Genetic Analysis of α-Latrotoxin Receptors Reveals Functional Interdependence of CIRL/Latrophilin 1 and Neurexin 1α*

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α-Latrotoxin triggers massive neurotransmitter release from nerve terminals by binding to at least two distinct presynaptic receptors, neurexin 1α and CIRL/latrophilin1 (CL1). We have now generated knockout (KO) mice that lack CL1 and analyzed them alone or in combination with neurexin 1α KO mice. Mice lacking only CL1, or both CL1 and neurexin 1α, were viable and fertile. Ca2+-independent binding of α-latrotoxin to brain membranes was impaired similarly in CL1 single and in CL1/neurexin 1α double KO mice (75% decrease) but not in neurexin 1α single KO mice. In contrast, Ca2+-dependent binding (~2 times above Ca2+-independent binding) was altered in both CL1 (~50% decrease) and neurexin 1α single KO mice (~35% decrease) and was decreased further in double KO mice (~75% decrease). Synaptosomes lacking CL1 exhibited the same decrease in α-latrotoxin-stimulated glutamate release in the presence and absence of Ca2+ (~75%). In contrast, synaptosomes lacking neurexin 1α exhibited only a small decrease in α-latrotoxin-activated release in the absence of Ca2+ (~20%) but a major decrease in the presence of Ca2+ (~75%). Surprisingly, synaptosomes lacking both CL1 and neurexin 1α displayed a relatively smaller decrease in α-latrotoxin-stimulated glutamate release than synaptosomes lacking only CL1 in the absence of Ca2+ (~50 % versus ~75%), but the same decrease in the presence of Ca2+ (~75%). Our data suggest the following two major conclusions. 1) CL1 and neurexin 1α together account for the majority (~75%) of α-latrotoxin receptors in brain, with the remaining receptor activity possibly due to other CL1 and neurexin isoforms, and 2) the two receptors act additively in binding α-latrotoxin but not in triggering release. Together these data suggest that the two receptors act autonomously in binding of α-latrotoxin but cooperatively in transducing the stimulation of neurotransmitter release by α-latrotoxin.

α-Latrotoxin is a 130-kDa component of black widow spider venom that is initially synthesized as a 160-kDa precursor protein and is then processed proteolytically at the N and C terminus (1–3). α-Latrotoxin is a potent neurotoxin, causing massive release of neurotransmitter in nerve terminals by exocytosis (4, 5). It seems that the toxin triggers release by first binding to neuronal surface receptors and then directly inserting into the presynaptic plasma membrane (3). Most studies imply that the toxin is active in the absence of extracellular Ca2+ and directly stimulates the secretory apparatus (6–8). Two classes of cell surface receptors for α-latrotoxin have been identified. Neurexins are neuron-specific proteins with a single transmembrane domain (9, 10). They function at least partly as cell adhesion molecules; one isoform, neurexin 1α, binds α-latrotoxin with high affinity in a Ca2+-dependent manner. CLs (CIRLs and latrophilins)1 are G-protein-coupled receptors with seven transmembrane domains and unusually large intra- and extracellular domains (11, 12). Currently, three CL isoforms are known (13, 14). CL1 and CL3 are highly enriched in brain, whereas CL2 is expressed ubiquitously. Although CL1 and neurexin 1α appear to be the most important receptors for α-latrotoxin, most of the other neurexin and CL isoforms also constitute functional α-latrotoxin receptors (10–15). Furthermore, consistent with the α-latrotoxin binding data, experiments on neurexin 1α KO mice showed that release triggered by α-latrotoxin does not require neurexin 1α in the absence of Ca2+ but does require neurexin 1α in the presence of Ca2+ (16).

