

Drosophila Neurexin IV stabilizes neuron-glia interactions at the CNS midline by binding to Wrapper

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Ensheathment of axons by glial membranes is a key feature of complex nervous systems ensuring the separation of single axons or axonal fascicles. Nevertheless, the molecules that mediate the recognition and specific adhesion of glial and axonal membranes are largely unknown. We use the *Drosophila* midline of the embryonic central nervous system as a model to investigate these neuron-glia interactions. During development, the midline glial cells acquire close contact to commissural axons and eventually extend processes into the commissures to wrap individual axon fascicles. Here, we show that this wrapping of axons depends on the interaction of the neuronal transmembrane protein Neurexin IV with the glial Ig-domain protein Wrapper. Although Neurexin IV has been previously described to be an essential component of epithelial septate junctions (SJ), we show that its function in mediating glial wrapping at the CNS midline is independent of SJ formation. Moreover, differential splicing generates two different Neurexin IV isoforms. One mRNA is enriched in septate junction-forming tissues, whereas the other mRNA is expressed by neurons and recruited to the midline by Wrapper. Although both Neurexin IV isoforms are able to bind Wrapper, the neuronal isoform has a higher affinity for Wrapper. We conclude that Neurexin IV can mediate different adhesive cell-cell contacts depending on the isoforms expressed and the context of its interaction partners.

KEY WORDS: *Drosophila*, Midline glia, Neurexin IV, Wrapper, Neuron-glia interaction

INTRODUCTION

In complex nervous systems neuron-glia interactions play pivotal roles in forming and maintaining neuronal circuits. From early developmental stages onwards, reciprocal signaling between neurons and glial cells ensures the balanced formation of correct cell numbers and cell types and their subsequent differentiation. During neuronal differentiation, glial cells often act as intermediate targets or guidepost cells, instructing neuronal growth cones on their path towards their final destination (Bastiani and Goodman, 1986; Bentley and Caudy, 1983; Whittington et al., 2004). Subsequently, once the axonal trajectories are established, glial cells migrate along these tracts to ensure that all axons are regularly covered with glial cells. Using signals that remain largely elusive, the glia starts to differentiate into the different insulating glial cell layers (Birchmeier and Nave, 2008; Brinkmann et al., 2008).

The CNS midline of *Drosophila*, which comprises only 22 cells with known lineage and transcriptional profile, provides a valuable model with which to study the manifold interactions between neurons and glial cells (Bossing and Technau, 1994; Jacobs, 2000; Kearney et al., 2004; Klämbt et al., 1991; Wheeler et al., 2006; Wheeler et al., 2008). Early during CNS development, the midline glial cells provide important information for the correct establishment of the intricate axonal lattice, as they secrete both Netrin and Slit, which subsequently instruct the navigation of commissural growth cones towards and across the CNS midline (Brankatschk and Dickson, 2006; Dickson and Gilestro, 2006; Kaprielian et al., 2001). Within the midline, the glia interact not only

with the crossing commissural axons but also with specific midline neurons (Jacobs, 2000; Jacobs and Goodman, 1989; Klämbt et al., 1991). These neuron-glia interactions initially guide the formation of distinct anterior and posterior commissures, and later allow the ensheathment and subdivision of the segmental commissures into several discrete fascicles (Stollewerk and Klämbt, 1997; Stollewerk et al., 1996).

Although a number of genetic screens identified components of major signaling cascades regulating midline glia development (Hummel et al., 1999; Seeger et al., 1993), no mutation was recovered that directly affected the interaction of glial cells with commissural axons at the CNS midline. The *wrapper* gene was identified in a reverse genetic screen for secreted and transmembrane proteins expressed in the *Drosophila* CNS (Noordermeer et al., 1998). The GPI-anchored immunoglobulin (Ig) domain protein Wrapper is expressed on the surface of midline glia and loss of the protein leads to defects in the wrapping of commissural axons and impairs viability (Noordermeer et al., 1998). The Wrapper protein is present in all *Drosophilidae* (72–95% identity over the entire protein length) and the honeybee (38% identity over the entire protein length); however, no clear Wrapper orthologs can be identified in mammals. How Wrapper mediates neuron-glia interaction at the CNS midline and what molecular interaction partners might be involved remains unclear.

Here, we show that Wrapper mediates its effects through the evolutionary conserved Neurexin IV protein expressed on axonal membranes. Neurexin IV has been previously known for its role during the formation of septate junctions in epithelial tissues, which resemble the septate junctions found at the paranode of vertebrates (Baumgartner et al., 1996; Bhat, 2003; Peles et al., 1997a; Poliak and Peles, 2003). In this work, we demonstrate that *Neurexin IV* (*Nrx-IV*) mutants have defects in neuron-glia interaction in the CNS midline. In *Nrx-IV* mutant embryos, Wrapper-expressing midline glia processes do not wrap individual fascicles within the commissures. Cell type-specific rescue experiments indicate that

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Neurexin IV is not required in the midline glia, but is rather provided by the neurons. Subsequent genetic and cell culture experiments demonstrate that neuronally supplied Neurexin IV binds to the glial expressed Wrapper protein and is able to attract and stabilize glial processes. The interaction between these two proteins does not result in the formation of septate junctions. In line with this observation, typical septate junction proteins are not recruited to sites of Neurexin IV-Wrapper contact at the CNS midline. We show that *Neurexin IV* encodes two isoforms that differ only in their extracellular Discoidin domain (Neurexin IV^{exon3} or Neurexin IV^{exon4}), and that both isoforms are differentially expressed in the nervous system. The Neurexin IV^{exon3} protein is expressed in cells that form septate junctions, whereas the Neurexin IV^{exon4} isoform is expressed by neurons, which contact the Wrapper-expressing midline glial cells but do not form septate junctions. Further cell aggregation and rescue experiments indeed show that Wrapper preferentially binds to the Neurexin IV^{exon4} isoform. This study demonstrates that Neurexin IV can participate in adhesive functions other than septate junctions and we present Wrapper as the first trans binding partner that mediates the novel adhesive properties.

MATERIALS AND METHODS

DNA work

The *Neurexin IV* cDNA RE18634 (obtained from the Drosophila Genomics Resource Center) carried a point mutation in the open reading frame. The cDNA was corrected following site-directed mutagenesis towards the published genomic sequence. No cDNA corresponding to the exon 4 variant was available from the DGRC. To generate such a cDNA, we isolated mRNA from stage 14–16 embryos. Following reverse transcription, the exon 1- to 4-containing DNA fragment was amplified: forward primer, CACCATGA-GGCCGCCCAGGAGCAATACG; reverse primer, GATGGCGAGTTCT-GGCGCTCCT. All 3' exons were added by standard cloning technologies to generate the full-length 'cDNA' was generated by standard cloning technologies. The Myc-tags were generated via custom-made DNA. All clones generated were verified by sequencing. Further details on primers and the generation of constructs are available upon request. The pUASTattB vector was kindly provided by K. Basler (Bischof et al., 2007). The constructs were initially generated in pEntry vector and then recombined using the gateway system into the pUASTattB-rfA vector that was generated by standard cloning technology. For mRNA isolation, embryos were fixed in 4% PFA/n-heptane for 30 minutes at 4°C. After several washes at 4°C, tissue-specific mRNA was isolated according to a previously published protocol (Yang et al., 2005) using a human flag-tagged PABP.

