

Extracellular Domains of α -Neurexins Participate in Regulating Synaptic Transmission by Selectively Affecting N- and P/Q-Type Ca^{2+} Channels

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Neurexins constitute a large family of highly variable cell-surface molecules that may function in synaptic transmission and/or synapse formation. Each of the three known neurexin genes encodes two major neurexin variants, α - and β -neurexins, that are composed of distinct extracellular domains linked to identical intracellular sequences. Deletions of one, two, or all three α -neurexins in mice recently demonstrated their essential role at synapses. In multiple α -neurexin knock-outs, neurotransmitter release from excitatory and inhibitory synapses was severely reduced, primarily probably because voltage-dependent Ca^{2+} channels were impaired. It remained unclear, however, which neurexin variants actually influence exocytosis and Ca^{2+} channels, which domain of neurexins is required for this function, and which Ca^{2+} -channel subtypes are regulated. Here, we show by electrophysiological recordings that transgenic neurexin 1 α rescues the release and Ca^{2+} -current phenotypes, whereas transgenic neurexin 1 β has no effect, indicating the importance of the extracellular sequences for the function of neurexins. Because neurexin 1 α rescued the knock-out phenotype independent of the α -neurexin gene deleted, these data are consistent with a redundant function among different α -neurexins. In both knock-out and transgenically rescued mice, α -neurexins selectively affected the component of neurotransmitter release that depended on activation of N- and P/Q-type Ca^{2+} channels, but left L-type Ca^{2+} channels unscathed. Our findings indicate that α -neurexins represent organizer molecules in neurotransmission that regulate N- and P/Q-type Ca^{2+} channels, constituting an essential role at synapses that critically involves the extracellular domains of neurexins.

Key words: transgenic mouse; synapse; transmission; calcium channels; cell adhesion; brainstem

Introduction

Neurotransmission requires formation and differentiation of presynaptic and postsynaptic compartments, including the assembly of an extensive molecular machinery for exocytosis and signal transduction. The number, ultrastructure, and physiological properties of synapses vary considerably between individual neurons and brain regions and can change dramatically during synaptogenesis and synaptic plasticity. Neurexins were proposed as candidate molecules to mediate some of the diversity (Missler and Südhof, 1998) because (1) their structure indicates a receptor-like transmembrane protein (Ushkaryov et al., 1992,

1994; Ushkaryov and Südhof, 1993), (2) they bind to postsynaptic cell-surface proteins (Ichtchenko et al., 1995; Sugita et al., 2001), (3) they are receptors for α -latrotoxin, which induces neurotransmitter release (Geppert et al., 1998; Sugita et al., 1999), and (4) they are polymorphic in their extracellular sequences because of significant alternative splicing (Ichtchenko et al., 1995; Missler et al., 1998; Sugita et al., 1999, 2001). Mammalian neurexins are encoded by three genes that contain independent promoters for α - and β -neurexins (Rowen et al., 2002; Tabuchi and Südhof, 2002). α -Neurexins contain substantially longer extracellular sequences than the much shorter β -neurexins, but share with β -neurexins the same C terminus (Missler and Südhof, 1998). Consequently, available biochemical evidence has revealed overlapping C-terminal (intracellular) binding partners (Hata et al., 1996; Butz et al., 1998; Biederer and Südhof, 2000), but distinct extracellular interactions for α - and β -neurexins (Ichtchenko et al., 1995; Missler et al., 1998; Sugita et al., 2001).

To determine the role of neurexins, we previously produced knock-out (KO) mice that lack one, two, or all three α -neurexins (Missler et al., 2003). Mutants with different combinations of knock-out alleles mostly died prematurely as a result of respiratory problems. Analyses of newborn mice deficient for multiple

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α -neurexins revealed almost no changes in brain architecture or synapse structure but uncovered a severe reduction in neurotransmitter release. As the presumptive cause of the inefficient exocytosis in mutant mice, we suggested that voltage-dependent Ca^{2+} channels (VDCCs) were impaired because the response pattern of synaptic transmission to specific Ca^{2+} -channel blockers was altered, and whole-cell Ca^{2+} currents were reduced (Missler et al., 2003). Although no knock-out mice are available yet for β -neurexins, their putative function was addressed recently by cell culture assays: overexpression of neurexin 1 β induced differentiation of postsynaptic receptors (Graf et al., 2004), and its trans-synaptic partner neuroligin stimulated *de novo* formation and differentiation of presynaptic terminals *in vitro* (Scheiffele et al., 2000; Dean et al., 2003; Sara et al., 2005). These studies established a prominent role for neurexins at synapses but also raised important questions: is the phenotype of reduced neurotransmitter release in knock-out mice specific for the deletion of α -neurexins? Are there functional differences between various neurexin genes? Is the highly variable extracellular domain of neurexins required for regulating VDCCs? Why were the β -neurexins that are still abundantly expressed in α -neurexin KO mice apparently unable to compensate for the loss of α -neurexins? Which subtypes of high voltage-activated Ca^{2+} channels are impaired in α -neurexin KO mice?

Here, we show that (1) a single transgenically expressed neurexin 1 α splice variant rescued the impaired neurotransmission at excitatory and inhibitory synapses in all deletions of α -neurexins tested; (2) comparable transgenic expression of β -neurexins had no effect, indicating that extracellular sequences of α -neurexins are important for regulating VDCCs; and (3) α -neurexins selectively altered N- and P/Q-type Ca^{2+} -channel function but left L-type Ca^{2+} channels unchanged.

Materials and Methods

Generation and breeding of transgenic mice. Transgenic vectors for neurexin 1 α ($\alpha\text{Nrx}::\text{HRP}$) (Missler et al., 2003) and neurexin 1 β ($\beta\text{Nrx}::\text{HRP}$) (generated *de novo* for this study) were constructed using cDNA plasmids pCMVL2 and pCMVL13, respectively. Plasmid pBS-HRP served as the source of the horseradish peroxidase (a gift from Dr. H. Kraemer, University of Texas Southwestern Medical Center). Two internal restriction sites (the 5' *NotI* site in pCMVL2 and the *PvuII* site in pBS-HRP) had to be destroyed by silent mutagenesis. A 980 bp HRP fragment was cloned into the 3' *NotI* site of the modified pCMVL2 and of pCMVL13 [resulting junctional amino acid sequence: neurexin . . . GRQPAMQLT.HRP.NSNSGQPRGR . . . neurexin (italicized residues represent the HRP cDNA)]. The chimeric neurexin–HRP cDNAs were blunt-end cloned into the unique *XhoI* site of the mouse Thy1.2 expression vector pEX21 (a gift from Dr. M. Goedert, Medical Research Council, Cambridge, UK) to generate the transgenic vectors. The Thy1.2 promoter was chosen because of its developmentally early widespread neuronal expression (Andra et al., 1996). Transgenic mice were produced by pronucleus injection, and founder mice and progeny were genotyped using dot/Southern blotting with *EagI/EagI*-isolated ^{32}P -labeled HRP cDNA probes. Because our rescue experiments required crossing mice from FVB strains (transgenics) into the mixed 129Sv \times C57BL/6 background (α -neurexin KOs), each set of experiments was done on littermate mice with and without additional transgene. Genotyping a large number of newborn offspring [$n = 265$ pups killed at postnatal day 1 (P1)] confirmed a normal mendelian ratio for all combinations of mutated neurexin alleles (data not shown). In addition, 52% of pups ($n = 96$ of 183) inherited the $\alpha\text{Nrx}::\text{HRP}$ transgene, and 51% inherited the $\beta\text{Nrx}::\text{HRP}$ transgene ($n = 42$ of 82). All analyses were performed with hemizygous transgenic mice on wild-type or α -neurexin KO backgrounds, as described below.

HRP antibodies and biochemical procedures. Expression of neurexin–HRP transgenic fusion proteins was tested in brains of hemizygous mice

using antibodies against HRP epitopes. Most commercial antibodies were generated against the plant HRP and cross-react with endogenous mouse brain proteins (data not shown). Therefore, we produced a recombinant HRP–glutathione S-transferase (GST) fusion protein (from pET28-HRP) in BL21 cells to affinity purify a commercially available polyclonal serum (Nordic) using nitrocellulose membrane stripes containing HRP separated by SDS-PAGE (Olmsted, 1981). Immunoprecipitations and GST pull-downs were performed with P₂ brain membrane proteins solubilized in 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate with affinity-purified HRP antibodies (anti-HRP_{aff}) or recombinant calcium/calmodulin-dependent serine kinase (CASK) immobilized on protein A and glutathione agarose beads, respectively (Hata et al., 1996). To measure the enzymatic activity of transgenic neurexin–HRP fusion proteins, mouse brain membrane fractions (nonsolubilized P₂ fractions) were incubated for 15 min with diaminobenzidine (1 mg/ml) and H₂O₂ (0.04%), and the absorbance at 595 nm was determined with reactions from nontransgenic mice serving as controls.

Morphological studies. To analyze the distribution of transgenic expression of both $\alpha\text{Nrx}::\text{HRP}$ and $\beta\text{Nrx}::\text{HRP}$ proteins in the brainstem of newborn mice, brains were freshly dissected in PBS, and brainstem preparations were immersion fixed in 2% paraformaldehyde in 0.1 M phosphate buffer for 2 h, postfixed for 2 h in the same solution, and cryoprotected in 25% sucrose. For light microscopy of transgenic mice, 12 μm cryosections were thaw mounted on poly-L-lysine-coated glass slides and labeled overnight with anti-HRP_{aff} antibodies in blocking buffer (0.1% Triton X-100, 50% normal goat serum, and PBS). Secondary goat anti-rabbit antibody and PAP antibodies (rabbit PAP) were incubated with sections in blocking solution (both antibodies were from Sternberger Monoclonals, Lutherville, MD) and visualized with diaminobenzidine (0.05% w/v) plus H₂O₂ (0.005% v/v) and heavy metal enhancement (NiCl₂, 0.15% w/v). To compare tissues from different genotypes, sections from several mice were usually processed in parallel. Overview panels of newborn brainstem (see Fig. 2) are montages from up to 15 individual pictures captured via an AxioCam HR digital camera system mounted on an AxioScope 2 microscope (Zeiss, Oberkochen, Germany) and composed using Adobe Photoshop 6.0 software (Adobe Systems, San Jose, CA).

