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Neurexin IV, caspr and paranodin – novel members of the neurexin family: encounters of axons and glia

Hugo J. Bellen, Y. Lu, R. Beckstead and M.A. Bhat

Axonal insulation is of key importance for the proper propagation of action potentials. In *Drosophila* and other invertebrates, it has recently been demonstrated that septate junctions play an essential role in axonal insulation or blood–brain-barrier formation. Neurexin IV, a molecular component of *Drosophila* septate junctions, has been shown to be essential for axonal insulation in the PNS in embryos and larvae. Interestingly, a vertebrate homolog of Neurexin IV, caspr – also named paranodin – has been shown to localize to septate-like junctional structures. These vertebrate junctions are localized to the paranodal region of the nodes of Ranvier, between axons and Schwann cells. Caspr/paranodin might play an important role in barrier formation, and link neuronal membrane components with the axonal cytoskeletal network.

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RAPID PROPAGATION of action potentials in neurons can be accomplished by keeping distances short, as seen in many insect species, by increasing the diameter of the axons, as exemplified in the squid giant axon, or by clustering of voltage-gated channels to unensheathed areas of axons flanked by glial sheaths, as seen in vertebrates (Fig. 1A,B). This organization is characterized by the presence of nodes of Ranvier and extensive myelination of axons by Schwann cells or oligodendrocytes. These cells provide insulation and promote Na^+ and K^+ channel clustering, allowing vertebrate axons to propagate action potentials rapidly through saltatory conduction (for review see Ref. 1). In the periphery, Schwann cells ensheath not only the large myelinated axons, but also the thin unmyelinated axons (PNS) (Fig. 1C). A group of fascicles of axons and their associated Schwann cells are surrounded by a perineurium, which is composed of specialized fibroblasts that are connected to each other with tight junctions, forming a protective barrier against the diffusion of substances into the peripheral nerve fascicle.

The mechanisms underlying axonal insulation in invertebrate species are less understood than those in vertebrates, as axons are not myelinated and the protein composition of the insulating glia is unknown. The axons also lack the typical nodes of Ranvier[1]. However, unmyelinated axons in *Aplysia* also exhibit clustering of Na^+ channels, suggesting that nodes of Ranvier are not a prerequisite for clustering, and that clustering is probably important to optimize action potential conduction in many species[6]. Invertebrate axons are insulated from their environment through a glial-dependent blood–brain barrier, which plays a crucial role in electrical and chemical insulation[5,7].

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Electron microscopy studies have suggested that septate junctions (SJs) between perineurial glial cells form the structural basis for the blood–brain barriers of some insects and primitive vertebrates. These SJs typically consist of an extensive ladder-like structure, about 15 nm in width, which connects adjacent cells (Fig. 1D; for review see Refs 8,9). Hence, in the absence of normal glia[7], or in the absence of functional SJs, propagation of action potentials should be impaired[8,9]. Genetic manipulations in *Drosophila* have verified the hypothesis that SJs form barriers *in vivo*, and have implicated a novel protein, neurexin IV, in barrier formation and insulation (see below).

Myelin is one of the most important components of axonal insulation in vertebrate glial cells. It has been shown to contain a variety of proteins required for proper nerve conduction velocity, such as peripheral myelin protein 22 (PMP22), myelin P zero (MPZ) and connexin 32 (Cx32). Defective functioning or alteration of the levels of these proteins causes reduced nerve conduction velocities, abnormal myelination and human neuropathies such as Charcot–Marie–Tooth disease and Dejerine–Sottas syndrome, as well as similar syndromes in mice (for review, see Refs 10,11). Similarly, loss of ceramide galactosyltransferase, an enzyme required for lipid biosynthesis of the myelin sheath, causes loss of saltatory conduction in mice[12]. Given the lack of nodes of Ranvier and myelin in invertebrates, and given that none of the proteins implicated in the neuropathies mentioned above has known homologs in invertebrate species, it has generally been assumed that the molecular machinery providing insulatory properties to vertebrate and invertebrate glia is fundamentally different. It therefore came as a surprise that vertebrate homologs of *Drosophila* Neurexin IV might play similar structural and functional roles in axonal insulation in vertebrates[13–15]. We propose to name this novel protein family the NCPs, after their founding members: human and *Drosophila* Neurexin IV (Ref. 5), also named human caspr[16], rat caspr[16] or rat paranodin[14].

