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Abstract

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Key words
Proteolysis, synaptic plasticity, synapse formation, long-term potentiation, activity-dependent exocytosis, coincidence detector, Hebb’s rule, Hebbian learning

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SUMMARY

The synaptic serine protease neurotrypsin is essential for cognitive function, as its deficiency in humans results in severe mental retardation. Recently, we demonstrated the activity-dependent release of neurotrypsin from presynaptic terminals and proteolytical cleavage of agrin at the synapse. Here we show that the activity-dependent formation of dendritic filopodia is abolished in hippocampal neurons from neurotrypsin-deficient mice. Administration of the neurotrypsin-dependent 22-kDa fragment of agrin rescues the filopodial response. Detailed analyses indicated that presynaptic action potential firing is necessary for the release of neurotrypsin, whereas postsynaptic NMDA receptor activation is necessary for the neurotrypsin-dependent cleavage of agrin. This contingency characterizes the neurotrypsin-agrin system as a coincidence detector of preand postsynaptic activation. As the resulting dendritic filopodia are thought to represent precursors of synapses, the neurotrypsin-dependent cleavage of agrin at the synapse may be instrumental for a Hebbian organization and remodeling of synaptic circuits in the CNS.
INTRODUCTION

Synaptic plasticity is a fundamental phenomenon contributing to cognitive functions, such as learning and memory. The molecular and cellular mechanisms underlying activity-dependent synaptic plasticity induce structural and functional changes in preexisting synapses and generate new synapses (Malenka and Nicoll, 1999; Nikonenko et al., 2002; Stepanyants et al., 2002; Chklovskii et al., 2004). The serine protease neurotrypsin is crucial for cognitive brain function, as a 4-base-pair deletion in the coding region, which generates a truncated protein lacking the protease domain, causes severe mental retardation in humans (Molinari et al., 2002). In the adult central nervous system (CNS), neurotrypsin mRNA is prominently expressed in neurons of the cerebral cortex, the hippocampus, the lateral amygdala, and in motor neurons of the brain stem and the spinal cord (Gschwend et al., 1997; Wolfer et al., 2001). Neurotrypsin protein was localized by immunoelectron microscopy to presynaptic boutons (Molinari et al., 2002; Stephan et al., 2008). Live imaging studies with cultured hippocampal neurons indicated that a major fraction of synaptic neurotrypsin is contained in internal stores and that both recruitment to synapses and exocytosis are regulated by neuronal activity (Frischknecht et al., 2008). These studies further revealed that externalized neurotrypsin-pHluorin remains visible at the synapse for minutes.
before it disappears due to diffusion, degradation, or re-endocytosis.

At present, the only known proteolytic substrate of neurotrypsin is the proteoglycan agrin (Reif et al., 2007). Neurotrypsin cleaves agrin at two homologous, highly conserved sites, resulting in a 90-kDa fragment (agrin-90) confined by the two cleavage sites, and a 22-kDa fragment (agrin-22) consisting of the C-terminal laminin G domain. Agrin is widely expressed in the CNS and it is abundant at and in the vicinity of synapses (Koulen et al., 1999; Kziazek et al., 2007). Subcellular fractionation and isolation of synaptosomes revealed that neurotrypsin-dependent agrin cleavage is concentrated at synapses (Stephan et al., 2008).

Here, we identify activity-dependent presynaptic exocytosis of neurotrypsin and the resulting proteolytic cleavage of agrin at CNS synapses as a mechanism promoting the activity-dependent formation of dendritic filopodia. Furthermore, we found that presynaptic exocytosis of neurotrypsin depended on action potentials and P/Q/N-type calcium channels, while neurotrypsin-dependent cleavage of agrin required the additional activation of the postsynaptic cell. Therefore, neurotrypsin-dependent cleavage of agrin could represent a molecular coincidence detector for concomitant pre- and postsynaptic activation. Because dendritic filopodia have been proposed as crucial precursors of new CNS synapses (Jontes and Smith, 2000; Yuste and Bonhoeffer,
2004), our results suggest a role for the neurotrypsin-agrin system in the activity-controlled regulation of synaptogenesis and circuit reorganization in the CNS.
RESULTS

Synaptic exocytosis of neurotrypsin-pHluorin requires presynaptic but not postsynaptic activation

To monitor exocytosis from synaptic intracellular stores in CNS slices, we generated transgenic mice expressing a neurotrypsin-pHluorin fusion protein in neurons (Fig. S1). pHluorin is a pH-sensitive variant of green fluorescent protein (GFP) (Miesenböck et al., 1998), with strong fluorescence at neutral pH, but very low fluorescence in an acidic environment, such as the lumen of secretory vesicles (pH 5 - 6). Therefore, fusion with pHluorin generates a means to monitor exocytosis of a protein from acidic secretory vesicles.

For the histological validation of correct synaptic sorting of neurotrypsin-pHluorin, we investigated the pathway from subiculum-CA1 neurons to the deep layers (III – VI) of the entorhinal cortex in 400 µm-thick acute slices taken from 4-6 week-old neurotrypsin-pHluorin expressing transgenic mice under permeabilizing conditions (Fig. 1 A,B,C). We found that all prominent neurotrypsin-pHluorin puncta larger than 0.8 µm$^2$ (arrows in Fig. 1A) colocalized with a prominent synapsin I-immunopositive punctum, indicating their synaptic localization (Fig. 1B,C). In contrast, the vast majority of small neurotrypsin-pHluorin puncta were not colocalized with the
synaptic marker and were therefore extrasynaptic. This observation is consistent with our live-imaging studies of dissociated hippocampal cultures, where we found that the fluorescence intensity was on average 3-5 times higher for synaptic neurotrypsin-pHluorin than for extrasynaptic transport packages (Frischknecht et al., 2008). Therefore, we defined fluorescent puncta as large or synaptic, if their area was between 0.8 and $3 \mu m^2$. Smaller puncta were qualified as extrasynaptic.

We found relatively few extracellular neurotrypsin-pHluorin puncta in tissue slices kept at neutral pH in artificial cerebrospinal fluid (Fig. 1D and S3A). To visualize the intracellular pools of neurotrypsin, we substituted 50 mM NaCl of the ACSF with ammonium chloride to neutralize the lumen of naturally acidic secretory vesicles (Miesenböck et al., 1998). Vesicle neutralization increased the number of large (synaptic) neurotrypsin-pHluorin puncta almost four-fold (Fig. 1D and S3B). This indicated that most synaptic neurotrypsin was intracellular and remained undetected at neutral pH and that approximately 75% of the neurotrypsin-pHluorin-containing synapses exhibited no or very little neurotrypsin on their extracellular side.

Cell depolarization with high extracellular potassium (Fig. 1D and S3D,E) or blockade of K\textsuperscript{+}-channels with tetraethylammonium chloride (TEA; Fig. 1D and S3F) increased the number of large extracellular fluorescent puncta more than two-fold. The
same results were found for a second neurotrypsin-pHluorin-expressing line (Fig. S4).

