Presynaptic α₂δ-2 Calcium Channel Subunits Regulate Postsynaptic GABA<sub>A</sub> Receptor Abundance and Axonal Wiring

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Presynaptic α<sub>2</sub>δ subunits of voltage-gated calcium channels regulate channel abundance and are involved in glutamatergic synapse formation. However, little is known about the specific functions of the individual α<sub>2</sub>δ isoforms and their role in GABAergic synapses. Using primary neuronal cultures of embryonic mice of both sexes, we here report that presynaptic overexpression of α<sub>2</sub>δ-2 in GABAergic synapses strongly increases clustering of postsynaptic GABA<sub>A</sub>Rs. Strikingly, presynaptic α<sub>2</sub>δ-2 exerts the same effect in glutamatergic synapses, leading to a mismatched localization of GABA<sub>A</sub>Rs. This mismatching is caused by an aberrant wiring of glutamatergic presynaptic boutons with GABAergic postsynaptic positions. The trans-synaptic effect of α<sub>2</sub>δ-2 is independent of the prototypical cell-adhesion molecules α-neurexins (α-Nrxns); however, α-Nrxns together with α<sub>2</sub>δ-2 can modulate postsynaptic GABA<sub>A</sub>R abundance. Finally, exclusion of the alternatively spliced exon 23 of α<sub>2</sub>δ-2 is essential for the trans-synaptic mechanism. The novel function of α<sub>2</sub>δ-2 identified here may explain how abnormal α<sub>2</sub>δ subunit expression can cause excitatory–inhibitory imbalance often associated with neuropsychiatric disorders.

Key words: auxiliary subunits; cacna2d; cultured hippocampal neurons; imaging; immunocytochemistry; voltage-gated calcium channels

Introduction

Auxiliary α<sub>2</sub>δ subunits are traditionally envisioned as potent modulators of voltage-gated calcium channels (Felix et al., 1997; Klugbauer et al., 1999). Recently, synaptic functions of all four α<sub>2</sub>δ subunit isoforms have been suggested, which may be partly...
or entirely independent of the channel complex: αδ-1 has been identified as postsynaptic receptor for glia-secreted thrombospondins, which mediate excitatory synaptogenesis (Eroglu et al., 2009; Risher et al., 2018). In mice, loss of αδ-2 affected morphology and function of cerebellar Purkinje neurons (Barclay et al., 2001; Brodbeck et al., 2002), as well as structure and function of auditory hair cell synapses (Fell et al., 2016). Additionally, αδ-2 has been identified as a suppressor of axonal regeneration in the adult CNS (Tedeschi et al., 2016). The invertebrate homologs of αδ-3 are crucial for the presynaptic development of motoneurons (Kurshan et al., 2009; Caylor et al., 2013) and, in mice, loss of αδ-3 results in aberrant synapse formation of auditory nerve fibers (Pirone et al., 2014). Finally, αδ-4 is required for the organization of rod and cone photoreceptor synapses (Wang et al., 2017; Kerov et al., 2018). The overall importance of αδ subunits is emphasized by their involvement in various neurological disorders: CACNA2D1 and CACNA2D2, the genes encoding αδ-1 and αδ-2, have been linked to epilepsy (Choza et al., 2009; Edvardson et al., 2013; Pippucci et al., 2013; Vergult et al., 2015; Butler et al., 2018); CACNA2D3 is a potential risk gene for autism spectrum disorders (Lossilov et al., 2012; De Rubeis et al., 2014); and all three genes may be associated with schizophrenia (Purcell et al., 2014; Moons et al., 2016). Mutations in CACNA2D4 can cause retinal dysfunction in humans (Ba-Abbad et al., 2016) and a partial deletion of CACNA2D4 was identified in patients with late-onset bipolar disorder (Van Den Bossche et al., 2012).

Despite the increasing knowledge, it is still largely elusive how the individual αδ isoforms contribute to the formation and function of central synapses. The fact that αδ subunits constitute large extracellular and highly glycosylated proteins (Davies et al., 2007; Bauer et al., 2010) puts them in an ideal position for extracellular functions, as previously suggested (Fell et al., 2016; Wang et al., 2017). Indeed, a recent study provided evidence for an interaction of αδ-1 with NMDARs in dorsal horn neurons (Chen et al., 2018) and presynaptic α-neurexins (α-Nrxns) are potential candidates for mediating synaptic functions of αδ subunits (Tong et al., 2017; Brockhaus et al., 2018). However, the role of αδ subunits has until now only been addressed in excitatory glutamatergic synapses. This is surprising because αδ subunits are strongly expressed in GABAergic neurons (Cole et al., 2005; Schlick et al., 2010) and aberrant GABAergic signaling is primarily implicated in the etiology of the abovementioned neurological disorders (e.g., epilepsy; Bonansco and Fuenzalida, 2016). Finally, the brain robustly expresses αδ-1, αδ-2, and αδ-3 (Schlick et al., 2010), however, whether and how the individual isoforms contribute to specific functions in neurons simultaneously expressing all isoforms is elusive.

Therefore, to address αδ subunit-mediated synaptic functions, we analyzed the consequences of presynaptic αδ subunit expression in glutamatergic and GABAergic synapses. We show that presynaptic overexpression of αδ-2 specifically induces the formation of mismatched glutamatergic synapses by trans-synaptically recruiting postsynaptic GABA<sub>R</sub>Rs. Moreover, αδ-2 recruits postsynaptic GABA<sub>R</sub>Rs also in GABAergic synapses and thus independently of the presynaptic neurotransmitter identity. Most importantly, the mismatched synapse formation is explained by an altered wiring of glutamatergic axons to GABAergic postsynaptic positions. Interestingly, the αδ-2-induced GABA<sub>R</sub> clustering is further upregulated in glutamatergic neurons lacking all three α-Nrxns. Therefore, α-Nrxns can modulate the presynaptic effect of αδ-2, however, they are not required for the trans-synaptic role of αδ-2 in recruiting GABA<sub>R</sub>Rs. Together, our findings prove that presynaptic αδ-2 acts trans-synaptically on postsynaptic GABA<sub>R</sub>s. The fact that increased αδ-2 expression triggers aberrant axonal wiring is particularly interesting in light of neurological disorders associated with axonal wiring defects and altered excitatory–inhibitory balance (Huang and Hsueh, 2015; Lee et al., 2017).

**Materials and Methods**

**Breeding and genotyping procedures**

**Animals.** Animal procedures for WT BALB/c and αδ mutant mice were performed at the Medical University Innsbruck in compliance with government regulations and approved by the Austrian Federal Ministry of Science, Research and Economy (license numbers BMFWF-66.011/0113-WF/V/3b/2014 and BMFWF-66.011/0114-WF/V/3b/2014). Regular reports including the mouse numbers used for this project were given to the Austrian Federal Ministry of Science, Research and Economy (bmfwe). Animal experiments at the University of Münster [α-Nrxn triple knock-out (TKO) and control mice] were performed in accordance with government regulations for animal welfare and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV, NRW, Germany, license numbers 84-02.05.20.11.209 and 84-02.04.2015.A423). Mice were maintained at the central animal facilities in Innsbruck and Münster under standard housing conditions with food and water available *ad libitum* on a 12 h light/dark cycle.

**Breeding and genotyping of mutant mice.** WT and αδ-3/-/- mice used for qRT-PCR and LacZ reporter expression were obtained from breeding double heterozygous αδ-1/-/-, αδ-3/-/- mice having a mixed 129×C57BL/6 background. The αδ-1 knock-out mouse strain (αδ-1/-/-) was previously generated and characterized (Fuller-Bicer et al., 2009; Patel et al., 2013; Mastrolia et al., 2017). Genotyping for the Cacna2d1 gene was done as published previously (Fuller-Bicer et al., 2009) with some modifications by use of standard PCR conditions (annealing at 52°C for 30 s). Primers were as follows: WT-F1: 5'-GAGCTTCTCTTCCCTGATCCATGG-3', mutant-F2: 5'-CTGCCAGAGCAACTGTTAGAGC-3', R: 5'-ACCATCTCAGAAGCTGAGAGACG-3'. Expected band sizes were 346 bp for WT (αδ-1/-/-) and 635 bp for knock-out (αδ-1/-/-) animals, respectively, and heterozygous mice showed both bands. The αδ-3 knock-out mice (αδ-3/-/-) generated by Deltangen (B6.129P2-Cacna2d1tm1Denb, Neely et al., 2010) were originally purchased from The Jackson Laboratory and provided by Jutta Engel (Saarland University, Germany). Knock-out was obtained by targeted insertion of a bacterial LacZ cassette into the Cacna2d3 gene such that the endogenous promoter drives the expression of β-galactosidase. The following primers were used for detecting the WT allele (F1-R, 183 bp fragment) and the knock-out allele (F2-R, 331 bp fragment): F1: 5'-AGACGCTCATTACCACTGCACCGAGCATG-3', R: 5'-GGGGCCGCTTGGTTACTGAGCACTGGA-3'. Expected band sizes were 364 bp for WT (αδ-3/-/-) and 396 bp for knock-out (αδ-3/-/-) mice used by standard PCR conditions (annealing at 35°C for 30 s). α-Nrnx TKO mice were generated as described previously (Missler et al., 2003; Dudanova et al., 2007). Genotyping of α-Nrnx-deficient mice was performed at the same time of neuronal preparation and as described previously (Missler et al., 2003).

**Cell culture and transfection procedures**

**Lentiviral production.** Lentiviruses were produced by transient transfection of confluent tsa201 cells with the lentiviral expression vectors containing pHR-βA-eGFP, pHr-βA-mcherry, or pHr-βA-eGFP αδ-2 in combination with pSAX2 (packaging plasmid) and the pVSV (envelope plasmid) using Metatfectene (Biontex Laboratories). The following day, medium was changed to neuronal plating medium (NPM; consisting of MEM, 10% horse serum, 0.2% glucose, and 1 mM sodium pyruvate) and, after 24 and 48 h, supernatants containing the viruses were harvested, sterile filtered (0.20 μm), aliquoted, and stored at −20°C. Cultured hippocampal and striatal neurons were infected immediately after plating with the lentiviral medium supernatant diluted 1:4 in NPM and incubated for 4 h in a humidified incubator (95% air and 5% CO<sub>2</sub>) at 37°C. More details for infection procedures are given in the individual sections below.

**Primary cultures of hippocampal neurons for fluorescence imaging.** Low-density cultures of hippocampal neurons were obtained from 16.5- to 18-d-old embryonic BALB/c mice of either sex as described previously.
In brief, hippocampi were dissected in cold Hank’s balanced salt solution (HBSS) following dissociation by 2.5% trypsin-EDTA treatment and trituration. Dissociated neurons were plated at a density of ~3500 or 7000 cells/cm² on 18 mm glass coverslips (#1.5; GFL) coated with poly-L-lysine (Sigma-Aldrich) in 60 mm culture dishes. After allowing the neurons to attach for 3–4 h, coverslips were transferred neuron-side down into a 60 mm culture dish containing a glial feeder layer. Maintenance of neurons and glia was done in serum-free neurobasal medium supplemented with Glutamax and B-27 (NBKO, all ingredients from Thermo Fisher Scientific). Three days after plating, Ara-C (5 μM) was added to stop glial proliferation and 1/3 of the medium was replaced weekly with fresh maintenance medium. Plasmids were introduced into neurons at 6 days in vitro (DIV) with Lipofectamine 2000-mediated transfection (Thermo Fisher Scientific) as described previously (Obermair et al., 2004). For cotransfection of pβA-eGFP plus pβA-αδ or mCherry plus pβA-αδ, 1.5 μg of total DNA was used at a molar ratio of 0.7:1. Control neurons from the same culture preparations were transfected with 1–2 μg of pβA-eGFP or mCherry, respectively. Labeling of cells for super-resolution gated stimulated emission depletion (gSTED) microscopy (see Fig. 1A–C) was done by introducing mCherry or mCherry plus αδ-2 at 6 DIV and pβA-eGFP at 7 DIV with lipofection. Cells were processed for immunostaining experiments between 20 and 30 DIV.

For α-Nrx-TKO, WT and mutant mice of either sex were used for neuronal cultures derived from timed pregnant dams at embryonic day 17 (E17). Dissociated primary neurons were prepared in HBSS from single hippocampi as described previously (Neupert et al., 2015). Briefly, cell suspensions obtained after 0.25% trypsin treatment and trituration were plated onto 18 mm glass coverslips (Menzel-Glasco) coated with poly-L-lysine (Sigma-Aldrich) at a density of 55,000 cells/coverslip. After 4 h at 37°C in plating medium (MEM, 10% horse serum, 0.6% glucose, and 1 mM sodium pyruvate), coverslips were inverted onto a 70–80% confluent monolayer of astrocytes grown in 12-well plates (Falcon) and incubated in Neurobasal medium supplemented with B27, 0.5 mM glutamine, and 12.5 μM glutamate. 3 d, media were refreshed with Neurobasal medium supplemented with B27, 0.5 mM glutamine, and 5 μM AraC. Cultures were maintained at 37°C in a humidified incubator with an atmosphere of 95% air and 5% CO₂. Neurons were transfected at 14 DIV using lipofectamine (Thermo Fisher Scientific) and experiments were performed between 20 and 25 DIV.

Primary cocultures of WT cortical and striatal neurons. Because the inclusion of glutamatergic neurons is required for the proper development of GAβAergic medium spiny neurons (MSNs) in culture (Segal et al., 2003), we modified the protocol for hippocampal neurons and adapted the striatal–cortical coculture published previously (Penrod et al., 2011). Cocultures of cortical and striatal MSNs were prepared in Neurobasal medium supplemented with 4000 or 7000 cells/cm², respectively, and infected with a lentiviral pHR-A-eGFP construct and activated using the Lipofectamine 2000-mediated transfection protocol (Thermo Fisher Scientific). Total mRNA amounts and molar ratios were the same as described above for hippocampal neurons. Infection of striatal neurons was stopped by replacing the virus-plating solution with 4 ml of fresh, prewarmed NPM. The cell suspension containing the cortical neurons and transfection solution was centrifuged for 5 min at 1000 rpm at 4°C. Finally, cortical neurons were resuspended in 1 ml of NBKO, seeded onto striatal neurons, and allowed to attach for 3–4 h before coverslips were transferred neuron-side down into a 60 mm culture dish containing a glial feeder layer. Ara-C treatment and maintenance of neurons and glia were as described above. Cells were processed for immunostaining and patch-clamp experiments at 21–27 and 13–14 DIV, respectively.

Neuronal cultures for paired recordings. Culture protocols were modified to enable electrophysiological recordings from pairs of isolated neurons. Poly-L-lysine solution (Sigma-Aldrich) was sprayed on glass coverslips, producing uniformly distributed single dots of neuronal substrate. Hippocampal neurons were seeded at a density of 880 cells/cm² and infected with pHβ-αδ-eGFP αδ-2. Cells were processed for electrophysiological experiments at 13–18 DIV.

