav-Gal4 UAS-Nrx-IV*, Nrx-IV was concentrated at the points of contact between neurons and both AMG (white arrow) and PMG (yellow arrow). (**K**) In stage 15 wild-type embryos, the AC (BP102) was ensheathed by AMG (Wrapper) and an AMG (arrow) was inserted between the AC and PC. (**L**) In *Nrx-IV$^-$*, AMG (Wrapper) failed to surround the AC (BP102) or migrate between the commissures; arrow indicates the position of the missing AMG. (**M**) Similar to wild type, *Nrx-IV$^-$ elav-Gal4 UAS-Nrx-IV* AMG (Wrapper) ensheathed the AC (BP102) and moved between the AC and PC (arrow).

embryos. In *wrapper* mutants, Nrx-IV was not concentrated at the contact points of neurons with MG, but was instead distributed uniformly around neuronal cell bodies (Fig. 5A,B) and axons (Fig. 5C,D) at higher levels than observed in wild-type embryos.

To determine whether Nrx-IV localization could be influenced by misexpressing *wrapper*, *en-Gal4* was used to express *UAS-wrapper* in a subset of CNS cells. In *en-Gal4 UAS-GFP-lacZ.nls* control embryos, cells that contacted GFP$^+$ cells did not show concentrations of Nrx-IV at their membranes (Fig. 5E). However, when *wrapper* was misexpressed in these cells, the Wrapper$^+$ cells induced high levels of adjacent Nrx-IV (Fig. 5F). Similar results were observed when *UAS-wrapper* was misexpressed in a different set of cells using *apterous-Gal4* (data not shown). These data indicated that Wrapper has the ability to induce Nrx-IV membrane localization on adjacent cell membranes. Interestingly, Nrx-IV accumulation only occurred in Wrapper$^-$ cells that bordered Wrapper$^+$ cells; Nrx-IV did not accumulate at high levels between adjacent Wrapper$^+$ cells (Fig. 5F). The ectopic neuronal Wrapper protein appeared more punctate than MG Wrapper, whereas the apposing Nrx-IV appeared relatively uniform. However, close

Table 2. *Nrx-IV* rescue experiments

Genotype	% Defective segments	
	Stage 15	Stage 17
Nrx-IV^{4304}/Nrx-IV^{4304}	93 (27/29)	90 (29/32)
elav-Gal4/+; UAS-Nrx-IV/+; Nrx-IV^{4304}/TM6	3 (1/34)	4 (1/28)
elav-Gal4/+; UAS-Nrx-IV/+; Nrx-IV^{4304}/Nrx-IV^{4304}	22 (7/32)	83 (25/30)
UAS-Nrx-IV/+; slit-Gal4 Nrx-IV^{4304}/TM6	3 (1/33)	0 (0/30)
UAS-Nrx-IV/+; slit-Gal4 Nrx-IV^{4304}/Nrx-IV^{4304}	92 (22/24)	96 (24/25)

Values in parentheses indicate the number of affected segments/total number of segments examined. MG migration was assayed using MG position, shape and anti-Wrapper staining. Additionally, in the *slit-Gal4* experiments, anti-Nrx-IV staining identified MG expressing UAS-Nrx-IV. Homozygous *Nrx-IV* mutants were identified by the absence of Nrx-IV staining from the epidermis.

examination (Fig. 5F) showed that Wrapper was also uniformly present along the membrane at low levels in addition to the presence of puncta. Nevertheless, the apparent difference in Wrapper levels between MG (endogenous Wrapper) and neurons (ectopic Wrapper) might indicate the existence of co-factors present in MG, but absent from neurons, that stabilize Wrapper at the membrane or prevent its degradation.