The number of extracellular neurotrypsin-pHluorin puncta was also increased by other chemical enhancers of network activity, such as glutamate and the GABA\textsubscript{A} receptor blocker bicuculline (Fig. 1E). The effect of glutamate was prevented by tetrodotoxin, a blocker of voltage-dependent sodium channels, indicating an essential role of action potentials.

We pharmacologically characterized the mechanism of secretion using several ion channel inhibitors (Fig. 1F). The K\textsuperscript{+}-induced increase in the number of large pHluorin puncta was completely abolished by ω-agatoxin (ATX) and ω-conotoxin (CTX), which block P/Q- and N-type calcium channels, respectively. Activation of AMPA and NMDA-type glutamate receptors (NMDA-R) was not necessary for neurotrypsin exocytosis, as the combination of CNQX and MK-801 had no effect. Together, these results indicated that synaptic exocytosis of neurotrypsin-pHluorin required action potentials and presynaptic activation, but did not depend on activation of postsynaptic glutamate receptors.

The time course of the activity-dependent synaptic externalization of neurotrypsin-pHluorin is shown in Fig. 1G. The number of pHluorin puncta increased significantly 2 min after addition of K\textsuperscript{+} or TEA. At its peak after 5-10 min, the number of
pHluorin puncta was about 3 times higher than before stimulation. After the end of the stimulation period, the number of puncta gradually decreased. A significant increase of pHluorin puncta was also elicited by a short (40 s) stimulation with 50 mM KCl. These results characterized extracellular neurotrypsin as a transient response to presynaptic activation.

**Activity-induced synaptic exocytosis of neurotrypsin results in proteolytic cleavage of agrin**

To assess neurotrypsin-dependent cleavage of agrin after synaptic exocytosis of neurotrypsin, we used whole hippocampi from P10 mice for two reasons: Firstly, expression levels of both neurotrypsin and agrin are highest during the first two postnatal weeks (Reif et al., 2007; Stephan et al., 2008) and, secondly, tissue at this age can be maintained in ACSF for several hours (Khalilov et al., 1997; Li et al., 2000). We used agrin-90 to monitor neurotrypsin activity, because its presence indicated cleavage at both neurotrypsin-dependent sites (Fig. 2A). Agrin-90 was readily detectable after incubation in ACSF for 10 min without stimulation (Fig. 2B). It was completely absent in neurotrypsin-deficient mice. Thus, cleavage of agrin *in vivo* strictly depended on neurotrypsin.
A 10-min exposure of whole hippocampi to the potassium channel blocker 4-aminopyridine (4-AP) resulted in a significant increase of agrin-90 (Fig. 2B), indicating that neurotrypsin-dependent cleavage of agrin was up-regulated by increased neuronal activity. Absence of agrin-90 in neurotrypsin-deficient mice after 4-AP stimulation indicated that neurotrypsin was the only protease cleaving agrin in an activity-dependent manner.

The time course of the stimulation-induced increase of agrin-90 was studied for KCl, 4-AP, and TEA. As shown in Fig. 2C and D, all three K+ -based stimulations resulted in a transient increase of agrin-90. The response patterns were similar, with a ~40% increase peaking at the end of the 10 min stimulation period. After stimulation the intensity of agrin-90 gradually decreased and reached pre-stimulation levels after ~30 min. Under control conditions without stimulation (Fig. 2D), levels of agrin-90 gradually decreased during the first 20 min and then remained constant for the following 20 min. These results indicate a close correlation between activity-dependent synaptic exocytosis of neurotrypsin and cleavage of agrin. Further tests showed that the TEA-induced increase of agrin-90 was abolished by the combination of ω-agatoxin and ω-conotoxin (Fig. 2E,F). Therefore, as with the stimulation-induced exocytosis of neurotrypsin, the stimulation-induced increase of agrin-90 required the activation of
presynaptic P/Q and N-type Ca\textsuperscript{2+} channels. Together, these results indicate that presynaptic exocytosis of neurotrypsin is necessary for agrin cleavage.

**Cleavage of agrin by externalized neurotrypsin requires postsynaptic activation**

TEA evokes global bursting and produces long-term potentiation (LTP) of synaptic transmission with similar properties as tetanus-induced LTP (Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995), indicating the concomitant activation of the pre- and the postsynaptic neuron by TEA, because this is a prerequisite for the induction of LTP (Malenka and Nicoll, 1999). We thus tested for a postsynaptic component in TEA-induced neurotrypsin-dependent agrin cleavage. We found that the TEA-induced increase of agrin cleavage was prevented by the AMPA and NMDA-R inhibitors CNQX and MK-801 (Fig. 3A,B). In addition, a significant decrease of agrin cleavage was also found with nifedipine, a selective inhibitor of L-type voltage-dependent Ca\textsuperscript{2+} channels (VDCC). Therefore, TEA-induced neurotrypsin-dependent agrin cleavage exhibited a postsynaptic contribution from both NMDA-Rs and L-type VDCCs, as previously reported for the TEA-induced LTP (Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995).
We further confirmed this postsynaptic component with another chemical LTP protocol, using the combination of picrotoxin, forskolin, and rolipram at high extracellular Ca$_{2+}$ and no Mg$_{2+}$ (PFR). PFR stimulation induces LTP in the hippocampal CA1 region in an NMDA-R-dependent manner by enhancing neuronal network activity via reduction of GABAergic inhibition in combination with enhancing cAMP-mediated intracellular signaling (Otmakhov et al., 2004; Kopec et al., 2006). We found that the PFR-induced increase of agrin cleavage was prevented by the AMPA and NMDA-receptor inhibitors CNQX and MK-801 (Fig. 3C,D). Only a small, but insignificant reduction of PFR-induced agrin cleavage was found with nifedipine, a blocker of L-type VDCCS (Fig. 3C,D), which is in line with the reported NMDA-R dependence of PFR-induced LTP (Otmakhov et al., 2004).

Together, these results indicate that neurotrypsin-dependent agrin cleavage requires activation of presynaptic P/Q- and N-type calcium channels that are essential for presynaptic exocytosis of neurotrypsin. However, in contrast to neurotrypsin exocytosis from presynaptic boutons, neurotrypsin-dependent agrin cleavage also requires the activation of the postsynaptic neuron, with the indispensable activation of NMDA-Rs. These results indicate that neurotrypsin is externalized in an inactive form and that NMDA-R-driven activity of the postsynaptic cell is required for its activation.
The dependence of neurotrypsin-dependent agrin cleavage on postsynaptic activation was also found with hippocampal slices from juvenile (4-6 week-old) mice (Fig. S5, S6). Therefore, the dependence of neurotrypsin activation on postsynaptic mechanisms was found to be an age-independent process.

