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PLASTICITY OF THE DEVELOPING GLUTAMATE SYNAPSE IN THE HIPPOCAMPUS

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Abstract
Synapses are highly plastic, i.e. they have the ability to change their signaling strength both in the short- and long-term (e.g. long-term potentiation - LTP) in response to specific patterns of activity. In the developing brain synaptic plasticity promotes activity-dependent development, whereas in the mature brain synaptic plasticity forms the basis for learning and memory. Although both development and learning involve organization and reorganization of synaptic circuits, the extent to which the plasticity behind these two phenomena uses the same mechanisms is unknown. The glutamate synapse which represents > 90 % of the brain synapses signals mainly via postsynaptic AMPA and NMDA receptors. In the developing brain, sparse synaptic activation can make the synapse lose its AMPA signaling capacity, i.e. make it AMPA silent, while LTP can reinstall the AMPA signaling (unsilencing). The aim of this study was to investigate the possible role of the AMPA silent synapse, and its unsilencing, in developmental and mature synaptic plasticity. Electrophysiological recordings of synaptic transmission in the CA1 region and in the dentate gyrus of acute hippocampal slices were used for these studies.

A new and unexpected finding was that AMPA unsilencing can also be induced by not activating the AMPA silent synapse for tens of minutes. Together with previous findings this suggests a model in which the glutamate synapse is born with a single AMPA labile module, i.e. the synapse cycles between an AMPA silent state, induced by sparse synaptic activity, and an AMPA signaling state, induced by the absence of synaptic activity. The results further suggest that AMPA silencing is a prerequisite for developmental LTP to occur. In other words, developmental LTP does not potentiate synaptic transmission but rather stabilizes the AMPA labile module. It can, however, transiently potentiate the synapse by the addition of a labile AMPA module to an existing synapse with a single stable AMPA module. After this initial period of synaptic stabilization there is an increase in synaptic connectivity between pre- and postsynaptic neurons. It is proposed that this increased connectivity can be explained, at least partly, by the addition of stable AMPA modules to existing synapses promoted by mature LTP. This thesis thus proposes that, using the same principle mechanism, namely the addition of stable AMPA modules, developmental LTP promotes initial synaptic stabilization while mature LTP promotes synaptic growth.

Keywords: synaptic plasticity, long-term potentiation, short-term potentiation, silent synapse, development, glutamate, hippocampus

POPULÄRVETENSKAPLIG SAMMANFATTNING

Nervcellerna i hjärnan signalerar till varandra via så kallade synapser. Dessa synaptiska kopplingar har förmågan att förändra sin signaleringsstyrka beroende på vilka signaleringsmönster de utsätts för, ett fenomen som kallas för synaptisk plasticitet. Den mänskliga hjärnan innehåller tusen biljoner synapser, vilka förbinder nervcellerna i funktionella nervcellsnätverk. Synapserna bildas under fostertiden samt under de första åren i barnets liv. Dock pågår en kontinuerlig omarbetning och mognad av det synaptiska nätverket, först för att dessa nätverk skall bli ändamålsenliga, sedan under resten av vår livstid för att lära oss nya fakta och färdigheter som lagras som minnen i vår hjärna. Denna process är ofrånkomligt beroende av att synapserna utsätts för ”rätt” sorts signaleringsmönster. En ökning av synapsens effektivitet kallas långtidspotentiering (LTP) och tros ligga bakom vår förmåga till minne och inlärning. LTP är emellertid också viktig tidigt i utvecklingen för att skapa funktionella nervcellsnätverk. Felaktig utveckling av synapserna anses ligga bakom ett flertal sjukdomstillstånd, såsom mental retardation, schizofreni och demens, således är anläggningen av det synaptiska nätverket en oerhört viktig process.

Glutamat är den dominerande kemiska substans som nervcellerna använder sig av vid signalering, eftersom ca 90% av alla hjärnans synapser är så kallade glutamatsynapser. Dessa synapser innehåller två huvudtyper av receptorer dit glutamat kan binda, AMPA- samt NMDA-receptorer. AMPA-receptorerna används vid den normala signaleringen medan NMDA-receptorerna behövs för synaptisk plasticitet. En radikal form av synaptisk plasticitet är total avstängning och aktivering av AMPA-signaleringen som kan få glutamatsynapsen att bli AMPA-tyst respektive AMPA-signalerande. Betydelsen av AMPA-tysta synapser under hjärnans utveckling är dåligt utredd. Syftet med denna avhandling är att få ny kunskap om detta.

Studien är utförd på tunna hjärnskivor som hålls vid liv i vävnadsvätska. Dessa skivor är tagna från nyfödda till vuxna råttor, vilket motsvarar den mänskliga hjärnan från strax före födelsen till vuxen ålder. Jag har studerat två olika synapsgrupper i ett område i hjärnan som kallas hippocampus, en struktur som har avgörande betydelse för vår förmåga till minne och inlärning. Genom att med hjälp av tunna elektroder nedstuckna i hjärnskivan elektriskt stimulera och registrera nervcellsaktivitet har synapsernas funktion och plasticitet studerats.
Ett nytt viktigt resultat är att om AMPA-tysta synapser inte aktiveras kontinuerligt, så blir de AMPA-signalerande. Denna AMPA-signalering är dock labil, dvs den kan tystas med några få synaptiska aktiveringar. Detta är, vad vi vet, en helt ny form av aktivering och avstängning av AMPA-signalering, dvs den nyfödda glutamatsynapsen saknar den stabila signalöverföring som återfinns hos mogna synapser. Jag fann vidare att funktionen för LTP under den tidiga utvecklingen är att omforma den omogna, labila, glutamatsynapsen till en mogen mer stabil synaps. Under utvecklingen sker en drastisk minskning av de labila glutamatsynapserna medan de stabila ökar i antal samtidigt som de synaptiska kopplingarna mellan två givna nervceller blir fler. Denna senare synaptiska tillväxt är sannolikt ett uttryck för minne och inlärning, till skillnad från den tidiga synaptiska stabiliseringen som snarare reflekterar skapandet av funktionella nervcellsnätverk. En viktig slutsats i denna avhandling är att LTP under den tidiga utvecklingen ansvarar för den synaptiska stabiliseringen medan den mogna formen av LTP ansvarar för den synaptiska tillväxten.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Abrahamsson T., Gustafsson B. and Hanse E.
Synaptic fatigue at the naïve perforant path-dentate granule cell synapse in the rat.
Journal of Physiology (2005) 569.3 pp 737-750