Electrophysiological recordings. All electrophysiological analyses were performed on brainstem neurons of mice whose genotype was unknown to the experimenter, essentially as described previously (Zhang et al., 1999; Missler et al., 2003). Acute slices containing the pre-Botzinger complex (PBC) and hypoglossal motor nucleus from newborn (P1) littermate mice were used for whole-cell recordings. The bath solution in all experiments consisted of the following (in mM): 118 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 25 NaHCO₃, 1 NaH₂PO₄, and 20 glucose, pH 7.4 (aerated with 95% O₂ and 5% CO₂ and kept at 28–30°C). Evoked EPSCs (eEPSCs) and evoked IPSCs (eIPSCs) were recorded from hypoglossal motor neurons in the presence of 1 μM strychnine and 1 μM bicuculline or from PBC neurons in the presence of 10 μM CNQX, respectively. EPSCs were evoked by 0.1 Hz field stimulations of axons of interneurons close to the PBC using a custom-built bipolar platinum electrode. An isolation unit, IsoFlex (A.M.P.I., Jerusalem, Israel), with a custom-built power supply, was used to apply currents of supramaximal stimulation strength (~ 1 mA actual current near the slice). During the long time course of investigations represented in this study (extending over 3 years), different stimulation setups and types of electrodes had to be used, preventing the direct comparison of absolute EPSC amplitudes between the $\alpha\text{Nrx}::\text{HRP}^{\text{tg}/+}$ and $\beta\text{Nrx}::\text{HRP}^{\text{tg}/+}$ experiments. This, however, does not affect our conclusions regarding their respective rescue potential. The pipette solution for eEPSC and eIPSC measurements contained the following (in mM): 140 K-gluconate (eEPSCs) or 140 KCl (eIPSCs), 1 CaCl₂, 10 EGTA, 2 MgCl₂, 4 Na₃ATP, 0.5 Na₃GTP, and 10 HEPES, pH 7.3. Peak amplitudes were averaged from 25 consecutive responses. To monitor changes in input resistance, current responses to a -10 mV voltage step (20 ms) from a holding potential of -70 mV were recorded before every fifth stimulus. In all experiments, the distance between the stimulation and recording electrodes was similar between slices of different genotypes, and we could not find any significant differences in latency of evoked responses between the genotypes. The supra-

maximal stimulation protocol using constant current amplitudes (see above) differs slightly from the one applied in our previous paper for the KO analysis (Missler et al., 2003), in which we adjusted the stimulation strength according to the failure rate obtained. The current approach was chosen to emphasize differences between mice that are homozygous for the same α -neurexin mutation and that either lack or contain the neurexin transgene. Using the supramaximal stimulation strength in all cases produced a higher resolution of rescue effect even in the case of multiple α -neurexin KOs. However, using supramaximal stimulation strength obscured comparison of absolute responses between the different genotypes in these experiments, explaining why the differences between KO combinations are less pronounced than in our previous study.

Miniature postsynaptic current analysis. Spontaneous miniature IPSCs (mIPSCs) and miniature EPSCs (mEPSCs) were recorded from neurons of the PBC and hypoglossal nucleus at a Cl^- reversal potential of ~ 0 mV in $10 \mu\text{M}$ CNQX or $1 \mu\text{M}$ strychnine and $1 \mu\text{M}$ bicuculline, respectively; signals with amplitudes of at least 2.5 times above background noise were selected, and statistical significance was tested in each experiment. Based on our selection criteria, miniature postsynaptic current (mini) events close to the detection level could be missed; however, the noise levels and amplitudes were similar between genotypes. Voltage-activated Ca^{2+} currents were measured from neurons of the PBC with electrodes containing (in mM) 110 CsCl₂, 30 TEA-Cl, 1 CaCl₂, 10 EGTA, 2 MgCl₂, 4 Na₃ATP, 0.5 Na₃GTP, and 10 HEPES, pH 7.3, and with $0.5 \mu\text{M}$ tetrodotoxin in the bath solution. Serial and membrane resistances were estimated from current transients induced by 20 mV hyperpolarization voltage commands from a holding potential of -70 mV. The serial resistance was compensated by 80%, and patches with a serial resistance of >20 M Ω , a membrane resistance of <0.8 G Ω , or leak currents of >150 pA were excluded. The membrane currents were filtered by a four-pole Bessel filter at a corner frequency of 2 kHz and digitized at a sampling rate of 5 kHz using the DigiData 1200B interface (Axon Instruments, Union City, CA). Currents were recorded in response to voltage step changes from the -70 mV holding potential to test potentials between -80 and $+30$ mV and quantified as peak currents in response to voltage steps from -70 to 0 mV. Ca^{2+} -current measurements were corrected using the P/4 protocol that subtracts leak currents measured during four leak-subtraction prepulses applied immediately before each voltage step. Blocking experiments using specific VDCC inhibitors were always performed in the same sequential order, applying $10 \mu\text{M}$ nifedipine for L-type channel contributions, $0.5 \mu\text{M}$ ω -conotoxin MVIIA for N-type channel contributions, $0.2 \mu\text{M}$ agatotoxin IVA for P/Q-type channel contributions, and $100 \mu\text{M}$ Cd²⁺ to probe for residual Ca^{2+} channel activities. Data acquisition and analysis were performed using commercially available software [pClamp 6.0 and AxoGraph 4.6 (Axon Instruments); Prism 3 software (GraphPad Software, San Diego, CA)].

Miscellaneous. Primary antibodies to neurexins (A473), neuroligin (4C12), and synaptotagmin 1 (W855) were characterized previously (Ushkaryov et al., 1992; Butz et al., 1998; Song et al., 1999) or obtained commercially: Sy38, anti-synaptophysin (DakoCytomation, Hamburg, Germany), anti-P/Q Ca^{2+} -channel subunit and anti-NMDA R1 (Chemicon, Temecula, CA), and heat shock protein 70 (Affinity BioReagents, Golden, CO). Statistical significance was tested with a two-tailed Student's *t* test in Excel (Microsoft, Redmond, WA) spreadsheets or with InStat (GraphPad Software) and, in case of mini events, with the Kolmogorov–Smirnov test integrated in MiniAnalysis (Synaptosoft, Decatur, GA).

Results

Transgenic mice expressing neurexins that differ solely in their extracellular sequences

To elucidate the molecular determinants that link neurexins to Ca^{2+} channels, we used a comparative transgenic rescue analysis. This analysis allows us to explore the role of structurally distinct α - and β -neurexins in synaptic transmission. In our initial characterization of α -neurexin KO mice, we showed that the decrease in whole-cell Ca^{2+} currents caused by deletion of α -neurexins

can be partially reversed by transgenic neurexin 1α expressed as a fusion protein with HRP under control of the Thy1 promoter (Missler et al., 2003). This finding raised questions as to whether the reduced neurotransmitter release was also reversed by the transgene and whether the partial rescue was a specific consequence of transgenic neurexin 1α expression (or could be extended to β -neurexins). To address these questions, here we have compared the physiological effects mediated by two sets of transgenic mice that express two different neurexins (Fig. 1A): (1) the original neurexin 1α transgene ($\alpha\text{Nrx::HRP}$) (Missler et al., 2003) and (2) a newly generated neurexin 1β transgene ($\beta\text{Nrx::HRP}$).

Both sets of transgenic mice were produced with identical vectors in which an HRP cDNA was inserted at the same position into neurexin 1α or 1β to monitor expression (Fig. 1A). HRP was chosen as an internal epitope tag, because studies in *Drosophila* demonstrated that HRP did not interfere with membrane traffic when inserted into the extracellular sequences of a cell-surface protein (Sunio et al., 1999; Dubois et al., 2001). The major difference between the two sets of transgenic mice arises from variations in neurexin 1α and 1β sequences, which are identical apart from their extracellular domains, the former containing a large extracellular sequence consisting of multiple domains and the latter consisting of a shorter extracellular region (Ushkaryov et al., 1992; Missler and Sudhof, 1998) (Fig. 1A). To ensure that the differences observed between the activity of these transgenes were not attributable to insertion effects, we generated multiple lines of both transgenes, which, as described below, produced similar transgene protein levels and expression patterns and thus allowed a direct comparison of the functions of the two types of neurexins.

Heterozygous ($\alpha\text{Nrx::HRP}^{\text{tg}/+}$ and $\beta\text{Nrx::HRP}^{\text{tg}/+}$) and homozygous ($\alpha\text{Nrx::HRP}^{\text{tg}/\text{tg}}$ and $\beta\text{Nrx::HRP}^{\text{tg}/\text{tg}}$) transgenic mice were viable and fertile (data not shown), indicating that the transgenic neurexins did not strongly interfere with normal neurexin function or the role of an unidentified essential gene close to the insertion site of the transgenes. To examine protein expression, we affinity-purified commercially available HRP antibodies on immobilized recombinant HRP and used anti-HRP_{aff} for immunoblotting. The anti-HRP_{aff} reacted with a single protein on immunoblots from adult transgenic mice and detected no cross-reactive bands in nontransgenic littermates (Fig. 1B). Comparable immunoblots of α -neurexin transgenic mice (data not shown) indicated that expression levels of the transgenic $\beta\text{Nrx::HRP}$ were approximately the same as those for transgenic $\alpha\text{Nrx::HRP}$ (for a direct comparison of their expression in newborn mice, see Fig. 2). We next investigated whether the HRP-tagged neurexins expressed in transgenic mice were capable of normal protein–protein interactions typical for endogenous neurexins. Pull-down experiments using a GST fusion protein containing the postsynaptic density-95/Discs large/zona occludens-1 (PDZ) domain of CASK, a protein that binds to the C terminus of neurexins (Hata et al., 1996), revealed that both transgenic neurexins bound CASK efficiently (Fig. 1C). This observation suggested that endogenous (Fig. 1C, asterisks) and transgenic (Fig. 1C, arrows) neurexins engage in similar intracellular binding activities, as predicted from their identical C terminus. To explore the integrity of specific extracellular interactions, we performed immunoprecipitations of brain proteins from transgenic mice with anti-HRP_{aff} antibodies. These experiments showed that the $\beta\text{Nrx::HRP}$ fusion protein is present in the brain in a complex with neuroligin 1, as predicted for this particular splice variant (Fig. 1D) (Ichtchenko et al., 1995). In contrast,