Molecular biology

**TaqMan qRT-PCR gene expression analysis.** For expression analysis of αδ subunits (αδ-1 to αδ-4), RNA was isolated from 3 individual preparations of mono-cultured MSNs and adult male mouse striatum (7–8 weeks) as described previously (Schlick et al., 2010). Briefly, striatal neurons were prepared from 16.5- to 18-old embryonic BALB/c mice of either sex and plated on poly-L-lysine-coated glass coverslips in four 60 mm culture dishes at a density of 10,500 cells/cm². After 24–25 d in culture, neurons were harvested for subsequent RNA extraction by tripsin treatment and homogenized with Qiachredder columns (Qiagen). For RNA isolation from brain tissue, WT mice obtained from double-heterozygous αδ-1/−, αδ-3/− breedings were killed by CO₂ exposure, decapitated, and brains were dissected in cold HBSS. After separating the cerebral hemispheres and stripping away the meninges, hemispheres were placed medial surface up, showing the hippocampus as a clearly visible structure. A region of the prefrontal cortex was dissected, minced, and transferred to a 15 ml Falcon tube containing HBSS (see Fig. 7A). The remaining cortical tissue was peeled along the line of the hippocampus to reveal the striatum, which was scooped out using small curved scissors and transferred to a separate tube. Striatal and cortical tissue of at least 2 hemispheres was separately collected and dissociated by 2.5% trypsin-EDTA treatment and trituration as described for hippocampal neurons (see above). Plasmid DNA–lipid complexes were prepared according to the Lipofectamine 2000-mediated transfection protocol (Thermo Fisher Scientific) and ~2.4 × 10⁷ striatal neurons were transfected for 20 min in a 37°C water bath keeping the total volume to 1 ml with NBKO. Subsequently, the cell suspension was directly seeded on poly-L-lysine-coated glass coverslips within a 60 mm culture dish containing 4 ml of prewarmed NPM and striatal neurons were allowed to attach at 37°C. For the entire transfection procedure, dissociated cortical neurons were maintained in HBSS 18–15 ml tube at 37°C and occasionally swirled. After 2 h, transfection of striatal neurons was stopped by replacing the transfection-plating solution with 5 ml of fresh, prewarmed NPM and untransfected cortical neurons were seeded onto striatal neurons in a ratio of 2 (cortical neurons) to 3 (MSNs) at a total density of ~14,000 cells/cm². For the immunostaining experiments and electrophysiological recordings shown in Figure 7, striatal neurons were plated at a density of 4000 or 7000 cells/cm², respectively, and infected with a lentiviral pHβ-αδ-eGFP construct. Following attachment of MSNs and viral infection for 4 h, untransfected cortical neurons were plated at a density of 2800 cells/cm². Cortical cells were allowed to attach for 3–4 h and coverslips were transferred neuron-side down into a 60 mm culture dish containing a glial feeder layer. In cocultures for superresolution gSTED microscopy (see Fig. 11E), striatal and cortical neurons were labeled with eGFP and mCherry, respectively. For this purpose, striatal neurons were plated at a density of 7000 cells/cm² on poly-L-lysine-coated glass coverslips in a 60 mm culture dish containing 4 ml of prewarmed NPM. MSNs were virally infected using a lentiviral pHβ-αδ-eGFP construct and allowed to attach for 3–4 h. In the meantime, ~2.0 × 10⁵ cortical neurons were transfected at 37°C in a water bath, keeping the total volume to 1 ml with NBKO. To this end, mCherry or mCherry plus αδ-2 were introduced using the Lipofectamine 2000-mediated transfection protocol (Thermo Fisher Scientific). Total DNA amounts and molar ratios were the same as described above for hippocampal neurons. Infection of striatal neurons was stopped by replacing the virus-plating solution with 4 ml of fresh, prewarmed NPM. The cell suspension containing the cortical neurons and transfection solution was centrifuged for 5 min at 1000 rpm at 4°C. Finally, cortical neurons were resuspended in 1 ml of NBKO, seeded onto striatal neurons, and allowed to attach for 3–4 h before coverslips were transferred neuron-side down into a 60 mm culture dish containing a glial feeder layer. Ara-C treatment and maintenance of neurons and glia were as described above. Cells were processed for immunostaining and patch-clamp experiments at 21–27 and 13–14 DIV, respectively.

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To compare the relative expression of distinct αδ subunits in different preparations, data were normalized to the preparation with the highest Hpr1 expression.

Expression vectors and cloning procedures. To facilitate neuronal expression, all constructs were cloned into a eukaryotic expression plasmid containing a neuronal chicken β-actin promoter, pβA. Cloning of all constructs was confirmed by sequencing (Eurofins Genomics).

For pβA-αδ-1: Mouse αδ-1 was cloned from genomic DNA derived from mouse cerebellum. Primer sequences were selected according to GenBank NM-001110844. Briefly, the cDNA of αδ-1 was amplified by PCR in three fragments. The forward primer used for amplifying fragment 1 introduced a NotI site and the Kozak sequence (CCTACC) upstream of the starting codon and the reverse primer used for amplifying fragment 2 introduced a KpnI and a Sall site after the stop codon. Fragment 2 (nt 1442–2564) was MfeI/BamHII digested and fragment 3 (nt 2335–3276) was KpnI/BamHII digested and co-ligated in the corresponding MfeI/KpnI sites of the pβA vector, yielding an intermediate construct. Fragment 1 (nt 1–1575) was NotI/MfeI digested and co-ligated with the Sall/MfeI-digested intermediate construct containing fragments 2 and 3 and the NotI/Sall-digested pβA vector, yielding pβA-αδ-1 (GenBank accession number MK327276).

For pβA-2HA-αδ-1: The putative signal peptide (aa1–24) was predicted using Signal P (SignalP 4.0: discriminating signal peptides from transmembrane regions; Petersen et al., 2011). A double hemagglutinin tag (2HA) followed by a TEV cleavage site was introduced between the third and fourth amino acids after the predicted signal peptide cleavage site of mouse αδ-1; that is, residue F27. Introduction of this sequence did not alter the predicted cleavage site. Briefly, the cDNA sequence of αδ-1 (nt 1–516) was PCR amplified with overlapping primers introducing the double HA tag and the TEV cleavage site in separate PCRs using pβA-αδ-1 as a template. The two separate PCR products were then used as templates for a final PCR with flanking primers to connect the nucleotide sequences. The resulting fragment was then NotI/BglII digested and ligated into the corresponding sites of pβA-αδ-1, yielding pβA-2HA-αδ-1.

For pβA-αδ-2 (v1): Mouse αδ-2 was cloned from genomic DNA from mouse brain. Primer sequences were selected according to GenBank NM-001174049. The cDNA of αδ-2 was amplified by PCR in four fragments. The forward primer used for amplifying fragment 1 introduced a HindIII site and the Kozak sequence (CCTACC). Fragment 1 was isolated from cerebellum, whereas the other three fragments were isolated from hippocampus. Fragment 1 (nt 1–686) and Fragment 2 (nt 323–1294) were HindIII/BamHII and BamHII/EcoRI digested, respectively, and co-ligated in the corresponding HindIII/EcoRI sites of the pBS (Bluescript) vector, yielding the intermediate construct pBS-αδ-2-part1. Fragment 3 (nt 1137–2359) was EcoRI/PstI digested and ligated into pBS-αδ-2-part1, yielding the intermediate construct pBS-αδ-2-part2. Fragment 4 (nt 2226–3444) was BsnI/XbaI digested and ligated into the corresponding sites of pSPORT-part2, yielding the intermediate construct pSPORT-αδ-2-part3. pSPORT-αδ-2-part3 (v1) was EcoRI/XbaI digested and the band containing fragments 3–4 (bp 1137–3444) was ligated into pBS-αδ-2-part2, yielding pβA-αδ-2. This construct was then HindIII/XbaI digested and the cDNA of αδ-2 was ligated into the pβA vector, yielding pβA-αδ-2 (v1) (GenBank accession number MK327277).

For pβA-2HA-αδ-2 (v1): A putative signal peptide was not reliably predicted using Signal P (SignalP 4.0: discriminating signal peptides from transmembrane regions; Petersen et al., 2011); however, the highest prediction showed that the signal peptide comprises residues 1–64. The 2HA tag followed by a thrombin cleavage site was therefore introduced after the predicted signal peptide cleavage site of mouse αδ-2; that is, residue D28. Introduction of this sequence did not alter the predicted cleavage site. Briefly, the cDNA sequence of αδ-2 (nt 1–653) was PCR amplified with overlapping primers introducing the double HA tag and the thrombin cleavage site in separate PCRs using pβA-αδ-2 as a template. The two separate PCR products were then used as templates for a final PCR with flanking primers to connect the nucleotide sequences. The resulting fragment was then HindIII/AHII digested and ligated into αδ-2 (v1). For pβA-αδ-2 (v2): An alternative splice variant was isolated from cerebellum, corresponding to GenBank NM-020263. Fragment 3 contained an additional 21 nt at position nt 1992 and was cloned into pSPORT together with fragment 4, yielding pSPORT-αδ-2-part3 (v2). pSPORT-αδ-2-part3 (v2) was digested with Clal/BglII and cloned into the corresponding sites of pβA-αδ-2 (v1), yielding pβA-αδ-2 (v2) (GenBank accession number MK327278).

For pβA-αδ-2 (v3): An alternative splice variant was isolated from hippocampus, corresponding to GenBank NM-001174048. Fragment 4 contained an additional 3 nt at position nt 2598 (resulting in an additional Q residue) and an additional 6 nt at position 3219 nt (translated in CPA instead of S) and was cloned into pSPORT together with fragment 3, yielding pSPORT-αδ-2-part3 (v3). pSPORT-αδ-2-part3 (v3) was digested with Clal/XbaI and cloned into the corresponding sites of pβA-αδ-2 (v1), yielding pβA-αδ-2 (v3) (GenBank accession number MK327279).

For pβA-αδ-3: Mouse αδ-3 was cloned from genomic DNA from mouse hippocampus. Primer sequences were selected according to GenBank NM-009785. Briefly, the cDNA of αδ-3 was amplified by PCR in four fragments. The forward primer used for amplifying fragment 1 introduced a NotI site and the Kozak sequence (CCTACC) upstream of the starting codon. Fragment 3 (nt 1520–2817) was then SacI/PstI digested and fragment 4 (nt 2727–3276) was Dral/PstI digested and co-ligated in the corresponding SacI/Stal sites of the pSPORT vector, yielding an intermediate construct. Fragment 1 (nt 1–653) was then NotI/BamHII digested and fragment 2 (nt 353–1636) was SacI/BamHII digested and co-ligated with the SacI/NotI digested intermediate construct containing fragments 3 and 4, yielding pSPORT-αδ-3. The cloned cDNA of αδ-3 was then NotI/RsrII digested and ligated into the corresponding sites of the pβA vector, yielding pβA-αδ-3 (GenBank accession number MK327280).

For pβA-2HA-αδ-3: The putative signal peptide (aa1–28) was predicted using Signal P (SignalP 4.0: discriminating signal peptides from transmembrane regions; Petersen et al., 2011). The 2HA tag followed by a thrombin cleavage site was therefore introduced after the predicted signal peptide cleavage site of mouse αδ-3; that is, residue D28. Introduction of this sequence did not alter the predicted cleavage site. Briefly, the cDNA sequence of αδ-3 (nt 1–653) was PCR amplified with overlapping primers introducing the double HA tag and the thrombin cleavage site in separate PCRs using pβA-αδ-3 as a template. The two separate PCR products were then used as templates for a final PCR with flanking primers to connect the nucleotide sequences. The resulting fragment was then NotI/BglII digested and ligated into the corresponding sites of pβA-αδ-3, yielding pβA-2HA-αδ-3.

For pHR-pβA-eGFP*2HA-αδ-2 (v1): To remove the HA tag from pβA-eGFP*2HA-αδ-2, pβA-eGFP*2HA-αδ-2 was XhoI/RsrII digested to isolate part of the promoter and the eGFP coding sequence. The obtained fragment was then inserted in the corresponding sites of pβA-αδ-2, yielding pβA-eGFP*2HA-αδ-2. For generating the viral vector pHR-pβA-eGFP*αδ-2, the eGFP*αδ-2 coding sequence was introduced with HindIII/XbaI into a custom-built pENTR vector and inserted into a custom-built destination vector, pHR-βA-DEST, using the LR Clonase II enzyme mixture (GATEWAY; Invitrogen), yielding pHR-pβA-eGFP*αδ-2 (v1).

For pβA-eGFP*2HA-αδ-2 (v1): The eGFP* coding sequence, followed by a stop codon and the Kozak sequence (CCTACC), were introduced by PCR between the Kozak sequence and the coding sequence of 2HA-αδ-2. The resulting fragment was then HindIII/PpuMI digested and ligated into the corresponding sites of pβA-2HA-αδ-2, yielding pβA-eGFP*2HA-αδ-2. This construct was then transfected in hippocampal cultures with lipofectamine to insure that both the eGFP and HA signals were present in transfected neurons.

For pHR-pβA·eGFP: The cloning procedure to generate this plasmid was described previously (Subramanyam et al., 2014). For pHR-βA-mcherry: The coding sequence of mcherry was inserted into a custom-built destination vector, pHR-βA-DEST, using LR Clonase II enzyme mixture (GATEWAY; Invitrogen) as described previously (Etemad et al., 2014).
Immunocytochemistry and microscopy

**Antibodies.** Information on primary and secondary antibodies is summarized in Table 1.

**High-resolution fluorescence microscopy.** Immunolabeling of permeabilized or live-cell-stained neurons was performed as described previously (Obermaier et al., 2004, 2010; Stanika et al., 2016; Folci et al., 2018) and information on antibodies is given in Table 1. For PSD-95 labeling of eGFP-transfected neurons, cultures were fixed in 4% paraformaldehyde/4% sucrose (pF) in PBS for 20 min at room temperature, rinsed in PBS followed by a second fixation with absolute methanol for 10 min at −20°C. For all other antibody combinations, neurons were fixed with pF only. Subsequent to fixation and washing, cultures were incubated for 30 min in 5% normal goat serum in PBS containing 0.2% bovine serum albumin (BSA) and 0.2% Triton X-100 (PBS/BSA/Triton), enabling membrane permeabilization. Primary antibodies were applied overnight in PBS/BSA/Triton at 4°C and detected by fluorochrome-conjugated secondary antibodies incubated for 1 h at room temperature. For surface staining of HA-tagged α2δ constructs, transfected neurons were incubated with rat-anti-HA antibody for 20 min at 37°C following rinsing in warm HBSS and fixation with pF for 10 min at room temperature. Subsequent washing and blocking steps were done with PBS and PBS/BSA, respectively, followed by 1 h incubation with fluorochrome-conjugated secondary goat-anti-rat Alexa Fluor 594 antibody. After rinsing and post-fixation of cells in pF for 5 min, neurons were permeabilized by blocking with PBS/BSA/Triton. Primary mouse-anti-synapsin antibody was applied overnight at 4°C and detected with goat-anti-mouse Alexa Fluor 350 antibody. Covesslips were either mounted in p-phenylenelediamine glyceral (Flucher et al., 1993) or DAKO fluorescence mounting medium (Agilent Technologies) for α-Nrxn knock-out and control cultures. Hippocampal cultures were mainly viewed with an Axio Imager microscope (Carl Zeiss) using a 63× 1.4 numerical aperture (NA) oil-immersion objective lens and 14-bit grayscale images were recorded with a cooled CCD camera (SPOT; Diagnostic Instruments) using MetaMorph software (Molecular Devices). α-Nrxn knock-out cultures and MSNs were observed with a BX53 microscope (Olympus) using a 60× 1.42 NA oil-immersion objective lens and 14-bit grayscale images were recorded with a cooled CCD camera (XM10; Olympus) using cellSens Dimension software (Olympus). To analyze presynaptic and postsynaptic differentiation cover glasses were systematically screened for contact sites between axons of presynaptic neurons [transfected with the fluorescent marker alone (eGFP or mCherry only) or cotransfected with the marker and target construct (eGFP or mCherry plus αδ)] and dendrites or somas of nontransfected postsynaptic neurons. Images of randomly selected well differentiated cells were acquired with the same exposure and gain settings for all conditions within an experiment. These settings were initially adjusted to facilitate optimal visualization of peripheral cell compartments (axons and presynaptic boutons) also in weakly expressing neurons. Therefore, overly saturated neurons (based on eGFP and mCherry levels) were excluded from analysis and only cells with medium to low eGFP or mCherry expression were selected for further analysis. Images were analyzed with MetaMorph software (Molecular Devices) as described below. Figures were assembled in Adobe Photoshop CS6 and linear adjustments were done to correct black level and contrast.

