Neurexins (NRXNs) are synaptic cell adhesion molecules that have essential roles in the assembly and maturation of synapses into fully functional units. Immunocytochemical and electrophysiological studies have shown that specific binding of the synapses to postsynaptic neurones can potentially have a bi-directional coordinate and trigger synapse formation. Moreover, *in vivo* studies as well as genome-wide association studies pointed out implication of NRXNs in the pathogenesis of cognitive disorders including autism spectrum disorders and different types of addictions including opioid and alcohol dependencies, suggesting an important role in synaptic function. Despite extensive investigations, the mechanisms by which NRXNs modulate the properties of synapses remain largely unknown. We report here that α- and γ-secretases can sequentially process NRXN3β, leading to the formation of two final products, an ~80-kDa N-terminal extracellular domain of Neurexin-3β (sNRXN3β) and an ~12-kDa C-terminal intracellular NRXN3β domain (NRXN3β-ICD), both of them being potentially implicated in the regulation of NRXNs and neuroligins functions at the synapses or in yet unidentified signal transduction pathways. We further report that this processing is altered by several PS1 mutations in the catalytic subunit of the γ-secretase that cause early-onset familial Alzheimer disease.

Neurexins (NRXNs) are type I transmembrane neuronal adhesion receptors that exit and function predominantly at presynaptic terminals. In mammals, NRXNs are encoded by three genes, NRXN1, NRXN2, and NRXN3. From each single gene and by virtue of two independent promoters, two NRXN isoforms are synthesized; that is, the longer α-NRXNs and the shorter β-NRXNs (essentially N-terminal-truncated α-NRXNs). Moreover, each of the six transcripts undergoes extensive alternative splicing, potentially giving rise to thousands of different Neurexin isoforms (1–3). At the protein level, NRXNs have several known extracellular ligands: neurexophins, dystroglycans, neuroligins (NLGNs) (for review, see Ref. 4), the recently identified LRRTM2 (5, 6), the complex Cbln1/GluRδ2 (7), and γ-aminobutyric acid, type A receptors (8). Insights into the role of NRXNs-NLGNs complexes for synapse development came from a study by Scheiffele et al. (9). In the latter, the development of an artificial synapse assay involving co-culture of non-neuronal and neuronal cells demonstrated that NRXNs-NLGNs interaction is sufficient to trigger postsynaptic and presynaptic differentiation. In this context, ligated neurexins not only signal to recruit on the presynaptic side neurotransmitter vesicles associated with the exocytotic machinery but also instruct postsynaptically the recruitment of N-methyl-D-aspartate or γ-aminobutyric acid receptors and scaffolding proteins PSD-95 and gephyrin (10, 11). Taken together, NRXNs and NLGNs appear to interact and signal bi-directionally between pre- and postsynaptic sites to assemble some of the elements required for fully functional synapses.

Based on the findings that particular variants of NRXNs preferentially bind to NLGN1 or NLGN2 and that NLGN1 and NLGN2 are differentially localized to excitatory or inhibitory synapses (12, 13), a concept emerged where differential assembly of specific postsynaptic elements dictated by NRXNs and their physical interaction with NLGNs regulates the balance between excitatory and inhibitory synapses (E/I ratio) (12). Consequently, any alteration of this process might have important implications in synaptic activity and plasticity. Alterations in this process might also have important implications on neurodevelopmental psychiatric disorders such as autism, which is thought to result from an imbalanced E/I ratio (for review, see Ref. 14). Strong evidence also exists supporting essential roles for the α-secretase TACE/ADAM17, β-secretase BACE1, and γ-secretase in regulating synaptic functions.

The α-secretase TACE/ADAM17 (or tumor necrosis factor-α-converting enzyme) is a metalloprotease that was previously shown to be essential for the processing of the extracellular ligand neurexophin-1 (15) and to be involved in the processing of neurexophin-3 (16). In addition, this protease is responsible for the cleavage of several other proteins involved in synaptic plasticity and neuronal development (17–19). The role of TACE/ADAM17 in the processing of NRXNs and NLGNs is currently unknown. Here, we characterize the role of TACE/ADAM17 in NRXN3β processing and its potential implication in the regulation of NRXNs-NLGNs complexes. We show that the presence of a transmembrane domain is required for the proteolytic processing of NRXN3β by TACE/ADAM17 and that the C-terminal intracellular domain (ICD) is the major substrate for this protease. Moreover, we demonstrated that the presence of the γ-secretase component nicastrin is required for the processing of the C-terminal intracellular domain by TACE/ADAM17 and that this processing is critically dependent on the presynaptic localization of NRXN3β. The role of TACE/ADAM17 in regulating the local availability of NRXN3β in the presynaptic membrane is critically dependent on the presence of NLGN1 or NLGN2 and suggests a key role for these proteins in the localization of NRXNs to the presynaptic membrane. Finally, we show that the presence of a transmembrane domain is required for the processing of the C-terminal intracellular domain of NRXN3β by γ-secretase and that the presence of the γ-secretase component nicastrin is required for the processing of the C-terminal intracellular domain by γ-secretase.
marily identified by its ability to cleave the proinflammatory cytokine TNFα (15, 16). TACE/ADAM17 proteolytic activity releases by “ectodomain shedding” functional domains from a growing list of membrane-anchored substrates including the Notch receptor, the epidermal growth factor receptor, the amyloid precursor protein (APP), and other cytokine receptors and adhesion receptors (for review, see Ref. 17). At the synaptic level, adhesion receptors such as L1, NCAM, or SynCAM1, but also the neuronal pentraxin receptor were identified as TACE/ADAM17 substrates (18–22). Although TACE-dependent processing of L1 and NCAM was shown to be involved in neurite outgrowth, the analysis of mechanisms regulating neuronal pentraxin receptor cleavage revealed the role of TACE/ADAM17 in synaptic plasticity. TACE is indeed required for cleaved neuronal pentraxin receptor to act in hippocampal and cerebellar synapses by promoting AMPAR endocytosis, a process that is essential for durable long term depression (18).