Nrx-IV and Wrapper proteins physically interact

The cellular and genetic experiments described above strongly suggested that Nrx-IV and Wrapper interact at the MG-neuron interface. To test this hypothesis, we carried out immunoprecipitation experiments from embryonic lysates using both Nrx-IV and Wrapper antibodies (Fig. 6A). Since *wrapper* is expressed in only a small number of embryonic cells, immunoprecipitation experiments were carried out in an *arm-Gal4 UAS-wrapper* strain, in which *wrapper* is expressed at high levels. Under these conditions, anti-Wrapper immunoprecipitated Nrx-IV, and anti-Nrx-IV immunoprecipitated Wrapper. Together with the localization of the proteins, these data strongly suggest that Nrx-IV and Wrapper directly bind at the MG-neuron interface.

Nrx-IV and Wrapper interact to induce cell adhesion in S2 cells

To test the hypothesis that Nrx-IV and Wrapper act as heterophilic cell adhesion partners, S2 cells were used to assay cell adhesion (Hortsch and Bieber, 1991). Immunoblot analysis of S2 cell

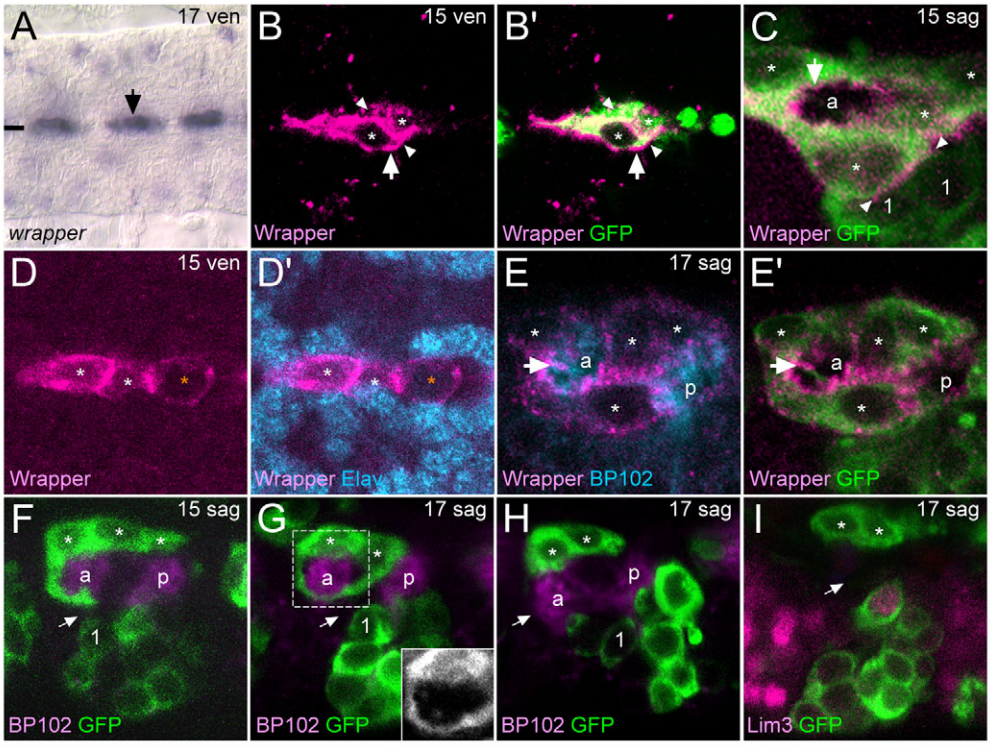

Fig. 4. *wrapper* is required for AMG migration and commissure ensheathment and subdivision. (**A**) *wrapper* in situ hybridization on wild-type *Drosophila* embryos showing expression in AMG (arrow). (B-E′) Wild-type and (F-I) *wrapper*$^{-}$ embryos containing *sim-Gal4 UAS-tau-GFP*. (**B**,**B′**) Wrapper protein was observed throughout the AMG, including the membrane (arrow), which lies just outside of cytoplasmic Tau-GFP staining. Wrapper is also present at MG-MG boundaries (between the arrowheads). (**C**) During AMG migration, Wrapper localized to AMG membranes surrounding the AC (arrow) and at the boundary with the MP1 neurons (between the arrowheads). (**D**,**D′**) Wrapper was present but not concentrated at the interfaces with Elav^{+} neurons. (**E**,**E′**) Wrapper localized along the MG projections (arrow) within the BP102^{+} AC and PC. (**F**) At stage 15, AMG failed to completely ensheath the AC, and migrated toward the PC. A gap was present between the AMG and MP1 neurons (arrow). (**G**) At stage 17, AMG loosely surrounded the AC, but not the PC. MG projections were absent from the AC (inset). (**H**) AMG failed to ensheath either commissure. (**I**) Lim3 showed the position of one MP1 neuron and its dissociation from the AMG.

