Neurotrypsin cleaves agrin locally at the synapse

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ABSTRACT **The synaptic serine protease neurotrypsin is considered to be essential for the establishment and maintenance of cognitive brain functions, because humans lacking functional neurotrypsin suffer from severe mental retardation. Neurotrypsin cleaves agrin at two homologous sites, liberating a 90-kDa and a C-terminal 22-kDa fragment from the N-terminal moiety of agrin. Morphological analyses indicate that neurotrypsin is contained in presynaptic terminals and externalized in association with synaptic activity, while agrin is localized to the extracellular space at or in the vicinity of the synapse. Here, we present a detailed biochemical analysis of neurotrypsin-mediated agrin cleavage in the murine brain. In brain homogenates, we found that neurotrypsin exclusively cleaves glycanated variants of agrin. Studies with isolated synaptosomes obtained by subcellular fractionation from brains of wild-type and neurotrypsin-overexpressing mice revealed that neurotrypsin-dependent cleavage of agrin was concentrated at synapses, where the most heavily glycanated variants of agrin predominate. Because agrin has been shown to play an important role in the formation and the maintenance of excitatory synapses in the central nervous system, its local cleavage at the synapse implicates the neurotrypsin/agrin system in the regulation of adaptive reorganizations of the synaptic circuitry in the context of cognitive functions, such as learning and memory.—Stephan, A., Mateos, J. M., Kozlov, S. V., Cinelli, P., Kistler, A. D., Hettwer, S., Rülicke, T., Streit, P., Kunz, B., Sonderegger, P. Neurotrypsin cleaves agrin locally at the synapse. *FASEB J.* 22, 1861–1873 (2008)**

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Neurotrypsin is a neuronal serine protease that has been localized to the presynaptic boutons of the central nervous system (CNS) synapses in several regions, including the cerebral cortex, the hippocampus, and the amygdala (1–3). It was recognized to play an indispensable role for cognitive processes in humans when a four-nucleotide deletion in the coding region of neurotrypsin, which disabled the expression of the proteolytic domain, was identified as the cause of severe mental retardation (2). The affected children exhibited severe deficits in cognitive development after normal psychomotor development during the first 18 months, indicating that neurotrypsin does not play a critical role for early neural development or for the formation of synapses. Rather, neurotrypsin may be crucial for the reorganizations of synapses and neuronal circuits, which are required to establish and maintain higher cognitive functions during later developmental and adult stages.

In presynaptic boutons of mature neurons neurotrypsin is predominantly localized to the area lining the synaptic cleft (2). Its synaptic recruitment as well as its externalization is regulated by neuronal activity, and externalized neurotrypsin lingers at the synapse for minutes before disappearing due to diffusion or degradation (4). These observations suggest an activity-dependent extracellular proteolytic function of neurotrypsin at the synapse.

The so far unique proteolytic target of neurotrypsin is agrin (5). Agrin, a large heparan sulfate proteoglycan, is expressed in neuronal, as well as nonneuronal tissues (6). Nerve-derived agrin has been demonstrated to play an essential role in development and maintenance of the neuromuscular junction (7, 8), but recent reports also implicate agrin as a regulator of CNS synapses. Lack of agrin in the CNS has recently been reported to result in loss of synapses and agrin has been proposed to play a role in the formation and/or maintenance of synapses *via* a mechanism involving the mitogen-activated protein (MAP) kinase signaling pathway (9). Other synaptic functions of agrin include inhibition of the α3-subtype of the Na^+-K^+-ATPase,

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thereby evoking synaptic depolarization (10), and the formation of filopodia (11, 12), which, in turn, may provide the basis for the formation of new synapses (13, 14). Cleavage by externalized neurotrypsin could thus regulate agrin's action at the CNS synapse.

To study whether synaptic release of neurotrypsin resulted in spatially concentrated cleavage of agrin in the synaptic extracellular space, we isolated synaptosomes from brains of wild-type and neurotrypsin-overexpressing mice by subcellular fractionation and analyzed their neurotrypsin-agrin relationship by Western blot analysis. We found that the heavily glycanated variants of agrin predominate at synapses and that neurotrypsin-dependent cleavage of agrin is indeed more pronounced at synapses when compared with other subcellular fractions. Local agrin cleavage at or in the vicinity of synapses may be a mechanism for regulating the synaptic functions of agrin.

MATERIALS AND METHODS

Generation of transgenic mice

To generate the pThy1-loxP-STOP-loxP-neurotrypsin construct, the human neurotrypsin cDNA was subcloned into the *Xho*I site of the murine Thy1.2 genomic expression cassette (15). The loxP-flanked transcription termination cassette from the pBS302 vector (Invitrogen AG, Basel, Switzerland) was inserted between the Thy1 promoter and the translational start codon of neurotrypsin. To generate the pThy1-neurotrypsin construct containing the serine-to-alanine mutation, a PCR fragment was amplified using mouse neurotrypsin cDNA as a template and 5′-CGTGTGGACAGC-TGCCAGGGAGACGCTGGAGGA-3′ and 5′ CTCAAGCTTAG-TTACAGACTGGTGACACTTTTTATC-3′ as primers. This fragment was reintroduced into the vector containing full-length neurotrypsin. The mutated neurotrypsin cDNA was excised and subcloned into the *Xho*I site of the Thy1.2 cassette. The two resulting vectors were digested with *Pvu*I, and the fragments were purified from agarose gels for pronuclear injections into fertilized oocytes according to standard protocols (16). Zygotes were produced by mating B6C3F1 hybrids. The offspring of transgenic founders of each transgenic line [human neurotrypsin, Tg(PRSS12)491Zbz; inactive mouse neurotrypsin, Tg(Prss12^{S711A})785Zbz] were mated with C57BL/6-Tg(CMV-Cre) mice (17) to delete the floxed transcription termination cassette (18) and activate the dormant transgene. Subsequent offspring of constitutively overexpressing mice were backcrossed for more than 10 generations to C57BL/6 to produce congenic lines. For genotyping, animals were screened by PCR on genomic DNA using the primers 5′-CCCATCCACATGGAT-AATGTGAA-3′ and 5′-CCCATGTTCTGAGATATTGGAAG-3′. Neurotrypsin-deficient mice were generated as described before (5). In brief, a targeting vector, containing the genomic DNA segment comprising exons 10 and 11 of murine neurotrypsin, was generated by inserting a loxP site 0.22 kb upstream of neurotrypsin exon 10 and a floxed neomycin resistance sequence 0.12 kb downstream of neurotrypsin exon 11. In addition, homology arms of 7.5 kb and 3.5 kb were added at the 5′- and 3′-regions, respectively. For negative selection, a diphtheria toxin cassette was inserted at the 3′ end. The linearized targeting vector was electroporated into C57BL/6-derived embryonic stem cells. Stem cell lines with homologous recombination were injected into blastocysts and a germ-line chimeric male was crossed with C57BL/6 females to generate heterozygous animals. These animals were crossed with CMV-Cre mice to excise the floxed region, resulting in a truncated neurotrypsin gene lacking the region, encoding the proteolytic domain. All experimental protocols were approved by the Veterinary Department of the Canton of Zurich, Switzerland.

Antibodies

Goat antiserum G93 was raised against full-length mouse neurotrypsin (5). Rabbit antiserum R132 was raised against the C-90 kDa fragment and goat antiserum G92 was raised against the C-22 kDa fragment of agrin (5). Both antibodies were purified by affinity chromatography. Depending on the goal of the analysis, we used either the anti-C-22 or the anti-C-90 antibody. The anti-C-22 antibody was used to make sure that the detected signals corresponded to full-length agrin, because it was directed *vs.* the most C-terminal domain of agrin. Because of its higher affinity, the anti-C-90 antibody was superior to the anti-C-22 antibody when detection of low amounts of antigen was the primary goal. In Western blot analysis experiments, both the anti-C-90 and the anti-C-22 antibodies detected the variants of full-length agrin in an identical band pattern. The slight differences in the relative intensity of the upper, middle, and lower variants obtained with the anti-C-22 (**Fig. 3*B***), compared to the anti-C-90 antibody (**Fig. 2*B***), most likely reflected a differential preference of the antibodies for distinct variants of agrin rather than contributions from neurotrypsin-dependent cleavage fragments, although fragments that originate from cleavage only at the β site are expected to colocalize with the full-length forms of agrin on Western blots in the Mr range above 250 kDa. Coexpression of agrin and neurotrypsin in HeLa cells resulted in the release of not only the 22- and the 90-kDa, but also the 110-kDa fragment that originated from partial cleavage at the α site (5). This pattern demonstrated that neurotrypsin-dependent cleavage at the β site was not a necessary condition for cleavage at the α site and suggested that agrin cleavage at the α site preceded β site-cleavage or that cleavage occurred at both sites in parallel. Therefore, we assumed that the large agrin fragment generated by partial cleavage at the β site and, thus, lacking only the 22-kDa fragment, occurred only in negligible amounts if at all.

