Mini-Review

Deciphering the Function of Neurexins at Cellular Junctions

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THE mechanisms that allow the nervous system of animals to interpret and respond to their environment requires an astonishing complexity of neuronal connections and signaling pathways. The past decade has seen the emergence of a common theme in the mechanisms mediating these seemingly complex tasks: during evolution, nervous system development has taken components of less complex cells and pathways and modified them to fit the requirements of neuronal signaling. Neurexins were originally identified as a polymorphic family of neuronal-specific type 1 cell surface membrane proteins that were postulated to serve a unique role in specifying synaptic specificity and docking synaptic vesicles at the active zone. However, recent genetic and molecular analyses have provided novel insights that suggest neurexins are evolutionarily conserved and mediate many aspects of cellular function.

Molecular Biology of Neurexins

Neurexins were first identified by Südhof and colleagues as cell surface receptors for α-latrotoxin (19). This component of black widow spider venom is well known for its potent effects at the synapse, causing massive calcium-independent release of neurotransmitters. Neurexins were also found to interact with the synaptic vesicle calcium-binding protein synaptotagmin (7, 19). These studies provided the framework for an attractive model in which α-latrotoxin binds to neurexins, relaying a signal from the outside of the synapse to the synaptic vesicle fusion machinery by activating synaptotagmin. This model also suggested an in vivo correlate that has been propagated throughout the synaptic literature: neurexins may be involved in the formation of the active zone by binding to postsynaptic receptors or extracellular matrix components and subsequently docking synaptic vesicles at these sites through an interaction with synaptotagmin. A second clue to neurexin's function was the report that they were selectively enriched at synapses (24) and exhibited a striking number of alternatively spliced forms (22). It was proposed that the differential localization of various populations of alternatively spliced neurexins may allow them to function in synaptic targeting (22).

Recent data have challenged these models of neurexin function. These findings include the following observations: (*a*) Although Neurexin Iα binds α-latrotoxin, their interaction is calcium-dependent. α-Latrotoxin's effects are calcium independent, and a novel calcium-independent ligand has now been identified as a candidate physiological α-latrotoxin receptor (4, 12). (*b*) α-Latrotoxin still causes exocytosis in mutant mice lacking Synaptotagmin I (6), raising questions about whether a neurexin–synaptotagmin interaction has a physiological role. (*c*) *Drosophila* and *Discopyge* homologues of neurexins have now been identified, and they are expressed in cells other than neurons and are not found at synapses (2, 19a). In addition, new studies have failed to repeat the reported presynaptic localization of mammalian neurexins (23). Given the challenges to the widely held views on neurexin function, we will review the recent literature and discuss the possible role of these proteins in cell function.

To date, the neurexin family includes three related vertebrate genes (NRX I, II, and III) (23, 24), a *Drosophila* gene (NRX IV), and its human homologue (hNRX IV) (2), which has recently been shown to be a contactin-associated protein, Caspr (17, 18). A homologue of Neurexin IIIα has also been recently reported in the marine ray, *Discopyge ommata*, and a second *Drosophila* neurexin is being characterized (Yuan, L., and B. Ganetzky, personal communication). As shown in Fig. 1 *A*, the three vertebrate neurexins encode an α-isoform (1,507–1,578 amino acids, 160–220 kD) and a β-isoform (437–471 amino acids). Neurexins have a large extracellular domain, a single transmembrane, and a short cytoplasmic segment (40–55 amino acids). The extracellular portions of the α-isoform contain three EGF repeats, six repeats with low homology to the G domain of laminin A, and an O-linked sugar domain in proximity to the transmembrane segment. *Drosophila* Neurexin IV contains two EGF repeats, five laminin G domains, and an NH_2-terminal Discoidin domain conserved in hNRX IV/Caspr, but not present in Neurexins I, II, or III. EGF and laminin G domains are present in a variety of proteins that are components of the extracellular matrix or involved in cell signaling, including laminin A, agrin, perlecan, and the *Drosophila* developmental proteins Crumbs and Slit, and are thought to function in protein–protein interactions within the extracellular environment. The COOH-terminal intracellular segment of neurexins contains a conserved four–amino acid tail that functions as a recognition sequence for the PDZ domains of membrane-associated guanylate kinase (MAGUK)[1] proteins (21). In addition,

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1. *Abbreviations used in this paper*: DLG, discs large protein; MAGUK, membrane-associated guanylate kinases.