The β-secretase BACE1 is a membrane-tethered aspartyl protease that cleaves the APP to generate APP-C99, a 99-amino acid-long C-terminal domain of APP, which, after processing by γ-secretase, leads to the formation of the pathogenic β-amyloid peptides implicated in Alzheimer disease (23). However, characterization of BACE1 substrates other than APP (including the voltage-gated sodium channel Na(v)1 β2-subunit and neuregulin 1) pointed out the role of BACE1 in neuronal functions (24, 25). Studies analyzing BACE1 knockout mice indeed highlighted dysfunctions in synaptic transmission and plasticity (26–28). In addition, BACE1 seems to be essential for cognitive and emotional functions as BACE1 null mice displayed behavioral changes (26, 29, 30).

γ-Secretase is an intramembrane-cleaving multisubunit protease complex made of at least four different proteins: presenilin1 or 2 (PS1 or PS2), nicastrin, APH-1, and PEN-2 (23). Mutations in PS1, the catalytic subunit of the protease, are the most common cause of early-onset familial Alzheimer disease, causing synaptic dysfunction leading to memory loss and neurodegeneration. Indeed, behavioral tests as well as electrophysiological studies on conditional knock-out PS1 and PS2 null mice revealed impairments in hippocampal memory and age-dependent neurodegeneration due to neuronal loss (31). Interestingly, and based on a study focusing on PS1 cDKO mice, Zhang et al. (32) assigned a specific presynaptic function to PS1/γ-secretase. They further demonstrated that loss of PS1 impairs the activity-dependent regulation of neurotransmitter release in mature neurons (32). Collectively, these findings suggest a substantial role of PS1/γ-secretase in synaptic plasticity and neuronal survival.

Considering the essential roles of the presynaptic NRXNβ, TACE/ADAM17, BACE1, and γ-secretase in both neurotransmitter release and synaptic plasticity, we investigated whether NRXN3β, the most widely expressed variant of β-neurexin (33), and γ-secretase are functionally associated and assessed whether NRXN3β can be processed by α-, β-, and γ-secretases.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**—Human NRXN3β (KIAA0743) cDNA lacking any insert at splice site 4 was purchased from Openbiosystems, sequenced, and subcloned (with a FLAG tag at the N terminus) into the mammalian expression vector pCDNA 3/Neo (+) (Invitrogen) or pSin lentiviral transfer vector (34). The sequences encoding for the NRXN3β-CTFs were subcloned into the pet21b (+) (Novagen) expression vector with a FLAG tag at the C terminus. The PS1 WT, PS1 L166P, PS1 P436Q, and the PS1 ΔE9 constructs were subcloned into the lentiviral transfer vector pSin. For generation of NRXN3β-FL-Gal4-VP16 (NRXN3β-FL-GV), the Gal4 DNA binding domain and the transactivation domain of the herpes simplex virus protein VP-16 were fused to the C-terminal end of the human NRXN3β. The NRXN3β-GV-encoding sequence was then inserted into the pcDNA transmembrane 5/TO vector (Invitrogen), and its expression was placed under the control of two tetracycline operator sequences (TO). The APP-C99-GV construct was a gift from M. Wolfe (35). The luciferase reporter vector pLG4.31 (Promega) contains the synthetic firefly luciferase gene (lac2) placed under the control of the GAL4/US response element.

**Cell Lines and Cultures**—Chinese hamster ovary (CHO) cell lines, human embryonic kidney cells (HEK293T), WT, and DKO PS1/PS2 mouse embryonic fibroblasts (MEFs) were routinely grown in DMEM with 10% fetal bovine serum (FBS) and penicillin/streptomycin in a humidified 5% CO2 atmosphere. The CHO cell line expressing stably FLAG-tagged NRXN3β was maintained in DMEM, 10% FBS supplemented with 300 μg/ml Geneticin G418 (Invitrogen). T-Rex HeLa cells stably expressing the repressor of tetracyclin (Invitrogen) were maintained in Eagle’s Minimum Essential Medium with 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, and 5 μg/ml blasticidin. Mouse primary cortical neurons (PCN) were prepared from embryonic day 17 Of1 mouse fetal brains. Cortices were dissociated by repeated passages through a fire-polished Pasteur pipette. The dissociated cells were plated in DMEM + 10% FBS on poly-D-lysine coated plates at 1 × 10^5 cells/cm². After 4–6 h, the medium was changed, and the cells were maintained in neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen) and 2 mM L-glutamine at 37 °C in a humidified 5% CO2 atmosphere. The cells were treated with 10 μM cysteine arabinoside after 4 days in vitro (DIV), PCN treatments with 10 μM N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyler ester (DAPT, Sigma), 10 μM Compound E (Alexis), 0.5 μM phorbol 12-myristate 13-acetate (PMA, Sigma), 20 μM TAPI-1 (Calbiochem), 5 μM GL189 (Calbiochem) were carried out for 16 h at DIV 13. PCN treatments with either 50 μM KCl or 50 μM l-glutamate were carried out at DIV 13. Briefly, fresh neurobasal medium (Invitrogen) supplemented with either l-glutamate or KCl or an equal volume of phosphate-buffered saline (PBS) was added to cells, and cells were incubated for 15 min at 37 °C in a humidified 5% CO2 atmosphere. Neurons were then washed with PBS, and microsomal membranes were prepared as described below.
Neurexins Processing by α- and γ-Secretases

