MOLECULAR MECHANISMS REGULATING SPINE REMODELLING

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Abstract

N-Cadherin plays an important role during dendrite arborisation, axon guidance and synaptogenesis. In particular, at synaptic sites, it is required for activity-dependent synaptic plasticity and dendritic spine remodeling. Recent studies have shown that N-Cadherin can be cleaved by the metalloproteinase ADAM10.

Here we demonstrate that modulating ADAM10 localization and activity at synaptic sites regulates its processing of N-Cadherin. This indices modification of dendritic spines morphology and of composition and function of AMPA receptors. In particular, inhibition of ADAM10 synaptic localization and activity leads to an accumulation of the full-length form of N-cadherin, to an increase in spine head width and to modifications of number and function of glutamate receptors of AMPA-type, both in vitro and in vivo. Conversely, the stimulation of ADAM10 activity towards N-Cadherin reduces dendritic spine size and AMPA receptor localization at postsynaptic sites.
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Introduction
**Synapses.**

The specialised contact sites between two contiguous neurons are called Synapses; they connect the axonal terminals on the “pre-synaptic” neurons to the dendritic spines of the “post-synaptic” ones. The term Synapse, coined from the Greek words "syn-" ("together") and "haptein" ("to clasp"), was introduced for the first time by Sir Michael Foster and Sir Charles Sherrington in their “A text book of physiology” and described as “a contact without the continuity of substances between two neuronal cells”. In 1959, with the advent of electron microscopy, George Gary provided the first clear evidence of synaptic contacts between neurons in the CNS (GRAY, 1959). He also described two different types of synapses in the CNS: asymmetric, found onto dendritic spines, and symmetric, present mostly onto cell bodies (Fig. 1a,b). Now these two types of synapses are called Gray type I and Gray type II synapses, corresponding to excitatory and inhibitory synapses, respectively (Colonnier, 1968).

From a structural point of view, a synapse is a polarised neuronal cell junction with specific membrane specialisations at the transmitter-releasing pre-synaptic site and, in close opposition, the afferent post-synaptic part (Boeckers, 2006). It can be divided into several domains (Fig. 1c): the pre-synaptic terminal, the synaptic cleft and the post-synaptic density. The pre-

![Figure 1.](image-url)
synaptic terminal consists of the mitochondria, synaptic vesicles and active zone. The synaptic vesicles are filled with neurotransmitters and fuse to a thickening of the pre-synaptic plasma membrane where release occurs. The gap between the pre-synaptic and post-synaptic membrane is called synaptic cleft into which neurotransmitters are released. At the post-synaptic side, just below the plasma membrane and directly opposed to active zones, electron-dense structures are found, which are referred to as post-synaptic densities (PSDs). They represent the location of neurotransmitter-activated ion channels anchored by scaffolding proteins (Scannevin and Huganir, 2000; Dresbach et al., 2001; Sheng, 2001). Excitatory synapses (type I synapses), which use glutamate as a neurotransmitter in the CNS, have a wider synaptic cleft than inhibitory synapses (type II synapses). The cleft of excitatory synapses are around 30 nm wide and contain electron-dense extracellular materials, active zones measure 1–2 μm² in area and PSDs 30–40 nm thickness. These PSDs are large protein complexes, which include glutamate-responding ion channels. In contrast, at type II inhibitory synapses, the synaptic cleft is 20 nm wide, and there is little or no electron-dense extracellular materials within the cleft. The active zone is smaller (less than 1 μm²), and the pre-synaptic membrane specializations and dense regions are less obvious. In addition, PSDs at the post-synaptic site are less obvious compared to those at excitatory synapses (GRAY, 1959).

**Dendritic Spines: Structure.**

In mature brain about 90% of the excitatory synapses is located on dendritic spines, morphological specialisations formed by small membrane protrusions with a total volume ranging from less than 0.01 μm³ to 0.8 μm³ (Harris and Kater, 1994; Harris, 1999). Dendritic spines are present on most of the principal glutamate- or GABA-releasing neurons, such as hippocampal pyramidal neurons, but not on many other neuron classes, such as GABA-releasing interneurons. Although dendritic spines are essentially formed by a neck and head attached to the dendritic membrane, they can have different shapes: thin, stubby or mushroom-shaped (Fig. 2) (Chang and Greenough, 1984; Harris et al., 1992; Harris and Kater, 1994; Hering and Sheng, 2001).

**Figure 2.** Schematic representation of different spine shapes.

<table>
<thead>
<tr>
<th>Spine types</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thin</td>
<td>Small, narrow neck, head is more than 0.6 μm³</td>
</tr>
<tr>
<td>Mushroom</td>
<td>Larger head, neck is wider than 0.6 μm³</td>
</tr>
<tr>
<td>Stubby</td>
<td>Thin head, narrow neck</td>
</tr>
</tbody>
</table>

All these different classes of spines can be found at the same time on the same dendrites (Spacek and Harris, 1998; Harris, 1999). In this classification the form and dimension of the neck and head are taken into account: the mushroom-shaped spines have a large head exceeding 0.6 μm³ and a narrow neck, whereas thin spines have a smaller head and narrow neck and stubby spines have no obvious constriction between the head and the dendritic shaft. However this static classification does not reflect the real in vivo situation which is

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**Introduction**
characterised by more plasticity as the majority of the spines, at least in developing neurons, change their shape over periods of minutes or hours (Parnass et al., 2000). Usually mature spines have a single excitatory synapse at their head, but the same spine may have inhibitory synapses (Knott et al., 2002). The intracellular composition of dendritic spines mainly consists of the post-synaptic density (PSD) facing the pre-synaptic button and a cytoskeletal structure formed almost exclusively by F-actin. The PSD surfaces vary from small discs to large irregular shapes which usually occupy about 10% of the surface area exactly opposite to the pre-synaptic active zone. Other organelles are localised at larger spines: Smooth Endoplasmic Reticulum (REM) (Spacek and Harris, 1997), polyribosomes (Ostroff et al., 2002), endosomal compartments (Cooney et al. 2002, Park et al. 2006) and also peri-synaptic astroglia (Witcher et al., 2007). Large spines usually have a proportionally large synapse and contain different types of organelles which are formally absent in small spines (Spacek and Harris, 1997). Moreover, mushroom-shaped spines usually have larger, more complex post-synaptic densities with a higher density of glutamate receptors (Matsuzaki et al., 2001; Nicholson et al., 2006). All these features suggest that larger spines are functionally stronger in their response to glutamate, local regulation of intracellular calcium, endosomal recycling, protein translation and degradation, and interaction with astroglia. Smaller spines may be more flexible, rapidly enlarging or shrinking in response to subsequent activation (Segal, 2005). AMPA and NMDA receptors number, and density, within the post-synaptic density is proportional to the latter’s area and spine volume (Nusser et al., 1998; Takumi et al., 1999; Racca et al., 2000). Post-synaptic density and spine volume are also proportional to the area of the active zone, which is itself proportional to the number of docked vesicles (Schikorski and Stevens, 1997), which in turn correlates with the amount of neurotransmitter release per action potential (Murthy et al., 1997). All of these data suggest that large spines represent stronger synapses for both pre-synaptic and post-synaptic properties, and that the growth of the spine head during development probably correlates with a strengthening of synaptic transmission. In adult hippocampal CA1 pyramidal cells, dendritic spines density ranges from two to four spines/μm of dendrite (Trommald and Hulleberg, 1997; Sorra and Harris, 1998), whereas it is around 3-4 spines for each 10 μm in dissociated cultures (Papa et al., 1995). In the brain, the density of spines is not homogeneous throughout the dendritic tree but increases at each layer, thus suggesting that theafferent system independently regulates different parts of the dendritic tree (Valverde, 1967; Marin-Padilla and Stibitz, 1968).

### Dendritic Spines: Formation and Stabilisation.

Early spines are often very long and have frequent filopodia-like shape but, later during development, their mean length decreases and the number of filopodia is greatly reduced. Three major changes can be observed during the maturation process: an increase in spine density, a decrease in overall length and a decrease in the number of dendritic filopodia with a simultaneous decrease in spine motility (Dunaevsky et al. 1999, Nimchinsky et al. 2001) (Fig. 3).

Several studies have shown that filopodia rapidly protrude and retract from dendrites, especially during the early stages of synaptogenesis (Dailey and Smith 1996, Ziv and Smith 1996, Fiala et al. 1998), and it is widely believed that dendritic filopodia are the precursors of dendritic spines, although
various hypotheses as to how the transition from filopodia to spines takes place have been put forward. In the first proposed model, filopodia actively seek out synaptic partners in the developing neuropil and when a filopodium makes contact with an axon, it becomes shorter and draws the axonal terminal closer to the dendrite shaft. Subsequently, a fully mature synapse is formed on the spine head, spine motility gradually decreases and the structure is stabilized (Dailey and Smith, 1996; Ziv and Smith, 1996; Fiala et al., 1998). However, this model does not explain why the density of asymmetric synapses is much higher on dendritic shafts than on filopodia during early development (Adams and Jones, 1982; Dailey and Smith, 1996; Ziv and Smith, 1996; Fiala et al., 1998). Another model has proposed that, after their contacts with axons, the filopodia retract completely, thus leading to the formation of an asymmetric shaft synapse from which a spine emerges with a mature synapse at its head (Fiala et al., 1998; Harris, 1999). In a recent study using two-photon time-lapse microscopy, Parnass and colleagues (Parnass et al., 2000) demonstrated that stubby and other types of spines can originate from filopodia in developing hippocampal neurons and, depending on the state of the afferent input, spines turning into filopodia were also observed. In this model a filopodium is like a spine in a state of morphological instability and is not a necessary intermediate for spine formation. In other words, the transition from filopodia to spines is less likely to be predestined than a reversible process regulated by local factors, such as synaptic activity.

In two recent time-lapse studies spine morphogenesis and PSD-95 fused to green fluorescent protein (GFP), a PSD marker, were simultaneously imaged. In both cases the authors suggested that synapses initially form on dynamic filopodia-like spines that soon convert directly into stable spines at the same time as the formation of postsynaptic specialization (Marrs et al., 2001; Okabe et al., 2001). However, only Marrs and colleagues showed the emergence of stable spines from shaft synapses. Overall, these data suggest that it is probably still incorrect to assume that all spines go through the same stages (beginning as filopodia, proceeding to thin or stubby spines, and ending as mushroom spines), and that spine maturity does not necessarily correlate with spine morphology. After the axodendritic contact took place, it must be stabilised (Fig. 4). An important role in stabilising the nascent spines and their synapses is played by cell-adhesion molecules, such as, N-Cadherins, catenins, neurexin and neuroligins, and members of the immunoglobulin superfamily. The N-Cadhein-based homophilic cell-adhesion system has a key role in maintaining pre- and postsynaptic apposition and is important for spine morphogenesis (Salinas and Price, 2005). The role of N-Cadherin in spine formation, maturation and plasticity is widely discussed in chapter 3.3.

SynCAM is the latest addition to the brain-specific Ig superfamily enriched at the synaptic junctions (Biederer et al., 2002). SynCAM contains an extracellular Ig domain and an intracellular PDZ-interaction motif, features that are expected of a potential synapse adhesion protein. Indeed SynCAM

**Figure 3.** Shape changes occurring during spine maturation.
mediates homophilic binding via its three extracellular Ig domains; the cytoplasmic domain associates with CASK and syntenin, which are PDZ-domain proteins that also interact with intracellular domains of other synaptic cell surface proteins, neurexins (see below) and syndecans. SynCAM is able to promote synapse formation instructing pre-synaptic differentiation, whereas its role in triggering post-synaptic differentiation is presently not clear. The ability of a homophilic adhesion protein to induce asymmetric synapse formation implies that other asymmetric cues translate the “symmetrical” association into differential responses on opposite sides of the cell contact. A potential synaptogenic cell surface interaction that satisfies the necessary asymmetry of pre- and post-synaptic differentiation is provided by the heterophilic adhesion interaction between neurexins and neuroligins (Missler and Sudhof, 1998; Rao et al., 2000). Neuroligins were the first cell surface molecule in which ectopic expression in non-neuronal cells was reported to induce pre-synaptic assembly in contacting axons in vitro (Scheiffele et al., 2000). The synaptogenic activity of neuroligins was blocked by overexpression of exogenous neurexins, suggesting that neurexins on the axonal plasma membrane mediate the pre-synaptic differentiation.

β-neurexin is enriched at the pre-synaptic terminals (Dean et al., 2003), and post-synaptic multimers of neuroligins (at least tetramers) are required to cluster neurexin in the pre-synaptic membrane, which in turn recruits synaptic vesicles via its cytoplasmic domain. The lateral clustering of synaptic adhesion molecules plays a key role in nucleating the pre-synaptic assembly process. Indeed, a critical density of neurexin cytoplasmic domains is required for organizing the pre-synaptic molecular scaffold, which is mediated by proteins such as CASK and syntenin that bind directly the cytoplasmic domain of neurexin.

After the axo-dendritic contact has been stabilised, synapse must be assembled and molecular component of both the pre- and post-synaptic compartments recruited. Synapse assembly in a very complex and well regulated event that occurs within 1 or 2 hours from initial axo-dendritic contact proceeding at a surprising rapid pace (Friedman et al., 2000; Antonova et al., 2001; Colicos et al., 2001; Okabe et al., 2001). Pre-assembled synaptic components are rapidly recruited to the site of cell contact, thereby obviating the need for building a synapse from scratch. Transport packets containing active zone components, such as Piccolo, Bassoon and N-Cadherin, traverse along the developing axon (Ahmari et al., 2000; Zhai et al., 2001) and insert into the pre-synaptic plasma membrane to deliver active zone components prior to the appearance of synaptic vesicles proteins required for exocytosis (Friedman et al., 2000; Shapira et al., 2003). The active zone components delivered by such transport packets have been proposed to provide a scaffold for recruiting exocytically competent synaptic vesicles, possibly by either trapping preformed synaptic vesicle clusters (Ahmari et al., 2000; Friedman et al., 2000) or de novo formation of vesicle clusters at the site of active zone (Okabe et al., 2001). In considering this model, it is important to note that the cytoplasmic surface of an active zone transport packet does not appear to recruit synaptic vesicles while in transit.

The first step of post-synaptic specialisation assembly is the appearance of NMDA receptors and PSD95 clusters that can serve as a molecular scaffold opposite the pre-synaptic active zone (McGee and Bredt, 2003). After PSD95 clusters formation, accumulation of AMPA receptors occurs (Friedman et al., 2000). There is a timing difference between pre- and post-synaptic assembly which is thought to depend on reciprocal signalling, which begins with the
retrograde activation of the axon by the motile dendritic filopodia, followed by an anterograde signal from the presumptive pre-synaptic loci that induce post-synaptic differentiation. Accordingly, the motile filopodia that trigger pre-synaptic assembly do not have PSD95 (Okabe et al., 2001). Live imaging of fluorescently tagged PSD95 demonstrates that post-synaptic PSD95 clusters originate from a diffuse cytoplasmic pool (Bresler et al., 2001; Marrs et al., 2001). Moreover, post-synaptic AMPA receptors can be recruited from the diffuse plasma membrane pool by lateral migration (Borgdorff and Choquet, 2002). Nevertheless, discrete dendritic transport packets of PSD95 (Prange and Murphy, 2001) and NMDA receptors (Washbourne et al., 2002) have been reported. Furthermore, AMPA receptors are present in a cytoplasmic vesicular pool that participates in rapid modulation of synaptic AMPA receptor number by an exo-endocytic mechanism (Lüscher and Frerking, 2001; Malinow and Malenka, 2002) synaptic assembly may involve both the delivery of prefabricated transport packets and de novo clustering of component proteins, including the lateral migration of plasma membrane proteins. When and how these different mechanisms are employed might depend on both the particular synaptogenic inducers involved in different neurons and the particular developmental environment.

Figure 4. A Model of Excitatory Central Synapse Formation. From the top: a) Early synaptogenic signaling events involving secreted factors precede cell contact, and motile filopodia search for potential partners. Neurotransmitters are released from exocytic hot spots where small clusters of synaptic vesicles are found (blue circles). Transport packets that contain active zone elements traverse along the axon (yellow circle). b) Cell adhesion molecules (red rectangles) stabilize select cell contact sites. c) Active zone elements (yellow) and synaptic vesicles accumulate at the presynaptic terminal. Postsynaptic terminal assembly follows presynaptic assembly by recruiting neurotransmitter receptors (double ellipses) and postsynaptic scaffolds (green triangle). d) In the assembled synapse, the presynaptic terminal has docked, and reserve pool of synaptic vesicles and the postsynaptic terminal show neurotransmitter receptors embedded with the scaffold proteins. See text for details.
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**Introduction**

Dendritic Spines: Activity-dependent Maturation.

Once formed, new synapses are usually thin and elongated and in general have a small head containing few glutamate receptors; at this stage their challenge is to become mature synapses, a process which is considered to be induced and regulated by neuronal activity. Newly formed spines are often referred to as learning spines (Fig. 5), in opposition to classical mushroom-shape spines that are representing more stable structures. During this early phase of stabilization, when newly formed thin spines acquire a PSD, their spine head enlarges, a phenomenon that probably shows similarities with the spine head enlargement associated with LTP induction (Matsuzaki et al., 2004; Harvey and Svoboda, 2007; Kopec et al., 2007; De Roo et al., 2008a). Increase in spine volume closely correlates with the accumulation of additional AMPA receptors (Zito et al., 2009) and reorganization of the actin cytoskeleton (Honkura et al., 2008). The mechanisms regulating these size changes start to be clarified. Following induction of plasticity at single spines, enlargement was linked to a destabilization of the PSD, an increased dynamics of PSD proteins such as PSD-95 and SHANK2, and a contribution through phosphorylation of CaMKII (Steiner et al., 2008). This enlargement process however also involves a structural reorganization controlled by several signalling systems, including proteolysis of the extracellular matrix by proteases, and signalling to the cytoskeleton through adhesion molecules. Recently, evidence was provided that MMP-9 activity is required for both LTP expression and spine enlargement and that this may involve signalling through a beta1-integrin receptor, cofillin, phosphorylation and actin polymerization (Wang et al., 2008). Additionally, in hippocampal cell cultures, a signalling pathway mediated by N-Cadherin and involving the scaffold protein AF6/afadin, the Rho GTPase exchange factor Kalirin-7, Rac1, p21-activated kinases (PAKs) and the cytoskeleton has been proposed to regulate the size of the spine head (Xie et al., 2008). This pathway could allow synaptic adhesion molecules to rapidly coordinate spine remodelling associated with synapse matura-

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**Figure 5.** Learning spines. During early phases of stabilisation, newly formed thin spines acquire PSD and change their shape towards mushroom-like stable structures.


**INTRODUCTION**

Dendritic Spines: Elimination.

In the central nervous system, the maturation, refinement and modulation of neural circuitry is driven not only by synapse formation and maturation, but also by regulated elimination of existing functional synapses. The pruning of initially excessive number of synapses is common during the early activity-dependent refinement of neural circuitry (Katz and Shatz, 1996; Sanes and Lichtman, 2001). Moreover, the mechanism of regulated synapse disassembly persists in the mature brain, although the number of remodelling events declines with age (Gan et al., 2003). It has been demonstrated that synapse elimination involves the disassembly of previously functional synapses. There are two phenomenological extremes that require dismantling previously functional synapses; they are “input elimination” and synapse disassembly.

During the input elimination a presynaptic neuron loses all synaptic contacts with a post-synaptic target by functionally and anatomically uncoupling (Sanes and Lichtman, 1999), preserving synaptic con-
contacts with other target cells (Keller-Peck et al., 2001). Input elimination requires rapid and complete disassembly of multiple individual synapses. At the other extreme is synapse disassembly, the elimination of an individual synapse, or a small number of synapse, without eliminating connectivity between two neurons. Synapses have been described as single intercellular junctions composed of the pre-synaptic active zone and the post-synaptic density capable of transducing pre-synaptically released neurotransmitters. Therefore, regulated synapse elimination represents a mechanism to modulate the strength of connection and synaptic transmission.

Synaptic Plasticity in Memory and Cognition.

Activity-dependent synaptic plasticity plays a crucial role in synapse maturation during early developmental stages, and has been identified in several synaptic pathways. Although, it occurs also in adult brain where memory formation is dependent on changes in synaptic efficiency that permit strengthening of associations between neurons; indeed, activity-dependent synaptic plasticity at appropriate synapses during memory formation is believed to be both necessary and sufficient for storage of information.

The modern theory about how memory works has been proposed at the beginning of the last century, when Cajal hypothesized that information storage relies on changes in strength of synaptic connections between neurons that are active (Cajal YR., 1913.). Hebb, supporting this idea, proposed that if two neurons are active at the same time the synaptic efficiency of the appropriate synapse will be strengthened (Hebb DO., 1949.). In the last 50 years, enormous efforts have been done to understand the mechanism by which strengthening of synaptic connections can be achieved and, in this effort, the importance of one model, above all others, cannot be overestimated; this model is long-term potentiation (LTP).

LTP was fully described for the first time by Bliss and Lomo in 1973 (Bliss and Lomo, 1973). They reported that trains of high-frequency stimulation to the rabbit perforant path caused a sustained increase in efficiency of synaptic transmission in the granule cells of the dentate gyrus. This report, and others which followed during the 1970s, confirmed the Hebbian nature of this form of synaptic plasticity, and it was immediately recognized that the synaptic changes that underpin certain forms of learning and memory may be similar to those upon which expression of LTP relied. The three well-described characteristics of LTP, cooperativity, associativity and input specificity (see Bliss and Collingridge, 1993), and the durability of LTP (Abraham et al., 1995), have been identified as solid arguments that support the hypothesis that LTP may be a biological substrate for at least some forms of memory. Several other pieces of evidence have consolidated this view.

1) LTP is most easily demonstrable in the hippocampus, an area of the brain known to be fundamentally important in memory acquisition.
2) Rhythmic bursts of activity that induce LTP mimic naturally occurring theta rhythm recorded in the hippocampus during exploratory behaviour (Larson et al., 1986; Rose and Dunwiddie, 1986; Greenstein et al., 1988; Diamond et al., 1988).
3) Inhibitors of hippocampal LTP also block hippocampal learning and retention of tasks (Morris et al., 1986).
4) Several biochemical changes that occur after induction of LTP also occur during memory acquisition (see below).
However, a definitive demonstration indicating that memory consolidation requires induction of changes that resemble those necessary for induction of LTP remains elusive. Similarly, it remains to be clearly shown that induction of LTP will result in some form of memory consolidation. At least two components of memory can be discerned: short-term memory, which endures for a few hours, and long-term memory, which persists for several days and often much longer. At the cellular level, the storage of long-term memory is associated with gene expression, de novo protein synthesis, and formation of new synaptic connections. Consistently, protein synthesis inhibitors can block persistent memory but leave short term memory unaffected, suggesting that stable, long lasting memories rely on gene activation that is triggered at, or close to, the time of the experience. Here, there is an interesting parallel between memory and LTP, since it has been revealed that LTP consists of distinct phases involving different molecular mechanisms. The early phase (ELTP), which lasts 2–3 hours, is independent of protein synthesis, while more persistent long-lasting LTP (L-LTP), which lasts several hours in vitro and weeks in vivo, requires synthesis of new proteins. The best known example of Hebb's canonical basis for learning and memory (Hebb, 1949) is long term potentiation of excitatory synapses in the hippocampus (Bliss and Lomo, 1973). The link between LTP and spine structure was suggested by the finding that the size of the post-synaptic density (PSD) is related to the size of the spine head (Harris et al., 1992) and the number of AMPA-type glutamate receptors within it (Nusser et al., 1998; Takumi et al., 1999; Kharazia and Weinberg, 1999). Moreover it has been shown that spine enlargement is associated with LTP in single identified spines (Matsuzaki et al., 2004), indicating that Hebb’s learning rule applies even at the level of a single synapse. Many studies have confirmed that the induction of LTP or long term depression (LTD), another form of activity-dependent plasticity, induces structural plasticity of spines in stimulated dendritic branches (for review see Segal, 2005). It is important to note that spine structural dynamics include broader phenomena than LTP/LTD. Namely, they include the generation and elimination of spines (Grutzendler et al., 2002; Trachtenberg et al., 2002; Engert and Bonhoeffer, 1999; Lendvai et al., 2000; Harvey et al., 2008) and long-term, activity-independent fluctuations (Yasumatsu et al., 2008). In addition, spines become larger in response to the force of actin polymerization (Honkura et al., 2008), which occurs within seconds (Matsuzaki et al., 2004) of LTP induction (Gustafsson and Wigström, 1990).