II. Abrahamsson T., Gustafsson B. and Hanse E.
A reversible synaptic depression in developing rat CA3-CA1 synapses explained by a novel cycle of AMPA silencing-unsilencing.
Submitted

III: Abrahamsson T., Gustafsson B. and Hanse E.
AMPA silencing: a prerequisite for LTP at developing CA3-CA1 synapses.
In manuscript

IV. Abrahamsson T., Gustafsson B. and Hanse E.
Hebbian induction adds an AMPA labile signaling module to developing AMPA signaling CA3-CA1 synapses.
In manuscript

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ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
AMPA	α-methyl-4-isoxazoleproprionic acid
CA	Cornu Ammonis
αCaMKII	α-Calcium-calmodulin-dependent kinase II
CV	Coefficient of variation
D-AP5	D(-)-2-amino-5-phosphonopentanoic acid
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
GABA	γ- aminobutyric acid
GDP	Giant depolarizing potential
GluR	AMPA receptor subunit
HFS	High-frequency stimulation
IPSP	Inhibitory postsynaptic current
LTP	Long-term potentiation
LTD	Long-term depression
m	Quantal content

mGluR	Metabotropic glutamate receptor
n	Number of functional release sites
NMDA	N-methyl-D-aspartate
NR	NMDA receptor subunit
NSF	*N*-ethylmaleimide-sensitive fusion protein
PKA	Protein kinase A
p	Release probability
P	Postnatal
p_{ves}	Release probability of a single vesicle
PPR	Paired-pulse ratio
PSD	Postsynaptic density
q	Quantal size
STP	Short-term potentiation
TARP	Transmembrane AMPA receptor regulatory protein

INTRODUCTION

The brain consists of 10^{11} neurons communicating with one another via specialized connections called synapses, a term introduced by Sherrington more than a century ago for the connection where information is transferred from one neuron to the other. Each neuron makes approximately 10^4 synapses onto other neurons, giving the brain about 10^{15} synaptic connections. A most salient feature of the brain is its ability to adapt to an ever-changing environment. An important basis for this ability is the plasticity of this myriad of synapses, that is, their capacity to change their signaling strength, both in the short- and in the long-term, in response to specific patterns of synaptic activity. In the immature brain synaptic plasticity forms the basis for its activity dependent development (Katz and Shatz, 1996), while in the more mature brain synaptic plasticity is a basis for learning and memory (Martin et al., 2000). These two phenomena (brain development and learning) both basically involve brain organization and reorganization, and it has been suggested that the synaptic plasticity in the adult brain, underlying learning, is a remnant of the more ubiquitous synaptic plasticity in the developing brain (Kandel and O'Dell, 1992). However, even though the synaptic plasticity that occurs during development in many respects resembles the adult plasticity qualitative discrepancies between the two have been found (see section on developmental LTP). These discrepancies suggest that the synaptic plasticity may in itself adapt to the different requirements for synaptic reorganization during brain development vs. learning.

The vast majority of synapses in the brain uses glutamate as transmitter. Although not restricted to the glutamate synapse, synaptic plasticity, especially long-term plasticity, has been studied mostly in glutamate synapses, and preferentially in a region important for explicit learning, the hippocampus (see Methods). These studies have established the existence of prolonged (minutes to months) increases, as well as decreases, in synaptic efficacy in response to specific and differential synaptic activation patterns, termed long-term potentiation (LTP) and long-term depression (LTD), respectively. Studies of hippocampal LTP/LTD have pointed to a number of possible expression mechanisms for these phenomena, suggesting a potential for plasticity in many components of the synaptic transmission process. However, studies of LTP/LTD have been performed on a number of different hippocampal preparations, including acute hippocampal slices taken from animals of various ages, hippocampal slice cultures and cultures of hippocampal neurons. The array of expression

mechanisms involved in synaptic plasticity that have been suggested on the basis of findings in these different models may reflect actual mechanisms used by a given synapse at any given time, or alternatively reflect an adaptation of plasticity to different developmental or experimental conditions.

In the present work I have studied hippocampal glutamate synapses at different developmental stages using a single experimental preparation, the rodent acute hippocampal slice. Whereas, as noted above, LTP has been associated with manifold expression mechanisms, a commonly proposed mechanism is an all or none (binary) switch of the synapse, from a non-signaling (silent) to a signaling (unsilenced) state. Such a switch in signaling state is commonly thought to relate to an activity-dependent acquirement of glutamate receptors to the postsynaptic membrane where none existed before. In my work I will specifically examine to what extent this unsilencing process may contribute to LTP at various developmental stages, i.e., whether it represents an actual expression mechanism at any given time, or an adaptation of plasticity present only within a specific developmental period.

A brief outline of ionotropic synaptic transmission

The synapse consists of a presynaptic bouton and a postsynaptic receptor structure physically tightly connected to each other via proteins bridging a synaptic cleft of about 15 nm. The presynaptic bouton contains all of the machinery required for the release of neurotransmitter-containing vesicles. When the action potential reaches the presynaptic bouton, Ca^{2+} enters through voltage-gated calcium channels. The increase in Ca^{2+} concentration at the release site (from < 1 μM to > 100 μM) increases the probability that a vesicle will fuse with the presynaptic membrane and release its content of neurotransmitter, the so called quantal release. The probability of release following an action potential can vary between synapses from almost zero to almost one depending on the synapse type, the animal age, the recent synaptic activity, as well as on the presence of release-modulating substances (such as endocannabinoids, acetylcholine, monoamines, neuropeptides, gliotransmitters and hormones). When the transmitter is released into the synaptic cleft it diffuses to the abutting postsynaptic structure and binds to its receptors. These receptors are located in the postsynaptic density (PSD), an area of the postsynaptic membrane containing a high concentration of neurotransmitter receptors as well as structural and signaling proteins.

Depending on what type of receptor that is activated, ligand-gated channels, permeable for different kinds of ions, are opened resulting in an ion flux causing changes in the postsynaptic membrane potential. In glutamate synapses the released transmitter gives rise to a depolarizing excitatory postsynaptic potential (EPSP) preferentially due to the influx of Na^+. On the contrary, an inhibitory transmitter, e.g. GABA, gives rise to a hyperpolarizing inhibitory postsynaptic potential (IPSP), preferentially due to the influx of Cl^-. These postsynaptic potentials (PSPs) will then spread through the postsynaptic dendrite towards the cell soma where they are summated in the initial segment and, if there exceeding a threshold depolarization, give rise to an action potential. The probability to elicit an action potential is not only controlled by the PSPs, but also by various kinds of voltage-gated and calcium-gated ion channels intrinsic to the soma-dendritic membrane. These ion channels, in common with the presynaptic release probability, are continuously subject to regulation by modulatory transmitters (cf. above)

In addition to the ionotropic synaptic transmission briefly outlined above, transmitters, including glutamate, bind to G-protein-coupled receptors, the so called metabotropic synaptic transmission. In this type of transmission the transmitter-receptor interaction does not directly lead to ion fluxes but to the production of 2^{nd} messengers, like cAMP, which activate enzymes such as protein kinases that phosphorylate e.g. ion channels. The metabotropically acting transmitter can thus modulate the functional properties, and thus the activity, of ligand-gated, voltage-gated and calcium-gated ion channels (see above).