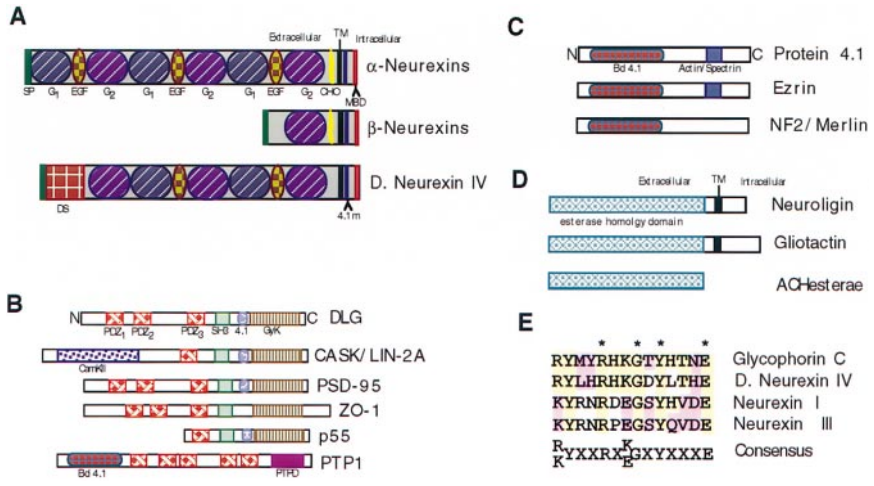

Figure 1. Domain structure of neurexins and interacting proteins. (*A*) Neurexins are type I integral membrane proteins with a classical signal peptide and an extracellular domain with EGF and laminin G repeats. The intracellular domain contains a conserved recognition sequence for PDZ domains and a motif homologous to glycophorin C that has been shown to bind protein 4.1. (*B*) Domain structure of the MAGUK family and related proteins. (*C*) The protein 4.1 family includes a conserved 30-kD domain shown to bind glycophorin C, calmodulin, and p55, and a COOH-terminal 10-kD domain that mediates interactions with the actin/spectrin cytoskeleton. (*D*) The neuroligin/gliotactin family are type 1 transmembrane proteins with a large extracellular domain homologous to the esterase family (e.g., acetylcholinesterase). (*E*) Sequence alignment of the protein 4.1 interacting domain of glycophorin C with the neurexins. Yellow boxes represent identities with glycophorin C, while pink boxes indicate sequence similarities. Amino acids conserved in all four proteins are indicated by asterisks. No sequence gaps have been introduced. This sequence motif is present intracellularly in all four proteins shortly after the transmembrane-spanning segment ends and includes residues 65–80 of glycophorin C, 1239–1254 of *Drosophila* Neurexin IV, 1455–1470 of Neurexin I, and 1417–1432 of Neurexin III. *SP*, signal peptide; G_1/G_2, laminin G domains; *CHO*, O-linked sugar domain; *TM*, membrane-spanning segment; *4.1m*, protein 4.1 binding motif; *MBD*, MAGUK binding domain; *N*, NH_2 terminus; *C*, COOH terminus; *4.1*, protein 4.1 binding site; *GyK*, guanylate kinase domain; *CAMKII*, calcium–calmodulin kinase II homology domain; *PTP1*, protein tyrosine phosphatase domain; *Bd 4.1*, protein 4.1 30-kD homologous domain; *Actin/Spectrin*, 10-kD cytoskeletal interacting domain; *D.*, *Drosophila.*

the intracellular domain also contains a putative protein 4.1 binding motif.