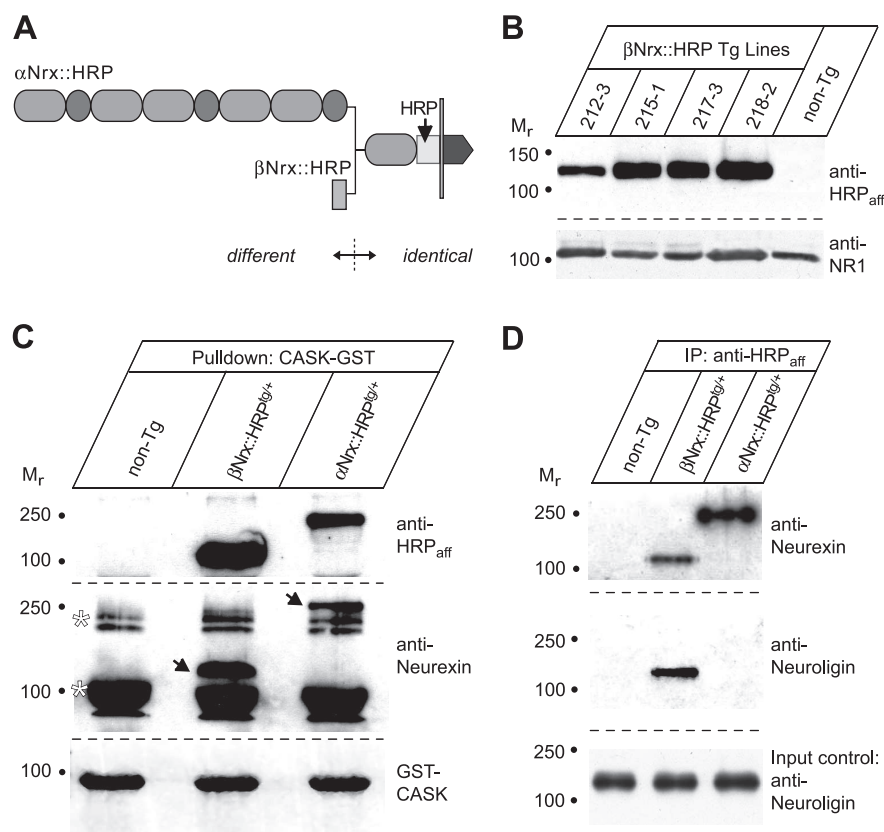


Figure 1. Transgenic mice expressing two distinct neurexins under control of a heterologous promoter. **A**, Domain model of transgenically introduced α Nrx::HRP and β Nrx::HRP proteins with an internal HRP epitope to monitor expression. The transgenic α - and β -neurexins under control of the Thy1.2 promoter differ exclusively in their extracellular sequences. Gray sheet, transmembrane domain (Missler and Sudhof, 1998). **B**, Comparative immunoblot analysis of several transgenic (Tg) mouse lines expressing β Nrx::HRP. Brain membrane proteins (20 μ g/lane) were probed with anti-HRP_{aff}. Loading control blots were probed with the protein CASK was analyzed by immunoblotting with affinity-purified HRP antibodies (top) or pan-neurexin antibodies (middle). Both transgenically expressed (arrows) and endogenous (asterisks) neurexins interact with their intracellular ligand CASK. Equivalent amounts of recombinantly expressed GST–CASK were used for the pull-down procedures (bottom; Coomassie stain). **D**, Immunoprecipitation (IP) of brain membrane proteins from transgenic mice expressing α Nrx::HRP and β Nrx::HRP and from control mice (non-Tg). Transgenic neurexin–HRP fusion proteins were precipitated with anti-HRP_{aff} and analyzed by immunoblotting with antibodies to neurexins (top) or neuroigin (middle; bottom shows control samples before immunoprecipitation). Only β Nrx::HRP binds to neuroigin, confirming that isoform-specific interactions are intact in brains of transgenic animals.

no neuroigin 1 coimmunoprecipitated with α Nrx::HRP was detectable.

To ensure a meaningful comparison of the effects of the transgenic neurexins (see below), we confirmed by immunohistochemistry that transgenic neurexin 1 α and 1 β are expressed in similar patterns in the brainstem of newborn mice in areas used for the electrophysiological rescue analysis (Fig. 2A–C, RVLN, XII). Although activity measurements revealed that the HRP moiety of both transgenic proteins was enzymatically active (Fig. 2D), the peroxidase activity conferred by HRP could not be used for these morphological studies because of the low sensitivity of the technique (data not shown). For functional analyses, we bred mice that are homozygous for the neurexin 2 α KO allele (Nrx2 α ^{-/-}) and heterozygous for the two other neurexin KO alleles (Nrx1 α ^{+/-} and Nrx3 α ^{+/-}) and, in addition, either lack or contain a hemizygous transgene (nontransgenic, α Nrx::HRP^{tg/+}, or β Nrx::HRP^{tg/+}) (Fig. 2E). Mating of this parent generation resulted in littermate homozygous neurexin 2 α KO offspring that additionally carried various combinations of the neurexin 1 α and 3 α alleles

and also (in 50% of the cases) a single copy of the respective transgene.

Transgenic neurexin 1 α enhances synaptic transmission and Ca²⁺ currents in α -neurexin KO mice

To explore the potential of one α -neurexin isoform to rescue the synaptic phenotype of multiple α -neurexin KO mice, we first asked whether α Nrx::HRP^{tg/+} was able to alter the amplitude of evoked synaptic responses. Because α -neurexin KO mice homozygous for two or three mutant alleles die early postnatally, we analyzed all mutant mice immediately after birth (P1) in acute brainstem slices, because the synaptic connections in this area mature relatively early and are involved in breathing control [normal breathing was disrupted in α -neurexin KO mice (Missler et al., 2003)]. Synaptic responses were monitored in hypoglossal motor neurons after afferent fiber stimulation near the PBC by measuring eEPSCs in littermate α -neurexin mutant mice with and without the α Nrx::HRP transgene.

Representative EPSC traces in Figure 3A and their quantification in Figure 3B show that transgenic expression of α Nrx::HRP caused a dramatic increase in evoked synaptic responses in α -neurexin KO mice (Fig. 3B). In single neurexin 2 α (SKO2) and double neurexin 2 α /3 α (DKO2/3) or 1 α /2 α KO (DKO1/2) mice, α Nrx::HRP doubled the mean EPSC to ~198–223% compared with their nontransgenic littermates. Higher absolute EPSC amplitudes in SKO mice expressing α Nrx::HRP, compared with DKO and triple KO (TKO) mice containing α Nrx::HRP, suggested that a single transgene was not sufficient to overcome deletion of multiple α -neurexins (Fig. 3B).

Only a limited number of TKO mice could be analyzed in these experiments because TKO mice are difficult to obtain by breeding (the probability of obtaining a TKO plus α Nrx::HRP^{tg/+} was 3.125%). Consistent with the increased amplitude of eEPSCs, the α Nrx::HRP^{tg/+} also decreased the failure rate in all genotypes (from 19.2 to 7.2% in SKO2 mice when the α Nrx::HRP transgene was expressed and from 30.0 to 7.3% in DKO1/2 mice; data not shown). To test whether this rescue also extended to evoked responses at inhibitory terminals, eIPSCs were recorded from PBC neurons after field stimulations of axons close to the PBC in the presence of 10 μ M CNQX. For these experiments, because of the difficulty of breeding a sufficient number of mice with the correct genotype, quantifications were restricted to littermate mice of one α -neurexin genotype (DKO2/3) with and without α Nrx::HRP^{tg/+}. As for excitatory synapses, eIPSCs increased significantly when an α -neurexin transgene was present (DKO2/3, 165.4 \pm 38.11 pA, n = 6; DKO2/3 and α Nrx::HRP^{tg/+}, 347.7 \pm 68.55 pA, n = 5; p < 0.05).

To analyze whether the increase in evoked synaptic responses by the neurexin 1 α transgene reflects a general improvement of

vesicle release, we next measured spontaneous frequencies and amplitudes of minis in PBC neurons (Fig. 3C,D). Confirming the results from the evoked release experiments, we observed that the α Nrx::HRP^{tg/+} transgene caused a significant enhancement of the spontaneous neurotransmitter release in all α -neurexin KO combinations studied. Mini frequencies at inhibitory synapses increased to ~160% in SKO2 and α Nrx::HRP^{tg/+} neurons, to ~174% in DKO2/3 and α Nrx::HRP^{tg/+} neurons, to ~233% in DKO1/2 and α Nrx::HRP^{tg/+} neurons, and to ~274% in TKO and α Nrx::HRP^{tg/+} neurons compared with their respective nontransgenic littermate controls (Fig. 3D). Although this proportional increase in mini frequency by the α Nrx::HRP transgene appears stronger the more α -neurexin alleles are missing, the lower absolute mIPSC frequencies in rescued DKO and TKO versus SKO2 neurons are consistent with our previous notion that a single transgene is not enough to replace multiple α -neurexins (see above). In contrast to the frequency, no changes in mini amplitudes were observed in rescued neurons (Fig. 3C and data not shown), supporting the idea that neurexins primarily function presynaptically. To test whether the rescue of mini release also extends to excitatory synapses, we measured minis in hypoglossal motor neurons in the presence of 1 μ M strychnine and 1 μ M bicuculline, again by restricting quantitative analysis to littermate mice of one particular α -neurexin genotype (DKO2/3) with and without α Nrx::HRP^{tg/+}. Frequencies of mEPSCs increased from $0.7 \pm 0.013 \times 10^{-1}$ Hz (DKO2/3; $n = 4$) to $3.9 \pm 0.24 \times 10^{-1}$ Hz (DKO2/3 and α Nrx::HRP^{tg/+}; $n = 4$), confirming that transgenic α -neurexin potentially enhances spontaneous and evoked synaptic neurotransmitter release in both excitatory and inhibitory synapses of knock-out mice.