What determines synaptic efficacy - quantal parameters

That synaptic release is quantal, i.e., that the postsynaptic response is made up of multiples of one single quantum, corresponding to the action of a single vesicle, was worked out in the 1950s using the neuromuscular junction (Del Castillo and Katz, 1954a, b).Thus, the strength, or the efficacy, of a synapse is determined by the following parameters, n (the number of functional release sites), p (the release probability at these sites) and q (the quantal size, i.e. the size of the synaptic response elicited from the release of a single vesicle). Hence, the amount of transmitter released, $n \times p$, is referred to as m (quantal content), and the synaptic strength is equal to $n \times p \times q$, and is represented by the mean amplitude of the evoked synaptic response.

In general, changes in *n* or *p* are believed to stem from a presynaptic locus, while an alteration in *q* is believed to be of postsynaptic origin. However, as indicated above and as will be discussed below there are exceptions. Most notably, a change in *n* can also be of postsynaptic origin, e.g. unsilencing of silent synapses (see below).

Quantal parameters for hippocampal synapses

For hippocampal synapses these three quantal parameters vary considerably between synapses and there are also important developmental changes. For the experimentally most commonly used hippocampal synapse, the glutamate synapse between CA3 and CA1 pyramidal cells, the CA3-CA1 synapse, *n* increases from 1 during the first two postnatal weeks to, on average, about 5 in adults (Hsia et al., 1998). The release probability (*p)* can vary between zero and almost one for these synapses, a reasonable average value being 0.1-0.3 (Hessler et al., 1993; Rosenmund et al., 1993; Dobrunz and Stevens, 1997; Hanse and Gustafsson, 2001c). *p* is further determined by the release probability of a single vesicle, p_{ves}, and by the size of the immediately releasable pool of vesicles (pool) such that $p = 1 - (1 - p_{ves})^{pool}$ (Hanse and Gustafsson, 2001c). Quantal size, *q,* is determined by a number of factors including the amount of glutamate in the vesicles, the diffusion distance of glutamate in the synaptic cleft and the number of functional postsynaptic receptors. Vesicle glutamate transporters control the concentration of glutamate, which may vary substantially between vesicles and contribute to the variation in quantal size at a given synapse (Wilson et al., 2005; Wu et al., 2007). A variation in the volume of the vesicle, and thereby glutamate content, may also contribute to the quantal variance (Bekkers et al., 1990). It should be noted that even if the diameter of vesicles is fairly constant, in the 30-35 nm range, even small differences in diameter lead to large differences in volume. On the other hand, the width of the synaptic cleft shows remarkably little variation, and it has been argued that this width is optimized for maximal quantal size (Savtchenko and Rusakov, 2007). Glutamate uptake (preferentially into astrocytes) and diffusion barriers in the synaptic cleft may potentially also influence the quantal size. Finally, the number of functional postsynaptic glutamate receptors of the AMPA type, the AMPA receptor (AMPAR, see section Glutamate receptor types), or rather the density of AMPARs opposite to the release site, is a main determinant of quantal size (Lisman et al., 2007). The number of AMPARs varies greatly between hippocampal synapses, numbers between 0 and 140 having been reported (Nusser et al., 1998). Each AMPAR has four binding sites for glutamate and the conductance of the channel increases with increased

number of bound glutamate (Rosenmund et al., 1998). The conductance of AMPAR channels thus depends on the concentration of glutamate, the conductance ranging from a few pS to about 12 pS when increasing the glutamate concentration from 200 nM to 20 mM (Gebhardt and Cull-Candy, 2006). It has been estimated that during synaptic activation of hippocampal synapses the mean unitary conductance is around 8 pS at the peak of the synaptic response (Benke et al., 1998). Maximal open probability for AMPARs, at least as judged from extrasynaptic AMPARs, has been estimated to 0.7 (Momiyama et al., 2003). Thus, with 0 to 140 AMPARs with a mean conductance of 8 pS , a maximal open probability of 0.7, and at a membrane potential of -80 mV the quantal size should vary between 0 and maximally about 80 pA, which is also what is generally found for glutamate synapses (e.g. Raastad et al., 1992; McAllister and Stevens, 2000; Hanse and Gustafsson, 2001b; Groc et al., 2002a).

Quantal analysis in central synapses

When analyzing synaptic function and synaptic plasticity it is often desirable to determine the quantal parameters. However, classical quantal analysis using amplitude histogram is usually not feasible at central synapses due to the variability in release probability and mean quantal size (McAllister and Stevens, 2000; Hanse and Gustafsson, 2001c, b). Moreover, quantal variance, i.e. the trial-to-trial variability in quantal size at a given synapse, is often large and also varies between synapses (McAllister and Stevens, 2000; Hanse and Gustafsson, 2001b; Franks et al., 2003; Chen et al., 2004).Instead, other methods such as paired-pulse ratio, miniature excitatory postsynaptic current (EPSC) analysis, coefficient of variation (CV) analysis and failure analysis have to be used in order to deduce quantal parameters for CNS synapses.

Paired-pulse ratio

A common method to deduce whether a change in presynaptic release probability has occurred is to measure the paired-pulse ratio (PPR) (for review, see Zucker and Regehr, 2002). When two presynaptic stimulations are given in rapid succession, the size of the second postsynaptic response relative to that of the first is related to the release probability of the activated synapses. Synapses with low release probability show paired-pulse facilitation, i.e. the second response is larger than the first response, whereas synapses with high release probability show paired-pulse depression. However, while the PPR method is sensitive to

changes in the release probability caused by a change in release probability of a single vesicle, it is much less sensitive to a change in release probability caused by a change in the vesicle pool size (Hanse and Gustafsson, 2001a). Moreover, when examining a population of synapses varying in release probability (and thus in PPR) changes in PPR may be related to postsynaptic rather than presynaptic mechanisms if these postsynaptic mechanisms affect synapses with different release probability differentially (Poncer and Malinow, 2001). In addition, if the same synapse releases transmitter to both stimuli (presumably a rather rare event), the second response may be affected by desensitization, i.e. that the postsynaptic receptors have transiently entered a non-conducting, desensitized, state after the first exposure to neurotransmitter.