Despite extensive work that includes the identification of multiple receptors, the mechanism by which α-latrotoxin triggers release has remained elusive (reviewed in Ref. 17). Studies using a recombinant mutant of α-latrotoxin revealed that high affinity binding of α-latrotoxin to its receptors is essential but not sufficient to trigger neurotransmitter release (18). Expression of neurexin 1α or CL1 in PC12 cells highly sensitizes them to α-latrotoxin; this occurs even when deletion mutants of both receptors lacking intracellular sequences are expressed (10, 13, 14). Because the mutant receptors lack intracellular sequences, they are presumably incapable of transducing a surface signal into the cell interior, suggesting that α-latrotoxin does not trigger release by activation of intracellular signal transduction pathways dependent on neurexin 1α and CL1. Moreover, the in vivo importance of the various proposed α-latrotoxin receptors is largely unclear. The fact that deletion of neurexin 1α severely impaired the α-latrotoxin response in the presence but not in the absence of Ca2+ confirms the importance of neurexin 1α as an α-latrotoxin receptor, although it is also puzzling. Specifically, because α-latrotoxin-triggered release is similar in the presence and absence of Ca2+ (8), it would have been expected that because neurexin 1α only binds to α-latrotoxin in the presence of Ca2+, CL1 should be sufficient to elicit a complete α-latrotoxin response. Thus the fact that the neurexin 1α KO had an effect at all is puzzling, even though as predicted, a large effect was only observed in the presence of Ca2+. Together these data raise exciting new questions. For

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1 The abbreviations used are: CL, CIRL/latrophilin; KO, knockout.
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FIG. 1. Partial structure of the CL1 gene and KO strategy. The top diagram shows the partial structure of the murine CL1 gene derived from a genomic λ-clone. The positions of two exons (exon 2 and x) are displayed by striped boxes, of which exon x is either exon 4 or 5. In none of the genomic λ-clones was exon 1 present. The structure of the targeting vector used for homologous recombination is depicted 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 CL1 gene is drawn with locations of the polymerase chain reaction primers used for the identification of homologously recombined embryonic stem cell clones (arrows labeled P1 and P3). Primer P2 located in the deleted region of the CL1 gene (see top diagram) and P1 were used for detection of heterozygous and wild type animals. 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 mutated gene are marked by dotted lines. Restriction enzyme cleavage sites for BgIII, BamHI, NotI, SalI, and ClOI were used for construction of the targeting vector and are therefore depicted in the diagram.

**Experimental Procedures**

**Cloning of the CL1 Gene and Generation and Maintenance of KO Mice**—A mouse genomic library was screened for the 5′-end of the CL1 gene by high stringency hybridization, and clones were isolated, mapped, and sequenced using general molecular biology techniques (19, 20). A KO vector was constructed from the genomic clone of CL1 as diagrammed in Fig. 1. Exon 2 and flanking introns were replaced by a neomycin resistance gene cassette. Embryonic stem cells were electroporated with the vector and selected with G418 (Invitrogen) and FIAU essentially as described (21). Resistant embryonic stem cell clones were analyzed by polymerase chain reaction for homologous recombination (primers used: P1, GCCTTGGAAACCTCTAGTGTTGCC; and P3, GAGGCGCCGGCGCGGAGTTGGTGCAG). The resulting PCR products were subcloned into pBluescript SK II, and its identity was confirmed by sequencing. Positive clones were injected into blastocysts, resulting in the generation of a single mouse line that was bred to homoygosity and genotyped by polymerase chain reaction using primers P1, P2 (CCGGCCGATCAACCCGATGAGCC), and P3. Mice heterozygous for neurexin 1α (16) were bred with homozygous CL1 mice, resulting in offspring mice heterozygous for both CL1 and neurexin 1α. Mice of this genotype were mated with each other, creating a small number of mice double homoygous for CL1 and neurexin 1α.