The CHO cells stably expressing NRXN3β were washed with ice-cold PBS and lysed in 1% Nonidet P-40 HEPES buffer (50 mM Hepes, 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, 1% Nonidet P-40, and a mixture of protease inhibitor (Roche Diagnostics)). Protein concentrations were determined by using the BCA protein assay kit (Pierce), and whole cell protein extracts were separated on 4–12% Bis-Tris gels and transferred onto nitrocellulose membranes to detect FLAG-tagged NRXN3β (M2 anti-FLAG, Sigma) as described below. For the PCN, membrane fractions were prepared by osmotic shock. After 2 washes with PBS and 2 washes with a hypotonic buffer (50 mM HEPES), cells were passed through a 25-gauge syringe, and the homogenates were centrifuged at 3000 × g for 10 min at 4 °C. The supernatants were collected and centrifuged at 100,000 × g for 1 h at 4 °C, and the pellets resuspended in 1% Nonidet P-40 HEPES. Solubilized cellular membranes were separated on 4–12% Bis-Tris gels (Invitrogen) and transferred onto PVDF membranes to detect endogenous NRXNs as described below.

Ectodomain Shedding Assays—16 h after transfection of HEK293T cells with FL-NRXN3β, DMEM, 10% FBS medium was replaced with fresh serum-free DMEM medium containing TAPI-1 (20 μM) or PMA (0.5 μM). After 24 h of incubation, the media were collected and centrifuged at 1000 × g for 3 min, and 1 ml of each supernatant was collected for TCA precipitations.

Protein Expression and Purification—For the purification of cellular NRXN3β-CTF substrates, 10 × 10-cm dishes of HEK293T cells transiently transfected with FL-NRXN3β-FLAG were incubated for 16 h with 10 μM DAPT and lysed in ice-cold 1% CHAPSO-HEPES lysis buffer (50 mM Hepes, 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, 1% CHAPSO, protease inhibitor mixture). The lysate was centrifuged at 14,000 rpm for 1 h at 4 °C, and the supernatant was incubated overnight with the M2 anti-FLAG affinity resin. After 2 washes in 1% CHAPSO-HEPES and 1 wash in 0.2% CHAPSO-HEPES, the bound proteins were eluted in 100 μl of 0.2% CHAPSO-HEPES containing 0.2 μg/ml FLAG peptides, pooled, and used for the in vitro γ-secretase assays. For the expression and purification of recombinant NRXN3β-CTF substrates, Escherichia coli strain BL21 (DE3) (Invitrogen) was transfected with pet21b (+) (Novagen) expressing the FLAG-tagged NRXN3β-CTFs. The expression of the proteins was induced for 4 h at 37 °C in 1 liter of LB/ampicillin containing 1 mM isopropyl-1-thio-β-D-galactopyranoside. After extraction of inclusion bodies (as described in Ref. 36) the NRXN3β-CTFs were refolded by dialysis in 10 mM Tris, pH 7.0, 150 mM NaCl, 1% Triton X-100 and purified using the M2 anti-FLAG affinity resin. Finally, the FLAG-tagged NRXN3β-CTFs were eluted in five 1-ml fractions in 1% Nonidet P-40, 100 mM glycine, pH 2.7. The purity of the CTFs was confirmed by SDS-PAGE using Coomassie staining.

Cell-free γ-Secretase Activity Assays—In vitro γ-secretase activity assays with purified γ-secretase were performed as previously described (37). Briefly, the reactions contained recombinant APP-C99 or NRXN3β-CTF (substrates expressed in E. coli as a fusion protein including a Met for translation initiation and the FLAG tag sequence, each at 1 μM), and γ-secretase (either purified or from solubilized membranes), 0.025% phosphatidylethanolamine (PE) and 0.10% phosphatidylcholine (PC). All the reactions were stopped by adding Laemmli sample buffer, and the samples were assayed by Western blot for intracellular domains (ICD)-FLAG as described below.

γ-Secretase activity assays with isolated membranes were performed as described in Marambaud et al. (38). Briefly, CHO cells stably overexpressing NRXN3β were collected in a hypotonic buffer (10 mM MOPS, pH 7.0, 10 mM KCl, protease inhibitors mixture (Roche Diagnostics)) and homogenized on ice by 10 passages through a 25-gauge needle. The homogenates were centrifuged at low speed (1000 × g, 15 min, 4 °C), and the postnuclear supernatants were further centrifuged at 16,000 × g for 40 min at 4 °C to pellet the membranes. These were resuspended in the assay buffer (150 mM sodium citrate, pH 6.4, protease inhibitor mixture) and incubated for 3 h at 37 °C with a gentle shaking (300 rpm) in the presence or absence of 1 μM DAPT or 1 μM Compound E. Alternatively, isolated membranes were prepared from cells incubated for 16 h in the presence or absence of 10 μM DAPT and incubated for 2 h at 37 °C. Membrane preparations from KCl or L-glutamate-stimulated PCN were incubated for 4 h in assay buffer (150 mM sodium citrate, pH 6.4, protease inhibitor mixture) at 37 °C. Next, membrane protein extracts were prepared by incubating the membranes in radioimmune precipitation assay buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) for 1 h on ice before adding Laemmli buffer and Western blotting.

Transactivation Assays—T-Rex HeLa cells were grown in 24-well plates (2.5–5 × 10⁵ cells/well), and each well was co-transfected with NRXN3β-FL-GV encoding plasmid and the firefly luciferase reporter plasmid. Transfected cells were incubated for 24 h with 1 μg/ml tetracycline in the presence or absence of 10 μM DAPT. The addition of tetracycline relieves transcriptional repression from the tetracycline operator and, therefore, allows expression of FL-NRXN3β-GV or APP-C99-GV. Cells were then harvested and used for luciferase activity assays. Briefly, cells were lysed for 5 min in 100 μl of Glo lysis buffer, and the luciferase activity was measured by mixing 90 μl of each lysate and Bright-Glo luciferase assay reagent (Promega) in 96-well Nunc Maxisorp plates (Nunc, Roskilde, Denmark). After 5 min of incubation at room temperature, the luminescence emitted in individual wells was monitored by using an automated plate reader (Tecan Infinite F500; Tecan, Grödig, Austria).