Protein work

The following peptide sequence was used to generate specific rabbit anti-Neurexin IV antiserum: HSTGHQVRKRTIEFI. Immunizations were performed by Davids (Regensburg, Germany). The following antibodies were used: anti-GFP (1/1000, Invitrogen); anti-HRP Cy5 (1/150 Dianova); anti-β-Spectrin [1/200 (Hulsmeier et al., 2007)]; 10D3 anti-Wrapper 1/5, (Noordermeer et al., 1998); anti-Coracle, 1/50 [CBI antibody facility, Southwestern University, AZ (now Abcam)]; and anti-β-Galactosidase (1/1000, Cappel). Antibody staining was performed as described (Stork et al., 2008) except for Wrapper staining, where embryos were incubated in methanol for 60 minutes at 37°C after fixation.

Cell culture

Drosophila S2 cells (obtained through the *Drosophila* genomics resource center) were grown and transfected as published previously (Bogdan et al., 2005). Generally, transfection efficiencies around 30% were obtained. Cells were co-transfected with UAS-based constructs and act::Gal4 DNA and kept for 2 days at 25°C before the aggregation experiments. For aggregation, 2 × 10⁶ cells per ml Sf9 medium (PAA laboratory, Austria) were incubated at room temperature on a shaker at 100 rpm for 1.5 hours. Cell aggregates were transferred onto coverslips and stained according to standard procedures (Bogdan et al., 2005).

Genetics

Flies were raised on standard food at 25°C or room temperature. The following genotypes were used: *Df(3L)Exel6116*; *nrxIV^{Δ304}*; *nrxIV^{EY06647}*; *nrx^{EP604}*; *nrg¹⁴*; *nrg¹⁷* (Bloomington); *nrxIV^{Δ025}* (Baumgartner et al., 1996); *wrapper^{Df(2R)02114}* (Noordermeer et al., 1998); *nrg^{RA35}* (Hall and Bieber, 1997); *cora⁴*; *cora⁵* (Lamb et al., 1998); *lac^{BG01462}*; *lac²* (Llimargas et al., 2004); *nrv^{2k13315}*; *nrv^{223B}* (Genova and Fehon, 2003); *slitGal4*; *elavGal4*; *enGal4*; *daGal4*; *eagleGal4* (all from the Bloomington stock center); *AA142Gal4* (U. Lammel and C.K., unpublished); *connectinGal4* (Lattemann et al., 2007); UAS Wrapper (Noordermeer et al., 1998); *nrg^{GFP}* (Morin et al., 2001); *lac^{GFP125}*; *nrxIV^{GFP454}* (Edenfeld et al., 2006); and *nrv^{2GFP173}* (Stork et al., 2008). To generate transgene insertions in the same chromosomal landing site, we employed the *phiC31* system and used the 51D landing site according to published protocols (Bischof et al., 2007).

RESULTS

Midline glia wraps commissural axons

The CNS axon patterning of a *Drosophila* embryo is characterized by two segmental commissures that connect the two halves of the nervous system and by the longitudinal connectives that bridge the individual neuromeric units. During development, commissures are initially formed across the cells of the CNS midline. The posterior commissure is established first, followed by the anterior commissure. Initially, axon bundles of the two segmental commissures form in close proximity but ultimately they are separated spatially by the midline glial cells. Briefly, midline glial cells are born anterior to the forming commissures, migrate to a position between the presumptive anterior and posterior commissure, and finally separate these axon bundles in two clear and distinct axon tracts (Fig. 1A,C,E). In mutants with no functional midline glial cells, the commissures cannot be separated and appear fused (Klamt et al., 1991).

In stage 16 embryos long after distinct commissures are formed, the midline glial cells ensheath individual axon fascicles (Fig. 1E,F). The gene *wrapper* has previously been identified to be required for the wrapping of commissural axons and encodes a GPI-anchored Ig-domain protein (Noordermeer et al., 1998). The Wrapper protein is expressed by the midline glia throughout their development. Initially, it is evenly distributed within the midline glial cell membrane but later appears to concentrate at sites of direct contact of glial wrapping processes with commissural axons (Fig. 1). Although its molecular function remains unclear, it does not appear to act as a homophilic adhesion protein (Noordermeer et al., 1998).

Neurexin IV is expressed in the embryonic CNS midline

In a previous screen for GFP-tagged exon-trapped proteins, we identified a Neurexin IV::GFP insertion line (Edenfeld et al., 2006). Using this expression reporter or antibody staining, we noted Neurexin IV expression in the CNS midline of stage 12 embryos. To determine which cell type expressed Neurexin IV, we employed a set of specific marker strains. In embryos expressing CD8::GFP under the control of the midline glial drivers *slitGal4* or *AA142Gal4* (data not shown) or by staining for Wrapper protein, the outline of the midline glial cells can be visualized. We found Neurexin IV expression was enriched at contact sites of neuronal cell bodies and midline glia (Fig. 1C,G). During later stages, the expression pattern refines and Neurexin IV was found to be strongly co-localized with Wrapper at axon contact sites (Fig. 1E,F, arrowhead). Low levels of Neurexin IV expression could also be detected in the neuropil and along nerve tracts, arguing that Neurexin IV is expressed by neuronal lineages (Fig. 1A,E, see below).