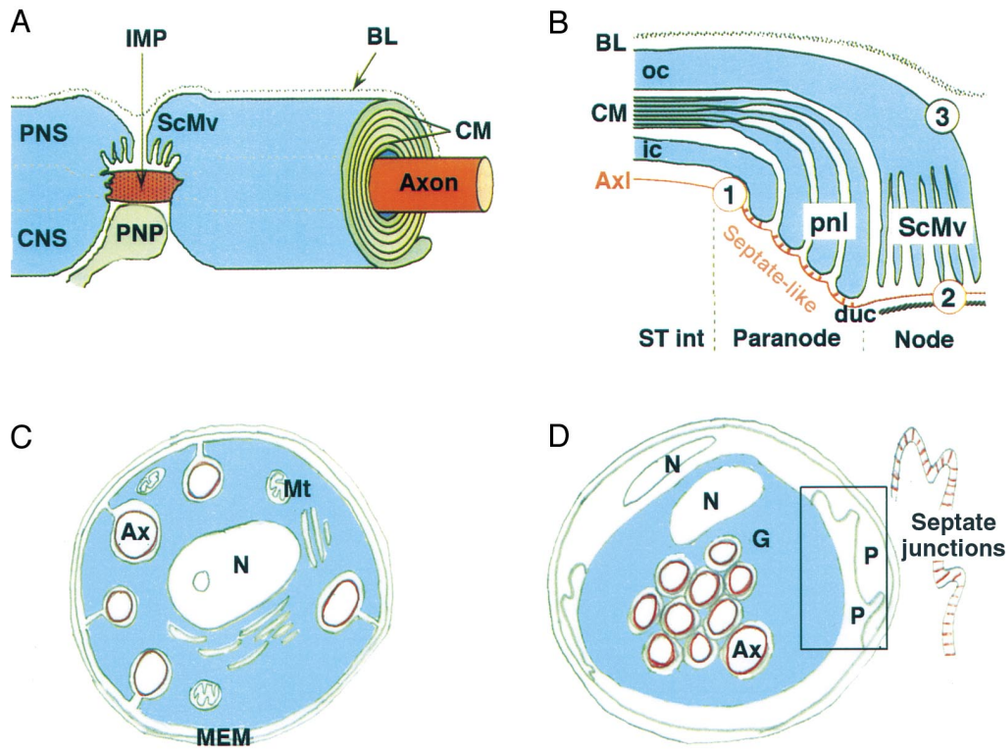


Fig. 1. ***Schematic representation of the node of Ranvier, unmyelinated vertebrate axons and an invertebrate nerve.*** **(A)** *The node of Ranvier* (reproduced from Ref. 1; copyright Cell Press). *This diagram illustrates the structure and the cellular components of a vertebrate node in both the PNS* (top half) *and CNS* (bottom half). *In the PNS, the axon is myelinated by Schwann cells that project numerous microvilli (ScMv) into close proximity of the axon. The Schwann cells are themselves covered by a basal lamina (BL). In the CNS, the myelin sheath is formed by oligodendrocytes. Astrocytes send out perinodal processes (PNP) that contact the naked axon. The diameter of the axon at the node in both the PNS and CNS is constricted and there is an enrichment of intramembranous particles (IMP). CM, compact myelin sheath.* **(B)** *Longitudinal section through the node* (reproduced from Ref. 1; copyright Cell Press). *This longitudinal section through a myelinating Schwann cell shows three distinct segments: the stereotypic internode (ST int), the paranodal region and the node itself. The Schwann cell is separated into an outer collar of cytoplasm (oc), a compact myelin sheath, an inner collar of cytoplasm (ic) and the axolemma (Axl). Paranodal cytoplasmic loops (pnl) contact a thickening of the axolemma at the paranodes and form septate-like junctions. Numerous Schwann cell microvilli contact the axolemma at the node. A dense cytoskeletal undercoating (duc) is also seen at the node. Voltage-gated channels are located to distinct nodal regions, with K^+ channels Kv1.1 and 1.2 (1) and Na^+ channels (2) in the axolemma and Kv1.5 (3) in the abaxonal Schwann cell membrane.* **(C)** *Unmyelinated vertebrate peripheral axons* (modified from Ref. 2). *In the unmyelinated fiber, small axons (Ax) are located in troughs formed by invaginations of the plasmalemma of the Schwann cell (MEM). The nucleus (N) and mitochondria (Mt) are labeled for reference.* **(D)** *Diagram of the* Drosophila *abdominal nerve* (modified from Ref. 3). *The invertebrate nerve consists of bundles of axons surrounded by a sheath formed by a single glial cell (G) similar to oligodendrocytes in the vertebrate CNS. This cell is surrounded by multiple perineurial glial cells (P) that form SJs (boxed area) with each other. For a description and classification of* Drosophila *glial see Ref. 4. The enlargement of the boxed area shows the contact area between two perineurial cells. These SJs have been shown to form the blood–brain barrier and to contain* Drosophila *Neurexin IV (Ref. 5).*

