Neurexin Iα Is a Major α-Latrotoxin Receptor That Cooperates in α-Latrotoxin Action*

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α-Latrotoxin is a potent neurotoxin from black widow spider venom that binds to presynaptic receptors and causes massive neurotransmitter release. A surprising finding was the biochemical description of two distinct cell surface proteins that bind α-latrotoxin with nanomolar affinities; Neurexin Iα binds α-latrotoxin in a Ca\(^{2+}\)-dependent manner, and CIRL/latrophilin binds in a Ca\(^{2+}\)-independent manner. We have now generated and analyzed mice that lack neurexin Iα to test its importance in α-latrotoxin action. α-Latrotoxin binding to brain membranes from mutant mice was decreased by almost 50% compared with wild type membranes; the decrease was almost entirely due to a loss of Ca\(^{2+}\)-dependent α-latrotoxin binding sites. In cultured hippocampal neurons, α-latrotoxin was still capable of activating neurotransmission in the absence of neurexin Iα. Direct measurements of \(^{3}H\)glutamate release from synaptosomes, however, showed a major decrease in the amount of release triggered by α-latrotoxin in the presence of Ca\(^{2+}\). Thus neurexin Iα is not essential for α-latrotoxin action but contributes to α-latrotoxin action when Ca\(^{2+}\) is present. Viewed as a whole, our results show that mice contain two distinct types of α-latrotoxin receptors with similar affinities and abundance but different properties and functions. The action of α-latrotoxin may therefore be mediated by independent parallel pathways, of which the CIRL/latrophilin pathway is sufficient for neurotransmitter release, whereas the neurexin Iα pathway contributes to the Ca\(^{2+}\)-dependent action of α-latrotoxin.

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EXPERIMENTAL PROCEDURES

Genomic Cloning of Neurexin Genes—A mouse genomic library was screened for the 5’ ends of the neurexin genes by high stringency hybridization as described (14). Clones were isolated, mapped, and sequenced using general molecular biology techniques (14, 15). Sequences were analyzed on a personal computer using DNA-STAR software.

Generation and Maintenance of Knockout Mice—A knockout vector was constructed from the genomic clone for neurexin Iα as diagrammed in Fig. 1. Embryonic stem cells were electroporated with the vector and
selected with G418 (Life Technologies, Inc.) and FIAU essentially as described (16). Resistant embryonic stem cell clones were analyzed by polymerase chain reaction for homologous recombination (primers used: 676 [GAGCGCGCGCGCGCGGATGTTGAC] and 918 [AGC-CAATCTTCGGAACAGACAGCT] and confirmed by Southern blotting. The genotype of each cell clone was determined by genotyping. To analyze the effect of the mutation in the neurexin Iα gene on mouse survival, mice heterozygous for the neurexin Iα mutation were mated with each other, and the number of adult surviving offspring was determined by genotyping.

Antibodies and Immunoblot Analysis—Antibodies against the cytoplasmic tails of neurexins were raised in rabbits using recombinant bacterially expressed proteins in which the N terminus of the cytoplasmic tail sequence was fused to a hexahistidine sequence for purification. Antibodies were affinity-purified on immobilized glutathione S-transferase fusion proteins of the same sequences, and their specificity was confirmed using recombinant neurexins. All other antibodies were described previously (17).

α-Latrotoxin Binding Measurements—α-Latrotoxin binding measurements were performed by a rapid centrifugation assay essentially as described (18). Mouse brains were homogenized in 0.15 M NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.8, and crude membranes were prepared by centrifugation. The specific binding of 0.5 nM 125I-labeled α-latrotoxin to crude brain membranes from mice was analyzed in triplicates in a 0.15-ml volume with 0.2 mg of protein in the presence of 2 mM Ca2+ or 2 mM EDTA. A 50-fold excess of unlabeled α-latrotoxin was added to control for nonspecific binding. Ca2+-independent binding was defined as α-latrotoxin binding observed in the presence of EDTA, and Ca2+-dependent binding was defined as the difference between total α-latrotoxin binding measured with Ca2+ and Ca2+-independent binding. Binding assays for Scatchard analyses were performed similarly except that α-latrotoxin concentrations from 0.17 to 17.0 nM were used.