Activity-dependent Plasticity and Receptor Trafficking.

Functional plasticity of the synapses involved either structural dynamics or receptor trafficking at post-synaptic compartment. As spine enlargement occurs within a minute (Matsuzaki et al., 2004) it well matches the rapid induction of LTP. On the other hand, enlarged spines also explain the long-term maintenance of LTP, given that the number of functional AMPA-type receptors closely correlates with spine volume (Matsuzaki et al., 2001; Smith et al., 2003; Zito et al., 2009). Furthermore, spine enlargement (Tanaka et al., 2008; Yang et al., 2008), like the late phase of LTP and long-term memory itself (Kelleher et al., 2004), depends on protein synthesis, and spine structures are stable for days in cultured hippocampal slices (De Roo et al., 2008b; Yasumatsu et al., 2008) and for years in the cortex in vivo (Grutzendler et al., 2002; Trachtenberg et al., n.d.; Zuo et al., 2005). These data support the idea that
structural plasticity is the central cellular mechanism that underlies memory formation. Glutamate receptor trafficking also can affect functional plasticity by facilitating the readout of dendritic spine structure. During LTP induction, AMPA-type receptors in vesicles are inserted into the plasma membrane (Kessels and Malinow, 2009; Newpher and Ehlers, 2009) and diffuse laterally to supply new receptors to the PSD (Heine et al., 2008; Makino and Malinow, 2009). These receptors are anchored to PSD proteins in an activity-dependent manner (Lisman and Zhabotinsky, 2001; Nakagawa et al., 2004; Kato et al., 2008). LTD protocols also affect the endocytosis of AMPA receptors (Kessels and Malinow, 2009). Hence, numerous experiments support the link between LTP/LTD induction, receptor trafficking and the acquisition of long-term memory in behaving animals (Kessels and Malinow, 2009). It is important to note, however, that the rapid exchange of AMPA receptors between synaptic and extra-synaptic regions (Heine et al., 2008) means that trafficking mechanisms by themselves cannot account for long-term changes in synaptic function. Recently, some authors speculated that receptor trafficking can not support the long maintenance of plasticity in the absence of structural modifications (Kasai et al., 2010) because of the turnover rates of individual molecules compared to dendritic lifetime. The latter, which can be more than a year in vivo (Grutzendler et al., 2002; Yang et al., 2009), is more than a thousand times greater than that of its molecular constituents. Even PSD95, among the most stable molecules in the spine, redistributes within 3 hours in both dissociated cultures and the intact brain in vivo (Okabe et al., 2001; Gray et al., 2006). Thus, maintenance of the molecular status of the spine can require some association with its structure.

Long-Term Intrinsic Fluctuations and Maintenance of Spines.

Neuronal networks reflect the properties of spine populations, rather than those of a single spine, because the generation of action potentials in a neuron requires the activation of many synapses. Recently, a key phenomenon has been identified that affects the long-term behaviours of spine populations. Through the observation and systematic quantification of spine dynamics over periods of days, it become evident that spine volumes grow and shrink spontaneously (Yasumatsu et al., 2008). These volumetric changes occur even when NMDA receptors and Na⁺ channels are completely blocked to prevent activity-dependent plasticity. Such changes, termed ‘intrinsic fluctuations,’ encompass all phenomena (other than activity-dependent plasticity) that contribute to the structural dynamics of spines (Yasumatsu et al., 2008), and represent at least a part of fluctuations of spine volumes reported in vivo (Zuo et al., 2005b; Holtmaat et al., 2006; Xu et al., 2009). These fluctuations reflect an inevitable lack of structural stability in spines whose molecular components turn over with a time constant of less than 3 hours (Okabe et al., 2001; Gray et al., 2006). Despite this constant change, the average daily volume change is close to zero for all spine sizes. Thus, spine populations at various sizes are in equilibrium, and individual spines can act as analogue memory elements despite the stochastic changes in spine volume (up to 20% per day in young hippocampus). Spines volume distribution shows high proportion of small spines (Benavides-Piccione et al., 2002) which can be explained by a positive correlation between the size of the spine and the amplitude of its intrinsic fluctuations. Small spines accumulate because of their smaller fluctuations, while larger spines become
less common because of their greater fluctuation amplitude (Yasumatsu et al., 2008; Kasai et al., 2010). Similar spine volume distribution reflects the distribution found in various conditions in vivo and in vitro. In contrast to the magnitude of intrinsic fluctuations, the frequency of activity-dependent structural modifications is greater in smaller spines, which are thought to correspond approximately to the notion of “silent synapses” (Malenka and Nicoll, 1999), synapses that express NMDA but not AMPA receptors (Matsuzaki et al., 2001; Zito et al., 2009; Noguchi et al., 2005). Small spines show greater increases in cytosolic Ca\(^{2+}\) induced by NMDA receptor stimulation (Noguchi et al., 2005; Sobczyk et al., 2005), and are preferential sites for LTP induction (Malenka and Nicoll, 1999; Matsuzaki et al., 2004). The structural modifications accompanying LTP include the enlargement of smaller spines and a consequent increased number of medium-sized spines. Such effects on the volume distribution, however, is relatively weak because intrinsic fluctuations occur constantly and appear to be the dominant mechanism in determining spine-volume distribution. On the other hand, large spines form only gradually, because activity-dependent enlargement acts preferentially on small, rather than medium, spines. For this reason, older spines will tend to be larger and have longer life expectancies (Yasumatsu et al., 2008) Thus, the history of a dendritic spine is reflected in its volume, and this history effect can explain a feature of memory, that older memories are more persistent. In 1885, Ebbinghaus estimated the decay of memories by memorising a list of nonsense syllables. After a variable period, he memorised the list again and quantified memory as the decrease in memorisation time. He found a rapid loss of memory in the first day, followed by a slower decline over the next 31 days. This non-exponential pattern of memory decay suggested that longer-lasting memories were more persistent. A graph of the time savings was fitted by a logarithmic curve that was later called the “savings function” or “forgetting curve” (Wixted and Ebbing, 1997). Thus, the nature of memory depends on its history, which can be explained in turn by the history effect of the dendritic spine. Intrinsic fluctuations are random forces that physiologically strengthen or destroy the smallest spines. The stochastic elimination of existing dendritic spines, by clearing space and recycling molecular resources, can enable the spontaneous generation of new spines also in adulthood (Yasumatsu et al., 2008). The creation of new spines accelerates 20–60 minutes after neuronal activity (Maletic-Savage et al., 1999; Engert and Bonhoeffer, 1999), but the sprouting cannot be synapse-specific as there is no synapse before its generation, in principle. Thus, activity-dependent synapse formation seems to reflect considerable chance (De Roo et al., 2008b; Yasumatsu et al., 2008). Perhaps the random nature of this make-and-test process enables animals to adapt to an unexpected environment. These new spines could be seeds of new memory (Holtmaat and Svoboda, 2009; Yang et al., 2009). All the above studies are consistent with a model in which activity-dependent spine volume changes regulate new memory acquisition (by enlarging/stabilising or eliminating the smallest spines) and existing-memory persistence (by changing volumes of spines) (Kasai et al., 2010). Thus, activity-dependent plasticity selects memory content and modifies memory strength, supporting the random-generation-and-test model of new-memory acquisition. In contrast, intrinsic fluctuations in spine volume may change the strength of a memory but seldom affects its content.
N-Cadherin: Structure.

Cadherins are Ca\(^{++}\) -dependent adhesion molecules expressed virtually by all cells that form solid tissue, during development as well as in adult life. They are type I transmembrane proteins, composed of an extracellular region, or ectodomain, a single-pass transmembrane anchor and a C-terminal intracellular domain (Fig.6a; Pokutta and Weis, 2007). The Cadherin superfamily is defined by the presence of characteristic 110 amino-acid domains in the ectodomain and the sensitivity of their interactions to Ca\(^{++}\). The homology and number of these domains, designated extracellular Cadherin (EC) domains, determine the division of the Cadherin superfamily into subfamilies. One of the most important and well studied subfamilies is family of classical Cadherins, which is in turn divided into type I and type II on the basis of sequence homologies. N-Cadherin, the most abundant Cadherin in the central nervous system belongs to type I classical Cadherins.

The ‘classical’ type I Cadherins have been most extensively studied and their binding specificities have been shown to be primarily homophilic (Nose et al., 1990; Gumbiner, 1996; Tamura et al., 1998). It means that, cells expressing a particular Cadherin (N-Cadherin, for example) will preferentially adhere to other cells expressing the same Cadherin, rather than to cells expressing a different Cadherin (E-Cadherin, for example). Homophilic adhesion provides a simple mechanism by which genetically identical cells form cell layers and homogeneous tissues. Recent studies, however, indicate that interaction between Cadherins can be more promiscuous (Shimoyama et al., 1999; Shan et al., 2000; Niessen and Gumbiner, 2002; Duguay et al., 2003). Although homophilic binding still appears to be preferred, many type I Cadherins can form productive cell adhesive interactions with other type I Cadherins.

Some type I Cadherins (such as N-Cadherin and E-Cadherin) induce the formation of separate cell aggregates when transfectants are mixed, whereas others (such as N-Cadherin and R-Cadherin) will co-aggregate. The single in-depth study on the adhesive specificity of type II Cadherins yields similar results for this subfamily (Shimoyama et al., 1999). Despite the cross-reactivity of Cadherins within the same subfamily, it appears that the binding specificities of type I and type II Cadherins are orthogonal. That is to say, type I Cadherins may have graded affinities for other type I Cadherins and type II Cadherins have graded affinities for others of their class (Niessen and Gumbiner, 2002). However, current data suggest that type I and type II Cadherins do not interact. The ‘orthogonality’ of the type I and type II Cadherins adhesion systems may have important consequences for how these molecules function in tissues where many different Cadherins may be expressed (Price et al., 2002). Type I Cadherins, including N-Cadherin, contain five tandem repeated domains (EC1-EC5) in the extracellular region. The N-terminal, membrane-distal EC1 domain has been demonstrated to be responsible for the homophilic binding and for determining the specificity of cell aggregation (Nose et al., 1990; Shan et al., 2000). Additionally, Cadherins are synthesized as pro-proteins with a pre-domain at the N-terminus of EC1; this must be removed to activate adhesive function (Ozawa and Kemler, 1990). The proximity of this small pre-domain to EC1 provides additional support for the notion that binding and specificity are dominated by interactions involving the EC1 domain and/or regions close to it. Recently, the three-dimensional structure of several EC domains has been determined by crystallography, including the N-Cadherin EC1 (Shapiro et al., 1995) and EC2 (Tamura et al., 1998) and the complete C-Cadherin...
ectodomain EC1-5 (Boggon et al., 2002). The structure reveals a pair of molecules in a symmetric dimer formed through the interaction of the partner EC1 domains (Fig. 6b). The interface is mediated by the exchange of the N-terminal β-strands between the partner EC1 domains. A central feature is the insertion of the conserved Trp2 (W2) side chain from one molecule into the hydrophobic core of the other (Fig. 6b). This interface, called Cadherin “strand dimer”, plays a crucial role in Cadherin-mediated cell adhesion. W2, and the residues lining its receptor pocket, are highly conserved among Cadherins. Moreover, Cadherin-mediated aggregation is abolished by mutation of this tryptophan to alanine (W2A) or by mutation of one of the alanine residues of the acceptor pocket to encode a larger residue, methionine (A80M). Such a residue would be expected to block tryptophan insertion (Tamura et al., 1998). Furthermore, a zebrafish lethal developmental mutation, glass onion, has been shown to encode a W2G mutant of N-Cadherin (Malicki et al., 2003). Cadherins are able to dimerise in cis orientation (that means between molecules presented on the same cell) as well as trans (between molecules on opposite cells), an ability which has an important role in the biology of Cadherins (Takeda et al., 1999; Klingelhöfer et al., 2002). Specifically, it is possible that Cadherins may exist as cis-strand dimers on a single cell surface, and that these cis-dimers may be replaced by or exist in equilibrium with trans-strand dimers in the presence of a juxtaposed Cadherin-presenting cell. In order to mediate cell adhesion, Cadherins must bind together from two opposing cells, and the adhesive bond must form at the cell surface between Cadherins that have been synthesized and processed in different cells. It is not then

Figure 6. a) Schematic representation of N-Cadherin structure. b) Structure of the hydrophobic core of EC1 domain.
surprising that monomeric forms of Cadherins should exist and indeed have been observed in high-resolution structural studies (Tamura et al., 1998; Pertz et al., 1999). Although EC1 is the primary site of adhesion binding in Cadherins, the involvement of other domains is required, in fact several cell aggregation assays show that EC1-EC3 domains per se mediate strong adhesion, but EC1–EC2 is only weakly adhesive and EC1 alone not at all (Chappuis-Flament et al., 2001). The molecular basis of the requirement for at least two domains in cell aggregation assays is not evident from the crystallographic studies. One possibility is that additional domains are required for a minimum clearance from the cell surface, which of course contains myriad other proteins. Another possibility involves a potential active role for Ca$$^{2+}$$ ligation (which requires more than the EC1 domain) in inducing a conformational change to an adhesion-competent state.

The intracellular C-terminal region of Cadherins binds several proteins that functionally link Cadherins to the underlying cytoskeleton. They include p120, plakoglobin (also known as γ-catenin) and β-catenin which, in turn, binds α-catenin (Ozawa et al., 1989). α-catenin has a number of binding partners, including actin; therefore α-catenin mediates a structural and functional link between he Cadherin–β-catenin complex and actin cytoskeleton. The cytoplasmic region of classical Cadherins, roughly 150 amino acids long, is the most highly conserved portion of these proteins (Nollet et al., 2000). The juxtamembrane region binds to p120, and the C-terminal, around 100 amino acids, binds to β-catenin and to plakoglobin. The Cadherin cytoplasmic domain, which is unstructured in absence of a binding partner, becomes ordered when complexed with β-catenin (Huber et al., 2001; Huber and Weis, 2001). A portion of the juxtamembrane region presumably becomes ordered on forming a stable complex with p120, but this has yet to be verified experimentally. Newly synthesised Cadherin binds β-catenin in the endoplasmic reticulum and they move together to the cell surface. The catenin-binding domain of Cadherins contains a sequence motif that is recognised by ubiquitin ligases but would be inaccessible in the complex with β-catenin (Huber et al., 2001; Huber and Weis, 2001). Thus, it appears that β-catenin prevents proteosomal destruction of Cadherin, insuring delivery of Cadherin-β-catenin complexes to the cell surface (Hinck et al., 1994). Moreover, the Ser/Thr kinase CamKII can positively modulate the interaction between Cadherins and β-catenin by phosphorylating the binding site on Cadherin (Lickert et al., 2000; Choi et al., 2006).

**N-Cadherin: Cytoplasmic Binding Partners.**

β-catenin. At the cytoplasmic region, Cadherins bind to β-catenin which in turn binds to α-catenin. The primary structure of the 781 amino acid β-catenin consists of an amino-terminal region of about 150 amino acids, a central 520 residue domain composed of 12 armadillo (arm) repeats, and a carboxy-terminal 100 residue region. E-Cadherin binds to the arm domain, whereas α-catenin binds to residues 118-149, just before the start of the arm domain (Aberle et al., 1996). The arm domain is an elongated superhelical structure formed by the successive packing of helical arm repeats (Huber et al., 1997). The superhelix features a groove that forms part of the binding site for β-catenin ligands (Choi et al., 2006). In contrast to the effect of Ser/Thr phosphorylation of Cadherin, phosphorylation of β-catenin Tyr654 (repeat 12) by Src kinase produces a sixfold reduction in affinity (Roura et al., 1999) with Cadherin C-terminal region. The car-
boxy-terminal tail is highly acidic and functions as a transcriptional activator in Wnt signaling. Analysis of the Drosophila protein indicates that this region is essential for adhesion (Cox et al., 1999).

p120. P120 catenin is a member of the armadillo-family whose arm repeat domain binds to the juxtamembrane region of the Cadherin cytoplasmic tail (Ohkubo and Ozawa, 1999; Thoreson et al., 2000); this arm domain is preceded by an amino-terminal sequence. The protein has four different splicing variants characterised by different length of the amino-terminal region (Anastasiadis and Reynolds, 2000). p120 regulates Cadherin levels by controlling the rate of Cadherin turn over (Ohkubo and Ozawa, 1999; Thoreson et al., 2000); furthermore it controls small GTPases and their effectors that regulate the actin cytoskeleton (Anastasiadis, 2007). Thus, the interaction with Cadherin is thought to localize the small GTPase regulatory activity of p120 at adherents junctions to affect actin organization.

α-catenin. α-catenin is a 906-aminoacids protein present in three different isoforms: E(epithelial), N(neuronal), and T (found in heart and testis). Both α-E- and α-N-catenin are coexpressed in a number of tissues, often at different levels and at different developmental stages. Alternative splicing generates two variants of α-N-catenin, the significance of which is unknown. α-catenin presents three major regions responsible for protein-protein interaction; they are the dimerisation/β-catenin-binding domain, the M-domain and the actin-binding domain. The first domain lies near the N-terminus of the protein, in residues 57–264 (Pokutta and Weis, 2000). This domain is responsible for the formation of a homodimer, that, upon binding to β-catenin, dissociates to form a 1:1 heterodimer (Koslov et al., 1997; Pokutta and Weis, 2000). The M-domain spans residues 377–633 in the middle of the α-catenin sequence (Pokutta et al. 2002, Yang et al. 2001) and is composed of two tandem four-helix bundles connected by a short linker. It is the binding site for the protein afadin (Pokutta et al. 2002), an actin-binding protein associated with the Ig-superfamily adhesion molecule nectin. The last domain is responsible for the binding to filamentous (F-) actin, providing a stable mechanical linkage between Cadherin-β-catenin complex to the actin cytoskeleton.

The Cadherin-Catenin Complex.

Until recently, the Cadherin-catenin complex was believed to link Cadherins to the actin cytoskeleton via binding of β-catenin to α-catenin, which in turn links to the actin cytoskeleton. By contrast, recent studies have demonstrated that α-catenin binds to β-catenin or F-actin in a mutually exclusive manner (Drees et al., 2005; Yamada et al., 2005), with the monomeric form preferentially binding β-catenin and the homodimeric form preferentially binding F-actin. As α-actin binds numerous actin-binding proteins, these observations do not challenge the function of α-catenin as a link between Cadherins and the actin cytoskeleton. The ability of Cadherins to bind to catenins serves to link the cell-adhesion complex to a multitude of intracellular signalling pathways, affecting the cytoskeleton, cytoplasmic signalling pathways and gene expression.

Distribution of Cadherin-Catenin Complex.

The N-Cadherin-catenin complex distribution depends on the brain area and the developmental stage. At young hippocampal synapses N-Cadherin is evenly distributed along the nascent synapse, but it clusters within the pre-synaptic active zone.
during maturation. This distribution is seen either in young hippocampal neurons in culture (DIV 5-6) (Elste and Benson, 2006) or in developing hippocampal slices in vivo (Rubio et al., 2005). The distribution of αN-catenin and β-catenin at synapses at different developmental stages mirrors that of N-Cadherin (Uchida et al., 1996). p120 is localised to both membrane and cytosolic fractions associated with N-Cadherin clusters; this distribution is maintained in older neurons (Chauvet et al., 2003). During development (DIV 7-14), the proportion of synapses that contain p120 decreases slightly (Rubio et al., 2005). It is not clear how this alteration in distribution with synaptic maturity controls the Cadherin complex at the synapse.

The Cadherin-catenin complex regulates multiple aspects of synaptogenesis and synaptic plasticity. Cadherins are well suited to promote adhesive functions and, by virtue of association with catenins, link cell adhesive interactions to a variety of intracellular pathways. Cadherins and catenins are localised on both sides of the synapse and bind a wide range of partners in each compartment to accomplish their compartment-specific roles (Fig. 7). These binding partners include PDZ-domain-containing proteins, protein phosphatases and kinases, and F-actin and microtubule-

![Figure 7. Scheme of N-Cadherin binding partners.](image-url)
associated proteins (for review see Arikkath and Reichardt, 2008). Thus, N-Cadherin accomplishes its functional role by adhesion-dependent as well as -independent mechanisms.

As a cell-adhesion molecule, N-Cadherin is well suited to provide a link between structural changes and synaptic plasticity. Consistently, synaptic activity promotes dimerisation of N-Cadherin, acquisition of protease resistance and strong adhesion across the synapse (Tanaka et al., 2000). These responses can be differentially modulated by activity, because KCl depolarisation, but not NMDA stimulation, leads to dispersion of Cadherins, but in both cases Cadherin dimerisation is observed. Hence, N-Cadherin is dynamic and cadherin-mediated adhesion can respond to local changes in synaptic activity. N-Cadherin is also required for long-term plasticity since using blocking antibodies prevents the induction of late-phase LTP (Bozdagi et al., 2000). Morphological changes in spines have been linked to synaptic plasticity. The activation of AMPA receptors causes a lateral expansion of the spine head and redistribution of Cadherins along the expanding spine head. Cadherin adhesion and actin polymerization are essential for this process, because over-expression of a Cadherin mutant that lacks the adhesive activity prevents spine head expansion (Okamura et al., 2004). Interestingly, N-Cadherin is directly involved in AMPAR subunits recruitment and stabilisation. Recent studies have reported interactions between AMPARs (in particular, the GluR21 and GluR2 subunits) and N-Cadherin, suggesting that the accumulation of either N-Cadherin or AMPARs at the synapse may promote the reciprocal recruitment of the other (Nuriya and Huganir, 2006; Saglietti et al., 2007). Both direct and indirect interaction between N-Cadherin and AMPAR subunits have been demonstrated. Direct extracellular interaction between N-Cadherin and the GluR2 subunit of AMPAR has been demonstrated by Saglietti and colleagues. However, co-overexpression and co-immunoprecipitation experiments in heterologous cells from Nurya and colleagues suggest the possibility of multiple interactions among several subunits of AMPAR with N-Cadherin. Alternatively, Cadherin-β-catenin and AMPARs may be preassembled by a GRIP-liprin-α scaffold and targeted to the post-synaptic membrane (Dunah et al., 2005). Once at the post-synaptic site, the association of these molecules may serve to stabilize AMPARs at the synapse as immobilized N-Cadherin molecules decrease the lateral diffusion of GluR2(Saglietti et al., 2007). Additional Cadherin-GluR2 interactions have been observed via their intracellular binding partners, neural plakophilin-related arm protein (NPRAP/d-catenin) and AMPA receptor-binding protein (ABP)/glutamate receptor-interacting protein (GRIP) of AMPARs (Silverman et al., 2007). NPRAP also binds PSD-95, another major molecule in the post-synaptic compartment, which tethers this complex to the post-synaptic scaffold and ultimately binds NMDA receptors.