We used the anti-C-22 antibody for detection of full-length agrin in our initial analyses of neurotrypsin-dependent agrin cleavage in brain homogenates of both neurotrypsin-overexpressing and neurotrypsin-deficient mice (Fig. 3), in order to exclude detection of partially cleaved agrin variants. As these analyses were done with P9 mouse brain tissue, the agrin signals were sufficiently strong to be detected by this relatively low affinity antibody. In contrast, for all experiments that required sensitive detection of weak signals, including the analysis of the time course of agrin cleavage (Fig. 2) and the analyses of synaptosomal preparations (**Figs. 4** and **5**), we used the anti-C-90 antibody that has a higher affinity. We also used the anti-C-90 antibody for our immunohistochemical analyses because of its higher affinity. This antibody detects both full-length agrin and isolated C-90 fragments. Because of the high-affinity interaction between a region of agrin composed in the C-90 kDa fragment and the transmembrane protein dystroglycan (20), diffusion of the 90-kDa fragment in the extracellular space is unlikely. Therefore, the immunohistological localizations of full-length agrin and the C-90 fragment are expected to be identical.

The monoclonal anti-β-actin antibody (clone AC-74) and the peroxidase-conjugated secondary antibodies for immunoblotting were purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA). The monoclonal anti-PSD-95 antibody (clone10) was purchased from BD Biosciences (Allschwil, Switzerland).

SDS-PAGE and Western blot analysis

The forebrain and midbrain regions of either C57BL/6 wild-type mice, neurotrypsin-deficient mice, mice overexpressing catalytically active human neurotrypsin or catalytically inactive mouse neurotrypsin were dissected and homogenized in a glass-Teflon homogenizer (1000 rpm, 10 strokes) in a solution of 320 mM sucrose in 5 mM HEPES, pH 7.5, containing protease inhibitors (P-8340; Sigma Aldrich Co.). After brief centrifugation of the homogenates at 1000 *g*, supernatants were collected, and protein concentrations were measured by a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples with equal protein concentrations were resolved by SDS-PAGE on 4–12% NUPAGE gels using MOPS running buffer, on 10–20% NOVEX Tricine gels (both from Invitrogen AG, Basel, Switzerland) for analyses of agrin or on conventional 10% gels for detection of neurotrypsin. Proteins were subsequently blotted to polyvinylidene fluorid membranes (Immobilon P membrane; Millipore, Billerica, MA, USA). The membranes were dried and incubated with primary antibodies in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) and 2.5% Western blocking reagent (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany), for 60 min. After washing with TBST, the blots were incubated with peroxidase-conjugated secondary antibodies for 45 min and washed again. Immunoreactive protein bands were detected using ChemiGlow (Alpha Innotech GmbH, Kasendorf, Germany). For reprobing, membranes were incubated twice for 30 min in stripping solution (0.1% SDS, 1% Tween-20, in 200 mM glycine-HCl, pH 2.2) and afterward extensively washed in TBST before incubation with primary antibodies.

Heparitinase digestion

The forebrain/midbrain regions of one P6 C57BL/6 mouse were homogenized with a glass-Teflon homogenizer (1000 rpm, 10 strokes) in enzyme buffer (20 mM Tris, 0.1 mg/ml BSA, 4 mM $CaCl_2$, protease inhibitors, pH 7.5). After brief centrifugation of the homogenate at 1000 *g*, the supernatant was collected and the protein concentration was measured by a Bradford assay (Bio-Rad Laboratories, Inc.). Aliquots of 150-μg protein were incubated with 1 U heparitinase (heparinase III, reconstituted in enzyme buffer; Sigma-Aldrich Co.) for either 2 or 5 h at 37°C. For the control sample, 150 μg of protein were incubated in enzyme buffer alone. Reactions were stopped by freezing the samples in liquid nitrogen. Samples were finally analyzed by SDS-PAGE and Western blot analysis for full-length agrin detection.

Preparation of synaptosomes

Synaptosomes were prepared from P12 to P15 neurotrypsin-overexpressing transgenic mice or their wild-type littermates as described before (21). In brief, the forebrain/midbrain regions from 6 mice were homogenized with a glass-Teflon homogenizer (800 rpm, 12 strokes) in buffer A (320 mM sucrose, protease inhibitors, in 5 mM HEPES, pH 7.5) and centrifuged at 1000 *g* for 5 min. The resulting supernatant was further centrifuged at 12,000 *g* for 20 min to produce the crude synaptosomal pellet. To reduce the content in mitochondria, only the upper part of the pellet was resuspended and rehomogenized in buffer A using a hand-held glass-homogenizer (5 strokes) and the centrifugation at 12,000 *g* was repeated. Again, only the upper part of the pellet was resuspended and rehomogenized in buffer A, loaded on a 7.5%/12% Ficoll step gradient, and centrifuged at 68,000 *g* for 1 h. The synaptosomes, concentrated at the 7.5%/12% Ficoll gradient interphase, were well separated from a mainly myelin-containing band in the upper part of the 7.5% Ficoll phase and the mainly mitochondria-containing pellet. The synaptosomal fraction was collected by puncturing the tube with an 18-gauge needle and further analyzed by Western blot analysis or electron microscopy.

Electron microscopy of the synaptosomal fraction

Purified synaptosomes were washed and resuspended in Krebs solution, incubated for 10 min at 37°C, and fixed overnight at 4°C in 4% formaldehyde and 0.05% glutaraldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). After several washes in PB, the samples were osmicated in 1% OsO_4 in PB for 30 min, washed thoroughly with PB, and dehydrated in increasing concentrations of ethanol. Subsequently, fractions were embedded in Epon resins (Epon 812, Fluka, Buchs, Switzerland). Ultrathin sections from synaptosomes were imaged using a digital camera (Gatan 791 multiscan; Gatan Inc., Pleasanton, CA, USA) attached to an EM10C electron microscope (Zeiss, Oberkochen, Germany).

Immuno-electron microscopy of brain sections

Two 6-day old mice, deeply anesthetized with a mixture of Dormicum and Hypnorm (15 μl/g body weight), were transcardially perfused with phosphate-buffered saline (PBS), followed by 150 ml of a fixative containing 4% formaldehyde and 0.05% glutaraldehyde in PB (0.1 M, pH 7.4). Brains were postfixed for 1 h and, after several washes in PB, immersed in increasing concentrations of sucrose: 10%, 20% and left overnight in 30% sucrose in PB at 4°C. Brains were frozen by immersion in N-methylbutane (Fluka, Buchs, Switzerland) at −40°C and quickly thawed in PBS. Afterward, 100-μm-thick vibratome sections were obtained from the cerebral cortex and hippocampus. Sections were preincubated in 10% goat serum in PBS for 1 h and incubated with 2 μg/ml of R132 in 2% goat serum/PBS for 2 days at 4°C. Subsequent washes were followed by preincubation in 2% goat serum and an incubation for 2 h in anti-rabbit secondary antibody conjugated to 1.2-nm gold particles (Nanoprobes, Inc., Yaphank, NY, USA) at a concentration of 1:100 in 2% goat serum/PBS. Sections were washed in PBS and postfixed in 1% glutaraldehyde for 10 min. After washes with double-distilled water, sections were silver intensified with the HQkit (Nanoprobes, Inc.) for 12 min, osmicated in 1% OsO_4 for 30 min, dehydrated in increasing concentrations of ethanol, and embedded in Epon resins. Serial sections from the hippocampus and cerebral cortex were analyzed and imaged with the electron microscope as described above. Detection of neurotrypsin in adult C57BL/6 mouse brains was performed as described before (2).

Mass spectrometric analysis

Both agrin C-90 fragments were affinity purified from P25 to P50 neurotrypsin-overexpressing transgenic mouse brains. At this age, both bands appear with almost equal intensity and, because of overexpression of active neurotrypsin, in substantial amounts. In addition, at ages above P15, bands detected in mouse brain tissue appear well separated (Fig. 2*B*). In brief, the soluble fraction of brain homogenates from 50 mouse brains was used for heparin-sepharose CL-6B affinity chromatography (Amersham Biosciences, GE Healthcare UK Ltd., Little Chalfont, UK). Proteins were eluted with 1M NaCl, in 20 mM HEPES, pH 7.5, and the eluate was concentrated with Vivaspin20 (MWCO–50 kDa) centrifuge cartridge concentrators (Sartorius AG, Goettingen, Germany). Samples

were further purified by immunoaffinity chromatography, using immobilized affinity-purified antibody R132. Bound proteins were eluted by boiling the affinity matrix in 10% SDS/PBS. The eluted proteins were concentrated by methanol/chloroform protein precipitation (22), resuspended in Laemmli buffer and separated by 10% SDS-PAGE to yield maximal partition of the upper and lower 90-kDa agrin bands. Separated proteins were visualized using the colloidal blue staining kit (Invitrogen AG, Basel, Switzerland). Bands corresponding to both upper and lower 90-kDa agrin (see Results; Fig. 2) were analyzed by LC-ESI-MS/MS analysis on a Qutof Ultima API (Waters S.A.S., Saint-Quentine, France) at the Functional Genomics Center Zurich, University and ETH Zurich.