**gSTED microscopy.** For gSTED microscopy of mCherry-transfected permeabilized hippocampal and striatal MSNs (see Fig. 3), immunolabeling was done as described for high-resolution fluorescence microscopy with some modifications. Briefly, mouse-anti-GABA_R12/3 antibody was used at higher concentrations (1:250) and detected with secondary goat-anti-mouse Oregon Green (OG) 488 (1:500). Covesslips were mounted in DABCO/glycerol to retard photobleaching (2% DABCO, 90% glycerol, buffered in Tris-HCl, pH 8.5; DABCO and glycerol were purchased from Carl Roth). Samples were visualized using a TCS SP8 gSTED microscope equipped with an HCXPL APO 100× 1.4 NA oil-immersion objective (Leica Microsystems) and images were scanned at a pixel size of 15 nm. For hippocampal neurons, individual fluorophores were recorded in the following sequence (excitation/detection range

| Table 1. List of primary and secondary antibodies |
|-----------------------------------------------|
| **Primary antibodies**                        |
| Species | Dilution | Source |
|-----------------------------------------------|
| Anti-HA Rat, monoclonal, clone 3F10 | 1:100 (LIVE/AS94) | Roche (catalog #11867423001, RRID:AB_390918) |
| Anti-GABA R12/3 Mouse, monoclonal, clone bd17 | 1:500 (AS94) | Millipore (catalog #AB341, RRID:AB_2109419) |
| Anti-GABA R12/3 Rabbit, polyclonal | 1:250 (AS95/O6488) | (Hörtnagl et al., 2013) |
| Anti-GABA R12/3 Rabbit, polyclonal | 1:1000 (AS94) | (Hörtnagl et al., 2013) |
| Anti-GABA R12/3 Rabbit, polyclonal | 1:500 (AS94) | (Hörtnagl et al., 2013) |
| Anti-GABA R12/3 Rabbit, polyclonal | 1:500 (AS94) | (Hörtnagl et al., 2013) |
| Anti-GABA R12/3 Rabbit, polyclonal | 1:1000 (AS50) | (Hörtnagl et al., 2013) |
| Anti-GABA R12/3 Rabbit, polyclonal | 1:2000 (AS94) | (Hörtnagl et al., 2013) |
| Anti-gephyrin Mouse, monoclonal, clone mab7a | 1:2000 (AS94) | Synaptic Systems (catalog #147021, RRID:AB_2232546) |
| Anti-GLUR1 Rabbit, polyclonal | 1:1000 (AS94) | Upstate (catalog #06-306) |
| Anti-PSD-95 Mouse, monoclonal, clone 666-1TC9 | 1:1000 (AS50) | Thermo Fisher Scientific (catalog #MAA1-045, RRID:AB_325399) |
| Anti-synapsin1/2 Rabbit, polyclonal | 1:2000 (AS50) | Synaptic Systems (catalog #106002, RRID:AB_887804) |
| Anti-synapsin1/2 Mouse, monoclonal, clone 46.1 | 1:500 (AS50) | Synaptic Systems (catalog #106011, RRID:AB_2619771) |
| Anti-vGLUT1 Rabbit, polyclonal | 1:2000 (AS50) | Synaptic Systems (catalog #135002, RRID:AB_2315546) |
| Anti-vGLUT1 Mouse, monoclonal, clone 31766 | 1:500 (AS94) | Synaptic Systems (catalog #153511, RRID:AB_2507797) |
| Anti-vGAT Rabbit, polyclonal | 1:500 (AS50) | Synaptic Systems (catalog #131002, RRID:AB_887871) |
| Anti-gephyrin Mouse, monoclonal, clone mab7a | 1:1000 (AS50/STAR440) | (Hörtnagl et al., 2013) |

| **Secondary antibodies**                        |
|-----------------------------------------------|
| Species | Dilution | Source |
|-----------------------------------------------|
| Alexa Fluor 350 Goat anti-mouse | 1:500 | Thermo Fisher Scientific (catalog #A-21049, RRID:AB_2535717) |
| Alexa Fluor 594 Goat anti-rabbit | 1:500 | Thermo Fisher Scientific (catalog #A-21068, RRID:AB_2535729) |
| Oregon Green 488 Goat anti-mouse | 1:500 | Thermo Fisher Scientific (catalog #A-11032, RRID:AB_2534091) |
| STAR 440 Goat anti-mouse | 1:500 | Thermo Fisher Scientific (catalog #A-11037, RRID:AB_2534095) |
| Abberior (catalog #2-0002-003-7, RRID:AB_2756514) | 1:500 | (Hörtnagl et al., 2013) |

**Notes:** All antibodies were purchased from SYNaptic Systems (catalog numbers for primary antibodies start with 147). In parentheses are species and dilutions taken from the antibody manufacturer, followed by the catalog number. Source is the supplier of antibody. For STAR 440 and Oregon Green 488, the supplier used was Thermo Fisher Scientific (catalog numbers start with 135). For Alexa Fluor 350, the supplier was Thermo Fisher Scientific (catalog number start with A).
galactosidase activity was analyzed in male
To study the localization of excitatory synapses on postsynaptic dendrites
GABAAR
600 – 690; gate 0.20 – 6 ns, in confocal mode) and OG 488 (mouse-anti-
mCherry (presynaptic axon: mCherry only or mCherry
in the following sequence (excitation/detection range wavelength in nm): mCherry (presynaptic axon: mCherry only or mCherry + α,δ-2; 580/600–688; gate 0.20–6 ns in confocal mode) and eGFP (postsynaptic dendrite: eGFP only, 490/508–584 or 520–584; gate 1–6.2 ns, STED laser at 592 nm). To enable clear visualization of dendritic structures, STED laser intensity was adjusted according to the eGFP signal. To study the localization of excitatory synapses on postsynaptic dendrites of inhibitory GABAergic neurons (see Fig. 11E), cortical neurons and MSNs were labeled with mCherry and eGFP, respectively, as described above. Primary mouse-anti-gephyrin (1:1000) was applied overnight in PBS/BSA/Triton at 4°C and detected with secondary goat-anti-mouse STAR440 (1:500). Fluorophores were recorded in the following sequence (excitation/detection range wavelength in nm): mCherry (presynaptic axon: mCherry only or mCherry + α,δ-2; 580/600–690; gate 0.5–6 ns in confocal mode), eGFP (postsynaptic dendrite: eGFP only, 470/508–580; gate 1–6.0 ns, STED laser at 592 nm), and STAR440 (mouse-anti-gephyrin; 470/480–570; gate 1–6.0 ns, STED laser at 592 nm). STED laser intensity was adjusted according to the eGFP signal to enable clear visualization of dendritic structures and to reduce cross-activation during the detection of STAR440. Channel dye separation was performed to further split the eGFP and STAR440 signals. Images were recorded with LAS AF software (Leica Microsystems), analyzed using MetaMorph software (Molecular Devices), and figures were assembled in Adobe Photoshop CS6 and linear adjustments were done to correct black level and contrast.

LacZ reporter expression. Using the LacZ reporter function of the mutated Cacna2d3 gene (Cacna2d3tm1Dgen; Neely et al., 2010), β-galactosidase activity was analyzed in male α,δ-3/- knock-out mice. Eight-week-old mice were killed by CO2 asphyxiation, decapitated, and brains were removed from the skull. Subsequently, hemispheres were separated with a sagittal cut along the midline, placed medial side down on a flat piece of thin acrylic glass, and frozen in 2-methylbutan (Carl Roth) and xylene (Carl Roth) and mounting with Eukitt (Christine Gröpl, Tulln, Austria). Representative images were recorded with a BX53 microscope (Olympus) equipped with a SC100 10 color camera (Olympus). Eight-bit panorama photographs were created by scanning specimens with a 4× 0.16 NA objective and using the manual multiple image alignment (MIA) function in cellSens Dimension software (Olympus).

Image analysis and quantification
Colocalization of synaptic proteins. The synaptic localization of HA-tagged α,δ subunits, presynaptic (vGAT, vGLUT1) and postsynaptic proteins (GABAAR subtypes, gephyrin, GLUR1) were analyzed with the “line scan” function in MetaMorph software (Molecular Devices) (Di Biase et al., 2009). Average fluorescence intensities of green (A488), blue (A350), and red (A594) signals were measured along a line of 3 or 8 µm length, background subtracted, and plotted in Microsoft Excel.

Quantification of fluorescent clusters in single boutons. To analyze the effects of presynaptic expression of α,δ subunits in GABAergic synapses of cultured neurons, 14-bit grayscale images from triple-fluorescence labeling were acquired from the eGFP (green), GABAAR, and vGAT (blue) channels. Images were analyzed with a custom programmed and semiautomated MetaMorph journal (Molecular Devices) as follows. Corresponding eGFP and vGAT images were superimposed, eGFP/vGAT-positive varicosities (putative GABAergic synapses) were randomly chosen as regions of interest (ROIs), and a region for background subtraction was selected. Axonal varicosities were defined as prominent swellings with higher fluorescence signal compared with the adjacent axonal shaft. Subsequently, eGFP, GABAAR, and vGAT grayscale images were thresholded to include the entire area of individual varicosities (eGFP) and synaptic clusters (GABAAR, vGAT). Selected ROIs were then transferred to the eGFP image and, by applying the “shrink region to fit” tool, automatic boundaries were drawn according to the threshold enabling only colocalized clusters to be analyzed. ROIs and background regions were further transferred to the thresholded GABAAR and vGAT channel images and ROI staining intensities were recorded. Therefore, the following parameters could be measured for the individual synapses in each of the channels in a blinded manner: eGFP threshold area as a measure for bouton size and average and integrated fluorescence intensities providing information on the size and intensity of clusters. To address the effects of presynaptic expression of α,δ subunits in glutamatergic synapses of α-Ntrα TKO neurons, 14-bit grayscale images from triple-fluorescence labeling were acquired from the eGFP (green), GABAAR, and vGLUT1 (blue) channels. Images were analyzed with a custom programmed and semiautomated journal as described above. Corresponding eGFP and vGLUT1 images were superimposed and eGFP/vGLUT1-positive varicosities (putative glutamatergic synapses) were randomly selected as ROIs. To analyze the effects of presynaptic expression of α,δ subunits in glutamatergic synapses of cultured hippocampal WT neurons, 14-bit grayscale images from triple-fluorescence labeling were acquired from the eGFP (green), GLUR1 (red), and GABAAR (blue) channel. Images were analyzed in the same manner as mentioned above but without the use of a semi-automatic MetaMorph journal. For all experiments, background subtractions for individual channels and further data organization and analyses were done with Microsoft Excel. Mean values for individual cells were calculated from individual boutons and normalized to the control (eGFP only) or α,δ-2 condition within each culture preparation. Cumulative fluorescence intensity distribution of GABAAR was described above. Corresponding eGFP signals from single boutons was done to visualize the effect of α,δ subunits on the whole GABAAR population. Two to four independent culture preparations were analyzed and details are given in the respective figure legends.

Quantification of gephyrin content in mismatched and neighboring synapses. To compare gephyrin content of α,δ-2-induced mismatched synapses with endogenous clusters, 14-bit grayscale images from triple-fluorescence labeling were acquired from the eGFP (green), GLUR1 (red), and gephyrin (blue) channel and analyzed with MetaMorph software (Molecular Devices). First, corresponding eGFP, GLUR1, and gephyrin images were superimposed and eGFP-positive varicosities expressing α,δ-2 (putative mismatched synapses) were selected as ROIs and a region was selected for background subtraction. eGFP, GLUR1, and gephyrin grayscale images were thresholded to include the entire area of individual varicosities (eGFP) and synaptic clusters (GLUR1, gephyrin). Regions were transferred to the eGFP image, shrunk to fit the threshold area, and further transferred to the thresholded gephyrin channel images. After measuring the integrated gephyrin intensity of eGFP-positive mismatched synapses, ROIs of 10 untransfected neighboring WT synapses located on the same dendrite were selected and regions were superimposed and the average fluorescence intensity was measured. The GLUR1 staining served as orientation to outline dendritic morphology of untransfected neurons. Per image, one mismatched gephyrin cluster induced by α,δ-2
was compared with the mean of 10 endogenous clusters. Dots in graph represent values for individual boutons (mismatched synapses) and means of 10 endogenous clusters measured per image. Values were normalized to endogenous gephyrin intensities of neighboring synapses within each culture preparation. Three independent culture preparations were done and eight to 10 images per preparation were analyzed.