N-cadherin has also been shown by proteomic analysis to physically associate with NMDA receptors in large multi-protein complexes (Husi et al., 2000). This type of complex formation offers the possibility for cross-talk between post-synaptic signalling and adhesion/anchoring molecules.

β-catenin is responsible for the recruitment and stabilisation of several intracellular partners, at both pre- and post-synaptic compartments. In the absence of β-catenin, the number of reserve pool vesicles is reduced, with concomitant reduction in exocytosis in response to repetitive stimulation. β-Catenin promotes synaptic vesicle localisation through binding to Cadherin and recruitment of PDZ-containing proteins via its C-terminal PDZ-binding motif (Bamji et al., 2003). Post-synaptically, loss of β-


catenin after synaptogenesis results in profound alteration in spine morphology (Okuda et al., 2007), leading to spines that are thin and elongated with no alterations in spine density. This is accompanied by reduction in amplitude, but not frequency, of mEPSCs. The ability of β-catenin to modulate synapse structure and function requires both its ability to bind Cadherin and PDZ proteins. In addition, loss of β-catenin inhibits chronic activity blockade-regulated quantum scaling. Murase and colleagues (Murase et al., 2002) showed that β-catenin localisation and function are dynamically regulated by synaptic activity. Depolarisation regulates the redistribution of β-catenin and increases the association of N-Cadherin with β-catenin, suggesting that the translocation of β-catenin to spines may increase cadherin-mediated adhesion. The overexpression of β-catenin phosphorylation mutants, showing higher Cadherin-binding affinity, in post-synaptic neurons is sufficient to trigger coordinated pre- and post-synaptic modifications. Even though he mutant β-catenin is only expressed in the post-synaptic compartment, both PSD-95 and Synapsin clusters (post- and pre-synaptic marker, respectively) increase. Functionally, the frequency of spontaneous excitatory synaptic transmission also significantly increases, reflecting an enhanced release probability. This work shows trans-synaptic activity-induced changes in the structure and function of synapses mediated by β-catenin and N-Cadherin, suggesting a role of these binding interactions in synaptic plasticity.

Also αN-catenin is crucial for spine stability; in fact, in the absence of αN-catenin spines are more motile, differently its over-expression promotes spine stability and increases dendritic spines density (Abe et al., 2004). The ability of αN-catenin to bind both β-catenin and cytoskeletal proteins is necessary for αN-catenin to regulate spine stability. Moreover, overexpression of αN-catenin also prevents conversion of spines to filopodia when neuronal activity is inhibited, suggesting that αN-catenin can function to coordinate activity to structural alterations in spine structure.

N-Cadherin surface expression and turnover are modulated by synaptic activity. NMDA receptor activation reduces the endocytosis of N-Cadherin, resulting in retention of N-Cadherin at the cell surface (Tai et al., 2007). This is mediated by a decrease in phosphorylation of tyrosine 654 of β-catenin, which then enhances association of N-Cadherin with β-catenin, stabilising surface N-Cadherin. Stabilisation of N-Cadherin-β-catenin association through mutation of tyrosine 654 to phenylalanine prevents NMDAR-dependent LTD in hippocampal neuron cultures, indicating that N-Cadherin endocytosis controls synaptic efficacy (Tai et al., 2007). Arcadlin, a protocadherin induced by activity (Yamagata et al., 1999), binds to and promotes endocytosis of N-Cadherin. Loss of arcadlin leads to increase in spine density that can be rescued by concomitant knockdown of N-Cadherin (Yasuda et al., 2007). Thus, activity-promoted regulation of arcadlin expression provides a mechanism through which synaptic density and stability can be regulated through control of N-Cadherin surface stability. Although these two results appear contradictory, with activity either promoting or inhibiting endocytosis, they describe effects of two different phases of synaptic stimulation. The surface stabilization of N-Cadherin is a more rapid (100 minutes) and protein-synthesis-independent response to activity, whereas activity-induced Cadherin internalization is initiated 4 hours after stimulation and requires protein synthesis. Members of the p120 family of proteins have also been shown to stabilize the surface levels of Cadherins through inhibition of endocytosis (Davis et al., 2003). Consistent with this, the expression of N-Cadherin is reduced in the hippocampi of mice.
lacking p120 (Elia et al., 2006), and the loss of p120 in neurons results in a reduction of spine head width that can be partially overcome by over-expression of N-Cadherin. Thus, regulation of p120 family functions provides another pathway for control of N-Cadherin levels at synapses, which in turn might regulate synaptic structure and function.

The Cadherin-Catenin Complex in Spine Morphogenesis.

N-Cadherin acts with β-catenin at both side of the synapse to regulate spine morphogenesis and synapse assembly. N-cadherin and β-catenin are diffusely distributed along the length of free dendritic filopodia that are not in contact with axons, but rapidly accumulate at filopodia-axon contact sites following target recognition (Figure 8; Jontes et al., 2004; Togashi et al., 2002). The localization of these molecules at filopodia-axon interfaces suggests that they may be involved in mediating contact stabilization, which may trigger spine maturation and synaptic differentiation. To study the role of the N-Cadherin-mediated adhesion in spine morphogenesis, a dominant negative form of N-Cadherin, carrying a deletion of the extracellular region, has been created. In hippocampal neurons, the expression of the dominant negative affects spine morphology without affecting

Figure 8. Model for the Development of Excitatory CNS Synapses. From the top: a) Prior to cell-cell contact, cadherin-β-catenin complexes are expressed in a diffuse pattern along the length of filopodia, and in small clusters along the length of axons. Preassembled packets of synaptic vesicle proteins and Piccolo-Bassoon transport vesicles (PTVs) containing N-cadherin and cytoskeletal proteins of the active zone, are translocated along the axon on microtubule mo- tors. b) Following cell-cell contact, cadherins cluster in cis, and cadherin-catenin-actin complexes are formed. At early stages of synapse formation, cadherins and catenins are expressed through- out the active zone. c) As synapses mature, β-catenin clusters act as scaffolds to recruit presynaptic PDZ domain-containing proteins to sites of cell-cell contact, thereby mediating the localization of synaptic vesicles to presynaptic compartments. In maturing glutamatergic synapses, cadherins and catenins are excluded from the active zone, and dendritic protrusions mature into short-necked spines. Postsynaptic differentiation is effected by the sequential recruitment of postsynaptic scaffold proteins and receptors to sites of cell-cell contact, and may involve cadherins and associated proteins.
the outgrowth of axon or dendrites (Togash
shi et al., 2002). Instead of the normal ma-
ture mushroom-shaped spines, dominant nega-
tive N-Cadherin expressing neurons exhibit ei
ther spine with filopodia-like mor-
phology or smaller elongated spine heads.
Although these neurons still appear to make
contact with axons, β-catenin is no longer con
centrated at spine–axon contacts or synapses
and PSD-95 puncta are reduced in number and
more diffuse. Corresponding-
ly, the pre-synaptic marker synapsin I has a
more diffuse distribution and there are fewer synap
tic puncta compared to control neurons.
Consistent with these find-
ings, the number of vesicle recycling
sites labelled by FM4-64, the membrane
selective dye used as a marker of endocy-
tosis, is significantly decreased. Therefore,
dominant negative N-Cadherin expression
disrupts N-Cadherin complex within the
dendritic spine resulting in the partial dis
assembly of the synapse. Dominant nega-
tive N-cadherin could act directly on synap
tic assembly or sequester the intracellular
binding partner β-catenin. Consistent with
the view that dominant negative N
-Cadherin expression might act directly on
Cadherin complex within the spine, loss of
function of αN-catenin also results in the in
creased presence of long and thin elon
gated spines (Togashi et al., 2002), al
though spine density is not affected (Abe
et al., 2004). The morphology of spines is
reminiscent of that produced by the depoly
merisation of actin in neurons (Fukazawa
et al., 2003). It was thus postulated that the
link of the Cadherin complex to the actin
cytoskeleton through αN-catenin is crucial for
the normal formation of dendritic spines.
In a recent study, the effects of the
overexpression of the wild type and domi
nant negative forms of N-Cadherin have
been compared (Mendez et al., 2010). The
overexpression of Wild type N-Cadherin in
duces an enlargement of dendritic spine
accompanied by an increase of post-
synaptic density and PSD95 puncta. N-
Cadherin also promotes the stability of
both pre-existing and newly formed den
dritic spines. Contrarily, the dominant
negative form of N-Cadherin reduces den
dritic spines size, PSD dimension and PSD95
puncta. It also increases the turn over and
decreases the stability of both pre-existing
and newly formed dendritic spines (Mendez
et al., 2010). Interestingly, N-Cadherin is
more expressed in larger spines and acti
vated spines. Thus, N-Cadherin represents
a key molecule regulating long-term syn
apse stability and its enhanced expression
during synaptic plasticity is required for the
structural remodelling of the synapse and
its persistence (Mendez et al., 2010).

The assembly of the pre-synaptic sites
was largely unperturbed; however, as as
essed by Synapsin I localisation, loss of
function of αN-catenin results in a decrease
in the number and size of synapsin I puncta
at synaptic sites (Togashi et al., 2002; Abe
et al., 2004). Interestingly, the number of
vesicle recycling sites and post-synaptic
sites labelled with PSD95 does not change.
Thus, loss of function of αN-catenin mimics
the effects of expression of dominant nega
tive N-Cadherin on spine morphology but,
in contrast to that found with dominant
negative N-cadherin expression, pre- and
post-synaptic markers distribution is largely
unaffected (Togashi et al., 2002). However,
αN-catenin deficient neurons seem to form
less stable synaptic contacts, possibly be
cause of their higher motility and/or an in
crease in spine turnover.

Conversely, overexpression of αN-
catenin stabilises spines and increases the
number of spines per unit length (Abe et
al., 2004). αN-catenin requires β-catenin
for its functions, in fact the expression of a
mutant form of αN-catenin carrying a dele
tion of the β-catenin-interacting domain
blocks the increase in spine density (Abe et
al., 2004). In summary, these studies dem
onstrate that N-Cadherin and αN-catenin
play a crucial role in the formation of dendritic spines, and that perturbations in spine morphology affect the assembly of the pre- and post-synaptic junctions. Cadherins do not play merely a structural role in the correct formation of the pre- and post-synaptic terminals, but they are also active participants in signalling across the synapse. The Cadherin–catenin system signals to the Rho family of GTPases by way of their interaction with p120 catenin (Anastasiadis and Reynolds, 2001; Fukata and Kaibuchi, 2001; Magie et al., 2002; Goodwin et al., 2003); this interaction is believed to increase the lifetime of the N-Cadherin complex at the membrane (Ireton et al., 2002). The regulation of Rho GTPases is crucial for spine morphogenesis and for the assembly of the pre- and post-synaptic terminals (Bonhoeffer and Yuste, 2002).

β-catenin acts also recruiting a protein complex that is important for pre- and post-synaptic assembly and for synaptic transmission. Selective gene ablation of β-catenin in the hippocampus results in a significant disruption in the localisation of a reserved pool of synaptic vesicles normally located in proximity of the active zone (Bamji et al., 2003). β-catenin could function directly through Cadherin-mediated adhesion or alternatively through the WNT signalling pathway, which also is involved in pre-synaptic differentiation (Hall et al., 2000). However, this function of β-catenin is mediated through its PDZ-binding domain rather than through the armadillo repeat that is required for Cadherin interaction, suggesting that WNT β-catenin-mediated signalling is not involved.

In summary, Cadherins and catenins are required on both sides of the synaptic junction for synaptic assembly. On the post-synaptic neuron, these molecules are essential for the formation, shape and dynamics of spines, which results in the recruitment of post-synaptic molecules. On the pre-synaptic neuron, the Cadherin–catenin complex regulates the assembly of the pre-synaptic machinery required for neurotransmitter release. These findings raise the question of the role of Cadherin–catenin in synchronised assembly of the synaptic machinery on both sides of the synapse.

The Cadherin-Catenin in Synaptic Activity.

As previously described (see chapter 2.3) synaptic activity plays a crucial role in the formation and function of synapses; since N-Cadherin is required for synaptogenesis, the role of synaptic activity in Cadherin function has been examined. Depolarisation of neurons or NMDA receptors activation induces both the dimerisation of N-Cadherin and the formation of a protease resistant complex of N-Cadherin, a hallmark of enhanced cell adhesion (Tanaka et al., 2000). Also Long-Term Potentiation increases the number of N-Cadherin puncta at synaptic sites in hippocampal slices (Bozdagi et al., 2000). Neuronal activity also regulates the distribution of αN-catenin. The neural activity blocker Tetrodotoxin (TTX) decreases the levels of αN-catenin at synaptic sites. By contrast, blockade of GABA receptors increases the level of αN-catenin at synaptic sites, although the total levels of αN-catenin within the cell remain unchanged (Abe et al., 2004). Thus, electrical activity regulates the distribution of N-Cadherin and αN-catenin at synapses. Neuronal activity also regulates the distribution of β-catenin. In fact, potassium chloride-mediated depolarisation induces the accumulation of endogenous β-catenin which is mobilised from the dendritic shaft to the spines. Depolarisation also induces the association of β-catenin with N-Cadherin, suggesting that the mobilisation of β-catenin to spines increases Cadherin-mediated adhesion (Murase et al., 2002).
Moreover, mobilisation of β-catenin to spines regulates the size of the pre-synaptic and post-synaptic terminals, as determined by the size and intensity of puncta for synapsin and PSD95, respectively. Importantly, decreased tyrosine phosphorylation of β-catenin, which drives the latter to spines, increases the frequency of miniature excitatory post-synaptic potentials, suggesting that pre-synaptic activity is increased (Murase et al., 2002). These results are consistent with studies in which the loss of β-catenin function results in deficits in the localisation of synaptic vesicles to the synaptic terminals (Bamji et al., 2003). In summary, electrical activity that regulates synaptic function induces the mobilisation of Cadherin and catenin to synaptic sites, which in turn regulate spine morphology and the assembly of the pre- and post-synaptic terminals. Thus, the Cadherin–catenin complex has profound effects on the activity-mediated structural and functional plasticity of neurons. Synaptic activity also regulates the surface expression of Cadherins. N-Cadherin undergoes a unique type of activity-regulated endocytosis, which temporarily retrieves N-Cadherin from the surface, decreasing adhesion and promoting the dynamics of the molecule (Tai et al., 2007; Yasuda et al., 2007). At basal state, 50% of surface N-Cadherin is internalized within 30 minutes, indicating a surprisingly high rate of turnover (Tai et al., 2007). Many key synaptic molecules, such as AMPA receptors (Shepherd and Huganir, 2007), also utilize this strategy of rapid endocytosis to achieve fast turnover during synaptic plasticity (Shepherd and Huganir, 2007). Furthermore, overexpression of a mutant form of β-catenin with enhanced cadherin-binding affinity in cultured neurons prevents the internalization of N-Cadherin and blocked Long-Term Depression (LTD), a NMDA-dependent form of synaptic plasticity (Tai et al., 2007). Another type of regulated N-Cadherin endocytosis is mediated by arcadin, a member of the protocadherin family (Yasuda et al., 2007). Here, arcadin signalling through the MAPK pathway plays little to no role in adhesion but rather promotes N-Cadherin endocytosis four hours after the induction of chemical LTP. Likely, the late-onset of N-Cadherin turnover is part of a homeostatic response. Clearly, there are distinctive molecular mechanisms mediating the endocytosis of N-Cadherin, which illuminate the importance of this type of regulation. The surface expression of N-Cadherin is also regulated by sequential proteolytic cleavages mediated by two different secretases; these events are widely discussed in chapter 3.4.

The Cadherin-Catenin in Long-Term Potentiation.

Cadherins-mediated cell-adhesion has also a role in the regulation of synaptic long-term plasticity; in particular N-Cadherin is required for Long-Term Potentiation induction. Two phases in LTP have been described: an early phase LTP (E-LTP), which is independent of transcription and translation and can last from several minutes to an hour, and a late phase LTP (L-LTP), which is dependent on transcription and translation and can last from several hours to, possibly, several days. Using a peptide containing the three amino-acids of the distal extracellular domain of N-Cadherin (called HAV-peptide), a region involved in homophilic interactions, it is possible to disrupt the N-Cadherin-mediated adhesion (Tang et al., 1998). The HAV-peptide does not affect normal synaptic function, but is able to attenuate Long-Term Potentiation only when applied during the induction or early-phase LTP. The attenuation of Long-Term Potentiation induction is not caused by changes in post-synaptic response or alterations in inhibi-
tory or NMDA receptor-meditated responses (Tang et al., 1998).

The addition of calcium prevented the inhibitory effect of the HAV-peptide on LTP, indicating that the activity of the HAV-peptide is dependent upon the extracellular concentration of calcium. The requirement of calcium is not surprising as Cadherin-mediated adhesion is calcium dependent. These results suggest that new Cadherin interactions are formed during E-LTP. However, another study reported that N-Cadherin plays a crucial role in L-LTP (Bozdagi et al., 2000). The number of the pre-synaptic marker synaptophysin and N-Cadherin puncta increases during LTP with this increase requiring protein kinase A activity and new protein synthesis, thus sharing the requirements for expression of L-LTP (Bozdagi et al., 2000). L-LTP increases both the synthesis and the dimerisation of N-Cadherin, suggesting an increase in the homophilic interaction of N-Cadherins between membranes. Interestingly, addition of anti-N-Cadherin antibodies to brain slices does not block the increase in synaptic puncta induced by L-LTP, suggesting that N-Cadherin blockade does not affect the number of synaptic boutons. However, anti-N-Cadherin antibodies block the induction of L-LTP but do not block E-LTP (Bozdagi et al., 2000). Although different protocols were used to elicit E-LTP in these two studies (Tang et al., 1998; Bozdagi et al., 2000), the reason for this apparent discrepancy remains unclear. Despite these differences, both studies demonstrate that Cadherin-mediated interaction is important for Long-Term Potentiation induction.

N-Cadherin Metabolism.

The release of the extracellular domain, which contains the homophilic binding site, is functionally of major importance for the regulation of cell adhesion, cell migration and neurite outgrowth (Paradies and Grunwald, 1993; Nakagawa and Takeichi, 1998). This ectodomain cleavage of N-Cadherin can be inhibited with matrix metalloproteases (MMP) inhibitors, including the tissue inhibitors of metalloproteases (TIMPs). TIMPs promote fibroblast adhesion through stabilisation of focal adhesion contacts, which is correlated with an increase in N-Cadherin expression at the cell surface (Ho et al., 2001). The extracellular domain released through metalloprotease activity has been shown to retain biological function and to promote neuronal cell adhesion and neurite outgrowth (Bixby and Zhang, 1990; Paradies and Grunwald, 1993; Utton et al., 2001). The proteolytic ectodomain cleavage leads to a membrane-bound carboxy-terminal fragment which is a substrate for regulated intramembrane proteolysis (RIP) (Marambaud et al., 2003). In this process, the carboxy-terminal membrane-bound fragment becomes a substrate for a presenilin/y-secretase mediated intramembrane proteolysis, resulting in the release of the cytoplasmic carboxy-terminal fragment. In case of N-Cadherin, this intracellular fragment is apparently involved in signal transduction, since it promotes, in a not yet clarified way, the degradation of the transcriptional co-activator CREB-binding protein (CBP) (Marambaud et al., 2003). The regulation of RIP (e.g. Notch, APP, CD44) occurs at the level of the first ectodomain-shedding processing step, which has been well characterised (Reiss et al., 2005; Uemura et al., 2006). The metalloprotease ADAM10, a member of the ADAMs family of proteins (see chapter 4), has been demonstrated to be responsible for the initial proteolytic event of N-Cadherin, leading to the release of the extracellular soluble domain and the production of the membrane-bound carboxy-terminal fragment CTF1 (Reiss et al., 2005). ADAM10 is required for physiological as well as activity induced cleavage of N-
Cadherin. ADAM10 mediated N-Cadherin cleavage occurs between the aminoacids R\textsuperscript{714} and I\textsuperscript{715} in the extracellular domain, nine aminoacids from the membrane (Uemura et al., 2006). Two different mutants were created: GD mutant by substituting both R\textsuperscript{714} → G\textsuperscript{714} and I\textsuperscript{715} → D\textsuperscript{715} and IRD mutant by substituting D\textsuperscript{713} → I\textsuperscript{713} and I\textsuperscript{715} → D\textsuperscript{715} (Fig. 9).

Both GD and IRD mutations completely abolished Ncad/CTF1 production after ionomycin treatment, indicating that calcium influx triggers ADAM10-mediated ectodomain shedding of N-Cadherin between R\textsuperscript{714} and I\textsuperscript{715}. The ADAM10-mediated ectodomain shedding is a prerequisite for the subsequent PS1/γ-secretase-mediated cleavage which leads to the formation of soluble carboxy-terminal fragment CTF2 (Reiss et al., 2005). In order to study the PS1/γ-secretase cleavage site, two kinds of N-Cadherin mutants (Fig. 10) were created: GG mutant by substitution of V\textsuperscript{743} → G\textsuperscript{743} and V\textsuperscript{744} → G\textsuperscript{744} (located inside the trans-membrane domain), and ED mutant by substitution of K\textsuperscript{747} → E\textsuperscript{747} and R\textsuperscript{748} → D\textsuperscript{748} (located at the membrane-cytosol interface). In contrast to wt N-Cadherin, Ncad/CTF2 production was compromised in the presence of GG mutation and abolished by ED mutation, indicating that N-cadherin is cleaved by PS1/γ-secretase at the membrane-cytosol interface (Uemura et al., 2006). These results reveal that the ADAM10-mediated ectodomain shedding of N-Cadherin, which would disrupt cell-cell (synaptic) contacts, is a prerequisite for the secondary PS1/γ-secretase cleavage. Further, the ADAM10-mediated shedding is a regulated process in response to calcium influx via NMDA-type glutamate receptor, whereas PS1/γ-secretase cleavage is a constitutive one. This proteolysis influences cell–cell adhesion as well as cell signalling in physiological, but probably also in pathological, conditions. The coordinated interaction of ADAM10 and N-Cadherin may be significant for the coordinated interplay between cell–cell adhesion, cell detachment,
cell proliferation and cell survival during embryogenic development, in wound healing and during tumour invasion.

A Disintegrin And Metalloprotease Family.

Proteins belonging to the ADAM family have metalloprotease, integrin-binding, cell-adhesion and intracellular signalling functions which put them in a position to coordinate various cellular processes that are required for neuronal development, plasticity and repair (Wolfsberg et al., 1995). ADAMs have an important role in cell-adhesion and cell-cell fusion, the latter playing a crucial role in vesicular trafficking and fertilisation (Evans, 2001). Other important roles have been identified in cell signalling (Wolfsberg and White, 1996) and in proteolysis of a wide range of cellular substrates, including: APP, Prp, Notch, insulin-like growth factor-binding proteins (IGFBPs) and N-Cadherin. From a structural point of view (Fig. 11), they are type I transmembrane proteins and have an N-terminal signal peptide, a propetide domain containing a cysteine switch, a cleavage site for the pro-hormone convertases, a metalloprotease domain, a cystein-rich region, a disintegrin domain, an epidermal growth factor (EGF)-like sequence, a transmembrane region and a short cytoplasmic tail. The propeptide domain is removed, in some cases by a furin-like endopeptidase (Loechel et al., 1998), leading to the activation of the metalloprotease domain, since ADAMs are synthesised as inactive precursors. Most of them contain the catalytic protease domain (HEXGHXXGXXHD) whose activity is dependent on the presence of zinc ions.