Quantification and statistical analysis

Western blots were recorded with a LAS-3000 Fujifilm imager (Fujifilm Europe GmbH, Duesseldorf, Germany), and densitometric analyses of the protein bands were performed using AIDA software (Raytest GmbH, Straubenhardt, Germany). We were careful to avoid measurement of saturated signals. For analysis of P9 mouse brain homogenates, at least 3 animals per genotype were analyzed and compared to signals of at least 3 different corresponding wild-type littermates. Signals of two independent blots per genotype were quantified and used for statistical analyses. For densitometric analyses of synaptosomal preparations, five separate preparations were analyzed. For all analyses, agrin signals were first normalized to actin signals of the corresponding sample, serving as an internal loading control. Then, adjusted values obtained from transgenic samples of each experiment were compared to the corresponding adjusted signals from the samples of wild-type littermates, whereby the wild-type signal was set to 100%. Data are presented as means ± SE. Statistical analyses were performed using unpaired Student's *t* test (Sigmaplot, Systat Software, Inc., San Jose, CA, USA).

RESULTS

Neurotrypsin and agrin localize to synaptic structures in the murine brain

Neurotrypsin has been localized to presynaptic nerve endings of excitatory synapses in the human brain (2). Similarly, we found neurotrypsin localized to presynaptic nerve endings in the hippocampus and the cerebral cortex of the murine brain based on pre-embedding immunogold labeling (**Fig. 1*A*, *B***). In both regions neurotrypsin immunoreactivity was most prominent in presynaptic terminals of excitatory synapses. Most immunogold particles were found in the presynaptic region along the membrane segment lining the synaptic cleft, confirming the presynaptic localization of neurotrypsin in the murine brain.

To determine the precise localization of agrin at the subcellular level, we performed pre-embedding immunogold experiments with P6 mouse brain samples using an affinity purified polyclonal antibody against the C-terminal 90-kDa agrin fragment (5). At the light microscopic level, immunohistochemistry with this antibody revealed an intense staining of the neuropil in many brain areas, including the hippocampus and the cerebral cortex (unpublished observations). Ultrathin sections from the stratum radiatum in the CA1 area of the hippocampus showed a strong staining in the basal lamina of the blood vessels (Fig. 1*E*), confirming the published expression of agrin by blood-brain barrier microvessels (23, 24). In the stratum radiatum of the hippocampal CA1 area (Fig. 1*C*) and in the cerebral cortex (Fig. 1*D*), the neuropil was decorated with silver-intensified gold particles associated with membranes. Clusters of silver-intensified gold particles were found at perisynaptic, as well as at extrasynaptic posi-

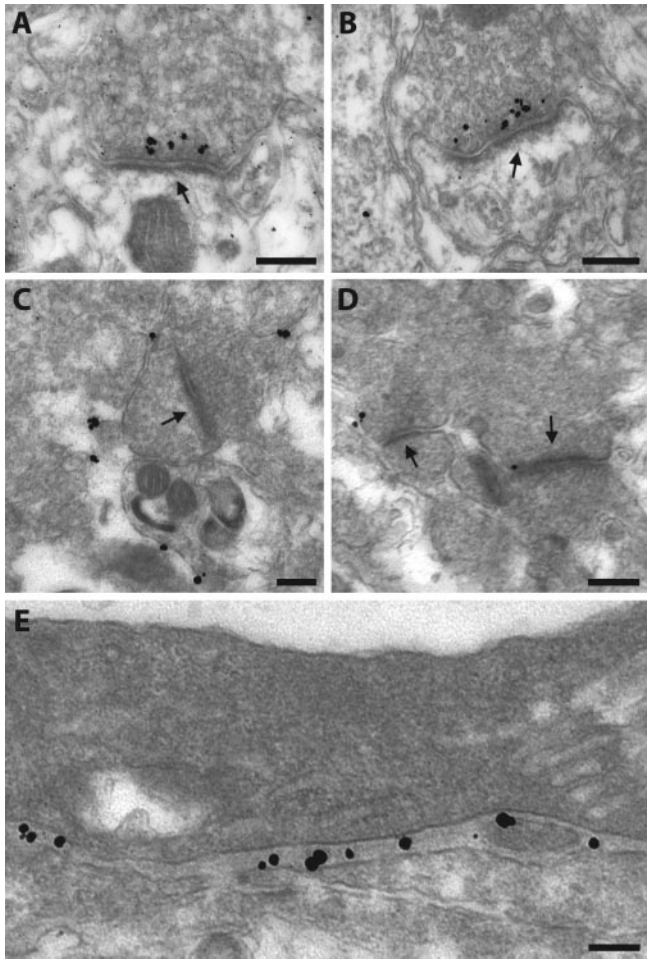

Figure 1. Subcellular localization of neurotrypsin and agrin in hippocampus and cerebral cortex of wild-type mice. *A, B*) Immuno-electron microscopy of the neuropil of the hippocampal CA1 area and the cerebral cortex localized neurotrypsin in presynaptic terminals, close to presynaptic membranes lining the synaptic cleft. Silver-intensified gold particles reporting neurotrypsin immunoreactivity were accumulated at vesicles close to the presynaptic membrane opposite to the postsynaptic density (arrows). *C, D*) Silver-intensified gold particles indicating agrin immunoreactivity were found at perisynaptic, as well as extrasynaptic locations in the neuropil of the hippocampal CA1 area and the cerebral cortex. *E*) The basal lamina of blood vessels in the stratum radiatum in the CA1 area of the hippocampus was strongly labeled with agrin-specific antibodies. The complete absence of gold particles in the adjacent tissue indicates a high specificity of the antibody. Arrows point to postsynaptic densities. Scale bars = 0.2 μm.

tions (Fig. 1*C, D*). These results confirm previous reports demonstrating agrin at or in the vicinity of synapses (9, 25). In the context of the present study, these results demonstrate that neurotrypsin and its as yet unique proteolytic target protein agrin are both located at synapses.

Expression of neurotrypsin and agrin, as well as neurotrypsin-dependent agrin cleavage predominate during neural development

We recently demonstrated that the abundance of the neurotrypsin-dependent fragments of agrin in the murine brain is highest during the peak of neurotrypsin expression in the first two postnatal weeks (5). To obtain a detailed view of neurotrypsin-dependent processing of agrin, we analyzed the expression pattern of full-length agrin together with neurotrypsin and the neurotrypsin-dependent fragments of agrin by Western blot analysis of brain homogenates from mice of various developmental and adult stages. After SDS-PAGE, based on a Bis-Tris buffer adjusted to neutral pH and gradient gels, we detected full-length agrin as three distinct groups of bands between 220 and 500 kDa with an antibody against the C-terminal 90-kDa fragment (Fig. 2*B*). The identity of these distinct groups of bands as variants of agrin was confirmed with several different polyclonal antibodies against the 90- or the 22-kDa agrin fragments (data not shown). On Western blots made from standard SDS-PAGE gels, the variant forms of agrin were not resolved but appeared as a broad smear ranging from 220 to 500 kDa. This feature was suggested to reflect the existence of differently glycanated agrin variants (6). We confirmed differential glycanation as the molecular basis underlying all of these resolved agrin variants by heparitinase digestion of brain homogenates, which resulted in a shift of all upper and middle bands to the level of the lower bands