Western Blotting and Antibodies—For Western blot analysis of FL-NRXN3β, PS1-NTF, APP, and β-actin, the samples were run on 4–12% Bis-Tris PAGE gels, transferred onto nitrocellulose membranes, and probed with the rabbit polyclonal anti-neurexin (Synaptic Systems) or M2 anti-FLAG (Sigma) (for FL-NRXN3β), anti-PS1 (1563, Millipore), and anti-APP C-terminal antibody (Sigma). A rabbit antiserum was raised against an N-terminal NRXN3β-specific sequence (Eurogentec). NRXN3β-GV and APP-C99-GV were detected by using an anti VP16 antibody (Abcam).

Lentiviruses Production—The production of lentiviral particles was performed by transient transfection of HEK293T...
Neurexins Processing by α- and γ-Secretases

Several requirements specifying γ-secretase substrates are shared by almost all substrates. First, the substrates are type I transmembrane proteins, with the N-terminal ectodomains facing the extracellular side. Next, they undergo an initial shedding by α- and/or β-secretases, thus leading to the formation of an extracellular soluble protein as well as a C-terminal membrane-embedded fragment (CTF). Finally, the CTFs are further processed by γ-secretase into ICD (for review, see Refs. 39 and 40). Based on these requirements and to assess whether NRXN3β can be processed by α-, β-, and γ-secretases, intracellular accumulation of neurexin CTFs in response to ectodomain shedding by α- and/or β-secretases activities in conjunction with γ-secretase inhibition (a common characteristic among known γ-secretase substrates) was probed in HEK293T cells transiently expressing human NRXN3β (Fig. 1A) inCHO cells stably expressing NRXN3β (Fig. 1B) as well as in untransfected mouse PCN at DIV 14 (Fig. 1C). FLAG-tagged NRXN3β-CTFs were detected with an anti-FLAG antibody (Fig. 1, A and B), whereas an antibody targeting the C-terminal part of neurexin was used to detect endogenous NRXN-CTFs (Fig. 1C). As shown in Fig. 1, treatments with γ-secretase specific inhibitors DAPT and Compound E (CpdE) triggered the accumulation of a major ~16-kDa FLAG-tagged NRXN3β-CTF in HEK293T and CHO cells (Fig. 1, A and B) and a major ~15-kDa endogenous NRXN-CTF in PCNs (Fig. 1C), migrating at the size of NRXN3β-CTF purified from CHO cells (Fig. 1C, lane 1). Because of the high homology existing among the different isoforms of Neurexin CTFs (Fig. 1D) and because the anti-NRXN antibody used in this experiment has been generated with a mixture of recombinant cytoplasmic tails of rat NRXNs 1, 2, and 3, it is likely that the latter also targets in PCNs neurexin CTFs derived from other NRXN isoforms. Next, DAPT and CpdE, in combination with PMA (a PKC activator stimulating α-secretases), further elevated the cellular levels of NRXN3β-CTFs (Fig. 1, A, lane 4, B, lanes 5 and 6, C, lanes 5 and 6). As shown in Fig. 1, A, lanes 3 and 4, and B, lanes 5 and 6, PMA stimulation of HEK293T and CHO cells was associated with increased levels of NRXN3β-FL. Because PMA alone does not affect the levels of NRXN3β-CTFs (compare lane 3 with lane 1 in Fig. 1A), changes observed at the levels of these CTFs in cells incubated with DAPT or CpdE in combination with PMA should be attributed to the inhibition of γ-secretase. To further confirm that the shedding of NRXN3β is α-secretase-specific, cells were treated with TAPI-1, a metalloprotease inhibitor with some specificity for TACE, in combination with DAPT and CpdE. In sharp contrast to the BACE inhibitors GL189 (Fig. 1) and C3 (not shown), TAPI-1 strongly reduced the DAPT/CpdE-induced accumulation of NRXN3β-CTFs (Fig. 1, A, compare lanes 6 and 2, B, compare lanes 7 and 3, lanes 8 and 4, lanes 9 and 3, and lanes 10 and 4, C, compare lanes 6 and 2, lanes 7 and 3, lanes 8 and 4, and lanes 9 and 3). Together, these data indicate that a protease of the α-secretase family, most likely TACE/ADAM17, is involved in full-length NRXN3β (FL-NRXN3β) cleavage. Furthermore, co-treatment of cells with γ-secretase inhibitors DAPT/CpdE and α-secretase inhibitor TAPI-1 suggest, when compared with cells treated with DAPT or TAPI-1 only, that NRXN3β cleavage by α-secretase occurs before γ-secretase NRXN3β-CTF processing.