Given their amino acid sequence, neurexins are predicted to play a role in extracellular signaling or cell adhesion. The splice variants of the vertebrate neurexins are expressed in distinct populations of neurons, but their subcellular localization is unknown. *Drosophila* Neurexin IV is not expressed in neurons but instead localizes to septate junctions of glia, epithelia, and ectodermally derived cells (2). Septate junctions are thought to be the invertebrate equivalent of tight junctions and represent sites of cell contact and intercellular signaling. Septate and tight junctions are also responsible for forming the blood–brain barrier, suggesting a role for glial-expressed neurexin in this process. Human NRX IV/Caspr is widely expressed, including in kidney, lung, and brain, and localizes to axons (2, 17). hNRX IV/Caspr was identified by its interaction with the axonally expressed Ig superfamily member contactin, which interacts with glial receptor tyrosine phosphatase β, again suggesting a role in neuronal–glial interactions (18). A newly identified *Discopyge* neurexin is found in neurons and perineural fibroblasts but does not localize to synapses (19a). Instead, it localizes at sites of Schwann cell–neuron contacts along the axon and at sites of perineural fibroblast contact. A second neurexin homologue has been identified in *Drosophila*, and it is also expressed in glia (Yuan, L., and B. Ganetzky, personal communication). These localization studies indicate that the role of neurexins is not confined to neurons and suggests that they may participate in a variety of cellular interactions.

Neurexins: A Link Between the Extracellular Environment and Intracellular Signaling Pathways

Both molecular and genetic analysis suggests that neurexins interact with several intracellular protein families of known function. Yeast two-hybrid screening for interacting partners with the rat neurexin intracellular domain identified a ligand that was named CASK (8). The NH_2-terminal third of CASK has homology to the calmodulin-binding and autoinhibitory domains of CAMKII. The remaining COOH-terminal region of the protein shares homology with the MAGUK family of proteins and contains a PDZ domain, a Src homology 3 domain, and a COOH-terminal catalytically inactive guanylate kinase domain. CASK is expressed not only in neurons but in many tissues, raising the possibility that additional neurexins may interact with CASK outside the nervous system. CASK interacts with all three vertebrate neurexins, and this binding is abolished by deletion of the last three amino acids of the intracellular COOH-terminal region of neurexin (8). Several subclasses of sequence-specific interactions involving the PDZ domains of MAGUK proteins and the COOH-terminal amino acids of interacting proteins have been defined and include the COOH-terminal sequence $EY^Y/_F{}^I/_V$, which is conserved in vertebrate and *Drosophila* neurexins and is also present in the erythrocyte integral membrane protein glycophorin C (21). Genetic studies also support an interaction with the MAGUK family of proteins. In *Drosophila*, the discs large protein (DLG), another MAGUK member, is localized to both synapses and septate junctions (13, 25), and genetic analysis has shown that both DLG and neurexin function in the assembly of septate junctions (2, 26). Interestingly, a new protein with PDZ domains has been recovered in a yeast two-hybrid screen using the *Drosophila* neurexin intracellular domain as a bait (Bhat, M.A., unpublished data). Thus, members of the PDZ-containing MAGUK family represent a group of intracellular ligands for neurexins.