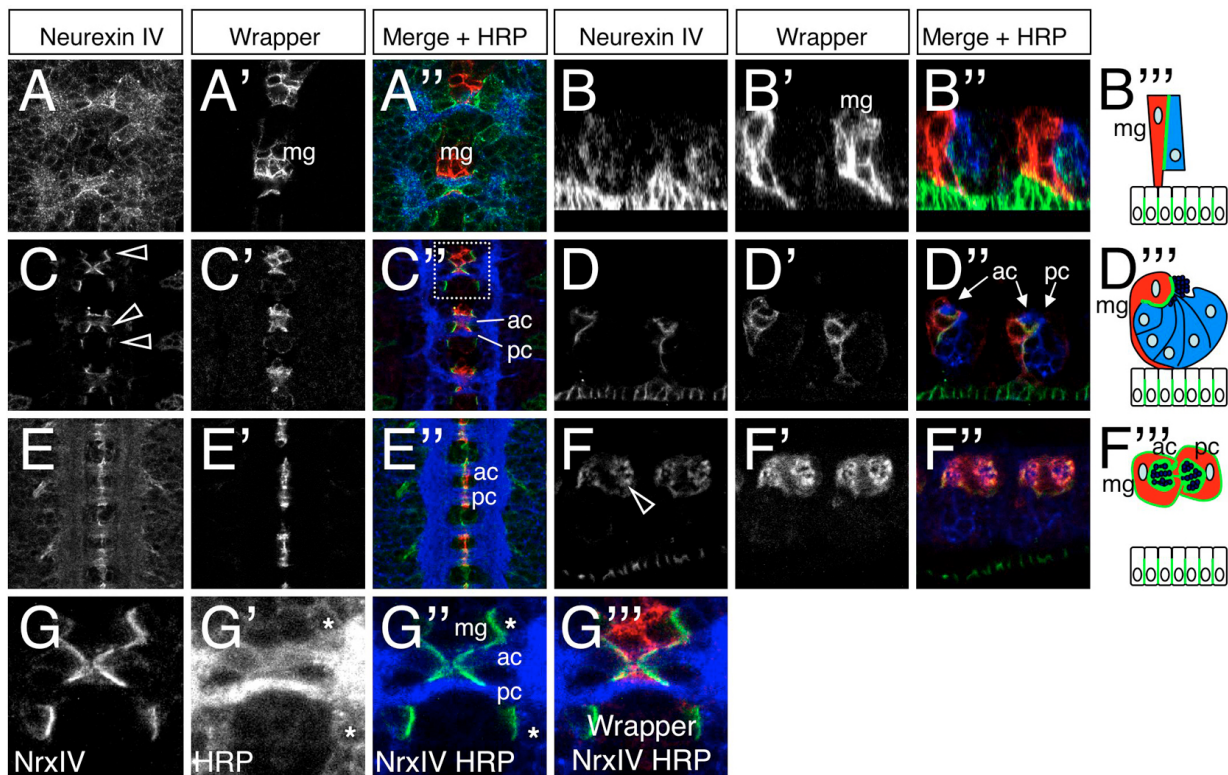


Fig. 1. Localization of Neurexin IV during the formation of commissures. Frontal, anterior upwards (A,C,E,G), and sagittal, anterior leftwards (B,D,F), views of developing commissures in the *Drosophila* embryo. To detect Neurexin IV localization, we used the GFP-exon trap insertion 454 and subsequent GFP staining (green in A",B") or used specific Neurexin IV antibodies (green in C",D",E",F",G",G"). The shape of the midline glial cells (mg) is highlighted by staining for Wrapper expression (red). Neuronal membranes are stained by anti-HRP antibodies (blue). Single confocal sections are shown. B",D",F" schematic representations of B", D" and F". The midline glia (mg) are shown in red, midline neurons are depicted in blue and green highlights Neurexin IV expression. (A,B) In stage 12 embryos, the midline glial cells are still anterior to where the commissures cross the midline. Prominent Neurexin IV expression can be detected in neurons and their axonal projections. (C,D) In stage 14 embryos, both segmental commissures (ac, anterior commissure; pc, posterior commissure) have been established. The midline glial cells are migrating between the commissures. Neurexin IV expression accumulates at neuron-glia contact sites at the CNS midline (arrowheads). The outlined area in C" is enlarged in G-G". (E,F) In stage 16 embryos, the midline glial cells have finished the wrapping of the segmental commissures and have extended processes within the commissural tracts. An intense overlap of Neurexin IV and Wrapper at axon-glia contact sites can be observed (arrowhead). (G) Neurexin IV accumulates at the interface of neuronal cell bodies (asterisks) stained with HRP (G', blue in G" and G") and the midline glia are highlighted by Wrapper staining in red (G").

Neurexin IV is required for wrapping of commissural axons

The above noted expression patterns suggested a requirement of *Neurexin IV* for commissural development. We thus analyzed several *Neurexin IV* mutants as homozygotes or in trans to a deficiency uncovering the *Nrx-IV* locus [*Df(3R)Exel6116*]. All embryos derived from these *Neurexin IV* mutants showed severe defects in midline glial commissural wrapping (Fig. 2B). Although the initial commissure development was normal, in stage 16 *Neurexin IV* mutant embryos the midline glial cells failed to extend processes into the commissures and did not properly wrap individual axon fascicles (Fig. 2B; similar results were obtained when following glial processes using a *slit>lacZ* reporter in *Neurexin IV* mutants, data not shown). In contrast to wild type, *Neurexin IV* mutant midline glial cell membranes were found around the segmental commissures but not within the axon bundles. Although midline glial cells often still surrounded the anterior commissure, more than 90% of the posterior commissures were affected and lacked a glial sheath altogether, or were covered only by processes on the dorsal or ventral side (Fig. 2A',B'; $n > 100$ neuromeres). Given its apparent neuronal

expression, Neurexin IV is thus required for normal neuron-glia interaction at the CNS midline and mediates the wrapping of commissural axons.

Neurexin IV has been extensively characterized as an essential component of septate junctions (Baumgartner et al., 1996; Faivre-Sarrailh et al., 2004; Schulte et al., 2003). In order to test whether the *Neurexin IV* mutant phenotype could be ascribed to its role in septate junction formation, we analyzed whether other septate junction components were also required for the wrapping of commissural axons. We determined both the CNS midline expression and phenotype of *neuroglian*, *coracle*, *lachesin* and *nervana2* mutants (Genova and Fehon, 2003; Lamb et al., 1998; Llimargas et al., 2004; Paul et al., 2003; Strigini et al., 2006). In no case we could detect co-expression of any of these molecules with Neurexin IV at the midline nor did we detect any phenotypic abnormalities (Fig. 2C-E; see Fig. S1 in the supplementary material; data not shown), indicating that Neurexin IV does not participate in the formation of septate junctions at the CNS midline. In line with these observations, no septate junctions between midline glia and commissural axons were detected in ultrastructural analyses (Jacobs and Goodman, 1989; Stollewerk and Klämbt, 1997; Stollewerk et al., 1996).

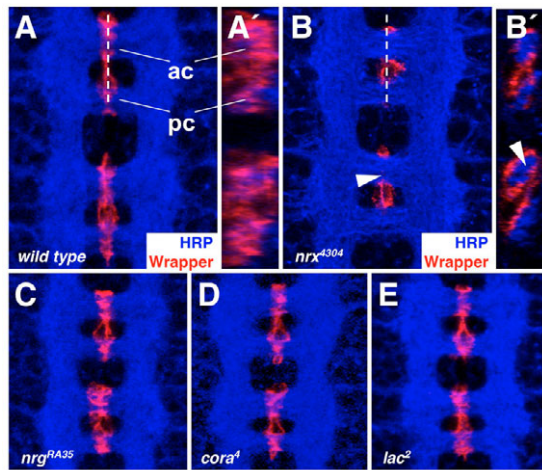


Fig. 2. Neurexin IV is required for axonal wrapping. Frontal views of CNS preparations of stage 16 embryos, the genotype is indicated, anterior is upwards. Axonal membranes are shown in blue (HRP staining), the shape of the midline glial cells is marked by Wrapper expression (red). Single confocal sections are shown. (A', B') Orthogonal sections through the z-stack of confocal images. The plane of section is indicated by a broken line in A, B. (A) In the wild-type CNS, anterior (ac) and posterior (pc) commissures are clearly established and axonal membranes are wrapped by Wrapper-positive midline glial cell processes. (A') In the orthogonal view of the midline the intense ingrowth of midline glial cell processes into the commissural tracts can be seen. (B) In *Neurexin IV* mutant embryos, commissures form normally, but the midline glial cells fail to establish processes within the commissural axon tracts (arrowhead). (B') In the orthogonal section, it becomes obvious that the midline glial cells grow around the commissural axon bundle but do not establish wraps of individual axonal fascicles (arrowhead). (C–E) In mutant *neuroglian* (C), *coracle* (D) or *lachesin* (E) embryos, neither the formation of commissures nor the wrapping of the commissural axons is affected. The midline glial cells establish intensive contacts with commissural axons.

Neurexin IV is provided by the CNS axons

Neurexin IV expression is enriched where the midline glia contacts neurons, especially commissural axons (Fig. 1), and loss of Neurexin IV results in a midline glial wrapping phenotype. To test whether Neurexin IV was provided by the midline glia or by the neurons contacting the glia, we performed cell type-specific rescue experiments. To express *Neurexin IV* in a mutant background we used an EP Insertion in the 5' region of *Neurexin IV* (*EP604*), which disrupts endogenous expression of the gene but allows directed expression via the UAS sites in the transposable element (Rorth, 1996) (Fig. 5A). This approach allowed tissue-specific expression of *Neurexin IV* from the endogenous locus in a *Neurexin IV* mutant background (Fig. 3). Embryos of the genotype *EP604/Df(3L)Exel6116* or *EP604/nrx⁴³⁰⁴* showed the typical *Neurexin IV* mutant phenotype (Fig. 2B, data not shown). When we expressed *Neurexin IV* in midline glia cells of such embryos using the *slit*Gal4 driver or *A1142*Gal4, no phenotypic rescue was observed (data not shown). Likewise, expression of *Neurexin IV* in all midline cells (neurons and glial cells) from early stages on with the *sim*Gal4 driver (Scholz et al., 1997) did not rescue the wrapping behavior (Fig. 3A), demonstrating that Neurexin IV is not required in the midline during axonal wrapping. In addition, when we provided Neurexin IV expression in all lateral glial cells using the *repo*Gal4 driver no rescue was observed (data not shown). However, when we used the *elav*Gal4

driver, which is active in all neurons and some glial cells (Berger et al., 2007), we obtained a full phenotypic rescue and observed normal wrapping of commissural axons (Fig. 3B).