**Measurement of Glutamate Release from Synaptosomes**—Synaptosomes were prepared from brains of 2-month-old mice by a ficoll gradient centrifugation method essentially as described (22). Synaptosomes from mice with identical genotype were not pooled because of the possibility of a wrong genotyping result. Glutamate release from synaptosomes of individuals was then monitored according to a standard procedure (23, 24). For this purpose, synaptosomes were resuspended in 1 ml of incubation buffer (140 mM NaCl, 5 mM KCl, 5 mM NaHCO3, 1.2 mM Na2HPO4, 1 mM MgCl2, 10 mM glucose, 20 mM HEPES-NaOH, pH 7.4) and stirred for 15 min at 37 °C (protein concentration 0.1 μg/μl). Either CaCl2 (1.3 mM) or EDTA (0.5 mM) was then added together with glutamate dehydrogenase (Sigma type II, 34 units) and 1 mM NADP. After further incubation for 5 min, either 50 mM KCl or α-latrotoxin (Latoxan, France) were added, and the incubation extended for an additional 10 min. Generation of NADPH was monitored by absorbance at 360 nm using an Amino DW 2000 dual wavelength spectrophotometer with 390 nm as reference wavelength.

**Antibodies and Immunoblot Analysis**—Antibodies against the extracellular part of CL1 and the cytoplasmic tail of neurexin 1α were described previously (13, 16). Monoclonal antibodies against synaptobrevin (cl. 69.1), syntaxin (cl. 78.1), SNAP25 (cl. 71.1), synaptophysin (cl. 7.2), rab3A (cl. 42.2), the GDP dissociation inhibitor GDI (cl. 81.1), and a polyclonal antibody against rabphilin (R44) were a gift of Dr. R. Jahn (MPI for Biophysical Chemistry, Göttingen, Germany). SDS-polyacrylamide gel electrophoresis and immunoblotting were performed with minor modifications as described (25, 26). Immunoreactive bands were visualized with enhanced chemiluminescence (Amersham Biosciences, Inc.). Prior to detection, CL1 was biochemically enriched by wheat germ agglutinin affinity chromatography as described (27).
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The long arm of the vector was obtained from further 3′-sequences (Fig. 1). As a result, the entire exon 2 with surrounding introns was deleted in the targeting vector. Exon 1 that was not present in the genomic CL1 clone encodes the signal peptide of CL1. In the case of homologous recombination, replacement of exon 2 would cause a shift in the reading frame of the corresponding RNA, resulting in the synthesis of a nonsense CL1 protein.

We transfected embryonic stem cells with the targeting vector. Clones emerging after positive and negative selection (with neomycin and FIAU, respectively) were analyzed by the polymerase chain reaction using primers annealing outside of the short arm and at the 5′-end of the neomycin resistance gene cassette (P1 and P3 in Fig. 1). Several clones with putative homologous recombination were obtained and injected into blastocysts. In this manner we generated a mouse line that transmitted the mutation through the germline (Fig. 2A).

**Mice Lacking CL1 Are Viable and Fertile**—Mice carrying the deletion of the second CL1 exon were bred to homozygosity and analyzed. Homozygous mutant mice were indistinguishable in appearance from wild type mice. They were fertile and survived for more than 1 year. The only abnormality observed was that female KO mice were less able than control mice to attend to litters. As a consequence, when mouse pups were cared for by CL1-deficient females, most pups died within a week independently of the genotype. These data indicate that the CL1 mutation does not cause a major impairment in mouse survival or brain function but may have more subtle behavioral effects. The exact definition of these potential behavioral changes will require extensive behavioral analyses, but similar phenotypes have been observed for many KO mice of synaptic proteins.

The lack of a strong phenotype in the CL1-deficient mice raised the possibility that we mutated an inactive pseudogene instead of an active gene. To address this possibility, we analyzed the expression of CL1 protein in wild type and KO mice by immunoblotting using a polyclonal antibody raised against an N-terminal fragment of CL1 (see “Experimental Procedures”). Analysis of synaptosomal proteins from these mice showed that the signal for CL1 was completely absent (Fig. 2B). The neurexin 1α and 1β signals, however, were unchanged in CL1 KO mice (Fig. 2B). Together these data confirm that we introduced a mutation into an active CL1 gene, resulting in the complete loss of CL1.

