Neurexophilin Binding to α-Neurexins

A SINGLE LNS DOMAIN FUNCTIONS AS AN INDEPENDENTLY FOLDING LIGAND-BINDING UNIT*

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α-Neurexins (Iα, IIA, and IIIa) are receptor-like proteins expressed in hundreds of isoforms on the neuronal cell surface. The extracellular domains of α-neurexins are composed of six LNS repeats, named after homologous sequences in the Laminin A G domain, Neurexins, and Sex hormone-binding globulin, with three interspersed epidermal growth factor-like domains. Purification of neurexin Iα revealed that it is tightly complexed to a secreted glycoprotein called neurexophilin 1. Neurexophilin 1 is a member of a family of at least four genes and resembles a neuropeptide, suggesting a function as an endogenous ligand for α-neurexins. We have now used recombinant proteins and knockout mice to investigate which isoforms and domains of different neurexins and neurexophilins interact with each other. We show that neurexophilins 1 and 3 but not 4 (neurexophilin 2 is not expressed in rodents) bind to a single individual LNS domain, the second overall LNS domain in all three α-neurexins. Although this domain is alternatively spliced, all splice variants bind, suggesting that alternative splicing does not regulate binding. Using homologous recombination to disrupt the neurexophilin 1 gene, we generated mutant mice that do not express detectable neurexophilin 1 mRNA. Mice lacking neurexophilin 1 are viable with no obvious morbidity or mortality. However, homozygous mutant mice exhibit male sterility, probably because homologous recombination resulted in the co-insertion into the neurexophilin gene of herpes simplex virus thymidine kinase, which is known to cause male sterility. In the neurexophilin 1 knockout mice, neurexin Iα is complexed with neurexophilin 3 but not neurexophilin 4, suggesting that neurexophilin 1 is redundant with neurexophilin 3 and that neurexophilins 1 and 3 but not 4 bind to neurexins. This hypothesis was confirmed using expression experiments. Our data reveal that the six LNS and three epidermal growth factor domains of neurexins are independently folding ligand-binding domains that may interact with distinct targets. The results support the notion that neurexophilins represent a family of extracellular signaling molecules that interact with multiple receptors including all three α-neurexins.

Neurexins are neuron-specific cell surface proteins. There are at least three neurexin genes in mammals, each of which contains two independent promoters (reviewed in Ref. 1). As a consequence, the three genes generate six principal neurexins (neurexins Iα, Iβ, IIA, IIIa, IIIb, and IIIβ) that differ in their N-terminal structures but share the same C-terminal sequences (shown schematically in Fig. 1A). All neurexins are subject to extensive alternative splicing, resulting in hundreds of isoforms (2). Neurexins are synthesized in the brain in a heterogeneous expression pattern. Not only different α- and β-neurexins but also various splice forms are differentially distributed, conferring onto each group of neurons a characteristic combination of neurexins (2).

Neurexins are composed of an interesting domain structure (Fig. 1A). Their dominant component is the LNS domain. LNS domains were named after repeated sequences Laminin A, Neurexins, and Sex hormone-binding protein (3). They are also referred to as G domain repeats with reference to laminin A (4). However, the term G domain repeat is confusing because, as demonstrated below, individual LNS domains are independently folding domains. As a result, the G domain in fact consists of five separate domains. In α-neurexins, LNS domains are part of a cassette that is repeated three times (1, 2). Each copy of the cassette contains two LNS domains, LNS(A) and LNS(B), separated by an EGF1-like sequence. This gives α-neurexins a total of six LNS and three EGF domains (Fig. 1A). In β-neurexins, only the last LNS domain is present and is preceded by a short β-neurexin specific sequence. In all neurexins, the LNS domains are followed by a carbohydrate attachment sequence, a single transmembrane region, and a short cytoplasmic tail (Fig. 1A).