The MAGUK family is concentrated at cellular junc-

tions and involved in clustering ion channels and organizing sites of intercellular communication (for review see reference 20). Most MAGUK proteins contain one to three PDZ domains, a Src homology 3 domain, a protein 4.1 recognition sequence, and a catalytically inactive guanylate kinase domain. Members include the tight junction–associated ZO-1 and ZO-2, the synaptic and septate junction–associated DLG, the postsynaptic PSD95/SAP90, the synaptic and junctional–associated human DLG homologue, SAP97, the synaptic chapsyn 110/PSD93, the erythrocyte membrane–associated p55, and the *Caenorhabditis elegans* LIN-2A cellular junction signaling protein (Fig. 1 *B*). DLG and PSD95 cluster Shaker-type K^+ channels and *N*-methyl-D-aspartate receptors at synapses via their PDZ domains (10, 11). PSD95 has been shown to bind and localize neuronal nitric oxide synthase to synaptic membranes through an interaction between PDZ domains (3). One missing link in this pathway is how the MAGUK proteins themselves are clustered at sites of contact given their intercellular location. If neurexins were to localize to sites of cell contact via interactions with extracellular or transmembrane ligands, the intracellular domain could then recruit members of the MAGUK family and assist in clustering and organizing ion channels and associated junctional proteins.

One question that arises from these studies is how a neurexin–MAGUK complex is linked to the underlying cell cytoskeleton, a likely requirement for organizing sites of cell contact. One candidate family of proteins performing such a task is the protein 4.1 family. In *Drosophila*, the protein 4.1 homologue, Coracle (5), along with DLG and neurexin, are localized to septate junctions. In *neurexin* mutants, protein 4.1 fails to localize to septate junctions, suggesting that the intracellular COOH terminus of *Drosophila* neurexin is required to localize protein 4.1 (2). This intracellular domain is 68% similar to glycophorin C and contains a conserved 12–amino acid protein 4.1 recognition sequence ([16]; Fig. 1 *E*). The protein 4.1 recognition sequence, along with surrounding amino acids, is also conserved in vertebrate neurexins and suggests a consensus sequence of $^{R}/_{K}YXXRX^{K}/_{E}GXYXXE$ (Fig. 1 *E*). Another mechanism that may recruit protein 4.1 to this complex is the presence of a distinct protein 4.1 binding sequence identified in p55, and which is conserved in many MAGUK members ([16]; Fig. 1 *B*). The human DLG homologue has also been shown to interact with protein 4.1 and its homologue ezrin (14, 15), consistent with the MAGUK family binding members of the protein 4.1 family. Thus, an interaction with CASK, which also contains a protein 4.1 binding motif, may provide an independent mechanism for vertebrate neurexins to merge into this pathway and serve as a membrane anchor to the underlying cytoskeleton. The protein 4.1 family is widely expressed in many tissues, including the brain, and includes ezrin, radixin, moesin, talin, neurofibromatous 2/merlin, and protein 4.1 (Fig. 1 *C*). These proteins are involved in modulating cytoskeletal–membrane interactions, allowing regulation of cell adhesion, shape, and mobility. The erythrocyte complex of glycophorin C and protein 4.1 are required in vivo to localize the MAGUK family member, p55, to the membrane and to maintain cytoskeletal–membrane stability (16). The potential for this pathway to organize sites of cell contact is of general interest, but the finding that the MAGUK protein, DLG, and the protein 4.1 homologue, neurofibromatous 2/merlin, are involved in tumor formation should draw widespread attention. Thus, disrupting junctions required for cell communication and adhesion is a new pathway for cellular overgrowth and tumor formation.

Extracellular Ligands for Neurexins

We have reviewed several intracellular protein families suspected to be involved in neurexin's ability to link the extracellular environment to the cytoskeleton (protein 4.1) and intracellular clustering/signaling proteins (MAGUK). An additional question is whether extracellular ligands interact with neurexins at cellular junctions and maintain or modify neurexin function. Using a recombinant splice-specific Neurexin Iβ as a ligand, a type 1 transmembrane protein family (neuroligins) with homology to esterase proteins (e.g., acetylcholinesterase and carboxylesterase) was identified as a calcium-dependent extracellular ligand (9). Neuroligins (836–848 amino acids, 95 kD) are expressed within neurons and have a large extracellular esterase domain, a single transmembrane domain, and a cytoplasmic tail of ~100 amino acids (Fig. 1 *D*). The extracellular domain lacks a serine residue at the active site, suggesting that the protein is catalytically inactive, similar to the *Drosophila* esterase proteins neurotactin and gliotactin. Gliotactin (956 amino acids, 109 kD) is 50% similar to neuroligin and is expressed in glial and epithelial cells, as is *Drosophila* neurexin (1). Electrophysiological analysis of *gliotactin* mutants have demonstrated a breakdown of the blood–brain barrier similar to that caused by mutations in *neurexin* (1). Thus, in vitro studies in mammals suggest a physical interaction between neurexins and the neuroligin/gliotactin family, while in vivo genetic manipulations in *Drosophila* indicate both proteins function in a similar pathway. This interaction might form a transcellular scaffold allowing neurexins to organize intracellular proteins or may act in a ligand-receptor fashion, modifying neurexin's interactions with the MAGUK and protein 4.1 families.