**Generation and Analysis of Mice Lacking Both CL1 and Neurexin 1α**—α-Latrotoxin binds to two distinct cell surface receptors, referred to as CL1 and neurexin 1α (9–13). Because the phenotypes of CL1- and neurexin 1α-deficient mice are weak, we were interested as to whether a genetic deletion of both α-latrotoxin receptors would cause a stronger phenotype, maybe accompanied by a complete loss of the α-latrotoxin response. Therefore heterozygous neurexin 1α KO mice were bred with homozygous CL1 mice, resulting in offspring heterozygous for both CL1 and neurexin 1α. Mice of this genotype were mated with each other to generate mice that are double heterozygous for CL1 and neurexin 1α (Fig. 2A).

The phenotype of the double KO mice was not stronger than that of the single KOs in terms of morbidity or mortality. Neurexin 1α/CL1 double KO mice are viable and healthy. Immunoblot analysis of their synaptosomal proteins confirmed the loss of neurexin 1α and CL1 (Fig. 2B). Other synaptic proteins such as synaptobrevin, syntaxin, SNAP25, synaptophysin, rab3A, rabphilin, and the GDP-dissociation inhibitor GDI were unchanged when compared with wild type animals (Fig. 2C).

**Binding of α-Latrotoxin to Brain Membranes Lacking CL1, Neurexin 1α, or Both**—To examine if deletion of CL1 and/or neurexin 1α changes the amount of α-latrotoxin that can be bound to brain membranes, we iodinated purified α-latrotoxin, and measured its binding to crude brain membranes prepared from wild type mice and from KO mice deficient for CL1, neurexin 1α, or both. Binding of 125I-labeled α-latrotoxin was studied upon standardization of brain membranes to equal protein amounts. In the absence of Ca2+, membranes from neurexin 1α KO mice and wild type mice bound the same amount of 125I-labeled α-latrotoxin, whereas membranes from CL1 single KO and neurexin 1α/CL1 double KO mice bound a much smaller amount (~75% decrease in binding; Fig. 3A). In the presence of Ca2+, membranes from neurexin 1α KO mice bound ~25% less α-latrotoxin than membranes from wild type mice, membranes from CL1 KO mice ~50% less, and membranes from CL1/neurexin 1α double KO mice ~75% less (Fig. 3B).
Glutamate release from synaptosomes was stimulated by K⁺ from mice that lack either neurexin 1α, CL1, or both (28). Synaptosomes were prepared from the brains of wild type mice and double KO mice, implying that for the binding of latrotoxin no cooperativity between its receptors is necessary. Data represent means ± S.E. (n = 6).

3B). Because the total amount of 125I-labeled α-latrotoxin bound was approximately two times higher in the presence of Ca²⁺ than in the absence of Ca²⁺, the total amount of 125I-labeled α-latrotoxin binding due to CL1 is rather similar in the presence or absence of Ca²⁺. The additive nature of α-latrotoxin binding to CL1 and neurexin 1α revealed by these data are consistent with previous results suggesting that CL1 and neurexin 1α bind α-latrotoxin independently of each other (9–15).

Neurotransmitter Release from Synaptosomes from Neurexin 1α/CL1 Double KO Mice Induced by K⁺-depolarization—Synaptosomes were prepared from the brains of wild type mice and from mice that lack either neurexin 1α, CL1, or both (28). Glutamate release from synaptosomes was stimulated by K⁺-depolarization in the presence of Ca²⁺ and monitored online using a photometric assay and a two wavelength photometer (23). Synaptosomes from all KO mice exhibited similar glutamate release in response to membrane depolarization (data not shown). In particular, synaptosomes from neurexin 1α/CL1 double KO mice displayed a glutamate release kinetics that was indistinguishable from that of wild type synaptosomes (Fig. 4). These data suggest, as previously shown for neurexin 1α KO mice (16), that deletion of CL1 or the double deficiency of CL1 and neurexin 1α does not result in a major disturbance of the exocytotic machinery for neurotransmitter release.

