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

Tobias Stork*, Silke Thomas, Floriano Rodrigues, Marion Silies, Elke Naffin, Stephanie Wenderdel and Christian Klämbt†

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 axon 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; Klambt 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 (Brandtschck 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; Klambt 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 impaired 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 parane 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...
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 IVexon3 or Neurexin IVexon4), and that both isoforms are differentially expressed in the nervous system. The Neurexin IVexon3 protein is expressed in cells that form septate junctions, whereas the Neurexin IVexon4 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 IVexon4 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 RE16834 (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 DGRG. 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-GGCACGCCAGGACAAATACG; reverse primer, GATGGCGAGTTCT-GGCCGCTCCT. 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 were generated were verified by sequencing. Further details on primers and the generation of constructs are available upon request. The pUAS TattB 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 pUAS TattB-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 antiseraum: HSTGHQVRKETIEFI. 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)); 1D3 anti-Wrapper 1/5, (Noordermeer et al., 1998); anti-Coracle, 1/50 (CIBI 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 (Bodgan 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 x 10⁶ cells per ml S9 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 (Bodgan et al., 2005).

Genetics

Flies were raised on standard food at 25°C or room temperature. The following genotypes were used: Df(3L)Exel6116; nrxIV94364; nrxIV96647, nrxIV29660; nrg14; nrg14 (Bloomington); nrxIV1025 (Baumgartner et al., 1996); wrapperDP85B2014 (Noordermeer et al., 1998); nrv2435 (Hall and Bieber, 1997); cora; cora (Lamb et al., 1998); lacB10146; lac (Llomargas et al., 2004); nrxG11135; nrv2138 (Genova and Fehon, 2003); slitGal4; erGal4; erGal4; daGal4; eaglali4 (all from the Bloomington stock center); AAI42Gal4 (U. Lammel and C.K., unpublished); connectinGal4 (Lattmann et al., 2007); UAS Wrapper (Noordermeer et al., 1998); nrgGFP (Morin et al., 2001); lacGFP125; nrxIVGFP454 (Edenfeld et al., 2006); and nrv2GFP175 (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 (Klambt 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 AAI42Gal4 (data not shown) or by staining for Wrapper protein, the outline of the midline glial cells was 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).
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 slt-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).
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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/nrx<sup>5604</sup> 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 slitGal4 driver or AA142Gal4, 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 simGal4 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 repoGal4 driver no rescue was observed (data not shown). However, when we used the elavGal4 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 eagleGal4 (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 connectinGal4 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-glial 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...
mRNA in the neuronal RNA pool, which matches the expression we consistently found less amount of cDNA. Within the glial-specific RNA pool, we detected a substantial the neuronal RNA pool, we found only a small amount of (Berger et al., 2007). The gene expression in a few lateral glial cells and all neuronal lineages (et al., 1995; Xiong et al., 1994), and expressed in only lateral glial cells but not in the midline glia (Halter et al., 2005) (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

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 Neurexin 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
expression of neither membrane protein altered growth behavior. Likewise, when we mixed cells expressing Neurexin IVexon3 with cells expressing Neurexin IVexon4, 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 IVexon4 compared with Neurexin IVexon3**

To further test the interaction between Wrapper and Neurexin IV we performed competitive adhesion experiments. To follow Nrx-IVexon3 independently from Nrx-IVexon4 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 interfered with binding characteristics and would mask the normal interaction preference, we mixed S2 cells expressing Wrapper with those expressing Nrx-IVexon3-myc and Nrx-IVexon4. Again, the proportion of Nrx-IVexon4-expressing cells is significantly increased (30 cell aggregates with more than 20 cells; 87.5% of all Nrx-positive cells were Myc positive and thus expressed Nrxexon4; 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-IVexon4-myc-expressing cells were found nine times as often in cell aggregates than in cells expressing the Neurexin IVexon3 isoform (30 cell aggregates with more than 20 cells; 82.5% of all Nrx-positive cells were Myc negative and thus expressed Nrxexon4; two independent experiments; Fig. 6G-I). 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 IVexon3 and UAS::Neurexin IVexon4 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.
IVexon4, no specific binding to any cell structure could be observed (Fig. S2 in the supplementary material). Likewise, when we expressed a secreted form of Nrx-IVexon3 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 IVexon3 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 IVexon4 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 IVexon4 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-IVexon3, 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-IVexon3 isoform is predominantly expressed in glial cells that can form septate junctions, the Nrx-IVexon4-specific isoform is enriched in neurons. Both proteins differ...
only in the sequence of their N-terminal Discoidin-like domain that mediates interaction with carbohydrates present on many adhesion proteins (Kiedzierska 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-IVexon4 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 IVexon3 is less pronounced compared with Neurexin IVexon4. 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 Disc 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

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Fig. 6. Wrapper preferentially interacts with Neurexin IVexon4. Drosophila S2 cells expressing the different proteins as indicated were tested for aggregation. (A-L) Red, Wrapper expression; blue, Neurexin IV expression detected with an antibody recognizing both isoforms; green, Myc-tag expression. (A) Upon mixing of Nrxexon3- and Nrxexon4-expressing cells, no aggregation of S2 cells can be detected. (B) Likewise, no aggregation was observed upon expression of Wrapper. (C) When Nrxexon3 and Wrapper-expressing cells were added, aggregates form. (D) Similar aggregates formed when Nrxexon4, 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 IVexon3, and Wrapper-expressing cells, or (F) Neurexin IVexon4 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 Nrxexon3 or Nrxexon4-myc, aggregates were noted that almost exclusively contained Wrapper- and Nrxexon4-myc-expressing cells. (J-L) When Wrapper-expressing cells were mixed with cells that either express Nrxexon3-myc or Nrxexon4, aggregates form. Again, the aggregates comprise almost exclusively Wrapper- and Nrxexon4-expressing cells. In J, a single Nrxexon3-myc-expressing cell can be seen attached to a cluster of Wrapper and Nrxexon4 cells (arrowhead).
processes are associated with the posterior commissures (pc, arrow). In segmental commissures (48 out of 50 neuromeres). No midline mutant Neurexin IV exon4 protein from the 51D landing site in the both anterior and posterior commissures (ac, pc; arrowhead). (4) 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-Ivexon3 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 anterior commissures (arrow). Arrowhead indicates commissure partially covered by midline glial processes. (D) In Neurexin IV mutant embryos expressing the Nrx-Ivexon4 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 Feohon, 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, Klingen (Butler et al., 1997; Matsumo 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/Casp, 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-glial 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-glial 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-glial interaction.

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Supplementary material
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