The neurexin family: neurexins I, II and III versus neurexin IV, caspr and paranodin

Our work on neurexins, and that of others[17,18], stems from a general interest in the molecular mechanisms of neurotransmitter release. Originally it was reported that neurexins were enriched at synapses[17] and that the neurexin I-alpha isoform was the alpha-latrotoxin receptor[19], suggesting that neurexins might play a role in neurotransmitter release as this toxin causes Ca^{2+}-independent synaptic vesicle exocytosis. Recently, several aspects of these observations have been challenged[18,20–22]. In addition, it is now becoming apparent that two novel proteins, neurexin IV and caspr/paranodin[5,14,16], which display significantly similar structural and biological properties to those of neurexins, play a role in the interactions between glia and the axons that they ensheathe.

The neurexins form a family of proteins that can be subdivided into two major classes on the basis of domain structure (Fig. 2A) and similarities (Fig. 2B,C). The first class consists of neurexins I, II and III-alpha[17] and a *Caenorhabditis elegans* gene derived from genomic sequences (C29A1); the second class consists of neurexin IV and caspr/paranodin. The first member of this novel class of neurexins was discovered as several overlapping human expressed sequence tags (ESTs) that were isolated, sequenced and mapped to

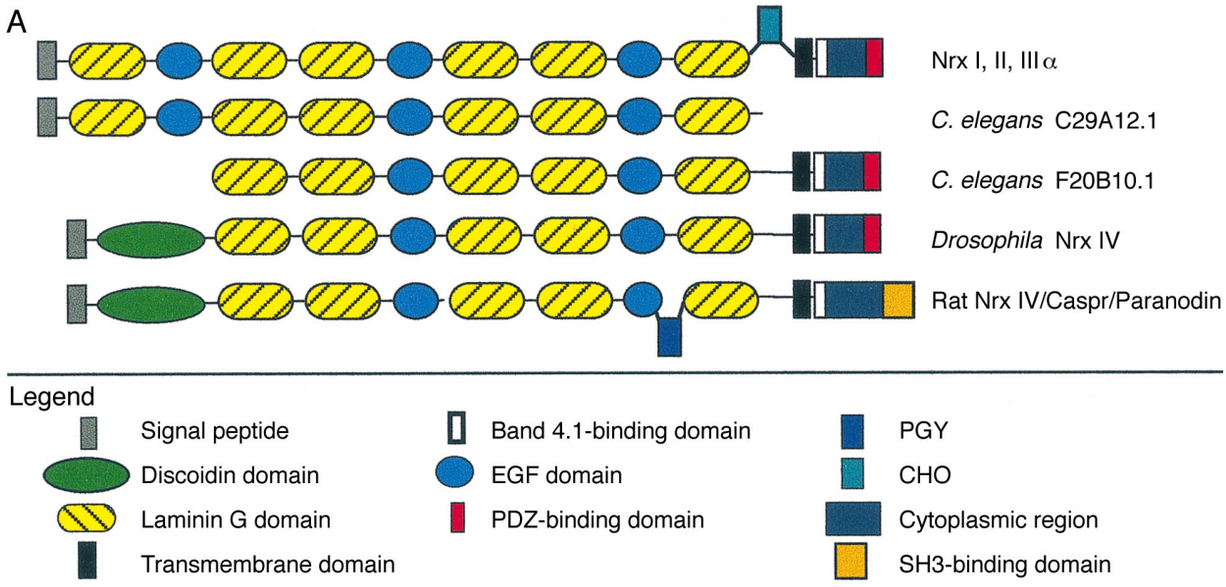


Fig. 2. *The neurexin family.* (A) *Structural comparison of the members of the neurexin family of proteins.* **(B)** *Summary of the results of phylogenetic analysis on selected members of the neurexin family. An exhaustive search was performed using the phylogenetic analysis program PAUP (Ref. 23) with alignments generated from both the full-length protein and laminin G–EGF–laminin G domains (neurexin motif). The same tree structure was obtained with each input method, resulting in similar trees with consistency indexes of 0.959 and 0.966 and retention indexes of 0.729 and 0.799, respectively.* **(C)** *BLAST searches performed using protein sequences from both* Drosophila *neurexin IV and rat neurexin I. The output sequences shown represent the most similar proteins only. BLAST searches were performed using a BLOSUM62 matrix looking at the following databases: nonredundant GenBank CDS translations, PDB, SwissProt, SPupdate and PIR.*