Embryonic Cultures and Electrophysiology—Cultures from embryonic hippocampus from wild type or mutant mice were prepared as described (19) and analyzed by electrophysiological recordings (20). Spontaneous miniature excitatory postsynaptic currents were monitored as a function of the application of 1 μM α-latrotoxin in the presence or the absence of Ca2+. To analyze the currents when there was a high degree of overlap of miniature excitatory currents (especially after α-latrotoxin application), currents were integrated over 200-ms intervals. The charge transfer obtained in this manner is proportional to the frequency of miniature excitatory postsynaptic currents, the quantal release rate (21).

Measurements of [3H]Glutamate Release from Synaptosomes—Synaptosomes were prepared from the neocortex of adult mice by a Percoll gradient centrifugation method modified from Ref. 2. The crude mitochondrial fraction (P2) was resuspended in 5.5% (v/v) Percoll, 250 mM sucrose, 10 mM Tris-NaOH, pH 7.4, and layered on top of an 11- to 17.5% (v/v) Percoll step gradient in the same buffer. After centrifugation at 16,000 rpm for 20 min, synaptosomes were recovered from the Percoll-tissue interface. Percoll was removed by the addition of 30 mM NaCl 118, KCl 3.5, CaCl2 1.25, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, 10 mM HEPES-NaOH 5 at pH 7.4, glucose 11.5, 0.1 g/liter bovine serum albumin (Sigma catalog number A-6793). Synaptosomes were resuspended in 2 ml of ice-cold gassed (95% O2/5% CO2) Krebs-Henseleit-HEPES buffer (KHH buffer), pH 7.4 (composition in mM: NaCl 116, KCl 3.5, CaCl2 1.25, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, HEPES-NaOH 5 at pH 7.4, glucose 11.5, 0.1 g/liter bovine serum albumin [Sigma catalog number A-6793]). Synaptosomes were loaded with 3H-labeled glutamate by a 5-min incubation in KHH buffer containing 0.17 μM [3H]glutamate (24 mCi/mmol; NEN Life Science Products) in a 95% O2/5% CO2 atmosphere at 34 °C. Afterward synaptosomes (100 μl) were captured on a glass fiber filter (GF/B) in a superfusion chamber, overlaid with 50 μl of a 50% slurry of Sephadex G-25 in KHH buffer, and superfused (flow rate, 0.8 ml/min) at 34 °C using two stimulation protocols: 1) Synaptosomes were superfused with KHH buffer for at least 12 min for equilibration, and then the perfusate was collected for 2 min to establish the base-line release rate of 125I-glutamate, and then the perfusate was collected for 2 min to establish the base-line release rate of [3H]glutamate, after which 0.5 mM sucrose was added to the superfusion buffer for 30 s to stimulate release of glutamate. Thereafter the synaptosomes were superfused for an additional 2.5 min with Ca2+-free KHH buffer to re-equilibrate them, and finally a 1-min pulse stimulation of α-latrotoxin in the same buffer was applied followed by continued superfusion without α-latrotoxin. The perfusate was continuously collected, [3H]Glutamate levels in the perfusate were determined by scintillation counting. The fractional release rate was calculated by dividing the amount of radioactivity released at any given time point by the total amount of radioactivity remaining with the synaptosomes at that point. Two mice from each genotype were analyzed in independent experiments.

RESULTS

Structure of the 5′ End of the Neurexin Iα Gene—Upon screening a mouse genomic library, we isolated a clone containing a single large exon from the 5′ end of the neurexin Iα mRNA (Fig. 1). Sequence analysis revealed that the exon encoded the N terminus of neurexin Iα, including the signal peptide, the first LNS domain (LNS domains are repeat sequences found in laminins, neurexins, and sex hormone-binding globulins; Ref. 26), and the first epidermal growth factor-like sequence. The exon ended at the 5′ boundary of the first site of alternative splicing in neurexin Iα corresponding to residue 253 (Fig. 1). In addition to the 5′ end of the coding region, the exon also contained the entire 5′-untranslated region that we previously sequenced in rat and bovine cDNA clones (0.89 kilobase pairs; Refs. 5, 7), suggesting that the exon present in this genomic clone constitutes the first exon of the gene and that the 5′-flanking sequences present in the genomic clone represent the neurexin Iα promoter.