To put our data into the physiological context of the synapse, we next assessed whether cleavage of endogenous neuronal neurexin by α- and γ-secretases can be modulated by neuronal activity. Treatment of primary neuronal cultures with KCl, resulting in membrane depolarization and neurotransmitter release at synaptic endings, has previously been shown to stimulate, via activation of glutamate receptors, γ-secretase cleavage of N-cadherin (38). We, therefore, examined the effects of 50 mM KCl or 50 μM 1-m-glutamate-mediated stimulation of PCN on NRXN processing. After 15 min of stimulation, membranes were prepared from the PCN and used in vitro activity assays in the presence or absence of 1 μM DAPT. As shown in Fig. 1E, endogenous NRXN-CTF levels were elevated by ~1.7-fold when cells were treated with 1-m-glutamate (Fig. 1E, lanes 3 and 4) or KCl (Fig. 1E, lanes 5 and 6) when compared with NRXN-CTFs in nontreated cells (Fig. 1E, lanes 1 and 2). Intracellular accumulation of CTFs in response to the combined effect of N-terminal shedding and γ-secretase inhibition is a common feature of known γ-secretase substrates. Therefore, and consistent with both α-secretase activation and γ-secretase inhibition hypotheses, the accumulation of NRXN-CTFs in cells can be attributed to α-secretase activation, γ-secretase inhibition, or a combination of both. Yet DAPT increases the levels of
NRXN-CTF levels in L-glutamate/KCl-stimulated neurons, further supporting enhanced \(\alpha\)-secretase activity and excluding the possibility that the observed increase in NRXN-CTFs after stimulation is solely due to reduced \(\gamma\)-secretase activity (Fig. 1E, compare lane 2 to lanes 4 and 6). Together, these results suggest that NRXN processing by \(\alpha\) - and \(\gamma\)-secretases is subjected to a regulation by synaptic activity.

**NRXN3\(B\) Ectodomain Shedding and Extracellular Soluble NRXN3\(B\) (sNRXN3\(B\)) Production**—To assess whether full-length NRXN3\(B\) undergoes ectodomain shedding, serum-free media of HEK293T cells transiently expressing NRXN3\(B\) were collected, and total proteins were TCA-precipitated and probed for soluble NRXN3\(B\) (sNRXN3\(B\)) with an N-terminal-specific NRXN3\(B\) antibody (Fig. 2A). As a control, the same samples were further analyzed by Western blotting using an antibody targeting the C-terminal FLAG-tagged full-length NRXN3\(B\) (Fig. 2B). We found a prominent \(\sim80\)-kDa band consistent with the expected position of sNRXN3\(B\) to be detected by the N-terminal (Fig. 2A)- but not C-terminal (Fig. 2B)-specific NRXN3\(B\) antibodies. These data demonstrate that a C-terminal-truncated NRXN3\(B\) species, but not the full-length protein, is detected in the conditioned media of HEK293T cells expressing transiently FL-NRXN3\(B\). Further supporting the NRXN3\(B\) ectodomain shedding by \(\alpha\)-secretase, elevated levels of sNRXN3\(B\) were observed in cells incubated for 16 h with PMA (Fig. 2A, compare lane 4 with lane 3), whereas reduced levels of sNRXN3\(B\) were found in cells incubated for 16 h with TAPI-1 (Fig. 2A, compare lane 5 with lane 3). However, the substantial amount of sNRXN3\(B\) remaining in the conditioned medium after TAPI-1 treatment (Fig. 2A, lane 5) indicates either incomplete action of the inhibitor or the contribution of other \(\alpha\)-secretase-like proteases responsible for cleaving NRXN3\(B\). Fully consistent with \(\alpha\)-secretase cleavage, changes seen in the levels of extracellular NRXN3\(B\)
ectodomains released after shedding mirrored changes seen in intracellular CTF levels (Figs. 1 and 2).

**Alzheimer Disease-causing Presenilin Mutations Impair NRXN3β Processing**—To further support the γ-secretase-mediated cleavage of NRXN3β, we assessed the impact on NRXN3β processing of PS1-L166P, PS1-P436Q, and PS1-ΔE9, mutations/deletion in the catalytic core of γ-secretase, causing early-onset forms of Alzheimer disease. To do so, CHO cells stably expressing NRXN3β were transduced with increasing amounts (to highlight the dose-dependent effect) of PS1 WT or the mutants PS1-L166P, PS1-P436Q, and PS1-ΔE9 (Fig. 3A). For accurate quantification of both NRXN3β-CTF and APP-CTF and because overexpression of PS1 (especially PS1-L166P and PS1-P436Q) apparently affects the levels of full-length NRXN3β (Fig. 3A, top panel), the levels of CTFs were normalized to these of full-length NRXN3β (8). A total cell lysate from HEK293T cells transiently expressing FL-NRXN3β-FLAG (hNRXN3β control; lane 1) and conditioned medium from untransfected HEK293T cells (−) were loaded as controls.

**Loss of γ-Secretase Activity Impairs NRXN3β Processing**—We further investigated the levels of NRXN3β-CTFs in PS1/PS2 double knock-out mouse embryonic fibroblasts (MEFs PS1/PS2 DKO, lacking functional presenilins-1 and -2 (41, 42)) transduced with NRXN3β lentiviruses (Fig. 3B). When compared with wt MEFs, elevated NRXN3β-CTF levels were found in the MEFs PS1/PS2 DKO (Fig. 3B, compare lane 2 with lane 1). To demonstrate that this failure to process NRXN3β-CTFs was a consequence of PS loss, human PS1 WT was reintroduced in the MEFs PS1/PS2 DKO by lentiviral transduction. To confirm the rescued γ-secretase activity in these cells, both expression and activity levels of human PS1 were assessed by Western blot using a PS1 N-terminal-specific antibody (to follow PS1-NTF, the mature and active form of PS1) and an anti-APP-CTF antibody (to follow the levels of APP-CTFs). Reduced APP-CTF levels in PS1-expressing MEFs PS1/PS2 DKO (when compared with that of non-transduced cells) confirmed the rescued γ-secretase activity (Fig. 3B, compare lane 3 with lane 2). Further supporting an essential role of PS1 in the processing of NRXN3β, the accumulation of NRXN3β-CTFs observed in MEFs PS1/PS2 DKO cells was reversed in the rescued cells.