LNS domains are found in a large number of proteins, including serum proteins, components of the extracellular matrix, and cell surface receptors involved in cell-cell interactions (3). These proteins share few properties and functions making it difficult to postulate a general function for LNS domains. Although LNS domains have been studied in different contexts, no activity of an individual LNS domain has been discovered. At present, it is unclear if LNS domains form autonomous domains or are components of larger structures.

β-Neurexins bind to neuroligins that are also neuron-specific cell surface proteins (5, 6). The interaction between β-neurexins and neuroligins induces cell-cell adhesion (7). Intracellularly, β-neurexin-neuroligin complexes are coated by PDZ domain proteins; neurexins interact on the intracellular side with CASK whereas neuroligins interact with PSD-95 and related molecules (8, 9). These data suggest that β-neurexins serve as cell adhesion molecules whose interaction participates in creating an intercellular junction between neurons. Recent results

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‡The abbreviations used are: EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; kb, kilobase(s); bp, base pair.
show that this junction most likely corresponds to synapses. Quantitative immunogold electron microscopy revealed that neuroligins are specific for postsynaptic densities and that CASK is highly concentrated in synapses (8, 10). Furthermore, the neurexin-neuroligin complex biochemically co-purifies with synaptic junctions (11). Thus β-neurexins may contribute to the formation or maintenance of synaptic junctions by serving as bridges between the presynaptic machinery and the postsynaptic membrane. Interestingly, the binding of β-neurexins to neuroligins is tightly regulated by alternative splicing (5, 6). As a result, the β-neurexin-neuroligin junction is regulated and could serve as a mechanism to establish specificity as originally proposed (12).

The functions of α-neurexins are better characterized. Although the functions of α-neurexins are less well defined than those of β-neurexins, α-neurexins are clearly important molecules because knockout mice that lack multiple α-neurexins are not viable. Two potential ligands for neurexin Iα have been identified that may also bind to other neurexins and give clues to their functions. These potential ligands are α-latrotoxin, an excitatory neurotoxin from black widow spiders, and neurexophilin, an endogenous ligand in the brain. Neurexin Iα binds with high affinity in a Ca2+-dependent manner to α-latrotoxin, which induces synaptic vesicle exocytosis and neurotransmitter release (12, 13). The binding of α-latrotoxin to neurexin Iα led to the original identification of neurexins (12) and agrees well with the notion that neurexins participate in synaptic functions. However, although neurexin Iα was shown to contribute to the effects of α-latrotoxin in triggering neurotransmitter release, neurexin Iα is not the only α-latrotoxin receptor (14). A second Ca2+-independent receptor called CL1 (CIRL/latrophlin 1) may mediate part or all of the effects of α-latrotoxin in the brain (15, 16). Furthermore, no direct demonstration is available that neurexin Iα is in fact a functional α-latrotoxin receptor. Thus it is unclear if neurexin Iα is simply a co-receptor or a genuine receptor for α-latrotoxin and if it is indeed co-localized with the synaptic release apparatus.

Neurexophilin was discovered as a 29-kDa protein that is purified in a tight complex with neurexin Iα on immobilized α-latrotoxin (17). The neurexophilin-neurexin Iα complex cannot be dissociated by urea but only by guanidinium thiocyanate, suggesting that binding is very tight (18). Molecular cloning revealed that there are at least four genes related to neurexophilins in mammals, referred to as neurexophilins 1–4 (18, 19). Rodents contain all four genes but express at detectable levels only neurexophilins 1, 3, and 4, which are primarily synthesized in the brain (19). The structure of neurexophilins predicts that they are composed of four domains: a signal peptide, a nonconserved N-terminal region, a highly conserved central N-glycosylated domain, and an equally conserved C-terminal cysteine-rich domain. Expression and protein purification experiments revealed that neurexophilins are secreted glycoproteins that are proteolytically processed from the primary translation product, first by signal sequence cleavage and then by cleavage at the boundary between the N-terminal nonconserved and the central conserved domain (19). The proteolytic processing of neurexophilins is reminiscent of maturation processes observed for neuropetides, and their tight binding to neurexins indicates that they represent endogenous ligands for α-neurexins.