Thus the 5′ end of the neurexin Iα gene is composed of a large exon (>1.5 kilobase pairs) that includes the entire 5′-untranslated region and extends to the first site of alternative splicing. Interestingly, the first site of alternative splicing in neurexin Iα is among the most polymorphic and exhibits at least seven different variants (7). The presence of a large exon preceding this site may facilitate regulation of alternative splicing. Preliminary studies on the structures of the neurexin IIα and IIIα genes suggest that their 5′ ends also contain a single large first exon that extends until the position of the first site of alternative splicing (not shown). This result agrees well with the fact that these neurexins are also extensively alternatively spliced at this position.

Generation of Knockout Mice for Neurexin Iα—Using the neurexin Iα genomic clone, we constructed a targeting vector in which the entire first exon and several kilobases of 5′-flanking sequence (presumably containing the neurexin Iα promoter) were replaced by a neomycin gene cassette as a positive selectable marker (Fig. 1). The short arm of the targeting vector was constructed from the sequence of the first intron and followed by two copies of a herpes simplex virus thymidine kinase gene cassette for negative selection. The long arm of the vector was obtained from further 5′-flanking sequences (Fig. 1). As a result, the entire first exon and part of the promoter of the neurexin Iα gene were deleted in the targeting vector.

We transfected embryonic stem cells with the targeting vector. Clones emerging after positive and negative selection (with neomycin and FIAU, respectively) were analyzed by polymerase chain reaction using primers flanking the short arm (A and B in Fig. 1). Several clones with putative homologous recombination were obtained and injected into blastocysts. In this manner we generated a single mouse line that transmitted the mutation through the germline. Southern blotting demonstrated that in the mutants, the wild type and mutant neurexin Iα genes were allelic and were transmitted in a Mendelian fashion (Fig. 2).

Mice Lacking Neurexin Iα Are Viable and Fertile—Mice carrying the deletion of the first exon of the neurexin Iα gene were bred to homozygosity and analyzed. Homozygous mutant mice were indistinguishable in appearance from wild type mice.
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They were fertile and survived for more than a year. The only abnormality that we observed was that female knockout mice were less able than control mice to attend to litters, either their own mutant litter or wild type substituted control litter. As a consequence, when mouse pups were cared for by neurexin Iα-deficient females, more pups died independent of the genotype. These data indicate that the neurexin Iα mutation does not cause a major impairment in mouse survival or brain functions but may have subtle behavioral effects. Exact definition of these potential behavioral changes will require extensive behavioral analyses.

The lack of a strong phenotype in the neurexin Iα-deficient mice raised the possibility that we mutated an inactive pseudogene instead of an active gene. To address this possibility, we raised affinity-purified polyclonal antibodies to the cytoplasmic tails of neurexins I and III and analyzed membrane proteins from wild type and knockout mice by immunoblotting. Because these antibodies were raised against the cytoplasmic tails of neurexins, they recognize both α- and β-neurexins, and because the cytoplasmic tails of neurexins are so similar, the antibodies are weakly cross-reactive with other neurexins. Immunoblotting revealed that in wild type mice, a cluster of bands of 160–200 kDa and a less diverse set of bands of 90–100 kDa were reactive with neurexin I and III antibodies (Fig. 3). The larger proteins correspond in size to α-neurexins, and the smaller proteins correspond to β-neurexins. The multitude of bands that we observed for the α-neurexins agrees well with their extensive alternative splicing (7) and provides evidence that this alternative splicing indeed results in different protein products. Analysis of the knockout mice by immunoblotting showed that the signal for the neurexin Iα bands was greatly diminished (Fig. 3). The neurexin IIIα, IIIb, and IIIβ signals, however, were unchanged. Some residual 160–200-kDa reactivity was observed with the neurexin I antibodies in the neurexin Iα knockouts, probably because of the cross-reactivity of the neurexin I antibody with neurexins II and III (5). Together these data confirm that we introduced a mutation into an active neurexin Iα gene that interferes with the expression of neurexin Iα.