17q21 in a search to identify the *BRCA1* gene[24,25]. The human ESTs were referred to as neurexin-like[24] and named hNRXIV (Ref. 5) or human caspr (Ref. 16) later. The second gene was isolated by degenerate polymerase chain reaction to identify *Drosophila* homologs of neurexins and named Neurexin IV (Ref. 5). The third member of the family, named rat caspr, was isolated as a contactin-associated protein[16]. Rat caspr was also independently isolated by Menegoz *et al.*[14] as a major rat brain glycoprotein that binds to specific lectins. The protein was named paranodin as it is localized to the nodes of Ranvier, in the paranodal space[14]. A fourth member of the family, the mouse homolog of NRXIV, was isolated by low stringency hybridization using the human ESTs and named *MHDNIV* (mouse homolog of *Drosophila neurexin* IV; GenBank accession number AF039833). Finally, genome database searches revealed the presence of a putative *C. elegans* homolog named Z69636 in genomic cosmid F20B10.1.

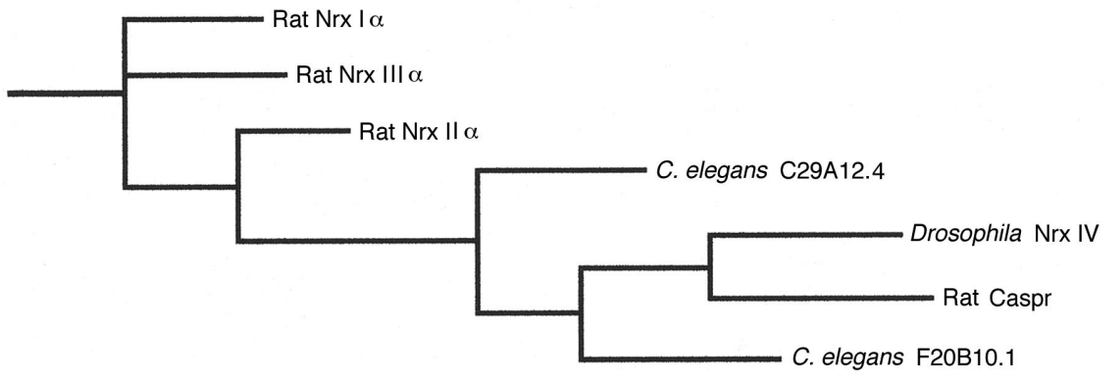
The proteins of the family typically contain two or three repeats of a laminin G domain–epidermal growth factor (EGF)–laminin G domain, also named neurexin motifs, followed by a transmembrane domain and a short cytoplasmic tail (Fig. 2A). Interestingly, the N-terminus domains within and between neurexins I, II and III and the novel members of the neurexin family are the most variable domains. The major difference between the novel members and the original neurexins is that they contain an N-terminus discoidin domain which is not found in neurexins I, II and III (Fig. 2A). Although the overall structure of the proteins of the family is quite similar, the overall identity between neurexins I, II and III and the novel members is only 21–29% throughout their entire length. Hence, it has been argued that neurexin IV should not be named neurexins on the basis of this and several other observations[26]. However, phylogenetic analyses and BLAST searches (see Fig. 2A,B) show not only that all these proteins are evolutionarily related, but also that they are more similar to each other than to any other protein in the databases, which now contain more than a million entries[23,27,28]. In addition, the BLAST probability data for *Drosophila neurexin* IV and vertebrate neurexins (Fig. 2C) are among the highest (top 10%) of a list of 66 human genes (see www.tigem.it) that are listed as *Drosophila* homologs[29]. We therefore propose that neurexins I, II and III and the novel members (NCPs) are all members of the neurexin family.