α-Latrotoxin Response in Nerve Terminals from Mice Lacking CL1 and/or Neurexin 1α—To test whether deletion of CL1 affects the ability of α-latrotoxin to elicit neurotransmitter release, and to compare the relative importance of CL1 and neurexin 1α as α-latrotoxin receptors, we prepared synaptosomes from wild type, CL1 KO, neurexin 1α KO, and double KO mice. We then measured glutamate release triggered from these synaptosomes in response to α-latrotoxin in the presence and absence of Ca²⁺. The results of an exemplary experiment performed at a single α-latrotoxin concentration are depicted in Fig. 5, and the summary data of the total release obtained after 15 min in multiple experiments with three different α-latrotoxin concentrations are represented in Fig. 6.

We first studied the α-latrotoxin response in the single CL1 and neurexin 1α knockouts. As shown in Figs. 5 and 6, deletion of CL1 alone caused a major decrease in the amount of glutamate release stimulated by α-latrotoxin (~75%). The decrease in glutamate release caused by the CL1 KO was very similar in the presence and absence of Ca²⁺, but significant residual α-latrotoxin-stimulated glutamate release remained, consistent with the presence of multiple α-latrotoxin receptors. These data demonstrate that CL1 is indeed a major physiological receptor for α-latrotoxin that is essential for a normal response to the toxin. In contrast to the CL1 KO, the neurexin 1α KO caused only a small but significant decrease in α-latrotoxin evoked glutamate release in the absence of Ca²⁺ (~20% decrease). However, in the presence of Ca²⁺ a major decrease in release was observed in the neurexin 1α KO (~75% decrease; Figs. 5 and 6). The fact that the CL1 and the neurexin 1α knockouts caused very similar decreases in release triggered by α-latrotoxin is surprising, and indicates that both receptors are required for most of the α-latrotoxin response. Thus both receptors are major α-latrotoxin receptors under physiological conditions, and cannot be independent of each other. Although the fact that only three different concentrations of α-latrotoxin
were used in our experiments limits the scope of analysis, the apparent affinity of the remaining α-latrotoxin response in the CL1 and neurexin 1α knockouts does not appear to be significantly different from each other, or from that of wild type synaptosomes, suggesting that the remaining α-latrotoxin receptors have similar affinities (Fig. 6).

We next analyzed the α-latrotoxin response in the double CL1/neurexin 1α KO in the absence of Ca\(^{2+}\). Under this condition, α-latrotoxin triggered the release of glutamate from synaptosomes lacking both CL1 and neurexin 1α with a unique time course that differed from that observed for all KO and/or conditions (Fig. 5). In the neurexin 1α/CL1 double-deficient synaptosomes, release was initially delayed for at least 5 min instead of the usual 2 min delay, but the amount of subsequent glutamate release was higher in CL1/neurexin 1α double-deficient synaptosomes (~50% decrease in response compared with wild type) than in CL1-deficient synaptosomes (~75% decrease; Fig. 5A). The altered time course of release in the double KO synaptosomes in the absence of Ca\(^{2+}\) was reproducibly obtained with four independent synaptosome preparations (data not shown). Furthermore, the increase in release in the double KO compared with the CL1 single KO was independent of the α-latrotoxin concentration (Fig. 6). This unexpected result suggests that even in the absence of Ca\(^{2+}\) (when neurexin 1α does not bind α-latrotoxin; Ref. 15), neurexin 1α participates in α-latrotoxin action. To confirm the genotypes of these mice, aliquots of their synaptosomes were analyzed for CL1 and neurexin 1α by immunoblotting (Fig. 2B).