α-Latrotoxin Binding Is Impaired in the Neurexin Iα Knockouts—We prepared brain membranes from wild type mice, neurexin Iα knockout mice, and two different control knockout lines and studied binding of 125I-labeled α-latrotoxin to these membranes in the presence and the absence of Ca2+ (Fig. 4). In the knockouts, a major decrease of Ca2+-dependent binding of α-latrotoxin was observed. In contrast, no major changes in Ca2+-independent binding were detected. To gain insight into the relative affinities of Ca2+-dependent and Ca2+-independent α-latrotoxin binding sites, we performed Scatchard plot analyses of α-latrotoxin binding to brain membranes from wild type and knockout mice. These analyses revealed that Ca2+-dependent and Ca2+-independent binding exhibit similar affinities (Fig. 5). Ca2+-dependent binding accounted for a major

FIG. 1. Structure of the 5′ end of the neurexin Iα gene and knockout strategy. The top diagram shows the structure of the 5′ end of the neurexin Iα gene. The subclones of the genomic λ clones that were used to map the gene (pLL13–1a and -1b, pLL3–1a) are indicated below the gene structure. The position of the exon is displayed by a shaded box, and the location of the initiator methionine is indicated by a vertical arrow. The structure of the targeting vector used for homologous recombination is drawn below in the middle, with the location of the neomycin resistance gene cassette (Neo) and the two Herpes simplex virus thymidine kinase gene cassettes (2 × HSV-TK) represented by striped boxes. At the bottom, the structure of the mutant neurexin Iα gene is represented with locations of the polymerase chain reaction primers used for the identification of homologously recombined embryonic stem cell clones (arrows labeled A and B) and of the probe used for Southern blot analysis. The scale of the drawing is shown in the bottom right corner, and the relative positions of the sequences in the wild type gene, the targeting vector, and the mutant gene are highlighted by dotted lines. The letters designate restriction enzyme cleavage sites for the following enzymes: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; K, KpnI; n, NotI; S, SpeI.

FIG. 2. Southern blot analysis of neurexin Iα knockout mice. Genomic tail DNA from mice offspring from matings between heterozygous mutant neurexin Iα mice was analyzed by digestion with SpeI and hybridization with the probe shown in Fig. 1. WT, wild type; KO, knockout.
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Fig. 3. Immunoblot analysis of neurexin Iα knockout mice.

Brain membranes from wild type mice (+/+) and neurexin Iα knockout (k/o) mice (−/−) were analyzed by immunoblotting with antibodies to the cytoplasmic tail of neurexin I (A) or of neurexin II (B), to dynamin (C), and to rab3A (D). The antibodies to neurexins I and III used in A and B were raised against their highly homologous cytoplasmic tails and still exhibit partial cross-reactivity after affinity purification. The antibodies recognize in brain membranes proteins of 160–200 kDa in size that are likely to correspond to α-neurexins (Nrx Iα, neurexins Iα; Nrx IIIα, neurexin IIIα), and proteins of 90–100 kDa that are probably made up of β-neurexins (Nrx Iβ, neurexin Iβ; Nrx IIIβ, neurexin IIIβ). Especially the α-neurexins exhibit size heterogeneity in agreement with their extensive alternative splicing (7). Note that there is no major change in the immunoreactivities of the proteins shown in the knockout except for a loss of neurexin Iα.

part of the total binding capacity but was largely absent in the knockout mice, suggesting that neurexin Iα constitutes the major Ca2+-dependent high affinity binding site for α-latrotoxin in mice. In addition, a smaller decrease in the Ca2+-independent binding of α-latrotoxin was occasionally observed in the knockout mice (Fig. 5). The Ca2+-dependent binding site has the same affinity and a similar abundance as the high affinity Ca2+-independent binding site that is probably provided by the CIRL/latrophilin protein (12, 13).