Investigation of α -neurexin KO mice had prompted the hypothesis that the impairment in neurotransmission is essentially a result of an impairment of Ca²⁺-channel function (Missler et al., 2003). Here, we performed whole-cell recordings of total high voltage-activated Ca²⁺ currents in KO neurons of the PBC with and without the transgenically expressed α Nrx::HRP. Consistent with our previous results, we found that Ca²⁺ currents were low in α -neurexin KO mice, but transgenic expression of α -neurexin (α Nrx::HRP^{tg/+}) robustly increased peak amplitudes (Fig. 4A). To compare the current data with our previous study, we quantified the whole-cell Ca²⁺ current as current densities (Fig. 4B), which confirmed and extended our previous observation that the α Nrx::HRP transgene can increase Ca²⁺-current densities approximately two-fold in all α -neurexin KO combinations. Although we noted that the increase in whole-cell Ca²⁺ currents paralleled the increase in the evoked and spontaneous responses (shown above), it remains an open question to what extent these presumably somatodendritic currents reflect the activity of presynaptic Ca²⁺ channels. Even the best rescue by α Nrx::HRP in single and double KO mice (to 17–18 pA/pF) (Fig. 4B), however, did not completely reach the Ca²⁺-

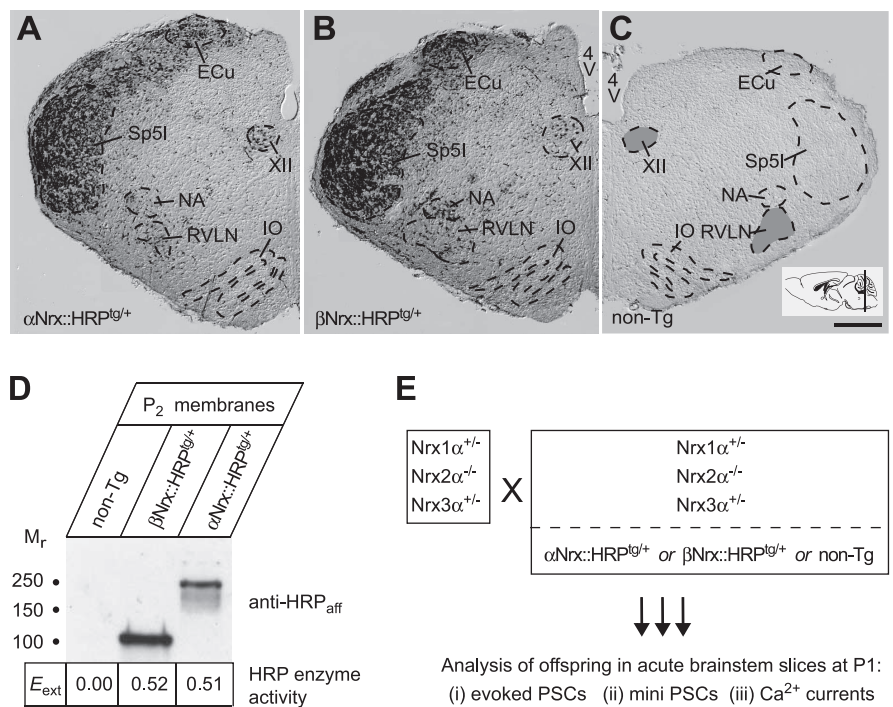


Figure 2. Transgenically expressed α - and β -neurexin can be explored for their potential to rescue the knock-out phenotype. **A–C**, Overviews of brainstem areas from newborn α Nrx::HRP^{tg/+} (**A**) or β Nrx::HRP^{tg/+} (**B**) mice or from nontransgenic (non-Tg) littermates (**C**), assembled from sections at the indicated level (see inset). Transgenic neurexins visualized by immunohistochemistry with anti-HRP_{aff} antibodies are present in areas used for functional analyses [PBC, part of the rostroventrolateral nucleus (RVLN), shadowed in **C**; and the nucleus of the hypoglossal nerve, nucleus of the 12th cranial nerve (XII), shadowed in **C**]. NA, Nucleus ambiguus; IO, inferior olive complex; Sp5I, spinal trigeminal nucleus interpolar part; 4V, fourth ventricle; ECu, external cuneate nucleus. Scale bar: **A–C**, 250 μ m. **D**, A calorimetric enzyme assay of membrane fractions from adult nontransgenic mice and mice expressing β Nrx::HRP or α Nrx::HRP was used to assess the integrity of the HRP moiety. Peroxidase activity is depicted below the corresponding Western blot lanes and expressed as extinction at OD₅₉₅ (means from $n = 3$ experiments). **E**, Mouse breeding strategy for electrophysiological experiments. Animals homozygous for the neurexin 2 α and double heterozygous for neurexin 1 α and 3 α genes ($Nrx1\alpha^{+/-}2\alpha^{-/-}3\alpha^{+/-}$) were bred to mice of the same KO genotype but carrying an additional α Nrx::HRP^{tg/+} or β Nrx::HRP^{tg/+} or no transgene. All functional analyses were done on their newborn offspring (P1).

current densities observed in corresponding neurons of genetically matching wild-type mice (~20 pA/pF) (Missler et al., 2003).

Transgenic neurexin 1 β has no effect on the α -neurexin KO phenotype

We next examined whether the neurexin 1 β transgene (β Nrx::HRP^{tg/+}) caused rescue activity similar to that seen for the neurexin 1 α transgene. A successful rescue effect with β Nrx::HRP^{tg/+} would suggest that the difference in extracellular sequences between neurexin 1 α and 1 β is unimportant with respect to synaptic transmission and that the strong phenotype in α -neurexin KO mice in the continued presence of β -neurexins is attributable to the differential expression or localization of α - and β -neurexins. Conversely, if the neurexin 1 β transgene is unable to rescue the phenotype despite similar expression levels and patterns, an essential function of the α -neurexin-specific extracellular domains in affecting presynaptic neurotransmitter release seems likely.

We found that the β Nrx::HRP^{tg/+} transgene had no effect on the size of evoked synaptic responses or on the failure rate in any of the α -neurexin KO mice tested (Fig. 5A,B and data not shown). In these experiments, we compared littermates that carry the same KO combinations with or without an additional β Nrx::HRP^{tg/+} transgene and measured the amplitudes in hypoglossal motor neurons after field stimulation of their inputs from the PBC. However, no significant differences in mean currents

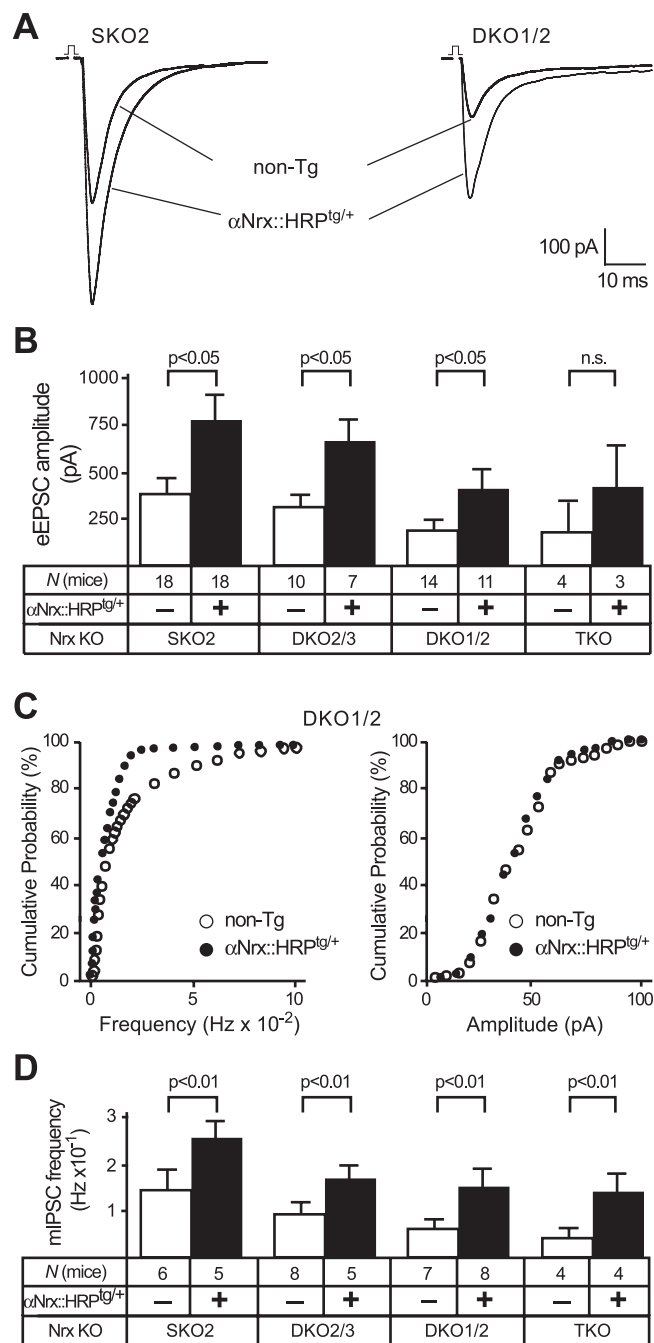


Figure 3. Transgenically expressed α Nrx::HRP increases evoked and spontaneous neurotransmitter release in neurons of KO mice. **A**, Sample traces of eEPSCs, representing an average from 25 consecutive responses to field stimulations recorded under 1 μ M bicuculline and 1 μ M strychnine. Recordings were obtained in hypoglossal motor neurons from newborn SKO2 and DKO1/2 mice without [nontransgenic (non-Tg)] and with an additional α Nrx::HRP^{tg/+} transgene. **B**, Corresponding mean EPSC amplitudes of multiple α -neurexin KO littermate mice (SKO2, DKO2/3, DKO1/2, and TKO) with and without the α Nrx::HRP^{tg/+} transgene. All recordings were performed on newborn mice (P1) before genotyping in this and all subsequent experiments. n.s., Not significant. **C**, Cumulative histogram of miniature frequency (left) and amplitudes (right) of spontaneous inhibitory neurotransmitter release (mIPSCs) in brainstem neurons, recorded from double KO (DKO1/2) neurons with (filled symbols) and without (open symbols) an additional α Nrx::HRP^{tg/+} transgene. **D**, Corresponding mean frequency of mIPSCs in brainstem neurons recorded in the presence of TTX and CNQX from multiple α -neurexin KO littermate mice (SKO2, DKO2/3, DKO1/2, and TKO) without (open bars) and with (filled bars) the α Nrx::HRP^{tg/+} transgene. In all graphs and panels, statistical significance is indicated above the bars. Data shown are means \pm SEM. Error bars represent SEM.