In a final set of experiments, we measured the release of glutamate triggered from synaptosomes from the CL1/neurexin 1α double KO mice in the presence of Ca\(^{2+}\). No additivity was observed with the single knockouts, and no change in the time course of release was observed (Figs. 5B and 6B). The decrease in release in the double KO synaptosomes was identical to that observed in the two separate single knockouts (~75%). This suggests that α-latrotoxin utilizes both neurexin 1α and CL1 in triggering release in the presence of Ca\(^{2+}\). Again, this result was reproducibly obtained in multiple independent experiments from several mice whose genotypes were confirmed by immunoblotting.
**DISCUSSION**

α-Latrotoxin is a potent excitatory toxin in black widow spider venom that triggers massive neurotransmitter release. α-Latrotoxin stimulates secretion of classical transmitters contained in small clear synaptic vesicles (e.g. GABA, glutamate, and acetylcholine) in the presence and absence of Ca$^{2+}$. In contrast, transmitters and neuropeptides contained in dense core vesicles (e.g. norepinephrine and neuropeptides) are secreted in response to α-latrotoxin only in the presence of Ca$^{2+}$ (see Refs. 8 and 17 and references cited therein). α-Latrotoxin acts by binding to specific high-affinity cell surface receptors (5), and inserting partially into the plasma membrane (3). α-Latrotoxin forms pores in the plasma membrane, but the pores alone do not appear to explain α-latrotoxin action because cadmium blocks the pore conductance (29) but enhances α-latrotoxin action (18). The precise mechanism of action of α-latrotoxin has remained unclear. One surprising discovery was that α-latrotoxin binds to at least two distinct high affinity receptors on neurons (9–15). These receptors, neurexin 1α and CL1, share no sequence similarity and have no obvious common properties. Furthermore, neurexin 1α binds to α-latrotoxin only in the presence of Ca$^{2+}$ (10, 15), whereas CL1 binding is Ca$^{2+}$-independent (11, 12). When the two different receptors for α-latrotoxin were discovered, three possible explanations for the existence of double receptors were raised. The first explanation was that neurexin 1α is responsible for dense-core vesicle exocytosis, whereas CL1 mediates release of classical neurotransmitters. However, the finding that neurexin 1α is widely expressed in most neurons but largely absent from chromaffin cells (9) and that KO of neurexin 1α impairs α-latrotoxin-triggered release of classical neurotransmitters in the presence of Ca$^{2+}$ invalidated this possibility (16). A second explanation was that neurexin 1α is simply not a functional α-latrotoxin receptor. Again, this possibility was ruled out by the demonstration that the ability of α-latrotoxin to trigger neurotransmitter release is impaired in neurexin 1α-deficient neurons (16), and by the finding that expression of neurexin 1α conferred an enhanced α-latrotoxin response onto neuroendocrine PC12 cells (10). A third possible explanation for the presence of two α-latrotoxin receptors was that neurexin 1α and CL1 are co-receptors, which act as heteromultimers, analogous to some G-protein-linked receptors (reviewed in Ref. 30). This possibility, however, was made improbable by the demonstration that each receptor separately binds to α-latrotoxin with the requisite high specificity and affinity expected of a physiological receptor (10–15); thus the receptors do not function as heteromultimers in binding to α-latrotoxin. Furthermore, both receptors were shown separately to cause a similar sensitization of α-latrotoxin-triggered release in transfected PC12 cells, and no increase in release was observed upon co-expression of both receptors (10). Together these findings document that other explanations have to be found for the unusual presence of two receptors for this toxin, and for the uncommon properties of how this toxin works. In an attempt to address this, we have now generated KO mice that lack the second receptor, CL1, analyzed the action of α-latrotoxin in these KO mice, and compared the changes in α-latrotoxin response in these mice to those observed in KO mice that lack neurexin 1α alone or both CL1 and neurexin 1α.

Our data demonstrate that single and double KO mice that lack CL1 and/or neurexin 1α are viable and fertile, although they probably exhibit more subtle neuronal and behavioral phenotypes that have not been analyzed in the present experiments. Furthermore, we show that α-latrotoxin binding to brain membranes from these animals is decreased in a pattern that largely follows expectations based on previous studies (11, 12, 15, 16): Total binding of α-latrotoxin to wild type membranes in the presence of Ca$^{2+}$ was two times higher than the binding observed in the absence of Ca$^{2+}$ as described (16); CL1 accounts for the majority of both the Ca$^{2+}$-dependent and -independent binding whereas neurexin 1α only contributes to Ca$^{2+}$-dependent binding; and the deficits in binding observed in the individual knockouts are additive in the double KO (Fig. 3). However, our data reveal surprising effects of the knockouts on the ability of α-latrotoxin to trigger neurotransmitter release. These effects cannot be explained in terms of α-latrotoxin binding alone. Specifically, we found the following.