Increased neuronal Neurexin IV expression can redirect glial processes

Interestingly, after strong overexpression of *Neurexin IV* in a mutant background in all neurons (*EP604 elavGal4/EP604 elavGal4*), we frequently noted an expanded expression domain of Wrapper within the commissures at the CNS midline when compared with wild-type embryos (data not shown). To address whether Neurexin IV could act in an 'axon-autonomous' way, we expressed *Neurexin IV* in a subset of commissural axons using *eagle*Gal4 (Dittrich et al., 1997) in the *Neurexin IV* mutant background. In this situation, we frequently observed restored glial wrapping specifically in the anterior *eagle* positive fascicle (Fig. 3C). In other cases, we observed ectopic protrusions of midline glial processes along the Neurexin IV-expressing neurites (Fig. 3E). When we re-expressed Neurexin IV only in a few ipsilateral-projecting neurons using the *connectin*Gal4 driver (Lattemann et al., 2007), we were also able to redirect Wrapper localization. Expression of Neurexin IV in the *connectin* pattern caused the extension of long cell protrusions of the midline glial cells along the axon bundles expressing Neurexin IV (Fig. 3D). These glial cell processes probably originate from gliopodia that explore the environment and search for appropriate neuronal targets (Vasenkova et al., 2006). In conclusion, we show that Neurexin IV is a key factor for recruitment and stabilization of individual midline glial processes and that this neuron-glia recognition occurs locally on the level of individual axons.

Ectopic Wrapper expression redirects Neurexin IV localization

Above, we have demonstrated that neuronally expressed Neurexin IV can recruit glial processes expressing the Wrapper protein, suggesting that the two proteins bind to each other to stabilize neuron-glia interaction at the midline. To test whether loss of Wrapper expression in the midline glia would also result in changes in neuronal Neurexin IV expression, we stained Wrapper-deficient embryos for Neurexin IV expression. Whereas the epidermal expression of Neurexin IV in such embryos was normal, Neurexin IV expression at the midline was lost (Fig. 4B). Reciprocally, overexpression of Wrapper in all neurons resulted in a depletion of Neurexin IV from the midline (Fig. 4C, arrowhead) and a redirection of Neurexin IV expression to neuronal cell bodies (compare Fig. 4A, C, asterisks). We conclude that, within the nervous system, localized expression of Wrapper at the midline recruits neuronal Neurexin IV to the midline.

Additionally, when we expressed Wrapper ectopically in the epithelial tissue of the hindgut, we frequently observed a recruitment of Wrapper to sites of Neurexin IV expression in the apical region of the basolateral membrane compartment (Fig. 4D). These regions have been shown to correspond to sites of septate junction formation. Thus, within epithelia, ectopic Wrapper can be recruited to regions of septate junctions, further suggesting an interaction with Neurexin IV.

Neurexin IV encodes two differentially expressed isoforms

The type I transmembrane protein encoded by the *Drosophila Neurexin IV* gene is not a typical member of the well-known vertebrate Neurexin family of synaptic proteins (Missler et al., 1998). Rather, it shares structural features with the vertebrate

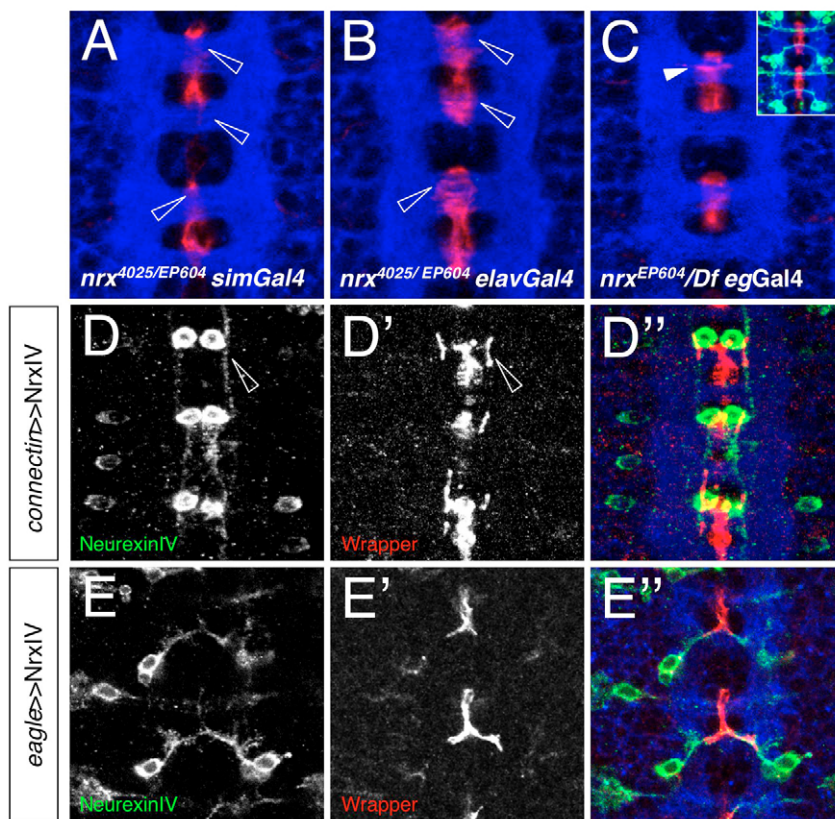


Fig. 3. Neuronal expression of Neurexin IV rescues the *Neurexin IV* midline glial phenotype.

Frontal views of CNS preparations of stage 16 embryos, anterior is upwards, the genotypes are indicated. Axonal membranes are shown in blue (HRP staining), the shape of the midline glial cells is marked by Wrapper expression (red). Neurexin IV expression is shown in green. Single confocal sections are presented. (A) The phenotype of *Neurexin IV* mutants is not rescued by re-expressing Neurexin IV in all midline cells using the EP-insertion *EP604* and *simGal4* activation. (B) Re-expressing Neurexin IV in all neurons using the EP-insertion *EP604* and *elavGal4* activation gives full phenotypic rescue. Note that, sometimes, glial processes extend slightly further into the commissures, as observed in wild type (arrowheads in A,B; Fig. 2A). (C) A partial rescue of the *Neurexin IV* mutant phenotype is observed by re-expressing Neurexin IV in the *eagle* pattern (the pattern of expression is shown in the inset in the top right-hand corner). Arrowhead indicates Wrapper-positive midline glial cell processes extending along the Neurexin IV-positive commissural fascicles. (D-D'') After re-expression of Neurexin IV on few ipsilateral-projecting neurons using the *connectinGal4* driver, we frequently noted extension of midline glial cell processes into the longitudinal connectives (arrowheads). (E-E'') Likewise, we noted extensions of midline glial cells along commissural axons following *eagleGal4*-mediated activation of Neurexin IV expression.

paranodal protein Caspr, such as extracellular EGF-domains, Laminin G domains and an N-terminal Discoidin domain known to mediate binding of extracellular carbohydrates typical for adhesion proteins (Baumgartner et al., 1998; Peles et al., 1997a; Peles et al., 1997b) (Fig. 6A). Within the epidermis, Neurexin IV is localized in pleated septate junctions and is required for their formation (Baumgartner et al., 1996). Alternative splicing at the *Neurexin IV* locus generates two transcript classes that differ in the exon encoding the Discoidin domain (Edenfeld et al., 2006) (Fig. 5A).