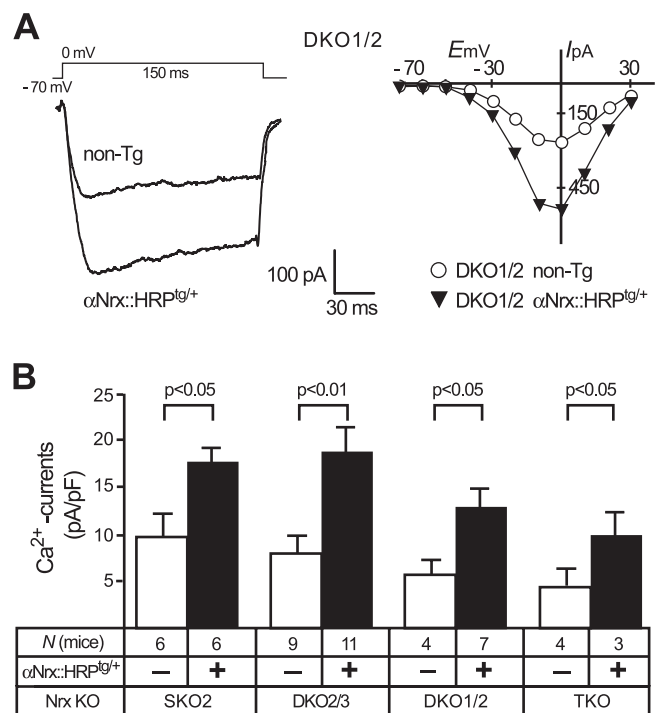


Figure 4. The α Nrx::HRP^{tg/+} transgene improves Ca²⁺ currents in all combinations of α -neurexin KO mice. **A**, Individual sample traces (left) and current–voltage relationships (right) of whole-cell Ca²⁺ current recordings from PBC neurons in DKO1/2 mice without [nontransgenic (non-Tg)] and with additional α Nrx::HRP^{tg/+} transgene. High voltage-activated Ca²⁺ currents were evoked by 150-ms-long voltage steps (10 mV each) from a holding potential of -70 mV to various test potentials ranging from -70 to $+30$ mV. **B**, The mean current densities show quantification of maximal depolarization steps to 0 mV (as shown in **A**, left). Whole-cell Ca²⁺ current increases significantly in neurons of all combinations of α -neurexin KO mice when an α Nrx::HRP^{tg/+} transgene is present (filled bars). Data shown are means \pm SEM. Statistical significance is indicated above the bars. Error bars represent SEM.

were observed (Fig. 5B). To test whether β Nrx::HRP had a detectable influence on spontaneous release at inhibitory synapses, we measured inhibitory mini events in PBC neurons with and without a β Nrx::HRP^{tg/+} transgene. However, mini frequencies were unchanged between all KO mice with or without the β Nrx::HRP^{tg/+} transgene present (Fig. 5C). In addition, transgenically expressed β -neurexin caused no alterations of postsynaptic receptor function, as evidenced by unchanged mini amplitudes in all KO combinations tested (SKO, 57.3 ± 5.98 pA, $n = 7$; SKO and β Nrx::HRP^{tg/+}, 55.6 ± 11.1 pA, $n = 6$; DKO2/3, 52.1 ± 10.9 pA, $n = 4$; DKO2/3 and β Nrx::HRP^{tg/+}, 49.7 ± 8.8 pA, $n = 4$; DKO1/2, 48.1 ± 6.9 , $n = 7$; DKO1/2 and β Nrx::HRP^{tg/+}, 45.9 ± 4.2 pA, $n = 6$; TKO, 47.2 ± 12.9 pA, $n = 3$; TKO and β Nrx::HRP^{tg/+}, 48.3 ± 11.2 pA, $n = 4$). Finally, to exclude the possibility that a potential rescue effect by the extracellularly short β -neurexin was restricted to increasing the VDCC activity without significantly improving presynaptic function, we also measured whole-cell Ca²⁺ currents (Fig. 5D,E). No difference in traces or current–voltage curves, however, was apparent from littermate mice with or without the β Nrx::HRP^{tg/+} transgene (Fig. 5D), and the respective current densities were unchanged (Fig. 5E). The experiments with β Nrx::HRP established that extracellular sequences of α -neurexins are required for their role in neurotransmission. It should be noted, however, that the failure of β Nrx::HRP to improve any of the KO parameters does not exclude an important function of the C-terminal domains of neurexins but demonstrates that they are not sufficient to regulate Ca²⁺ channels.

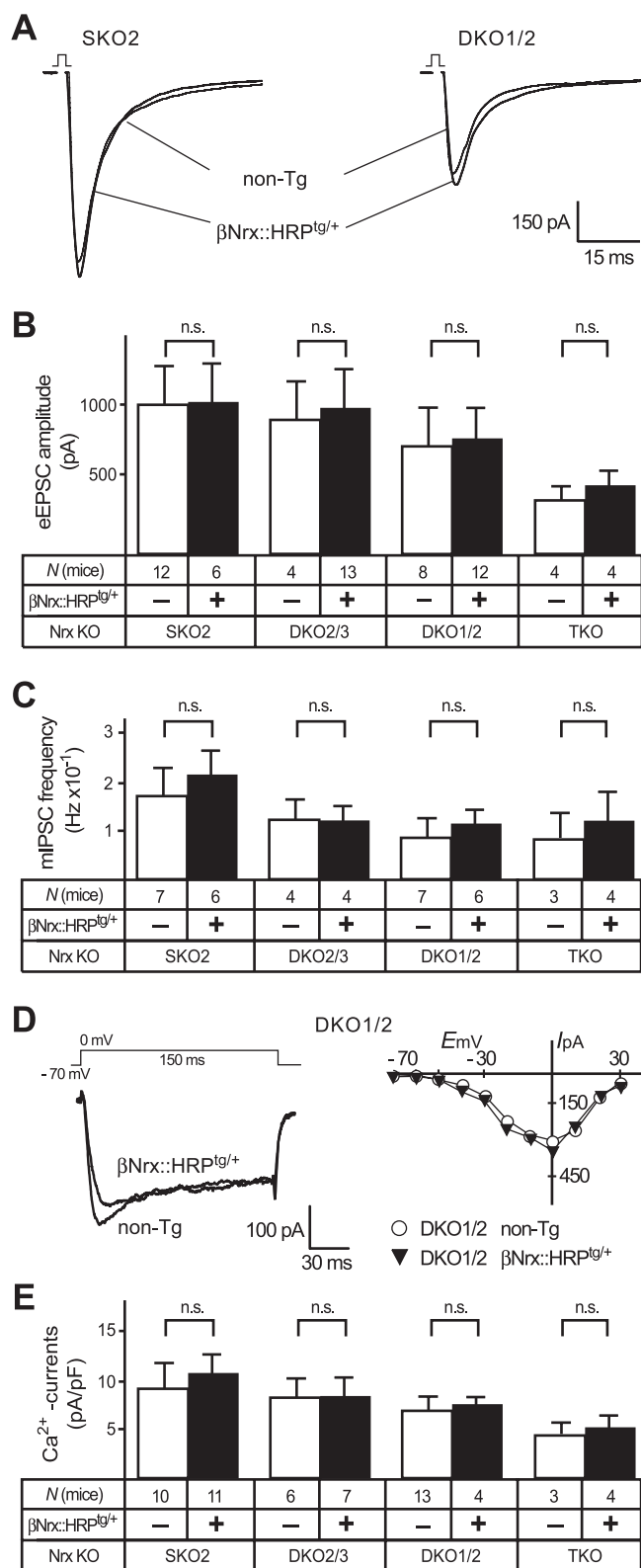


Figure 5. Transgenically expressed β Nrx::HRP is not sufficient to improve synaptic function and Ca²⁺ currents. Experiments similar to those described in Figures 3 and 4, with the exception that a different neurexin variant (β -neurexin) with distinct extracellular but identical C-terminal sequences was transgenically expressed and tested for its rescue potential (β Nrx::HRP; see Fig. 1A). **A–E**, Evoked EPSC amplitudes (**A**, **B**), spontaneous IPSC frequencies (**C**), and high voltage-activated Ca²⁺ currents (**D**, **E**) are unchanged in KO mice (SKO2, DKO2/3, DKO1/2, and TKO) without [nontransgenic (non-Tg); open bars] and with (filled bars) the β Nrx::HRP^{tg/+} transgene present. In all graphs and panels, statistical significance is indicated above the bars. Data shown are means \pm SEM. Error bars represent SEM.

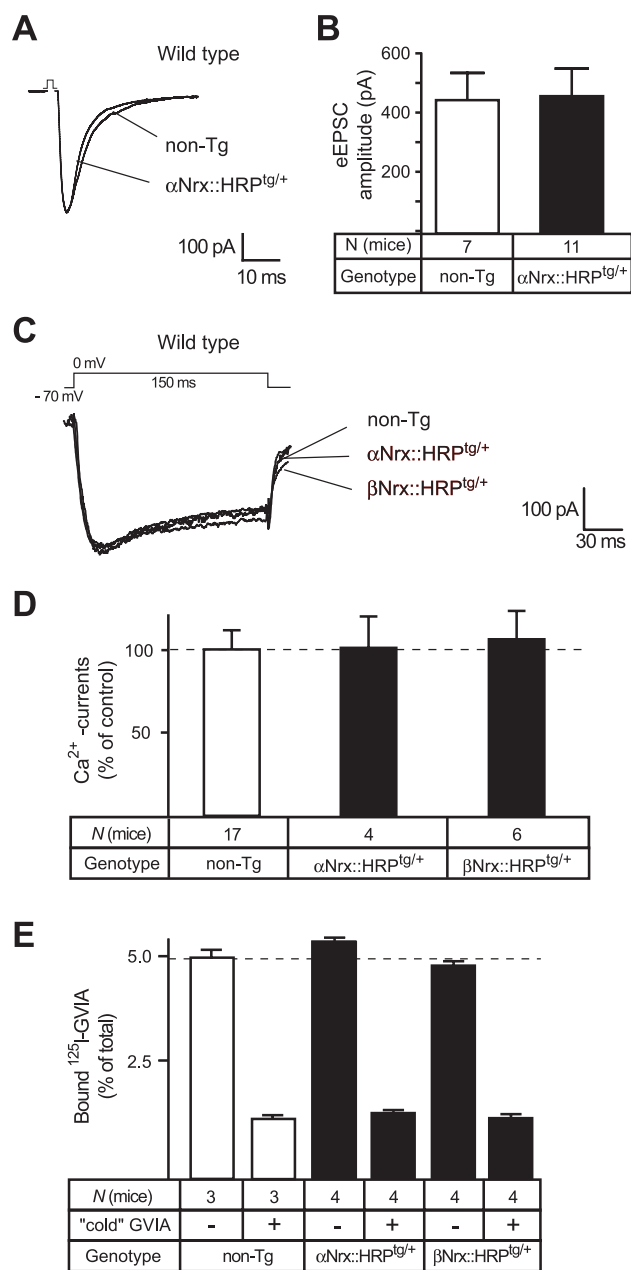


Figure 6. Transgenic α - and β -neurexins do not induce a general gain-of-function in wild-type neurons. **A**, **B**, Sample traces (**A**) and averaged evoked excitatory postsynaptic responses (**B**) from wild-type neurons (carrying no neurexin deletions) without [nontransgenic (non-Tg); open bar] and with (filled bar) an additional α Nrx::HRP^{tg/+} transgene, exhibiting no effect of transgenically expressed α -neurexin on wild-type levels of synaptic transmission. **C**, Representative traces of high voltage-activated Ca²⁺ currents from wild-type neurons without (non-Tg) and with an additional α Nrx::HRP^{tg/+} or β Nrx::HRP^{tg/+} transgene. The depolarization protocol was identical to the one used in Figures 4A and 5D. **D**, Quantitative comparison of current densities in littermate wild-type mice without (non-Tg, 100%) and with an additional α Nrx::HRP^{tg/+} or β Nrx::HRP^{tg/+} transgene, indicating that the expression of neurexin transgenes did not increase Ca²⁺ currents beyond wild-type levels. Data shown are means \pm SEM. **E**, Binding of ¹²⁵I-labeled ω -conotoxin GVIA at 30 pM to brain membranes from wild-type control (non-Tg) and transgenic mice expressing α Nrx::HRP^{tg/+} or β Nrx::HRP^{tg/+}. All experiments were done with and without a 100-fold excess of unlabeled ("cold") toxin to account for unspecific binding. Data shown are means \pm SEM. Error bars represent SEM.