ADAMs differ from most of the MMP and ADAMTS proteins in that they have an integrin-binding (disintegrin) domain and by their juxtamembrane cleavage of other membrane-associated proteins. This cleavage often activates the substrate protein either by release of the active extracellular peptide (e.g., tumor necrosis factor a, TNFα) or by enabling intracellular activation or further processing of the transmembrane protein (e.g., Notch). Because of this ectodomain shedding activity, the ADAMs are also known as sheddases. ADAMs may also play a role in cleavage-dependent inactivation of proteins (e.g., ADAM10 inactivating the ephrin/Eph complex by shedding ephrins (Hattori et al., 2000; Janes et al., 2005)) or extracellular matrix molecules (e.g., in Xenopus where ADAM13 regulates neural crest cell migration by cleaving fibronectin (Alfandari et al., 2001)). The metalloprotease activity of some ADAMs is inhibited by tissue inhibitors of metalloproteases (TIMP) which are thought to play a dynamic role in regulating metalloprotease activity in cellular plasticity.

Many of the ADAMs have an ECD motif (functionally equivalent to the classical RGD) in the disintegrin loop which can bind to various integrins (Bigler et al., 2000).

Normally, extracellular binding of an

Figure 11. Schematic representation of ADAMs structure.
integrin can promote cell adhesion through focal adhesions and can also activate transmembrane intracellular signalling by regulating growth factor receptors, ion channels, intracellular kinases, and actin organization (Giancotti and Ruoslahti, 1999). However, almost nothing is known about the potential of ADAMs to activate intracellular signalling by integrins. When the ADAMs lacking metalloprotease activity are biologically active, they most likely act through the integrin-binding disintegrin domain. There is some specificity of ADAMs in integrin-binding, although our understanding of the extent of distinction and overlap between the ADAMs is limited at present. The ADAMs may also have classical cell adhesion properties by binding of their cysteine rich domain to other proteins such as syndecan (Iba et al., 2000) and fibronectin (Gaultier et al., 2002), as well as dimerise with other ADAMs (Cho et al., 2000). The cytoplasmic tail of some of the ADAMs contains SH3-binding sites which can interact with and potentially activate SH3 domain containing intracellular signalling molecules such as Src and Grb (Howard et al., 1999; Suzuki et al., 2000; Huang et al., 2002). ADAMs can also bind to other intracellular proteins such as endophilin I and SH3PX1 (Howard et al., 1999) and ArgBP1, beta-cop, and ubiquitin (Huang et al., 2002). However, little is known about the extracellular events that might lead to activation of intracellular signalling pathways. One possibility would be the conformational change of the ADAM that would be caused by binding to an integrin, which would mean that ADAMs can be bidirectional signalling molecules. Overall, the signalling functions of ADAMs are unknown but are expected to contribute to the regulation of cellular behaviours.

ADAM10.

A disintegrin and metalloprotease 10, ADAM10, originally came into focus in genetical and biochemical research as a peptide sequence purified from bovine brain myelin membrane preparations (Chantry et al., 1989), and was referred to as MADM (i.e. mammalian disintegrin-metalloprotease). Further studies revealed that ADAM10 is expressed in a wide variety of tissues either in Bos taurus (Howard et al., 1996) and, more interestingly, in distinct areas of the human brain (Kärkkäinen et al., 2000; Marcinkiewicz and Seidah, 2000) and peripheral structures (McKie et al., 1997; Dallas et al., 1999). Initially, ADAM10 was suggested to be an enzyme, shaping the extracellular matrix by cleavage of collagen type IV, or to be a tumour necrosis factor α (TNF-α) convertase. In a relatively short time, a wide variety of additional substrates (with amyloid precursor protein probably being the most prominent) has been identified and the search is still ongoing. The enzyme belongs to the subgroup of metzincins within the zinc proteinases family. The typical multidomain structure of ADAM family proteins as a type I integral transmembrane protein consists of a prodomain, a catalytical domain with a conserved zinc binding sequence, a cysteine-rich disintegrin-like domain, a transmembrane domain and a rather short cytoplasmic domain (Fig. 11). The nascent protein itself is not functional and is produced as a zymogene. After cleavage of the signal sequence, ADAM10 enters the secretory pathway to be processed and thereby activated by the proprotein convertases furin or PC7 (Anders et al., 2001), as demonstrated for the prodomains of several ADAMs (Loechel et al., 1998; Lum et al., 1998; Roghani et al., 1999). ADAM10 prodomain exhibits a dual function: the separately expressed prodomain is capable of inactivating endogenous ADAM10 in cell
cultures while overexpressed ADAM10 without its prodomain is inactive (Anders et al., 2001). By contrast, coexpression of the prodomain in trans rescues the activity of the deletion mutant of ADAM10 without the intramolecular prodomain (Anders et al., 2001). In addition, the recombinant murine prodomain purified from Escherichia coli acts as a potent and selective competitive inhibitor in experiments performed in vitro (Moss et al., 2007). This implicates that the prodomain of ADAM10 acts not only as a transient inhibitor, but also as an internal chaperone in the maturation of the enzyme. The catalytic domain of ADAM10 contains a typical zinc-binding consensus motif (HEGXHXX GXHHD); the mutation E384A, compromising this motif, leads to a substantial decrease in APPs-\(\alpha\) secretion (Fahrenholz et al., 2000; Postina et al., 2004). Although the removal of the disintegrin domain of ADAM10 did not grossly affect shedding of APP in cell cultures (Fahrenholz et al., 2000), cleavage of some substrate molecules is likely to be influenced by non-catalytic domains. For example, epidermal growth factor cleavage is at least partially impaired in ADAM10\(^{-}\) cells overexpressing a cytoplasmic domain deletion mutant of ADAM10 (Horiuchi et al., 2007). Accordingly, the cytoplasmic domain of ADAM10 contains an IQ consensus binding site for calmodulin that afflicts maturation of the proteinase (Horiuchi et al., 2007). Moreover, ADAM10 has been shown to be activated by a calcium ionophore and the calmodulin inhibitor trifluoroperazine (Nagano et al., 2004; Sanderson et al., 2005). The cytoplasmic domain of ADAM10 contains two proline-rich putative Src homology 3 (SH3) binding domains, from which the juxtamembrane domain affects basolateral localization of ADAM10 in epithelial cells (Wild-Bode et al., 2006). In neurons, the SH3 binding domain directs ADAM10 to the post-synaptic membrane via binding to synapse-associated protein-97 (Marcello et al., 2007).

For the enzyme ADAM10, more than 40 substrates have been identified that belong to three different classes of membrane bound proteins (Pruessmeyer and Ludwig, 2009). Most of them are type I transmembrane proteins such as APP (Lammich et al., 1999), APP-like protein 2 (Endres et al., 2005) or the receptor for glycosylation end products (Raucci et al., 2008; Zhang et al., 2008). Type II transmembrane proteins such as the apoptosis-inducing Fas ligand (Schulte et al., 2007; Kirkin et al., 2007) or Bri2 (Martin et al., 2008) have also been reported to be shed by ADAM10. Additionally, at least three glycosylphosphatidylinositol (GPI)-anchored proteins are candidate substrates for ADAM10: the metastasis-associated protein C4.4A was characterized by a proteome technique as a substrate of ADAM10 (Esselens et al., 2008). Furthermore, the GPI-anchored neuronal guidance molecule ephrin A5 is cleaved by ADAM10 upon binding to its receptor EphA3, leading to termination of the receptor–ligand interaction (Janes et al., 2005). Third, from cell culture experiments, the prion protein PrPc was suggested to be processed by ADAM10 (Vincent et al., 2001; Cissé et al., 2005) and the abundance of the PrP cleavage product C1 was associated with mature ADAM10 within a small set of human cerebral cortex samples (Laffont-Proust et al., 2005). However, in vivo overexpression of ADAM10 in mice reduced all cellular prion protein species instead of generating enhanced amounts of cleavage products (Endres et al., 2009).

In the adult brain ADAM10 is widely distributed and expressed in astrocytes, microglial cells and neurons (Goddard et al., 2001; Skovronsky et al., 2001; Kieseier et al., 2003). In the CNS, “good” substrates can be found that participate in neuroprotection and regeneration and include:
amyloid precursor protein (APP), prion protein (PrP), N-Cadherin, neuroregulin, ephrins, L1 adhesion molecule, transmembrane chemokines, Notch and its ligand Delta. By cleavage of these substrates, ADAM10 is thought to play critical roles in protective processes in neurodegenerative diseases and in regenerative events after CNS injury.

ADAM10 mediated N-Cadherin cleavage has been extensively described in chapter 3.4.

APP. The amyloid precursor protein undergoes a similar metabolic pathway. is cleaved at several sites termed α-, β- and γ-sites and the involved proteases are designated as α-, β- and γ-secretases. ADAM10 is probably the most relevant α-secretase for APP but can be compensated by ADAM17 (Buxbaum et al., 1998; Lammich et al., 1999; Hartmann et al., 2002). Cleavage at the β- and γ-sites involves the β-amyloid cleaving enzyme (BACE) and the γ-secretase complex containing presenilin (De Strooper et al., 1998).

PrP. The cellular prion protein PrP is essential for the pathogenesis and transmission of prion diseases. Whereas the majority of PrP is bound to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor, a secreted form of the protein has been identified. PrP can be released into the medium by both protease- and phospholipase-mediated mechanisms (Parkin et al., 2004). PrP undergoes constitutive cleavage involving ADAM10 and phorbol ester-regulated proteolytic cleavage mediated by ADAM17 (Vincent, 2004). Therefore, activation of ADAM10 and ADAM17 could be seen as putative therapeutic strategy aiming at increasing normal PrP breakdown and thereby depleting cells of the putative toxic domain of PrP (Vincent, 2004).

Ephrin family. Ephrins are neuronal guidance molecules that bind to receptor tyrosine kinases of the Eph family. When the growth cone of a neuron that expresses Eph receptors encounters ephrin ligands on the surface of another cell, this initiates bidirectional signalling cascades that regulate cell adhesion, axonal guidance and neuronal plasticity. The growth cone then overcomes these adhesive forces and breaks away from the ephrin surface by proteolytic shedding of ephrin. A cleavage-inhibiting mutation within ephrin A2 delays axon detachment, suggesting that shedding is critical for axon guidance in the CNS (Hattori et al., 2000). ADAM10 can cleave ephrin A5 bound to EphA3 and thus terminate binding via ephrin (Janes et al., 2005). Notably, the ephrin receptor EphB2 also undergoes calcium-influx and n-methyl-d-aspartate (NMDA)-induced cleavage that is sensitive to ADAM10 inhibition (Janes et al., 2005). Thus, by cleavage of ephrins as well as their receptors, ADAM10 can be regarded as promoter of axon guidance and extension in the CNS.

Notch. The receptor Notch and its ligand Delta 1 are required for neuroepithelial development during embryogenesis, but also function in neuronal stem cell maintenance and self-renewal of the adult CNS. ADAM10 cleaves the extracellular domain of Notch (Brou et al., 2000; Hartmann et al., 2002) which is then directly followed by an intramembranous cleavage of the remaining cell-associated Notch molecule via γ-secretase. This results in the generation of a cytoplasmic cleavage fragment which can translocate into the nucleus and function as transcription factor (De Strooper et al., 1999). Thus ADAM10 is critically involved in the transcriptional signalling pathway of Notch and is required for its functions in neurogenesis even in the adult CNS. Other than N-Cadheirin, at least two classes of cell-adhesion molecules are cleaved by ADAM10, γ-protocadherins and L1 adhesion molecule.

γ-Protocadherins (Pcdh-γ) are abundantly expressed in the nervous system. They are enriched at synapses and involved
in synapse formation, specification, and maintenance. Pcdh γ C3 and Pcdh γ B4 are constitutively cleaved within their ectodomains by ADAM10 inhibiting cell aggregation (Reiss et al., 2006).

The immunoglobulin superfamily recognition molecule L1 promotes neuronal migration, neuronal survival and neurite outgrowth (see Kamiguchi, 2003). L1 undergoes constitutive cleavage at the cell surface which can be enhanced by stimulation with PMA, cholesterol depletion or NMDA-treatment (Mechtersheimer et al., 2001; Maretzky et al., 2005). Constitutive and NMDA-induced shedding of L1 is mediated by ADAM10 while PMA stimulation or cholesterol depletion leads to ADAM17-mediated L1 cleavage (Maretzky et al., 2005). ADAM10-mediated release was found to occur in intracellular vesicles that are subsequently released while cleavage of L1 in response to PMA occurred at the cell surface (Stoeck et al., 2006). Soluble L1 has been shown to stimulate cellular migration, neurite outgrowth and recovery after spinal cord injury (Doherty et al., 1995; Roonprapunt et al., 2003).

ADAM10 plays a crucial role in the development of central nervous system. Indeed, ADAM10−/− mice die at embryonic day (E) 9.5 as a result of major defects in development of the body, including the cardiovascular system. Their brains have multiple and major morphological defects, including a shortened forebrain primordium (Hartmann et al., 2002).

Potential substrate of ADAM10’s proteolytic activity involved in neural development are Notch1 and EGF receptor ligands. Without juxtamembrane cleavage by an ADAM, Notch cannot be activated by the subsequent proteases (Mumm et al., 2000). Notch1−/− mice die by embryonic day 11.5 with widespread cell death occurring before this time (Swiatek et al., 1994). This raises the possibility that ADAM10 is a major regulator of Notch activation and signalling, which is needed for cell proliferation and specification of cell fate (Cornell and Eisen, 2005). ADAM10 has been shown to trans-activate EGF receptor by cleavage of pro-heparin-binding EGF ligand (Yan et al., 2002; Prenzel et al., 1999). However, EGF receptor knockout mice have only minor brain defects during embryogenesis, including ectopic migration of hippocampal neurons and reduction in the number of astrocytes (Sibilia et al., 1998). These latter results suggest that ADAM10 does not play a major role in regulating EGF signalling during early embryonic times. The ADAM10−/− phenotype could also be explained by effects on neural progenitor proliferation, cell migration or survival. In Drosophila, the ADAM10 homolog kuzbanian sheds heparin-binding EGF-like growth factor (HB-EGF) (Yan et al., 2002) but its major role appears to involve proteolytical activation of Notch, which is involved in lateral inhibition of neuronal differentiation (Pan and Rubin, 1997; Lieber et al., 2002). During neurogenesis in the embryonic mouse brain, excessive Notch1 signalling reduces proliferation and generation of neurons, whereas during gliogenesis, Notch1 increases the number of glial fibrillar acidic protein-expressing cells (Chambers et al., 2001). Thus, the effects of ADAM10 may depend on the timing of its activation of EGF and Notch signalling, and the cell types that express these proteins. The late embryonic, early postnatal expression of ADAM10 in CNS neurons (Marcinkiewicz and Seidah, 2000), would be consistent with its role in regulating astroglial or oligodendroglial proliferation and/or differentiation, which occurs largely over the same time period. ADAM10 could regulate proliferation of late developing neuronal populations through EGF signalling. For example, ADAM10 mRNA is present within the E17-postnatal day (P)4 mouse hippocampal formation (Marcinkiewicz and Seidah, 2000), HB-EGF is also expressed here.
(Mishima et al., 1996) and EGF receptors are located within the neurogenic granule layer of the dentate gyrus (reviewed by (Wong and Guillaud, 2004)). Other studies suggest that processing of Notch by ADAM10 is important for downregulating its signalling (Mishra-Gorur et al., 2002; Sapir et al., 2005). It has been proposed that this mechanism enables an asymmetry in Notch signalling between adjacent cells promoting lateral inhibition and tissue patterning (Sapir et al., 2005) as was shown in Xenopus (Pan and Rubin, 1997). These findings suggest that inhibition of the metalloprotease domain of ADAM10 might indirectly promote neuronal differentiation, which is relevant to the neural stem cell and cell replacement fields.

As has been shown for other classes of metalloproteases and for integrins, ADAM10 plays a very important role in axon outgrowth. It is important for development of motor axon pathways in Drosophila embryos where kuzbanian mutant axons exhibit stalling and a failure to extend, with later-extending axons being more severely affected than pioneer axons (Fambrough et al., 1996). ADAM10 also plays a role in repelling longitudinally-projecting axons away from the midline. For example, expression of a dominant-negative ADAM10 in CNS midline cells causes aberrant axon growth across the midline (Schimmelpfeng et al., 2001). Based on comparisons of genetic mutants, the authors proposed a model where ADAM10 (kuzbanian) proteolytically activates the slit/round-about (Robo) receptor which would normally repel those axons. ADAM10 can also proteolytically terminate binding between ephrinA2 and Eph receptor, thus enabling release of the growth cone during its collapse (Hattori et al., 2000). ADAM10 initiates ephrinA5 cleavage (in trans) only after the ligand ephrinA5 binds to an EphA3 receptor which is constitutively associated with ADAM10 (Janes et al., 2005). This mechanism may ensure that only receptor-bound ephrins are cleaved. ADAM10 might also promote axonal fasciculation by shedding L1 neural cell adhesion molecule. ADAM10 can cleave L1 (Mechtersheimer et al., 2001; Gutwein et al., 2003) while L1+/- mice exhibit decreased overall size and defasciculation of axons in the corpus callosum and corticospinal tract (Dahme et al., 1997). L1 mRNA and protein has been observed within late embryonic/early postnatal cortical neurons and their fibers in the corpus callosum and corticospinal tract (Fujimori et al., 2000). Therefore, ADAM10 may regulate axonal behaviour depending on the location of the substrate it sees.

The Post-Synaptic Density.

The dynamic rearrangement of spines and PSDs seems to be the structural basis for the synaptic regulation and synaptic plasticity that might be involved in long-term memory formation. For this reason, the regulation and functional interplay between PSD components leading to a fast alteration of synaptic structures are two of the current exciting fields in synaptic research (Hering and Sheng, 2001; Yuste and Bonhoeffer, 2004; Carlisle and Kennedy, 2005). Type 1 glutamatergic excitatory synapses are characterised by large post-synaptic densities. Post-synaptic densities are either located directly on the dendritic shaft or at the tip of tubular, thin, cup-shaped or mushroom-shaped dendritic spines and lie just below the post-synaptic membrane. They form a 30- to 40-nm-thick protein meshwork with a diameter of a few hundred nanometres and exhibit filamentous structures that extend deeply into the spine cytoplasm. Here, they exist in intimate relationship with the cytoskeletal meshwork of spines and/or dendrites suggesting the existence of a sub-synaptic do-
main at this interface. In spiny synapses, other organelles such as the smooth endoplasmic reticulum (SER) or the spine apparatus are linked to post-synaptic densities via these filamentous structures (Spacek and Harris, 1997) creating an enlarged post-synaptic subcompartment that is remarkably dynamic and that can be re-arranged according to synaptic activity-dependent Ca$^{2+}$ concentrations (Toni et al., 1999; Hering and Sheng, 2001; Yuste and Bonhoeffer, 2004). According to one proposed model, an activated simple synapse can alter rapidly in shape (via enlarged spines with horseshoe and/or segmented PSDs) and can finally transform into two separated synapses. From a molecular point of view, the PSD is composed of a huge complex protein network consisting of several hundred different proteins (Collins et al., 2006; Cheng et al., 2006) which can be divided into classes of (1) cell-adhesion proteins, (2) cytoskeletal proteins, (3) scaffolding and adaptor proteins, (4) membrane-bound receptors and channels, (5) G-proteins and modulators and (6) signalling molecules including kinases and phosphatases (Klauck and Scott, 1995; Ziff, 1997; Husi et al., 2000; Kennedy, 2000; Sheng, 2001).

Recently, the mass of a single post-synaptic density was measured (1.1 ± 0.36 GDa) and the relative or absolute amounts of single proteins determined (Chen et al., 2005; Sugiyama et al., 2005). A single post-synaptic density has been calculated to contain about 200–400 molecules of prominent PSD proteins such as ProSAP/Shank or Homer (see also below), indicating that the amount of individual PSD proteins per PSD is not accidental but is determined according to a specific PSD protein stoichiometry (Kennedy, 2000; Sugiyama et al., 2005). The resulting complex protein assembly, which is built from highly enriched PSD core proteins and proteins that are only loosely attached to the PSD, is the molecular basis for locally distinct but diverse intracellular events. These tasks include the clustering and transport of membrane-bound receptors, the temporal and spatial organization of signalling cascades, the dynamic organization of cytoskeletal components and the induction and structural maintenance of intercellular contact sites. Glutamatergic excitatory synapses express different type of ionotropic and metabotropic glutamate receptors (GluRs), which are specifically targeted and clustered at the post-synaptic membrane (Mayer and Armstrong, 2004). All these different type of receptors are components of individual, tightly associated, multiprotein complexes that regulate synaptic targeting or removal from synaptic sites, local expression, signal transduction and clustering (Kim and Sheng, 2004; Kneusssel, 2005).

An NMDA complex (NRC), an AMPAR complex (ARC) and an mGluR complex (mGC) have been defined and described (Husi et al., 2000; Farr et al., 2004; Collins et al., 2006). The most important modular protein interaction domains that are characteristic and essential for the organization of these complexes are PDZ (PSD-95/DLG/ZO1) protein-protein interaction domains (Cho et al., 1992; Kornau et al., 1995), that are found in large number of synaptic proteins.

**PSD-MAGUKs.**

Post-Synaptic Density-Membrane Associated GUanylate Kinase proteins form a family sharing a multi-domain structure. They contain three PDZ (PSD-95/ Discs large/Zona occludens 1) domains, an src-homology 3 (SH3) domain and a C-terminal guanylate kinase (GUK) domain (Fig. 12). In the mammalian brain, which is the tissue expressing the greatest diversity of MAGUK proteins, four homologous have been described. Synapse-associated proteins
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Figure 12. Schematic representation of multi-domain structure of PSD-MAGUK proteins.

(SAP)90/PSD95, SAP102, SAP97/hDlg and chapsyn-110/PSD93, all of which have been localised at CNS glutamatergic synapses (Garner et al., 2000; Aoki et al., 2001) as well as at cholinergic synapses (Conroy et al., 2003).

As modular proteins, it has often been hypothesized that the most likely function of this subfamily of MAGUKs is as central organizers of CNS synapses. To a large extent, this conclusion is based on the spatial distribution of these proteins, their range of binding partners and genetic studies. As a family, all have been localized to either the pre- and/or post-synaptic face of excitatory and in some cases inhibitory CNS synapses (Garner et al., 2000; Aoki et al., 2001; Conroy et al., 2003). Each member of PSD-MAGUK protein family is distributed differently in the brain cell compartments. PSD95 and PSD93 are highly enriched in the post-synaptic density, especially due to their high palmitoylation degree (El-Husseini et al., 2000). SAP102 and SAP97 are found in dendrites and axons and are abundant in the cytoplasm as well as at synapses. PSD-MAGUK proteins are also expressed in different stages of life: SAP102 is highly expressed in early postnatal development, whereas PSD95 and PSD93 predominate at later stages (Kim and Sheng, 2004). Indeed, in mammalian brain, PSD95 and related neuronal PSD-MAGUKs are also suggested to play roles in post-synaptic development. The PDZ domains of PSD-MAGUKs bind voltage- and ligand-gated ion channels as well as cell adhesion molecules, such as the Shaker voltage-gated K+ channels (Kim et al., 1995a), subunits of the NMDA (Kornau et al., 1995; Müller et al., 1996), AMPA (Leonard et al., 1998) and kainate (Garcia et al., 1998) glutamate receptors, neuroligin (Irie et al., 1997) and synGAP (Kim et al., 1998; Chen et al., 1998). Although they show similar specificities of protein interaction in vitro, PSD-MAGUKs interact with different, but overlapping, sets of proteins in vivo. The C- terminal cytoplasmic tails of NR2 subunits of NMDA receptor directly interact with PDZ domains of all members of the PSD-MAGUK family (Kim and Sheng, 2004). In particular, the last three amino acids of the carboxyterminal of NR2A and NR2B subunits contain a consensus motif threonine/serine-X-valine (T/ SXV, where X is any amino acid) that has been demonstrated to be responsible for efficient binding to PDZ domains of PSD-MAGUK protein members such as PSD95 (Niethammer et al., 1996). PSD95, in turn, binds to the amino terminal of neuronal nitric oxide synthase (nNOS), a Ca2+-
activated form of NOS, through its PDZ domain. Therefore, PSD95 may concentrate nNOS near the NMDA receptor at postsynaptic sites in neurons (Christopherson et al., 1999) thereby connecting NMDA receptors to specific signal transduction pathways (Kennedy, 2000).