The small size of the differentially spliced *Neurexin IV* exons precluded in situ hybridization to determine their expression patterns. To test which of the two *Neurexin IV* mRNA isoforms is expressed in CNS neurons, we isolated tissue-specific RNA following expression of a Flag-tagged poly A binding protein (Yang et al., 2005) (see Materials and methods). Cell type-specific mRNA was then used to generate cDNA, and the exons corresponding to different mRNA species were amplified by PCR (Fig. 5B, the primers were designed to bridge large introns, avoiding the amplification of possible genomic DNA contamination). To control the efficiency of the RNA isolation, we used *repoGal4*, which is expressed in only lateral glial cells but not in the midline glia (Halter et al., 1995; Xiong et al., 1994), and *elavGal4*, which drives expression in a few lateral glial cells and all neuronal lineages (Berger et al., 2007). The gene *crumbs* was included as epidermally expressed gene and served as a negative control. In both the glial and the neuronal RNA pool, we found only a small amount of *crumbs* cDNA. Within the glial-specific RNA pool, we detected a substantial amount of *repo* mRNA and found only little *elav* message, whereas we consistently found less *repo* mRNA but higher levels of *elav* mRNA in the neuronal RNA pool, which matches the expression

pattern reported for both Gal4 driver strains (Fig. 5B). In both the neuronal and the glial mRNA pools, we were able to detect *Neurexin IV* mRNA (Fig. 5B).

The *Neurexin IV* primer pair was designed to span the alternatively spliced region comprising exons 3 and 4. Owing to the identical length of exon 3 and exon 4, no size difference was predicted for both PCR amplification products (Fig. 5A). However, the inclusion of exon 3 generates an *XbaI* restriction site, whereas the inclusion of exon 4 generates an *NcoI* restriction site within the cDNA. To determine which of the two different *Neurexin IV* isoforms was expressed in glial or neuronal lineages, the PCR products were restricted with *XbaI* and/or *NcoI* (Fig. 5C). The almost exclusive presence of the *XbaI* site in the glial cDNA pool suggested that glial cells predominantly generate the exon 3 form (Fig. 5C), whereas the cDNA pool generated from neuronal cells DNA with an *XbaI* restriction site is under-represented, indicating that neurons predominantly generate the *Nrx-IV^{exon4}*. Thus, the *Neurexin IV* locus shows cell type-specific differential splicing, by which the exon 4 isoform appears enriched within neurons.

Both *Neurexin IV* isoforms can interact with Wrapper

To further study the relationship of Neurexin IV and Wrapper, we generated full-length cDNAs encoding the different protein isoforms. An exon 3-containing cDNA was available from the DGRC, the exon4 containing cDNA clone was generated following a RT PCR reaction from neuronal mRNA (see Materials and methods). The corresponding UAS constructs were first used for transfection of S2 cells to test possible heterophilic interaction of Neurexin IV and Wrapper. Neither Neurexin IV nor Wrapper-expressing S2 cells showed a homophilic adhesion and the

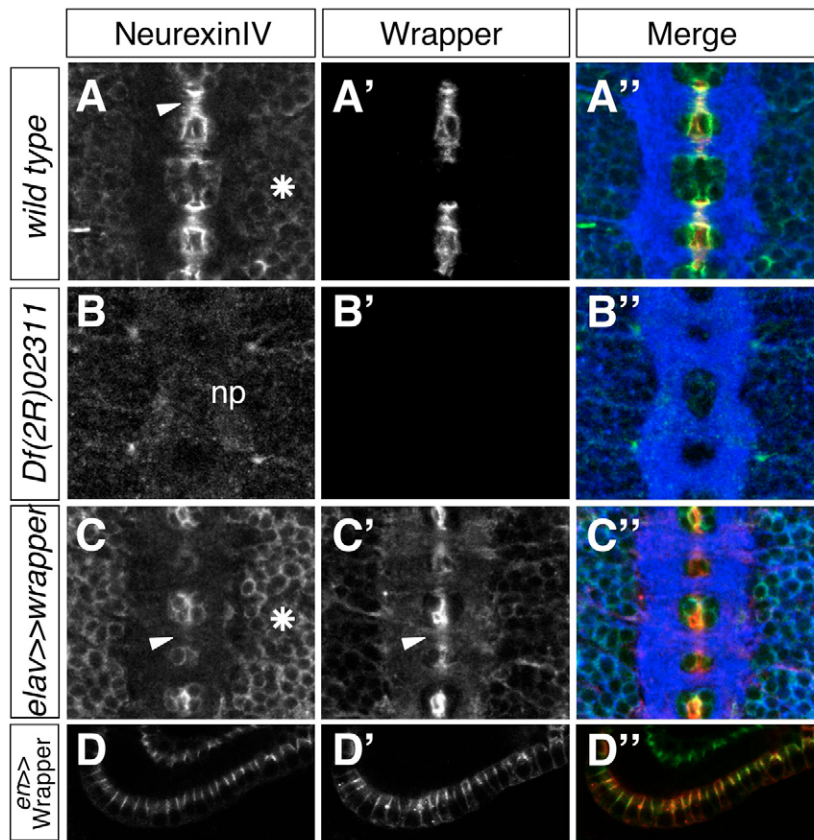


Fig. 4. Glial Wrapper expression stabilizes neuronal Neurexin IV.

(A–C'') Frontal views of CNS preparations of stage 16 embryos, anterior is upwards, the genotypes are indicated. (D–D'') Hindgut epithelium. Axonal membranes are shown in blue in the merge (HRP staining), the shape of the midline glial cells is marked by Wrapper expression (red). Neurexin IV expression is shown in green. Single confocal sections are presented. (A–A'') In wild-type embryos, an intensive overlap of Neurexin IV and Wrapper expression at the CNS midline can be seen. Note the presence of Neurexin IV expression in the commissural axon tracks (arrowhead). The asterisk in A indicates weak Neurexin IV staining on neuronal cell bodies. (B–B'') In *Df(2R)02311* mutant embryos that lack *wrapper* expression, Neurexin IV expression is lost at the midline. By contrast, Neurexin IV expression in CNS neurons can still be detected. Note the weak Neurexin IV expression in the neuropil (np). (C–C'') Following overexpression of Wrapper in all CNS neurons using *elavGal4*, Neurexin IV is depleted from the CNS midline despite the presence of normal levels of Wrapper expression at the midline glial cells. No Neurexin IV expression can be detected in commissural axons at the midline (arrowheads). Instead Neurexin IV expression is redirected to the neuronal cell bodies (asterisk). (D–D'') Hindgut of a wild-type embryo ectopically expressing Wrapper protein in the ventral cells using the *enGal4* driver. Wrapper is recruited to the septate junctions that normally express Neurexin IV.

expression of neither membrane protein altered growth behavior. Likewise, when we mixed cells expressing Neurexin IV^{exon3} with cells expressing Neurexin IV^{exon4}, no aggregation of cells could be observed (Fig. 6A,B).

However, when cells expressing Wrapper were mixed with S2 cells expressing either of the two Neurexin IV isoforms, we noted the formation of cell aggregates (Fig. 6C,D). Although transfection efficiency was around 30%, only occasionally were untransfected cells trapped in the Wrapper/Neurexin IV cell clusters (in 54 cell aggregates with 1215 cells total, we found 50 untransfected cells), demonstrating the specificity of the cell-cell binding. In this aggregation assay, both Neurexin IV isoforms interact equally well with Wrapper.

Wrapper prefers interaction with Neurexin IV^{exon4} compared with Neurexin IV^{exon3}

To further test the interaction between Wrapper and Neurexin IV we performed competitive adhesion experiments. To follow *Nrx-IV^{exon3}* independently from *Nrx-IV^{exon4}* we generated constructs that carry a Myc tag just C-terminal to the cleavage site of the signal peptide. We had previously shown that Neurexin IV tolerates the inclusion of a GFP moiety (Edenfeld et al., 2007) and thus expected that the inclusion of a Myc tag at the same site would not change binding behavior. To further control for minor effects, we generated both a *Nrx-IV^{exon3-myc}* and a *Nrx-IV^{exon4-myc}* protein.