LTP is intact, but LTP-associated formation of dendritic filopodia is abolished in neurotrypsin-deficient mice

Activation of NMDA-Rs and postsynaptic Ca\textsuperscript{2+} influx is essential for LTP induction (Malenka and Nicoll, 1999). Therefore, the recognition of neurotrypsin activation as an NMDA-R-dependent process prompted the question whether neurotrypsin plays a role in LTP expression. To test this possibility, we compared LTP in acute hippocampal slices from 4-6 week-old wildtype and neurotrypsin-deficient mice (Fig. 4A,B). LTP was evoked in the CA1 region with 4 1-s trains of 100Hz stimuli. Significant LTP was observed in slices from both wildtype littermates and neurotrypsin-deficient mice (150.5 ± 8.8%, n = 10, p < 0.001 and 169.1 ± 38%, n = 7, p = 0.05, respectively). There was no difference in the extent of LTP between wildtype and neurotrypsin-deficient mice (p = 0.66) (Fig. 4B), indicating that neurotrypsin was not essential for LTP expression.
Among the LTP-associated cellular phenomena, the formation of dendritic filopodia is particularly intriguing, because filopodia have been characterized as early forms of spines and, thus, precursors of synapses (Ziv and Smith, 1996; Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Knott et al., 2006; Toni et al., 2007). Therefore, we studied whether agrin cleavage is involved in activity-dependent generation of filopodia in the mature hippocampus. To visualize dendritic filopodia in hippocampal slices, we used the transgenic mouse line L15 expressing membrane-targeted GFP (mGFP) in sparse neurons (De Paola et al., 2003). Filopodia were counted in reconstructed 3-dimensional images of secondary apical dendrites by inspection over a length of 30-40 µm (Fig. 4C-E). Dendritic filopodia (arrows in Fig. 4E) were identified according to the following morphological criteria: 1) a protrusion with a length of at least twice the average length of the spines on the same dendrite, 2) a ratio of head to neck diameter smaller than 1.2:1, and 3) a ratio of length to neck diameter larger than 3:1 (Grutzendler et al., 2002).

First, we investigated the effect of chemical LTP on filopodia number, again using TEA- or PFR-induced global bursting as a means to mimic tetanus-induced LTP. Our electrophysiological recordings after TEA and PFR stimulation confirmed that both protocols induce robust LTP and corroborated our results obtained with electrical LTP
induction that LTP was intact in slices from neurotyspin-deficient mice (Fig. S7). In addition, tests with propidium iodide indicated that neither TEA nor PFR stimulation induced significant apoptosis in hippocampal slices (Figure S8). Quantification of filopodia in non-stimulated control samples indicated that the average number of filopodia was 0.114-0.118/µm (Fig. 4F,H). Following TEA or PFR stimulation, filopodial density was significantly increased to 0.156 or 0.152/µm, respectively ($p < 0.001$ vs No Stim by ANOVA with Tukey’s post hoc test). Administration of the glutamate receptor blockers CNQX or MK-801, or nifedipine, abolished the filopodial response to TEA (Fig. 4F). The dependence of the filopodial response on both NMDA-Rs and L-type VDCCs is consistent with previous studies indicating that TEA-induced LTP consists of two mechanistically distinct forms of LTP, one depending on NMDA-Rs and the other on L-type VDCCs (Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995). The blockade by MK-801 and nifedipine indicates that the LTP-associated filopodial response to TEA exhibits the same inhibitor profile as TEA-induced LTP and TEA-induced neurotyspin-dependent agrin cleavage. Likewise, the filopodial response to PFR stimulation was also blocked by the glutamate receptor blockers CNQX and MK-801, but no significant inhibition was found after blockade of L-type VDCCs with nifedipine (Fig. 4H). Again, this is consistent with the inhibitor profile of PFR-induced
chemical LTP (Otmakhov et al., 2004).

To examine the role of neurotrypsin in LTP-induced generation of filopodia (Fig. 4G,I), we crossed neurotrypsin-deficient with transgenic L15 mice expressing mGFP in sparse neurons. The number of filopodia along secondary apical dendrites of CA1 pyramidal neurons was counted in mGFP-positive, neurotrypsin-deficient mice and in littermate (neurotrypsin-wildtype) controls. CA1 neurons of wildtype mice had 0.107-0.120 filopodia per µm (Fig. 4G,I). Filopodia number was significantly increased by both TEA and PFR-stimulation, reaching 0.172/µm and 0.166/µm, respectively (Fig. 4G,I). In contrast, neither TEA nor PFR-stimulation induced a significant increase in filopodial density (0.117/µm and 0.119/µm, respectively) in hippocampal slices of neurotrypsin-deficient mice (Fig. 4G,I). Together, these results indicate that neurotrypsin is not required for induction and expression of LTP, but rather for the LTP-associated generation of filopodia.

The C-terminal fragment of agrin restores the LTP-induced increase of filopodia in neurotrypsin-deficient mice

Because agrin is the only proteolytic target of neurotrypsin and because LTP-dependent induction of dendritic filopodia was abolished in neurotrypsin-deficient
mice, we wondered whether the released agrin fragments (agrin-22, agrin-90, and agrin-110; see also Fig. 2A) might function as filopodial inducers. To test this hypothesis, we produced these fragments in HEK293T cells, purified them, and added 50 nM of each to neurotrypsin-deficient hippocampal slices (Fig. 5). We found that administration of agrin-22 (z0) with PFR induced a significant increase of filopodia, reaching levels observed in PFR-stimulated wildtype hippocampi (0.182/µm; Fig. 5A-E). Almost the same effect was found when agrin-22 was applied without PFR (0.167/µm). These results identify agrin-22 as the mediator of the filopodia-generating response associated with LTP.

Agrin-90 was unable to rescue PFR-induced filopodia stimulation in neurotrypsin-deficient mice when administered alone or in combination with PFR (0.128/µm or 0.120/µm, respectively; Fig. 5F). We also tested agrin-110 that was generated by partial proteolytic cleavage at the cleavage site and comprised both agrin-90 and agrin-22. Agrin-110 (y0z0) showed only a weak, statistically unsignificant rescue (0.136/µm; Fig. 5G) even though it contains the C-terminal part of agrin. Therefore, we concluded that the C-terminal domain of agrin was only active when isolated in the form of agrin-22, and thus that β cleavage was important to exert a maximal filopodia-inducing effect.
The role of agrin splice variants at the z site has been well characterized for NMJ formation and AChR clustering (Burgess et al., 1999). Because agrin-22 contains the z site, we investigated the effects of all splice variants (z0, z8, z11, z19) in wildtype mice without chemical stimulation. Administration of all splice variants of agrin-22 for 10 min induced a significant increase in the number of filopodia (Fig. 5H). This is in contrast to results from the NMJ where the z0 isoform differed from the other isoforms.

In summary, we have shown that the neurotrypsin-dependent generation of agrin-22 is essential for LTP-dependent filopodia induction and that the agrin-22-derived signals resulting in NMJ development and those resulting in the generation of filopodia from dendrites of CNS neurons are distinct.

**DISCUSSION**

The neurotrypsin-dependent 22-kDa fragment of agrin is instrumental for the promotion of dendritic filopodia

We found activity-dependent exocytosis of neurotrypsin from presynaptic terminals and cleavage of its substrate agrin to be crucial for the formation of dendritic filopodia in the context of NMDA-R-dependent postsynaptic signaling. In neurotrypsin-deficient mice, in which the cleavage of agrin was abolished, no
activity-dependent generation of dendritic filopodia was found. However, filopodia formation was completely restored by exogenous administration of agrin-22, the neurotrypsin-dependent C-terminal 22-kDa fragment of agrin.