Miniature EPSC analysis

Changes in quantal properties can also be estimated by recording miniature PSC events which derive from action potential independent release of vesicles. The average amplitude of these spontaneous events represents the average quantal size, whereas the frequency of these events is correlated with the quantal content, *m*. However, some caution must be exercised since spontaneous and evoked release can be differentially affected (Maximov et al., 2007) (see further Developmental LTP in Discussion).

CV analysis

Another method to estimate quantal changes, using evoked responses, is to measure the trial-to-trial variability of the response and to calculate the $1/CV^2$, where CV is defined as the standard deviation divided by the mean. A change in *n* or *p* generally results in a corresponding change in the $1/CV^2$ value whereas a change in *q* should not affect it. Evoked responses also lend themselves to a failure analysis. If the stimulation is weak enough to activate only a few synapses, response failures will occur. An alteration in the frequency of failures indicates a change in *n* or *p*. The use of CV analysis for central synapses has been criticized (Korn and Faber, 1991). The most important argument for this critique is that the mean quantal size often varies substantially between synapses and that quantal size often varies substantially in a given synapse (Hanse and Gustafsson, 2001b; Franks et al., 2003; Chen et al., 2004). Nevertheless, it has been shown empirically that the CV analysis when

applied to central synapses faithfully report changes in *n* and *p*, but not in *q* (Manabe et al., 1993; Chen et al., 1998).

Glutamate receptor types

The glutamate synapse is by far the most common type of synapse in the brain constituting about 90% of all synapses (Megias et al., 2001). Two morphologically distinct glutamate synapses exist, termed spine and shaft synapses. The postsynaptic densities of spine synapses are located on small dendritic protrusions, spines, and this type of synapse dominates on principal cells in the adult brain. In contrast, shaft synapses are formed directly onto the dendritic shafts and are common on GABAergic interneurons and on principal cells when the first synapses are formed early in development. There are generally two distinct ionotropic glutamate receptor types in the postsynaptic density, AMPA and NMDA receptors.

When glutamate binds to the AMPAR the channel pore opens and cations, mostly Na^+ and K^+ diffuse in and out of the cell, respectively, and give rise to an excitatory current that lasts for a few ms. AMPARs are responsible for most of the fast excitatory synaptic transmission in the brain. AMPARs are tetramers and can be composed from four different subunits, GluR1 – 4, (also called GluRA-D) (Hollmann and Heinemann, 1994). The subunits consist of an extracellular N-terminus, four membrane-associated domains and an intracellular C-terminus (Bredt and Nicoll, 2003), of which the latter contains one or several PDZ-domains, which are important binding sites for cytosolic proteins. The AMPARs also contain auxiliary subunits, the so called TARPs, which are important for receptor trafficking and channel function (Nicoll et al., 2006). The GluR2 subunit is important for the control of channel properties, and receptors lacking the GluR2 subunit exhibit Ca^{2+} permeability and an inward rectification (Isaac et al., 2007). In fact, if not Q/R edited (a substitution of glutamine for arginine at a single site in the GluR2 subunit) also GluR2-containing AMPARs exhibit these properties. The AMPAR transcripts may also undergo alternative splicing, resulting in either a flip or a flop version, exhibiting partly different characteristics, e.g. the flip isoform desensitize with slower kinetics than the flop isoform (Sommer et al., 1990; Mosbacher et al., 1994). In adult hippocampal principal cells, GluR1, 2 and 3 are the dominating subunits expressed, with the dominant subtype combinations being receptors made of GluR1 and GluR2, or GluR2 and GluR3 (Wenthold et al., 1996).

NMDARs differ from AMPARs in several ways (for review, see Dingledine et al., 1999). Most importantly, their activation is both ligand- and voltage dependent. The voltage-dependent block by Mg^{2+} means that the postsynaptic membrane needs to be depolarized for the channel to conduct ions. In contrast to most AMPAR channels, NMDAR channels are highly Ca^{2+} permeable. The NMDARs also have a much higher affinity for glutamate, which results in a more long-lasting synaptic current, about 100 ms or more. The NMDAR acts as a coincidence detector, signifying that ion flow is only permitted through the channel when both the pre- and the postsynaptic cells are excited, a feature that is decisive for synaptic plasticity (see below). Similar to AMPARs, NMDARs are heterotetramers consisting of two NR1 subunits, which bind the co-agonist glycin, and two NR2 subunits which bind glutamate. Four different NR2 subunits have been identified, NR2A-D, which provide for NMDARs with different functional properties, e.g., various durations of the synaptic response. In the hippocampus, NR2A and B are the major subunits expressed.

Glutamate also acts as a modulatory transmitter through the activation of kainate receptors and metabotropic glutamate receptors (mGluRs). Kainate receptors can modulate presynaptic release probability via both ionotropic and metabotropic mechanisms (Lerma, 2003; Lauri et al., 2006). Activation of presynaptic mGluRs, generally belonging to group II (mGluR2-3) or group III (mGluR4 and mGluR6-8) reduce release probability (Cartmell and Schoepp, 2000). Activation of postsynaptic mGluRs, generally belonging to group I (mGluR1 and mGluR5), produce the PKC activator diacyglycerol and IP_3, releasing Ca^{2+} from intracellular stores, and has, in addition to NMDARs, been implicated in the postsynaptic induction of synaptic plasticity (Bortolotto et al., 1999).

Glutamate receptor trafficking

Glutamate receptors are not stable within the PSD, but are subjected to a continuous turn-over on time scales that can range from ms to hours. The number of synaptic glutamate receptors thus relies on a dynamic equilibrium between synaptic and non-synaptic (intracellular and extrasynaptic membrane) receptor pools. The glutamate receptors traffic laterally, i.e. by surface diffusion both within the synapse, and between synaptic and extrasynaptic membrane (Choquet and Triller, 2003). They also undergo vertical trafficking, i.e., to and from the plasma membrane through exocytosis and endocytosis, respectively (Malinow and Malenka, 2002). This trafficking is regulated by a number of proteins in the PSD that interact directly or

indirectly with the receptors (for details, see Discussion). These proteins are specific not only with respect to glutamate receptor type (e.g AMPA) but also with respect to the subunit composition (e.g. GluR1), and their posttranslational state (e.g. phosphorylated, or not), thereby providing for a very high degree of specificity in the trafficking of the glutamate receptors. Modulation of this trafficking is now considered a major plasticity mechanism for the glutamate synapse.