α-Latrotoxin Still Activates Neurotransmission in Neurons Lacking Neurexin Iα—To test if α-latrotoxin still stimulated neurotransmitter release in neurexin Iα-deficient neurons, we cultured hippocampal neurons from knockout and wild type mouse embryos and studied miniature excitatory postsynaptic currents as a function of α-latrotoxin. 1 nM α-latrotoxin, a concentration considerably higher than the KD values of the Ca2+-dependent and Ca2+-independent binding sites (Fig. 5), was applied in the presence or the absence of Ca2+. An increase in neurotransmitter release was observed in both types of mice, with or without Ca2+ (data not shown). These data demonstrate that neurexin Iα-deficient mice still respond to relatively high concentrations of α-latrotoxin, suggesting that neurexin Iα is not required for α-latrotoxin stimulated neurotransmitter release. However, it is difficult to quantitate the number of synapses whose release events give rise to the signal in electrophysiological experiments under the particular conditions used here. As a consequence, the electrophysiological results give no insight into the relative magnitude of the effect of α-latrotoxin in the two types of mice.

Quantitation of [3H]Glutamate Release from Synaptosomes Triggered by α-Latrotoxin—To directly measure [3H]glutamate release from nerve terminals, we purified synaptosomes from the neocortex of wild type and knockout mice and loaded them with [3H]glutamate. We suspended the [3H]glutamate-loaded synaptosomes in a superfusion chamber, measured base-line release of [3H]glutamate during superfusion with either Ca2+-containing or Ca2+-deficient buffer, and then stimulated release with α-latrotoxin at low concentration (0.5 nM) or with sucrose (0.5 M). When we superfused wild type synaptosomes with α-latrotoxin in the presence of Ca2+, we observed a large prolonged increase in [3H]glutamate release (Fig. 6A). [3H]Glutamate release was activated by α-latrotoxin within 30 s and lasted for at least 10 min, the length of the experiment. In
Neurexin Iα-deficient synaptosomes, however, the extent of [3H]glutamate release stimulated by α-latrotoxin was significantly decreased (Fig. 6A). These data could either mean that neurexin Iα contributes to α-latrotoxin action in the presence of Ca2+ and is required for full action of α-latrotoxin or that there is a general decrease in the amount of [3H]glutamate that can be released from neurexin Iα-deficient synaptosomes. To differentiate between these two hypotheses, we quantitated the amount of [3H]glutamate release triggered by hypertonic sucrose and α-latrotoxin in the absence of Ca2+. Hypertonic sucrose was used because it had been shown in electrophysiological studies to trigger exocytosis of docked vesicles and can therefore be used as an indirect measure of the releasable pool of glutamate (23). A 30-s pulse of 0.5 M sucrose stimulated similar amounts of [3H]glutamate release from wild type and neurexin Iα-deficient synaptosomes, suggesting that there is no principal impairment of neurotransmitter release in the mutants and no major difference in pool size (Fig. 6A). Furthermore, when we applied α-latrotoxin in the absence of Ca2+, no difference in [3H]glutamate release between wild type and neurexin Iα-deficient synaptosomes was observed. Thus the neurexin Iα-deficient synaptosomes exhibit a selective impairment of α-latrotoxin-stimulated glutamate release in the presence of Ca2+.

DISCUSSION

Neurexins are a family of highly polymorphic cell surface proteins with a receptor like structure. There are three α- and three β-neurexins, each of which is alternatively spliced. Neur-
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LUTEXINS interact at least two endogenous ligands: α-neurexins bind to neurexophilin (10), and β-neurexins bind to neurolins (8, 9). In addition, neurexin Iα but not other neurexins bind α-latrotoxin with high affinity (3, 4, 11). The structures and known ligands of neurexins indicate the possibility of dual functions for neurexins as cell adhesion molecules and signal transduction receptors (24, 25). However, the exact in vivo roles of neurexins are unclear.