Aggregation experiments indicated that the inclusion of a Myc tag does not interfere with the ability to form cell aggregates with Wrapper-expressing cells and *Nrx-IV^{exon3-myc}*. Wrapper aggregates formed with comparable characteristics to *Nrx-IV^{exon4-myc}*. We then mixed S2 cells expressing Wrapper with S2 cells expressing *Nrx-IV^{exon3}* and S2 cells

expressing *Nrx-IV^{exon4-myc}*. In this triple aggregation experiment, Neurexin IV^{exon4-myc}-expressing cells were found nine times as often in cell aggregates than in cells expressing the Neurexin IV^{exon3} isoform (30 cell aggregates with more than 20 cells; 87.5% of all *Nrx*-positive cells were Myc positive and thus expressed *Nrx^{exon4}*; two independent experiments; Fig. 6G–I). To exclude that the addition of the Myc-tag interfered with binding characteristics and masked the normal interaction preference, we mixed S2 cells expressing Wrapper with those expressing *Nrx-IV^{exon3-myc}* and *Nrx-IV^{exon4}*. Again, the proportion of *Nrx-IV^{exon4}*-expressing cells is significantly increased (30 cell aggregates with over 20 cells; 82.5% of all *Nrx-IV*-positive cells were Myc negative and thus expressed *Nrx-IV^{exon4}* and not *Nrx^{exon3-myc}*; two independent experiments; Fig. 6J–L). These data demonstrate that the exon4 containing Neurexin IV isoform preferentially interacts with Wrapper.

Isoform specific rescue of the Neurexin IV phenotype

The above data indicate that the midline-derived Wrapper protein binds to the neuronally derived Neurexin IV protein to ensure stabilizing of glial wrapping. To further dissect a possible differential requirement of Neurexin IV in the CNS we generated transgenic flies, allowing for the expression of individual Neurexin IV isoforms. As the transgene expression of constructs introduced into the germline via P-element-based vectors is generally very much dependent on the chromosomal insertion site, we employed the *phiC31* system and inserted a *UAS::Neurexin IV^{exon3}* and *UAS::Neurexin IV^{exon4}* construct in the 51D landing site on the second chromosome (Bischof et al., 2007; Venken and Bellen, 2007). *Neurexin IV* mutants are characterized by a lack of glial cell processes around the posterior commissure

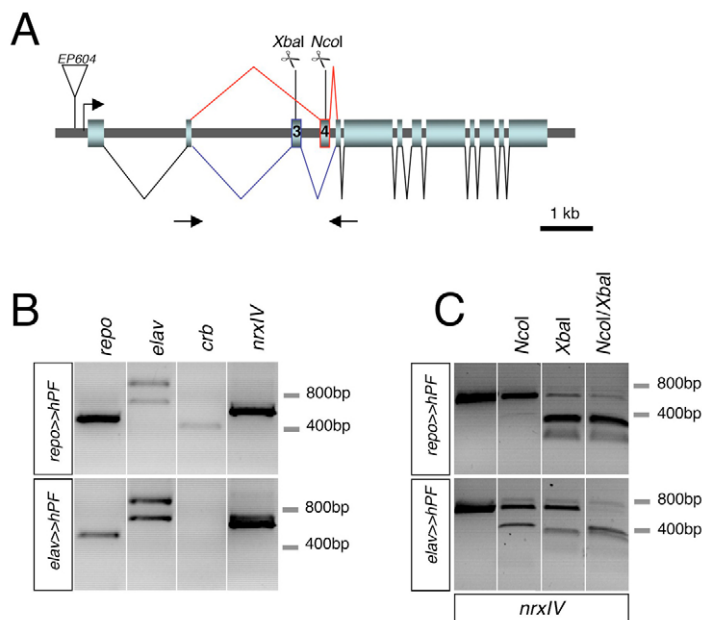


Fig. 5. Neurexin IV encodes two differentially expressed protein isoforms. (A) Schematic representation of the Neurexin IV locus. Transcription is from left to right. The position of the EP604 insertion and the differentially spliced exons 3 and 4 is indicated. Exon 3 has a unique XbaI site, exon 4 carries a unique NcoI site. The arrows indicate the position of the primers used in the RT-PCR experiments shown in B,C. Scale bar: 1 kb. (B) Following expression of a human Flag-tagged PABP protein in either glial cells (*repo>hPF*) or neuronal cells (*elav>hPF*), tissue-specific mRNA was isolated. The expression of several genes was assayed using RT-PCR. As expected, *repo* mRNA is enriched in the *repo>hPF* RNA pool, whereas *elav* mRNA is enriched in the *elav>hPF* pool. *crumbs* mRNA is specifically expressed in epidermal cells and was used as a negative control and only some *crumbs* mRNA could be detected in the *repo>hPF* RNA pool. *Neurexin IV* mRNA was identified in both the glial and the neuronal mRNAs. (C) To discriminate which of the two *Neurexin IV* mRNA isoforms was amplified in the two RNA pools, we digested the DNA with *NcoI* or *XbaI*, or both. This analysis demonstrates that in the glial mRNA pool, the *Neurexin IV* exon 3 isoform predominates, whereas in the neurons, the exon 4 predominates.

(Fig. 2B, Fig. 7B; only two out of 50 neuromeres have few glial processes in the posterior commissure). Ubiquitous expression of *Nrx-IV^{exon3}* with the help of the *daGal4* driver is able to rescue the septate junction phenotype associated *Nrx-IV* mutants (not shown). However, expression of Neurexin IV^{exon3} in the nervous system using the *elavGal4* only partially rescues the CNS midline phenotype (Fig. 7C, 12 out of 60 neuromeres have glial processes in the posterior commissure). By contrast, an almost full rescue of the *Neurexin IV* mutant CNS phenotype resulted from expression of the Neurexin IV^{exon4} variant in all neurons using the *elavGal4* driver (Fig. 7D, 50 out of 52 neuromeres have glial processes in the posterior commissure). As both Neurexin IV isoforms are expressed from the same landing site, this differential rescue argues for a distinct requirement of Neurexin IV^{exon4} in binding the midline glial-derived Wrapper protein.

To further test the relevance of the extracellular domain, we also expressed a Neurexin IV variant lacking all extracellular regions of the protein (see Fig. S2 in the supplementary material). Such a membrane-anchored cytoplasmic domain localizes mostly to cytoplasmic vesicles and is not able to provoke any phenotype. In turn, when we expressed a Neurexin IV protein lacking the intracellular domain, the truncated Neurexin IV protein fails to be properly integrated into the septate junctions and sometimes decorates the entire membrane (see Fig. S2 in the supplementary material). Likewise, when we expressed a secreted form of *Nrx-IV^{exon3}*, no specific binding to any cell structure could be observed (see Fig. S2 in the supplementary material). Thus, both the cytoplasmic and the extracellular domains are required to control normal protein localization.

DISCUSSION

Neuron-glia interaction plays a crucial role for the development and function of neuronal circuits. Here, we have used the midline glia of the *Drosophila* embryonic central nervous system to elucidate mechanisms that govern neuron-glia recognition and the establishment of glial ensheathment of axonal fascicles.