Analysis of synaptic localization in gSTED images. Scans of mCherry-labeled boutons (control or α,δ-2) contacting eGFP-labeled postsynaptic dendrites were acquired from the eGFP (green, gSTED mode) and mCherry (red, confocal mode) channels. Axonal varicosities were visible as prominent swellings with higher fluorescence signal compared with the adjacent axonal shaft. Initially, linear contrast adjustments were done with LAS AF software (Leica Microsystems), thus enabling clear visualization of postsynaptic dendritic spines and the potential overlap of fluorescence signals, which was used to define the contact zone of putative synapses. Superimposed images were exported into a TIFF format and further analyzed using MetaMorph software (Molecular Devices). To determine the position of synapses on postsynaptic elements, two types of analysis were used. First, the postsynaptic contact sites of presynaptic boutons were manually classified as dendritic shafts, dendritic spines, or unclear localizations. Second, the distances from the postsynaptic dendritic shafts to the contact points with the presynaptic boutons were calculated as follows. First, the distances between the center of the dendritic shafts and the shaft surfaces were determined and measured. Second, the distances between the center of dendritic shafts and the contact zone (presynaptic bouton with postsynaptic dendrite) were determined and measured. Third, the actual distance from the dendritic shaft surface to the contact zone was determined by subtracting the individual center surface distances from the center contact zone distances. Dots in graph (Fig. 11C) represent values for individual boutons and means (± SEM) are shown. Two independent culture preparations were analyzed, of which one experiment was performed in a blinded manner.

Electrophysiology

Induced (evoked) AMPAR-mediated synaptic responses were recorded from paired hippocampal neurons (holding potential −70 mV) using the whole-cell patch-clamp technique. Neurons were stimulated with 10 ms depolarization pulse from a holding potential of −70 to 60 mV. Patch pipettes were pulled from borosilicate glass (Harvard Apparatus), fire-polished (Microforge MF-830; Narishige), and had resistances of 2.5–4 MΩ when filled with the following (in mM): 130 K-gluconate, 1 MgCl2, 10 HEPES, 5 EGTA, 4 Mg-ATP, and 0.3 Na-GTP, pH 7.2 with KOH. The bath solution contained the following (in mM): 137 NaCl, 3 KCl, 10 HEPES, 2 MgCl2, 1.8 CaCl2, 0.05 DL-AP5, and 10 glucose, pH 7.4 with NaOH. Currents were recorded with an EPC 10 amplifier controlled by PatchMaster software (HEKA Elektronik Dr. Schulze). To test synaptic plasticity (or paired-pulse facilitation), we applied a pair of pulses with varying interpulse intervals (10, 25, 50, 100, 250, or 500 ms) and calculated the paired-pulse amplitude ratio (P2/P1).

To test spontaneous activity of mixed cortical-MSN cultures, TTX (1 μM) and bicuculline (10 μM) or CNQX (10 μM) were added to bath solution and miniature EPSCs (mEPSCs; holding potential −70 mV) or miniature IPSCs (mIPSCs; holding potential 0 mV), respectively, were recorded.

Sequence alignment and homology modeling

Information on α,δ-2 splice sites was obtained from the Uniprot (Q6PH99) and Ensemble (ENSMUSG00000010066) databases. Protein sequences of the distinct variants were aligned with CLUSTAL O (1.2.4) multiple sequence alignment tool. The high-resolution structure of α,δ-1 (PDB code: 5GJV; Wu et al., 2016) was used as a template to model the structure of α,δ-2 splice variants. The pdb files were generated with the Swiss-Model server (http://www.expasy.org/swissmod/SWISS-MODEL.html) and models were exported and further analyzed using UCSF Chimera (http://www.rbvi.ucsf.edu/chimera).

Experimental design and statistical analysis

According to the RRR principle, the number of mice used was kept to the minimum necessary for a statistical representative analysis, which was comparable to numbers reported in previous studies. Where indicated and possible, investigators were partially blinded during experiments and analyses. Moreover, analysis of presynaptic and postsynaptic proteins was done in a blinded manner as described above. Two to four independent culture preparations were analyzed per experiment and details on cell or bouton numbers are given in the respective figure legends. The graphs show values of individual cells/boutons (dots) and means (line) ± SEM. The numbers (n) used to calculate SEMs were either given by number of animals used (qRT-PCR) or the number of analyzed cells/boutons. Before statistical analysis, the distribution of datasets was evaluated with histograms. Whenever datasets were strongly skewed or normality was experimentally excluded (e.g., a large proportion of “0” values), raw data were log10 transformed and data distribution was reevaluated by histograms. For normally distributed raw or log10-transformed datasets, unpaired t test or one-way ANOVA with Holm–Sidak post hoc test was used. Alternatively, when log10 transformation did not result in normally distributed data, the Mann–Whitney U test or Kruskal–Wallis ANOVA with Dunn’s post hoc analysis was applied. Significance levels (p-values) of statistical tests and post hoc analysis are presented in the respective figure legends. The model in Figure 3 was generated with Maya software (version 2018; Autodesk). Data, graphs and figures were organized, analyzed, and assembled using Microsoft Excel, GraphPad Prism 6, SigmaPlot (Systat Software), and Adobe Photoshop CS6.

Results

Presynaptic overexpression of α,δ-2 induces the formation of mismatched synapses in excitatory hippocampal neurons

To date, the synaptic functions of α,δ subunits have been identified in cells and tissues primarily expressing a single dominant α,δ isoform (Eroglu et al., 2009; Pirone et al., 2014; Fell et al., 2016; Wang et al., 2017). To study the involvement of the individual α,δ isoforms in glutamatergic synapse formation and differentiation, we homologously overexpressed mouse α,δ-1, α,δ-2, and α,δ-3 cDNAs in WT mouse hippocampal cultures together with soluble eGFP. To analyze the consequences on synapse differentiation, transfected neurons were immunolabeled against the vesicular glutamate transporter (vGLUT1), a marker for presynaptic excitatory synapses, and the postsynaptic GABAAR subunit (GABAAR), which is typically absent in glutamatergic synapses. Immunofluorescence analysis revealed that, as expected, control hippocampal neurons (eGFP only) axonal varicosities were positive for presynaptic vGLUT1 and negative for postsynaptic GABAAR labeling (Fig. 1A,B, first column). Surprisingly, presynaptic expression of α,δ-2 induced a robust localization of GABAAR clusters opposite vGLUT1-positive presynaptic boutons (Fig. 1B, blue arrowheads). These apparently mismatched synapses are atypical because they are formed between excitatory nerve terminals and inhibitory postsynaptic receptors. Importantly, mismatched postsynaptic receptor localization was not observed opposite terminals expressing α,δ-1 or α,δ-3.

Excitatory and inhibitory neurotransmitter transporters can coexist in glutamatergic hippocampal and cerebellar mossy fiber terminals, possibly leading to the corelease of glutamate and GABA and the localization of postsynaptic GABAARs in spines (Bergeren et al., 2003; Münzer-Wandowski et al., 2016). Therefore, we next tested whether vesicular GABA transporters (vGATs) are present in synapses overexpressing presynaptic α,δ subunits. As expected and in contrast to neighboring untransfected GABAergic synapses (Fig. 1D, asterisks), presynaptic vGAT and postsynaptic GABAARs were not found in putative excitatory synapses expressing α,δ-1, α,δ-3, or eGFP only (Fig. 1C,D). Most importantly, presynaptic vGAT labeling was also absent in synaptic presynaptic α,δ-2, which again showed strong postsynaptic GABAAR clustering (Fig. 1D, + α,δ-2, blue arrowheads). Therefore, the mismatched localization of postsynaptic GABAARs cannot be explained by the exis-
tence of inhibitory neurotransmitters within glutamatergic nerve terminals.

To further characterize our observation, we next quantitatively assessed the postsynaptic receptor composition of mismatched glutamatergic synapses by double labeling transfected hippocampal neurons with markers for excitatory and inhibitory synapses (Fig. 2A–C). Immunofluorescence analysis revealed that, in control hippocampal neurons (eGFP only) postsynaptic AMPARs (GLUR1) were expressed opposite transfected presynaptic terminals, as expected for putative...
Figure 2. Presynaptic expression of $\alpha_2\delta-2$ alters the postsynaptic composition of glutamatergic synapses. A, B, Representative immunofluorescence micrographs of cultured hippocampal neurons (24–30 DIV) cotransfected with distinct $\alpha_2\delta$ subunits and soluble eGFP (control condition with eGFP alone is summarized in sketch, C). Colocalization of fluorescence signals was analyzed using line scans. A, Colabeling of the AMPA receptor subtype GLUR1 and the GABA$\text{A}$R. Presynaptic expression of $\alpha_2\delta-2$ (blue arrowheads) induces postsynaptic GABA$\text{A}$R localization with a concomitant reduction of the GLUR1 fluorescence signal. In contrast, postsynaptic GABA$\text{A}$Rs are not expressed opposite putative glutamatergic synapses expressing $\alpha_2\delta-1$, $\alpha_2\delta-3$, or eGFP only (control). B, Colabeling of GLUR1 and gephyrin, the scaffolding protein of GABAergic synapses. Presynaptic expression of $\alpha_2\delta-2$ (blue arrowheads) induces postsynaptic gephyrin localization with a concomitant reduction of the GLUR1 fluorescence signal. In contrast, postsynaptic gephyrin immunoreactivity is absent opposite putative glutamatergic synapses expressing $\alpha_2\delta-1$, $\alpha_2\delta-3$, or eGFP only (control). D–F, Quantitative analysis of GABA$\text{A}$R (D), GLUR1 (E), and gephyrin (F) fluorescence intensities. Values were normalized to $\alpha_2\delta-2$ (D, F) or eGFP only (control, E) fluorescence intensities within each culture preparation. Note the significant increase of postsynaptic GABA$\text{A}$R and gephyrin clusters opposite presynaptic boutons expressing $\alpha_2\delta-2$. In contrast, integrated intensity of GLUR1 was significantly reduced opposite axonal varicosities transfected with $\alpha_2\delta-2$ or $\alpha_2\delta-3$. Values for individual cells (dots) and means (line) ± SEM are shown. Data are shown from three independent culture preparations. 11–25 (D), 22–30 (E), and 11–28 (F) cells were analyzed in each condition. Statistics: D, Kruskal–Wallis ANOVA with Dunn’s post hoc analysis: $H_{(4)} = 51.6, p < 0.0001$, post hoc: *** $p < 0.0001$ between $\alpha_2\delta-2$ and all other conditions; E, ANOVA on log10-transformed data with Holm–Sidak post hoc analysis: $F_{(3,100)} = 18.6, p < 0.001$, post hoc: *** $p < 0.001$ between control/$\alpha_2\delta-1$ and $\alpha_2\delta-2$/$\alpha_2\delta-3$; F, Kruskal–Wallis ANOVA with Dunn’s post hoc analysis: $H_{(4)} = 64.6, p < 0.0001$, post hoc: *** $p < 0.0001$ between $\alpha_2\delta-2$ and all other conditions. Asterisks in graphs indicate the significant difference compared with control. Scale bars, 3 μm.
glutamatergic synapses (Fig. 2A, control). Consistent with our initial observations (Fig. 1), postsynaptic GABA<sub>R</sub> fluorescent intensities were strongly and significantly increased opposite α<sub>2</sub>δ-2-expressing presynaptic terminals (Fig. 2A, D). The elevated GABA<sub>R</sub> receptor abundance (Fig. 2A) was accompanied by a 76% reduction of GLUR1 clustering compared with control (Fig. 2E). Surprisingly, presynaptic expression of α<sub>2</sub>δ-3, which did not alter postsynaptic GABA<sub>R</sub> clustering, also caused a significant reduction of GLUR1 receptors (66% reduction vs control). Finally, we also observed a slight but not significant tendency for a reduced GLUR1 receptor expression opposite α<sub>2</sub>δ-1-overexpressing boutons (Fig. 2E). We next tested whether gephyrin, a key organizer of GABAergic synapses that anchors, clusters, and stabilizes GABA<sub>R</sub>s and other postsynaptic elements of inhibitory synapses (Choii and Ko, 2015), was also recruited to mismatched GABA<sub>R</sub>-positive synapses. Indeed, we found that the strong increase in GABA<sub>R</sub> expression opposite α<sub>2</sub>δ-2-expressing terminals was accompanied by similarly increased gephyrin abundance, whereas it was missing from control and α<sub>2</sub>δ-1- or α<sub>2</sub>δ-3-expressing synapses (Fig. 2B, F).

**Presynaptic α<sub>2</sub>δ-2 recruits synaptic GABA<sub>R</sub> subtypes**

Because immunofluorescence analysis suggested an effect on postsynaptic GABA<sub>R</sub> clusters opposite presynaptic boutons, we next used superresolution gSTED microscopy to verify their subsynaptic localization (Fig. 3A). Postsynaptic GABA<sub>R</sub> clusters appear closely opposed to mCherry-positive, α<sub>2</sub>δ-2-overexpressing presynaptic boutons: depending on the orientation of the imaged synapses, the GABA<sub>R</sub>-labeling pattern is either visible as a thin line (side view; Fig. 3A, first column and Fig. 3B, top) or as a ring-like structure (planar view; Fig. 3A, second column and Fig. 3B, bottom). These labeling patterns support a confined localization of α<sub>2</sub>δ-2-recruited GABA<sub>R</sub>s in the postsynaptic membrane.

GABA<sub>R</sub>s are pentameric ligand-gated chloride channels consisting typically of α, β, γ, and δ subunits (Hörtnagl et al., 2013). Because the antibody used in our colocalization experiments (Figs. 1, 2) detects only the GABA<sub>R</sub>β2 and 3 subunits, we next analyzed whether and which additional GABA<sub>R</sub> subtypes can be recruited by presynaptic α<sub>2</sub>δ-2. Immunofluorescence analysis revealed robust labeling of the typically synaptic GABA<sub>R</sub> subunits α<sub>1</sub>, α<sub>2</sub>, β<sub>2</sub>, and γ<sub>2</sub> opposite α<sub>2</sub>δ-2 transfected vGLUT1-positive nerve terminals (Fig. 4A). In contrast, the mainly extrasynaptic subunits α<sub>3</sub>, α<sub>4</sub>, β<sub>3</sub>, and δ (Hörtnagl et al., 2013) were either not detectable in these α<sub>2</sub>δ-2-expressing synapses or diffusely and extrasynaptically located (Fig. 4C). Importantly, mismatched postsynaptic receptor localization of all distinct subtypes was neither observed opposite terminals expressing eGFP only (control) nor those expressing α<sub>2</sub>δ-1 or α<sub>2</sub>δ-3 (Fig. 4B, D, examples for α<sub>1</sub> and α<sub>4</sub> subunits are shown). Because there was no apparent difference between postsynaptic α<sub>1</sub>, α<sub>2</sub>, β<sub>3</sub>, γ<sub>2</sub>, and β<sub>2/3</sub> labeling, we continued using the commercially available and extensively characterized β<sub>2/3</sub> antibody for all subsequent experiments (Goffin et al., 2010; Arama et al., 2015; Stefanits et al., 2018).