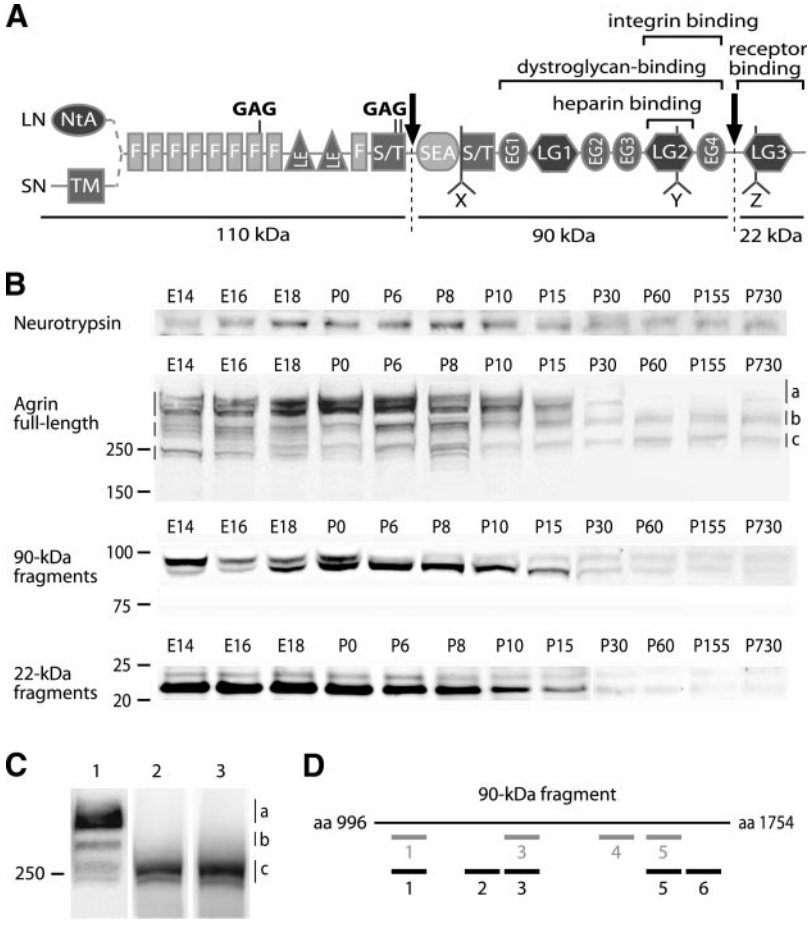

Figure 2. Expression of neurotrypsin and agrin, as well as neurotrypsin-dependent agrin cleavage, are developmentally regulated. *A*) Schematic overview of agrin's protein structure (modified from refs. 6, 19). Alternative splicing leads to the expression of a variety of transmembrane (SN) or secreted (LN) agrin variants, containing variable inserts of several amino acids at the x, y, and z splice sites. Binding sites within the C-terminal moiety of agrin are indicated. Neurotrypsin-dependent cleavage of agrin (indicated by arrows) generates two C-terminal fragments of 90 and 22 kDa. NtA, N-terminal agrin domain; TM, transmembrane domain; F, follistatin-like domains; GAG, glycosaminoglycan side chains; S/T, serine-threonin-rich region; LE, laminin-epidermal growth factor (EGF) -like domains; SEA, sperm protein, enterokinase and agrin domain; EG, EGF-like domains; LG, laminin-globular domains. *B*) Western blot analyses of mouse brain homogenates obtained from animals of E14 to P730. Peak expression of neurotrypsin and agrin is found during the first postnatal week. The protein bands above 220 kDa, detected with the anti-C90-agrin antibody, represent differentially glycosylated variants of full-length agrin. They can be categorized into three distinct groups, termed upper (a), middle (b), and lower (c) variants of agrin. Neurotrypsin-dependent cleavage of agrin also appear strongest around the first postnatal week. Both cleavage products of agrin appear as double bands. Molecular mass references are indicated in kDa. *C*) Heparitinase digestion of P6 mouse brain homogenates. In control samples, full-length agrin was detected as three distinct groups of bands, running above 220 kDa (1: control). Digestion with heparitinase III resulted in the disappearance of the upper and middle variants, characterizing them as glycanated variants of agrin (2: 2 h digestion; 3: 5 h digestion). *D*) Mass spectrometric analysis of the purified 90-kDa fragments from brains of P25-P50 mice overexpressing active neurotrypsin confirmed that both bands correspond to the C-terminal region of agrin. The detected tryptic peptides of the isolated protein bands (gray: upper 90-kDa band; black: lower 90-kDa band) were aligned within the designated C-terminal region of agrin ranging from amino acid (aa) 996 to aa 1754 (NCBI protein accession no. AAA40703.1). The identified peptides were 1-SIESTLDDLFR (pos. 1061–1071), 2-ALETEGLLLYNGNAR (pos. 1293–1307), 3-FDTGSGPAVLTSLVPVEPGR (pos. 1325–1344), 4-GPSGLLLYNGQK (pos. 1563–1574), 5-EPLYVGGAPDFSK (pos. 1656–1668), and 6-GHQLLTQEHVLR (pos 1690–1701).

(Fig. 2*C*). On the basis of these results, we concluded that the lower group of bands reflected agrin variants without or with little glycanation, while the middle and upper groups were composed of variants with intermediate and strong glycanation.

Western blot analysis of brain homogenates of mice from E14 through P730 demonstrated that the expression of full-length agrin was developmentally regulated (Fig. 2*B*), with maximal expression between E18 and P10. Subsequently, expression declined until P30 but persisted at a low level throughout adult life. Similar overall expression profiles of agrin mRNA have been reported previously (26, 27). Our analysis allowing resolution of the glycanation-dependent variants of agrin revealed that the stage-dependent regulation of agrin expression was most pronounced for the upper group of variants, consisting of the most heavily glycanated forms of agrin. These variants exhibited the most pronounced developmental rise, as well as a stronger decline during adulthood. In contrast, the expression of the middle and lower groups of agrin variants was only slightly enhanced during development and less reduced during adulthood. The abundance of the neurotrypsin-dependent agrin fragments roughly reflected the temporal expression pattern of full-length agrin and the expression pattern of neurotrypsin, which also peaked between E18 and P10 (Fig. 2*B*; ref. 5).

Both neurotrypsin-dependent fragments of agrin were detected as double bands (Fig. 2*B*). The two bands of the 90-kDa fragment showed a differential regulation between E14 and P15. The upper 90-kDa band was strongest in the samples from E14 brain, and its intensity declined in the first postnatal days. In contrast, the lower 90-kDa band was strongest between E18 and P15. Beyond P15, both bands persisted throughout adult life at equal but weak levels. To confirm their identity, we purified both 90-kDa bands by immunoaffinity isolation from mouse brain homogenates, subjected them to tryptic digestion, and analyzed the peptides by mass spectrometry. Several sequences of agrin-derived peptides, covering the 90-kDa fragment, were found in both bands (Fig. 2*D*). The two bands of the 22-kDa fragment, detected with an antibody exhibiting higher affinity compared to the antibody used in our previous study (5), were of equal relative intensity at all timepoints with much stronger signals for the lower band. Highest levels of the 22-kDa fragment were found between E14 and P8. The decline in the intensity of the 22-kDa fragment between P8 and P15 correlated well with a drop in expression of both full-length agrin and neurotrypsin. Similar to the 90-kDa fragment, the 22-kDa fragment remained detectable throughout adult life. In contrast, the high levels of the 22-kDa fragment detected already at E14 do not correlate well with the relatively low expression levels of neurotrypsin. This discrepancy could be due to particularities in the spatial locations of agrin and neurotrypsin at early developmental stages, which are not reflected in the results of Western blot analyses based on homogenates of whole brains. The molecular basis underlying the differential electrophoretic mobility of the two variants of the 90- and 22-kDa fragments of agrin is currently not clear. On the basis of the highly specific nature of the proteolytic activity of neurotrypsin (5), differential proteolytic cleavage is unlikely. Rather, the variants of the two agrin fragments may reflect different splice variants of agrin or differential post-translational modifications (6), such as potential O-linked glycosylation as predicted for the region of agrin giving rise to the 90-kDa fragment (28).

The glycanated variants of CNS agrin are preferred substrates of neurotrypsin

To analyze the neurotrypsin-dependent cleavage of agrin in detail, we performed quantitative densitometry of Western blots of mouse brain homogenates. Brain samples were prepared at P9 from neurotrypsin-deficient (Ntd) mice, mice overexpressing catalytically active (Nto-act) or a catalytically inactive (Nto-inact) neurotrypsin and were compared with brain samples from their wild-type littermates. The analyses of Nto-inact mice were included as a control to confirm that the catalytic activity of neurotrypsin is strictly required for neurotrypsin-dependent cleavage of agrin (see below). Representative Western blots of neurotrypsin and full-length agrin, detected with the anti-C-22 antibody, as well as its 90- and 22-kDa fragments are shown in Fig. 3*A–D*. Western blot analyses of neurotrypsin confirmed its absence in Ntd mice and its excess in neurotrypsin-overexpressing mice (Fig. 3*A*). Neither the 90- nor the 22-kDa fragment of agrin was found in samples from Ntd mice, confirming their origin from neurotrypsin-dependent cleavage of agrin (Fig. 3*C–F*). Accordingly, both fragments were increased in samples from Nto-act mice (Fig. 3*C–F*). Densitometry revealed that overexpression of active neurotrypsin resulted in a significant 4-fold increase of the 90-kDa fragment (392.0±44.1% of wild type; Fig. 3*E*). In contrast, the 22-kDa fragments showed an increment of only 1.5-fold (148.4±7.9% of wild type; Fig. 3*F*). This discrepancy may be explained by higher diffusive properties of the 22-kDa fragments or different stabilities of the respective fragments. In samples from transgenic Nto-inact mice, the signal intensities of both agrin fragments did not significantly differ from those of wild-type mice (92.6±5.0% of wild type for the 90-kDa fragment and 111.7±7.2% of wild type for the 22-kDa fragment; Fig. 3*E, F*. The neurotrypsin-dependent increase of the agrin fragments in samples from Nto-act mice coincided with a decrease of full-length agrin (Fig. 3*G–I*). We measured the signal intensities of the upper, middle, and lower groups of full-length agrin in samples from Nto-act, Nto-inact, and Ntd mice using an antibody against the 22-kDa fragment and normalized the results to the corresponding signals of wild-type littermates. The results indicated that the protein levels for upper and middle full-length agrin were significantly decreased in the brain of Nto-act mice (59.1±4.4 and 75.2±8.7% of wild