We show that wrapping of commissural axons by midline glia is dependent on the interaction between the GPI-linked protein Wrapper expressed on glial cells (Noordermeer et al., 1998) and the

neuronally expressed Neurexin IV protein. Mutants for *Neurexin IV* and *wrapper* show similar wrapping defects at the midline. In both mutants, the midline glia is unable to infiltrate the axonal neuropil of the commissures to ensure ensheathment of individual fascicles. Tissue-specific rescues of *Neurexin IV* mutants showed that Neurexin IV acts in neurons and functions as an axon-autonomous-specific recognition signal for midline glial processes, as only Neurexin IV-expressing fascicles show restoration of ensheathment. Ectopic expression of Neurexin IV was even able to recruit pronounced midline glial processes to ectopic places far away from their normal localization in wild-type embryos. This suggests that Neurexin IV is a key factor in the axon-glia recognition at the midline, and we propose a model in which Neurexin IV attracts and stabilizes midline glial processes in a contact-dependent manner.

It has been shown that midline glia exhibit thin, highly dynamic cell processes that explore neighboring neuronal substrates (Vasenkova et al., 2006). Upon binding to a Neurexin IV-expressing axon fascicle, these initially transient midline glial processes might then be stabilized. This stabilization itself may be required to establish a tight glial wrap or to promote the assembly of further signaling complexes that are required for glial cell development. One possible candidate for neuron-glia communication could be the EGF-receptor ligand Spitz, which is provided by the commissural neurons and needs to be transferred to the midline glia in order to promote their survival (Bergmann et al., 2002; Scholz et al., 1997; Sonnenfeld and Jacobs, 1995). A tight adhesion of the glial processes to the neuronal membranes might facilitate this transfer.

Our genetic analysis in vivo strongly suggested that Wrapper might act as the glial binding partner for neuronal Neurexin IV in this recognition process. Indeed aggregation assays in S2 cells revealed specific heterophilic binding of Wrapper and Neurexin IV. The *Neurexin IV* gene generates two distinct isoforms through alternative splicing (Edenfeld et al., 2006). Here we showed that the two different isoforms are differentially expressed in the nervous system. Whereas the *Nrx-IV^{exon3}* isoform is predominantly expressed in glial cells that can form septate junctions, the *Nrx-IV^{exon4}*-specific isoform is enriched in neurons. Both proteins differ

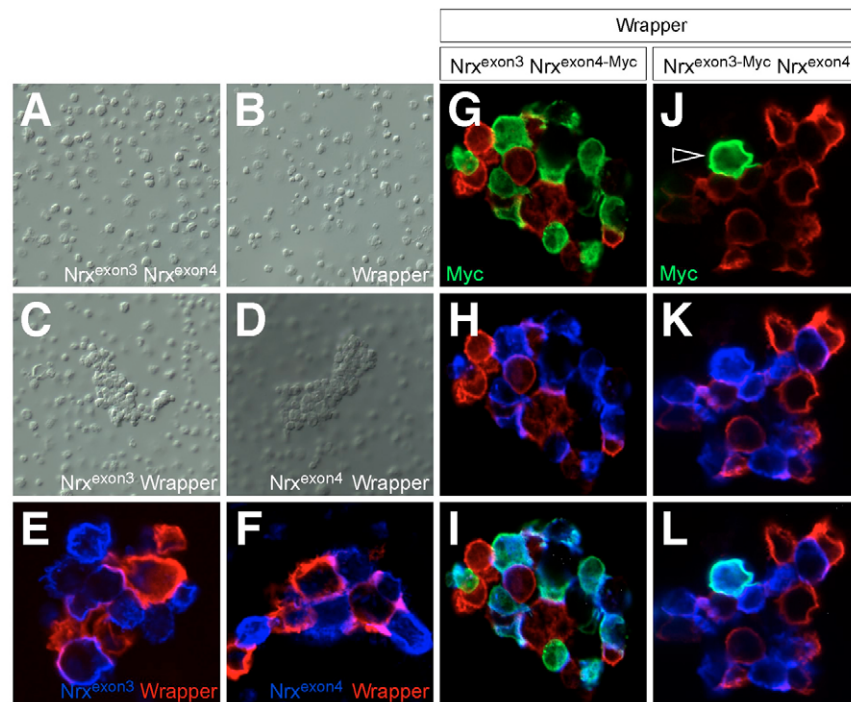


Fig. 6. Wrapper preferentially interacts with Neurexin IV^{exon4}. *Drosophila* S2 cells expressing the different proteins as indicated were tested for aggregation. (E-L) Red, Wrapper expression; blue, Neurexin IV expression detected with an antibody recognizing both isoforms; green, Myc-tag expression. (A) Upon mixing of Nrx^{exon3}- and Nrx^{exon4}-expressing cells, no aggregation of S2 cells can be detected. (B) Likewise, no aggregation was observed upon expression of Wrapper. (C) When Nrx^{exon3}- and Wrapper-expressing cells were added, aggregates form. (D) Similar aggregates formed when Nrx^{exon4}- and Wrapper-expressing cells are mixed. (E,F) To identify the different cells, we used antibodies against Wrapper (red) and Neurexin IV (blue). (E) When Neurexin IV^{exon3}- and Wrapper-expressing cells, or (F) Neurexin IV^{exon4}- and Wrapper-expressing cells were mixed, Neurexin IV- and Wrapper-expressing cells alternate in the cell aggregates. (G-I) Upon mixing of Wrapper-expressing cells with cells that either express Nrx^{exon3} or Nrx^{exon4}-myc, aggregates were noted that almost exclusively contained Wrapper- and Nrx^{exon4}-myc-expressing cells. (J-L) When Wrapper-expressing cells were mixed with cells that either express Nrx^{exon3}-myc or Nrx^{exon4}, aggregates form. Again, the aggregates comprise almost exclusively Wrapper- and Nrx^{exon4}-myc-expressing cells. In J, a single Nrx^{exon3}-myc-expressing cell can be seen attached to a cluster of Wrapper and Nrx^{exon4} cells (arrowhead).

only in the sequence of their N-terminal Discoidin-like domain that mediates interaction with carbohydrates present on many adhesion proteins (Kiedziarska et al., 2007). Although each isoform alone is able to interact with Wrapper in S2 cell aggregation experiments, we demonstrate a much higher affinity of the neuronally enriched *Nrx-IV^{exon4}* isoform to Wrapper in a competitive aggregation assay. The *in vivo* rescue experiments corroborate the results obtained by the tissue-specific mRNA isolation and the cell culture experiments. Although both isoforms are able to at least partially rescue the *Neurexin IV* mutant midline glial wrapping phenotype in the embryo, the rescuing abilities of Neurexin IV^{exon3} is less pronounced compared with Neurexin IV^{exon4}. The alternatively spliced exons 3 and 4 are conserved in all *Drosophilidae* and *Anopheles*, and, thus, probably have important functional purposes.

Neurexin IV and Wrapper interaction possibly has not only an impact on the midline glial cell but also on the commissural axon. Neurexin IV accumulates in commissural axons, and by recruiting additional adaptors through its cytoplasmic domain it could reorganize the cytoskeleton. In epithelia, Neurexin IV recruits Coracle (Cor), a member of the band 4.1 superfamily, to septate junctions (Lamb et al., 1998). No expression of Coracle is found at the midline and no mutant midline phenotype is detected in *coracle* mutant embryos. However, we have recently noted enhanced levels of β -Spectrin (Hulsmeier et al., 2007) and Discs large protein (Learte

et al., 2008) (T.S., unpublished) at the midline, which might hint towards a specific cytoskeletal connection established in commissural axons at the CNS midline.

In addition to a pure adhesive function of the Neurexin IV-Wrapper complex, Wrapper may also exert signaling properties in the glia cell. However, as Wrapper is a GPI-linked protein it would require a still unknown co-receptor for this function. In this respect, it is also interesting to note that Wrapper is more generally expressed in cortex glia (Noordermeer et al., 1998) and its binding to Neurexin IV may be a more general property of neuron-glia interaction.