**Potential mechanisms explaining an α<sub>2</sub>δ-2-induced mismatched synapse formation**

So far, our results show that the specific expression of a single α<sub>2</sub>δ subunit isoform, α<sub>2</sub>δ-2, in presynaptic glutamatergic terminals...
triggers a mismatched localization of postsynaptic GABAARs, which was accompanied by a strong reduction of postsynaptic AMPARs. The underlying mechanism could be explained by three hypotheses (Fig. 5): First, it is known that an elevated expression of \( \alpha_2/\delta-2 \) subunits increases presynaptic calcium channel abundance and current densities (Hoppa et al., 2012; Geisler et al., 2015) and thus likely enhances synaptic transmission (Zhou and Luo, 2015; Chen et al., 2018). Therefore, postsynaptic GABAARs could be recruited opposite these glutamatergic boutons in an attempt to suppress excessive glutamatergic excitation (Shrivastava et al., 2011). If this hypothesis applies, then GABAAR abundance at inhibitory synapses should not change upon \( \alpha_2/\delta-2 \) overexpression in GABAergic presynaptic terminals (Fig. 5A). Second, presynaptic \( \alpha_2/\delta-2 \), an extracellular protein extending far into the synaptic cleft, may contribute to the anchoring of postsynaptic GABAARs by a trans-synaptic mechanism. Such a specific role of \( \alpha_2/\delta-2 \) should be independent of the synapse type and thus GABAAR abundance should be affected when \( \alpha_2/\delta-2 \) is overexpressed in both glutamatergic and GABAergic synapses (Fig. 5B). Independent of the two hypotheses, the observed reduction in GLUR1 labeling opposite \( \alpha_2/\delta-2 \)-expressing glutamatergic terminals (Fig. 2) could be explained by GABAARs competing for AMPAR slots within dendritic spines. Alternatively, however, presynaptic \( \alpha_2/\delta-2 \) could also trans-synaptically induce an aberrant axonal wiring by guiding glutamatergic axons to GABAergic postsynaptic locations along dendritic shafts (Fig. 5C). In this scenario, the reduced GLUR1 expression would be a secondary effect because no GLUR1 receptors are to be expected in postsynaptic GABAergic synapses. To distinguish between these hypotheses, we next studied the consequence of presynaptic \( \alpha_2/\delta-2 \)-overexpression on the molecular composition of GABAergic synapses.

**MSNs express three presynaptic \( \alpha_2/\delta \) isoforms**

The cultures of hippocampal neurons used for the analysis of experiments presented in Figures 1, 2, 3, and 4 contain only 5–10% GABAergic interneurons (Obermaier et al., 2003). Consequently transfected GABAergic neurons are rarely observed.
Therefore, to quantitatively address the role of \(\alpha_3\delta\) subunits in GABAergic synapses, we established cultures of striatal neurons, which consist of ~95% inhibitory MSNs. Quantitative RT-PCR revealed that differentiated monocultured MSNs (24–25 DIV) expressed three neuronal \(\alpha_3\delta\) isoforms (\(\alpha_3\delta_1, \alpha_3\delta_2,\) and \(\alpha_3\delta_3\)) similar to the adult mouse striatum (Fig. 6A). Whereas \(\alpha_3\delta_2\) and \(\alpha_3\delta_3\) expression levels were similar in cultured neurons, the higher abundance of \(\alpha_3\delta_3\) transcripts in 8-week-old striatal tissue was consistent with previous studies (Cole et al., 2005). The strong expression of \(\alpha_3\delta_3\) was confirmed on cryosections of a mouse line carrying a lacZ reporter gene in the Cacna2d3 locus using \(\beta\)-galactosidase histochemistry (Fig. 6B). The \(\alpha_3\delta_4\) iso-
form, which is predominantly present in the retina, was hardly detectable and thus not considered for the following experiments. For studying presynaptic α2β-2 subunit expression in differentiated GABAergic neurons, we adapted a coculture system of GABAergic MSNs with glutamatergic cortical neurons (Penrod et al., 2011) (Fig. 7A). Lentiviral infection of MSNs with soluble eGFP just before starting the coculture allowed discriminating them from excitatory cortical neurons (Fig. 7B) based on their eGFP fluorescence. Immunolabeling of presynaptic and postsynaptic markers for excitatory (vGluT1, PSD-95) and inhibitory (vGAT) synapses demonstrated that glutamatergic and GABAergic synapses formed properly on dendritic spines and shafts, respectively (Fig. 7C–E). Moreover, patch-clamp recordings of spontaneous mEPSCs and mIPSCs in MSNs further confirmed the functionality of glutamatergic and GABAergic synapses in 14 DIV cocultures (Fig. 7F). Finally, live-cell labeling of MSNs cotransfected with HA-tagged α2δ subunits and soluble eGFP revealed that all α2δ isoforms are expressed on the surface of presynaptic axonal varicosities and thus can in theory contribute to synaptic functions in GABAergic neurons (Fig. 6C).

Presynaptic overexpression of α2δ-2 upregulates postsynaptic GABAαRs in inhibitory synapses

To distinguish between our hypotheses (Fig. 5), we coexpressed individual α2δ isoforms together with soluble eGFP in differentiated GABAergic MSNs. As expected, immunofluorescence analysis revealed that, in control MSNs (eGFP only), axonal varicosities were typically positive for presynaptic vGAT and postsynaptic GABAαR labeling (Fig. 8A, B). Postsynaptic GABAαRs were similarly localized opposite vGAT-positive terminals expressing the individual α2δ isoforms (Fig. 8B). However, quantitative analysis of GABAαR fluorescence intensities of individual cells revealed that GABAαR labeling was strongly (~2-fold) and significantly increased opposite boutons expressing the α2δ-2 isoform (Fig. 8C). Furthermore, presynaptic expression of α2δ-3 caused a 20% decrease of GABAαR labeling intensity compared with control (Fig. 8C). Plotting the cumulative frequency distribution of GABAαR fluorescence intensities of the single synapses demonstrates that presynaptic α2δ subunit isoforms differentially regulate postsynaptic GABAαR abundance in MSNs (Fig. 8D). GABAαR clusters were increased in size and intensity in the majority of synapses overexpressing α2δ-2 (blue line), causing a shift of the entire population to the right toward higher fluorescence intensities compared with control (eGFP only, green line). In contrast, ~24% and ~40% of all boutons expressing α2δ-1 (yellow line) and α2δ-3 (magenta line), respectively, lacked postsynaptic GABAαR labeling and the remaining population was left shifted toward smaller clusters. The slightly but significantly reduced GABAαR clustering opposite α2δ-3-overexpressing boutons (Fig. 8C) was associated with smaller presynaptic boutons (27% smaller than control; Fig. 8E) and decreased presynaptic vGAT content (46% lower than control; Fig. 8F). Conversely, presynaptic expression of α2δ-2, which strongly increased postsynaptic GABAαR clustering, affected neither bouton size nor vGAT labeling intensity.

Similar to mismatched glutamatergic synapses (see above), we used superresolution gSTED microscopy to visualize the subsynaptic localization of postsynaptic GABAαR clusters in cultured MSNs (Fig. 3C). MSNs were cotransfected with α2δ-2 and soluble mCherry or mCherry only (control). In both conditions, postsynaptic GABAαR clusters appeared closely opposed to mCherry-positive presynaptic boutons, thus confirming their localization in the postsynaptic membrane. Moreover, similar to hippocampal neurons, the GABAαR pattern was either visible as a thin line (side view; Fig. 3C, first and third column and Fig. 3B, top) or a ring-like structure (planar view; Fig. 3C, second and fourth column and Fig. 3B, bottom). Importantly, gSTED imaging further confirmed that presynaptic expression of α2δ-2 increased GABAαR density at the corresponding postsynaptic side.

α-Nrxns modulate postsynaptic GABAαR abundance set by presynaptic α2δ-2

Thus far, our results revealed that presynaptic α2δ-2 strongly induced postsynaptic GABAαR receptor clustering in both glutamatergic and GABAergic synapses, suggesting that α2δ-2 acts trans-synaptically according to our hypothesis outlined in Figure 5. This means that α2δ-2 could either directly interact with postsynaptic receptors or may act via other synaptic organizer molecules such as α-Nrxns. Presynaptic α-Nrxns are synaptic cell adhesion molecules whose general importance in a variety of synaptic functions is well established (Reissner et al., 2013; Stödhof, 2017). Several lines of evidence implicate α-Nrxns as interesting candidates for mediating a possible indirect interaction between α2δ-2 and GABAαRs. First, α-Nrxns have been linked functionally to high-voltage-dependent Ca2+ channels, which has strong effects on presynaptic neurotransmitter release (Misserl et al., 2003; Chen et al., 2017). Second, α-Nrxns have been implicated as organizing molecules at both excitatory glutamatergic and inhibitory GABAergic synapses by promoting clustering of postsynaptic AMPARs (Heine et al., 2008; Aoto et al., 2013, 2015) and GABAαRs (Kang et al., 2008). Third, the presynaptic complex of α-Nrxn and its ligand neurexin-1 is characteristic for inhibitory interneurons and can induce postsynaptic GABAαR recruitment when ectopically expressed at excitatory synapses (Born et al., 2014). Fourth, neurexin-1α has recently been shown to modulate P/Q-type (Brockhaus et al., 2018) and N-type (Tong et al., 2017) calcium channels by cooperating with α2δ subunits. Therefore, to test the possibility that upregulation of postsynaptic GABAαRs depends on α-Nrxns, we expressed α2δ-2 in glutamatergic synapses lacking all three α-Nrxn isoforms (Misserl et al., 2003). We transfected eGFP alone or in combination with α2δ-2 or α2δ-3 in hippocampal cultures from WT and α-Nrxn TKO mice. Immunofluorescence analysis revealed that, as expected, in control hippocampal neurons, axonal varicosities were typically positive for presynaptic vGluT1 but negative for postsynaptic GABAαR labeling (Fig. 9A–C, WT control). Consistent with our previous observations, presynaptic expression of α2δ-2 induced the formation of mismatched synapses (Fig. 9A–C, WT + α2δ-2), whereas expression of α2δ-3 had no effect (p = 0.64; Fig. 9A–C, WT + α2δ-3). Strikingly, expression of presynaptic α2δ-2 in α-Nrxn TKO neurons induced postsynaptic GABAαR clustering opposite glutamatergic boutons more strongly than in WT (Fig. 9A–C, α-Nrxn TKO + α2δ-2). Quantitative analysis revealed that this effect was three times higher compared with the WT + α2δ-2 condition, suggesting that the propensity of α2δ-2 in inducing mismatched synapses was enhanced in the absence of α-Nrxns. In fact, increased GABAαR abundance was already visible in nontransfected α-Nrxn TKO synapses in which the presence of endogenous α2δ-2 may be responsible for a significantly increased baseline level of mismatched GABAαRs (p = 0.023; Fig. 9A–C). This is an important result because it suggests that no overexpression of α2δ-2 is needed to induce postsynaptic GABAαR clustering, but an imbalance between α2δ-2 and α-Nrxns. These differential effects can be best compared by blotted the cumulative frequency distribution of GABAαR fluorescence intensities of
Figure 7. Presynaptic and postsynaptic differentiation of GABAergic MSNs. **A**, Schematic illustration of the coculture procedure: Cerebral hemispheres were dissected and stripped of meninges (1). Parts of the prefrontal cortex and striatum were dissected as shown (2). Striatal and cortical tissue was collected separately and dissociated using trypsin-EDTA and trituration (3). Cortical neurons were plated on poly-L-lysine-coated coverslips while striatal neurons were transfected with soluble eGFP (4). Subsequently, striatal neurons were plated onto cortical neurons in a ratio of 3:2 (5) and maintained above a glial feeder layer (6).

**B**, Lentiviral infection or lipofection with soluble eGFP allowed discriminating MSNs from cortical neurons.

**C**–**E**, Double immunofluorescence of striatal–cortical cocultures (24–26 DIV) with presynaptic and postsynaptic markers for excitatory and inhibitory synapses. Neuronal morphology is outlined by eGFP. **C**, GABAergic synapses of transfected eGFP-positive MSNs showed immunoreactivity for vGAT, whereas vGLUT1 was absent. **D**, Axons of cortical neurons formed excitatory synapses on MSN spine heads (vGLUT1, white arrowheads), whereas GABAergic synapses were located on the dendritic shaft (vGAT, blue arrowhead). **E**, Labeling of PSD-95 in spine heads opposite synapsin-positive presynaptic terminals further indicated the presence of functional excitatory synapses on MSNs. **F**, Patch-clamp analysis of mIPSCs and mEPSCs in 14 DIV neurons confirmed the functionality of GABAergic and glutamatergic synapses. Representative micrographs of two independent cultures are shown. Scale bars, 50 μm (**B**), 3 μm (overview), and 1 μm (magnified selections; **C**–**E**).
all individual synapses analyzed (Fig. 9D). Compared with WT (control, green line), the entire population of synapses was right-shifted toward larger GABAAR clusters in α-Nrxn TKO neurons (dashed green line). Presynaptic expression of transfected α₂δ-2 strongly shifted the distributions toward larger GABAAR clusters in WT (+α₂δ-2, blue line) and α-Nrxn TKO neurons (TKO + α₂δ-2, dashed blue line). Presynaptic expression of α₂δ-3 had no effect in WT neurons, which, due to the lack of mismatched synapses, basically displayed only background GABAAR intensity (+α₂δ-3, magenta line). Interestingly, overexpression of α₂δ-3 in α-Nrxn TKO neurons reduced the elevated GABAAR clusters to control levels (TKO + α₂δ-3, dashed magenta line). This possibly indicates that overexpression, abundant α₂δ-3 blocked the effect by endogenous α₂δ-2 on baseline GABAAR intensity in TKO synapses. Together, our data suggest that presynaptic α₂δ-2 potently induced mismatched synapse formation with GABAAR clustering in the absence of α-Nrxns. This demonstrates that the trans-synaptic role of α₂δ-2 in recruiting postsynaptic GABAARs is independent of α-Nrxns. However, the fact that GABAAR abundance was already increased in α-Nrxn TKO synapses without overexpression of α₂δ-2 and that the abundance could be differentially modulated by presynaptic α₂δ-2 and α₂δ-3 suggests a cooperative and trans-synaptic activity of α₂δ subunits and α-Nrxns in fine-tuning postsynaptic GABAAR levels.

**α₂δ-2 induces the aberrant wiring of glutamatergic axons to GABAergic postsynaptic positions**

Our experiments described above demonstrate that presynaptic α₂δ-2 induces the recruitment of postsynaptic GABAARs opposite glutamatergic and GABAergic presynaptic boutons. Moreover, this effect of α₂δ-2 is independent of the presynaptic neurotransmitter identity, suggesting a trans-synaptic activity in recruiting/anchoring postsynaptic GABAARs. As hypothesized (Fig. 5), such a trans-synaptic role of α₂δ-2 could result in two types of mismatched synapses: the GABAARs could either be recruited to postsynaptic compartments of dendritic spine synapses with a con-
comitant reduction of the resident glutamate receptors or α₂δ-2 could induce an altered wiring of glutamatergic axons to GABAergic postsynaptic locations along the dendritic shaft.