The robust rescue mediated by transgenic α Nrx::HRP raises the question of whether the α Nrx::HRP acts as a general enhancer of synaptic transmission that would also increase neurotransmitter release within a wild-type genetic background. We therefore

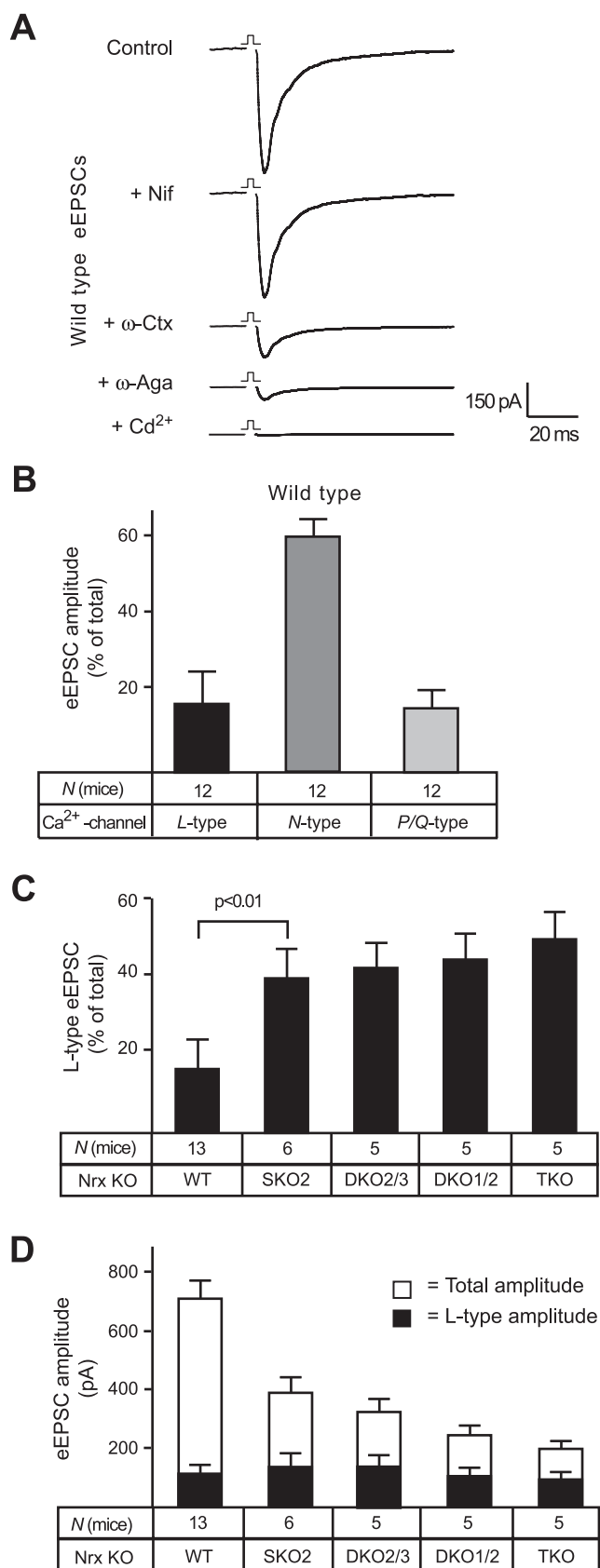


Figure 7. L-type Ca²⁺-channel-mediated EPSC components are unaffected by α -neurexins. **A**, Averaged traces of glutamatergic eEPSCs monitored in wild-type hypoglossal motor neurons before (control) and after sequential application of 10 μ M nifedipine (Nif) for L-type channel contributions, 0.5 μ M ω -conotoxin MVIIA (ω -Ctx) for N-type channel contributions, 0.2 μ M agatoxin IVA (ω -Aga) for P/Q-type channel contributions, and 100 μ M Cd²⁺

investigated whether transgenic neurexins potentiate evoked postsynaptic responses or Ca²⁺-channel function even in wild-type neurons that express endogenous α -neurexins normally. For this purpose, we measured eEPSCs in neurons from wild-type littermate mice that carried or lacked a transgenic α Nrx::HRP but found no differences in release (Fig. 6A,B). We also tested whether the normal Ca²⁺-channel activity could be facilitated (α Nrx::HRP) or inhibited (β Nrx::HRP) by recording high voltage-activated Ca²⁺ currents in neurons from wild-type littermate mice that carried or lacked an α Nrx::HRP^{tg/+} or β Nrx::HRP^{tg/+} transgene (Fig. 6C,D). However, current traces and quantitations revealed no changes (Fig. 6C,D), consistent with observations that expression levels of VDCC pore-forming subunits were neither reduced in α -neurexin KO mice (Missler et al., 2003) nor increased in the transgenic mice shown here (Fig. 6E). Thus, expression of additional transgenic neurexins by itself does not produce a gain-of-function or a dominant-negative phenotype.

α -Neurexins specifically affect non-L-type Ca²⁺ channels

Although transgenic neurexin 1 α increased synaptic transmission in all KO combinations, evoked postsynaptic responses are not completely abolished in KO neurons, even in neurons lacking all three α -neurexins (Figs. 3B, 5B). The residual synaptic transmission that is independent of α -neurexins could be explained by at least two hypotheses: that α -neurexins only partially modulate the activity of all VDCCs or that α -neurexins modulate only specific subtypes of VDCCs. To test which Ca²⁺ channels are affected by α -neurexins, we first analyzed the contribution of different VDCC subtypes to evoked neurotransmitter release in hypoglossal motor neurons from wild-type mice by sequentially blocking specific channels pharmacologically (Fig. 7A,B). Addition of nifedipine, ω -conotoxin, and agatoxin (blockers of L-, N-, and P/Q-type Ca²⁺ channels, respectively) reduced the EPSC to almost zero (Fig. 5A). The small residual EPSC after these additions was blocked by Cd²⁺, suggesting that it most likely was mediated by R-type Ca²⁺ channels (Fig. 5A). Therefore, we focused our quantitative analysis on L-, N-, and P/Q-type VDCCs that appear to be dominant at this synapse.

The amplitude of EPSCs in wild-type neurons depended >60% on an N-type Ca²⁺ channel-dependent component, supplemented by smaller but approximately equal contributions from L-type and P/Q-type Ca²⁺ channels (Fig. 7B). The contributions of these three VDCC subtypes to neurotransmission make the neonatal brainstem particularly suitable to study regulatory preferences. Control experiments in wild-type neurons using different mixtures of blockers instead of adding them sequentially resulted in a similar contribution of Ca²⁺-channel subtypes (data not shown). Because the participation of L-type channels (>15% of the total amplitude) (Fig. 7B) reported here is larger

to probe for residual Ca²⁺-channel activities (presumably mediated by R-type). **B**, Relative contributions of different VDCC subtypes to total EPSC amplitudes calculated from blocking experiments, as shown in **A**. The evoked synaptic response in the neonatal brainstem preparation depends mostly on N-type Ca²⁺ channels but also has significant contributions from L- and P/Q-type VDCCs. **C**, Comparison of L-type-mediated EPSC components in wild-type (WT) and multiple α -neurexin KO mice, uncovering a significantly increased proportion of the L-type VDCC component in mutant neurons, which is already evident in single KO (SKO2) mutant mice. **D**, Absolute currents of evoked postsynaptic responses (EPSCs; in picoamperes) during field stimulations of hypoglossal neurons. Total (open bars) and L-type-mediated (filled bars) amplitudes were analyzed in wild-type controls and in multiple α -neurexin KO mice. Statistical significance is indicated above the bars. Data shown are means \pm SEM. Error bars represent SEM.