PSD-MAGUKs may provide critical links between motor proteins and the vesicular cargos they carry to and from synapses. In particular, the interaction with SAP97 appears to be more relevant for processing AMPA receptor subunits (Rumbaugh et al., 2003; Mauceri et al., 2004) and NR2A subunits of NMDA receptors (Gardoni et al., 2003; Mauceri et al., 2007), whereas SAP102 is crucial for driving NR2B-containing NMDA receptors to spines (Sans et al., 2003).

At the post synaptic density, PSD-MAGUKs are involved in clustering ion channels and can influence the changes in glutamate receptor dynamics that occur during synaptic function and plasticity via their known interactions with these receptors. In addition, NMDA receptors' synaptic localization and binding to PSD-MAGUK protein family play a key role in the control of downstream signals resulting from receptor activation (Lau and Zukin, 2007; Elias and Nicoll, 2007). PSD95 directly determines the density of AMPA receptors at synapses, through its association with stargazin (Schnell et al., 2002), therefore this PSD-MAGUK complex can directly participate in controlling synaptic plasticity. Overexpression of PSD95 dramatically increases synaptic density of AMPA receptors and this overexpression prevents the recruitment of new AMPA receptors that normally occurs during Long-Term Potentiation (Stein et al., 2003; Ehrlich and Malinow, 2004). This occlusion of LTP is traditionally interpreted to mean that overexpression of PSD95 and endogenous LTP occur through similar mechanisms. On the other hand, prolonged low-intensity activation of NMDA receptors (long term depression, LTD) can decrease AMPA receptor density (Dudek and Bear, 1992). Interestingly, overexpression of PSD95 increases the magnitude of LTD induction, suggesting a role for PSD95 (Stein et al., 2003) also in this process.

In order for a synapse to grow and remodel, not only do glutamate receptors need to undergo dynamic changes in surface expression, but the PSD itself must be subject to dynamic changes in protein composition. Intriguingly, different PSD proteins appeared to be co-regulated in their expression patterns, such that specific protein 'sets' were either increased or decreased in a stable and reversible manner in response to altered activity. Co-regulated protein ensembles could result from the activity-regulated removal of scaffold proteins that subsequently destabilise groups of proteins. Obviously, given the known ability of PSD-MAGUKs to act as scaffolding proteins, these molecules could be key candidates for regulating such en masse movement. Of the MAGUKs measured, PSD95 and SAP102 show activity-dependent regulation, such that PSD95 and NR2A are enriched in active synapses and reduced in inactive synapses. However, not all PSD components are subject to multi-protein co-regulation; for example, neither SAP97 nor PSD93 is altered by global changes in synaptic activity.

**SAP97.**

Among MAGUKs, which are highly enriched at the post-synaptic density, SAP97 represents the atypical member, since it is broadly expressed (Kim and Sheng, 1996). Light microscopy studies on hippocampal neurons have shown that a majority of SAP97 immunoreactivity is present in the cytoplasm with less than one third being present at synaptic sites (Sans et al., 2001).
This might suggest a function outside the synapse for SAP97, perhaps in molecules trafficking from the soma to the synaptic plasma membrane. Indeed, SAP97 is associated more with light membrane fractions and cytosol and interacts specifically with GluR1 subunit of AMPA receptors (Leonard et al., 1998). In particular, this interaction occurs early in the secretory pathway, while the receptors are in the endoplasmic reticulum or cis-Golgi. In contrast, few synaptic receptors are associated with SAP97, suggesting that SAP97 dissociates from the receptor complex at the plasma membrane. On the other hand, internalisation of GluR1, as triggered by NMDAR activation, does not require SAP97 (Sans et al., 2001).

SAP97 has been also described to form a complex with NMDA receptor by binding its C-terminal tail. This interaction is regulated by CamKII phosphorylation of serine 232 residue in the PDZ-1 domain of SAP97 (Gardoni et al., 2003). This complex is not detectible in the triton insoluble fraction, which represents the synaptic fraction; thus indicating that like AMPA receptors, SAP97 is involved in the trafficking of NMDA receptors to synapse. Moreover, CamKII activation leads to increased targeting of SAP97 into dendritic spines, while CamKII-dependent SAP97-Ser39 phosphorylation determines a redistribution of the GluR1 subunit of AMPA receptors (Mauceri et al., 2004). The direct interaction between SAP97 and NMDA receptor has been determined by high-resolution NMR study; the NR2B subunit binds the first and the second PDZ domains of SAP97, with higher affinity for PDZ-2 (Wang et al., 2005). Intra-cellularly, SAP97 binds voltage-gated Kv1 channels and prevents further biosynthetic trafficking, causing an intracellular accumulation of these channels (Tiffany et al., 2000).

However, SAP97 performs tasks other than just trafficking receptors to the plasma membrane. Its versatility is due to the existence of at least seven different isoforms (see McLaughlin et al., 2002), arising via alternative splicing, which seem to be differentially distributed (McLaughlin et al., 2002; Rumbaugh et al., 2003; Hanada et al., 2003). Moreover, the insertion via alternative splicing, in the N-terminal region of two different proline-rich sequences found to bind the SH3 domains in three tyrosine kinases, Lck (Hanada et al., 2003) Crk and Abl (McLaughlin et al., 2002), influences homomultimerisation of SAP97 containing these inserts. In particular, this long N-terminal domain (187 aa) is composed of at least three distinct functional elements: one involved in subcellular targeting (Wu et al., 1998), one in homomultimerisation (Marfatia et al., 2000; McLaughlin et al., 2002), and one in regulating intramolecular and intermolecular interactions (Wu et al., 2000; Mehta et al., 2001). With regard to subcellular targeting, a L27 or MRE motif (present in the first 65 residues of SAP97) appears to be the most important, at least in non neuronal cells, where it has been shown to function in the retention of SAP97 and ion channels within the endoplasmic reticulum (Tiffany et al., 2000; Chetkovich et al., 2002) and the targeting of SAP97 to the epithelial lateral membrane (Wu et al., 1998; Karnak et al., 2002; Hanada et al., 2003). Binding partners important for these diverse functions include CASK (Karnak et al., 2002), myosin VI (Wu et al., 2002) and Hrs (an endosomal ATPase that regulates vesicular protein sorting) (Chetkovich et al., 2002). Moreover, N-terminal segment of SAP97 can bind the SH3 domain of PSD95, and overexpression of the latter triggers accumulation of SAP97 in dendritic spines and induces dendritic clustering of GluR1 containing AMPA receptors (Cai et al., 2006).

SAP97 and PSD95 contain alternative N-terminal regions expressing either double cysteines that normally are palitoylated (α-isoforms) or an L27 domain (β-isoforms).
Whereas α-isoforms of SAP97 and PSD95 influence AMPA receptor-mediated synaptic strength independent of activity, the effects of β-isoforms are regulated by activity in a CamKII-dependent manner (Schlüter et al., 2006). Between the SH3 and the GK domain, there is the Hook domain, which contains four alternatively spliced insertions, termed I2, I3, I4 and I5 (Thomas et al., 2000; Ruiz-Cañada et al., 2002). I2, I3 and I5 inserts are expressed in several tissues, whereas the I4 occurs only in brain and liver. Aminoacids coded in the I3 insert bind to the actin/spectrin-associated protein 4.1 binding site (Hanada et al., 2003) that not only facilitates the synaptic recruitment of SAP97, but also its association with the actin cytoskeleton within dendritic spines (Rumbaugh et al., 2003). SAP97 has a key role in synaptic plasticity and synapse development. As already describe, SAP97 is involved in the delivery and retention of AMPA receptors at synapses. Moreover, the neuronal A-kinase anchoring protein AKAP79/150 interacts with PKA and the calcium-dependent protein phosphatase PP2B is linked to GluR1 by SAP97 (Oliveria et al., 2003). Furthermore, SAP97 overexpression drives GluR1 to synapse leading to an increased synaptic localisation of AMPA receptors, dendritic spines enlargement and an increase in miniature EPSC (mEPSC) frequency and occluded LTP. The potentiation on synaptic transmission depends on multimerisation of SAP97 via its L27 domain. In fact, synaptic potentiation and GluR1 delivery are dissociated by L27 domain mutants that inhibit SAP97 multimerisation. Loss of potentiation is correlates with faster turn over of monomeric SAP97 mutants in dendritic spines, while L27-mediated interactions with itself or other proteins regulate synaptic delivery of AMPARs (Nakagawa et al., 2004). SAP97 has both pre- and post-synaptic effects on synaptic transmission. In fact, SAP97 may play a central role in the coordinated growth of synapses during development and plasticity by recruiting a complex of post-synaptic proteins that enhances pre-synaptic terminal growth and function via multiple trans-synaptic molecular interactions. Indeed, post-synaptic expression of SAP97 recruits a complex of additional post-synaptic proteins to synapse including glutamate receptors, Shank1a, SPAR (spine-associated RapGAP) and ProSAP2. Furthermore, inhibition of several different trans-synaptic signalling proteins including Cadherins, Integrins and EphB receptor/ephrinB significantly reduces the pre-synaptic growth caused by post-synaptic SAP97 (Regalado et al., 2006). A partial knockout mouse for SAP97 has been generated; this mutant mouse expresses a truncated form of SAP97 that has the three PDZ domains but lacks the SH3, GK and Hook region (Caruana and Bernstein, 2001). Mutant mice display in utero growth retardation, severe craniofacial abnormalities and die perinatally. Synaptically, this partial deletion has no effect on the synaptic clustering of glutamate receptors (Klöcker et al., 2002). However, as the SH3-GK domains do not appear to be necessary for synaptic targeting of SAP97 (Rumbaugh et al., 2003) and the PDZ domains are critical for interaction with AMPA receptor (Leonard et al., 1998), a full knockout of SAP97 will be necessary to fully understand its role in the development and maintenance of synapses.

The NMDA Receptor Complex.

PDZ-containing proteins of the MAGUK family are very important for the formation of the NMDA receptor complex (Fig.13). PSD95 is one of the first MAGUK proteins to be isolated and characterised. It tethers the NMDA receptors to the post-synaptic density by the interaction of the first two PDZ domains with NR2 subunit of the heteromeric NMDAR complex (Cho et al.,
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1992; Kistner et al., 1993; Kornau et al., 1995; Niethammer et al., 1996) and is involved in targeting and NMDAR signalling, as indicated by the analysis of various transgenic mouse models (Sprengel et al., 1998; Migaud et al., 1998). PSD95 also binds Kainate receptors (Garcia et al., 1998) and the Shaker K+ channel via its second PDZ domain (Kim et al., 1995b; Kim et al., 1995a), the same domain is also able to heterodimerize with a PDZ domain of neuronal nitric oxide synthase (nNOS; Brenman et al., 1996). Via this nNOS interaction, the PSD protein Kalirin7 (Penzes et al., 2000; Ma et al., 2003), which is a Rho-GEF protein that is essential for dendrite shape and spine and PSD formation, can be attached to the NMDA receptor complex (Kone et al., 2003). Furthermore, neuroligin, the neurexin transmembrane ligand, binds to the third PDZ domain of PSD95 (Irie et al., 1997; Song et al., 1999) and with the epithelial growth factor (EGF) receptor tyrosine kinase, ErbB4, interacts with the first two PDZ domains (Garcia et al., 1998) as do guidance molecules of the semaphorine family (Schultze et al., 2001; Burkhardt et al., 2005). PSD95 also binds several regulators or effectors of small GTPases influencing the shape and number of spines and PSDs. The GTPase activating protein for Ras, SynGAP, interacts with all three PDZ domains of PSD95 via its C-terminus (Kim et al., 1998) and co-operatively regulates spine and synapse formation in hippocampal neurons (Vazquez et al., 2004). SPAR, the spine-associated Rap-Gap, binds to the C-terminus of PSD95 regulating

**Figure 13.** The NMDA receptor complex.
the size and shape of spines through the reorganization of the actin cytoskeleton (Pak et al., 2001). Moreover, the GK domain serves as a docking site for the four members of a PSD protein family named GKAPs (guanylate-kinase-associated proteins) or SAPAPs (synapse-associated protein 90/post-synaptic density protein-95-associated proteins; Kim et al., 1997; Takeuchi et al., 1997). The four members of the GKAP/SAPAP are closely related but characterized by distinct spatiotemporal expression patterns during brain development (Kindler et al., 2004). GKAP/SAPAP play as linker molecules that indirectly attach PSD95 to Arg-binding protein 2 (nArgBP2; Kawabe et al., 1999) or to the scaffolding proteins of the ProSAP/Shank family binding to the PDZ domain. ProSAP/Shank proteins are also multidomain molecules that function as important backbone molecules of the post-synaptic density (Naisbitt et al., 1999; Boeckers et al., 1999; Sheng and Kim, 2000; Boeckers et al., 2002). Since ProSAP/Shank have the ability (1) to build huge platforms via self-association (Baron et al., 2006) and (2) to interact simultaneously with different GluR complexes, the cytoskeleton and signalling molecules in the PSD, they are described as the “master-scaffolding” molecules of the PSD (see below).

The mGluR Complex.

In the post-synaptic density mGluR complex (Fig. 14) is tightly linked to the NMDAR/PSD95 complex (Farr et al., 2004; Collins et al., 2006) via the interaction with the synaptic scaffolding protein Homer. Homer is an mGluR cytoplasmic binding protein (Brakeman et al., 1997) that exists in different splice variants and clusters and localizes mGluRs to synaptic sites (Brakeman et al., 1997; Xiao et al., 2000). Homer proteins, which are able to self-associate in a process mediated by coiled-coil domains, interact with a proline-rich region of ProSAP/Shank proteins (Tu et al., 1999; Thomas, 2002) thereby combining the mGluRs with the NMDAR complex. Importantly, in addition to N-type calcium channels and M-type potassium channels (McCool et al., 1998; Kammermeier, 2006), Homer also links the inositol-1,2,5-trisphosphate receptor (InsP3R), which is part of the mGluR downstream signalling pathway via a specific Homer-binding motif, PPXXF (Tu et al., 1998), to the PSD scaffold. InsP3R is localized at the membrane of the SER extending into the neck region of dendritic spines. These interactions closely link the intracellular calcium stores, the calcium-binding protein calmodulin (Minakami et al., 1997) and specific ion channels to the GluR complexes allowing an efficient coupling of signals from the membrane to the spine compartment (Tu et al., 1998; Xiao et al., 2000). Via the ProSAP/Shank interaction, NMDAR is also included in those signal transduction pathways and can participate in local responses to, for example, synaptic activation.

The AMPAR Complex.

The AMPAR GluR1 subunit interacts directly with the PDZ domain of ProSAP/Shank (Uchino et al., 2006) attaching the AMPAR complex (Fig. 15) to the other GluR complexes present at the post-synaptic density. An alternative possibility for attaching AMPAR to the NMDAR complex is via the AMPA-binding protein, Stargazin (TARPs: (Fukata et al., 2005), which has been shown to interact with PDZ domains of PSD95 (Chen et al., 2000). Proteomic analysis of PSD proteins, however, have indicated that AMPARs are localised in a separated distinct PSD subcompartment (Husi and Grant, 2001). This might be explained in part by the finding that the syn-
aptic levels of AMPARs are variable and depend upon synaptic activity as well as age (Liao et al., 1999; Carroll et al., 1999; Lüscher et al., 2000). There are even synaptic structures without “stable” AMPARs localized at the post-synaptic membrane (called silent synapses; Groc et al., 2006). Post-synaptic AMPARs are characterized by their ability to move in and out the post-synaptic membrane in a rapid receptor-subunit-dependent fashion (Shi, 2001). This AMPAR trafficking, which requires regulated endo- and exocytosis, depends not only upon receptor subunits and interacting proteins, but also upon the nature of synaptic stimulation or neuronal cell types (Sheng and Hyoung Lee, 2003). This recycling phenomenon seems to be part of an important molecular mechanism by which synaptic activity of a single synapse can be regulated, a prerequisite for all forms of synaptic plasticity. Therefore, the synaptic targeting and clustering mechanisms of AMPARs have to be different from that of other GluRs and require a separate set of molecules (Kim and Sheng, 2004). The C-terminal tails of the GluR2 and GluR3 subunits of the tetrameric AMPARs bind to the fifth PDZ domain of the scaffolding protein GRIP (GluR-interacting protein)/ ABP (AMPAR-binding protein) that harbours a total of seven PDZ domains (Dong et al., 1997). Via these domains GRIP/ABP is able to dimerise and colocalize another set of proteins involved in AMPAR stabilization, targeting and transport including the Ephrin

**Figure 14.** The glutamate metabotropic receptor complex.
receptor (Brückner et al., 1999; Kim and Sheng, 2004). Another GluR2/GluR3-binding protein via a PDZ domain is PICK-1 (Xia et al., 1999), a molecule initially characterized as a protein interacting with protein kinase C α but now known to be a molecule that is also able to bind to a set of pre- and post-synaptic proteins including the ErbB2 receptor tyrosine kinase (Kim and Sheng, 2004). More proximal regions of the AMPAR subunits can bind to the adaptor proteins AP-2 and the N-ethylmaleimide sensitive factor (NSF) involved in vesicle budding and fusion (Song et al., 1998; Noel et al., 1999). The C-terminal end of the GluR1 subunit has been shown to interact with a PDZ domain of the MAGUK SAP97 (Leonard et al., 1998).

ProSAP/Shanks Proteins: Master-scaffolding Molecules.

To organize the signal reception, modulation and transduction at the post-synaptic membrane and in the submembranous compartment effectively, the PSD of excitatory synapses seems to be arranged in a clear-cut laminar hierarchical structure (Fig. 16; Valtschanoff and Weinberg, 2001; Zuber et al., 2005); this hierarchy appears to be determined by the targeting and binding characteristics of the individual proteins. At the membrane, receptors and channels are organised as distinct subcompartments that are then combined into larger protein units, which are finally attached...
to the local cytoskeleton. In this respect, the three members of the ProSAP/Shank family can be seen as “master organizing” molecules of the post synaptic density (Boeckers et al., 2002; Kim and Sheng, 2004) as they are able to build up large sheets within the PSD (Baron et al., 2006) and can interact with a growing number of different proteins, large protein complexes, membrane-spanning and signalling proteins and cytoskeletal components (Gundelfinger et al., 2006). According to their prominent role within the organization of the post-synaptic density, ProSAP/Shank molecules are early components of post-synaptic specializations during synaptogenesis (Boeckers et al., 1999; Petralia et al., 2005) and are efficiently targeted to synaptic sites (Boeckers et al., 1999; Sala et al., 2001). Their structure includes a huge set of protein-protein interaction domains, namely ankyrin repeats, an SH3 and PDZ domain, proline-rich stretches and a SAM (sterile alpha motif) domain. As previously mentioned, the NMDAR complex is attached to ProSAP/Shank via SAPAP/GKAP proteins that interact with the PDZ domain. At the C-terminal end, Homer proteins can bind to a short proline-rich stretch connecting the mGluR complex. An interaction with AMPARs is also possible via stargazin/TARPs or SAP97 attached to the NMDA/SAP90/PSD95 complex. A direct interaction of the C-terminus of the GluR1 subunit (Uchino et al., 2006) and of a special GluR subunit (GluRδ2) that is solely ex-

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**Figure 16.** ProSAP/Shanks Proteins: Master-scaffolding Molecules.

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pressed in Purkinje cells of the cerebellum with the PDZ domain (and with the SH3 domain) of ProSAP/Shank has also been reported (Uemura et al., 2004). Other receptor or channel proteins that also bind the ProSAP/Shank PDZ domain and are therefore potentially clustered at synaptic membranes include somatotropin receptor 2 (SSTR2; Zitzer et al., 1999), the alpha-latrotoxin receptor (CIRL; Kreienkamp et al., 2000) and the Cav1.3 L-type Ca2+ channel (Zhang et al., 2005; Olson et al., 2005).

The described receptor complexes that are attached to ProSAP/Shank are finally affixed to the local actin-based cytoskeleton via the N-terminal ankyrin repeats or short proline-rich regions. Here, several classical actin-binding and modulating proteins of the PSD, such as brain α-fodrin, cortactin or Abp1, are linked to the scaffold (Boeckers et al., 2002; Qualmann et al., 2004; Kim and Sheng, 2004). Further components of the ProSAP/Shank master complex are the insulin receptor tyrosine kinase substrate IRSp53 (Bockmann et al., 2002; Soltau et al., 2002) Dynamin 2 and Densin-180 (Okamoto et al., 2001; Quitsch et al., 2005). All these molecules have the potential to alter the shape of spines and PSDs by recruiting and/or regulating small GTPases within the PSD, as does the spine-associated Rap-Gap SPAR, which is also indirectly included in the ProSAP/Shank complex by binding to GKAP/SAPAP1, or members of the Fezzin family of PSD proteins including PSD-Zip70 (Maruoka et al., 2005) or ProSAPIP1, a novel recently identified PSD component (Wendholt et al., 2006).

Cytoskeleton.

As already mentioned, actin (Figková and Morales, 1992; Adam and Matus, 1996) and actin-binding proteins are important components of dendritic spines and postsynaptic densities. The dynamic structural rearrangement is essentially based on the regulated remodelling of actin by actin-binding proteins (Matus, 2000). In addition to brain α-fodrin (Carlin et al., 1983; Böckers et al., 2001), Drebrin A and alpha actinin (Shirao and Sekino, 2001; Mizui et al., 2005), cortactin and Abp1 (Qualmann et al., 2004; Racz and Weinberg, 2004; Mizui et al., 2005), profilin1 (Neuhoff et al., 2005), dystrophin (Kim et al., 1992) and amyloid-precursor-like protein-1 (Kim et al., 1995b; Kim et al., 1995a) have been detected in the PSD. Interestingly, the overexpression or downregulation of most of these proteins alters significantly the number and/or shape of synaptic contacts. Alpha actinin 2 can also directly bind to NMDAR subunits, a binding that is antagonized by Ca2+/calmodulin and that might explain the Ca2+-regulated “release” of NMDARs from the cytoskeleton (Ehlers et al., 1996). In addition to myosin Va (Walinkonis et al., 2000), the actin-binding motor protein myosin VI is also found in PSD complexes together with AMPAR, SAP97 and AP-2 and might play a role in the clathrin-mediated endocytosis of AMPARs (Osterweil et al., 2005).

Kinases.

Several Kinases are present at the postsynaptic density which play key roles as regulatory elements.

Calmodulin and CaMKII were certainly among the first important regulatory proteins to be identified as integral members of the PSD protein network (Grab et al., 1979; Kennedy et al., 1983). CaMKII builds up heteromultimers from two closely related catalytic subunits and binds to the cytosolic tail of the NR2 subunit of the NMDAR and to several other PSD components including the novel scaffolding protein TANC (Suzuki et al., 2005). CaMKII is able to phosphorylate the NR2 subunit and
has been shown to be necessary for normal synaptic plasticity (Silva et al., 1992; Giese et al., 1998; Kennedy, 2000).