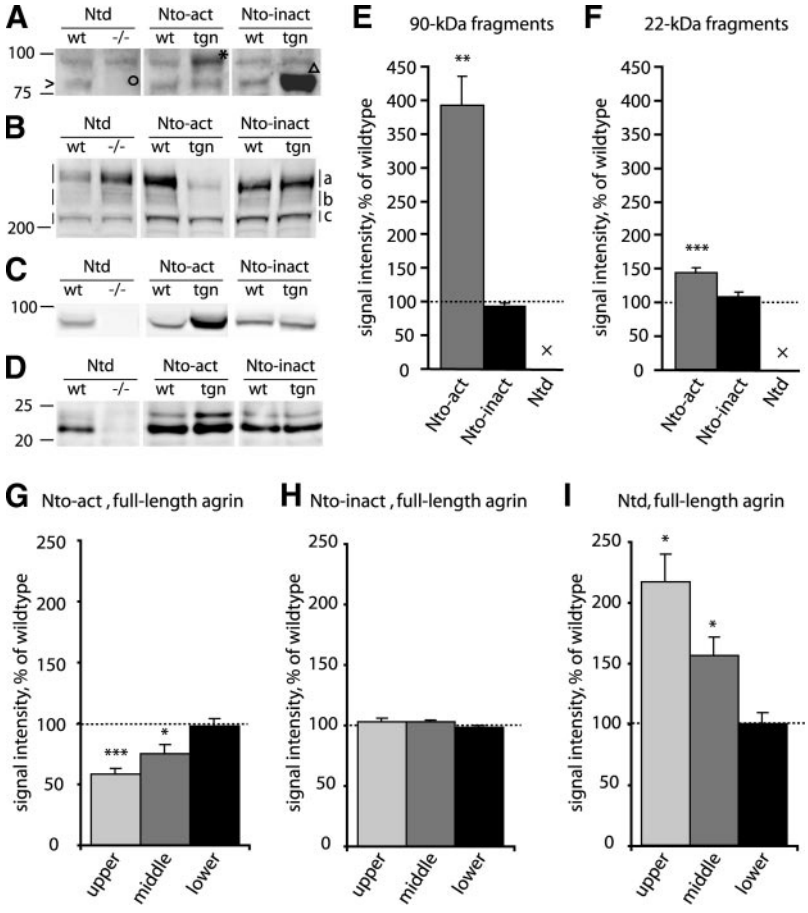

Figure 3. Neurotrypsin exclusively cleaves glycanated agrin variants in the mouse brain, generating the 90- and 22-kDa fragments. *A–D*) Representative images of Western blots from P9 mice confirm that agrin is a substrate for neurotrypsin. Brain homogenates from neurotrypsin-deficient (Ntd) mice, human neurotrypsin-overexpressing (Nto-act) mice, or mice that overexpress a catalytically inactive variant of mouse neurotrypsin (Nto-inact) were compared to brain homogenates from their wild-type (wt) littermates. Molecular mass references are indicated in kDa. *A*) Neurotrypsin detected in samples of Ntd, Nto-act, and Nto-inact brain homogenates. Endogenous neurotrypsin was detected as a single band at 85 kDa (arrowhead) in samples of all genotypes except Ntd (circle). In contrast to mouse neurotrypsin, human neurotrypsin contains an additional SRCR domain, resulting in an approximate Mr of 95 kDa. Transgenic human neurotrypsin was detected in samples of Nto-act mice as a double band at around 95 kDa (asterisk) with a much stronger signal for the upper band. This human neurotrypsin-specific double band was situated on top of a weak unspecific band visible in samples of all genotypes, including Ntd. Transgenic inactive mouse neurotrypsin was detected at 85 kDa, superimposed over endogenous neurotrypsin (triangle). *B–D*) Cleavage of the variants of full-length agrin [upper (a), middle (b), and lower (c) full-length agrin variants], as well as the appearance of the 90- and the 22-kDa fragments, detected in brain homogenates of Ntd, Nto-act, and Nto-inact mice. *E, F*) Quantitative densitometric Western blot analyses of the appearance of the 90- and the 22-kDa fragments, respectively, revealed that these fragments were only generated by active neurotrypsin. *G–I*) Quantitative analyses of full-length agrin signals confirmed that neurotrypsin exclusively cleaved the upper and middle variants of agrin, with preference for the most heavily glycanated upper variants. Signal intensities of the upper, middle and lower agrin variants in samples from Nto-act (*G*), Nto-inact (*H*), and Ntd (*I*) mice. Signal intensities of Ntd, Nto-act, or Nto-inact samples, normalized to signals of the corresponding wild-type littermate controls (wild-type values set to 100%), are shown. Data represent mean ± SE values. For each genotype, two Western blots with samples of at least three different animals per genotype were analyzed; $n \geq 6$. × = no signal detected. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

type, respectively; Fig. 3*G*), unaltered in Nto-inact mouse brains (103.6±2.7 and 103.7±0.3% of wild type, respectively; Fig. 3*H*), and increased in the brain of Ntd mice (216.4±23.2 and 156.4±14.0% of wild type, respectively; Fig. 3*I*). In contrast, no alterations of the lower group of full-length agrin were measured in all analyzed tissues (Nto-act, 99.0±6.2%; Nto-inact, 97.9±2.2%; and Ntd, 99.4±10.2% of wild type; Fig. 3*G–I*). These results demonstrated that only the glycanated variants of full-length agrin are cleaved by neurotrypsin. Furthermore, we registered a tendency for preferential cleavage of the variants of the upper group, as their increase in Ntd and decrease in Nto-act mice were stronger compared to the variants of the middle group. However, the differences were not statistically significant.

The most heavily glycanated variants of agrin are enriched in synaptosomes

Neurotrypsin localizes to presynaptic terminals *in vivo* (Fig. 1*A*) and results obtained with cultured hippocampal neurons indicated its activity-dependent release from synaptic terminals (4). Experiments with fluorescently tagged neurotrypsin indicated that neurotrypsin lingered in the synaptic extracellular space after activity-dependent externalization from presynaptic intracellular stores. Therefore, we speculated about a local proteolytic role of neurotrypsin in the synaptic or perisynaptic region. To test this hypothesis, we prepared synaptosomes from brains of wild-type mice (P12 to P15) and visualized their content of neurotrypsin, agrin, and neurotrypsin-dependent agrin fragments by SDS-PAGE and Western blot analysis (Fig. 4*A, C*). The quality of our synaptosomal preparations was evaluated by electron microscopy and Western blot analysis. The morphological analysis confirmed the integrity of the synaptosomal fraction that was strongly enriched in synaptic structures, mainly consistent of resealed presynaptic membranes associated with post- and perisynaptic structures (Fig. 4*B*). Western blot analysis of the initial brain homogenate (S1), the soluble fraction (S2), and the synaptosomal fraction (SS; Fig. 4*A*), using an antibody against the postsynaptic density protein