Plasticity of the glutamate synapse

The ability of a synapse to respond to changes in its activity with an increased or decreased synaptic efficacy is called synaptic plasticity. Plasticity changes can last for ms up to may be years and have been found to occur in most excitatory synapses in the brain, albeit with different types of induction and expression depending on factors such as animal age and brain region. Synaptic plasticity is broadly categorized in, on one hand, short-term and long term plasticity and, on the other hand, potentiation and depression. Beyond these categories there are also homeostatic plasticity (changes in global synaptic efficacy in response to global changes in activity) and metaplasticity (plasticity of synaptic plasticity).

Short-term plasticity

Short-term plasticity is a modulation of synaptic strength following repetitive synaptic activity that covers a time scale of ms up to at most a few minutes (for review, see Zucker and Regehr, 2002). Generally there are three types of short-term plasticity associated with an increase in transmission, namely facilitation, augmentation and post-tetanic potentiation, all of which are presynaptically located and thought to rely on an activity-dependent increase in the cytoplasmic Ca^{2+} level of the bouton. Facilitation is elicited by brief synaptic activations and decays within about 100 ms. An example of this kind of short-term plasticity, mentioned above, is paired-pulse facilitation. Augmentation has a fixed decay time constant of approximately 5 s whereas post-tetanic potentiation has a decay time constant that increases with increasing duration of a high-frequency train stimulation and can last up to a few minutes following long stimulus trains. Short-term plasticity is time dependent, i.e. it decays irrespective of whether the synapse is activated, or not. Synaptic activation can also result in a short-term depression, caused e.g. by depletion of readily releasable vesicles or by inactivation of presynaptic voltage-dependent calcium channels (Kavalali, 2007).

Long-term plasticity

In 1949, Donald Hebb postulated that simultaneous activation of the pre- and postsynaptic elements should trigger the reinforcement of the active input, or "cells that fire together wire together". Such synaptic strengthening was proposed to be the cellular basis for learning and memory (Hebb, 1949). In 1973, Bliss and Lømo discovered that a long-lasting change in synaptic strength occurred at the hippocampal perforant path-granule cell synapse in response to brief tetanic stimulation (Bliss and Lomo, 1973). This finding was the first of what has later become known as long-term potentiation (LTP). Generally, a burst of high-frequency activity increases the efficacy of the synapse, an increase that can last for minutes up to months, may be years. The induction of this potentiation requires Ca^{2+} influx through NMDAR channels, for example as revealed by the fact that blockade of NMDARs prevents the induction of LTP (Collingridge et al., 1983). High-frequency stimulation of a large population of presynaptic axons (strong stimulation) is the most common manner of inducing LTP. Such stimulation activates AMPARs at many synapses resulting in a large postsynaptic depolarization that together with the released glutamate open up NMDAR channels at the activated synapses. Thus, many synapses need to be active at the same time for the NMDARs to open and LTP to be induced, a characteristic of LTP called cooperativity (McNaughton et al., 1978). The NMDAR thus acts as a detector for coincident pre- and postsynaptic activity (Wigstrom and Gustafsson, 1986). Another important feature of LTP is that it is input specific (Andersen et al., 1977), meaning that the increased efficacy only occurs in those synapses that were active during the high-frequency stimulation. However, a weak input can be potentiated if its activation is paired with a tetanic stimulation to another input, a feature called associativity (Levy and Steward, 1979). In 1986 Hebbs' postulate was proven correct when it was directly shown that simultaneous activation of the pre- and postsynaptic neuron is sufficient for the induction of LTP (Wigstrom et al., 1986). It was observed that even low-frequency stimulation could induce LTP in single hippocampal CA1 pyramidal cells if the stimulation was given in conjunction with a strong depolarizing pulse. Hence, weak low-frequency stimulation is sufficient to induce LTP as long as the postsynaptic cell is adequately depolarized. Thus the LTP induction does not depend on high-frequency stimulation per se.

LTP induction

In a typical LTP experiment the synapses are first activated at a low frequency, the so called test frequency, generally between 0.01-0.2 Hz, a frequency assumed to maintain the synapse in its naïve state. When a stable baseline has been reached, a conditioning stimulation is given to induce plasticity, after which the test frequency is resumed. LTP can be induced in several ways, but most commonly using a high-frequency electrical stimulation of the afferent axons. Using this method high-frequency trains are repeated a few times, common protocols are to use a single pulse train at 100 Hz for 1 second or several trains repeated with seconds apart. The strong high-frequency stimulation gives rise to glutamate release and causes a depolarization of the postsynaptic cell; hence the conditions for LTP induction are fulfilled. By blocking $GABA_A$ receptors, thereby enhancing the train-induced depolarization by removing evoked postsynaptic inhibition, the induction of LTP is greatly facilitated and much shorter trains are sufficient for a powerful induction of LTP (Wigstrom and Gustafsson, 1983). Another manner to induce LTP is to use theta bursts (Larson et al., 1986) to mimic more physiologically relevant stimuli, i.e. to mimic the theta rhythm, an endogenous hippocampal rhythm. Theta bursts consist of ten short bursts of four or five pulses at 100 Hz, repeated at 5 Hz. When the whole-cell configuration is used the most common method to induce LTP is the pairing protocol where 1-2 Hz synaptic activation is paired for 1-2 minutes with current-induced depolarization of the postsynaptic cell.

LTP time course

The onset of NMDAR-dependent potentiation is fast, in the hippocampus potentiation begins within 2 - 3 s after a brief tetanus, reaches its peak after about 30 s, and then decays for about 5-15 min before reaching a more stable value (Gustafsson et al., 1989; Hanse and Gustafsson, 1994a). The NMDAR-dependent potentiation is often categorized by its different phases after the induction; a short-term potentiation (STP) covering the early decaying phase, an early LTP and a late LTP (> a few hours). Following an LTP induction there is thus usually an STP that also requires correlated pre- and postsynaptic activity for its induction (Gustafsson et al., 1987). STP lasts for about 5-15 min, decays in a stimulation dependent manner (Volianskis and Jensen, 2003) and appears to occur only when high-frequency stimulation has been used as induction protocol. For reasons that are not clear STP is not seen using the pairing protocol for LTP induction. The relationship between STP and LTP is unclear (Malenka and Nicoll, 1993; Hanse and Gustafsson, 1994a; Stevens et al., 1994; Lauri et al., 2007). Many

pharmacological and genetic interventions, for example of protein kinase activity (Lauri et al., 2007) that block LTP, often leave an isolated STP, suggesting a mechanistic separation between STP and LTP. However, since protein kinase inhibition affects an isolated STP to about the same extent as LTP (Hanse and Gustafsson, 1994b) it is doubtful whether the interpretation from the above studies, in which isolated STPs were not examined, holds. Moreover, in some studies, inhibition of αCaMKII has totally blocked both STP and LTP (e.g. Chen et al., 2001). Following the STP there is a fairly stable potentiation, the early-LTP, lasting for about an hour, or so. After this time gene transcription and protein synthesis are required to sustain the potentiation for longer periods, a state called late-LTP (Sajikumar et al., 2005; Schuman et al., 2006), which is then generally defined as the LTP persisting after 1-2 hours.