In light of its filopodia-inducing activity, agrin-22 can be considered as one of a growing number of cell function-regulating factors generated by proteolytic cleavage of extracellular matrix or non-matrix proteins. Most prominent among these are several endogenous inhibitors of angiogenesis, such as endostatin, derived from collagen XVIII, arresten, derived from collagen IV, and endorepellin, derived from the heparansulfate proteoglycan perlecan (Nyberg et al., 2005). Unlike the cell-derived cytokines, these factors are produced by extracellular proteolysis. They have little or no activity as long as they are part of the parent protein. Likewise, separation of agrin-22 from agrin by extracellular proteolysis was necessary for full expression of its filopodia-inducing activity.

The dendritic receptor mediating the filopodia-inducing function of agrin-22 is currently unknown. Based on recent investigations, the molecular mechanisms of agrin function at the NMJ and in the CNS are fundamentally distinct. The agrin signal at the NMJ is mediated via the receptor tyrosine kinase MuSK (Glass et al., 1996) and it promotes the formation, maturation, and maintenance of the synaptic specialization by
protecting it from a synapse-dispersing activity (Kummer et al., 2006). In contrast, the 3-subtype of the Na+/K+-ATPase was identified as a neuronal agrin receptor in the CNS, and it was demonstrated that its Na+/K+-pumping function was inhibited by agrin binding, thus suggesting a depolarizing function of agrin on CNS neurons (Hilgenberg et al., 2006). Other studies on agrin function in the CNS indicate that clustering of agrin by antibodies (Annies et al., 2006) and neuronal overexpression of agrin (McCroskery et al., 2006) stimulate filopodia formation.

**The neurotrypsin-agrin system may serve as a coincidence detector for concomitant pre- and postsynaptic activation**

Neurotrypsin exocytosis after presynaptic depolarization was not sufficient for agrin cleavage, which depended on the concomitant activation of the postsynaptic neuron. When LTP was induced by PFR stimulation, and therefore was NMDA-R dependent (Otmakhov et al., 2004), neurotrypsin-dependent agrin cleavage was blocked by the selective NMDA-R antagonist MK-801. Activation of NMDA-Rs requires strong depolarization of the postsynaptic membrane to remove the Mg^2+ block (Mayer et al., 1984). Thus, neurotrypsin activity induced by PFR required presynaptic activity for neurotrypsin exocytosis and postsynaptic depolarization resulting in NMDA-R signaling.
for its activation. Only then was agrin cleavage by neurotrypsin detected. In contrast, if
LTP was induced by TEA, which induces a combination of NMDA-R-dependent and
L-type VDCC-dependent LTP (Huang and Malenka, 1993; Hanse and Gustafsson,
1994; Huber et al., 1995), neurotrypsin-dependent agrin cleavage was blocked by both
MK-801 and nifedipine. As with the activation of NMDA-Rs, activation of L-type VDCCs
is linked to action potential firing in the postsynaptic cell. Therefore, these results
suggest that neurotrypsin-dependent agrin cleavage may serve as a coincidence
detector for correlated activity of the pre- and postsynaptic neuron. Correlated activity is
widely considered as an essential requirement for activity or experience-dependent
modification of activated synapses. According to Hebb's postulate, connections with
correlated activity will be strengthened, whereas those with uncorrelated activity are
weakend and eventually disassembled (Hebb, 1949). Based on its activation
mechanism, the NMDA-R serves as a molecular detector for the coincidence of
presynaptic activation (glutamate release) and postsynaptic depolarization (Bourne and
Nicoll, 1993) and is therefore thought to be the molecular master-switch that initiates
Hebbian learning (Tsien, 2000). Neurotrypsin-dependent agrin cleavage shares its
induction mechanism with LTP by its crucial dependence on both activity-controlled
presynaptic exocytosis and the concomitant activation of the postsynaptic cell.
The mechanism underlying NMDA-R-dependent activation of neurotrypsin is currently not clear. The most straightforward models suggest that neurotrypsin is released in an inactive form that requires NMDA-R activity for its activation or, alternatively, that the susceptibility of agrin for proteolytic cleavage by neurotrypsin is regulated by mechanisms requiring NMDA-R activity.

The neurotrypsin-agrin system as a potential mediator of LTP-associated synapse formation

It is widely accepted that dendritic filopodia serve as precursors of new spines in the context of activity-dependent synaptogenesis (Jontes and Smith, 2000; Yuste and Bonhoeffer, 2004; Knott and Holtmaat, 2008). Formation of filopodia is observed within minutes of LTP-inducing stimulation (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Jourdain et al., 2003; Nägerl et al., 2004; Holtmaat et al., 2005; 2006). The transition of filopodia to spines upon contact with a presynaptic bouton was directly observed (Ziv and Smith, 1996) and inferred from the observation of synapses on filopodia (Vaughn et al., 1974; Saito et al., 1997; Fiala et al., 1998; Marrs et al., 2001). Time course studies indicated that postsynaptic molecules, such as PSD-95, appear at the filopodial tip already 2 h after initial contact with a bouton (Friedman et al., 2000;
Okabe et al., 2001; Marrs et al., 2001). In contrast, it takes between 15 and 19 h until ultrastructural indicators of postsynaptic specialization appear (Nägerl et al., 2007). The time difference of >12 h from the first molecular signs of postsynaptic specialization to ultrastructural synaptic features may reflect the time-scale of synapse maturation.

These observations indicate that filopodia and resulting synapses are not essential for the synaptic enhancement recorded after LTP induction. Rather, dendritic filopodia may be considered as an epiphenomenon of LTP and the resulting synapse formation may represent a delayed effect that results in long-term structural stabilization of the enhanced synaptic function and/or the reorganization of the synaptic connectivity.

Consistent with this view, inactivation of neurotrypsin abrogated filopodia formation, but did not affect LTP.
**EXPERIMENTAL PROCEDURES**

**Neurotrypsin-pHluorin-expressing mice**

To generate a catalytically inactive reporter for neurotrypsin exocytosis, serine 711 of the catalytic triad of murine neurotrypsin was mutated to alanine by PCR mutagenesis. The mutated neurotrypsin cDNA was attached to the superecliptic variant of pHluorin (SpH, kindly provided by Dr. Miesenböck) via a -Ser-Gly-Ser-Gly-Gly- linker. The fusion construct was inserted into the Thy-1.2 vector (Caroni, 1997). After microinjection of the construct into the pronucleus of fertilized oocytes we obtained 15 transgenic lines. From these we selected lines B6;D2-\(Tg(Thy1-Prss12/SpH)\) 962 and 1099 Zbz based on their strong expression of neurotrypsin-pHluorin in hippocampal neurons, especially in the subiculum-CA1 area (Fig. S1A,B).