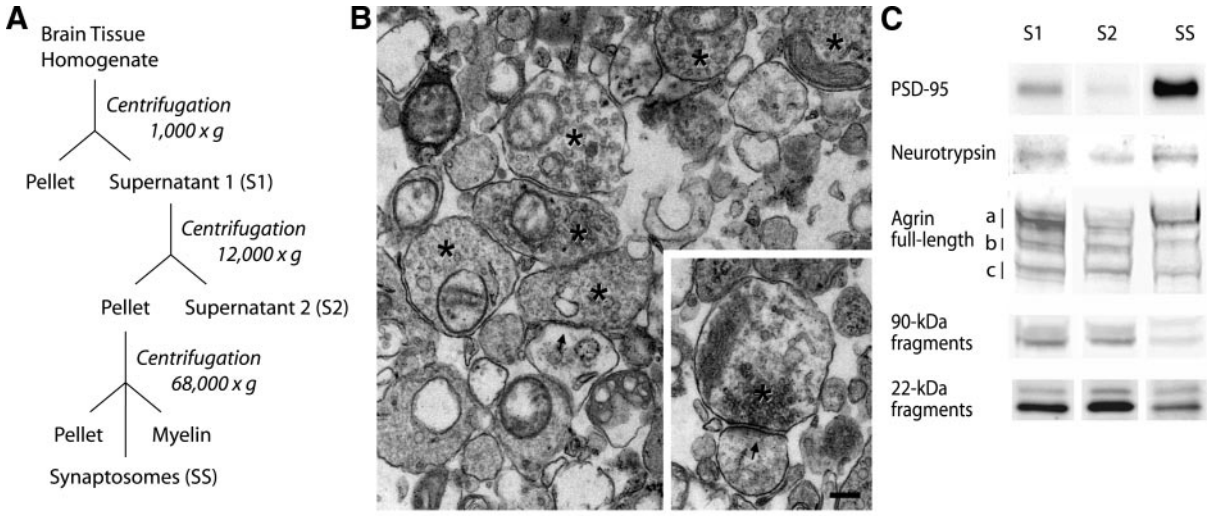

Figure 4. The upper variants are the predominant isoforms of agrin in synaptosomes. *A*) Schematic representation of the fractionation protocol used to prepare synaptosomes. *B*) Electron micrographs of a representative synaptosomal sample confirmed the integrity of the preparations. Presynaptic structures were prominent (asterisks), and in many cases attached postsynaptic structures were found (arrows). Scale bar = 0.2 μm. *C*) Representative Western blots of a synaptosomal preparation from brains of 12- to 15-day-old wild-type mice. Postsynaptic-density protein 95 (PSD-95) was strongly enriched in the synaptosomal fraction (SS) and served as a control for the quality of the preparation. Endogenous neurotrypsin was found in the synaptosomal, as well as in the other fractions of the preparation [soluble fraction 1 of the brain homogenate (S1), soluble fraction 2 (S2)]. Mainly the upper agrin variants were detected in synaptosomes, whereas all three groups of agrin variants were detected with similar intensities in the other fractions of the preparation [upper (a), middle (b), and lower (c) variants]. *D, E*) Quantitative densitometric Western blot analyses of synaptosomal preparations confirmed upper variants as the predominant isoforms of agrin in synaptosomes. On the basis of normalization to lower full-length agrin, only the upper (*D*) but not the middle variants (*E*) were found enriched in the synaptosomal fraction of wild-type mice. Data represent mean ± SE values; $n = 5$. $^{**}P < 0.01$.

PSD-95 as a synaptic marker, also demonstrated the synaptic enrichment of our synaptosomal preparations (Fig. 4*C*). Endogenous neurotrypsin was found in the synaptosomal, as well as in the other fractions of the preparation (Figs. 4*C* and 5*A*). This observation is in accordance with our immunohistological localization studies of neurotrypsin, as well as with our live imaging studies of intracellular trafficking of neurotrypsin. Both studies indicated that besides its synaptic localization, a fraction of neurotrypsin is localized to intracellular compartments ranging from the rough ER through the Golgi apparatus to axonal transport vesicles. However, the synaptosomal preparation protocol utilizes homogenization and therefore tissue disruption to yield a fraction enriched in synaptosomes. The intermediate fractions of the preparation therefore partially contain synaptic material. Hence, neurotrypsin detected in the soluble fraction (S2) represents a heterogenous pool of nonsynaptic as well as synaptic origin. Likewise, all three groups of full-length agrin were detected in all fractions (Fig. 4*C*). However, their relative amounts varied between the distinct fractions. As the distribution of all three groups of full-length agrin appeared to be different in the distinct fractions, we quantified the full-length variants of agrin in each fraction by densitometry of the Western blots and normalized the upper and middle group of variants to the lower group. We found that the upper group was the predominant form of full-length agrin in all analyzed fractions. In homogenate (S1) and in the soluble fraction (S2), we measured approximately 2 times more upper than lower full-length agrin (ratios to lower group of 1.95±0.25 and 1.70±0.25, respectively; Fig. 4*D*). In synaptosomes, the upper variant was more than 5-fold enriched (ratio to lower group of 5.25±1.11; Fig. 4*D*). In contrast, the relative amounts of the middle group were nearly identical with those of the lower group of variants in all fractions (Fig. 4*E*, ratio for S1=0.96±0.05, for S2=1.01±0.05, for SS=1.16±0.27). These results indicated that the upper group of agrin variants is the predominant agrin variant in synaptosomes. In addition, both neurotrypsin-dependent cleavage fragments of agrin appeared in all analyzed fractions, including synaptosomes (Fig. 4*C*).

Neurotrypsin-mediated cleavage of agrin is localized at synapses

The synaptic localization of neurotrypsin (Fig. 1*A*), its activity-dependent externalization from presynaptic terminals (4), and the appearance of the neurotrypsin-dependent agrin fragments in synaptosomes (Fig. 4) suggested a local proteolytic activity of neurotrypsin at synapses. To test this hypothesis, we compared the extent of neurotrypsin-dependent cleavage of agrin in synaptosomes and other subcellular brain fractions of neurotrypsin-overexpressing mice (Nto-act) and their wild-type littermates (P12 to P15; Fig. 5*A*). Evaluation by electron microscopy revealed no morphological differences between synaptosomes from wild-type littermates and Nto-act mice (data not shown). In addition, Western blot analysis confirmed the overexpression of human neurotrypsin in Nto-act mouse brains with a distribution similar to endogenous neurotrypsin (Fig. 5*A*).

The comparison of full-length agrin signals in synaptosomes and the other subcellular fractions from wild-type and Nto-act mice revealed both the predominant cleavage of agrin in the synaptosomal fraction and the preferred cleavage of the upper group of full-length agrin (Fig. 5*A*, arrow). The synaptosomal upper group of full-length agrin was identified as the predominant target for neurotrypsin at synapses, because its levels were reduced to 40.1 ± 4.7% in samples of Nto-act mice (Fig. 5*B*), while its reduction was only to 64.5 ± 3.3% in the soluble fraction and to 62.2 ± 4.7% in the homogenate of neurotrypsin-overexpressing mice. Neurotrypsin-dependent cleavage of full-length agrin was not as strong for the middle group as found for the upper group of bands, but the tendency was conserved (Fig. 5*C*). Signals for the middle variants of agrin signals