LTP expression

Ever since LTP was first discovered in the hippocampus there has been a controversy as to whether the mechanisms that directly enhance the synaptic efficacy are mainly pre- or postsynaptically located. Expression mechanisms that have been discussed include addition of AMPARs into the postsynaptic density, increases in AMPAR channel conductance or increased presynaptic release probability (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). Some controversy may arise from the fact that a variety of LTP induction protocols are used and that different phases of LTP has been examined. Moreover, additional controversy might be caused by the use of different developmental stages in different studies since the expression mechanisms of LTP may change during development (see Developmental LTP below).

LTD

Synapses can also undergo a long-lasting weakening of synaptic strength, termed long-term depression (LTD). LTD, discovered in the hippocampal CA1 region in the early 1990s (Mulkey and Malenka, 1992; Dudek and Bear, 1993), is typically induced by a low-frequency stimulation protocol (LFS), 600-900 stimuli at 1 Hz and its induction typically relies on activation of NMDARs or on mGluRs (Kemp and Bashir, 2001). As for LTP, the expression of LTD may involve both pre- and postsynaptic mechanisms and may vary during development. The threshold for inducing LTD is generally lower among developing,

compared to mature, synapses (Wagner and Alger, 1995; Wasling et al., 2002; Pavlov et al., 2004).

Homeostatic plasticity and metaplasticity

When the general activity level in a synaptic network is altered for a prolonged period of time (at least a day), a homeostatic mechanism takes place which upregulates the synaptic strength if the activity level has been low, and vice versa (Turrigiano and Nelson, 2000). This homeostatic plasticity, or synaptic scaling, has been suggested to promote network stability and a constant level of activity. For example, if a $GABA_A$ antagonist is applied the overall activity initially increases but will eventually return to the original level, possibly due to a reduction of surface AMPARs (Lissin et al., 1998; Turrigiano and Nelson, 2004).

Synaptic plasticity itself depends on the prior history of synaptic activity, a characteristic called metaplasticity (Abraham and Bear, 1996). The basis for this type of plasticity is an alteration of the induction threshold for synaptic plasticity due to prior activity that per se has not changed the synaptic efficacy. For example, if a given high-frequency stimulation is not strong enough to elicit an increase in synaptic strength this stimulation can still result in an inhibition of subsequent induction of LTP (Huang et al., 1992).

Development of the glutamate synapse

The CA1 and CA3 pyramidal cells proliferate in the rat between embryonic days 17 and 19 (Bayer, 1980). Newly generated neurons migrate from the subventricular zone to their final destination, where they start growing their neurites. The growth of these extensions is guided by molecular cues that either attract or repel the neurites. Interneurons are generated before pyramidal cells, and GABAergic synapses precede the birth of the glutamatergic synapses (Ben-Ari, 2001). In rats, the majority of the CA3-CA1 synapses are generated between P0 and P30 (Steward and Falk, 1991), with a slow increase in synaptic density during the first postnatal week, followed by a rapid increase up to puberty, around P30, when the brain is believed to have reached maturity (*Figure 1*). At birth, the development of the rat brain corresponds to that of the human brain of a half-term fetus (Hagberg et al., 2002). After approximately the second postnatal week the rat brain has reached the same stage of development as the human brain at birth.

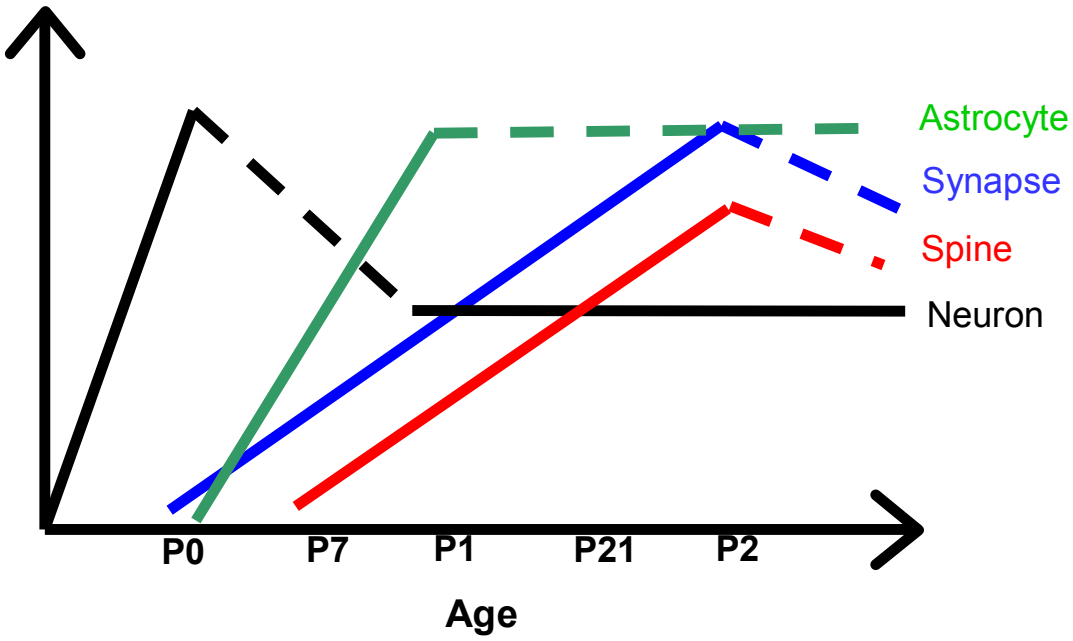

Figure 1. Schematic representation of the relative development of neurons, astrocytes, synapses and spines in the rat CA1 region.