The novel neurexins can be subdivided into two classes on the basis of their motifs. Along with the characteristic structure of neurexins, hNRXIV, rat *caspr/paranodin* and mouse *MHDNIV* have an amino acid repeat (PGY) between the last EGF and laminin A domains, as well as a proline-rich C-terminus SH3 binding domain[14,16]. These three neurexins are very similar to each other (more than 93% identity) and are almost certainly orthologs. The *Drosophila neurexin* IV and the *C. elegans* gene lack the PGY repeat, and the SH3 binding domain is replaced with a PDZ binding motif (M.A. Bhat, unpublished observations). We therefore believe that these novel neurexins should be subdivided into two classes.

Neurexin IV and caspr/paranodin are enriched at SJs

Subcellular localization of neurexin IV to specific junctions provided the first clue about the function of the novel neurexins. The *Drosophila* protein was shown to localize to SJs (Ref. 5) in all cells that were previously shown to contain pleated SJs by electron microscopy criteria[30]. Neurexin IV expression occurred just prior to the morphological appearance of the SJs, and colocalization studies with other proteins known to localize to SJs – coracle[31] and discs large

B



C

BLAST Search Using *Drosophila* Nrx IV

Sequence	Smallest sum
neurexin IV (*Drosophila melanogaster*)	0.0
caspr (*Homo sapiens*)	1.1e-131
caspr (*Rattus norvegicus*)	4.9e-129
neurexin III-alpha (*R. norvegicus*)	2.0e-46
F20B10.1 (*Caenorhabditis elegans*)	1.6e-45
neurexin I-alpha (*R. norvegicus*)	5.8c-44
neurexin II-alpha (*R. norvegicus*)	1.2e-40
C29A12.4 (*C. elegans*)	4.2e-27
crumbs protein (*D. melanogaster*)	1.9e-19
laminin A (*C. elegans*)	8.4e-15
agrin-related protein 1 (*Gallus gallus*)	1.2e-14

BLAST Search Using Rat Nrx I-alpha

Sequence	Smallest sum
neurexin I-alpha (*R. norvegicus*)	0.0
neurexin II-alpha (*R. norvegicus*)	0.0
neurexin III-alpha (*R. norvegicus*)	0.0
C29A12.4 (*C. elegans*)	1.1e-122
neurexin IV (*D. melanogaster*)	2.5e-44
caspr (*R. norvegicus*)	5.0e-37
caspr (*H. sapiens*)	1.7e-34
crumbs (*D. melanogaster*)	9.6e-23
laminin A (*C. elegans*)	5.9e-19
agrin (*G. gallus*)	4.4e-17

(DLG)[32] – confirmed that Neurexin IV is a specific marker for pleated SJs. One subpopulation of cells with prominent SJs are those between the outermost glial cells of the CNS and PNS, often called the perineurium[8,9,33]. As shown in Fig. 3, these cells express Neurexin IV in the PNS of embryos (Fig. 3A) and third instar larvae (Fig. 3B). These SJs have previously been proposed to form the blood–brain barrier in other insects, and to slow down paracellular transport. They were therefore proposed to substitute functionally for the vascular endothelium of higher organisms[8,9,33].