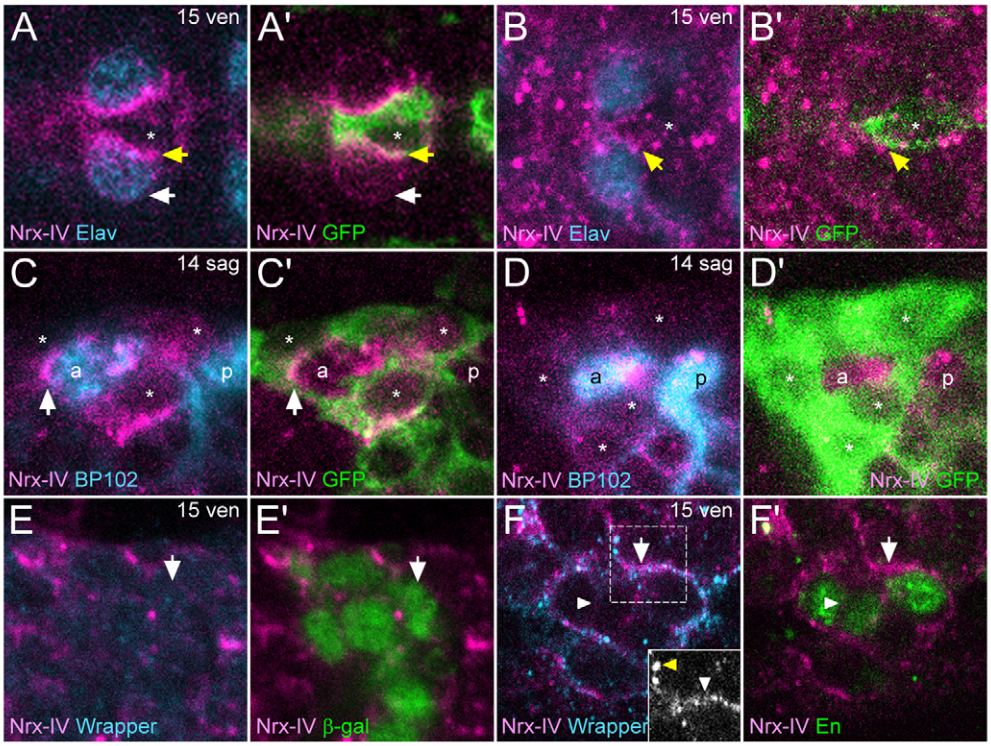

Fig. 5. Nrx-IV membrane localization is dependent on *wrapper*. (**A-D′**) *sim-Gal4 UAS-tau-GFP* (A,A′,C,C′) and *wrapper*$^{-}$ *sim-Gal4 UAS-tau-GFP* (B,B′,D,D′) *Drosophila* embryos. (A,A′) Nrx-IV was highly concentrated at the boundary of AMG and lateral CNS neurons (yellow arrow), and weakly present along the neuronal membrane not touching the AMG (white arrow). (B,B′) In *wrapper* mutants, Nrx-IV was not localized to the boundary with AMG (yellow arrow), but was instead uniformly localized around the entire membrane of lateral CNS neurons. (C,C′) Nrx-IV was concentrated at the boundaries between AMG and the BP102^{+} AC (arrow). (D,D′) In *wrapper* mutants, mislocalized Nrx-IV was present at high levels within the BP102^{+} AC and PC, rather than at the boundaries with AMG. Nrx-IV was not observed in AMG. (**E**,**E′**) In *en-Gal4 UAS-GFP-lacZ.nls*, Nrx-IV was not concentrated at the contact points between β-Gal^{+} and β-Gal^{-} cells (arrow). Wrapper was undetectable because it is not endogenously expressed in these cells. (**F**,**F′**) In *en-Gal4 UAS-wrapper* embryos, Nrx-IV was highly concentrated at the boundaries of Wrapper^{+} En^{+} cells at sites of contact with Wrapper^{-} En^{-} cells (arrow). Nrx-IV did not accumulate at the borders of adjacent Wrapper^{+} cells (arrowhead). Inset shows that Wrapper was either localized to the membrane (white arrowhead) or present in puncta (yellow arrowhead).