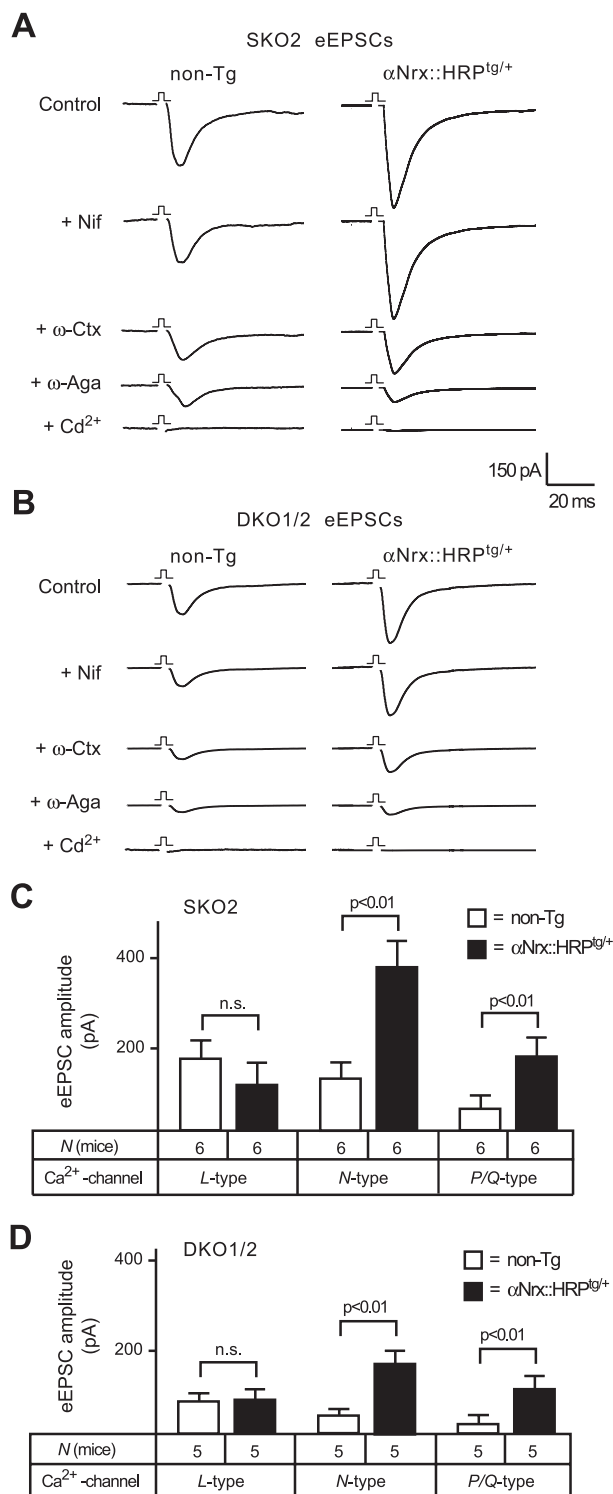


Figure 8. Transgenic α -neurexin increases evoked responses by selectively activating N- and P/Q-type Ca²⁺ channels. **A, B**, Averaged traces representing glutamatergic EPSCs from SKO2 and DKO1/2 mice without [nontransgenic (non-Tg)] and with additional α Nrx::HRP^{tg/+} transgene. Evoked responses were recorded before (control) and after sequential application of specific VDCC blockers (same protocol as in Fig. 7A). Nif, Nifedipine; ω -Ctx, ω -conotoxin; ω -Aga, agatoxin. **C, D**, Quantitative comparison of the contributions from different VDCC subtypes to EPSCs in single KO and double KO mice without (open bars) and with (filled bars) additional α Nrx::HRP^{tg/+} transgene shows an increased amplitude mediated by N- and P/Q-type calcium channels when a transgenic α -neurexin is present. The proportion of L-type-dependent current components is unchanged in rescued neurons. n.s., Not significant. In all graphs and panels, statistical significance is indicated above the bars. Data shown are means \pm SEM. Error bars represent SEM.

than the L-type-dependent component observed in other central synapses (Wu et al., 1999), we tested how this L-type dependence was affected in α -neurexin KO neurons. Analyzing the relative proportion of L-type-mediated EPSCs to the total postsynaptic response revealed that the L-type component increases from \sim 15% in wild-type neurons to $>$ 50% in TKO neurons (Fig. 7C). In contrast to most other parameters studied thus far in α -neurexin KO mice (survival rates, ventilation activity, evoked and spontaneous release, and Ca²⁺ currents) (Missler et al., 2003), the relative L-type contribution increases dramatically in SKO2 mice (Fig. 7C).

The augmented L-type dependence of EPSCs in KO neurons could reflect a true compensation caused by upregulated activity of L-type Ca²⁺ channels or merely constitute a relative increase caused by the dramatically reduced total EPSC. Evaluation of absolute amplitudes demonstrated that the L-type-mediated EPSC amplitudes did not differ significantly between α -neurexin genotypes (Fig. 7D, filled bars). This result suggests that the activity of L-type VDCCs is not dependent on α -neurexins, indicating that the impairment of N- and P/Q-type Ca²⁺ channels is primarily responsible for the phenotype.

To obtain direct evidence for a positive regulation of non-L-type Ca²⁺ channels by α -neurexins, we applied the sequential blocking protocol described above to two different α -neurexin KO combinations (SKO2 and DKO1/2) with and without the α Nrx::HRP^{tg/+} transgene (Fig. 8A,B). The presence of α Nrx::HRP^{tg/+} increased the contributions of N- and P/Q-type VDCCs to the total EPSC twofold to threefold (Fig. 8C,D). Consistent with the unchanged L-type-mediated EPSC in KO neurons (Fig. 7D), expression of an additional α Nrx::HRP^{tg/+} transgene did not change the size of L-type-dependent amplitudes in rescued neurons (Fig. 8C,D).

Our finding of a specific regulation of N- and P/Q-type VDCC activity by α -neurexins documents the molecular specificity of the neurexin–Ca²⁺-channel link. To further validate this result, we performed experiments that tested the effect of Ca²⁺-channel inhibitors on the frequency of mIPSCs. Recording mini frequencies in PBC neurons to study Ca²⁺-channel activity is meaningful, because in these neurons approximately one-half of the mini events depend on VDCCs and can be blocked by Cd²⁺ (45–55%) (Missler et al., 2003) (data not shown). We first recorded minis mediated by different VDCC subtypes in wild-type neurons (Fig. 9A) after a sequential blocking protocol described above (Figs. 7, 8). Consistent with the data on evoked responses, we found that N-type Ca²⁺ channels mediated most of the Ca²⁺ channel-dependent minis ($>$ 60%). However, the P/Q-type-dependent component of minis was slightly higher (26%) than the L-type-dependent component (6%) (Fig. 9B). Despite the small contribution of L-type VDCCs to wild-type minis, their relative proportion increased greatly in the different α -neurexin KO mice, reaching proportions similar to the EPSC data shown above (Fig. 9C). Corresponding to the EPSC analysis, however, this apparent compensation was not attributable to an actual increase in L-type-dependent minis, because the overall mini frequency is reduced in KO neurons (Missler et al., 2003) (Figs. 3D, 5C), supporting the notion that the function of L-type VDCCs is mostly unaffected by the absence of α -neurexins. We then determined how the VDCC subtype contributions to mini frequencies changed when an α Nrx::HRP^{tg/+} transgene was crossed into the same α -neurexin KO combinations used above (SKO2 and DKO1/2) (Fig. 10A,B). Consistent with the results from evoked release, the presence of the α Nrx::HRP^{tg/+} transgene signifi-

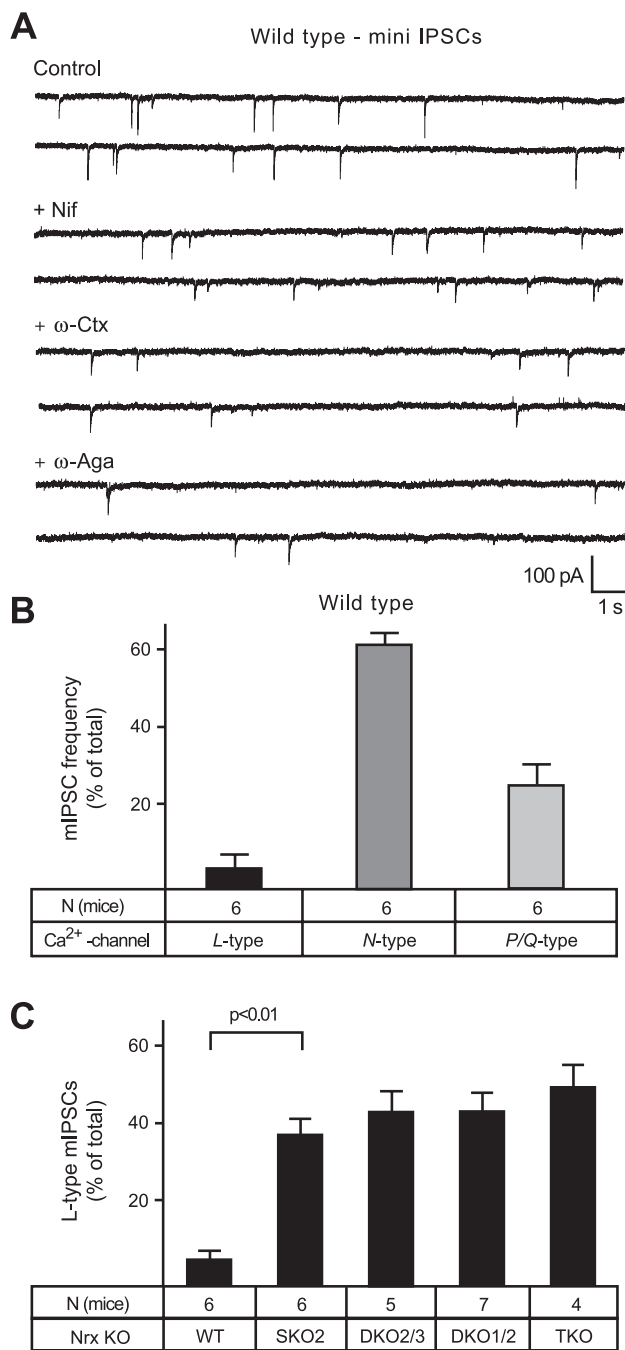


Figure 9. Ca²⁺-dependent minis at inhibitory synapses of wild-type and α -neurexin KO mice. **A**, Representative traces of spontaneous release events (mIPSCs) recorded under TTX and CNQX from neonatal PBC neurons of wild-type mice before (control) and after the sequential blocking of Ca²⁺ channels, as described in Figures 7 and 8 for evoked release. Nif, Nifedipine; ω -Ctx, ω -conotoxin; ω -Aga, agatoxin. **B**, Dependence of wild-type mIPSCs on different VDCC subtypes is expressed as a percentage of the respective Ca²⁺-dependent mini frequency, quantitated from blocking experiments, as shown in **A**. In wild-type neurons of this brainstem area, approximately one-half of all minis depend on Ca²⁺ influx (data not shown). **C**, In knock-out mice lacking one, two, or all three α -neurexins (SKO2, DKO2/3, DKO1/2, and TKO), the proportion of L-type-mediated minis appears significantly larger because of the strongly reduced overall mIPSC frequencies (compare with Figs. 3D and 5C). WT, Wild type. Statistical significance is indicated above the bars. Data shown are means \pm SEM. Error bars represent SEM.

cantly increased the N- and P/Q-type-mediated mini frequencies, whereas L-type-dependent mIPSC events were unchanged (Fig. 10C,D), indicating that L-type channels are also excluded from α -neurexin regulation at inhibitory synapses.