Menegoz *et al.*[14] partially purified a 180-kDa glycoprotein from rat brain using concanavalin A (a lectin) affinity chromatography. Immunohistochemical localization showed that the protein is present throughout the neuropil in the cerebellum and is highly enriched in the paranodal area of the nodes of Ranvier, hence the name paranodin. A similar paranodal localization was also observed in the sciatic nerve in the PNS. In all cases it was observed that this protein is initially expressed at the onset of myelination and becomes localized progressively to the paranodes during neuronal maturation[14]. These studies were confirmed and extended by Einheber *et al.*[15], who showed that the protein (named caspr in this study) is specifically expressed by neurons, not by Schwann cells or oligodendrocytes. The protein levels are downregulated during myelination and this downregulation and axonal localization is paired with a dramatic redistribution to the paranodal space during the formation of the nodes. Immunoelectron microscopy studies showed that the protein is indeed an axonal component localized to the septate-like junctions of the paranodal region in the corpus callosum and the facial nerve. It is also likely that the rat caspr/paranodin detected in the cerebellum is localized to septate-like junctions that have been reported in this tissue[14,34,35]. In addition, Einheber *et al.*[15] document that small-diameter nerve fibers that appear to be unmyelinated express high levels of caspr/paranodin in the CNS, suggesting that in the absence of myelination some neurons might continue to express high levels of the protein.

It is interesting to note that glial cells have been shown to form the blood–brain barrier not only in higher invertebrates[5] but also in primitive vertebrates, such as the elasmobranch fish[8,36]. An antibody raised against the electric elasmobranch fish neurexin III cytoplasmic domain stained the glial cells of the electromotor nerve[18]. These cells have been involved in the formation of the blood–brain barrier in this fish, indicating that, in addition to the structural similarities between the novel neurexins and neurexins I, II and III (see above), there are also functional similarities. Russell and Carlson[18] also showed that neurexin III is localized at the interface of axons and myelinating Schwann cells, similar to caspr/paranodin. Hence, in a primitive vertebrate, neurexin III is localized to two different cell populations – glial cells and neurons – to which *Drosophila* Neurexin IV and caspr/paranodin have been localized, respectively.

Neurexin IV and caspr/paranodin might form barriers

SJs have been proposed to play a role in cellular cohesion/cell adhesion[37], blood–brain barrier formation[8,9,33] and intercellular communication[32]. The morphology of SJs is obviously affected in *nrx IV* mutants and the electrophysiological analyses clearly indicate a defect in barrier formation, because varying the