1) In the absence of Ca$^{2+}$, deletion of CL1 depresses the majority of α-latrotoxin induced glutamate release, confirming the hypothesis that CL1 constitutes the major Ca$^{2+}$-independent α-latrotoxin receptor (11, 12). However, we also observed a small but significant effect of the neurexin 1α KO on release that may reflect a participation of neurexin 1α in the release process itself. This effect was small, explaining why it was not observed in a previous study (16), but was reproducible at different α-latrotoxin concentrations (Fig. 6A). Curiously, in the absence of Ca$^{2+}$, α-latrotoxin was more potent in triggering release from synaptosomes lacking both CL1 and neurexin 1α than from synaptosomes lacking only CL1 (Fig. 5A); this effect was independent of the α-latrotoxin concentration (Fig. 6A).

2) In the presence of Ca$^{2+}$, we observed a very simple effect of each single KO and of the double CL1/neurexin 1α KO on glutamate release: No matter which receptors were deleted, release was strongly inhibited, with the impairment in α-latrotoxin-triggered release being equal for all three genotypes.

Overall, these results allow two major conclusions. First, they unequivocally establish that both CL1 and neurexin 1α are physiologically the most important α-latrotoxin receptors. Minor receptor activity remains in the absence of CL1 and neurexin 1α, which may be explained by the presence of CL2 and CL3, and of neurexins 1δ, 2α, 2β, 3α, and 3β that are known to function at least partly as α-latrotoxin receptors (10, 13, 14) and have not been deleted in the CL1 and neurexin 1α knockouts. Second, these results demonstrate that the two types of α-latrotoxin receptors do not function independently of each other in vivo, despite their autonomous α-latrotoxin binding activities. Multiple lines of evidence support this conclusion. For example, both in the presence and the absence of Ca$^{2+}$, the effects of the CL1 and neurexin 1α knockouts on α-latrotoxin-triggered release were not additive, although their effects on α-latrotoxin binding were. In the presence of Ca$^{2+}$, both receptors are equally required, and the impairment observed in the double KO is no more severe than that of each single KO, suggesting that under these more physiological conditions the two receptors are essential components of the same machinery that mediates the action of α-latrotoxin. In the absence of Ca$^{2+}$, we also observed a non-additive effect which, however, went in the opposite direction. Although we do not currently understand the molecular basis for these intriguing observations, they clearly demonstrate that the two receptors act autonomously in α-latrotoxin binding, but interdependently in α-latrotoxin action in neurotransmitter release.

Apart from these major conclusions, however, our data raise important questions that we cannot currently answer. Why does the neurexin 1α KO decrease α-latrotoxin-triggered release in the absence of Ca$^{2+}$, even though the decrease is small, but increase release on the background of the CL1 KO? Because neurexin 1α does not bind α-latrotoxin without Ca$^{2+}$, the most plausible explanation is that neurexin 1α is part of the machinery by which α-latrotoxin binding to CL1 triggers neurotransmitter release, as described above. As part of this machinery, neurexin 1α has incongruous effects on α-latrotoxin...
action: It is required for full activation of release by α-latrotoxin binding to Ca\(^{2+}\)-independent receptors when all of these receptors are present, but slows down release when the major Ca\(^{2+}\)-independent receptor (CL1) is absent. A possible explanation for this finding would also be consistent with the equal requirement for both receptor types in the presence of Ca\(^{2+}\) that is the precise stoichiometry of Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent receptors (i.e. neurexins and CLs) is important, especially in the absence of Ca\(^{2+}\). Thus creating an excess of one over the other inhibits, whereas deleting both reactivates. However, precise definition of this hypothesis will require further insight into how precisely the two receptors types cooperate in α-latrotoxin action, the next experimental challenge in this interesting field.

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