To distinguish between these two possibilities, we focused more closely on the position of the mismatched synapses along the postsynaptic neurons. First, it is important to emphasize that α₂δ-2 induced the upregulation of postsynaptic GABAergic components only at the sites contacted by the presynaptic axon without affecting the neighboring synapses. This is best visualized by the observation that gephyrin fluorescence intensity was 4.5 times higher in α₂δ-2-positive mismatched synapses compared with control (eGFP only). This effect is much stronger in α-Nrxn TKO synapses compared with WT neurons (3-fold higher GABAAR fluorescence intensity).

**Figure 9.** α-Nrxn modulates the effect of α₂δ-2 on GABAARs. Representative immunofluorescence micrographs of cultured hippocampal neurons (20–25 DIV) prepared from WT or α-Nrxn TKO mice. **A, B.** In control boutons (eGFP only), potential glutamatergic synapses are positive for presynaptic vGLUT1 but negative for postsynaptic GABAAR staining (summarized in sketch, B). **C.** Consistent with our initial observation (see Fig. 1), presynaptic expression of α₂δ-2 (blue arrowheads) induces postsynaptic GABAAR localization opposite vGLUT1-positive nerve terminals in WT neurons. Most importantly, in α-Nrxn TKO synapses, the expression of α₂δ-2 strongly induced postsynaptic GABAAR localization (blue asterisks). **D.** Postsynaptic GABAARs clusters (integrated intensities) are significantly increased opposite glutamatergic nerve terminals expressing α₂δ-2 compared with control (eGFP only). This effect is much stronger in α-Nrxn TKO synapses compared with WT neurons (3-fold higher GABAAR fluorescence intensity). **D.** Cumulative frequency distribution further reveals that the vast majority of glutamatergic boutons are positive for postsynaptic GABAARs in WT + α₂δ-2 (88%) and TKO + α₂δ-2 (97%) and that the population is shifted toward larger and more intense clusters. However, this analysis also demonstrates that mismatched GABAARs already form opposite α-Nrxn TKO synapses, although at a lower overall intensity. Values for individual cells (dots) and means (lines) ± SEM are shown. Values were normalized to the WT control within each culture preparation. Data from two independent culture preparations and 12–19 cells were analyzed within each condition. Statistics: C, ANOVA on log10-transformed data with Holm–Sidak post hoc analysis: genotype: F(1,86) = 5.3, p = 0.023; transfection: F(2,86) = 49.0, p < 0.001; genotype × transfection: F(2,86) = 3.7, p = 0.03; post hoc: *p < 0.05, ***p < 0.001. Asterisks in graphs indicate the significant difference compared with control. Scale bar, 3 μm.
Figure 10. Mismatched synapses show characteristics of GABAergic shaft synapses. A, D, Representative immunofluorescence micrographs of cultured hippocampal neurons cotransfected with \( \alpha_2 \delta-2 \) and soluble eGFP. Co-labeling of transfected neurons (24 DIV) for postsynaptic GLUR1 and gephyrin is shown. A, Presynaptic expression of \( \alpha_2 \delta-2 \) induces a strong upregulation of postsynaptic gephyrin (asterisk) compared with neighboring endogenous GABAergic synapses situated on the same dendrite (arrowheads). Although GLUR1 expression was reduced in mismatched synapses (*; see Fig. 2), it was absent in endogenous GABAergic synapses (arrowheads, see sketch in B). C, Quantitative analysis of gephyrin fluorescence intensities in GABAergic neighboring and mismatched synapses. Dots represent values for individual boutons (mismatched synapses) and means of 10 endogenous clusters measured per image. Results were normalized to endogenous gephyrin intensities of neighboring synapses within each culture preparation. Data from three independent culture preparations are shown. Statistics: \( t \) test on log10-transformed data: \( t_{\text{GFP}} = 9.5; * * * p < 0.001 \). D, Left, Control boutons transfected with eGFP only form glutamatergic synapses on dendritic spines (gephyrin negative, GLUR1 positive; white arrowheads). Right, Expression of \( \alpha_2 \delta-2 \) in putative glutamatergic boutons induces the formation of mismatched synapses (gephyrin-positive, GLUR1-negative) along the shaft of the dendrite (blue arrowheads). Dendritic morphology of untransfected neurons was outlined according to GLUR1 labeling. Scale bars, 5 \( \mu \text{m} \) (A) and 4 \( \mu \text{m} \) (B).

corroborated by a highly significant reduction of the mean distance from the contacting presynaptic boutons to the postsynaptic dendritic shafts in \( \alpha_2 \delta-2 \)-transfected compared with control axons (mean distance \pm SEM: control: 0.92 \pm 0.05 \( \mu \text{m} \); \( \alpha_2 \delta-2 \): 0.54 \pm 0.05 \( \mu \text{m} \); Mann–Whitney \( U \) test: \( p < 0.001 \); Fig. 11C, bottom). Importantly, applying the same methodological approach for the coculture of GABAergic MSNs and glutamatergic cortical neurons (Fig. 11D, also see Fig. 7) revealed that axonal varicosities from control cortical neurons generally contacted dendritic spines of postsynaptic inhibitory MSNs (total of 65 synapses: 83% spine, 1.5% shaft, 15.5% unclear; Fig. 11 E, F, top). In contrast, the presynaptic expression of \( \alpha_2 \delta-2 \) shifted the preferential contact points of cortical boutons to dendritic shafts of MSNs (total of 51 synapses: 19.6% spine, 72.6% shaft, 7.8% unclear; Fig. 11 E, F, bottom, gephyrin labeling and position at dendritic shaft). These findings demonstrate that the \( \alpha_2 \delta-2 \)-induced aberrant wiring is independent of the identity of the postsynaptic neuron.

Together, our results provide strong evidence that overexpression of presynaptic \( \alpha_2 \delta-2 \) induces the aberrant wiring of glutamatergic axons to postsynaptic GABAergic locations, resulting in a mismatch of presynaptic excitatory neurotransmitters and postsynaptic inhibitory receptors.

Reduced synaptic transmission in aberrantly wired synapses

Excitatory and inhibitory synapses require matched presynaptic and postsynaptic specializations to enable efficient and synchronous neurotransmission. To assess the functional consequences of \( \alpha_2 \delta-2 \) overexpression in presynaptic boutons of aberrantly wired glutamatergic synapses, we performed whole-cell patch-clamp recordings on synaptically connected pairs of isolated neurons grown on poly-

\( l \)-lysine dots (Fig. 12A). We first investigated whether AMPAR-mediated evoked EPSCs (eEPSCs) between individual pairs of hippocampal neurons were altered by the lentiviral overexpression of \( \alpha_2 \delta-2 \) in the presynaptic neuron. Isolated synaptic currents were measured in the postsynaptic neuron in response to an action potential induced in the presynaptic neuron. The presynaptic action potential followed a given waveform recorded from WT hippocampal neurons (Fig. 12B). This experimental setup allowed to directly compare synaptic transmission in glutamatergic control synapses (presynaptic cell = control, postsynaptic cell = \( \alpha_2 \delta-2 \) overexpressing) and mismatched synapses (presynaptic cell = \( \alpha_2 \delta-2 \) overexpressing, postsynaptic cell = control). In each of seven neuronal pairs recorded, the eEPSC amplitude was lower in the mismatched compared with the control pair (on average reduced by 48%) (Fig. 12C). To discriminate whether the eEPSC reduction is the result of the reduced postsynaptic AMPA receptor expression associated with the aberrantly wired axons or if presynaptic \( \alpha_2 \delta-2 \) reduces release probability, we next measured synaptic paired-pulse response ratios (PPRs) (Katz and Miledi, 1968; Zucker and Regehr, 2002). eEPSCs were recorded in the postsynaptic neuron in response to two action potentials elicited in the presynaptic neuron at different interstimulus intervals. As previously reported for hippocampal pyramidal neurons, calculated PPRs displayed facilitation (Fig. 12D) with a decline in the magnitude at increasing intervals (Dobrunz et al., 1997). Overexpression of \( \alpha_2 \delta-2 \) in presynaptic neurons slightly increased facilitation at 25 and 50 ms compared with the initial 10 ms interval. The strong reduction of eEPSC amplitudes is thus consistent with a reduced number of functional synapses caused by the aberrant wiring of glutamatergic \( \alpha_2 \delta-2 \)-expressing boutons with GABAergic postsynaptic sites.
Conversely, the only moderately altered paired-pulse facilitation (PPF) indicates that the decreased amplitude of eEPSCs was not caused by a reduced presynaptic release probability. The observed slightly increased PPF may reflect an increased abundance of presynaptic calcium channels caused by the overexpression of α₂δ subunits, as has been previously demonstrated (Hoppa et al., 2012).

**Lack of exon 23 in α₂δ-2 splice variants mediates the trans-synaptic effect on GABA<sub>R</sub>s**

Our results revealed a specific trans-synaptic function of only one of the three expressed α₂δ isoforms, namely α₂δ-2. Sequence comparisons of the previously known alternatively spliced regions (Hobom et al., 2000) identified three potential splice sites in α₂δ-2 (Fig. 13A, scheme): inclusion or absence of exon 23 and two alternative splice sites at exons 30 and 38. Using homology modeling, we explored the potential implication of these sequence differences on the structural fold of α₂δ-2 (Fig. 14) based on the recently suggested α₂δ-1 cryo-EM structure (Wu et al., 2016). Inclusion of exon 23 resulted in the formation of an extra loop that might disrupt an α-helix (Figs. 13B, 14B), whereas alternative splicing of exons 30 and 38 predicted only small changes in the secondary structure (Fig. 14C,D). Therefore, to test whether alternative splicing at these sites is involved in the trans-synaptic function of α₂δ-2, we cloned and compared three splicing variants (α₂δ-2-v1, α₂δ-2-v2, and α₂δ-2-v3) induced the clustering of postsynaptic GABA<sub>R</sub>αs on dendrite shafts (GABA<sub>R</sub>MSNs, Fig. 10C). To determine the potential of the splice variants in inducing mismatched synapses, we transfected WT hippocampal neurons with the three distinct α₂δ-2 variants together with soluble eGFP. Strikingly, only the splice variants lacking exon 23 (α₂δ-2-v1 and α₂δ-2-v3) induced the clustering of postsynaptic GABA<sub>R</sub>αs opposite transfected glutamatergic nerve terminals (Fig. 13C, ΔExon 23). The fact that α₂δ-2-v1 and α₂δ-2-v3 both induced mismatched synapse formation excludes a major role of exons 30 and 38, which differ in α₂δ-2-v1 and α₂δ-2-v3. In contrast, α₂δ-2-v2, which contains the exon 23 insert, failed completely to induce mismatched synapse formation (Fig. 13C, + Exon 23), indicating that the trans-synaptic function
of α₂δ-2 on GABA<sub>R</sub>s is transcriptionally regulated. Together, our results have uncovered a highly specific role of the α₂δ-2 subunit of voltage-gated calcium channels in clustering postsynaptic GABA<sub>R</sub>s, an important process that is further regulated by the presence of α-Nrxns and by alternative splicing.

Discussion

The experiments described here reveal that presynaptic expression of a single splice variant of α₂δ-2 trans-synaptically regulates postsynaptic GABA<sub>R</sub> abundance. Therefore, presynaptic α₂δ-2 modulates postsynaptic GABA<sub>R</sub> clustering independently of the presynaptic neurotransmitter, and alters synaptic transmission in mismatched synaptic pairs. These results demonstrate for the first time that presynaptic α₂ sub-units are able to trans-synaptically regulate synapse specification and axonal wiring.

α₂δ-2 induces the formation of mismatched synapses via a trans-synaptic mechanism

Excitatory and inhibitory synapses are characterized by their distinct molecular composition and subcellular localization in neurons. The proper alignment of presynaptic and postsynaptic specializations is a hallmark of synapse formation and a prerequisite for efficient and synchronous excitatory and inhibitory synaptic transmission (Lardi-Studler and Fritschy, 2007). Only a small number of studies have reported mismatched synapses in vitro. In micro-island cultures of autaptic hippocampal neurons, the vast majority of GABA<sub>R</sub>s and gephyrin clusters are located opposite glutamatergic terminals, suggesting that postsynaptic compartments can be recruited without the proper presynaptic input (Rao et al., 2000; Christie et al., 2002). Moreover, in low-density cultures of hippocampal and cerebellar granule neurons, mismatches of presynaptic and postsynaptic proteins can form during early developmental stages, but are essentially absent in differentiated cultures (Brüning et al., 2002; Anderson et al., 2004; Studler et al., 2005). In contrast to these studies, the mismatched synapses reported in our study are specifically induced by the presynaptic expression of α₂δ-2 and are independent from neurotransmitter input, suggesting that α₂δ-2 operates via a different mechanism.

Mixed synapses with glutamatergic and GABAergic properties have also been detected in different brain areas, indicating a functional role in vivo. For example, postsynaptic GABA<sub>R</sub>s are present opposite presynaptic glutamatergic mossy fiber axon terminals in the cerebellar cortex and hippocampus (Nusser et al., 1996a,b; Bergersen et al., 2003). Physiologically, these receptors may be involved in fine-tuning excitatory synaptic transmission in response to GABA spillover from surrounding synapses (Isacson et al., 1993). Although we cannot completely rule out GABA spillover from adjacent neurons, it is important to note that the α₂δ-2-induced mismatched synapses do not express presynaptic vGAT. Therefore, the recruitment of postsynaptic GABA<sub>R</sub>s is entirely independent of presynaptic GABA release. These findings are consistent with a recent report showing that, even in the absence of presynaptic glutamate release, dendritic spines are formed and AMPARs are present in glutamatergic synapses (Siggler et al., 2017).