The current data suggest that neurexophilin serves as an endogenous ligand for neurexins. The question arises if all neurexophilins are ligands for all neurexins or if subsets of neurexins and neurexophilins interact. Furthermore, the complicated composition of neurexins with repeated domains raises the question if the various domains collaborate to form a binding site for neurexophilins, or if the repeats are specialized for different types of interactions, and a single repeat is sufficient for neurexophilin binding. In the current study we have explored the nature of the interaction of α-neurexins and neurexophilins and its biological significance. We have investigated the possibility that binding of neurexophilin to α-neurexins is mediated by a specific extracellular domain of neurexins and probed the specificity of the binding of neurexophilin to α-neurexins. Our data show that of six LNS domains a single LNS domain in all three neurexins is sufficient for binding neurexophilin and that neurexophilins 1 and 3 but not 4 bind to neurexins. This is the first demonstration that LNS domains function as autonomous ligand-binding sites with a high degree of specificity.

**EXPERIMENTAL PROCEDURES**

**Construction and Transfection of Expression Vectors**—We constructed a series of vectors that direct expression of various extracellular domains of neurexins fused to the Fc region of human IgG. Different extracellular regions of neurexins were amplified by polymerase chain reaction from characterized cDNAs using specific oligonucleotide primers and subcloned into pcMV-IgG vectors (6, 20). For neurexins 3 and 4, full-length expression vectors were constructed using cDNA clones previously identified (19). For pCMVPh3, a 1.8-kb fragment was cloned into the EcoRI and HindIII sites of pcMV5. Two different vectors were cloned for neurexophilin 4, a full-length expression vector by inserting a 1.2-kb EcoRI/BglII fragment into the same sites of pcMV5 (pCMVPh4-myc). COS cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and transfected using DEAE-dextran with chloroquine and a 2-min glycerol shock (21).

**Expression, Purification, and N-terminal Sequencing of Neurexin-IgG Proteins**—Medium from COS cells transfected with the various neurexin IgG expression vectors used to purify the IgG fusion proteins on protein A-agarose (6, 20). Recombinant neurexin Iα and IIB-proteins were subjected to N-terminal sequence analysis to determine the site of signal peptide cleavage. Neurexin IIA fusion proteins containing the native N-terminal neurexin IIA signal peptides could not be similarly processed because of low yields (data not shown).

**Binding of Neurexophilin 1 to Neurexin-IgG Fusion Proteins**—PC12 cells were infected with recombinant adenovirus expressing full-length neurexin Iα 1 for 2–3 days, followed by subcloning in M13 vectors (19). PC12 cells were washed, harvested into cold extraction buffer (40 mM Tris-HCl, pH 8.0, 0.15 mM NaCl, protease inhibitors), and processed and unprocessed forms of neurexophilin I were solubilized in 2% CHAPS in extraction buffer. The supernatant of the solubilization step was used for binding experiments using neurexin-IgG fusion proteins that were immobilized on protein A-agarose beads. Binding experiments were performed in 1% CHAPS in extraction buffer. Bound proteins and supernatants from binding reactions were analyzed by SDS-PAGE and immunoblotting.

**Genomic Cloning of Neurexophilin 1 and Generation of Knockout Mice**—A mouse genomic library was screened with a 1.4-kb KpnI fragment from rat cDNA of neurexophilin 1 as described (22, 23). Four independent clones were isolated, mapped, and partially sequenced after subcloning in M13 vectors (18). A targeting vector was constructed (14, 24) (see Fig. 4 for knockout strategy). Embryonic stem cells (SM1) were grown on STO cells, electroporated with the vector, and selected with G418 (Life Technologies, Inc.) and FIAU (1-(2-deoxy-2-fluoro-ß-D-arabinofuranosyl)-5-iodouracil). Resistant clones were analyzed by Southern blotting, and positive clones were injected into blastocysts resulting in the generation of a single mouse line that was bred to homozygosity and genotyped by Southern blotting.