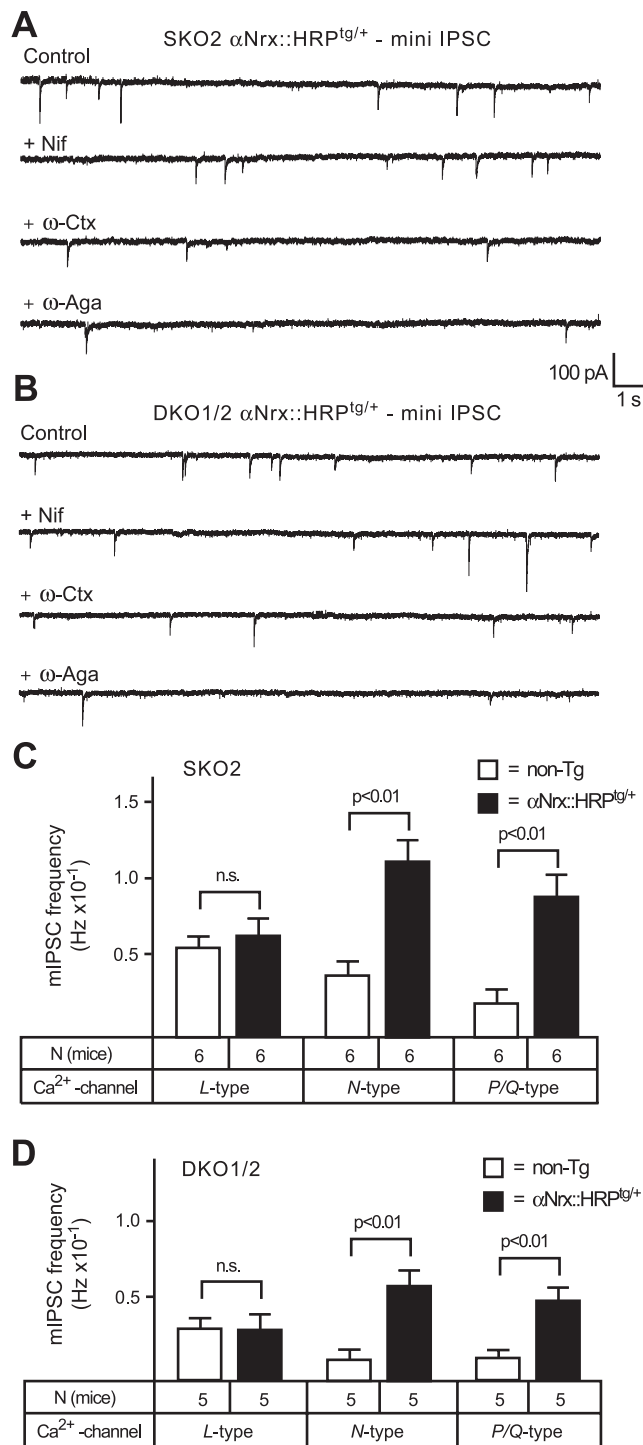


Figure 10. Transgenically expressed α -neurexin specifically increases N- and P/Q-type-mediated mini frequencies. **A, B**, Sample traces of mini events (mIPSCs) recorded under TTX and CNQX from SKO2 and DKO1/2 mice, both with an additional α Nrx::HRP^{tg/+} transgene present. Spontaneous postsynaptic responses were recorded before (control) and after sequential application of Ca²⁺-channel inhibitors (same as in Fig. 9A). To show representative changes in mini events after drug treatment, recordings with above-average frequencies were chosen for all conditions (compare with absolute frequencies in **C, D**). Nif, Nifedipine; ω -Ctx, ω -conotoxin; ω -Aga, agatoxin; n.s., not significant. **C, D**, Because transgenic α -neurexin rescued mini frequencies in all α -neurexin KO combinations studied (Fig. 3D), the contributions of different VDCC subtypes were compared in single KO (**C**) and double KO (**D**) mice without [nontransgenic (non-Tg); open bars] and with (filled bars) additional α Nrx::HRP^{tg/+} transgene. Increased mini frequencies reveal an augmented activity of N- and P/Q-type VDCCs but not of L-type channels. In all graphs and panels, statistical significance is indicated above the bars. Data shown are means \pm SEM. Error bars represent SEM.

Discussion

Neurexins are among several neuronal cell-adhesion molecules that were proposed to contribute to synaptic neurotransmitter release and/or synapse formation (for review, see Sudhof, 2001; Missler, 2003; Scheiffele, 2003). For many of these candidate cell-adhesion molecules, studies in knock-out mice have produced illuminative phenotypes, although their suggested functions are often only indirectly related to synapses (Tomasiewicz et al., 1993; Cremer et al., 1994; Evers et al., 2002; Togashi et al., 2002; Wang et al., 2002). The α -neurexin KO phenotype, in contrast, strongly points toward a specifically synaptic function, because their deletion severely impairs both excitatory and inhibitory neurotransmitter release, presumably as a result of impaired Ca^{2+} -channel function, but does not affect brain architecture and synaptic ultrastructure (Missler et al., 2003). Despite the present inavailability of β -neurexin knock-out mice, recent *in vitro* work on the β -neurexin/neurologin complex has suggested that it may have a role in promoting synapse formation and differentiation (Scheiffele et al., 2000; Dean et al., 2003; Graf et al., 2004).

We have now directly compared the putative roles of α - and β -neurexins and investigated the molecular specificity of the neurexin- Ca^{2+} -channel regulation using a comparative rescue strategy in transgenic mice. Such a strategy is rarely used because of the amount of work involved, although a similar approach has been followed, for example, to analyze the functional redundancy of the closely related growth cone proteins GAP43 and cytoskeleton-associated protein 23 (Frey et al., 2000). For questions relating to the normal regulation of Ca^{2+} channels by a cell-adhesion molecule in an intact brain, a transgenic strategy may be advantageous, because cell-adhesion events are best studied in acute slices of an intact neuronal network. The brainstem system was chosen because multiple α -neurexin KO mutants die early postnatally as a result of respiratory problems arising from brainstem defects, and data from the rescue analysis could be tested for consistency compared with the previous KO study. Furthermore, the background-free detection of transgenic neurexin::HRP proteins allows an unbiased estimation of expression levels and distribution patterns. In addition, choosing transgenic lines that express neurexin::HRP fusion proteins within the range of endogenous neurexins reduces the problems of dominant-negative effects and high variability, frequently associated, for example, with overexpression approaches *in vitro*. Our current study extends the previous analysis of neurexins by reporting three novel findings.

First, transgenic neurexin 1 α , but not transgenic neurexin 1 β , partly rescued the well characterized properties of the α -neurexin KO phenotype. Because transgenic neurexin 1 α and 1 β differ solely in their extracellular domains, but contain identical transmembrane regions and cytoplasmic sequences, we conclude that α - and β -neurexins have nonredundant functions that involve their distinct N-terminal domains. However, if the extracellular sequences of α -neurexin define their role as organizer molecules of presynaptic release, what then is the respective role of β -neurexins? The work by the Scheiffele and Craig laboratories suggests, as originally proposed when neurexins were first identified (Ushkaryov et al., 1992), that neurexins can promote the formation of *de novo* synapses and differentiation of postsynaptic receptors (Scheiffele et al., 2000; Dean et al., 2003; Graf et al., 2004). Because these reports were confined to the *in vitro* activities of β -neurexins, whereas we study the effects of transgenes *in vivo*, it is at present difficult to compare their results with ours.

However, we did not detect an effect of transgenic expression of neurexin 1 β on mini amplitudes or evoked responses (as would be expected if postsynaptic receptors were recruited or activated) and did not find that transgenic neurexin 1 β augmented mini frequencies (as would be expected from a synaptogenetic activity). A possible interpretation of this discrepancy could be that the synapse formation and differentiation observed by overexpression in cultures uncovers an additional (rather than essential) functional activity of neurexins that is redundant with other molecules and thus is not present in intact tissue. Alternatively, it is possible that a synaptogenetic function for neurexins is developmentally compensated, although this raises the question of why the essential function for α -neurexins in executing neurotransmitter release, as documented here and previously (Missler et al., 2003), is not compensated.

Second, the neurexin 1 α transgene ameliorated all facets of the α -neurexin KO phenotype, independent of which neurexin genes were mutated, demonstrating that the various α -neurexins do not exhibit fundamental functional differences. The fact that a single transgene with only one set of splice variants was active in reversing the various phenotypes suggests that α -neurexins mediate a single integrated activity. The increase in neurotransmitter release and the regulation of VDCCs mediated by α -neurexin were not simple gain-of-function activities, because the transgenic proteins did not further facilitate their activity in wild-type mice. This result suggests that neurexins may regulate VDCC activity only within their normal range but not beyond.

Third, our data now show that deletion of α -neurexins selectively impairs neurotransmitter release that is dependent on non-L-type Ca^{2+} channels. Other classes of extracellular molecules such as the matrix component tenascin-C may modulate L-type-dependent synaptic plasticity (Evers et al., 2002), but L-type-mediated EPSC amplitudes and mini frequencies in brainstem synapses were unaffected by α -neurexins, as shown here. The fact that a preference of α -neurexins for N- and P/Q-type Ca^{2+} channels exists is interesting, because the VDCCs involved in presynaptic Ca^{2+} entry are usually of the N- and P/Q-type (Dunlap et al., 1995; Reid et al., 1998; Wu et al., 1999), but R- and L-type channels may be involved at particular synapses as well (Wu et al., 1998; Brandt et al., 2003). Although all presynaptic terminals contain VDCCs, the release probability and Ca^{2+} dynamics of presynaptic terminals vary significantly (Hessler et al., 1993; Rosenmund et al., 1993; Koester and Sakmann, 2000), even at synaptic terminals of the same neuron (Maccaferri et al., 1998; Markram et al., 1998; Reyes et al., 1998; Scanziani et al., 1998). Because of the high Ca^{2+} cooperativity and Ca^{2+} sensitivity of neurotransmitter release (Bollmann et al., 2000; Schneggenburger and Neher, 2000), regulating VDCCs efficiently controls synaptic strength, and small changes in Ca^{2+} influx lead to magnified effects on synaptic release (Felmy et al., 2003). It comes as no surprise, therefore, that VDCCs are extensively regulated. In addition to the intracellular free calcium concentration itself, several helix-loop-helix Ca^{2+} -binding motifs containing proteins called EF-hand proteins (e.g., calmodulin, normal calf serum, calcium-binding protein) (for review, see Weiss and Burgoyne, 2002), G-protein-coupled receptor pathways (such as GABA_B or 5-HT) (for review, see Catterall, 2000; Zamponi, 2001), components of the exocytotic machinery (e.g., syntaxin 1 or synaptosomal-associated protein of 25 kDa) (for review, see Catterall, 1999; Atlas, 2001), or even signaling events induced by lipid bilayer components (Wu et al., 2002) facilitate or inhibit VDCCs.

Our findings on α -neurexins now put the first synaptic cell-adhesion molecule on the list of important VDCC regulators.

These results, however, raise additional questions. Why are there multiple α -neurexin genes with hundreds of splice variants if a single isoform, expressed in a single splice variant, is at least partly able to rescue different phenotypic effects produced by the deletions of multiple α -neurexin genes? What are the extracellular interactions that mediate the essential functions of α -neurexins, and how are these extracellular interactions coupled to intracellular effector pathways? What is the actual role of β -neurexins, including their splice variants that do not bind to neuroligin in a trans-synaptic complex? Addressing these questions will significantly promote our understanding of the organization of presynaptic terminals but will require new experimental approaches.

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