Generally, excitatory and inhibitory synapses are mostly targeted to different subcellular regions of the postsynaptic neuron (Lardi-Studler and Fritschy, 2007). Therefore, glutamatergic

Figure 12. Presynaptic expression of α₂δ-2 affects synaptic transmission in aberrantly wired synapses. A–C, Experimental paradigm to study the functional consequences of presynaptic α₂δ-2 overexpression in mismatched glutamatergic synapses of cultured hippocampal neurons. A, Neurons were cultured in pairs of two synaptically connected cells in which one represents an untransfected control neuron (arrowhead) and the other an α₂δ-2-overexpressing neuron (eGFP labeled). B, Whole-cell patch-clamp recordings of excitatory synaptic transmission (13–18 DIV). An action potential in neuron 1 elicited by a given AP waveform recorded from WT hippocampal neurons caused eEPSCs in neuron 2. Synaptic transmission was analyzed in both directions of synaptically connected pairs. Therefore, putative glutamatergic spine synapses (control innervating neuron 2) and putative mismatched synapses (α₂δ-2-overexpressing innervating control neuron) could be directly compared within the same pair. C, D, Quantitative analysis of mean eEPSC peak amplitudes (C) and paired-pulse response ratios (PPRs) as a measure for synaptic plasticity (D). C, eEPSCs were strongly reduced (48%) in mismatched synapses. D, Dashed line shows the boundary between paired-pulse depression (PPR < 1) and facilitation (PPR > 1). Note the slight significant increase in facilitation in mismatched synapses at 25 and 50 ms compared with the initial 10 ms interval. Values for individual pairs (dots) and means (lines) ± SEM are shown. Data from three independent culture preparations from seven (C) and six (D) pairs were analyzed. Statistics: C, paired t test: t<sub>60</sub> = 4.6; **<i>p</i> < 0.01; D, two-way repeated-measures ANOVA: condition: F<sub>(1,45)</sub> = 0.02, <i>p</i> = 0.9; interval: F<sub>(5,45)</sub> = 11.1, <i>p</i> < 0.001; condition × interval: F<sub>(5,45)</sub> = 2.7, <i>p</i> = 0.03; Holm–Sidak post hoc analysis: **<i>p</i> = 0.004, ***<i>p</i> < 0.001 compared with 10 ms within α₂δ-2 to control. Scale bar, 20 μm.
boutons predominantly contact postsynaptic dendritic spines (Peters, 2002), whereas GABAergic nerve terminals mostly connect to the shaft of the dendrite (Markram et al., 2004). Nevertheless, converging lines of evidence demonstrate morphologically mixed synapse types in different parts of the brain. In the adult neocortex, for example, two distinct types of GABAergic synapses are positioned either on the shaft or spine of the dendrite, which are suggested to have different roles in shaping dendritic activity (Chen et al., 2012). It is hypothesized that these constellations facilitate the shunting of excitatory currents, thereby balancing excitatory and inhibitory neurotransmission (Nusser et al., 1996a; Root et al., 2014). Along these lines, we proposed that the underlying mechanism of α2β-2-induced mismatched synapses could be explained by a compensatory upregulation of postsynaptic GABAARs due to increased excitatory transmission upon α2β overexpression (Zhou and Luo, 2015; Chen et al., 2018). However, several of our observations argue against this theory. First, using distinct experimental paradigms, we show here that α2-1 and α2-3, which both enhance excitatory synaptic transmission and increase presynaptic release probability (Hoppa et al., 2012), do not induce postsynaptic clustering of synaptic GABAAR subtypes. Second, the effect of α2-2 is independent of the presynaptic neurotransmitter identity, thus providing compelling evidence for a trans-synaptic function. Third, solely α2-2 splice variants lacking exon 23 specifically induced the recruitment of postsynaptic GABAARs opposite glutamatergic nerve terminals. Fourth, the effect of α2-2 is restricted to transfected synapses, emphasizing a synapse-specific role rather than a global alteration of GABAergic postsynaptic components.

Presynaptic α2β subunits differentially modulate postsynaptic GABAAR abundance via distinct mechanisms

We found diverging effects of presynaptic α2β-2 and α2β-3 on the postsynaptic GABAAR abundance of GABAergic MSNs because α2β-2 strongly increased and α2β-3 reduced postsynaptic
Evidence for an αδ-2-mediated trans-synaptic signaling cascade

To date, surprisingly little is known on the specific signals imperative for the proper alignment of inhibitory synapses and the targeting of GABAARs and gephyrin to the postsynaptic compartment. In vitro cultured neurons provide an excellent and reduced model system allowing us to address basic cell biological mechanisms. Most importantly, they are less prone to potential compensatory mechanisms compared with the in vivo situation (Dong et al., 2007; Brown et al., 2016; Chen et al., 2017). Likewise, overexpression of proteins in their native environment provides a powerful experimental tool but needs to be carefully discussed, particularly in relation to the specific physiological roles. Therefore, it is conceivable that an aberrantly high accumulation of presynaptic αδ-2 proteins may induce artificial synaptic connections. Nevertheless, our study provides several lines of evidence supporting a presynaptic role of αδ-2 in the trans-synaptic alignment of synapses and receptor clustering. First, we observed that presynaptic expression of a single splice variant of αδ-2 trans-synaptically regulates postsynaptic GABAAR abundance. Second, overexpression of αδ-2 specifically induces an aberrant wiring of glutamatergic axons to postsynaptic GABAergic sites independently of the postsynaptic neuron type (Fig. 11). Third, whereas mismatched synapses lack glutamate receptors, they contain different synaptic GABAAR subtypes and gephyrin. Importantly, the αδ subunits regulate presynaptic differentiation and postsynaptic GABAAR abundance by two independent mechanisms. Although expression of αδ-3 induces smaller synapses associated with a reduced postsynaptic receptor density, presynaptic αδ-2 modulates postsynaptic GABAAR abundance but does not affect presynaptic bouton size. Altered expression of mammalian αδ-3 (Pirone et al., 2014) and its invertebrate homologs (Kurshan et al., 2009; Caylor et al., 2013) has previously been shown to affect the size and morphology of presynaptic boutons of auditory nerve fibers and motoneurons, respectively.

GABAAR levels (Fig. 8). In contrast to presynaptic αδ-2 expression, which modulated the postsynaptic composition without apparently affecting the presynaptic side, the effect of αδ-3 was associated with smaller presynaptic boutons and decreased presynaptic vGAT content. Smaller presynaptic boutons may also explain the reduced GLUR1 content opposite αδ-3-expressing synapses (Fig. 2). Together, these data suggest that presynaptic strongly reduced GLUR1 expression (Fig. 2) in the mismatched and aberrantly wired synapses is a secondary effect because no AMPARs are to be expected in postsynaptic GABAergic synapses (Fig. 5C, model). Fourth, gephyrin abundance is higher in αδ-2-induced mismatched synapses compared with untransfected endogenous GABAergic synapses. Considering that gephyrin immobilizes synaptic GABAAR clusters in hippocampal neurons

Figure 14. Splicing sites of αδ-2 and implications on secondary structure. A, Using homology modeling, we tested the potential consequences of alternative splicing on the structure prediction of three distinct αδ-2 splice variants (v1–v3) based on the high-resolution structure of αδ-1 (PDB code: 5GU; Wu et al., 2016). Although some of the longer loops generally seemed to be quite flexible and differently orientated in all three models (gray arrows), alternative splicing of exons 23, 30, and 38 resulted in differences between secondary structure elements in distinct variants (boxed regions in B–D). B, Higher magnification showing that the inclusion of exon 23 in αδ-2-v2 suggests the formation of an extra loop (arrowhead and green selection in overlay), leading to the disruption of an α-helix present in αδ-2-v1 and αδ-2-v3 (see also Fig. 13). C, Structure modeling further proposes that the insertion of three base pairs in exon 30 of αδ-2-v3 causes the formation of a short α-helix in the depicted loop, which is absent in αδ-2-v1 and αδ-2-v2. D, Moreover, models implicate that alternative splicing of exon 38 affects the length of a β-sheet at this position, which is longer in αδ-2-v1 and αδ-2-v2.
First, the presynaptic effect of \( \alpha_{2}\delta-2 \) overexpression in TKO was already significantly increased, which shows that no other factors are required for its functional effect. Alternatively, the loss of \( \alpha_{2}\delta-2 \) is needed for inducing postsynaptic \( \text{GABA}_{\alpha}\text{Rs} \) clustering. This finding may indicate that the presence of \( \alpha_{2}\text{-Nrxn}\alpha \) negatively influences \( \text{GABA}_{\alpha}\text{Rs} \) recruitment by \( \alpha_{2}\delta-2 \). Therefore, alterations in the balance of presynaptic \( \text{GABA}_{\alpha}\text{Rs} \) may act as similarly specific trans-synaptic organizers in distinct synapses. Finally, our findings allow hypothesizing that presynaptic \( \alpha_{2}\delta \) subunits are critical determinants of synapse specificity and connectivity. Therefore, alterations in the balance of presynaptic \( \alpha_{2}\delta \) isoforms may underlie synaptic plasticity and, conversely, pathological axonal wiring observed in neurological disorders.

Future implications and potential relevance to neurological disorders

One important finding presented here is that only \( \alpha_{2}\delta-2 \) splice variants lacking exon 23 induced a mismatched localization of postsynaptic \( \text{GABA}_{\alpha}\text{Rs} \). This is interesting for several reasons. First, the presynaptic effect of \( \alpha_{2}\delta-2 \) lacking a single spliced exon is highly specific and thus argues against a simple unspecific effect caused by homologous overexpression of \( \alpha_{2}\delta \) isoforms in our experimental paradigm. Second, in light of discussing potential interaction mechanisms (see above), it is tempting to speculate that \( \alpha_{2}\delta-2 \) interacts with its partners via the region surrounding the lacking exon 23, which will be tested in future experiments. Third, our findings show that the trans-synaptic function of \( \alpha_{2}\delta-2 \) may be independent of the calcium channel complex. This is further supported by a previous study showing that heterologous coexpression of different \( \alpha_{2}\delta-2 \) splice variants with various \( \alpha_{\sub} \) subunits caused similar effects on calcium channel current densities and activation/inactivation kinetics (Hobom et al., 2000). Fourth, and most importantly, alternative splicing has also been described for mouse and human genes encoding for \( \alpha_{2}\delta-1 \) and \( \alpha_{2}\delta-4 \) (Lana et al., 2014; Bacchi et al., 2015). This is particularly interesting considering the previously identified associations between \( \alpha_{2}\delta \) subunits with neurological disorders (Chioza et al., 2009; Edwardson et al., 2013; Pippucci et al., 2013; Vergult et al., 2015; Butler et al., 2018; Valence et al., 2018). Two distinct splice site mutations in \( \text{CACNA2D2} \), for example, lead to the truncation of the protein, causing epilepsy, dyskinesia, and cerebellar atrophy in one patient (Pippucci et al., 2013), whereas the other mutation is associated with congenital ataxia (Valence et al., 2018). These clinically observed phenotypes are similar to the previously described phenotype of \( \text{Cacna2d2} \) mutant or null mouse models (Barclay et al., 2001; Brodbeck et al., 2002; Ivanov et al., 2004) and may be caused by the loss of functional \( \text{GABA}_{\alpha} \) synaptic vesicles and the consequential increased excitatory activity. Moreover, \( \alpha_{\sub} \) subunits are potential risk genes for autism spectrum disorders (Jossifov et al., 2012; De Rubeis et al., 2014) and schizophrenia (Purcell et al., 2014; Moons et al., 2016), diseases classically associated with altered neuronal connectivity (Belmonte et al., 2004; Fornito and Bullmore, 2015). The findings presented here provide a possible mechanistic explanation for why altered \( \alpha_{\sub} \) subunit expression may be linked to neurological disorders. However, for the future understanding of the precise underlying disease mechanisms, it will be necessary to reveal whether all \( \alpha_{\sub} \) subunit isoforms can act as similarly specific trans-synaptic organizers in distinct synapses.

References

Anderson TR, Shah PA, Benson DL. (2003) Muturation of glutamatergic and \( \text{GABA}_{\alpha} \)ergic synaptic composition in hippocampal neurons. Neuropharmacology 47:694–705.

Aoto J, Martielli DC, Malenga RC, Tabuchi K, Sudhof TC (2013) Presynaptic neurexin-3 alternative splicing trans-synaptically controls postsynaptic AMPA receptor trafficking. Cell 154:75–88.

Aoto J, Foldy C, Iclus SM, Tabuchi K, Sudhof TC (2015) Distinct circuit-dependent functions of presynaptic neurexin-3 at \( \text{GABA}_{\alpha} \)- and glutamatergic synapses. Nat Neurosci 18:997–1007.

Arama J, Abitbol K, Goffin D, Fuchs C, Sihra TS, Thomson AM, Jovanovic JN (2015) \( \text{GABA}_{\alpha} \) receptor activity shapes the formation of inhibitory synapses between developing medium spiny neurons. Front Cell Neurosci 9:290.

Ba-Abbad R, Arno G, Cars K, Stirrup K, Penkett CJ, Moore AT, Michaelides M, Raymond FL, Webster AR, Holder GE (2016) Mutations in \( \text{CACNA2D4} \) cause distinctive retinal dysfunction in humans. Ophthalmology 123:668–671.e2.

Bacchi N, Messina A, Bartischer V, Dassi E, Provenzano G, Bozzi Y, Demontis M, Raymond FL, Webster AR, Holder GE (2016) Mutations in \( \text{CACNA2D4} \) cause distinctive retinal dysfunction in humans. Ophthalmology 123:668–671.e2.

Bacchi N, Messina A, Bartischer V, Dassi E, Provenzano G, Bozzi Y, Demontis M, Raymond FL, Webster AR, Holder GE (2016) Mutations in \( \text{CACNA2D4} \) cause distinctive retinal dysfunction in humans. Ophthalmology 123:668–671.e2.

Barclay J, Balaguer N, Mione M, Ackerman SL, Letts VA, Brodbeck J, Canti C, Meir A, Page KM, Kasumi K, Perez-Reyes E, Lander ES, Frankel WN, Gardiner RM, Dolphin AC, Rees M (2001) Ducky mouse phenotype of \( \text{Cacna2d2} \) mutant or null mouse models. Curr Opin Neurobiol 11:609–6104.

Bauer CS, Tran-Van-Minh A, Kadurin I, Dolphin AC (2010) A new look at calcium channel \( \alpha_{2}\sub \) subunits. Curr Opin Neurobiol 20:563–571.

Belmonte MK, Allen G, Beckel-Mitchener A, Boulanger LM, Carper RA, Webb SJ (2004) Autism and abnormal development of brain connectivity. J Neurosci 24:9228–9231.

Bergersen L, Ruiz A, Bjalke JG, Kullmann DM, Gundersen V (2003) GABA and \( \text{GABA}_{\alpha} \) receptors at hippocampal mossy fibre synapses. Eur J Neurosci 18:931–941.

Bonansco C, Fuenzalida M (2016) Plasticity of hippocampal excitatory–inhibitory balance: missing the synaptic control in the epileptic brain. Neuroplast 2016:3607038.

Borger LS, Kirschner J, Sherrington J, Pettem D, Kiefer F, Heine M, Pape HC, Misserl M (2014) Mutations of synaptic function through the \( \alpha_{\sub} \)-neurexin-specific ligand neurexophilin-1. Proc Natl Acad Sci U S A 111:E1274–E1283.
subunits regulate CA2+ influx through CaV2.1 channels. J Neurosci 38:8277–8294.

Brodbeck J, Davies A, Courtney JM, Meir A, Balagurov N, Canti C, Moss FJ, Page KM, Pratt WS, Hunt SP, Barclay J, Rees M, Dolphin AC (2002) The diisulphide mutagenesis in CaV2.3 results in altered purkinje cell morphology and is associated with the expression of a truncated alpha 2 delta-2 protein with abnormal function. J Biol Chem 277:7684–7693.

Brown LE, Nicholson MW, Arama JE, Mercer A, Thomson AM, Jovanovic JN (2016) γ-aminobutyric acid type A (GABA(A) receptor) subunits play a direct structural role in synaptic contact formation via their N-terminal extracellular domains. J Biol Chem 291:13926–13942.

Brüning I, Suter A, Knuesel I, Lüscher B, Fritschi JM (2002) GABAergic terminals are required for postsynaptic clustering of dystrophin but not of GABA(A) receptors and gephyrin. J Neurosci 22:4805–4813.