**Northern and Southern Blots**—Total RNA was isolated from brains of adult mice using the guanidinium thiocyanate method (RNAstat-60), separated on 1.2% formaldehyde-agarose gels, and transferred to Nylon membranes (Hybond). The following cDNA fragments from the 5′-coding regions of the respective rat cDNAs were labeled with

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2 J.-Y. Song, K. Ichtchenko, T. C. Sudhof, and N. Brose, submitted for publication.

3 M. Müssler, R. E. Hammer, and T. C. Sudhof, unpublished observation.
Neurexophilins Bind to a Single α-Neurexin LNS Domain

**Fig. 1.** Domain structures of neurexins (A) and diagram of recombinant neurexins used for binding assays (B). A. structures of α- and β-neurexins. α-Neurexins contain three cassettes identified by roman numerals below the diagram. Each cassette is composed of two LNS domains (hatched boxes, LNS(A) and LNS(B)) separated by a central EGF-like domain. β-Neurexins only contain the LNS(B) domain of cassette III. All neurexins have a carbohydrate attachment domain (CO) just before the transmembrane region and a short cytoplasmic tail. α-Neurexins are extensively alternatively spliced at five canonical positions that are identified by numbered arrows above the drawing. Of these, splice sites 4 and 5 are also present in β-neurexins. B, neurexin domains included in recombinant IgG fusion proteins used for binding experiments. Fusion proteins of the Fc domain of human IgG with the indicated fragments from the extracellular sequences of neurexins were produced by transfection in COS cells. The domains present in the various fusion proteins are represented by bars, including the content of splice site 2 if applicable. A, full insert; B, half insert; C, no insert; dash, not alternatively spliced in neurexin IIIα. The different fusion proteins are identified on the left. The far right column summarizes the results of binding experiments of neurexophilin 1 (Nph1) to the fusion proteins. +, binding; −, no binding (see Figs. 3 and 4).

Production of Recombinant Neurexin-IgG Fusion Proteins; Determination of the Signal Peptidase Cleavage Sites in Neurexins Iα and IIIα—We generated fusion proteins of the Fc domain of human IgG with extracellular sequences from the various neurexins (Fig. 1). In previous experiments, we found that similar fusion proteins are very useful to study the binding of α-latrotoxin and neuroligin to neurexins (6, 13). For the current experiments, the locations of the extracellular neurexin sequences that were fused to IgG were chosen based on the domain structure of neurexins and the binding activities of increasingly smaller fragments (Fig. 1B). We expressed the fusion proteins by transfection in COS cells and purified them from the COS cell medium by affinity chromatography on protein A-Sepharose.
Neurexophilins Bind to a Single α-Neurexin LNS Domain

Synthesis of Recombinant Neurexophilin 1 in PC12 Cells—

To determine which sequence of neurexin Iα is required for binding neurexophilin 1, we utilized neurexin-IgG fusion proteins immobilized on protein A (Fig. 3). Pulldown experiments were performed with extracts from PC12 cells that overexpress neurexophilin. A fusion protein of the entire extracellular sequence of neurexin Iα (NxIα-1) efficiently bound neurexophilin 1 with enrichment of the processed form (Fig. 3, compare lanes 3 and 4). In contrast, the extracellular sequence of neurexin Iβ (NxIβ-1) bound neither processed nor unprocessed neurexophilin 1 (lanes 1 and 2). Shortening the extracellular neurexin Iα sequences to the first three or two LNS domains (NxIα-14 to NxIα-17 and NxIα-20) still allowed binding of neurexophilin 1 (Fig. 3, lanes 5 and 6) (data not shown). Further shortening revealed that the first LNS domain alone (NxIα-18) was unable to bind (Fig. 3, lanes 7 and 8). The second LNS domain of neurexin Iα (NxIα-21, -24, and -25), however, was fully active (lanes 9–14). Because the second LNS domain of α-neurexins is subject to alternative splicing with three variants in neurexin Iα, we tested all three splice variants (Fig. 1). No protein of alternative splicing on neurexophilin 1 binding was detected (Fig. 3).