extracts revealed that Nrx-IV was abundantly present as a 155 kDa protein (Fig. 6B); by contrast, Wrapper was undetectable (data not shown). S2 cells were transfected at high concentration with *pAc-wrapper* and *pAc-Nrx-IV*. Transfection of *Nrx-IV* alone did not result in the formation of aggregates (Fig. 6D), whereas transfection of *wrapper* alone resulted in the formation of small aggregates (2-15 cells/aggregate) (Fig. 6E). Mixing together of *Nrx-IV*- and *wrapper*-transfected cells resulted in the appearance of large aggregates (>100 cells/aggregate) (Fig. 6F), indicating that Nrx-IV and Wrapper bind in trans and mediate cell adhesion.

Since transfection of *wrapper* alone in S2 cells induced small aggregates, we tested whether this aggregation was due to the presence of endogenous Nrx-IV or a different adhesion molecule. RNAi was used to remove endogenous Nrx-IV in cells transfected with *wrapper* alone. Two distinct dsRNAs (Nrx1 and Nrx2) each depleted Nrx-IV protein levels by more than 95% as compared with control levels (Fig. 6B). S2 cells were then treated with either *Nrx-IV* or control dsRNAs, transfected with *wrapper*, and assayed for aggregation (Fig. 6C). Cultures depleted of *Nrx-IV* showed only 12.5±0.7% and 23.4±6.2% of Wrapper^{+} cells in aggregates, respectively. By contrast, in cells treated with negative control dsRNA, 93.1±0.1% of Wrapper^{+} cells were found in aggregates of two or more cells. These observations indicated that Wrapper and Nrx-IV were able to induce cell adhesion only when both proteins were present.

To analyze Nrx-IV and Wrapper protein localization in the aggregates, we examined the small aggregates generated by transfection of *wrapper* alone. *wrapper*-transfected cells were readily identifiable by their prominent cortical labeling with anti-Wrapper antibody (Fig. 6G-I). The sites of cell-cell contact with Wrapper^{-} cells displayed a pronounced recruitment of Nrx-IV into cortical patches (Fig. 6G-I); these were never observed in untransfected cells. We did not observe enrichment of Wrapper to Nrx-IV^{+} patches, and did not see Nrx-IV^{+} patch formation at sites where two Wrapper^{+} cells were in contact (Fig. 6H,I). This phenomenon was also observed in *en-Gal4 UAS-wrapper* embryos, in which Nrx-IV was absent from sites of contact between Wrapper^{+} cells (Fig. 5F). This result might be due to the ability of high levels of Wrapper to saturably bind pools of Nrx-IV intracellularly – this competition would not leave sufficient Nrx-IV to bind to Wrapper in adjacent cells and form observable Nrx-IV^{+} membrane patches. Alternatively, Nrx-IV–Wrapper intracellular interactions could inhibit Nrx-IV transport and assembly into the membrane, or promote its internalization or degradation. Overall, these data show that expression of *wrapper* causes membrane accumulation of Nrx-IV similar to that observed in the embryonic CNS.