**NRXN3β Processing in a Cell-based Luciferase Reporter Assay**—To further assess neurexin processing at the cellular level, we developed a cell-based reporter gene assay specific for NRXN3β (described in Fig. 4A), similar to that specific for APP-C99 (35). T-Rex HeLa cells constitutively expressing the tetracycline repressor protein (tetR) were co-transfected with pGL4.31, a plasmid encoding a Gal4-driven luciferase reporter gene and either with a plasmid encoding a tetracycline-driven FL-NRXN3β fused to the Gal4 DNA binding domain and to the VP16 activation domain (NRXN3β-GV) or encoding the C-terminal APP fragment fused to Gal4-VP16 (APP-C99-GV). In the absence of tetracycline, tetR binds to the tetacycline operator and represses the transcription of both NRXN3β-GV and APP-C99-GV, resulting in low expression of luciferase reporter and luminescence (Fig. 4B). Added tetracycline results in tetR release from the tetracycline operator and consequently to the expression of the chimeric proteins NRXN3β-GV or APP-C99-GV. As shown in Fig. 4B, we found a 10–20-fold increase in the luminescence after tetracycline treatment. This activation is largely dependent on γ-secretase activity, which releases NRXN3β-ICD-GV and APP-ICD-GV from the membrane and activates transcription of the luciferase reporter gene. Supporting a γ-secretase-dependent activation of the luciferase assay, the addition of 10 μM DAPT triggered a ~75 and ~52% reduction in the luminescence emitted in cells expressing NRXN3β-FL-GV and APP-C99-GV, respectively (Fig. 4B, left). DAPT further leads to the accumulation of both NRXN3β-CTF-GV and APP-C99-GV (Fig. 4B, right). Taken together, these data further support intracellular α- and γ-secretase-dependent NRXN3β cleavage and suggest the release into the cytosol of NRXN3β-ICD.
Neurexins Processing by α- and γ-Secretases

A

FIGURE 3. Partial loss of γ-secretase activity impairs NRXN3β processing. A, Alzheimer disease-causing mutants of PS1 impair NRXN3β processing are shown. NRXN3β-CTFs were analyzed in CHO cells stably expressing FL-NRXN3β and transiently expressing increasing amounts of WT PS1 (lanes 2 and 3) or the Alzheimer disease causing mutants PS1-L166P, PS1-P436Q, and PS1-ΔE9 (lanes 4–9). FL-NRXN3β, NRXN3β-CTFs, PS1-NTF, FL-APP, and APP-CTFs were probed by Western blotting using the M2 anti-FLAG (for FL-NRXN3β/NRXN3β-CTFs), anti-hPS1, and anti-amyloid precursor protein (for FL-APP/APP-CTFs) C-terminal antibody (upper panel). Lower panel, the levels of hNRXN3β- and APP-CTFs were quantified by densitometric analysis of the bands in the Western blot. For accurate quantification, the relative levels of CTFs were normalized to these of full-length NRXN3β and APP (CTFs/FL). LV indicates the volume of lentivirus preparation used to infect 2 × 10^6 CHO cells. β-Actin serves as a loading control. B, impaired NRXN3β processing in PS1/PS2 double knock-out MEFs is shown. Representative Western blot analysis is shown of NRXN3β-CTFs in wild-type mouse embryonic fibroblasts (MEF wt, lane 1) in PS1/PS2 double knock-out MEFs (MEF DKO, lane 2), and in PS1/PS2 double knock-out MEFs expressing hPS1 WT (MEF DKO + PS1, lane 3). *, minor band immunoreactive for NRXN3β.

branes isolated from CHO cells stably expressing NRXN3β (Fig. 5A). A second assay was developed using FLAG-tagged NRXN3β-CTFs purified from HEK293T cells and highly purified γ-secretase (Fig. 5B). A third assay was developed using recombinant FLAG-tagged NRXN3β-CTFs purified from E. coli and purified γ-secretase (Fig. 5C). As shown in Fig. 5A, top panel, incubation for 3 h at 37 °C of membranes from CHO cells stably overexpressing NRXN3β resulted, in contrast to membranes incubated on ice, in the generation of an ~12-kDa NRXN3β-ICD product (Fig. 5A, compare lane 3 with lanes 1 and 2). To test whether γ-secretase activity was responsible for the NRXN3β-ICD production, membranes were incubated at 37 °C in the presence or absence of 1 μM of the γ-secretase inhibitors DAPT (Fig. 5A, lane 4) or CpdE (Fig. 5A, lane 5). As expected, DAPT and CpdE both abolished the production of NRXN3β-ICD. Interestingly, we also observed elevated NRXN3β-CTFs levels in membranes incubated with DAPT or CpdE (Fig. 5A, compare lanes 4 and 5 with lanes 1–3). Elevated NRXN3β-CTFs levels were associated with reduced levels of full-length NRXN3β (Fig. 5A, lanes 3–5), indicating that de novo α-secretase-dependent CTFs are produced during the assays.

In an alternative γ-secretase membrane assay (Fig. 5A, lower panel), CHO cells stably overexpressing NRXN3β were first incubated for 16 h in the presence or absence of 10 μM DAPT before being used to prepare membranes. In contrast to membranes prepared from DAPT-treated cells, incubation for 2 h at 37 °C of membranes prepared from DAPT-free cells resulted in the production of NRXN3β-ICD (Fig. 5A, lanes 6 and 7).