Next we investigated if neurexophilin 1 also binds to other neurexins and if these interactions are also mediated by the isolated second LNS domain (Fig. 4). Using the same pulldown experiments described above, we found that the individual isolated second LNS domain of all three neurexins captured neurexophilin 1. All three neurexins appeared to be similarly active. This result agrees well with the high sequence conservation of the second LNS domain in neurexins (12, 25).

The Gene for Neurexophilin 1 Is Not Essential—To investigate if neurexophilin 1 is an essential gene, we generated neurexophilin 1 knockout mice. For this purpose, we constructed a targeting vector in which exon 2 of the neurexophilin 1 gene was replaced by a neomycin resistance gene (Fig. 5). Exon 2 encodes almost the entire coding region of neurexophilin and only lacks the sequence for the signal peptide (18). Embryonic stem cells were transfected with the targeting vector and analyzed for homologous recombination after double selection. In this manner we obtained embryonic stem cells in which the endogenous neurexophilin 1 gene was mutated. The embryonic stem cells were used to generate mutant mice by blastocyst injection and breeding of the resulting chimeric mice. Analysis of the offspring of crossing between mice heterozygous for the mutant neurexophilin 1 gene revealed that homozygous mutant mice were viable. However, when we bred homozygous mice, male homozygous mutant mice were found to be infertile, whereas female mice or male heterozygotes reproduced normally. This was a surprising finding for a protein expressed almost exclusively in the brain (19). Histological

Fig. 2. Recombinant neurexophilin 1 produced in PC12 cells; effect of reducing agents. Recombinant adenovirus containing full-length neurexophilin 1 was used to infect PC12 cells for 72 h. Cells were lysed in SDS-PAGE buffer containing β-mercaptoethanol (β-ME) (lane 1), dithiothreitol (DTT) (lane 2), or no reducing agents (lanes 4 and 5). Proteins were run on SDS-PAGE with four empty lanes between reducing and nonreducing conditions to prevent diffusion of reducing agents and analyzed by immunoblotting with ECL detection. Neurexophilin 1 is synthesized as a prepropeptide (unprocessed form) and cleaved to generate the mature protein (processed form) (19).
analysis of the testes of homozygous mutant mice revealed abnormalities of spermatogenesis that closely resembled the phenotype of transgenic mice expressing herpes simplex virus thymidine kinase in testis (28, 29) (data not shown). Subsequent analysis of genomic DNA from the knockout mice confirmed that homologous recombination had introduced the 5'9 part of the herpes simplex virus thymidine kinase cassette into the neurexophilin locus (data not shown).

Except for the male sterility and testis abnormalities, the homozygous mutant mice showed no obvious morbidity, premature mortality, or anatomical abnormalities. Analysis of the genotype of 183 offspring from heterozygous crossing detected 50 wild type mice, 96 heterozygotes, and 37 homozygous mutants (80% of expected). Thus the offspring exhibit a nearly normal Mendelian distribution with a possible small loss of homozygous mutants.

The Neurexophilin 1 Mutant Is a Null Mutant—A potential cause for the lack of a striking phenotype in a knockout experiment is that the introduced mutation does not actually abolish expression of the target gene. This could happen because a pseudogene was inadvertently targeted or because chromosomal rearrangements occur during gene targeting. Especially the second possibility is raised in the case of the neurexophilin 1 knockouts because part of the thymidine kinase gene is present in the targeted locus (see above). To test if neurexophilin 1 expression is abolished in the knockout, we analyzed wild type and mutant neurexophilin 1 mice by RNA blotting (Fig. 6). Hybridizations of RNA blots with a neurexophilin 1 probe showed that the neurexophilin 1 mRNA was reduced in heterozygous mutant mice and undetectable in homozygous mutant mice. Thus the knockout produced is a null allele.