Moreover, CaMKII phosphorylates SAP97 on two major sites in vivo; one located in the N-terminal domain, Ser39, and the other in the first PDZ domain, Ser232 (Mauceri et al., 2007). CaMKII-mediated phosphorylation of SAP97-Ser39 is necessary and sufficient to drive SAP97 to the post-synaptic compartment (Mauceri et al., 2004). CaMKII-dependent phosphorylation of SAP97-Ser232 disrupts the interaction of SAP97 with NR2A subunits thereby regulating synaptic targeting and insertion in the post-synaptic membrane of this NMDA receptor subunit (Gardoni et al., 2003; Mauceri et al., 2007) Other kinases that are enriched in PSD preparations include protein kinase C subtypes and MAPKs (Suzuki et al., 1993; Suzuki et al., 1995), Fyn tyrosine kinase (Suzuki and Okumura-Noji, 1995), cAMP-dependent protein kinase (PKA), protein phosphatase 2B/calcineurin (PP2B/CaN) and protein phosphatase 1 (Dosemeci and Reese, 1993). The functional relevance of these kinases is exemplified by the finding of Hori et al. (Hori et al., 2005) who have shown that the translocation of the synaptic protein and the key player of cytoskeletal dynamics, IRsP53, into the post-synaptic compartment is regulated via a NMDA/protein kinase C signalling pathway. Several of these kinases are anchored to the PSD via specific adaptors such as AKAP (A kinase anchoring protein) 79 and 150 (Carr et al., 1992), which bind to MaGuK proteins. These adaptors have recently been shown not to be stationary at the synapse but to take part in AMPAR recycling (Snyder et al., 2005); they are also able to move out of the PSD into the cytoplasm in response to synaptic activation (Smith et al., 2006).
AIM
The cell-cell adhesion molecule N-Cadherin has a key role during dendrite arborization, axon guidance and synaptogenesis (Benson and Tanaka, 1998). Moreover, at synapses, N-Cadherin regulates synaptic adhesion, spine morphology and activity-dependent plasticity (Okamura et al., 2004; Togashi et al., 2002; Xie et al., 2008; Mendez et al., 2010). Recent studies also showed that N-Cadherin is mandatory for an increase in AMPA receptor content and function at synaptic sites (Xie et al., 2008; Liao et al, 1999; Saglietti et al., 2007; Okuda et al., 2006) which is associated with spine enlargement (Kasai et al., 2003; Abe et al., 2004; Mendez et al., 2010). Thus, a temporal and functional relation between N-Cadherin, synaptic accumulation of AMPA receptors and spine growth has been put forward (Zito et al., 2009).

Recent data showed that N-Cadherin can be cleaved by ADAM10 (a disintegrin and metalloproteinase 10). Via its metalloproteinase domain, the enzyme is responsible for the initial step of N-Cadherin processing (Reiss et al., 2005; Uemura et al., 2006), which, extracellularly, releases the soluble active fragment and, intracellularly, generates a C-Terminal fragment (CTF). CTF initiates signaling pathways through the cytoplasmic β-catenin pool (Reiss et al., 2006). Therefore, ADAM10-dependent cleavage of N-Cadherin modulates cell–cell adhesion as well as signal transduction. ADAM10 shows post-synaptic localization (Janes et al., 2005; Marcello et al., 2007) permitted by association with synapse-associated protein-97 (SAP97), a member of the PSD-MAGUK protein family. Perturbing ADAM10/SAP97 association impairs ADAM10 localization in the postsynaptic membrane and decreases ADAM10 activity towards synaptic substrates (Marcello et al., 2007).

The aim of my study was to clarify which are the consequences of ADAM10 activity on N-Cadherin at synaptic sites and how may this processing contribute to spine remodelling and availability/stabilization of active glutamate receptors, which have been described as linked to N-Cadherin.

To tackle this issue, ADAM10 activity has been up- and down-regulated and the effects on N-Cadherin metabolism, dendritic spine morphology and AMPA receptors localisation and activity have been investigated. TIMP-1, an ADAM10 inhibitor, as well as a cell-permeable peptide (TAT-Pro) able to interfere in vitro and in vivo with ADAM10 synaptic localization and activity have used.
Material and Methods
Antibodies, Reagents and Cell-permeable Peptides.

The following antibodies (Ab) were used: mAb anti-GFP and mAb anti-pan Shank purchased from NeuroMab (University of California Davis, Davis, CA). mAb anti-α-Tubulin, anti-Actin IgG fraction of antiserum (20-33 amino acids) from Sigma (St. Louis, MO) and pAb anti-ADAM10 N-Terminal from Abcam (Cambridge, MA, USA); mAb anti-NMDA NR1, pAb anti-NR2B and mAb anti-NR2A from Zymed Laboratories (San Francisco, CA); pAb anti-AMPA Glur1, mAb anti-GluR2, goat pAb anti-AKAP150 and mAb anti-PYK-2y67 from Millipore (Billerica, MA, USA); mAb anti-SAP97 from Stressgen-Biotechnologies (Victoria, British Columbia, Canada) and mAb C-32 anti-N-Cadherin C-Terminal domain from BD Biosciences (San Jose, CA); pAb anti-HA-probe from Santa Cruz Biotechnology (Santa Cruz, CA); pAb anti-green fluorescent protein (GFP) and AlexaFluor secondary Abs were purchased from Invitrogen (Carlsbad, CA), peroxidase-conjugated secondary Abs were purchased from Pierce (Rockford, IL).

Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) and PACAP-38 were purchased from Merck KGaA (Darmstadt, Germany); (+)-Bicuculline was purchased from Tocris Biosciences (Ellisville, Missouri) and TTX was purchased from Ascent Scientific (Bristol, UK).

TAT-Pro 709-729 ADAM10 inhibitory peptide (TAT-Pro) was obtained linking the 11 amino acids human immunodeficiency virus TAT transporter sequence (Aarts et al., 2002) to the 21 amino acids sequence corresponding to ADAM10 proline rich domains (NH2-YGRKKRRQRRRPKLPPKPLPGTLKRRRPPQPP-COOH). TAT-Ala 709-729 ADAM10 peptide (TAT-Ala) in which all proline residues were mutated into alanine (NH2-YGRKKRRQRRR- AKLAALAGTLKRRRAAQA-COOH) was used as control (Marcello et al., 2007). Non cell-permeable peptide lacking TAT sequence mimics the 21 amino acids sequence of ADAM10 proline rich domains (Pro ADAM10 peptide, NH2-PKLPPKPLPGTLKRRRPPQPP-COOH). As negative control a similar peptide in which all proline residues were mutated into alanine (Ala ADAM10 peptide, NH2-AKLAALAGTLKRRRAAQA-COOH) was used. All peptides were synthesized by XigenPharma (Lausanne, Switzerland).

GFP-N-Cadherin plasmid was provided by Maria Passafaro; GFP-N-Cadherin cleavage-defective (GFP-N-Cadherin GD) was created by site-direct mutagenesis substitution of both R 714 → G 714 and 715 → D 715 as previously described (Uemura et al., 2006); HA-N-Cadherin Wt and HA-N-Cadherin GD were provided by Kengo Uemura.

Immunoprecipitation.

50 μg of homogenate from cultured hippocampal neurons, acute hippocampal slices or mouse brain treated with both TAT-Pro and TAT-Ala peptides were incubated in RIA buffer (200 mM NaCl, 10 mM EDTA, 10 mM Na2HPO4, 0.5% NP-40 and 0.03% SDS) in a final volume of 200 μl with antibody against the ADAM10 N-Terminal domain overnight at 4°C. Protein A-sepharose beads (Sigma-Aldrich) were added and incubation was continued for 2 hours, at room temperature, with shaking. Beads were collected by centrifugation at 1000g for 5 minutes and washed three times with RIA buffer before adding sample buffer for SDS-PAGE and boiling for 5 minutes. Beads were collected by centrifugation, all supernatants were applied onto 6% SDS-PAGE.
Animal Treatment.

Two weeks old mice (Charles River, Calco, Italy) received a single intraperitoneal injection of either TAT-Pro (3 nmol/g) or TAT-Ala peptide (3 nmol/g) diluted in sterile saline solution (Marcello et al., 2007). Animals were killed after 24 hours and the brains were rapidly removed. All experimental procedures were performed with care to minimized discomfort and pain to treated animals, in accordance with the guidelines of the European Communities Council (Directive of November 24, 1986, 86/609/EEC). One hemisphere of each animal was homogenized in ice-cold sucrose 0.32 M containing Hapes 1 mM, MgCl₂ 1 mM, EDTA 1 mM, NaHCO₃ 1 mM, PMSF 0.1 mM, at pH 7.4 in presence of a complete set of proteases inhibitors (Complete™, GE Healthcare; Mannheim, Germany) and phosphatases inhibitors (Sigma-Aldrich) The homogenized tissue was centrifuged at 1,000g for 10 minutes. The resulting supernatant (S1) was centrifuged at 13,000g for 15 minutes to obtain a crude membrane fraction (P2 fraction). The pellet was re-suspended in Hapes 1mM + Complete™ in a glass-glass homogenizer and centrifuged at 100,000g for 1hour. The pellet (P3) was re-suspended in buffer containing 75 mM KCl and 1% Triton-X 100 and centrifuged at 100,000g for 1hour. The final pellet (P4, TIF) was homogenized in 20 mM Hapes + Complete™ in a glass-glass homogenizer.

Acute Hippocampal Slices, Neuronal Cultures Preparation and Transfection.

Acute hippocampal slices were prepared as previously described (Gardoni et al, 2001, 2003). Briefly, brains were removed and placed into chilled (4°C) oxygenated Krebs’ buffer. After removal of meninges, hippocampal slices were prepared quickly with a McIlwain tissue chopper and placed in custom-made chambers equilibrated continuously with O₂ 95%-CO₂ 5% (v/v). Slices were then equilibrated at 37°C (O₂ 95%-CO₂ 5%) for 60 minutes. After the equilibration period, slices were incubated with pharmacological treatments. After pharmacological treatments slices were quickly frozen at -80°C. Slices were then thawed on ice and subcellular fractionation was performed.

Hippocampal neuronal primary cultures were prepared from embryonic day 18-19 (E18-E19) rat hippocampi as previously described (Piccoli et al., 2007). Neurons were transfected at DIV7 with GFP, DS-RED, GFP-N-Cadherin Wt, GFP-N-Cadherin GD, HA-N-Cadherin Wt and HA-N-Cadherin GD constructs using calcium-phosphate method.

Subcellular Fractionation and Surface Expression Assay.

Triton Insoluble postsynaptic Fractions (TIFs) were isolated from neurons as described previously (Gardoni et al., 2009).

Cross-linking experiments by means of bis (sulfosuccinimidyl) suberate (BS3) (Pierce) were performed as described previously (Hall and Soderling, 1997; Mauceri et al., 2004). Briefly, following a wash incubation of 20 minutes at 37°C, slices or cultures were incubated with 1 mg/ml BS3 in saline solution for 10 minutes with agitation at 37°C. Plates were then washed three times with ice-cold lysis buffer containing protease inhibitors (Complete™, GE Healthcare; Mannheim, Germany) before harvesting (ethanolamine is present in the harvest buffer to quench any unreacted BS3).
Immunocytochemistry.

Transfected neurons treated with both TAT-Pro and TAT-Ala peptide (10µM, 30 minutes) were fixed for 10 minutes in 4% paraformaldehyde plus 4% sucrose at room temperature and immunostained for GFP; primary and secondary antibodies were applied in GDB buffer (Sala et al., 2001) (30 mM phosphate buffer, pH 7.4, containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 M NaCl). Cells were chosen randomly for quantification from 4 different coverslips (2 independent experiments), 14 neurons for each treatment were analyzed. Fluorescence images were acquired by using Zeiss Confocal LSM510 Meta system with 63x objective and a sequential acquisition setting at 1024x1024 pixels resolution; for each image three to four 0.5 µm sections were acquired and z-projection was obtained.

For colocalization studies, hippocampal neurons treated with TAT-Pro and TAT-Ala peptide (10 µM, 30 minutes), TIMP-1 (15 nM, 30 minutes), or PACAP-38 (300nM, 30 minutes), were fixed for 10 minutes in 4% paraformaldehyde plus 4% sucrose at room temperature or for 15 minutes in methanol at -20°C and immunostained for GluR1, ADAM10 and Pan-Shank as postsynaptic marker; primary and secondary antibodies were applied in GDB buffer (Sala et al., 2001) (30 mM phosphate buffer, pH 7.4, containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 M NaCl). Cells were chosen randomly for quantification from 4 different coverslips (2 independent experiments), at least 10 neurons for each treatment were analyzed. Fluorescence images were acquired by using Zeiss Confocal LSM510 Meta system with 63x objective and a sequential acquisition setting at 1024x1024 pixels resolution; colocalization analysis was performed with the Zeiss Confocal Software. Morphological analysis of in vivo experiments was conducted by using a diolistic gene gun system to propel Dil-coated particles into sections of fixed brain tissue. Images of Dil-labeled structures in hippocampal CA1 stratum radiatum were acquired using the LSM510 Zeiss confocal microscope. At least 10 z-stack images were acquired for each animal and for each area of interest. Morphometric analysis of dendritic spines was performed with Metamorph software.

Live Imaging.

Time-lapse imaging occurred in an environmentally controlled chamber with 5% carbon dioxide at 37°C and images were obtained using the Zeiss Confocal LSM510 Meta system with 63x objective and zoom function set on 4; for each image three to four 0.5 µm sections were acquired and z-projection was obtained. Images were acquired every 3 minutes for 30 minutes of non-treated cells used as control, after injection of TAT-Pro or TAT-Ala peptides images were acquired every 3 minutes for 30 minutes. Head width of all spines was measured at each time point.

Electrophysiological Recordings from CA1 Pyramidal Cells.

Experiments were performed on parasaggital hippocampal slices from 14- to 16-day-C57Bl6 mice by using standard techniques. Whole-cell voltage-clamp recordings (3.5- to 4.5 MO electrodes) were made at 30-32°C from pyramidal cells of the CA1 field visualized by infrared videomicroscopy. Slices were perfused with extracellular solution composed of 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 2.3 mM CaCl2, 1.3 mM MgCl2, and 25 mM glucose saturated with 95% O2/5% CO2. Bicuculline (10 µM) was added to the bath to block GABA_A receptors. The intracellular solution was com-
posed of: 140 mM cesium methanesulfonate, 2 mM MgCl2, 4 mM NaCl, 5 mM phospho-creatine, 2 mM Na2ATP, 10 mM EGTA, 10 mM HEPES, 0.33 mM GTP (pH 7.3). In experiments in which the rectification indices (RIs) were measured 1 mM QX314 and 0.1 mM spermine were also added to the intracellular solution. Evoked postsynaptic responses were induced in CA1 pyramidal cells by stimulating Schaffer collaterals in stratum radiatum (0.1 Hz) with a monopolar glass pipettes. In experiments showed in Fig 7, non-permeable ADAM10 Pro and Ala peptides were delivered postsynaptically through the recording pipette. We waited 15 min after establishing the whole-cell configuration before starting the recording to ensure adequate diffusion of reagents into dendritic compartments. RIs were calculated by plotting the magnitude of the average EPSC at -60, 0, and +40 mV and taking the ratio of the slope of the lines connecting values at 0-40 and at -60–0 mV. This calculation, respect to the simple ratio of EPSC amplitude at +40/-60 mV, takes into account the variable AMPA receptor reversal potential of each recording (Adesnik and Nicoll, 2007). In experiments of figure 11, mice were injected intraperitoneally with ADAM10 TAT-Ala or TAT-Pro peptides (3 nmol/g) 24 hr before slice preparation. AMPA receptor EPSCs were recorded at -80 mV. NMDA receptor EPSCs were recorded at +40 mV and measured 40 ms after the initiation of the EPSC, a time point at which AMPA receptor-mediated currents are absent or minimal. Small, hyperpolarizing voltage steps were given before each afferent stimulus allowing on-line monitoring of input and series. Values are expressed as mean ± SEM.

Quantification and Statistical Analysis.

Quantification of WB analysis was performed by means of computer-assisted imaging (ImageJ) after normalization on actin or tubulin levels and values were expressed as mean ± SEM. Colocalization analysis was performed using Zeiss AIM 4.2 software.

Statistical evaluations were performed by using unpaired Student’s Test or, when appropriate, by one-way ANOVA (Figure 3e,f; 4; 6 d; 7; 13 b,c; 16), followed by Bonferroni’s as a post hoc test.

For electrophysiological experiments shown in figure 7 we used two way ANOVA among groups perfused with Pro and Ala peptide and unpaired Student’s t test for the last two minutes of recording. For electrophysiological experiments shown in figure 11, unpaired Student’s t test was used for data comparison.
Results
ADAM10/SAP97 Interaction is Required for ADAM10 Mediated Cleavage of Synaptic N-Cadherin.

Firstly, we asked whether constitutive ADAM10-mediated cleavage of N-Cadherin occurs at synapses of primary hippocampal neurons during neuronal development (DIV10). To this, we interfered with ADAM10 activity by means of a previously characterized cell-permeable peptide (see Marcello et al, 2007), in which TAT cell-permeable domain of the HIV is fused to the C-terminal proline-rich domains of ADAM10 (TAT-Pro; Fig. 1a). TAT-Pro peptide competes with endogenous ADAM10 for the binding to the SH3 domain of SAP97 and disrupts endogenous ADAM10/SAP97 complex (Marcello et al 2007). ADAM10/SAP97 complex is responsible for

**Figure 1. TAT-Pro peptide disrupts ADAM10/SAP97 complex.**

**a)** Schematic representation of ADAM10 and SAP97 structure and of the amino acidic sequence of cell-permeable TAT-Pro peptide corresponding to ADAM10709-729 proline-rich domains. **b)** DIV10 primary hippocampal neurons were treated with TAT-Ala and TAT-Pro ADAM10 peptides (10 µM, 30 minutes) and then lysed. ADAM10 was immunoprecipitated from total homogenate with an antibody raised against its N-terminal domain and Western Blot analysis was performed with an antibody against SAP97. Treatment with TAT-Pro was able to reduce co-immunoprecipitation of SAP97 with ADAM10 (p<0.005, n=3) when compared to TAT-Ala. **c)** Total homogenate from mice treated with TAT-Ala and TAT-Pro was immunoprecipitated with an antibody raised against AKAP150 and western blot analysis was performed with an antibody against SAP97. Animals treated with TAT-Pro showed no modification in SAP97 co-immunoprecipitation with AKAP150 compared to animals treated with TAT-Ala. **d)** Total homogenate from mice treated with TAT-Ala and TAT-Pro was immunoprecipitated with an antibody raised against PYK2 and western blot analysis was performed with an antibody against PSD95. Animals treated with TAT-Pro showed no modification in PSD95 co-immunoprecipitation with PYK2 compared to animals treated with TAT-Ala.
the enzyme localization and activity at synaptic membranes and treatment with the peptide is able to reduce both ADAM10 localization and activity at synapses (Marcello et al., 2007). As control, an analogous inactive peptide in which all proline residues were substituted by alanines was used (TAT-Ala). After treatment of primary hippocampal neurons (DIV10) with TAT-Pro or TAT-Ala peptides (10 μM, 30 minutes), total homogenates were immunoprecipitated with an antibody against ADAM10 and the co-immunoprecipitation of SAP97 evaluated by means of western blot analysis (Fig. 1b). A reduction of co-immunoprecipitation was observed in neurons treated with TAT-Pro inhibitory peptide, compared to TAT-Ala treated ones, indicating a decreased presence of ADAM10/SAP97 complex (Fig. 1b; TAT-Pro vs. TAT-Ala, -29.2% ± 1.1%, p<0.005; n=3). To check TAT-Pro specificity to disrupt ADAM10/SAP97 complex without influencing other protein-protein interactions, we tested SAP97 SH3 domain capability to bind to other partners in presence of TAT-Pro. No alterations were found in the association between AKAP150 and SAP97 (Fig. 1c). TAT-Pro did not either interfere with the binding of other synaptic SH3 to Proline rich domains, as assessed by co-immunoprecipitation of PYK2 and PSD-95 (Fig. 1d). On the whole, these data confirm that TAT-Pro is specific for its capability to interfere with ADAM10/SAP97 complex (Marcello et al., 2007).

Under these experimental conditions, we found no modifications of SAP97 (p=0.7692, n=7) and ADAM10 (p=0.4423, n=7) levels in total homogenate, suggesting that the observed uncoupling can be ascribed to a decreased association rather than to changes in levels of these proteins (Fig. 2a). Conversely, a significant reduction of ADAM10 in Triton Insoluble postsynaptic Fraction (TIF), enriched in proteins of the post-synaptic density (Gardoni et al., 2009), was observed (Fig. 2b; TAT-Pro vs. TAT-Ala, -39.6% ± 5.8%, p<0.0005; n=7), without any modification in SAP97 levels (p=0.8488, n=7); thus indicating a decreased ADAM10 synaptic localization. Biochemical data were further confirmed by immunocytochemical labeling of ADAM10 in hippocampal neurons (DIV10). As shown in Figure 2c, treatment with Tat-Pro (10 μM, 30 minutes) led to a reduction of ADAM10 co-localization with the postsynaptic marker Shank (TAT-Pro vs TAT-Ala, -45.3% ± 8.3%, p<0.05; n=10).

We then investigated whether the observed decrease in ADAM10 synaptic localization after TAT-Pro treatment was associated to inhibition of ADAM10 activity towards N-Cadherin. By using an antibody raised against N-Cadherin C-terminal intracellular domain (C-32; see Fig. 6 in chapter 3.1), we detected two main immunoreactive bands corresponding to the Full-Length N-Cadherin (FL) and to its C-Terminal Fragment (CTF), which has previously been described as deriving from ADAM10-mediated cleavage (Reiss et al., 2005; Uemura et al., 2006). After treatment with TAT-Pro, an increase of N-Cadherin FL (Fig. 2d; TAT-Pro vs. TAT-Ala, +42.6% ± 12.7%, p<0.05; n=7), a concomitant decrease of N-Cadherin CTF (Fig. 2d; TAT-Pro vs. TAT-Ala, -32.5% ± 8.6%, p<0.05; n=7) and a consequent increase of the FL/CTF ratio (Fig. 2e; TAT-Pro vs. TAT-Ala, +90.1 ± 10.7%, p<0.005; n=7) were observed, compared to TAT-Ala.

Thus, interfering with ADAM10 localization and activity at the post-synaptic membrane by means of TAT-Pro, we demonstrated that constitutive ADAM10-mediated cleavage of N-Cadherin indeed occurs at synapses of primary hippocampal neurons during development.
ADAM10 Localization and Activity at Synapse Influence Spine Morphology.

Since N-Cadherin signalling leads to modulation of spine morphology and synapse stability (Abe et al., 2004; Xie et al., 2008; Mendez et al., 2010), we asked whether the increased synaptic accumulation of N-Cadherin FL, as obtained by TAT-Pro treatment, could be paralleled by modifications of spine morphology.

DIV7 GFP-transfected hippocampal neurons were incubated, at DIV10, with TAT-Pro or TAT-Ala (10 μM, 30 minutes). After treatment, cells were fixed and immunolabelled with an anti-GFP antibody (Fig. 3a) and average spine head width and number of dendritic spines were evaluated. Statisti-
cal analysis revealed a significant increase in spine head width (Fig. 3b; p<0.0005, n>2600 spines from 14 different neurons for each group) after treatment with TAT-Pro and cumulative frequency plots of spine head width confirmed a significant shift towards bigger spine size (Fig. 3c). No changes in the mean spine density were found (TAT-Ala 3.75 ± 0.29 spines/10μm; TAT-Pro 4.10 ± 0.18 spines/10μm; p=0.1853).