The development of the glutamate synapse can be divided into two stages, the synaptogenesis, i.e. the birth of the synapse, and the synaptic maturation. The maturation can, in turn, be divided into collective maturation (seen as a change in the average behavior of a synaptic population during development) and individual maturation (seen as a specific change in an individual synapse). Examples of collective maturation are changes in subunit composition of postsynaptic receptors and a decrease in release probability. Such collective changes might be governed by neural activity, i.e. by synaptic plasticity that involves the majority of synapses, or they might proceed irrespective of neural activity, e.g. as a consequence of a genetic differentiation program or as the result of endocrine/paracrine signals. On the other hand, individual synaptic changes are purely activity dependent and their purpose is to develop appropriate neural networks, where "appropriate" synapses are strengthened and "inappropriate" ones eventually eliminated.

Synaptogenesis

As noted above, both activity-dependent and activity-independent mechanisms are involved in the development of synaptic networks, but the dependence on activity increases as the brain matures. The initial formation of synaptic connections, synaptogenesis, depends on cell-to-

cell contacts and is thus not dependent on neural activity. This was shown for example by Verhage et al, who produced a mouse strain that lacked munc-18, a protein important for synaptic transmitter release (Verhage et al., 2000). Even though the mice more or less lacked synaptic activity their nervous system, including synaptic contacts, developed normally until birth. For a synapse to form, dendritic filopodia make contacts with nearby axons and induce formation of a presynaptic terminal along the axon (Garner et al., 2002). The formation of the presynaptic bouton includes clustering of synaptic vesicles and the formation of an active zone, where the transmitter-containing vesicles dock and fuse with the cell membrane. Conversely, on the postsynaptic side, opposite the presynaptic active zone, a PSD, where receptors and other molecules are clustered and localized to the postsynaptic membrane, is formed.

Synaptic maturation

Following synaptogenesis neural activity becomes increasingly important for the maturation of synaptic circuits, for reorganization and refinement of the developing connections. During the early period of synaptic development in the hippocampus (mainly the first postnatal week) the neural activity largely consists of spontaneous, recurrent network-driven large synaptic events, or giant depolarizing potentials (GDPs), associated with intracellular Ca^{2+} oscillations (Ben-Ari, 2001). This activity probably governs the early synaptic maturation in the hippocampus and it has been found to affect neuronal incorporation into the neural network (Ge et al., 2006) and to affect the growth of dendrites (Groc et al., 2002b).

Collective synaptic maturation

During development several changes occur in the composition of the glutamatergic ionotropic receptors. For example, during the first two postnatal weeks GluR4 and $GluR2_{long}$ (a C-terminal splice form of GluR2) are expressed in hippocampal principal neurons (Zhu et al., 2000; Kolleker et al., 2003). The GluR4 subunit is otherwise only expressed in GABAergic interneurons. The level of GluR4 reaches its peak at postnatal day (P) 2, and at this time the amount of GluR1, 2 and 4 is about the same, whereas at P10 the expression of GluR4 is almost nil (Zhu et al., 2000). $GluR2_{long}$ is expressed during the embryonic stage, but peaks between P7 and P15, after which it decreases (Kohler et al., 1994; Kolleker et al., 2003). With respect to the NMDAR subunits, the NR2A/NR2B expression ratio increases during the third postnatal week in the hippocampus (Barria and Malinow, 2002). Thus, at early postnatal

stages NR2B-containing receptors are more important while NR2A has an essential role at the mature synapses. NR2B by its higher affinity for glutamate gives rise to a more prolonged EPSP than does NR2A, the subunit switching thus leading to a shortening of the NMDAR-mediated response with increasing age of the rat (Monyer et al., 1994). There may thus be enhanced coincidence detection by the NMDA receptors in developing synapses and a decreased ability for the induction of synaptic plasticity in the adult animal (Crair and Malenka, 1995). Also, NR2B containing receptors are located both synaptically and extrasynaptically, whereas NR2A containing receptors are generally found at the center of the glutamate synapse (Tovar and Westbrook, 1999).

Before the first ten postnatal days, or so, CA3 pyramidal cells are connected to CA1 pyramidal cells with maximally one release site (Hsia et al., 1998; Groc et al., 2002a). Thereafter there is a gradual increase in this connectivity until adulthood when the number of release sites is estimated to be around 5 (Hsia et al., 1998). This is a fundamental collective maturation of the CA3-CA1 synaptic connectivity possibly driven by LTP, but that remains to be tested. Another distinct collective maturation at these synapses is a decrease in the probability of transmitter release that occurs during the second postnatal week (Muller and Lynch, 1989; Bolshakov and Siegelbaum, 1995; Wasling et al., 2004).

Individual synaptic maturation

Individual synaptic maturation is the selective strengthening and elimination of "appropriate" and "inappropriate" synapses, respectively, to develop functional neural networks. Although much remains to be unraveled about the plasticity governing this synaptic maturation, the AMPA silent synapse and its associated plasticities as well as developmental LTP should have prominent roles.

AMPA silent synapses

One of the most salient features of the developing brain is that many glutamate synapses are functionally silent, i.e., they do not display any evoked transmission at the resting membrane potential. Hence when such synapses are activated NMDAR-mediated responses are found (when keeping the cell depolarized) while no AMPAR-mediated responses are found. These synapses are therefore being referred to as AMPA silent synapses (Isaac et al., 1995; Liao et

al., 1995; Durand et al., 1996). It is generally held that AMPA silence is explained by an absence of AMPARs (Malinow and Malenka, 2002), although alternative explanations have been forwarded (Kullmann and Asztely, 1998; Choi et al., 2000; Gasparini et al., 2000). Electrophysiological approaches indicate that in the neonatal hippocampus AMPA silent synapses should constitute at least half of the population of glutamate synapses (Liao et al., 1995; Durand et al., 1996; Hsia et al., 1998), while anatomical evidence points to a figure of about 30% of the population (Nusser et al., 1998; Petralia et al., 1999). The relative amount of AMPA silent synapses decreases throughout development, with about 17% of the synapses in the CA1 area in the mature rat being AMPA silent when investigated using immunogold labeling (Nusser et al., 1998).

AMPA silent synapses can be converted into AMPA signaling synapses by correlated pre- and postsynaptic activity (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996). This LTP based on AMPA unsilencing can be observed as a decrease in the failure rate or in the synaptic variance (CV) (Isaac et al., 1995; Liao et al., 1995; Xiao et al., 2004). The most likely expression mechanism behind AMPA unsilencing is a fast recruitment of AMPARs to the synapse (Malinow and Malenka, 2002; Ward et al., 2006). The unsilencing of the AMPA silent synapses can be seen as both collective and individual maturation, since the relative number of silent synapses decreases with age in the population and since the switch is activity-dependent and occurs in the individual synapse.