Another potential cause for the lack of a phenotype in knockouts is the presence of redundant genes or compensatory changes. Because mice contain three other neurexophilin genes that could potentially be redundant, we analyzed their expression in wild type and knockout mice by RNA blotting (Fig. 6). As reported previously (18, 19), we observed no expression of neurexophilin 2 in wild type mice while neurexophilins 3 and 4 mRNAs were
Neurexophilins Bind to a Single α-Neurexin LNS Domain

The data from the neurexophilin 1 knockout suggest that neurexophilin 1 acts as an α-neurexin ligand and its function is redundant with the functions of neurexophilins 3 or 4. Neurexophilins 3 and 4 should also bind to α-neurexins. Such a redundancy is not excluded by the absence of a compensatory increase in mRNA levels for these neurexophilins because the regular levels of neurexophilins 3 or 4 may be sufficient to compensate for the loss of neurexophilin 1. To address this question, we investigated if neurexins in the neurexophilin 1 knockout mice are complexed to other neurexophilins. This experiment can be performed relatively easily for neurexophilin 3 because neurexin Iα is efficiently purified on immobilized α-latrotoxin and because the previously produced antibody to neurexophilin 1 cross-reacts with neurexophilin 3. However, this antibody does not recognize neurexophilin 4, making it necessary to raise an antibody to this protein.

We immunized rabbits with a peptide from neurexophilin 4 and tested the resulting serum with COS cells transfected with a neurexophilin 4 that was epitope-tagged with a c-Myc sequence (Fig. 7). Unprocessed neurexophilin 4 was detected as a 43-kDa band only in neurexophilin 4-transfected COS cells, indicating that the antibody specifically reacts with neurexophilin 4.

We then utilized wild type and neurexophilin 1 knockout mice to purify neurexin Iα by affinity chromatography on immobilized α-latrotoxin (Fig. 8). Probing the purified protein with antibodies to neurexins confirmed that neurexin Iα was purified (lanes 6 and 7). The antibody that recognizes neurexophilins 1 and 3 reacted with a protein of the appropriate molecular mass in both wild type and knockout material (lanes 1 and 2). Because the knockout mice do not contain detectable neurexophilin 1 mRNA (Fig. 6), this result implies that neurexophilin 3 is present in a complex with neurexin Iα. The antibody to neurexophilin 4, however, failed to uncover any reactivity associated with purified α-neurexin, suggesting that neurexophilin 4 is not a significant component of purified neurexin Iα (Fig. 8, lanes 4 and 5).

Neurexin Iα Binds to Recombinant Neurexophilin 3 but Not 4—The data from the neurexophilin 1 knockout suggest that neurexophilins 1 and 3 but not 4 bind to α-neurexins. To
Neurexophilins Bind to a Single α-Neurexin LNS Domain

The extracellular sequences of α-neurexins are primarily composed of six LNS domains punctuated by EGF repeats (Fig. 1A). Many cell surface proteins contain repeated extracellular domains that perform either individual functions as discrete binding sites for separate ligands or shared functions as compound binding sites for single ligands. For example, the homophilic L1 cell adhesion molecule contains six tandem IgG domains. Of these, the second IgG domain is sufficient for cell adhesion (30, 31). Conversely, the low density lipoprotein receptor contains seven cysteine-rich repeats and three EGF domains. At least six of the cysteine-rich repeats and one EGF domain are required for low density lipoprotein binding, suggesting that cooperation between the various domains assembles a single ligand-binding site (32). It is unknown if the six LNS domains and three EGF domains in α-neurexins form separate, individual ligand-binding domains or cooperate to create a single ligand-binding site. Furthermore, although LNS domains are found in a large number of proteins, no functional activity has been demonstrated for an individual LNS domain (1, 2, 4, 33).

α-neurexins form a complex with neurexophilin 1 and its homologs as secreted glycoproteins that resemble neuropetides (19) suggested that neurexophilins may be endogenous peptide ligands for α-neurexins. This would imply that α-neurexins have a defined binding site for neurexophilins and that there should be a general mechanism that mediates binding between these two proteins. In the current study we have addressed these questions by investigating which neurexophilins bind to which neurexins and how the extracellular sequences of α-neurexins relate to neurexophilin binding.