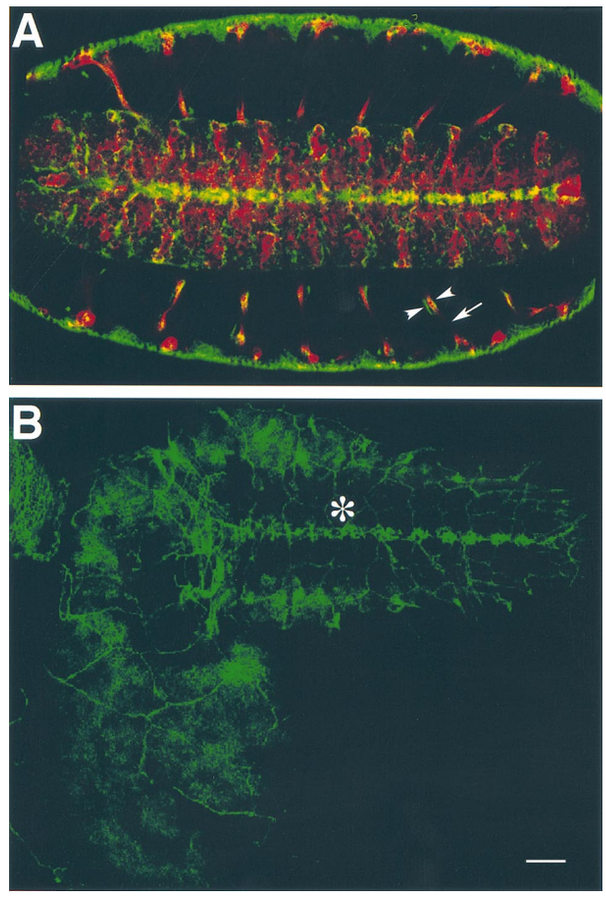


Fig. 3. Drosophila *Neurexin IV is localized to perineurial glia.* (A) *A stage 15 embryo double-labeled with anti-neurexin IV antibody (green) and mAb 22CI0 (red; this antibody labels neuronal membranes).* Drosophila *neurexin IV is expressed in ectodermal epithelial cells and the perineurial glial cells of the PNS and CNS. In the PNS, the protein is expressed in the perineurial glia (see Fig. 1D). An arrowhead indicates neurexin IV (green) next to the axon bundle which is shown in red (arrow). In the CNS, neurexin IV is expressed in the midline glia wrapping the commissures.* **(B)** *The whole third instar larval CNS. Immunolocalization of Neurexin IV in the larval ventral nerve cord and left lobe of the CNS shows that the protein is expressed in midline glia as well as at the edges of the interdigitating perineurial glial cells required for the maintenance of the blood–brain barrier in third instar larvae. Perineurial glia are very large cells that ensheath the whole CNS. In the ventral nerve cord, only eight cells per segment are required to circumference the nerve cord; for example, ∗ corresponds to a single perineurial cell. Note also the giant cells surrounding the brain lobes. Anterior is to the left. Scale bar, 50 μm* (A)*; 25 μm* (B).

extracellular K^+ concentration greatly affects the profile of spontaneous neural activity in mutants but not in wild-type embryos[5]. Hence, a major function of Neurexin IV in *Drosophila* embryos is to form a barrier to prevent ions and other molecules reaching other cells, probably by entrapping molecules that bind ions[8]. However, neurexin IV is not the only component of SJs, as transmission electron microscopy studies clearly show that, in the absence of the protein, pleated SJs take the shape of smooth SJs (Ref. 5). This implies that SJs might play a much broader role than suggested by the phenotype in *nrx IV* mutants. Indeed, mutant *nrx IV* embryos show no major abnormal cellular morphologies, or defects in cell adhesion, loss of cell polarity, or aberrant proliferation[5], suggesting that neurexin IV is not required for cell adhesion or intercellular communication in embryos.

The septate-like junctions of the paranodal space of the node of Ranvier have also been shown to provide a partial barrier to the diffusion of small ions, and an absolute barrier for the passage of large molecules into the internode[38]. It is thought that, by slowing down ionic flow, these junctions are likely to promote saltatory conduction[15]. In addition, the septate-like junctions might form a diffusion barrier for Na^+, channels which are concentrated in the nodal area, and an anchor for K^+ channels, which are localized adjacent to the paranodal space[1]. Analysis of mice mutant for caspr/paranodin could reveal the precise *in vivo* function of the septate-like junctions in vertebrates in the near future.

Neurexin IV anchors band 4.1 proteins to SJs

All members of the neurexin family contain a conserved band 4.1-binding domain[39], a few amino acids from the C terminus of the transmembrane domain[22] (Fig. 2A). Several observations indicate that this domain plays an important role in the localization of band 4.1 proteins. Colocalization at SJs of neurexin IV and coracle, the *Drosophila*-band 4.1 homolog[31], prompted experiments to determine whether coracle/band 4.1 was mislocalized in neurexin IV mutants. Baumgartner *et al.*[5] showed that, in *nrx IV* mutants, coracle/band 4.1 is not localized at SJs but is present in cytoplasm and cell membranes. In addition, lack of neurexin IV causes a phenotype very similar to that observed in *coracle* mutants. Hence, this neurexin is required for proper localization of coracle/band 4.1. It was recently demonstrated that partially purified band 4.1 from erythrocytes and rat brain was able to bind this sequence in rat caspr/paranodin[14], showing a direct interaction between band 4.1 and these neurexins. Based on these observations it seems likely that other neurexins will bind band 4.1 homologs.

Band 4.1 is a member of a superfamily of proteins including ezrin, radixin, moesin and merlin – the ERM protein family. These proteins act as linkers between the plasma membrane and the cytoskeleton by interacting with F-actin and with each other in vertebrate cells (for review see Ref. 40). In insects, SJs have been shown to be very enriched in coracle/band 4.1 protein[31] and F-actin, suggesting a similar role for these proteins as in vertebrates. Depolymerization of actin causes a disorganization of the intramembrane components of SJs (Ref. 37), further supporting a link between SJs and the cytoskeletal network. Mutations in the gene encoding the merlin protein are involved in tumor formation, including schwannomas and meningiomas, but the process by which these tumors arise is poorly understood (for review see Ref. 41). Recently, the members of the ERM family were shown to be downstream effectors of Rho- and Rac-dependent assembly of focal adhesion complexes and actin filaments, suggesting a role for ERMs in stress fiber assembly[42]. Hence, disrupting components of the complexes containing ERM proteins and F-actin might affect cellular adhesion and facilitate tumorigenesis. Since *Drosophila* Neurexin IV is required for proper localization of coracle/band 4.1, it will be of interest to determine if neurexin IV acts as a tumor suppressor as well.