**Chemical stimulation**

To prepare acute slices for chemical stimulation, the hippocampus, together with the adjacent cerebral cortex, was rapidly dissected from whole brains of 4-6 week-old mice, then cut vertically to the long axis of the hippocampus into 400 \(\mu\)m-thick slices using a McIlwain tissue chopper (Mickle Laboratory Engineering Co). The slices were transferred into artificial cerebrospinal fluid (ACSF) without calcium (120 mM NaCl, 3
mM KCl, 1.2 mM NaH$_2$PO$_4$, 23 mM NaHCO$_3$, 11 mM glucose, 2.4 mM MgCl$_2$) oxygenated with 95% O$_2$/5% CO$_2$, and incubated for one hour at room temperature, to provide sufficient time for the brain tissue to recover from the dissection. Before stimulation, the slices were incubated in ACSF with calcium (2.4 mM CaCl$_2$) for 20 min. For chemical stimulation, slices were incubated with either 30 mM KCl, 30 mM TEA, 1 mM glutamate, or 100 µM bicuculline for 10 minutes, or with 50 mM KCl for 40 s. Another stimulation protocol (PFR stimulation) used a combination of 50 µM picrotoxin, 50 µM forskolin, and 0.1 µM rolipram in ACSF with high Ca$_2+$(4 mM CaCl$_2$) and without Mg$_2+$ for 16 min. All inhibitors were added 20 min before stimulation. The following concentrations were used: ω-agatoxin IVA, 0.5 µM; ω-conotoxin GVIA, 1 µM; CNQX, 20 µM; MK-801, 20 µM; and nifedipine, 50 µM. The same chemical stimulation protocols were also applied to whole hippocampi dissected from P10 brains. After stimulation tissue was either immediately frozen for Western blotting or fixed by incubation in 4% paraformaldehyde, 4% sucrose in PBS, pH 7.4, overnight at 4°C for histological analyses.

**Analysis of neurotrypsin-pHluorin secretion**

Fixed acute slices were mounted on slides with Vectashield. The layer V region of the
entorhinal cortex was imaged with a 40x objective and fluorescence optics. The images were changed to gray and binary-mode to count positive puncta using Scion Image software. Puncta were defined as an area of 0.8-3 µm² to exclude small extrasynaptic signals and large cell body signals.

**Electrophysiological methods**

Parasagittal brain slices (350 µM thick) containing the hippocampus were prepared according to procedures approved by the Department for Veterinary Affairs of the Canton of Zurich, Switzerland. Briefly, mice were anesthetized with halothane and decapitated. The heads were immediately immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing: 125 mM NaCl, 1.25 mM Na₂HPO₄, 2.5 mM KCl, 1.0 mM MgCl₂, 25 mM NaHCO₃, 10 mM glucose, 2 mM CaCl₂, 1 mM MgCl₂ (pH 7.4, 305 ± 5 mOsm, saturated with 95% O₂/5% CO₂). After cooling for about 1 minute the brain was rapidly removed from the skull and placed ventral side down on an ice-cold surface. The cerebellum was cut away and the hemispheres were separated along the midline. The hemisphere was fixed with the cut side down on the stage of a vibratome (VT1000S Leica, Nussloch Germany) with cyanoacrylate glue. Sections containing the hippocampus were kept submerged in ACSF at 34 °C for 40 min then stored at room
temperature for 1-6 hours before use. Field recordings from the CA1 region were made in submersion-type chambers at 27 °C using ACSF–filled pipettes both to stimulate and record the excitatory postsynaptic potentials (fEPSPs) in the CA1 stratum radiatum. Axopatch 200B or Multiclamp amplifiers (Molecular Devices, Palo Alto, CA) were used to record the fEPSPs, and data were stored and analyzed using the pClamp 9.0 software package (Molecular Devices). The stimulation intensity was set to evoke a fEPSP 40-50% of the maximal amplitude without population spikes. Stimuli were applied at 30 s intervals. After obtaining a stable baseline for 30 min, long-term potentiation was evoked with four 1-s trains of 100 Hz stimuli delivered at the test intensity at 30 s intervals. The fEPSP amplitude was measured from the baseline for each sweep. The baseline amplitude was the average over a 10-min sampling period just prior to the high frequency stimulation and the average fEPSP amplitude was calculated as the average of a 10-min sampling period 3 hours after stimulation. Paired t-tests using raw values were used to determine whether there was significant potentiation within each group and unpaired t-tests on normalized values were used to compare the amount of potentiation between groups.

Quantification of filopodia
To visualize dendritic filopodia, we used the transgenic mouse line L15 overexpressing membrane-targeted GFP in sparse CA1 neurons (De Paola et al., 2003), or offspring of crosses between L15 and neurotrypsin-deficient mice. Tissue slices were produced as detailed above. Serial images of secondary apical dendrites of hippocampal CA1 pyramidal neurons expressing mGFP were collected at z-steps of 0.12 µm using a 100x objective (Leica SP1). We only analyzed healthy looking dendrites without swelling that could be traced back to the cell body. We reconstructed 3D images from z stacks using the Surpass Volume mode in the Imaris isoftware (Bitplane AG), and counted the number of filopodia over a length of 30-40 µm along 27-40 independent secondary apical dendrites (~1 mm in total length) from 3 independent experiments by inspecting the 3D images from all directions. Dendritic filopodia were identified according to the morphological criteria described by Grutzendler et al., 2002. Counting was done blind with respect to condition and genotype on images that were coded before counting.

**Preparation of recombinant agrin fragments**

The recombinant agrin fragments used in this study were cloned as secretory proteins into the pEAK8 or pcDNA3.1 vector using standard recombinant DNA procedures. The proteins were expressed in the eukaryotic cell line HEK293 EBNA. The secreted
proteins were purified from the culture supernatants using standard chromatographic procedures (for details see Supplemental Data).
REFERENCES


ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. Exocytosis of neurotrypsin is a transient response to presynaptic activation

Neurotrypsin-pHluorin was expressed in neurons of transgenic mice under the Thy-1 promoter. Its subcellular localization was monitored in slices of the deep layers of the entorhinal cortex of 4-6 week-old mice based on the pH-dependent fluorescence of pHluorin. (A-C) Neurotrypsin-pHluorin signals in the deep layers (III-VI) of the entorhinal cortex colocalized with the synaptic marker synapsin I. Note that all large neurotrypsin-pHluorin puncta (arrows in A) are colocalized with a prominent synapsin I signal (B,C). Bar 10 μm. (D,E) The numbers of large neurotrypsin-pHluorin puncta with an area between 0.8 and 3 m² were counted in visual fields of 0.08 mm² after NH₄Cl treatment and various chemical stimulations of synaptic activity, such as KCl for 10 min (KCl-L) or 40 s (KCl-S) or tetraethylammonium chloride (TEA), bicuculline (BCC), and glutamate (Glu). Tetrodotoxin (1 μM) was used to block action potentials. (F)

Pharmacological characterization of synaptic exocytosis of neurotrypsin. Presynaptic Ca²⁺-channels were blocked with ω-agatoxin IVA (ATX) and ω-conotoxin GVIA (CTX). AMPA and NMDA receptors were blocked with CNQX and MK-801, respectively. (G)

Time course of neurotrypsin-pHluorin exocytosis. Stimulation periods are shown by
Figure 2. Neuronal activity enhances agrin cleavage by neurotrypsin

Neurotrypsin-dependent cleavage of agrin was studied on Western blots of whole hippocampi from P10 mice after stimulation of neural activity by different protocols. (A)