The first aim of our study was to establish if neurexin Iα has a discrete binding site for neurexophilin 1 and if neurexophilin 1 also binds to the same site in neurexins Iα and IIIα. Because endogenous neurexophilin 1 is not detectable in total brain and may already be quantitatively bound to endogenous α-neurexins, this question could not be addressed using brain extracts as a source of neurexophilin 1. Instead, we had to use recombinant neurexophilin 1 produced by an adenovirus in PC12 cells. The results demonstrated that a single LNS domain of neurexins, the second overall LNS domain, is sufficient for binding. All three α-neurexins interacted with neurexophilin 1. The processed, cleaved form of neurexophilin 1 was preferentially bound, but the unprocessed precursor also interacted.
These data establish that all α-neurexins bind neurexinophil 1. More importantly, the results show that a single LNS domain from neurexins forms an independently folding ligand-binding domain. One of the implications from this observation is that LNS domains are probably general ligand-binding domains. It is possible that each of the six LNS domains of α-neurexins may have a different ligand.

We next investigated the question if other neurexophilins besides neurexinophil 1 bind to α-neurexins. We addressed this question by using two separate approaches. First, we generated neurexinophil 1 knockout mice. These mice expressed no detectable neurexinophil 1 mRNA but were viable and had no obvious phenotype related to the loss of neurexinophil 1. Neurexophilins 3 and 4 were expressed normally and showed no compensatory changes. Thus similar to α-neurexins (14), knockout of a single neurexinophil is not lethal. This observation indicates that neurexinophil 1 either has no function or its function is redundant with those of other proteins, e.g. other neurexophilins even without compensatory changes. The second possibility is supported by the finding that in α-neurexins, knockouts of more than one α-neurexin gene cause major phenotypes. This suggests that α-neurexins are functionally redundant. By analogy to α-neurexins it is likely that neurexophilins are redundant.

Although the knockout mice failed to yield insight into the in vivo function of neurexinophil because there was no obvious phenotype, the knockouts were useful in defining which neurexophilins bind to α-neurexins. Isolation of neurexin Iα from the neurexinophil 1 knockout mice revealed that neurexinophil 3 was complexed to neurexin Iα whereas neurexinophil 4 was not. This result showed that neurexophilins 1 and 3 can bind to neurexin Iα in vivo, suggesting that neurexophilins 1 and 3 but not 4 are endogenous ligands for α-neurexins. The lack of binding of neurexinophil 4 may be because of its more divergent structure, especially in the long loop connecting the two conserved domains (19). The conclusion that neurexinophil 3 but not 4 binds to α-neurexins was confirmed by a second approach. When we incubated neurexinophil 3 and 4 expressed as recombinant proteins in COS cells with recombinant α-neurexins, only neurexinophil 3 but not neurexinophil 4 bound. An implication of these results is that not all neurexophilins are ligands for α-neurexins and that the neurexinophil family may be functionally heterogeneous.

Our data support a model whereby neurexophilins 1 and 3 are physiological ligands for α-neurexins. Evidence for this model is as follows. 1) The structures of neurexophilins and α-neurexins strongly resemble neureptide ligands and receptors, respectively. 2) In the brain, neurexophilins 1 and 3 are present in a tight complex with neurexin Iα. This was revealed by the purification of the neurexin Iα-neurexophilins 1 and 3 complex from the brain on immobilized a-latrotoxin. 3) A single LNS domain of α-neurexins is sufficient for binding neurexophilins. Thus there is a specific, defined binding site at the N terminus of α-neurexins. This observation agrees well with the finding that a-latrotoxin binds to the C terminus of neurexin Iα (13). As a result, neurexophilins and α-latrotoxin can bind to neurexin Iα simultaneously. 4) The binding of neurexinophil to neurexin Iα is very tight, being resistant to urea denaturation (18). This suggests a very high affinity interaction. The major question that now arises is if neurexophilin binding to α-neurexins initiates an intracellular signal. Addressing this question will require production of copious amounts of native soluble neurexophilins, which are not currently available.

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