Caspr/paranodin and contactin binding

In addition to binding intracellular ligands such as band 4.1, it is likely that the extracellular domains of

neurexins will interact with specific proteins present in the extracellular matrix or other neuronal or glial cells. Peles *et al.*[43] showed that rat caspr binds contactin, a glycosylphosphatidylinositol (GPI)-anchored receptor with Ig and FIII domains, which is a neuronal specific cell surface receptor that binds to a receptor-like protein tyrosine phosphatase β (RPTP). RPTPβ, which is expressed in radial glia and astrocytes, is a transmembrane protein with an extracellular domain that contains a carbonic anhydrase domain and a FIII repeat. RPTPβ mediates cell adhesion and neurite outgrowth of specific neurons. These responses are blocked by antibodies against contactin, suggesting that interactions between glial RPTPβ and neuronal contactin can lead to bidirectional signals between neurons and glia[43]. However, contactin is a GPI-anchored protein that lacks a cytoplasmic domain, and it was therefore surmised that its biological response in the neuron might be mediated by binding a neuronal protein that has an intracellular signaling domain. Using coprecipitation experiments, Peles *et al.*[43] purified a 190-kDa protein associated with contactin and RPTPβ. Microsequencing led to cloning and sequencing of the full-length human and rat protein, named caspr (Ref. 16).

Contactin and caspr/paranodin form a complex when both proteins are present in the same membrane (i.e. in *cis*). In addition, both proteins seem to be constitutively complexed. Signaling via binding of RPTPβ to contactin and caspr could be transduced into the neuronal cytoplasm via the caspr SH3 binding site which is found at the C-terminus domain[16].

A number of issues need to be clarified with respect to the *in vivo* significance of the interaction of contactin and caspr/paranodin. It was recently shown that both proteins have a very different fate during myelination. Contactin is barely detectable in myelinated fibers, whereas caspr/paranodin is highly concentrated in the paranodes. Hence, at this developmental stage, the two proteins are not obligately associated[15]. In addition, a recent report has shown that contactin is expressed in nonneuronal cells (for example, oligodendrocytes)[15,44]. The expression on these glial cells and the neuronal expression of caspr/paranodin could promote the interaction between these two cell populations[15], but these *trans* interactions have not been observed to occur biochemically[16]. Finally, it should be noted that RPTPs have been implicated in growth cone guidance of motor neurons in *Drosophila*[45,46] and that *nrx IV* mutants have been shown to lack muscle innervation in about 10% of cases. This lack of synapses is associated with subtle morphological defects of motor neurons which resemble those seen in RPTP mutants[5,45,46].

Future prospects

The past two years have revealed a series of interesting observations and insights into the function of a novel subgroup of neurexins, which we term here NCPs. This protein family is likely to play a key role in axonal–glial interactions in invertebrates as well as in vertebrates, and several observations suggest that other members of the neurexin family will also play a role in interactions between neuron and glia. Subcellular localization to the paranodal septate-like junctions of vertebrate NCPs as well as phenotypic analyses of *Drosophila* mutants suggest that removal of these proteins in vertebrates might cause defects in saltatory responses. It will be of interest to determine if the localization of the paranodal potassium channels and the nodal Na^+ channels[1] is altered in mouse caspr/paranodin mutants. In addition, lack of this protein might cause a decrease in nerve conduction velocity and partial or complete demyelination, thereby mimicking some of the phenotypes associated with Charcot–Marie–Tooth disease and related peripheral neuropathies[11,47]. Human *NRXIV* appears to be a candidate gene for these diseases as >50% of the patients with these syndromes, who do not have a duplication of PMP22, have mutations in genes that remain to be identified[10,11,48].

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