Schematic representation of agrin and its neurotrypsin-dependent cleavage sites (arrows α and β). Cleavage of agrin at both sites generates a 22-kDa C-terminal fragment (agrin-22) and a middle 90-kDa fragment (agrin-90). Partial cleavage at the α site only generates a 110-kDa C-terminal fragment (agrin-110). Abbreviations: NtA, N-terminal agrin domain; TM, transmembrane segment; FS, follistatin-like domain; LE, laminin EGF-like domain; S/T, serine/threonine-rich region; SEA, sperm protein, enterokinase and agrin domain; EG, epidermal growth factor domain; LG, laminin globular domain; y and z, mRNA splicing sites. (B) Western blots for agrin from non-stimulated (NS) and 4-aminopyridine-stimulated (4AP) hippocampi from wildtype and neurotrypsin-deficient mice using anti-agrin antibody R132. Due to differential glycanation, full-length agrin appears as a smear in the range of 200 to 600 kDa (arrow). Agrin-90 is indicated by the arrowhead. β-actin loading controls are shown below. (C)
Western blots of agrin in KCl, 4AP, TEA-stimulated and non-stimulated hippocampi from P10 mice at various time points after onset of stimulation. Arrowheads indicate agrin-90. β-actin loading controls are shown below. (D) Quantification of agrin-90 levels of C. Levels of agrin-90 were normalized to β-actin. The average level before stimulation (0 min) was set to 1. The stimulation period is indicated by a black bar. Error bars indicate S.E.M.; *, p < 0.05; **, p < 0.01 vs non-stimulated controls by ANOVA with Tukey’s post hoc test (n = 3-8). (E,F) Neurotrypsin-dependent agrin cleavage requires activation of presynaptic Ca\textsubscript{2+} channels. TEA was used to stimulate neurotrypsin-dependent agrin cleavage and the response to blockade of presynaptic P/Q- and N-type Ca\textsubscript{2+} channels was studied by Western blotting of agrin-90 (E). No Stim: control hippocampi without stimulation. Presynaptic P/Q- and N-type Ca\textsubscript{2+} channels were blocked with ω-agatoxin IVA (ATX) and ω-conotoxin GVIA (CTX), respectively. (F) Quantification of agrin-90 levels in E. Relative levels of agrin-90 were normalized to β-actin. The average level found without stimulation was set to 1. Error bars indicate S.E.M.; *, p < 0.05; **, p < 0.01, ANOVA with Tukey’s post hoc test (n = 4-9).

**Figure 3. Neurotrypsin-dependent agrin cleavage requires postsynaptic activation**

The chemical LTP inducers TEA and PFR were used to stimulate
neurotrypsin-dependent agrin cleavage in whole hippocampi from P10 mice and the response to blockade of postsynaptic channels was studied by Western blotting of agrin-90. (A, B) Stimulation with TEA. (A) Representative Western blot for agrin-90. The β-actin loading control is shown below. No Stim: wildtype hippocampi without stimulation. AMPA and NMDA receptors were blocked with CNQX and MK-801, respectively. L-type VDCCs were blocked with nifedipine (Nife). (B) Quantification of agrin-90 levels under the conditions specified in A. Levels of agrin-90 were normalized to β-actin. The average level found without stimulation was set to 1. Error bars indicate S.E.M.; **, p < 0.01; ***, p < 0.001, ANOVA with Tukey’s post hoc test (n = 13-14). (C, D) Stimulation with PFR. (C) Representative Western blot for agrin-90 with β-actin loading control below. Ntd: neurotrypsin-deficient hippocampi. (D) Quantification of agrin-90 levels under the conditions specified in C. **, p < 0.01; ***, p < 0.001 by ANOVA with Tukey’s post hoc test (n =10-12).

Figure 4. LTP is intact, but LTP-associated formation of filopodia is abolished in neurotrypsin-deficient mice

LTP and LTP-associated promotion of dendritic filopodia were assessed in hippocampal slices of 4-6 week-old neurotrypsin-deficient mice. (A, B) LTP was studied by stimulation
of the Schaffer collaterals and electrophysiological recordings of Schaffer collateral-CA1 synaptic responses (A). Test stimuli were delivered at 30s intervals and LTP was induced by delivering four 1-s 100 Hz trains at 30s intervals. (B) Comparison of long-term potentiation (LTP) in the hippocampal CA1 area of neurotrypsin-deficient (white squares) and wildtype (black squares) mice. Data are shown as mean ± S.E.M. The results indicate that neurotrypsin-deficient mice have normal LTP. (C-I) Analysis of filopodia on dendrites of CA1 pyramidal neurons in wildtype and neurotrypsin-deficient mice expressing membrane-targeted GFP in sparse neurons. (C) Representative image of a GFP-expressing CA1 pyramidal neuron in a hippocampal slice. (D) Higher magnification of the secondary apical dendrite indicated by the arrow in C. (E) Reconstructed 3D image of the secondary apical dendrite boxed in D. Arrows show filopodia identified according to the criteria of Grutzendler et al. (2002). Bars: 50 µm in C; 20 µm in D; 10 µm in E. (F-I) Filopodia numbers on secondary apical dendrites of hippocampal CA1 pyramidal neurons (For representative images see Fig. S9). (F) Number of filopodia per 1 µm of dendrite after TEA stimulation and effect of AMPA and NMDA receptor blockade by CNQX and MK-801, respectively, and blockade of L-type Ca$^{2+}$-channels by nifedipine (Nife) in L15 mice (wildtype for neurotrypsin). (G) Comparison of filopodia formation after TEA stimulation in wildtype and
neurotrypsin-deficient mice. (H) Filopodia numbers after PFR and effect of CNQX, MK-801, and nifedipine. (I) Comparison of filopodia formation after PFR stimulation in wildtype and neurotrypsin-deficient mice. Error bars indicate S.E.M.; ***, p < 0.001, ANOVA with Tukey’s post hoc test. wt.: neurotrypsin wildtype littermate control. ntd: neurotrypsin-deficient mice.

**Figure 5. Isolated agrin-22 promotes the formation of dendritic filopodia**

The fragments of agrin generated by neurotrypsin-dependent cleavage were tested for their filopodia-promoting activity on secondary apical dendrites of CA1 pyramidal neurons in hippocampal slices from 4-6 week-old mice. (A-E) Densities of filopodia on dendrites in neurotrypsin-deficient hippocampi with or without agrin-22. (A-D)

Representative images of dendrites of CA1 pyramidal neurons of neurotrypsin-deficient mice with or without agrin-22. Arrows indicate filopodia. (E) Number of filopodia per 1 µm of dendrite. Note that the impairment of the activity-dependent increase of filopodia in neurotrypsin-deficient mice was restored by administration of agrin-22. (F,G)

Filopodia densities on dendrites after application of agrin-90 (F) and agrin-110 (G). Note that agrin-90 and agrin-110 had little or no activity. (H) Filopodia densities on dendrites in hippocampi from wildtype mice after application of the four splice variants of agrin-22
(z0, 8, 11, 19). Error bars indicate S.E.M.; ***; \( p < 0.001 \) by ANOVA with Tukey’s post hoc test. For representative images of dendrites studied in F-H see Fig. S10.
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Figure 4  K. Matsumoto-Miyai et al.
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Coincident pre- and postsynaptic activation induces dendritic filopodia via neurotrypsin-dependent agrin cleavage

Supplemental Data

Fig. S1. Transgenic neurotrypsin-pHluorin is sorted to synapses.