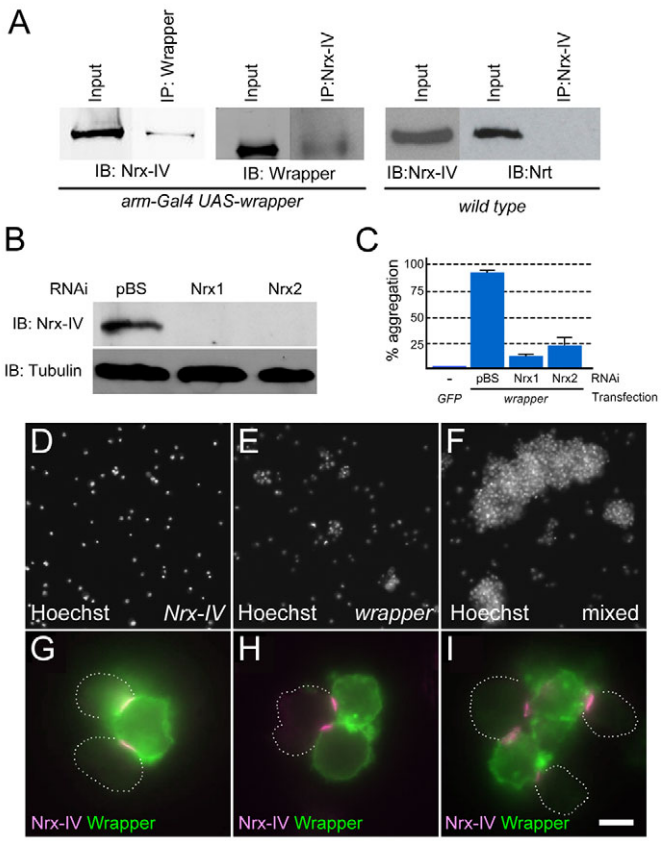

Fig. 6. Nrx-IV and Wrapper bind and mediate cell adhesion. (**A**) *arm-Gal4 UAS-wrapper* embryonic lysates were probed on immunoblots (IB) with anti-Nrx-IV after anti-Wrapper immunoprecipitation (IP), and with anti-Wrapper after anti-Nrx-IV immunoprecipitation. This showed that Wrapper and Nrx-IV exist in a protein complex. In negative control experiments on wild-type lysates, anti-Nrx-IV did not immunoprecipitate Neurotactin (Nrt). (**B**) Anti-Nrx-IV immunoblot showing the presence of Nrx-IV in cells treated with control pBluescript RNAi (pBS), and absence of Nrx-IV in cells treated with two different *Nrx-IV* dsRNAs (Nrx1, Nrx2). Anti-Tubulin controlled for input protein levels. (**C**) Summary of RNAi experiments. *y*-axis shows the percentage of Wrapper$^+$ cells found in aggregates with ⩾2 cells, averaged between two experiments. Error bars show s.d. (**D**,**E**) *Drosophila* S2 cells were transiently transfected with (D) *Nrx-IV* alone and (E) *wrapper* alone. (**F**) *Nrx-IV*- and *wrapper*-transfected cells were mixed together. (**G-I**) S2 cells were transiently transfected with *wrapper* and immunostained for Wrapper and Nrx-IV. Wrapper$^-$ cells are outlined (dotted lines). Scale bar: 5 μm

DISCUSSION

In this paper, we provide genetic, cellular and biochemical evidence that Nrx-IV and Wrapper physically interact to mediate cell adhesion between MG and neurons. The data presented here on *Nrx-IV* and *wrapper* mutant phenotypes are in close agreement with results previously published for *wrapper* (Noordermeer et al., 1998), and show that both genes have identical MG phenotypes, a likely outcome for two genes involved in heterophilic cell adhesion. The Nrx-IV–Wrapper interactions occur between MG and three neuronal cell types or structures: (1) MP1s, (2) cell bodies of lateral CNS neurons, and (3) commissural axons. Together, these interactions control the position of MG and the ensheathment and subdivision of commissures.