Butterwick SM, He XJ, Marsh SS, da Silva C, Alysson JJ, Escayg A (2018) Epileptiform encephalopathy and cerebellar atrophy resulting from compound heterozygous CACNA2D2 variants. Case Rep Genet 2018:3080283.

Caylor RC, JIn Y, Ackley BD (2013) The caenorhabditis elegans voltage-gated calcium channel subunits UNC-2 and UNC-36 and the calcium-dependent kinase UNC-43/CalMII regulate neuromuscular junction morphology. Neural Dev 8:10.

Chen JL, Villa KL, Cha JW, Mercer A, Thomson AM, Jovanovic JN (2016) γ-aminobutyric acid type A (GABA(A) receptor) subunits play a direct structural role in synaptic contact formation via their N-terminal extracellular domains. J Biol Chem 291:13926–13942.

Brown LE, Nicholson MW, Arama JE, Mercer A, Thomson AM, Jovanovic JN (2016) γ-aminobutyric acid type A (GABA(A) receptor) subunits play a direct structural role in synaptic contact formation via their N-terminal extracellular domains. J Biol Chem 291:13926–13942.

Chen JL, Villa KL, Cha JW, Mercer A, Thomson AM, Jovanovic JN (2016) γ-aminobutyric acid type A (GABA(A) receptor) subunits play a direct structural role in synaptic contact formation via their N-terminal extracellular domains. J Biol Chem 291:13926–13942.

Brown LE, Nicholson MW, Arama JE, Mercer A, Thomson AM, Jovanovic JN (2016) γ-aminobutyric acid type A (GABA(A) receptor) subunits play a direct structural role in synaptic contact formation via their N-terminal extracellular domains. J Biol Chem 291:13926–13942.

Chen JL, Villa KL, Cha JW, Mercer A, Thomson AM, Jovanovic JN (2016) γ-aminobutyric acid type A (GABA(A) receptor) subunits play a direct structural role in synaptic contact formation via their N-terminal extracellular domains. J Biol Chem 291:13926–13942.

Brown LE, Nicholson MW, Arama JE, Mercer A, Thomson AM, Jovanovic JN (2016) γ-aminobutyric acid type A (GABA(A) receptor) subunits play a direct structural role in synaptic contact formation via their N-terminal extracellular domains. J Biol Chem 291:13926–13942.

Chen JL, Villa KL, Cha JW, Mercer A, Thomson AM, Jovanovic JN (2016) γ-aminobutyric acid type A (GABA(A) receptor) subunits play a direct structural role in synaptic contact formation via their N-terminal extracellular domains. J Biol Chem 291:13926–13942.

Brown LE, Nicholson MW, Arama JE, Mercer A, Thomson AM, Jovanovic JN (2016) γ-aminobutyric acid type A (GABA(A) receptor) subunits play a direct structural role in synaptic contact formation via their N-terminal extracellular domains. J Biol Chem 291:13926–13942.

Chen JL, Villa KL, Cha JW, Mercer A, Thomson AM, Jovanovic JN (2016) γ-aminobutyric acid type A (GABA(A) receptor) subunits play a direct structural role in synaptic contact formation via their N-terminal extracellular domains. J Biol Chem 291:13926–13942.
Kaec S, Banker G (2006) Culturing hippocampal neurons. Nat Protoc 1:2406–2415.
Kang Y, Zhang X, Dobie F, Wu H, Craig AM (2008) Induction of GABAergic post-synaptic differentiation by α-neurexins. J Biol Chem 283:2323–2334.
Katz B, Miledi R (1968) The role of calcium in neuromuscular facilitation. J Physiol 195:481–492.
Kerov V, Laird JG, Joiner ML, Knecht S, Soh D, Hagen J, Gardner SH, Gutiérrez W, Yoshimatsu T, Bhattachari S, Puthussery T, Arteymy NO, Drack AV, Wong RO, Baker SA, Lee A (2018) α2β4 is required for the molecular and structural organization of rod and cone photoreceptor synapses. J Neurosci 38:6145–6160.
Klugbauer N, Lacinová L, Marais E, Hobom M, Hofmann F (1999) Molecular diversity of the calcium channel α2δ2α subunit. J Neurosci 19:6690–6691.
Kurshan PT, Orzant A, Schwartz TL (2009) Presynaptic α2δ2δ3 is required for synaptic morphogenesis independent of its Ca2+ channel functions. Nat Neurosci 12:1415–1423.
Lana B, Schlick B, Martin S, Pratt WS, Page KM, Goncalves L, Rahman W, Dickinson AH, Bauer CS, Dolenc AP (2014) Differential upregulation in DRG neurons of an α2δ2 Lama1 splice variant with a lower affinity for gabapentin after peripheral sensory nerve injury. Pain 155:522–533.
Lardi-Studler B, Frisch my JM (2007) Matching of pre- and post-synaptic specializations during synaptogenesis. Neuroscientist 13:115–126.
Lee E, Lee J, Kim E (2017) Excitation/Inhibition imbalance in animal models of autism spectrum disorders. Biol Psychiatry 81:838–847.
Li J, Han W, Pelkey KA, Duan J, Mao X, Wang YX, Craig MT, Dong L, Petralia RS, McBain CJ, Lu W (2017) Molecular dissection of Neuronin 2 and Slitrk3 reveals an essential framework for GABAergic synapse development. Nature 550:808–826.e8.
Markram H, Toledo-Rodríguez M, Wang Y, Gupta A, Silbergberg G, Wu C (2004) Interneurons of the neocortical inhibitory system. Nat Rev Neurosci 5:769–780.
Mastrolla V, Flucher SM, Obermair GJ, Drach M, Hofer H, Renfrst E, Schwartz A, Striessing J, Flucher BE, Tuluc P (2017) Loss of α2β6-1 calcium channel subunit function increases the susceptibility for diabetes. Diabetes 66:907–918.
Misserl M, Zhang W, Rohlmann A, Kattenstroth G, Hammer RE, Gottmann K, Sudhof TC (2003) α-neurexin II couples α2δ2 channels to synaptic vesicle exocytosis. Nature 423:939–948.
Moons T, De Hert M, Gellens E, Vandekerckhove P, Claes S (2016) Genetic evaluation of schizophrénia using the Illumina HumanExome chip. PLoS One 11:e0150464.
Münster-Wandowski A, Zander JF, Richter K, Ahnert-Hilger G (2016) Coexistence of functionally different vesicular neurotransmitter transporters. Front Synaptic Neurosci 8:40.
Neely GJ, et al. (2010) A genome-wide drosophila screen for heat nociception identifies α2δ3 as an evolutionarily conserved pain gene. Cell 143:626–638.
Neupert C, Schneider R, Gelmuth A, Bierman B, Niesmann K, Misserl M, Heine M (2015) Regulated dynamic trafficking of α-neurexins inside and outside of synaptic terminals. J Neurosci 35:13647.
Neupert C, Schneider R, Gelmuth A, Bierman B, Niesmann K, Misserl M, Heine M (2015) Regulated dynamic trafficking of α-neurexins inside and outside of synaptic terminals. J Neurosci 35:13647.
Neupert C, Schneider R, Gelmuth A, Bierman B, Niesmann K, Misserl M, Heine M (2015) Regulated dynamic trafficking of α-neurexins inside and outside of synaptic terminals. J Neurosci 35:13647.
Patel R, Bauer CS, Nieto-Rostro M, Margas W, Ferron L, Chaggar K, Crews K, Ramirez JD, Bennett DL, Schwartz A, Dickinson AH, Dolenc AP (2013) α2β6-1 gene deletion affects somatosensory neuron function and delays mechanical hypersensitivity in response to peripheral nerve damage. J Neurosci 33:16412–16426.
Penedo P, Kourrich S, Kearney E, Thomas MJ, Laierm LA (2011) An embryonic culture system for the investigation of striatal medium spiny neuron dendritic spine development and plasticity. J Neurosci Methods 200:1–13.
Peters A (2002) Examining neocortical circuits: some background and facts. J Neurocytol 31:183–193.
Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8:785–786.
Petitm KL, Yokomaku D, Takahashi H, Ge Y, Craig AM (2013) Interaction between autism-linked MDGAs and neurolnigins suppresses inhibitory synapse development. J Cell Biol 200:321–336.
Pippucci T, Parmeggianni A, Palombo F, Maresca A, Angius A, Crispino L, Cucca F, Liuzzo R, Valenitno ML, Seri M, Carelli V (2013) A novel null homozygous mutation confirms CACNA2D2 as a gene mutated in epi-leptic encephalopathy. PLoS One 8:e62154.
Pirona A, Kurt S, Zuccotti A, Rüttiger L, Pitz P, Brown DH, Franz C, Schweizer M, Rust MB, Rubasmen R, Frualie E, Knippie M, Engel J (2014) α2β3 is essential for normal structure and function of auditory nerve synapses and is a novel candidate for auditory processing disorders. J Neurosci 34:434–445.
Purcell SM, Moran JL, Fromer M, Ruderfer D, Soloviev N, Roussos P, O’Dushaline C, Chambert K, Bergen SE, Kahler A, Duncan L, Stahl E, Genoves G, Fernández E, Collins MO, Komiyama NH, Choudary JS, Magnunson PK, Banks E, Shaker K, et al. (2014) A polygenic burden of rare disruptive mutations in schizophrenia. Nature 506:185–190.
Rao A, Cha EM, Craig AM (2000) Mismatched appositions of presynaptic and postsynaptic components in isolated hippocampal neurons. J Neurosci 20:8344–8353.
Reissner C, Runkel F, Misserl M (2013) Neurexins. Genome Biol 14:213.
Risher WC, Kim N, Koh S, Choi JE, Miter P, Spence EF, Pilaz LJ, Wang D, Feng G, Silver DL, Soderling SH, Yin H, Erogul C (2018) Thrombospondin receptor α2δ1 promotes synaptogenesis and spino genesis via postsynaptic Rac1. J Cell Biol 217:3747–3765.
Root DH, Mejias-Aponte CA, Zhang S, Wang HL, Hoffman AF, Lupica CR, Morales M (2014) Single rodent mesolimbocortical axons release glutamate and GABA. Nat Neurosci 17:1543–1551.
Schlick B, Flucher BE, Obermair GJ (2010) Voltage-activated calcium channel expression profiles in mouse brain and cultured hippocampal neurons. Neuroscience 167:786–798.
Segal M, Greenberger V, Korkotian E (2003) Formation of dendritic spines in cultured striatal neurons depends on excitatory afferent activity. Eur J Neurosci 17:2573–2585.
Shrivastava AN, Triller A, Sieghart W (2011) GABAA receptors: post-synaptic co-localization and cross-talk with other receptors. Front Cell Neurosci 5:7.
Sigler A, Oh WC, Imig C, Ahsa B, Kawabe H, Cooper BH, Kwon HB, Rhee JS, Brose N (2017) Formation and maintenance of functional spines in the absence of presynaptic glutamate release. Nature 94:304–311.e4.
Stanika R, Campiglio M, Pinggera A, Minelli F, Hainfellner JA, Kovacs GG, Kowal S, Studler B, Sidler C, Fritschy JM (2005) Differential regulation of GABAA receptors at inhibitory and excitatory synapses of cultured mouse hippocampal neurons. J Neurosci 25:13406–13412.
Stefanits H, Milenkovic I, Mahr N, Pataraia E, Hainfellner JA, Kovacs GG, Kowal S, Studler B, Sidler C, Fritschy JM (2005) Differential regulation of GABAA receptors at inhibitory and excitatory synapses of cultured mouse hippocampal neurons. J Neurosci 25:13406–13412.
Stefanits H, Milenkovic I, Mahr N, Pataraia E, Hainfellner JA, Kovacs GG, Kowal S, Studler B, Sidler C, Fritschy JM (2005) Differential regulation of GABAA receptors at inhibitory and excitatory synapses of cultured mouse hippocampal neurons. J Neurosci 25:13406–13412.
}
Tedeschi A, Dupraz S, Laskowski CJ, Xue J, Ulas T, Beyer M, Schultze JL, Bradke F (2016) The calcium channel subunit Alpha2delta2 suppresses axon regeneration in the adult CNS. Neuron 92:419–434.

Tong XJ, López-Soto EJ, Li L, Liu H, Nedelcu D, Lipscombe D, Hu Z, Kaplan JM (2017) Retrograde synaptic inhibition is mediated by α-neurexin binding to the α2δ subunits of N-type calcium channels. Neuron 95:326–340.e5.

Valence S, Cochet E, Rougeot C, Garel C, Chantot-Bastaraud S, Lainey E, Afenjar A, Barthez MA, Bednarek N, Doummar D, Faiivre L, Goizet C, Haye D, Heron B, Kemlin I, Lacombe D, Milh M, Moutard ML, Riant F19, Robin S, et al. (2018) Exome sequencing in congenital ataxia identifies two new candidate genes and highlights a pathophysiological link between some congenital ataxias and early infantile epileptic encephalopathies. Genet Med. Advance online publication. Retrieved July 12, 2018. doi:10.1038/s41436-018-0089-2.

Van Den Bossche MJ, Strazisar M, De Bruyne S, Bervoets C, Lenaerts A-S, De Zutter S, Nordin A, Norrback K-F, Goossens D, De Rijk P, Green EK, Grozeva D, Mendlewicz J, Craddock N, Sabbe BG, Adolfsen R, Souery D, Del-Favero J (2012) Identification of a CACNA2D4 deletion in late onset bipolar disorder patients and implications for the involvement of voltage-dependent calcium channels in psychiatric disorders. Am J Med Genet Part B Neuropsychiatr Genet 159B:465–475.

Vergult S, Dheedene A, Meurs A, Faes F, Isidor B, Janssens S, Gautier A, Le Caignec C, Menten B (2015) Genomic aberrations of the CACNA2D1 gene in three patients with epilepsy and intellectual disability. Eur J Hum Genet 23:628–632.

Wang Y, Fehlhaber KE, Sarria I, Cao Y, Ingram NT, Guerrero-Given D, Throesch B, Baldwin K, Kamasawa N, Ohtsuka T, Sampath AP, Martemyanov KA (2017) The auxiliary calcium channel subunit α2δ4 is required for axonal elaboration, synaptic transmission, and wiring of rod photoreceptors. Neuron 93:1359–1374.e6.

Wu J, Yan Z, Li Z, Qian X, Lu S, Dong M, Zhou Q, Yan N (2016) Structure of the voltage-gated calcium channel Ca(v)1.1 at 3.6 Å resolution. Nature 537:191–196.

Zhou C, Luo ZD (2015) Nerve injury-induced calcium channel alpha-2-delta-1 protein dysregulation leads to increased pre-synaptic excitatory input into deep dorsal horn neurons and neuropathic allodynia. Eur J Pain 19:1267–1276.

Zucker RS, Regehr WG (2002) Short-term synaptic plasticity. Annu Rev Physiol 64:355–405.