Acute slices from hippocampus and adjacent entorhinal cortex of 4-6 week-old mice of the transgenic line 962 (A) exhibited strong fluorescence in the subiculum-CA1 region (B). (C) Relative amounts of neurotrypsin-pHluorin in synaptosomes were determined by Western blotting with an anti-neurotrypsin and an anti-GFP antibody. Synaptosomes were prepared as specified in Figure S2. From each fraction of the synaptosome preparation 50 µg were loaded: H, homogenate; S1, supernatant 1; S2, supernatant 2; P2, pellet 2; M, myelin fraction; SS, synaptosomes. The arrowhead indicates the 120-kDa band of neurotrypsin-pHluorin. The relative amounts of the synaptic marker protein PSD-95 are shown below. Note that a substantial amount of neurotrypsin-pHluorin was correctly sorted to synapses.
To verify that the transgenic neurotrypsin-pHluorin protein was correctly targeted to synapses, we performed subcellular fractionations and isolated synaptosomes from hippocampus and cerebral cortex. Hippocampi and cerebral cortices were homogenized in 0.32 M sucrose, 0.5 mM EDTA, 5 mM HEPES pH 7.4, including 1% protease inhibitor cocktail (Sigma P-8340). The homogenate was centrifuged at 1,000 \( \times \) g for 5 min to separate supernatant (S1 fraction) from pellet (P1 fraction). Further centrifugation (12,000 \( \times \) g for 20 min) of S1 generated the S2 (supernatant) and P2 (pellet) fractions. Subsequently, the P2 fraction was loaded onto a 7.5%/12% Ficoll gradient and ultra-centrifuged at 68,000 \( \times \) g for 1 hour. The synaptosomal fraction was concentrated between 7.5% and 12% Ficoll. The lighter fraction above 7.5% Ficoll was collected as the myelin fraction.
Fig. S3. Observation of exocytosis of neurotrypsin-pHluorin in response to neuronal activation.

The subcellular localization of neurotrypsin was monitored in slices of the deep layers of the entorhinal cortex of 4-6 week-old mice based on the pH-dependent fluorescence of pHluorin. (A-F) Representative images for the quantitative data presented in Figure 1D. (A,B) Neutralization of intracellular vesicles by ammonium chloride (NH₄Cl; B) revealed intracellular synaptic stores of neurotrypsin-pHluorin that were not detectable under control conditions (A). (C-F) Cell depolarization with high K⁺ (D,E) or blockade of K⁺-channels with TEA (F) induced a marked increase of extracellular synaptic neurotrypsin compared to unstimulated slices (C). Bar 20 µm.
Fig. S4. A second line expressing neurotrypsin-pHluorin in neurons under the Thy1 promoter confirmed the synaptic targeting of transgenic neurotrypsin-pHluorin, its storage in intracellular vesicles, and its activity-dependent exocytosis.

(A, B) High expression levels of neurotrypsin-pHluorin were found in the CA1-subiculum area also in the transgenic mouse line Tg1099. (C-E) Neurotrypsin-pHluorin puncta in entorhinal cortex of line Tg1099. After pH neutralization by ammonium chloride (D) or TEA stimulation (E) the number of puncta was increased. Bar: 20 µm. (F) The numbers of large neurotrypsin-pHluorin puncta with an area between 0.8 and 3 µm² were counted in visual fields of 0.08 mm² in the deep layers of the entorhinal cortex from mice of the transgenic line Tg1099 using Scion Image software. Error bars indicate S.E.M. **: p < 0.01 using ANOVA with Tukey’s post hoc test (n = 4-8).
Fig. S5. Studies with hippocampi from 4-6 week-old mice confirm that neurotrypsindependent agrin cleavage requires postsynaptic activation.

In the signaling cascades for LTP induction certain age-dependent differences have been reported downstream of the mandatory postsynaptic Ca\(^{2+}\) transient (Yasuda et al., 2003). Therefore, we tested for the dependence of neurotrypsin-induced agrin cleavage also in hippocampal slices from juvenile (4-6 week-old) mice. The chemical LTP inducers TEA and PFR were used to stimulate neurotrypsin-dependent agrin cleavage and the response to blockade of postsynaptic glutamate channels was studied by Western blotting of agrin-90. Four slices were prepared for each experimental condition and together subjected to the respective stimulation protocol. The pooled slices were then lysed for SDS-PAGE and
Western blotting. Levels of agrin-90 were normalized to β-actin. The average level found without stimulation was set to 1. Error bars indicate S.E.M.; *, p < 0.05; ***, p < 0.001 using ANOVA with Newman-Keuls’ post hoc test (n = 3-8). The results with hippocampal slices from 4-6 week-old mice demonstrated that neurotrypsin-dependent agrin cleavage after stimulation by TEA or PFR requires activation of postsynaptic glutamate receptors.

Reference:
Fig. S6. Parallel observation of neurotrypsin exocytosis and agrin cleavage in PFR-stimulated slices from 4-6 week-old mice.

Because the mechanism of LTP induction by PFR is distinct from that of TEA, we studied PFR-induced synaptic exocytosis of neurotrypsin and the associated cleavage of agrin in parallel in acute slices from hippocampus and entorhinal cortex of 4-6 week-old transgenic mice expressing neurotrypsin-pHluorin (line Tg1099). The slices from the same PFR stimulation experiment were divided into two groups for the parallel analysis of neurotrypsin-pHluorin exocytosis and agrin cleavage. (A-C) Neurotrypsin-pHluorin puncta in entorhinal cortex after PFR stimulation. Scale bar: 20 µm. (D) Western blots for agrin-90 in PFR-stimulated hippocampal slices. Bands of agrin-90 are shown in the upper part. β-actin loading controls are shown below. No Stim: non-stimulated condition. M/C: MK-801 and
CNQX. (E) The numbers of neurotrypsin-pHluorin puncta per visual field (0.08 mm$^2$) under conditions shown in A-C. Error bars indicate S.E.M. ***: $p < 0.001$ by ANOVA with Tukey’s post hoc test ($n = 12-13$). (F) Relative band densities of agrin-90 shown in D normalized to $\beta$-actin. The agrin-90 level in non-stimulated hippocampi was set to 1. Error bars indicate S.E.M. *: $p < 0.05$ using ANOVA with Newman-Keuls’ post hoc test ($n = 11-12$). PFR stimulation for 16 min resulted in both a significant increase in the number of neurotrypsin-pHluorin puncta in the entorhinal cortex (A-C,E) and a significant increase of the agrin 90-kDa fragment (D, F). Blockade of postsynaptic activation in PFR-stimulated hippocampal slices reduced the amount of the agrin 90-kDa fragment to the control level. Together, these results confirmed the sequence of events from neurotrypsin exocytosis to agrin cleavage after PFR stimulation in the same experiment and corroborated the dependence of agrin cleavage on activation of postsynaptic glutamate receptors with hippocampal slices from 4-6 week-old mice.
Fig. S7. Chemical stimulation with TEA or PFR confirmed that neurotrypsin-deficient mice have intact LTP.