MG-MP1 interaction

In the present paper, we provide direct evidence that MP1 neurons closely interact with AMG, suggesting an important role in their development. Beginning at late stage 12, there is a strong accumulation of Nrx-IV at the interface between the MP1s and a subset of AMG, and the Nrx-IV concentration is maintained as the AMG migrate and ensheath the commissures. Nrx-IV accumulation at the MG-MP1 boundaries was abolished in *wrapper* mutants, and both *Nrx-IV* and *wrapper* mutants had gaps between the AMG and MP1s. These results indicate that the MP1s physically adhere to the AMG, and this adhesive interaction is required for proper positioning of MG and ensheathment of the commissures.

MG-lateral CNS neuronal cell body interaction

Nrx-IV protein is present in most, if not all, CNS neurons. However, protein levels are generally low. The exceptions are the neurons that flank the MG. These cells show a strong accumulation of Nrx-IV at the interfaces with MG. This indicates an aspect of midline cell biology not commonly considered – that MG interact closely with adjacent lateral CNS neurons. This might act to physically constrain migrating MG at the midline and restrict their lateral movement. In this sense, the lateral CNS neurons, the MP1 neurons and axon commissures work together to construct the MG cytoarchitectural scaffold. Alternatively, the adhesion between lateral CNS neurons and MG might allow developmental signals to pass between these cell types.

MG-commissural axon interaction

A key functional role of MG is their interaction with commissural axons, and these interactions require complex MG movements and morphological changes. The AMG extend cytoplasmic processes between the commissures, followed by an AMG cell body. These MG structures effectively partition the AC from the PC. Previously, it was proposed that commissure separation is caused by the interposition of MG into the unseparated commissure (Klambt et al., 1991). However, we noted that in *wrapper* mutants, the AC and PC were well separated, even though MG processes were commonly absent between the commissures (Fig. 4F-H). It is possible that in *wrapper* mutants, MG initially caused commissure separation and then quickly retracted or underwent apoptosis, indicating that MG function was required only transiently. Alternatively, commissure separation could be independent of MG interposition and the MG partition already-separated commissures. In contrast to *wrapper* mutants, *Nrx-IV* mutants have poorly separated commissures. This difference is most likely to reflect an additional function of *Nrx-IV* because: (1) the MG phenotypes were similar between *Nrx-IV* and *wrapper* mutants, (2) the mutants of each gene were null, (3) neither had a recognizable maternal effect, and (4) *Nrx-IV* was more widely expressed.

Throughout commissure ensheathment, axons have strong accumulations of Nrx-IV along their interface with the AMG. This suggests a continual requirement of Nrx-IV and Wrapper to mediate MG-axonal adhesion and is consistent with the wide variety of MG-axon adhesion defects observed in both *Nrx-IV* and *wrapper* mutants and the inability of *elav-Gal4 UAS-Nrx-IV* to rescue late *Nrx-IV* mutant phenotypes. By contrast, MG remained relatively well associated with each other, suggesting that neither *wrapper* nor *Nrx-IV* plays an important role in MG-MG adhesion.

MG projections also subdivide each commissure into discrete compartments. Previous work employing electron microscopy proposed that the MG subdivided each commissure into three

dorsoventral regions (Stollewerk and Klambt, 1997). This subdivision also requires *Nrx-IV* and *wrapper* function because Nrx-IV and Wrapper accumulated in the AMG commissural projections, and the projections were absent in both *Nrx-IV* and *wrapper* mutants (Noordermeer et al., 1998). Both the organizing principles and the significance of these commissural subdomains are unknown, and it remains to be determined whether the MG are a cause of the subdivision or are filling in axonal regions that are already subdivided.