To confirm that the effects of ADAM10 inhibition on spine size are dependent on N-Cadherin cleavage, we repeated morphological analysis in neurons after transfection with GFP, GFP-N-Cadherin wild-type (Wt) or with the cleavage-defective GFP-N-Cadherin GD construct, mutated in the ADAM10 cleavage site (Uemura et al., 2006). Both constructs showed the same expression at the postsynaptic site as demonstrated by colocalization analysis with Shank-positive clusters (data not shown).

Statistical analysis revealed a significant

![Diagram showing relative average spines head width (p<0.0005 TAT-Pro vs TAT-Ala, n>2600 spines from 14 different neurons for each condition) and cumulative frequency plots from neurons treated with TAT-Ala and TAT-Pro.](image)

**Figure 3. ADAM10 localization and activity influence spine morphology.**

a) Primary Hippocampal neurons were transfected with GFP construct at DIV7 and treated at DIV10 with TAT-Ala and TAT-Pro ADAM10 peptides (10 mM, 30 minutes). Cells were fixed and Immunolabeled for GFP. Representative images show dendrites from neurons treated with TAT-Ala and TAT-Pro ADAM10 peptides; scale bar 10mm. b) Diagram showing relative average spines head width (p<0.0005 TAT-Pro vs TAT-Ala, n>2600 spines from 14 different neurons for each condition) and cumulative frequency plots from neurons treated with TAT-Ala and TAT-Pro. c) Neurons co-transfected at DIV7 with DS-RED and GFP, GFP-N-Cadherin Wt or GFP-N-Cadherin GD. Cells were fixed and images taken for DS-RED. d) Diagram showing relative average spines head width (p<0.0001 among groups, p<0.001 GD vs GFP, p<0.0001 GD vs Wt; n>500 spines from 10 different neurons for each group) and cumulative frequency plots from neurons transfected with GFP, GFP-N-Cadherin Wt or GFP-N-Cadherin GD.
increase of spine head width in GFP-N-Cadherin GD but not in GFP N-Cadherin Wt transfected neurons (Fig. 3e; p<0.0001 among groups, p<0.0001 GD vs GFP, p<0.0001 GD vs Wt; n=500 spines from 10 different neurons for each group), paralleled by a significant shift towards bigger spine size (Fig. 3f). Thus, these data confirm that blocking ADAM10-dependent cleavage of N-Cadherin leads per se to an increase of spine size.

After confirming that the increase in spine width is due to the blocking of ADAM10 cleavage of N-Cadherin, we investigated the onset of the effect by time-lapse imaging. Spine head width was measured every 3 minutes and expressed as percentage of initial values. In basal conditions, no significant modification of average spine head width was observed over 30 minutes (Fig. 4a,b). Similarly, treatment with TAT-Ala did not induce any modification of spine size. An increase of spine head width (Fig. 4a,b), that became statistically significant 18 minutes after treatment, was observed in TAT-Pro treated neurons if compared to control and TAT-Ala treated ones (Fig. 4b; p<0.001 among groups, p<0.05 TAT-Pro vs Control, p<0.05 TAT-Pro vs TAT-Ala; n>60 spines from 4 independent experiments for each group).

Is this morphological effect of TAT-Pro treatment dependent on spine size? Evaluation of spine head width in DIV10 primary hippocampal neurons revealed that 0.8 μm represents a cut-off value that allowed to split spines into two equal populations (Fig. 4c,d). As shown in Fig. 4e, even if the absolute differences were bigger for spines smaller than the cut-off value, both groups showed a significant increase of spine head width 30 minutes after treatment with TAT-Pro compared to control and TAT-Ala treated neurons (Width <0.8 μm: p<0.01 among groups, p<0.01 TAT-Pro vs Control, p<0.05 TAT-Pro vs TAT-Ala. Width ≥ 0.8μm: p<0.0001 among groups, p<0.0001 TAT-Pro vs Control, p<0.005 TAT-Pro vs TAT-Ala). The observed modifications of average values of spine head width induced by treatment with TAT-Pro inhibitory peptide could be ascribed to a general and collective change in the size of all spines or to a change of the percentage of shrinking spines compared to growing spines in TAT-Pro treated neurons. Spines having final values of width bigger than 105% of initial values were considered as growing spines, spines having final values of width smaller than 95% of initial values were considered as shrinking spines (Fig. 4f). Treatment with TAT-Pro for 30 minutes induced a significant increase of the percentage of growing spines (p<0.01 among groups, p<0.05 TAT-Pro vs Control; p<0.01 TAT-Pro vs TAT-Ala; n>60 spines from 4 independent experiments for each group) and a corresponding decrease of the percentage of shrinking ones (p<0.01 among groups, p<0.05 TAT-Pro vs Control; p<0.01 TAT-Pro vs TAT-Ala) compared to control and TAT-Ala.
Molecular Mechanisms Regulating Spine Remodelling

Results

Figure 4. Timing of ADAM10-dependent effect on spine morphology.

a) Representative time-lapse imaging of hippocampal neurons transfected with GFP (DIV7) and incubated (DIV10) with TAT-Ala or TAT-Pro ADAM10 peptides (10 µM). b) Spine head width was measured at all set times and expressed as percentage of initial values; TAT-Pro increased spine head width compared to control and TAT-Ala treated neurons (p<0.001 among groups, p<0.05 TAT-Pro vs Control, p<0.05 TAT-Pro vs TAT-Ala, n>60 spines from 4 independent experiments for each group). Dendritic spines from Control, TAT-Ala and TAT-Pro treated neurons analysed in time-lapse experiments have been classified on the basis of their head width before the 30 minutes treatment; c) Cumulative probability of dendritic spine head width. d) Number of dendritic spines having head width smaller or larger than 0.8 µm expressed as percentage of total number of spines. e) Dendritic spines have been divided into two groups depending on their head width (cut-off 0.8 µm). For each group of spines, the average head width at end point (30 minutes) has been calculated and expressed as percentage of the initial value. TAT-Pro increased average head width in both populations of dendritic spines when compared to control and TAT-Ala treated neurons (Width <0.8 µm: p<0.01 among groups, *p<0.01 TAT-Pro vs Control, *p<0.05 TAT-Pro vs TAT-Ala. Width ≥ 0.8µm: p<0.0001 among groups, #p<0.0001 TAT-Pro vs Control, #p<0.005 TAT-Pro vs TAT-Ala, n>60 spines from 4 independent experiments for each group). f) Diagram showing statistical analysis of growing and shrinking spines after 30 minutes treatment with TAT-Pro or TAT-Ala peptides as percentage of total number of spines. TAT-Pro ADAM10 induced a statistically significant increase of the percentage of growing spines (p<0.01 among groups, #p<0.05 TAT-Pro vs Control; *p<0.01 TAT-Pro vs TAT-Ala) and a statistically significant decrease of shrinking ones (p<0.01 among groups, #p<0.05 TAT-Pro vs Control; *p<0.01 TAT-Pro vs TAT-Ala; n>60 spines from 4 independent experiments for each groups).
ADAM10 Localization and Activity Influence Subunit Composition of Synaptic AMPA Receptors.

AMPA receptors at synapses (Togashi et al., 2002; Liao et al., 1999; Kasai et al., 2003) and can interact with specific subunits of AMPA receptors (Saglietti et al., 2003) and can interact with specific subunits of AMPA receptors. More recently, it has been shown that N-Cadherin inactivation results in fewer GluR1 synaptic clusters (Xie et al., 2008). To test whether this N-Cadherin-dependent modulation of AMPA receptor composition at synapses is associated with ADAM10-mediated N-Cadherin cleavage, hippocampal slices from three weeks old rats were incubated with TAT-Pro or TAT-Ala for 30 minutes. As for primary neurons (Fig.1b), we first validated our tool: in hippocampal slices, treatment with TAT-Pro decreased ADAM10 interaction with SAP97 (Fig. 5a; TAT-Pro vs TAT-Ala, -59.5% ± 9.9%, p<0.005; n=3), decreased ADAM10 localization in the TIF (Fig. 5b; TAT-Pro vs TAT-Ala, -35.3% ± 10.4%, p<0.05; n=5) and, as a direct consequence, increased N-Cadherin FL/CTF ratio (Fig. 5c; TAT-Pro vs TAT-Ala, +53.9% ± 16.4%, p<0.05; n=3).

In hippocampal slices treated with TAT-Pro, GluR2 subunit of AMPA receptor (Fig. 6a; p=0.9399, n=6), as well as all tested NMDA receptor subunits (Fig. 6b; NR1, p=0.9728; NR2A, p=0.4375; NR2B, p=0.9839; n=4), did not show any modification of their localization in the post-synaptic compartment. Instead, GluR1 levels showed a specific and significant increase in TIF (Fig. 6a; TAT-Pro vs TAT-Ala, +30.7% ± 3.2%, p<0.0001; n=6). This result was confirmed in hippocampal neurons treated with TAT-Pro where increased GluR1 localization in Shank-positive clusters was detectable (Fig. 6c; TAT-Pro vs TAT-Ala, +39.0% ± 6.7 %, p<0.0005; n=10 from 3 independent experiments for each group).

Also in this case, to confirm that the observed effect is dependent on N-Cadherin cleavage, we repeated colocalization analysis in neurons transfected with GFP, GFP-N-Cadherin wild-type (Wt) or with the cleavage-defective GFP-N-Cadherin GD construct. Statistical analysis revealed a significant increase of GluR1 localization in Shank-positive clusters in GFP-N-Cadherin GD but not in GFP N-Cadherin wt transfected neurons (Fig. 6d; p<0.0002 among groups, p<0.002 GD vs GFP, p<0.0002 GD vs}

Figure 5. ADAM10/SAP97 interaction in hippocampal slices.

a) Acute hippocampal slices were treated with TAT-Pro or TAT-Ala ADAM10 peptides (10 μM, 30 minutes). Total homogenate was immunoprecipitated with an antibody against ADAM10 and western blot analysis for SAP97 was performed: treatment with TAT-Pro inhibitory peptide reduced SAP97 co-immunoprecipitation with ADAM10 (p<0.005, n=3). b) Treatment with TAT-Pro induced a statistically significant reduction of ADAM10 localization in the TIF (p<0.05, n=5). c) TAT-Pro inhibitory peptide induced an increase in FL/CTF N-Cadherin ratio (p<0.05, n=3) when compared to TAT-Ala.
Wt; n=21 from 3 independent experiment for each group) compared to control.

**ADAM10 Localization and Activity Influence Synaptic AMPA Receptor Function.**

Since interfering with ADAM10 activity at synapses leads to modification of the composition of AMPA receptors, we tested if it is also capable of modifying AMPA receptor function. To this, we delivered postsynaptically non-permeable Pro and Ala peptides (same effector sequence as TAT-Pro and TAT-Ala, respectively, but not bound to TAT-cargo domain) through the patch pipette while monitoring AMPA receptor-mediated EPSCs evoked by stimulation of Schaffer collaterals in CA1 pyramidal cells in the presence of bicuculline (10 µM) and D-AP5 (50 µM). 30 minutes after intra-
cellular perfusion with Pro peptide, cells showed a significant increase of EPSC amplitudes if compared to cells dialyzed with Ala peptide (Fig. 7a,b; p<0.0001; n=10-11 recordings for each group). In a subset of experiments, we evaluated the contribution of AMPA receptors lacking the GluR2 subunit to the EPSCs measuring the rectification index (RI) at the beginning (0 minutes) and at the end (30 minutes) of the experiment, (Fig. 7c; at -60, 0 and +40 mV). RI values observed for both peptides at 0 min (Fig. 7c; Pro, 0.84 ± 0.06; Ala, 0.92 ± 0.05; n= 6-8 recordings for each group) were similar to those previously observed at this age in CA1 pyramidal cells (Adesnik and Nicoll, 2007). No significant change in RI was observed in cells dialyzed for 30 minutes with Pro or Ala ADAM10 peptides (Pro peptide p=0.4; Ala peptide p=0.37). Therefore, inhibition of ADAM10/SAP97 interaction not only induces a raise in GluR1 localization in the TIF but also increases the amplitude of AMPAR-mediated EPSCs, which does not appear to be mediated by an insertion of GluR2-lacking AMPARs.

**Figure 7 . Role of ADAM10 synaptic localization on AMPA receptors activity.**

a) Averaged AMPAR EPSCs (6 responses) recorded at -60 mV and + 40 mV from CA1 pyramidal cells with an intracellular solution containing spermidine (0.1 mM) and Pro or Ala ADAM10 peptides. Recordings were started after 15 minutes after entering in whole cell configuration to ensure an adequate diffusion of reagents into dendritic compartments and AMPA responses were pharmacologically isolated with bicuculline (10 µM) and D-AP5 (50 µM). (Scale bars: 50 pA , 50 ms). The amplitude of EPSCs was measured for the next 30 min. b) Summary of the changes in the amplitude of the AMPAR EPSCs in cells intracellularly perfused with Pro or Ala ADAM10 peptides (p<0.0001 with two way ANOVA among groups perfused with Pro and Ala peptide and p<0.005 by unpaired Student’s t test for the last two minutes of recording for Pro and Ala peptide; n=10-11). Responses were normalized respect to the first minute of recording. c) Summary of RIs of AMPAR EPSCs in cells intracellularly perfused with Pro or Ala ADAM10 peptides at 0 and 30 minutes (Pro peptide p=0.4; Ala peptide p=0.37; n=6-8).
Inhibition of ADAM10 Activity by Treatment with TIMP-1.

In order to confirm that the results obtained inhibiting ADAM10 localization at the post-synaptic membrane, by interfering with ADAM10/SAP97 interaction, were due to ADAM10 activity, we used a classic pharmacological approach. We performed a pharmacological inhibition of ADAM10 activity by using TIMP-1 (Amour et al., 2000) in DIV10 primary hippocampal neurons. As TAT-Pro peptide did, also TIMP-1 treatment (15 nM, 30 minutes) caused, in the TIF, an increase of N-Cadherin FL (Fig. 8a; TIMP-1 vs Control, +37.6% ± 14.5%, p<0.05; n=4), a concomitant decrease of N-Cadherin CTF (Fig. 8a; TIMP-1 vs Control, -38.6% ± 9.5%, p<0.01; n=4) and a consequent increase of the FL/CTF ratio (FL/CTF, Fig. 8a; TIMP-1 vs Control, +131.5% ± 23.7%, p<0.005; n=4). As expected, TIMP-1 did not induce any modification of localization in the triton insoluble fraction (Fig. 8b) of SAP97 (p=0.9118, n=5) and ADAM10 (p=0.2240, n=6). Biochemical data were further confirmed by immunocytochemical labeling of ADAM10 in hippocampal neurons (DIV10). Treatment with TIMP-1 (15 nM, 30 minutes) showed no modification of ADAM10 co-localization with the postsynaptic marker Shank (Fig. 8c; p=0.3637, n=10). Furthermore, the effects of the inhibition of ADAM10 activity towards N-
Cadherin on dendritic spines morphology and AMPA receptors localization have been investigated. Treatment with TIMP-1 significantly increased spine head width (Fig. 9a,b; p<0.0001; n>500 spines from 9 different neurons for each group), as confirmed by cumulative frequency plots analysis (Fig. 9c). As for TAT-Pro treatment, no changes in the mean spine density were found. Again, effects of TIMP-1 treatment on AMPA type receptors localization at synapse were evaluated. In TIMP-1 treated neurons, levels of GluR1 (Fig. 9d; TIMP-1 vs Control, +32.2% ± 5.3%, p<0.0001; n=5), but not of GluR2 (p=0.8481, n=5), were significantly increased in the postsynaptic membranes if compared to controls. Moreover, after treatment GluR1 localization was higher in Shank-positive clusters if compared to controls (Fig. 9e; TIMP-1 vs Control, +48.9% ± 7.6 %, p<0.005; n=10).

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**Figure 9. ADAM10 inhibition effects on spine morphology and AMPA receptors composition.**  
**a)** Primary Hippocampal neurons were transfected with EGFP construct (DIV7) and treated or not at DIV10 with TIMP-1 (15 nM, 30 minutes). Representative images show dendrites from control and TIMP-1 treated neurons.  
**b)** Diagram showing relative average spines head width (p<0.0001, TIMP-1 vs Control, n>500 spines from 9 different neurons for each condition) and **c)** cumulative frequency plots from control and TIMP-1 treated neurons.  
**d)** Western blot analysis performed in the TIF showed a statistically significant increase of GluR1 (p<0.0001, n=5) but no modification of GluR2 (p=0.8481, n=5) subunit of AMPA receptor.  
**e)** Primary hippocampal neurons immunolabeling for GluR1 (green) and Shank (red). Neurons treated with TIMP-1 showed a statistically significant increase of GluR1/Shank colocalization (p<0.005, n=10).
In vivo modulation of ADAM10/SAP97 interaction.

The above-described results indicate that inhibition of ADAM10 activity in the post-synaptic compartment in primary hippocampal neurons and in acute hippocampal slices leads to an increase of the synaptic levels of N-Cadherin FL and of spine head width and to a modification of AMPA receptor currents and composition at synapse.

To assess the role of ADAM10 activity in modulating long-lasting rearrangements of
glutamatergic synapse during development, we performed an *in vivo* treatment with TAT-Pro in young mice (2 weeks old).

24 hours after intraperitoneal administration of TAT-Pro (3 nmol/g, see Marcello et al., 2007), ADAM10 interaction with SAP97 was reduced (Marcello et al., 2007) as shown by co-immunoprecipitation assay (Fig. 10a). Also *in vivo*, we found no modifications of SAP97 (p=0.7474) and ADAM10 (p=0.9862, n=22 animals from 3 independent experiments) levels in total homogenate (Fig. 10b), suggesting that the observed uncoupling can be ascribed to a decreased association rather than to changes in levels of these proteins. Conversely, a significant reduction of ADAM10 in Triton Insoluble post-synaptic Fraction (TIF) was observed (Fig. 10c; TAT-Pro vs TAT-Ala, -34.7% ± 4.4%, p<0.05; n=22 animals from 3 independent experiments) with no modification of SAP97 levels (p=0.0533); thus indicating a decreased ADAM10 synaptic localization. As consequence, 24 hours after administration of TAT-Pro, N-Cadherin FL/CTF ratio was increased at synapse (Fig. 10d; TAT-Pro vs TAT-Ala, +110.3 ± 16.7%, p<0.05; n=22 animals from 3 independent experiments), indicating a reduction of ADAM10-mediated N-Cadherin cleavage.

Morphological analysis of the size of dendritic spines was conducted using a diolistic gene gun system to propel Dil-coated particles into hippocampal sections of fixed brain tissue of TAT-Pro and TAT-Ala treated mice (Fig. 10e). Statistical analysis revealed a significant increase of spine head width (Fig. 10f; p<0.005, n>900 spines from 18 neurons from 4 different animals for each group) after treatment with TAT-Pro and cumulative frequency plots of spine head width confirmed the significant shift towards bigger spines (Fig. 10g). A significant decrease in the mean spine density was also found after *in vivo* TAT-Pro treatment (TAT-Ala 6.72 ± 0.46 spines/10μm; TAT-Pro 5.53 ± 0.31 spines/10μm; p<0.05).

Moreover, mice treated with TAT-Pro peptide showed a significant increase of the levels of GluR1 subunit of AMPA receptor (Fig. 11c; TAT-Pro vs TAT-Ala, +38.1 ± 7.6%, p<0.05; n=22 animals from 3 independent experiments) in the post-synaptic compartment when compared to TAT-Ala treated ones. No changes of synaptic abundance of GluR2 AMPA receptor subunit (p=0.8703) and of all tested NMDA receptor subunits (Fig. 10d; NR1 p=0.1700, NR2A p=0.6748, NR2B p=0.7424; n=22 animals from 3 independent experiments) were found 24 hours after *in vivo* treatment with TAT-Pro. No modification was observed in the total homogenate neither for AMPA (Fig. 11a; GluR1 p=0.3755; GluR2 p=0.9241) nor for NMDA (Fig. 11b; NR1 p=0.3779; NR2A p=0.6611; NR2B p=0.6398) receptor subunits.

To confirm that the above described modification of GluR1 localization at synapse is mediated by ADAM10/N-Cadherin pathway and not by a nonspecific effect of TAT-Pro on SAP97 interaction with GluR1 (Gardoni et al., 2003; Waites et al., 2009) a co-immunoprecipitation assay was performed. Total homogenates from mice treated with TAT-Pro and TAT-Ala were immunoprecipitated with an antibody against SAP97 and GluR1 co-immunoprecipitation was evaluated. Mice treated with TAT-Pro showed no modification of GluR1/SAP97 complex compared to animals treated with TAT-Ala (Fig. 11e; p=0.5763, n=3).

Again, as we did *in vitro*, we tested whether impairing ADAM10-mediated cleavage of N-Cadherins *in vivo* was accompanied by changes in synaptic currents. Fourteen days-old mice were injected intraperitoneally with either TAT-Pro or TAT-Ala peptides (3 nmol/g), and hippocampal slices were prepared 24 hours later. AMPA and NMDA receptor-mediated EPSCs were recorded from CA1 pyramidal cells respectively at -80 mV and +40 mV.
Figure 11. In vivo effect of ADAM10 synaptic activity on AMPA receptor composition and currents. 

a) Western blot analysis performed with antibodies for AMPA receptor subunits showed no modification of GluR1 (p=0.3756) and GluR2 (p=0.9241) levels in total homogenate of brain from mice treated with TAT-Pro compared to brain from mice treated with TAT-Ala (22 animals from 3 independent experiments).  
b) Western blotting of NR1, NR2A and NR2B subunits of NMDA receptor shows no changes of NMDA receptor levels in total homogenate of brain from mice treated with TAT-Pro compared to brain from mice treated with TAT-Ala (NR1 p=0.3779; NR2A p=0.6611; NR2B p=0.6398; TAT-Pro vs TAT-Ala; 22 animals from 3 independent experiments).  
c) Western blot analysis performed in TIF with antibodies against AMPA receptor subunits; TAT-Pro increased GluR1 subunit level (p<0.05, 22 animals from 3 independent experiments), when compared to animals treated with TAT-Ala control peptide. No change of synaptic abundance of GluR2 AMPA receptor subunit was found (p=0.8703, 22 animals from 3 independent experiments) after 24h in vivo treatment with TAT-Pro inhibitory peptide.  
d) Western blot analysis performed on TIF with antibodies against NR1, NR2A and NR2B subunits of NMDA receptor (NR1 p=0.1700, NR2A p=0.6748, NR2B p=0.7424, 22 animals from 3 independent experiments).  
e) Total homogenate from mice treated with TAT-Ala and TAT-Pro was immunoprecipitated with an antibody raised against SAP97 and western blot analysis was performed with an antibody against GluR1 subunit of AMPA receptor. Animals treated with TAT-Pro showed no modification in GluR1 co-immunoprecipitation with SAP97 (p=0.5763) compared to animals treated with TAT-Ala.  
f) Averaged EPSCs recorded at -80 mV and + 40 mV from CA1 pyramidal cells from mice injected, 24 hr prior slicing, with Tat-Ala and Tat-Pro ADAM10 peptides (3 nmol/gr). (Scale bars: 50 pA, 50 ms).  
g) Bar graph summarizing the AMPA/NMDA ratio obtained from mice injected with Tat-Ala or Tat-Pro peptide (p<0.05; TAT-Pro, 2.05 ± 0.29, n=13; TAT-Ala, 1.18 ± 0.19, n=14). Decay times of responses at -80mV were 14.8 ± 0.1 and 14.5 ± 0.1 ms in TAT-Pro and TAT-Ala group respectively; p=0.86; decay times responses +40mV were 135.5 ± 10.1 and 147.3 ± 4.1 ms in TAT-Pro and TAT-Ala group respectively (n=13-14; p=0.26).
The NMDA receptor-mediated contribution was measured 40 ms after initiation of the EPSC, a time point at which AMPA receptor-mediated EPSCs are absent or minimal. Treatment of mice with TAT-Pro induced a significant increase of the AMPA/NMDA ratio compared to TAT-Ala treatment (Fig. 11 f,g; TAT-Ala 1.18 ± 0.19; TAT-Pro 2.05 ± 0.2; p<0.05; n=13-14 recordings for each group). No significant differences were observed in the decay times of EPSCs recorded at -80 (p=0.86) and +40 mV (p=0.26).