For chemical induction of LTP, TEA or PFR were bath applied to hippocampal slices from 4-6 week-old mice for 10 min and for 16 min, respectively. Synaptic responses were recorded in the stratum radiatum of the CA1 region in response to Schaffer collateral stimulation at 30 s intervals. During the chemical induction (indicated by a black bar) the electrical test stimulation was discontinued. (A) Chemical induction of LTP with TEA. Application of TEA for 10 min induced LTP in all of the 9 slices tested with similar amplitude in wildtype (+/+, black squares) and neurotrypsin-deficient (-/-, white squares) mice (P = 0.81). (B) Chemical induction of LTP with PFR. Application of PFR for 16 min also induced significant LTP with similar amplitude in wildtype (+/+, black squares) and neurotrypsin-deficient (-/-, white squares) mice (P = 0.52). However, in 2 out of 10 slices from wildtype and 2 out of 9 slices from neurotrypsin-deficient mice PFR application induced LTD (data not included in plot). As PFR-induced synaptic plasticity requires NMDA receptor activation, this finding is consistent with the fact that NMDA receptors can mediate either LTP or LTD depending on the magnitude of activation and the resulting increase in intracellular calcium. All data points are the mean normalized EPSC amplitude ± S.E.M. These results confirm that chemical stimulation with TEA and PFR induce robust LTP and that LTP is intact in neurotrypsin-deficient mice.
Fig. S8. Chemical induction of hippocampal LTP by TEA or PFR did not induce neuronal cell death.

To confirm that chemical LTP protocols are physiologically appropriate, we performed cell death assays in acute hippocampal slices from 4-6 week-old mice stimulated by TEA or PFR. After chemical stimulation, the slices were incubated in ACSF containing 2.3 µM propidium iodide (PI; Molecular Probes, Invitrogen) for 20 min. Images of PI fluorescence were taken with a fluorescence microscope (Olympus). As a control, oxygen and glucose deprivation (OGD) for 30 min was used to induce massive neuronal cell death (A). (B–D) Representative CA1 images of PI fluorescence in hippocampal slices of wildtype mice without stimulation (B),
after TEA stimulation for 10 min (C), and after PFR stimulation for 16 min (D). (E-G)

Representative CA1 images of PI fluorescence in hippocampal slices of neurotrypsindeficient mice without stimulation (E), after TEA stimulation (F), and after PFR stimulation (G).

Note that deprivation of oxygen and glucose resulted in massive neuronal cell death, as indicated by the intensive PI fluorescence in the principal cell layer of the CA1 area. All the other conditions did not induce substantial cell death. In particular, PI fluorescence after stimulation by TEA or PFR was not increased when compared with non-stimulated slices.

Scale bar: 200 µm. (H) The average intensities of PI fluorescence over an area of 0.64 mm² were measured with ImageJ software (National Institutes of Health, USA) using the same settings. A significant difference was detected between OGD and all the other conditions. No differences were found between stimulated and non-stimulated slices. These results indicated that chemical stimulation by TEA or PFR did not induce neuronal cell death in the hippocampal CA1 region. Error bars indicate S.E.M. ***: p < 0.001 versus all other conditions using ANOVA with Tukey’s post hoc test (n = 3-6).
Fig. S9. LTP-associated formation of filopodia is abolished in neurotrypsin-deficient mice.

Images of dendritic filopodia were acquired with a confocal microscope and processed using Imaris software. Dendritic filopodia were identified according to the morphological criteria...
established by Grutzendler et al. (2002) and indicated by arrows. Scale bar: 10 µm. (A-F)

Representative images corresponding to the quantitative data presented in Figure 4F. (G-J)

Representative images for the quantitative data presented in Figure 4G. (K-P)

Representative images for the quantitative data presented in Figure 4H. (Q-T)

Representative images for the quantitative data presented in Figure 4I.
Fig. S10. Dendritic filopodia are induced by agrin-22, but not by agrin-90 and agrin-110.

Images of dendritic filopodia were acquired with a confocal microscope and processed using Imaris software. Dendritic filopodia were identified according to the morphological criteria established by Grutzendler et al. (2002) and indicated by arrows. Scale bar: 10 µm. (A-D) Representative images corresponding to the quantitative data presented in Figure 5F. (E-H)
Representative images for the quantitative data presented in Figure 5G. (I-M) Representative images for the quantitative data presented in Figure 5H. Note that administration of agrin-90 or agrin-110 did not rescue PFR-induced dendritic filopodia in neurotrypsin-deficient mice (ntd; C,D,G,H). On the other hand, all the splice variants of agrin-22 (z0, z8, z11, and z19) without chemical stimulation induced an increase in filopodia in hippocampal slices from wildtype mice (wt; J-M).
Supplemental methods

Preparation of recombinant agrin fragments.

For the expression of the different C-terminal fragments of agrin (agrin-22 splice variants and agrin-110 y0z0; see Fig. 2A), corresponding coding regions were inserted into the pEAK8 vector (EdgeBio Systems); Ser_{1755} to Pro_{1940} of rat agrin (P25304-3, splice variant y4z0; for agrin-22 z0), Ser_{1755} to Pro_{1948} of rat agrin (P25304-4, splice variant y4z8; for agrin-22 z8), Ser_{1755} to Pro_{1951} of rat agrin (P25304-5, splice variant y4z11; for agrin-22 z11), Ser_{1755} to Pro_{1959} of rat agrin (P25304-1, splice variant y4z19; for agrin-22 z19), and Ala_{1103} to Pro_{2045} of human agrin (NP_940978.2, splice variant y0z0; for agrin-110 y0z0). The signal sequence of human calcyntenin-1 (Met_{1} to Asn_{32} of NP_001009566) and a (His)$_8$ tag were inserted Nterminally of the agrin sequence. To purify secreted recombinant agrin-22, the supernatant of transfected HEK293 EBNA cells was dialyzed against 400 mM NaCl in 20 mM Tris-HCl, pH 8.5, and loaded onto a HisSelect column (Sigma). Bound proteins were eluted in a gradient up to 250 mM imidazole. Fractions containing agrin-22 were pooled and concentrated to 0.5 ml with a centrifugal filter device (Millipore). The buffer was exchanged with a NAP 5 column (GE Healthcare) or Slide-A-Lyzer (10 kDa cut; PIERCE) to PBS or 100 mM NaCl in 20 mM MOPS, pH 8.

For the preparation of agrin-90, HEK 293T cells were co-transfected with the full-length transmembrane rat agrin y4z8 splice variant in pcDNA 3.1 (gift from K. Tsim) and full-length human neurotrypsin in pcDNA 3.1 using the calcium-phosphate precipitation method. After 4h, the transfection medium was removed and replaced by DMEM (Gibco) with 10% FCS. On the following day the medium was replaced by DMEM without FCS. The secreted rat agrin-90 generated from full-length agrin by neurotrypsin was purified from the supernatant using a Heparin Sepharose column (Amersham) and an anion exchange column (Poros 20 HQ/M) on the BioCAD HPLC system.