Perspectives on MG migration

The view of MG migration presented here builds on previous work, but also differs in several aspects. These include nomenclature, MG-neuron interactions and PMG migration. Klambt et al. (Klambt et al., 1991) proposed a model in which three pairs of MG (MGA, MGM and MGP) arise in the anterior of the segment and, during migration, separate and ensheath the AC and PC. The MGA and MGM migrate posteriorly and ensheath the AC; the MGA ultimately resides anterior to the AC and the MGM between the AC and PC. By contrast, the MGP migrate anteriorly from the adjacent posterior segment and partially ensheath the PC. More recent observations, including some from this paper, point toward a different view. Analysis of 52 genes expressed in MG (Kearney et al., 2004) indicates that (to date) only two distinct MG cell types can be identified, which we have termed AMG and PMG. There are six AMG in the anterior of the segment (this class includes MGA and MGM, which, to our knowledge, cannot be distinguished molecularly) and four PMG that reside in the posterior of the segment and are identical to MGP in terms of gene expression. Of the six initial AMG, only three survive (Bergmann et al., 2002). These cells migrate posteriorly, ensheath both the AC and PC, and elaborate projections into the commissures. By contrast, all PMG die by stage 17 (Dong and Jacobs, 1997; Sonnenfeld and Jacobs, 1995), and therefore do not ensheath the PC. Initially, it was proposed that PMG/MGP migrate from the adjacent posterior segment. In our experiments, we see no evidence for this. Instead, PMG arise in the En^+ posterior of the segment and migrate anterodorsally toward the commissure. Before undergoing apoptosis, a single PMG abuts the PC from the posterior side. Thus, the PMG are positioned to influence commissure development.

Neurexin IV and immunoglobulin superfamily protein interactions

The experiments described in this paper strongly support the view that Nrx-IV and Wrapper directly bind and mediate cell adhesion. By contrast, neither protein mediates homophilic cell adhesion (Baumgartner et al., 1996; Noordermeer et al., 1998). Wrapper is an Ig superfamily protein, and experiments in both flies and vertebrates indicate that Nrx-IV can bind to additional Ig superfamily proteins. In *Drosophila* septate junctions, Nrx-IV forms a complex with Contactin and Neuroglian (Faivre-Sarrailh et al., 2004), which are two Ig superfamily proteins. It was proposed that Nrx-IV binds to Contactin at the membrane in a cis configuration (Faivre-Sarrailh et al., 2004), and that Contactin is required for proper Nrx-IV membrane localization (Laval et al., 2008). Contactin is present in the CNS, and might play a similar role in neurons. It is unknown whether Nrx-IV binds Neuroglian or Contactin in trans, similar to the mechanism proposed here for Nrx-IV–Wrapper binding. However, at paranodal axo-glial junctions in mice, the Nrx-IV homolog Caspr binds in cis to contactin, and in trans to the Neuroglian homolog neurofascin. In summary, Nrx-IV binding to Wrapper indicates a general feature of Nrx-IV, which is its ability to bind diverse Ig superfamily proteins.

One of the remarkable aspects of Nrx-IV is its strong membrane accumulation at sites where neurons are apposed to MG. In one sense, this resembles the accumulation of Nrx-IV in septate junctions. However, from a mechanistic perspective the situation appears different. In septate junctions, Nrx-IV membrane localization is constrained by interactions with Contactin and Neuroglian (Faivre-Sarrailh et al., 2004; Laval et al., 2008), as well as with cytoskeleton-associated proteins important for membrane localization (Laval et al., 2008; Ward et al., 1998b; Wu et al., 2007). By contrast, the localization of Nrx-IV in neurons appears relatively fluid and dispersed, only accumulating at high levels when in contact with a $Wrapper^+$ membrane. It remains possible that once Wrapper and Nrx-IV bind, additional proteins might bind to Nrx-IV to stabilize its membrane localization. These interactions could further regulate the dynamics of MG-neuron interactions.

We thank Jasperina Noordermeer for fly stocks; Raehum Paik for generating the anti-Wrapper guinea pig antibody; the Developmental Studies Hybridoma Bank, the Bloomington *Drosophila* Stock Center, the East Asian Distribution Center for Segmentation Antibodies; and FlyTrap for strains and reagents. This work was supported by NIH grants R37 RD25251 to S.T.C., NS050356 to M.A.B., GM081645 to S.L.R. and an NRSA postdoctoral fellowship to S.R.W. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/7/1147/DC1

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