Obviously, neuron-glia interaction is not confined to the *Drosophila* CNS but is also of eminent importance during the insulation of all axonal trajectories in both invertebrates and vertebrates. In vertebrates, Schwann cells wrap axons by either forming a myelin sheath or Remak fibers (Nave and Salzer, 2006). Similarly, oligodendrocytes form myelin in the CNS (Sherman and Brophy, 2005). During myelination, the glial cell membranes form special contact zones with the axon, the paranodes, abutting the nodes of Ranvier (Girault and Peles, 2002; Poliak and Peles, 2003). These are characterized by septate-like junctions that prevent current leakage. The ultrastructural architecture of these cell-cell junctions and also the molecules establishing these junctions have been conserved between flies and mammals, suggesting an ancient evolutionary origin of this axonal insulation. As core components of septate or septate-like junctions, the Caspr/Paranodin, Contactin and

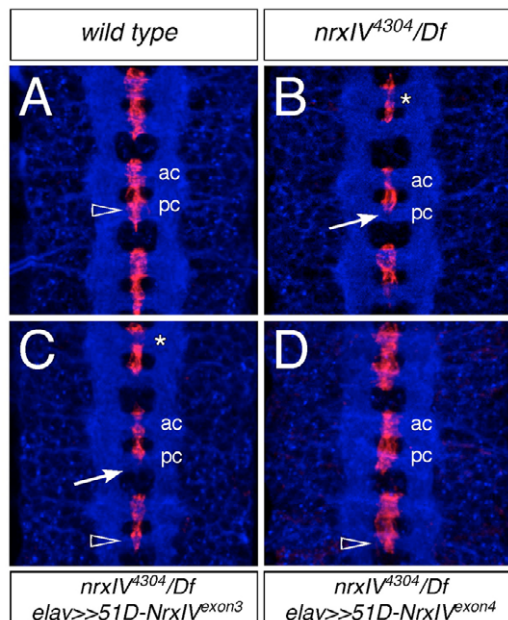


Fig. 7. Differential rescue abilities of the Neurexin IV isoforms.

Frontal views, anterior is upwards, projection of confocal stacks of stage 16 ventral nerve cords spanning the commissural width stained for the presence of Wrapper (red) to label the CNS midline cells and HRP (blue) to label axonal trajectories. (A) In wild-type embryos, the midline glia engulfs the entire commissure and sends processes across both anterior and posterior commissures (ac, pc; arrowhead). (B) In mutant *Neurexin IV* embryos, the midline glial cells fail to wrap the segmental commissures (48 out of 50 neuromeres). No midline processes are associated with the posterior commissures (pc, arrow). In addition, only few glial processes are found around the anterior commissure (asterisk). (C) In *Neurexin IV* mutant embryos expressing the *Nrx-IV^{exon3}* protein from the 51D landing site in the *elav* pattern, the commissural wrapping is only partially rescued and in 48 out of 60 neuromeres, the midline glia fails to cover the posterior commissures (arrow). Anterior commissures often contain only few glial processes (asterisk). Arrowhead indicates commissure partially covered by midline glial processes. (D) In *Neurexin IV* mutant embryos expressing the *Nrx-IV^{exon4}* protein from the 51D landing site in the *elav* pattern, the commissural wrapping is almost entirely rescued and, in 50 out of 52 neuromeres, the midline glia covers the posterior commissures (arrowhead).

Neurofascin/155 and their *Drosophila* counterparts Neurexin IV, Contactin and Neuroglian have been identified (Banerjee et al., 2006; Baumgartner et al., 1996; Bhat, 2003; Bhat et al., 2001; Bieber et al., 1989; Faivre-Sarrailh et al., 2004; Genova and Fehon, 2003; Peles et al., 1997a). Interestingly, Wrapper appears to be less conserved. Although it is present in all *Drosophilidae* and the *Drosophila* genome harbors a Wrapper-related protein, Klingon (Butler et al., 1997; Matsuno et al., 2009), no clear Wrapper orthologs can be identified in mammals. However, there are several GPI-linked Ig-superfamily proteins in the mouse genome whose expression profiles need to be determined.

Besides prior identification of direct cis-binding partners of Neurexin IV/Caspr, which act in the same cell (Faivre-Sarrailh et al., 2004; Peles et al., 1997b), we here identified Wrapper as the first factor that interacts with Neurexin IV in a trans fashion. Based on the tissue culture data, we anticipate a direct interaction but at present cannot exclude the involvement of additional complex partners. In

previous studies it has been shown that Neurexin IV is required to facilitate the secretion of Contactin to the membrane, thereby allowing the generation of adhesive septate junctions (Faivre-Sarrailh et al., 2004). Here, we show that Neurexin IV can directly perform adhesive functions by binding to the Wrapper protein decorating opposing cell membranes. Interestingly, Contactin and Wrapper are both similar Ig-domain proteins linked via GPI anchors to the plasma membrane (Faivre-Sarrailh et al., 2004; Noordermeer et al., 1998).

Within the nervous system, Neurexin IV has been extensively studied for its role in organizing the formation of septate junctions between glial cells, which constitute the major structural component of the *Drosophila* blood brain barrier (Banerjee et al., 2006; Baumgartner et al., 1996; Stork et al., 2008). Unlike in the vertebrate paranodes, septate junctions are found extensively at glial-glia cell contacts in the *Drosophila* nervous system (Bainton et al., 2005; Schwabe et al., 2005; Stork et al., 2008; Tepass and Hartenstein, 1994) and are only rarely detected between glial cells and axons (Banerjee et al., 2006). The midline glia is not part of this subperineurial glial sheath but rather belongs to the class of wrapping glia that ensures normal insulation of axon fascicles at the midline (Ito et al., 1995; Jacobs and Goodman, 1989; Klambt et al., 1991; Noordermeer et al., 1998). In line with this notion, midline glial cells do not form septate junctions visible at the electron-microscopic level (Jacobs and Goodman, 1989; Stollewerk and Klambt, 1997; Stollewerk et al., 1996). Additionally, major septate junction components such as Coracle, Neuroglian and Lachesin are not enriched at the midline glia (this work) (Kearney et al., 2004; Wheeler et al., 2006), and the corresponding mutants show normal midline glial wrapping behavior. For some septate junction components, midline expression has been previously reported. We found that, in these cases, expression is restricted to channel glia, which is part of the subperineurial sheath known to form epithelial-like pleated septate junctions and is not related to the midline glia (Beckervordersandforth et al., 2008; Ito et al., 1995; Schwabe et al., 2005; Stork et al., 2008).

Our results show that at the *Drosophila* midline Neurexin IV acts in a novel, septate junction-independent way to ensure neuron-glia adhesion; it will be interesting to determine whether similar adhesive interactions can be attributed to the mammalian homolog Caspr or to other members of the Caspr protein family (Poliak et al., 2003; Spiegel et al., 2002; Traka et al., 2003). Interestingly, it has been recently reported, that Neurexin IV and other canonical septate junction-associated proteins control the adhesive properties of cardiac and pericardial cells in the embryonic heart of *Drosophila* without forming septate junctions (Yi et al., 2008). Additionally, these noncanonical adhesive properties of septate junction proteins in the heart, and also the assembly of canonical septate junctions in the *Drosophila* blood brain barrier, are controlled by different heterotrimeric G protein signaling pathways (Bainton et al., 2005; Schwabe et al., 2005) and possibly Wrapper-Neurexin-IV-mediated adhesion at the CNS midline is also influenced by G protein signaling pathways. In the future, it will be interesting to determine the different roles of the Neurexin IV-Wrapper complex and to dissect the cellular responses triggered by this neuron-glia interaction.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/8/1251/DC1>

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