**Synaptic Activity Modulates ADAM10-mediated N-Cadherin cleavage.**

We demonstrated *in vitro* and *in vivo* that, under basal-unstimulated conditions, inhibition of ADAM10-dependent N-Cadherin cleavage leads to a specific and long-lasting modification of the morphology of the glutamatergic synapse as well as of AMPA receptor subunits composition and function.

We then asked whether these mechanisms could be modulated by synaptic activity. To this, DIV10 hippocampal neurons were treated with bicuculline (50 μM, 30 minutes). After treatment, TIF was purified and analyzed by western blot. As shown in Fig. 12a, neurons stimulated with bicuculline showed a significant increase of ADAM10 levels in the TIF (Bicuculline vs Control, +71.0% ± 20.5%, p<0.005; n=13).

Since ADAM10 is active only when inserted into the post-synaptic membrane, we performed a surface assay with the cross-linker B3 after treatment with bicuculline: in lysates of neurons treated with bicuculline, intracellular amount of ADAM10 was decreased when compared to control cells (Fig. 12b; Bicuculline vs Control, -36.3% ± 6.2%, p<0.05; n=3), indicating an increased insertion of the enzyme into the membrane. As expected, the increased ADAM10 localization at the post-synaptic membrane was associated with a decrease in N-Cadherin FL/CTF ratio (Fig. 12c; Bicuculline vs Control, -41.4% ± 6.7%, p<0.0005; n=13). On the other hand, treatment with TTX (2 μM), a neural activity blocker, for 30 minutes led to decreased ADAM10 levels at synapses (Fig. 12d; TTX vs Control, -31.3% ± 7.4%, p<0.01; n=7) that was not paralleled by any modification of N-Cadherin FL/CTF ratio (Fig. 12e; p=0.6729, n=7) and GluR1 levels at synapses (Fig. 12f; p=0.1408, n=7). Moreover 3 hours TTX treatment did not result in any modification of ADAM10 levels (Fig. 12g; p=0.7107, n=4) at synaptic sites as well as of N-Cadherin FL/CTF ratio (Fig. 12h; p=0.7852, n=4) and GluR1 (Fig. 12i; p=0.8705, n=4), suggesting that TTX induces only a transient effect on ADAM10 localization at synaptic sites not sufficient to affect N-Cadherin/GluR1 pathway.

**ADAM10 Activity is Needed for Activity-dependent N-Cadherin Processing.**

To verify whether the above-described bicuculline-induced N-Cadherin cleavage in neurons was mediated by ADAM10, bicuculline treatment was repeated in presence or absence of TIMP-1. DIV10 hippocampal neurons were treated or not with TIMP-1 (15nM, 1hour) and then stimulated with bicuculline (50 μM, 30 minutes). As expected, stimulation with bicuculline increased ADAM10 localization to the post-synaptic compartment in absence as well as in presence of TIMP-1 (Fig. 13a,b; p<0.01 among groups; bicuculline vs control, +108.6% ± 24.2%, p<0.005; TIMP-1 + bicuculline vs control, +72.0% ± 19.2%, p<0.05; n=7) but, pre-treatment with TIMP-1 prevented the increase of ADAM10-mediated N-Cadherin cleavage induced by bicuculline.
Figure 12. Synaptic activity modulates ADAM10-mediated N-Cadherin cleavage.

a) Primary hippocampal neurons were stimulated or not with Bicuculline (50 µM, 30 minutes). Stimulation with Bicuculline increased ADAM10 amount in the TIF (p<0.005, n=13).

b) Surface assay performed with the cross-linker BS3, a membrane impermeable amine-reactive cross-linker reagent, performed on primary hippocampal neurons after stimulation with Bicuculline (50 µM, 30 minutes). Western blot analysis shows a significant decrease of the intracellular amount of ADAM10 (p<0.05, n=3) in neurons treated with Bicuculline when compared to control cells; all data have been normalized using tubulin. ADAM10 surface pool was not detectable due to the formation of high molecular weight aggregates that did not enter the gel.

c) Stimulation with Bicuculline (50 µM, 30 minutes) induced a statistically significant decrease of N-Cadherin FL/CTF ratio (p<0.00005, n=13).

d) Western blot analysis performed in TIF after treatment with TTX (2 µM, 30 minutes); TTX treatment induced a statistically significant decrease of ADAM10 amount (p<0.01, n=7), but no modification of N-Cadherin FL/CTF ratio (p=0.6729, n=7) and of GluR1 (p=0.1408, n=7) and GluR2 (p=0.4258, n=7).

e) Western blot analysis performed in TIF after treatment with TTX (2 µM) for 3 hours; TTX treatment did not induce any modification of ADAM10 amount (p=0.7107, n=4), as well as of N-Cadherin FL/CTF ratio (p=0.7852, n=4) and GluR1 (p=0.8705, n=4).
(Fig. 13a,c; FL/CTF; p<0.05 among groups; bicuculline vs. control, -40.4% ± 11.3%, p<0.0005; TIMP-1 + bicuculline vs. bicuculline, +134.6 ± 40.6%, p<0.05; n=7). Thus, TIMP-1 blocks activity-dependent cleavage of N-Cadherin mediated by ADAM10 without affecting ADAM10 subcellular localization.

Since bicuculline increases ADAM10 localization to the post-synaptic membrane and its activity on N-Cadherin and since ADAM10 localization in TIF is regulated by SAP97, we asked if SAP97 was involved in ADAM10 localization to the postsynaptic membrane. To this end, primary hippocampal neurons (DIV10) were pre-treated with TAT-Pro or TAT-Ala (10 µM, 2 minutes) and then stimulated with bicuculline (50 µM, 30 minutes). TAT-Pro treatment reduced ADAM10 (Fig. 13d,e; TAT-Pro vs TAT-Ala, -45.6% ± 7.5%, p<0.005; n=5) localization in TIF also in neurons pre-treated with bicuculline. Accordingly, the reduced ADAM10 localization in the post-synaptic membrane was associated with an enhanced N-Cadherin FL/CTF ratio (Fig. 13d,f; TAT-Pro vs TAT-Ala, +103.2 ± 33.5%, p<0.05; n=5). These results indicate the binding to SAP97 as necessary also for activity-dependent localization and activity of ADAM10 at synaptic sites.

Figure 13. ADAM10 activity is needed for activity-dependent N-Cadherin processing.

a) Primary hippocampal neurons were incubated or not with the ADAM10 specific inhibitor peptide TIMP-1 (15 nM, 1 hour) and then stimulated with bicuculline (50 µM, 30 minutes); untreated cells were used as control. b) Neurons pretreated or not with TIMP-1 and then stimulated with bicuculline showed statistically significant increased amount of ADAM10 in the TIF when compared to controls (p<0.01 among groups, *p<0.005, Bicuculline vs. Control; #p<0.05, TIMP-1+Bicuculline vs. Control, n=7). c) Neurons stimulated with bicuculline showed statistically significant modification of N-Cadherin FL/CTF ratio (p<0.05 among groups, *p<0.0005, Bicuculline vs. Control; #p<0.05 TIMP-1+Bicuculline vs. Bicuculline, n=7; one-way ANOVA, followed by Bonferroni’s as a post hoc comparison test) when compared to control and TIMP-1 treated cells. d) Western blot analysis performed in TIF with antibodies against ADAM10 and the C-terminal region of N-Cadherin. Primary hippocampal neurons (DIV10) were incubated with both TAT-Ala and TAT-Pro ADAM10 peptides for two minutes and then stimulated with bicuculline (50 mM, 30 minutes). e) Neurons pretreated with TAT-Pro inhibitory peptide show a statistically significant decrease of ADAM10 localization in the TIF (p<0.005; TAT-Pro vs TAT-Ala, n=5) and f) a consequent increase of N-Cadherin FL/CTF ratio (p<0.05, TAT-Pro vs TAT-Ala, n=5) when compared to TAT-Ala pretreated cells.
**MOLeULAR MECHANISMS REGULATING SPINE REMODELLING**

**RESULTS**

![Diagram](image)

**Figure 14. PACAP-38 increases ADAM10 activity.**

a) ADAM10 activity as α-secretase was evaluated on APP metabolism by performing western blot analysis with 4G8 antibody raised against its C-terminal domain, able to recognize C-terminal fragments from both ADAM10 (CTF83) and BACE (CTF99) cleavage. Treatment with PACAP-38 showed a statistically significant increase of CTF83 production (p=0.0008; n=3), no modification of CTF99 (p=0.0669, n=3) and b) a consequent decrease of CTF99/CTF83 ratio (p=0.0072; n=3) when compared to control, indicating increased ADAM10 α-secretase activity. c) Western Blot analysis on TIF revealed, after treatment with PACAP-38, an increase of ADAM10 localization (p=0.0194; n=4) and no significant modification in SAP97 level (p=0.1871, n=4), when compared to control. d) DIV10 primary hippocampal neurons were immunolabeled for ADAM10 (green) and Shank (red). PACAP-38 (300nM) induced an increase of ADAM10/Shank colocalization (p=0.0035, n=10) if compared to control.

**ADAM10 Activation by PACAP-38 Influences N-Cadherin Metabolism and AMPA Receptor Composition.**

Bicuculline induces a nonspecific increase of synapse activity, in order to stimulate ADAM10 activity in a more specific manner, we treated neurons with the pituitary adenylate cyclase-activating polypeptide 38 (PACAP-38), recently shown to induce ADAM10 activity, particularly the α-secretase cleavage of the amyloid precursor protein (APP) (Kojro et al., 2006). PACAP-38 is a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) family and it is known to play an important role during brain development as well as in learning and memory processes (Miyata et al., 1989; Arimura, 1998). DIV10 primary hippocampal neurons were treated with PACAP-38 (300nM) and ADAM10-dependent effect on APP metabolism was at first checked to confirm the capability of PACAP-38 to activate ADAM10 also under our experimental conditions (Fig. 14). In the TIF, the amount of APP C-terminal fragments, CTF83, deriving from ADAM10 α-secretase activity, and CTF99, deriving from β-secretase pathway, were measured and CTF99/CTF83 ratio calculated (see Marcello et al., 2007). Statistic analysis showed a significant increase of ADAM10-mediated...
CTF83 production (Fig. 14a; PACAP-38 vs control, +86 ± 9.5%, p=0.0008; n=3), no modification of CTF99 (p=0.0669, n=3) and a consequent decrease of CTF99/CTF83 ratio (Fig. 14b; PACAP-38 vs control, -36 ± 7.1%, p=0.0072; n=3) in neurons treated with PACAP-38 compared to control, indicating that ADAM10 α-secretase activity was increased by the treatment with the neuropeptide. Notably, western blotting analysis performed in the TIF shows that treatment with PACAP-38 induced also ADAM10 trafficking to the post-synaptic compartment (Fig. 14c; PACAP-38 vs control, +79 ± 17.2%, p=0.0194; n=4), without affecting synaptic levels of its cargo protein SAP97 (PACAP-38 vs control, +30 ± 17.8%, p=0.1871; n=4). Biochemical data were further confirmed by immunocytochemical labeling of ADAM10 in hippocampal neurons (DIV10). Treatment with PACAP-38 (300 nM) significantly increased co-localization of ADAM10 with the post-synaptic marker Shank (Fig. 14.d; PACAP-38 vs control +63.7 ± 9.3%, p=0.0035; n=10).

This effect was associated to an increased ADAM10-dependent N-Cadherin metabolism as shown by the decreased N-Cadherin FL/CTF ratio (Fig. 15a; PACAP-38 vs control, -34 ± 4.6%, p=0.0186; n=3) in the TIF of PACAP-38 treated neurons compared to controls. To test the effect of PACAP-38 on AMPA receptor subunits localization at the post-synaptic site we treated acute hippocampal slices with PACAP-38 (300nM); TIF was then prepared and western blot analysis performed with antibodies against GluR1 and GluR2 (Fig. 15b). Treatment with the neuropeptide PACAP-38 did not affect GluR2 subunit level (p=0.6365, n=7), but induced a significant decrease of GluR1 level in the TIF.

Figure 15. ADAM10 activation by PACAP-38 influences N-Cadherin metabolism and AMPA receptors localization

a) Western blot analysis on TIF. Neurons treated with PACAP-38 showed a statistically significant decreased of FL/CTF ratio (p=0.0186; n=3), compared to non treated cells. b) Western blot analysis performed in the TIF showed a statistically significant reduction of GluR1 (p=0.0087; n=7) but no modification of GluR2 (p=0.6365, n=7) subunit of AMPA receptor after treatment with PACAP-38. c) DIV10 primary hippocampal neurons were immunolabeled for GluR1 (green) and Shank (red). PACAP-38 (300nM) induced a statistically significant reduction of GluR1/Shank colocalization (p=0.0336, n=6) if compared to control.
Figure 16. ADAM10 activation by PACAP-38 influences dendrite spines size
a) Primary hippocampal neurons (DIV7) were transfected with GFP (upper panels), GFP and HA-N-Cadherin Wt (middle panels) or with GFP and HA-N-Cadherin GD (lower panels). At DIV 10 they were treated (left panels) or not (right panels) with PACAP-38. b) Overexpression of the cleavage-defective HA-N-Cadherin GD mutant induced per se a significant increase of spine head width when compared to neurons transfected with N-Cadherin Wt or GFP alone (p<0.0001 among groups, p=0.0029 GD vs GFP, p=0.0132 GD vs Wt; n>500 spines from 10-12 neurons from two independent experiments for each group). Treatment with PACAP-38 significantly decreased spine head width in neurons transfected with GFP alone (p=0.0204, n>500 spines from 10-12 neurons from two independent experiments for each group) as well in neurons transfected with HA-N-Cadherin Wt (p<0.0004) if compared to non treated cells. Instead, overexpression of the cleavage-defective HA-N-Cadherin GD mutant prevented ADAM10-mediated modification of spine morphology after treatment with PACAP-38 (p=0.9735).

c) Representative time-lapse imaging of hippocampal neurons transfected with GFP (DIV7) and incubated with PACAP-38 (300nM) d) Spine head width was measured at all set times and expressed as percentage of initial values; PACAP-38 reduced spine head width compared to control neurons (p<0.005, n>80 spines from 4 independent experiments for each group).

e) Dendritic spines have been divided into two groups depending on their head width (cut-off 0.8 µm). For each group of spines, the average head width at end point (30 minutes) has been calculated and expressed as percentage of the initial value. PACAP-38 decreased average head width in both populations of dendritic spines when compared to control (Width < 0.8 µm: p<0.0001; Width ≥ 0.8 µm: p=0.0213).

f) Diagram showing statistical analysis of growing and shrinking spines after 30 minutes treatment with PACAP-38, expressed as percentage of total number of spines. PACAP-38 induced a statistically significant reduction of the percentage of growing spines (p=0.0492, n>500 spines from 10-12 neurons from two independent experiments for each group) and a statistically significant increase of shrinking ones (p=0.0386).
was observed over 30 minutes. An increase modification of average spine head width, I and expressed as percentage of initial values.

The spine head width was measured every 3 minutes. PACAP affected at primary hippocampal neurons transferred with GFP (DIV7) with PACAP-38 (300nM) for 30 minutes (Fig. 16a, upper panels). Also in this case, to confirm that the observed results were mediated by ADAM10-mediated N-Cadherin cleavage, we repeated the treatment with PACAP-38 in neurons transfected with GFP and HA-N-Cadherin Wt or the cleavage-defective HA-N-Cadherin GD mutant (Fig. 16a, middle and lower panels).

Treatment with PACAP-38 significantly decreased spine head width (Fig. 16b) in neurons transfected with GFP alone (p=0.0204, n>500 spines from 10-12 neurons from two independent experiments) as well as neurons transfected with HA-N-Cadherin Wt (p<0.0004) if compared to non treated cells. Instead, overexpression of the cleavage-defective HA-N-Cadherin GD mutant prevented ADAM10-mediated modification of spine morphology after treatment with PACAP-38 (p=0.9735).

Also in this case, to investigate more deeply the kinetic of these modification, we performed time-lapse imaging experiments on primary hippocampal neurons transfected at DIV7 with GFP treated with PACAP-38 (300nM; see Fig. 16c). Spine head width was measured every 3 minutes and expressed as percentage of initial values. In basal conditions, no significant modification of average spine head width was observed over 30 minutes. An increase of spine head width, that became statistically significant 21 minutes after treatment, was observed in PACAP-38 treated neurons if compared to controls (Fig. 16c,d; p<0.005; n>80 spines from 4 independent experiments for each group). Statistical analysis revealed that treatment with PACAP-38 induces a reduction of the percentage of growing spines (Fig. 16f; p=0.0492) and an increase of the percentage of shrinking ones (Fig. 16f; p=0.0386).

Moreover, dendritic spines smaller (Fig. 16e; p<0.0001) as well as larger (Fig. 16e; p=0.0213) than the cut off value of 0.8 μm undergo this morphological shrinkage. Thus, stimulating ADAM10 activity by PACAP-38 is able to induce a reduction on dendritic spine size, which is mediated by ADAM10-dependent N-Cadherin cleavage.
**Figure 17.** Diagrammatic representation of the complex sequence of events through which modulation of ADAM10-dependent N-Cadherin cleavage affects spine maturation and controls structure and function of glutamatergic synapses.
Discussion.
Here we demonstrate that synaptic activity of ADAMA10, a metalloprotease belonging to the disintegrin and metalloprotease family represents a new mechanism regulating morphological and functional maturation of the glutamatergic excitatory synapse (see Figure 17). Interfering with ADAM10 localization at the post-synaptic compartment is sufficient to induce a significant decrease of ADAM10-mediated N-Cadherin cleavage, leading to accumulation of N-Cadherin FL. This molecular event correlates with an increase in spine head width and with an increase of AMPA receptors expression and AMPA receptor-mediated currents both in vitro and in vivo. Conversely, stimulating ADAM10 activity induces a reduction of dendritic spine head width and of AMPA receptors localisation at post-synaptic membrane.

The existence of a close coordination among N-Cadherin, dendritic spine size and AMPA receptor content has been put forward: N-Cadherin activation results in more AMPA receptors and, consequently, in bigger and stable synapses (Kasai et al., 2003; Xie et al., 2008; Kopec et al., 2007); moreover, N-Cadherin is more expressed in potentiated synapses where is required for long-term stabilisation (Mendez et al., 2010). Here we show that ADAM10-dependent cleavage of N-Cadherin represents an upstream event of these pathways. Up- or down-regulation of ADAM10-mediated processing of N-Cadherin induces significant modifications in spine head width in both small-immature and large-mature spines, as observed within half an hour in time-lapse experiments. Interestingly, morphological modifications occur 18-20 minutes following N-Cadherin FL stabilisation/distabilisation and remain stable up to 30 minutes.

Notably, these observations have been confirmed by in vivo experiments. The TAT-Pro peptide utilized in the present study is a useful tool not only for in vitro but also for in vivo studies. This is of relevance because in vivo study of N-Cadherin processing was formerly hampered by the lack of sensitive tools. In particular, here we show that the in vivo effect of ADAM10 inhibition on spine morphology is quite long lasting and stable, being significant still 24 hours after treatment.

It has been shown that accumulation of N-Cadherin FL results in more GluR1 synaptic clusters, whereas N-Cadherin inactivation results in less GluR1 clusters (Xie et al., 2008). Accordingly, our results show that increased ADAM10-mediated N-Cadherin cleavage reduces GluR1 localisation at the post-synaptic compartment, while 30 minutes after stabilization of the full-length form of N-Cadherin a significant recruitment of GluR1 into the post-synaptic compartment is detectable. Electrophysiological experiments show that recruitment of GluR1 is paralleled by an increase in AMPA receptor-mediated currents. No significant changes in the rectification index were observed in cells dialysed with ADAM10 inhibitory peptide suggesting the absence of any significant modification in the percentage of GluR2 containing AMPA receptors. This is only an apparent discrepancy between molecular and electrophysiological analysis; in fact, molecular analysis performed in the TIF allows detecting modifications of AMPA receptor subunits’ levels at synaptic sites, but does not give information on modifications of AMPA receptor subunits insertion/endocytosis taking place within the synaptic compartment.

Long-term persistence of the effect of ADAM10 inhibition on synaptic GluR1-containing AMPA receptors and on AMPA receptor currents was demonstrated by 24 hours in vivo experiments, again confirming the close correlation between spine size and GluR1 levels.

The major issue of the present thesis is to analyse molecular, functional and morphological outcomes of the ADAM10/N-
Cadherin pathway. Recent papers provided indications that ADAM10 is responsible for the first step of the proteolytic processing of N-Cadherin, leading to the generation of the CTF1 fragment (see chapter 3.4). In the present study, we link ADAM10/N-Cadherin pathway to morphological and functional modifications of glutamatergic postsynapses. Moreover, we show that transfection of hippocampal neurons with the cleavage-defective N-Cadherin construct, mutated in the ADAM10 cleavage site, induces enlargement of spine size and recruitment of AMPA receptors, not observed after transfection with N-cadherin wild-type, possibly by adding at the cell membrane a new pool of N-cadherin that could not be cleaved by ADAM10 thus altering the N-cadherin FL/CTF ratio. This evidence strengthens the key role of N-Cadherin catabolism in modulating spine size and synaptic function.

It is noteworthy that ADAM10 is responsible for both constitutive and activity-dependent N-Cadherin cleavage. Treatment with bicuculline shows that it is possible to increase ADAM10 synaptic localization and consequently its activity towards N-Cadherin upon neuronal activity. Conversely, blocking neuronal activity with TTX, a significant decrease of ADAM10 localization at synapse is achieved. Further, both TAT-Pro peptide and TIMP-1 block in vitro ADAM10 activity stimulated by neuronal activity. Overall, these results confirm the already proposed role for N-Cadherin in homeostatic plasticity (Peng et al., 2009), suggesting that ADAM10-dependent N-Cadherin cleavage could represent a new modality regulating the strength of the excitatory synapse.

This is not the first report showing that a molecular and functional connection between the ADAMs family/adhesion molecules and scaffolding elements of the PSD-MAGUKs family (Fukata et al., 2006) leads to modifications of AMPA receptor-mediated currents. Thus, since several types of adhesion molecules and PSD-MAGUKs -with redundant functions- are present at synapse, it is likely that functional cross-talks between these key families of synaptic proteins are relevant to control synaptic maturation and stabilization in vivo. We have also previously shown such a functional correlation between PSD-MAGUKs and ADAMs family, describing a direct interaction between ADAM10 and SAP97-member of the PSD-MAGUK family (Marcello et al., 2007).

Here we have cleared up the morphological and functional outcomes of this interaction on the glutamatergic synapse, demonstrating an important role for ADAM10 in the complex and coordinated sequence of events through which N-Cadherin affects spine maturation.
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