We next assessed whether cellular NRXN3β-CTF can be processed by purified γ-secretase (Fig. 5B). HEK293T cells transiently transfected with FL-NRXN3β-FLAG were first incubated for 16 h with 10 μM DAPT to trigger intracellular accumulation of NRXN3β-CTFs (as shown in Fig. 1A, lane 2). Cell lysates were prepared, and FLAG-tagged proteins (including FL-NRXN3β-FLAG and FLAG-tagged NRXN3β-CTFs) were further purified with M2 anti-FLAG affinity resin. NRXN3β-based proteins were eluted in 0.2% CHAPSO-HEPES buffer for compatibility with our in vitro γ-secretase activity assay.
assay. As expected, Western blot analysis of the purified proteins displayed high levels of both the full-length NRXN3 as well as its CTF (Fig. 5B, lane 1). Using this partially purified substrate, in vitro γ-secretase assays were performed as previously described (37, 44). Basically, the reaction mixtures contained the cellular NRXN3β-CTF substrate, the purified γ-secretase solubilized in 0.2% CHAPSO-HEPES, pH 7.5, 0.025% PE, and 0.10% PC. After 4 h of incubation at 37 °C, the reactions were stopped by adding Laemmli buffer, and the samples were assayed for FLAG-tagged ICDs production by Western blot analysis. Confirming our results generated in the membrane assays described above, incubation of the purified NRXN3β with purified γ-secretase led to the production of an ~12-kDa NRXN3β-ICD (Fig. 5B, lane 2). This processing was γ-secretase-specific as DAPT and CpdE reduced by ~48 and ~65% NRXN3β-ICD production, respectively (Fig. 5B, compare lanes 3 and 4 with lane 2, and densitometric analyses).

To get new insight into the sequence requirements for NRXN3β-CTF intramembrane cleavage, we generated recombinant NRXN3β-CTFs of different lengths. Based on size estimation of the major NRXN3β-CTF accumulating in CHO cells stably expressing NRXN3β (see Fig. 1B), we expressed in E. coli recombinant NRXN3β-CTFs (rNRXN3β-CTFs) of different lengths that we extracted from inclusion bodies, purified using an M2 FLAG resin, and used as the substrate in our in vitro γ-secretase assays (not shown). We found that the recombinant NRXN3β C-terminal fragment that includes an

FIGURE 4. γ-Secretase-dependent processing of NRXN3β in a cell-based luciferase reporter assay. A, a schematic representation of the NRXN3β-specific luciferase reporter assay is shown. Full-length NRXN3β fused to the Gal4 DNA binding domain and to the VP16 activation domain (NRXN3β-FL-GV) was expressed under the control of the tetracycline operator (TO) in T-Rex HeLa cells constitutively expressing the tetracycline repressor (tetR) protein transfected with pGL4.31, a plasmid encoding a Gal4-driven luciferase reporter gene. In the absence of tetracycline (− Tet), tetR binds to the tetracycline operator and represses the transcription of NRXN3β-FL-GV, resulting in low expression of luciferase reporter. Added tetracycline (+ Tet) results in tetR release from the tetracycline operator and consequently to the expression of the chimeric proteins NRXN3β-FL-GV and high expression of luciferase reporter. In this system, sequential processing by α- and γ-secretase of NRXN3β-FL-GV leads to the formation of NRXN3β-ICD-GV, which gets released from the membrane and translocated into the nucleus, where it activates transcription of the luciferase reporter gene. Inhibition of α- or γ-secretase is, thus, expected to prevent expression of the luciferase reporter gene. B, the γ-secretase inhibitor DAPT blocks the proteolysis of NRXN3β-CTF-GV or APP-C99-GV and prevents the expression of the luciferase reporter gene. Left, shown is luminescence (counts per second) emitted by cells treated with tetracycline (1 μg/ml) in the presence or absence of DAPT (10 μM). Results are expressed as the means ± S.D. of triplicate luminescence measurements (n = 3, *, p < 0.05, t test). Right, a representative reaction was Western-blotted for NRXN3β-GV and APP-C99-GV by using an anti VP16 antibody. Light (top) and dark (bottom) exposures are shown.
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**A**  
N-terminal 13-amino acid-long short ectodomain stub (starting at amino acid residue 551 according to human FL-NRXN3β numbering) followed by the transmembrane domain and the FLAG-tagged cytoplasmic tail serves as a substrate for γ-secretase (Fig. 5C). Consistent with our membrane assays and *in vitro* assays performed with cellular NRXN3β-CTFs, rNRXN3β-CTF-551 was specifically processed by γ-secretase (Fig. 5C, lane 6) as DAPT and CpdE reduced this processing by ~58 and ~82%, respectively (Fig. 5C, compare lanes 7&8 with lane 6). Finally and consistently, the alignment of the transmembrane regions of NRXN3β with known γ-secretase substrates revealed a recently reported leucine residue well conserved in these transmembrane domains (45) (supplemental Fig. S1).

**DISCUSSION**

In the present study we report that NRXN3β, a cell adhesion molecule playing an important role in establishing and maintaining proper synaptic function, is a substrate for α- and γ-secretases. We further show that the sequential processing of NRXN3β by α- and γ-secretases generates a large extracellular ectodomain of ~80 kDa designated sNRXN3β and a ~12-kDa intracellular domain NRXN3β-ICD. Finally, we show that cleavage of endogenous neuronal neurexin by α- and γ-secretases can be modulated by neuronal activity stimulated by KCl or l-glutamate. The biological relevance of 1) the processing of full-length NRXN3β (FL-NRXN3β), 2) the generation of sNRXN3β, and 3) NRXN3β-ICD production needs to be investigated, but it is conceivable that cleavage of NRXNs by α- and γ-secretases has functional implications beyond the mere clearance of NRXNs in the context of protein turnover (hypotheses are depicted in Fig. 6).