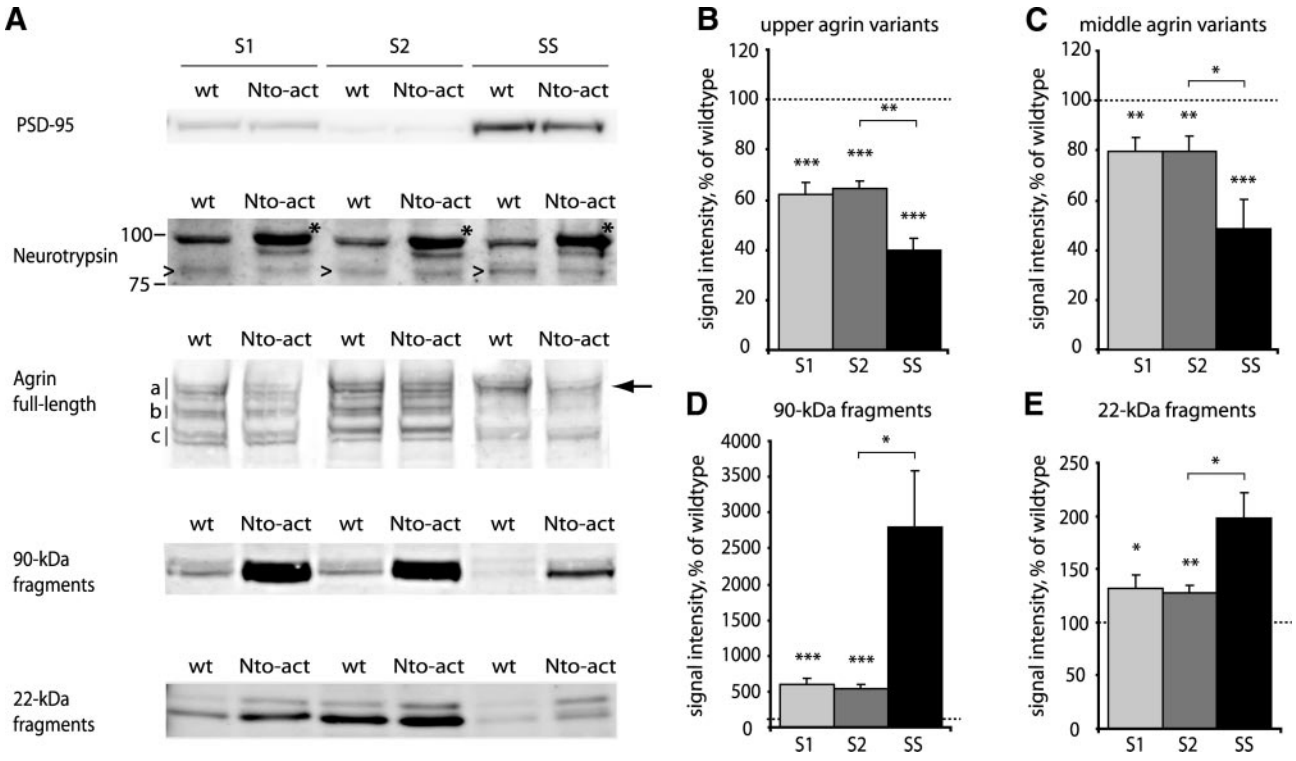

Figure 5. Neurotrypsin preferentially cleaves synaptic agrin. *A*) Western blot analysis of a representative synaptosomal preparation from brains of 12- to 15-day-old mice overexpressing active human neurotrypsin (Nto-act) compared to their wild-type (wt) littermate samples. Postsynaptic-density protein 95 (PSD-95) was strongly enriched in the synaptosomal fraction (SS) and served as a control for the quality of the preparation. Endogenous (murine) neurotrypsin (arrowheads), as well as overexpressed human neurotrypsin (asterisks), were found in the synaptosomal (SS) fraction, but as expected, they were also detected in the other fractions of the preparation [soluble fraction 1 of the brain homogenate (S1), soluble fraction 2 (S2)]. Human neurotrypsin was detected as a double band of ~100 kDa on top of an unspecific band that was also visible in the wild-type samples. The overexpression of neurotrypsin led to a major decline of upper full-length agrin, especially in synaptosomes [arrow; upper (a), middle (b), and lower (c) full-length agrin variants] and a corresponding increase of the 90- and the 22-kDa fragments in all fractions. Molecular mass references are indicated in kDa. *B–E*) Quantitative Western blot analyses of synaptosomal preparations from postnatal day 12 to 15 Nto-act mouse brains compared to their wild-type littermates. *B, C*) Upper agrin variants (*B*), as well as middle agrin variants (*C*), were more efficiently cleaved in all analyzed fractions of transgenic animals. For both the upper and middle agrin variants, cleavage was strongest in the synaptosomal fraction. *D, E*) The levels of both the 90-kDa (*D*) and the 22-kDa (*E*) fragment were increased in all analyzed fractions compared to wild-type levels. This increase was strongest in the synaptosomal fraction. Signal intensities of samples from Nto-act mice were normalized to corresponding signals of the wt littermate control samples (wt values set to 100%). Data represent mean ± SE values; $n = 5$. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

were reduced to ~80% of wild-type levels (79.3±5.8 and 79.8±6.1%) in the homogenate and soluble fraction, respectively, and to ~50% (48.2±11.9%) in the synaptosomal fraction. Signal intensities of the lower group of agrin variants were not altered between wild-type and transgenic samples (data not shown). In accordance with the results from the analysis in P9 mouse brains (Fig. 3), we concluded that lower full-length agrin is not a substrate for neurotrypsin in any of the analyzed fractions.

In parallel with the predominant cleavage of agrin in synaptosomes, we found that the increase of agrin fragments due to neurotrypsin-overexpression was strongest in the synaptosomal fraction for both fragments. The increase of the 90-kDa agrin fragment due to neurotrypsin overexpression was over 25-fold (2787.8±791.4% of wild type; Fig. 5*D*) in the synaptosomal fraction, but only 5- to 6-fold in brain homogenate and the soluble fraction (594.5±89.2 and 543.9±53.0%, respectively; Fig. 5*D*). A similar but less pronounced synaptosomal enrichment was found for the 22-kDa fragment. The 22-kDa fragment was enriched 2-fold (197.4±25.1%; Fig. 5*E*) in the synaptosomal fraction of Nto-act mice and appeared in the homogenate and the soluble fraction at ~130% of wild-type levels (132.6±12.0 and 127.6±6.9%, respectively; Fig. 5*E*).

Taken together, we observed that the upper group represents the main form of full-length agrin in synaptosomes and that this group of variants was predominantly cleaved by neurotrypsin. A similar enrichment in synaptosomes was not found for the middle group of agrin variants, but their cleavage was also localized predominantly in synaptosomes. Our data therefore indicated that neurotrypsin-dependent cleavage of agrin is concentrated at synapses.

DISCUSSION

We found that the synaptic serine protease neurotrypsin cleaves the extracellular proteoglycan agrin predominantly at or in the vicinity of synapses, resulting in increased concentrations of agrin fragments and decreased full-length agrin at synapses.

Neurotrypsin-dependent cleavage of agrin is concentrated at synapses

Agrin is a complex heparan sulfate proteoglycan expressed in a variety of different isoforms with distinct locations and functions (6). Using a variant of SDS-PAGE based on a Bis-Tris buffer adjusted to neutral pH, we were able to resolve brain-derived full-length agrin as three distinct groups of variants with different levels of glycanation. Comparison of the three groups of agrin variants with regard to their *in vivo* cleavage revealed that neurotrypsin-dependent cleavage of agrin affected only the glycanated agrin, *i.e.*, the upper and middle variants.

In the CNS, agrin is found on cell surfaces at various locations, including synapses (9, 24, 25, 29, 30, present study) and glial cells, as well as in the basal lamina lining blood vessels (9, 23, 24, present study). To investigate whether a particular isoform of agrin was predominant at CNS synapses, we isolated synaptosomes by subcellular fractionation and analyzed them with SDS-PAGE. We found that the upper group of agrin variants was the major form in synaptosomes, exceeding the lower variants 5-fold, while its excess in the other cellular fractions was less than 2-fold.

Synaptic agrin was clearly the preferred proteolytic target of neurotrypsin when compared with agrin found in other subcellular fractions. Synaptosomes from neurotrypsin-overexpressing mice exhibited a significantly stronger reduction of full-length agrin and showed the strongest increase for both agrin fragments. The locally concentrated increase of the fragments at the expense of full-length agrin that was found on increasing neurotrypsin supports the notion of a precursor-product relationship between full-length agrin and its neurotrypsin-dependent fragments and thus characterizes neurotrypsin-dependent cleavage of agrin as a locally concentrated event at or in the vicinity of the synapse.

The notion of a locally concentrated cleavage of agrin at synapses and, thus, a synaptic source of agrin fragments is corroborated by the stronger increase of the 90-kDa fragment as compared with the 22-kDa fragment in synaptosomes from neurotrypsin-overexpressing mice. Agrin binds with high affinity to α-dystroglycan through a binding site located between the two neurotrypsin-dependent cleavage sites (20, 31). Therefore, the 90-kDa fragment may be efficiently retained at the site of its production. The 22-kDa fragment, in contrast, appears to be more diffusible. Its appearance in the CSF indicates that it is either produced in excess over its potential receptors or that its receptor interactions are more volatile than those of the 90-kDa fragment. Because at least stoichiometric amounts are expected for the 22-kDa fragment based on the pattern of proteolytic cleavages that are required for generating the 90- and 22-kDa fragments, its minor increase observed in synaptosomes on increasing neurotrypsin indicates a synaptic source that has been cleared by a diffusional flux passing *via* the nonsynaptic tissue to the CSF. In accordance with this conclusion, the increase of the 22-kDa fragment observed under transgenic overexpression of neurotrypsin reaches considerably lower levels in all nonsynaptosomal fractions, as well as in brain homogenates.

Spatially concentrated cleavage of agrin by neurotrypsin at synapses is in accordance with our immunoelectron microscopic localization of neurotrypsin to presynaptic terminals (2, present study), and our recent live imaging studies with cultured hippocampal neurons showing that neurotrypsin are transported to synapses and released from presynaptic terminals in an activity-dependent manner (4). In addition, neurotrypsin was found lingering at its synaptic release sites for

minutes before disappearing. The molecular mechanism of transient retention of neurotrypsin at the synapse remains to be determined. Interactions with cell surface or extracellular matrix components are possible. Basic segments resembling the one found at the N terminus of neurotrypsin could exert a retaining function *via* interaction with glycosaminoglycans, possibly those of agrin, and other negatively charged surface components, as shown for other positively charged secreted molecules, such as the C-terminal basic segment of netrin (32). Restricted mobility by cell-surface and extracellular matrix (ECM) association is crucial for netrin's role as a chemotropic agent in axonal guidance (33). For neurotrypsin, the transient local lingering at its synaptic site of externalization may be essential for the spatially concentrated proteolytic action at or near the synapse. In addition, the transient nature of the extracellular presence of neurotrypsin after synaptic externalization might indicate a temporal restriction of neurotrypsin's synaptic actions.

Neurotrypsin-dependent cleavage of agrin at the synapse: loss of function or gain of function?