Recently, another extracellular ligand, contactin/axonin-1, has been identified that binds hNRX IV/Caspr. This axonally expressed member of the Ig superfamily has been shown to bind glial receptor tyrosine phosphatase β, an interaction which is thought to play a role in axonal growth (18).

Summary and Future Prospects

Recent in vitro and in vivo studies have provided exciting insights suggesting that the neurexin family may function in organizing cellular junctions. This model is supported by the phenotypic analysis of *Drosophila neurexin* mutations (2). These mutants lack the ladder-like transcellular septate characteristic of septate junctions. In addition, neurexin is required in glia to form the blood–brain barrier, consistent with an ability of neurexins to form transcellular barriers at cellular junctions. Neurexin also localizes protein 4.1 to sites of cell contact, and *neurexin* mutations show defects in dorsal closure of the epidermis, a process requiring extensive cellular movements and signaling. Fig. 2 presents a model of a cellular junction incorporating neurexins.

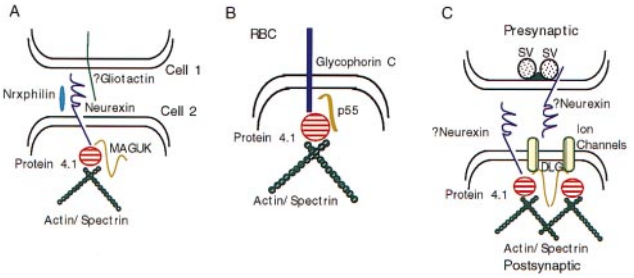

Figure 2. Model of neurexin interactions in cellular junctions. (*A*) Hypothetical junction incorporating a neurexin-gliotactin/neuroligin interaction with a link to the underlying cytoskeleton via protein 4.1 and to intracellular signaling/clustering MAGUK proteins. (*B*) A similar pathway connects the erythrocyte membrane to the cytoskeleton via glycophorin C. (*C*) MAGUK members have also been shown to cluster ion channels at synapses. It is currently unclear if neurexins exist at synapses and are involved in synapse formation, presynaptic vesicle docking, or postsynaptic channel clustering. Current data favor a role for neurexins in axonal–glial interactions and cellular junctions instead. *Nrxphilin*, Neurexophilin; *SV*, synaptic vesicle; *DLG*, discs large protein/PSD95.

Essential questions remain to be answered concerning the neurexins. Foremost, a precise subcellular location of the known vertebrate neurexins is required to determine if they may also be involved in axonal–glial interactions, similar to that reported for the NRX IV homologue, hNRX IV/Caspr. In addition, it will be important to extend the search for nonneuronal neurexins, as a more complete catalog of the neurexin family is required to begin to determine the variety of roles these proteins might play in cellular junctions. Do neurexins also play a role in the MAGUK's ability to cluster ion channels at synapses, or is their interaction with members of this protein family restricted to other sites of cell contact? Do extracellular ligands serve as activators or modulators of neurexin's link to the intracellular environment, and how does the extensive alternative splicing in vertebrate neurexins define or modify these interactions? And finally, what, if any, cellular signals can be relayed through the neurexins? These and other questions should inspire many interesting experiments in the near future, as dissection of the role of neurexins in cell junctions is just beginning.

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