First, *in vivo* studies shed light on the role of NRXNs in both the activity and plasticity of synapses (10, 46, 47). The analysis of α-NRXNs triple knock-out mice (which die shortly...
after birth due to respiratory failure) revealed that synapses were morphologically normal but characterized by dramatically reduced transmission (48). This supports α-NRXNs as proteins essential for proper synaptic function but not for the initial formation of the synapse. The extracellular N terminus of α-NRXNs contains three large repeats composed of two similar LNS (laminin, neurexin, sex hormone binding globulin) domains responsible for the physical interaction across the synaptic cleft with the cholinesterase-like domain of neuroligins (NLGNs) (49). The β-NRXNs, which are essentially N-terminal-truncated α-NRXNs, also display an LNS domain. The transmembrane domain and C-terminal cytoplasmic tail of α and β-NRXNs are strictly identical (with the very C-terminal part of all NRXNs cytoplasmic tails bearing a PDZ II binding motif that is essential for targeting NRXNs to the presynaptic terminals and for the recruitment of scaffolding proteins (47, 50)). Hence, both α- and β-NRXNs, through physical interactions with NLGNs on the extracellular side of the membrane and scaffolding proteins on the cytoplasmic side, are directly implicated in the assembly of synapses. Therefore, we can speculate that processing of FL-NRXN3β by α-secretase and γ-secretase is able to modulate the activity of synapses (Fig. 6).

The production of the soluble large ectodomain sNRXN3β drew our attention as well as several studies using different experimental approaches and aiming to induce NLGNs clustering by using soluble NRXN1βs fragments (rat amino acids 1–299 or mouse amino acids 1–272) have demonstrated inhibitory effects on NLGNs-associated synaptic functions; for instance, presynaptic vesicle clustering (9, 51). Moreover, five canonical alternative splicing sites have been identified in the α-isoforms of NRXNs (sites 1–5), two of which (4 and 5) are shared by the β-isoforms. At these sites, splicing results in the insertion (or not) of sequences ranging from few to up to 191 amino acid residues (1–3). Interestingly, and specifically for the NRXN3 gene, the alternative splicing in the site 5 can result in secreted NRXN3 proteins (3). In this context, a genome-wide study analyzing mutations associated with alcohol dependence recently reported the occurrence of mutations in the NRXN3 gene in a region that controls the generation of transmembrane or soluble NRXN3 proteins (52). Finally, functional interaction of neurexin ectodomain with γ-aminobutyric acid, type A receptors has been recently identified (8). Hence, it is likely that soluble neurexin NTFs can modulate this channel activity. Taken together, these observations suggest a mechanism by which sNRXN3β released into the

FIGURE 6. Model for NRXN3β processing by α- and γ-secretases and hypothetical implications on synaptic activity and plasticity. Full-length NRXN3β (FL-NRXN3β) is synthesized as a type I transmembrane protein forming trans-synaptic complexes with NLGNs required for synapse maturation and function (A). FL-NRXN3β can undergo cleavage at the α-secretase sites to release large ectodomains designated sNRXN3β and leave membrane-embedded fragments NRXN3β-CTFs (B). Proteolysis of these CTFs by γ-secretase releases intracellular domains NRXN3β-ICDs and extracellular NRXN3β peptides (NRXN3β-Ps). Considering the essential roles of NRXNs in synaptic activity and plasticity, one can speculate that the processing of FL-NRXN3β by α-secretase (1) and/or the release of soluble sNRXN3β into the synaptic cleft (2) can modulate NLGNs and NRXN-dependent activities. The intracellular release of NRXN3β-ICD (3) might trigger signaling pathways and/or affect neurotransmitter vesicle clustering and release.
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synaptic cleft, resulting either from mutations in the NRXN genes or FL-NRXNs processing by α-secretases, can affect synaptic activity (Fig. 6).

We also found that the processing by γ-secretase of NRXN3β-CTFs releases an ~12-kDa intracellular domain NRXN3β-ICD. Studies on different γ-secretase substrates including the Notch receptor, APP, p75, the insulin receptor, or the voltage-gated sodium channel B2 subunit have unraveled the role of γ-secretase cleavage and ICD release in inter- or intracellular signaling pathways and downstream transcriptional regulation of nuclear target genes (53–56). Yet, NRXN3β-ICD contains the PDZ II binding motif that is required for its binding to cytoplasmic partners, such as CASK and Mint, and functions as a scaffold protein for vesicle clustering (50, 57). Moreover, the NRXNβ intracellular domain has recently been reported to interact with and participate in the proper targeting of nicotinic receptors (58). Whether NRXN3β-ICD also triggers signaling pathways and/or affects neurotransmitter vesicle clustering and release remains unknown and needs further investigation.

Moreover, we found that several mutations in the catalytic core of γ-secretase known to cause aggressive early-onset forms of Alzheimer disease (59) impair the processing of NRXN3β. In total agreement with our findings, PS1-L166P and PS1-ΔE9 have been reported to cause a loss in APP and Notch substrate processing (60). Because more than 100 missense mutations in the PS genes are known to cause familial, early-onset Alzheimer disease, it remains important to investigate whether impaired NRXN3β processing and sNRXN3β/ NRXN3β-ICD production can participate to the neuronal defects associated with a loss of preasnin/γ-secretase function (31, 32). Similarly, mutations in NRXN1, which have been reported to cause autism spectrum disorders or schizophrenia (61–63), might cause impaired NRXN1 processing and sNRXN1/NRXN1-ICD production.

In conclusion, the present data demonstrate that NRXN3β can be processed by α- and γ-secretases, leading to the formation of two final products, an ~80-kDa N-terminal extracellular sNRXN3β and an ~12-kDa C-terminal intracellular NRXN3β-ICD, both being potentially implicated in the regulation of the activity and plasticity of synapses and signal transduction pathways.

Acknowledgments—We are grateful to B. De Strooper for the WT and PS1−/− PS2−/− mouse embryonic fibroblasts and to M. Wolfe and C. Saura for the APP-C99-GV and PS1-ΔE9 constructs. We also acknowledge L. Aeschbach and S. Reymond for excellent technical assistance.

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