Nerve-derived agrin plays a crucial and well-characterized role at the neuromuscular junction (34). However, studies about the functions of agrin in the CNS are just emerging, and only little is known about agrin's interplay with its potential interaction partners, also expressed at CNS synapses (**Fig. 6**). A peptide containing the LG-3 domain of agrin, closely resembling the 22-kDa agrin fragment, was shown to bind to and signal through a synaptic receptor (41) that was identified as the α3-subunit of the Na^+-K^+-ATPase (10). Its inhibitory activity on the Na^+-K^+-ATPase was demonstrated to result in membrane depolarization and increased

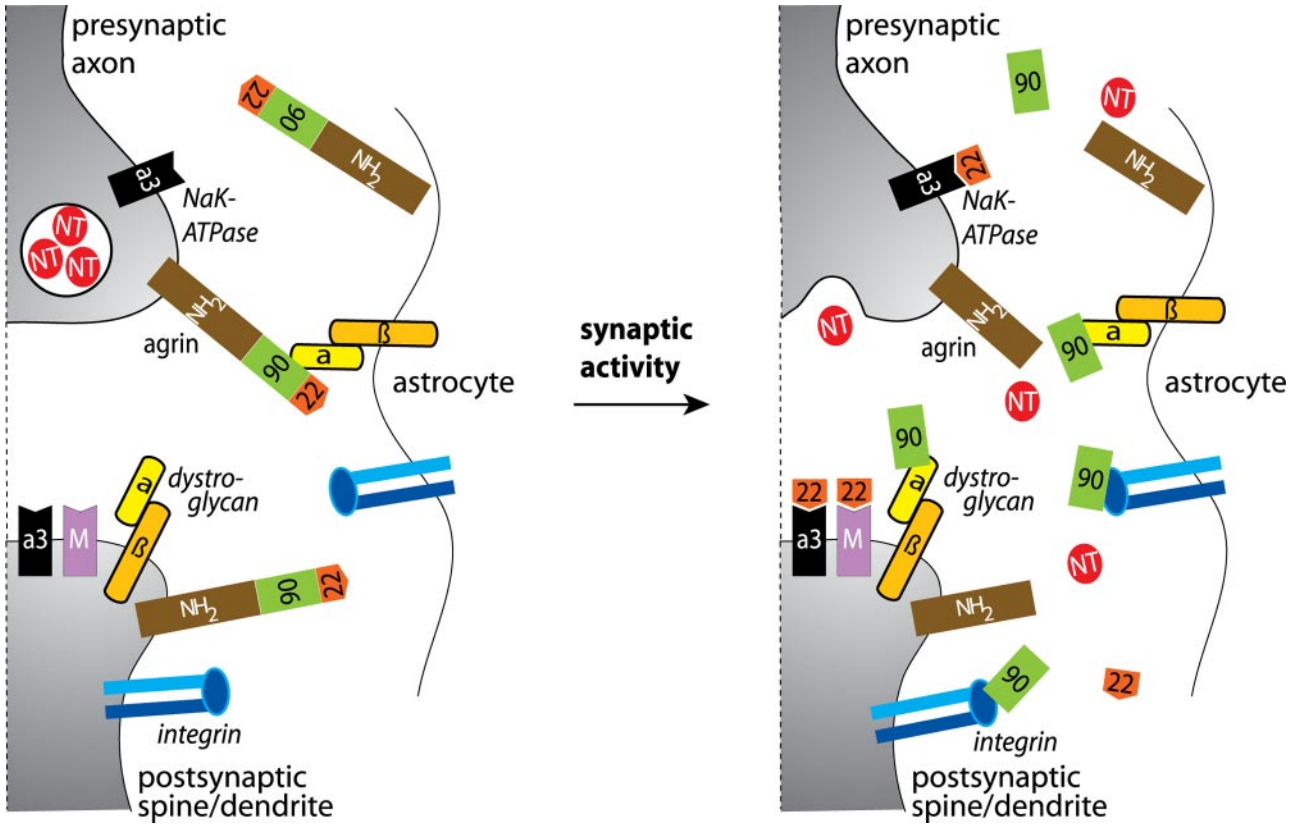

Figure 6. Model of the neurotrypsin-dependent cleavage of synaptic agrin. In the central nervous system (CNS), neurotrypsin (NT) is mainly localized to intracellular vesicles in presynaptic terminals. In contrast, synaptic agrin is mainly localized to extracellular perisynaptic sites. On synaptic activity, neurotrypsin is secreted from presynaptic terminals and cleaves synaptic agrin at two sites, generating the 90- and the 22-kDa fragments. Cleavage of agrin may either disrupt its interaction with synaptic partners or enable the released C-terminal fragments to bind to their respective receptors. The region of agrin corresponding to the 90-kDa fragment was shown to interact specifically with α-dystroglycan, integrins, and heparin (*i.e.*, heparan sulfate proteoglycans, not depicted, but see Fig. 2*A*; ref. 35) that are expressed by neurons or astroglia enclosing the synapse (36–38). Similarly, the 22-kDa agrin fragment was shown to bind to synaptic receptors. Recent evidence suggests that a C-terminal agrin fragment, closely resembling the 22-kDa fragment generated by neurotrypsin, specifically interacts with the α3-Na^+-K^+-ATPase (α3) at synapses, modifying its functional properties (see Discussion). This isoform of Na^+-K^+-ATPase was shown to localize to presynaptic sites, as well as to the postsynaptic plasma membrane (39, 40). Another well-characterized receptor for the 22-kDa agrin fragment is the receptor tyrosine kinase MuSK (M). MuSK was recently shown to be expressed in the CNS and localized to CNS synapses (9); however, its specific subcellular localization is not known. According to its exclusive location in the postsynaptic membrane of the NMJ, we assigned MuSK to the postsynaptic membrane of CNS synapses.

action potential firing. A role of agrin in the formation or maintenance of excitatory synapses through a mechanism activated *via* the MAP kinase signaling pathway has been suggested from the observation that agrin-deficient mice, rescued from perinatal death by transgenic expression of agrin in motoneurons, exhibited a reduced number of synapses in the cerebral cortex (9). The agrin receptor in this process has not been identified. However, the authors demonstrated a striking coincidence of agrin-dependent synapse loss and the expression of the "muscle-specific" kinase, MuSK, in a subpopulation of excitatory synapses and suggested MuSK, which previously was thought not to be expressed in the CNS, as a possible agrin receptor in the CNS. As MuSK activation at the neuromuscular junction was unequivocally shown to depend on the C-terminal LG-3 domain of agrin, this domain could also play a role in the activation of MuSK in the CNS.

The region covered by the 90-kDa fragment was shown to interact with heparin (*i.e.*, heparan sulfate proteoglycans), α-dystroglycan and different integrins (35). The diverse synaptic functions reported for these molecules may be modified by binding of agrin or the 90-kDa fragment alone.

Antibody-induced clustering and overexpression of agrin in cultured neurons were recently reported to induce dendritic filopodia (11, 12). Dendritic filopodia have been characterized as precursors of dendritic spines and therefore, their induction could indicate an early stage of synapse formation (13, 14, 42, 43). The N-terminal region of agrin, corresponding to the N-terminal part up to the first neurotrypsin cleavage site, was shown to be sufficient for filopodia induction (12). Neurotrypsin-dependent cleavage might destabilize agrin's N-terminal region, leading to a rapid degradation or internalization, and thus negatively affect its role in filopodia generation.

Neurotrypsin-dependent cleavage of agrin at the synapse may alter agrin's synaptic functions, either by abolishing functions of full-length agrin residing at synapses or by activating regions of agrin from synapse-resident agrin. Thus, the release of active fragments could promote interactions with their respective receptors that were inaccessible for full-length agrin. Further work will be required to elucidate the role of neurotrypsin-dependent cleavage on agrin's synaptic functions and to determine whether cleavage by neurotrypsin induces, modifies, or terminates the activity of agrin at the synapse.

CONCLUSION

The severe mental retardation found in humans with a loss-of-function mutation in the neurotrypsin gene (2) indicates neurotrypsin as an essential regulator of adaptive synaptic plasticity that is crucial for establishing and maintaining cognitive brain functions. We recently reported that neurotrypsin cleaves the proteoglycan agrin at two homologous, highly conserved sites, liberating a middle 90-kDa and a C-terminal 22-kDa fragment from the membrane or ECM-bound N-terminal moiety of agrin. Deciphering the regional and functional parameters of the interaction between neurotrypsin and agrin in the brain may thus lead us toward understanding neurotrypsin's essential role in cognitive functions. Along this path, we recently demonstrated that synaptic neurotrypsin is contained in intracellular stores and that both recruitment of neurotrypsin to synapses and synaptic exocytosis of neurotrypsin are enhanced by neuronal activity (4). In this study, we also found that externalized neurotrypsin transiently lingers at the synapse, before it disappears by diffusion or degradation. In the present study we found that neurotrypsin-dependent cleavage of agrin is focused at or in the vicinity of synapses, where it affects mainly the most heavily glycanated variants of synaptic agrin, which predominate at the synapse. Agrin is a key regulator of the maturation and the maintenance of the neuromuscular junction, and recent evidence suggests that agrin may also play an important role in the formation and the maintenance of CNS synapses. The selective activity-dependent externalization of neurotrypsin and its local cleavage of agrin at the synapse make neurotrypsin an ideal regulator of structural and functional reorganizations of the synaptic circuitry. FJ

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