As a first step to probe the functions of neurexins and their role in α-latrotoxin action, we have now produced mice that lack neurexin Iα. Our data show that these mice are remarkably normal, suggesting that neurexin Iα is not an essential gene and not required for basic nervous system functions. This finding suggests that neurexin Iα is functionally redundant or that it performs a more subtle function that is not immediately apparent in the analysis performed here. The presence of multiple neurexins with overlapping expression patterns (7) would agree well with functional redundancy. This indicates that neurexins may substitute for each other functionally. On the other hand we found that the maternal behavior of the neurexin Iα knockout mice appears to be abnormal, suggesting that subtle defects may exist in the single neurexin Iα knockout. Future experiments using knockouts of multiple neurexins and a detailed behavioral analysis will be required to resolve these questions.

α-Latrotoxin is an excitatory neurotoxin that is a component of black widow spider venom and produces massive neurotransmitter release after binding to presynaptic nerve terminals (reviewed in Ref. 1). Although α-latrotoxin binding to neurexin Iα requires Ca2+ (11), α-latrotoxin induces neurotransmitter release even in the absence of Ca2+, indicating that a second receptor for α-latrotoxin may mediate its toxicity in the nerve terminal. Such a receptor was recently identified in CIRL/latrophilin, a membrane protein that resembles G-protein-linked receptors and binds α-latrotoxin with high affinity in the absence of Ca2+ (12, 13).

It is puzzling that neurons should express two distinct α-latrotoxin receptors with different binding properties (Ca2+-dependent versus Ca2+-independent) but similar affinities. Although the two types of α-latrotoxin receptors have been characterized thoroughly biochemically, it is unknown if they function as α-latrotoxin receptors in vivo. To address this question, we analyzed α-latrotoxin action in the neurexin Iα-deficient mice that we had generated. Our data demonstrate that in the neurexin Iα-deficient mice, most of the Ca2+-dependent α-latrotoxin binding activity is lost, whereas Ca2+-independent binding is unchanged. The α-latrotoxin affinities of neurexin Iα and CIRL are very similar, as is the relative abundance of the Ca2+-dependent binding site due to neurexin Iα and the Ca2+-independent binding site presumably due to CIRL. Thus neurexin Iα accounts for the bulk of the Ca2+-dependent high affinity binding of α-latrotoxin. We found, however, that α-latrotoxin is still capable of triggering neurotransmitter release in cultured hippocampal neurons or synaptosomes from neurexin Iα-deficient mice. Therefore neurexin Iα is not absolutely required for the excitotoxic action of α-latrotoxin. Nevertheless, we observed that in the presence of Ca2+, glutamate release triggered by α-latrotoxin from synaptosomes is decreased in the knockout mice compared with wild type mice. If release is stimulated by α-latrotoxin or by sucrose in the absence of Ca2+, no change is observed, indicating that the general release apparatus is intact. This finding also agree well with the electrophysiological data and the mild phenotype of the knockout mice. Viewed together, our data demonstrate that neurexin Iα is not essential for the ability of α-latrotoxin to trigger neurotransmitter release but contributes to α-latrotoxin action in the presence of Ca2+.

What could the action of neurexin Iα be compared with that of CIRL? CIRL is the presumptive Ca2+-independent α-latrotoxin receptor that probably mediates the α-latrotoxin-dependent activation of neurotransmitter release observed in the neurexin Iα knockout mice. Our finding that in the presence of Ca2+, neurexin Iα is required for a full response to α-latrotoxin at low concentrations suggests two possibilities. Either neurexin Iα and CIRL represent independent pathways for α-latrotoxin action that work in parallel, or neurexin Iα assists CIRL in the presence of Ca2+ in triggering neurotransmitter release. In either case, neurexin Iα represents a target for α-latrotoxin. Furthermore, it is possible that α-latrotoxin may have as yet unidentified effects other than triggering neurotransmitter release, which could be mediated by neurexin Iα. Taken together, with the identification of two structurally different, presumably cooperative receptors the action of α-latrotoxin is much more complex than previously envisioned.

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