It was initially assumed that glutamate synapses are born AMPA silent (Durand et al., 1996). However, it was later found that spontaneous AMPAR and NMDAR-mediated responses in CA1 pyramidal cells occur in equal proportion throughout the first two postnatal weeks, indicating that newborn glutamate synapses signal via both types of receptors (Groc et al., 2002a). Xiao et al subsequently demonstrated that it is possible to create AMPA silent synapses by mere low-frequency stimulation (as low as 0.05 Hz), suggesting that the glutamate synapse is born AMPA labile, rather than AMPA silent (Xiao et al., 2004). This study used an unconventional way of recording in that the very first synaptic response evoked in the slice, referred to as the naïve response, was used as the reference, while in contrast the conventional way is to wait until obtaining a stable baseline of responses to be used as a reference for subsequent plasticity. Thus, using the naïve response as reference AMPA signaling in the neonatal CA3-CA1 synapse was quickly diminished by the low-frequency stimulation, while the NMDA responses remained stable, indicating that AMPA silent

synapses were created. To verify that the depression observed in fact was a total removal of a subset of the AMPA signaling synapses, the change in the variance of the EPSCs was established, along with an increased number of EPSC failures. This induction of AMPA silencing did not require NMDAR or mGluR activation. In these experiments only whole-cell recordings were performed. Hence the possibility remains that the whole-cell configuration promotes AMPA silencing, e.g. by a wash out of substances essential for the stability of AMPA signaling. Also, since AMPA silencing is very easily induced it is remarkable that when recording spontaneous EPSCs no AMPA silent synapses were found (Groc et al., 2002a). A possible solution to this apparent contradiction is that the AMPA silent state is not stable, but can revert back to an AMPA signaling state within tens of minutes. These two aspects of AMPA silencing were investigated in this thesis.

The question arises whether AMPA silencing is a feature unique for the developing CA3-CA1 synapses or if it can be observed in other synapses as well. It has previously been shown that AMPA silent synapses exist among developing perforant path-granule cell synapses (Ye et al., 2000; Poncer and Malinow, 2001), but whether they are created by AMPA silencing or born AMPA silent is not known. I have investigated also this issue in my thesis. The question of AMPA silencing in perforant path – granule cell synapses is of additional interest because of the cellular development of the granule cells in the dentate gyrus. While almost all neurons in the brain are born before the birth of the animal, the granule cells in the dentate gyrus of the hippocampus have their peak proliferation, when approximately 50000 cells are generated each day in the subgranular layer, between postnatal days 5 and 8 (Schlessinger et al., 1975; Altman and Bayer, 1990). Moreover, the dentate granule cells continuously proliferate throughout adulthood, albeit at a low rate (Altman and Das, 1965). This feature has been extensively studied during the last decades not the least because of its possible influence on our ability for learning and memory. Some of the newborn granule cells are incorporated into the synaptic network; hence the dentate gyrus also exhibits a prolonged synaptogenesis. Does this characteristic have an overall impact on the level of AMPA silencing? If AMPA silencing can be elicited in this synapse, is it then also found in adult animals? These adult-generated cells can exhibit characteristics different from their neighboring mature neurons, one of the most important being the lowered threshold for LTP induction (Snyder et al., 2001; Schmidt-Hieber et al., 2004).

Developmental LTP

It was long held that LTP could not be induced in animals below the age of 8 days (Harris and Teyler, 1984). However, it was later shown, using patch-clamp recordings, that it is perfectly possible to induce LTP in neonatal animals (Liao and Malinow, 1996), if sufficient depolarization of the postsynaptic cell can be provided. Is the mechanisms underlying this developmental LTP the same as those underlying the LTP that forms the basis for learning and memory in the more adult animal? It has been put forward that the synaptic plasticity in the adult nervous system is a remnant of the more ubiquitous synaptic plasticity in the developing nervous system (Kandel and O'Dell, 1992). One key aspect that also appears to be common for developmental and mature LTP is the requirement for NMDAR activation for their inductions (Durand et al., 1996; Liao and Malinow, 1996). However, even though the LTP that occurs during development resembles the adult LTP in some respects, important discrepancies between the two have been discovered.

Thus, conversion of AMPA silent synapses into AMPA signaling synapses has been proposed as an important mechanism explaining LTP (Malinow and Malenka, 2002). However, such a mechanism is not likely to explain much of LTP in mature animals since there should be very few AMPA silent synapses after the developmental period (Liao et al., 1995; Durand et al., 1996; Hsia et al., 1998). In this thesis I address the question of the relative importance of AMPA unsilencing as a mechanism for LTP at different developmental stages. It should be noted that irrespective of to what extent incorporation of AMPARs at previously silent synapses contributes to LTP at various developmental stages, the critical involvement of some form of AMPAR trafficking for LTP at any developmental stages now seems taken for granted (Malinow and Malenka, 2002). However, many key proteins involved in this trafficking change with development. With respect to AMPAR trafficking and LTP there seems to be a shift in the importance of different AMPAR subunits during development. The GluR1 subunit appears to have a central role in the expression of adult hippocampal LTP since GluR1 subunits are delivered to the synapse during LTP (Hayashi et al., 2000; Plant et al., 2006) and adult GluR1-/- mice are deficient in LTP in the CA3-CA1 synapse (Zamanillo et al., 1999). However, this mouse still exhibits developmental LTP (Jensen et al., 2003). Which are then the important AMPAR subunits for developmental LTP? Conversion of silent synapses into functional synapses is believed to be due to delivery of receptors containing the

GluR4 (Zhu et al., 2000) and $GluR2_{long}$ subunits (Kolleker et al., 2003). GluR4 and $GluR2_{long}$ can be inserted into the synapse by spontaneous synaptic activity or by LTP induction, GluR4 and $GluR2_{long}$ more important during the first and second postnatal week, respectively (see Collective synaptic maturation). For example, when $GluR2_{long}$ insertion was blocked in about two week old GluR1-/- mice LTP was abolished (Kolleker et al., 2003). Thus, it seems as if different AMPAR subunits are critical for AMPAR trafficking during LTP at different developmental stages; GluR4 and $GluR2_{long}$ during the first postnatal weeks whereas GluR1 becomes gradually more critical the more mature the synapses become.

Another important difference between developmental and mature LTP is that developmental LTP requires activation of protein kinase A (PKA), whereas mature LTP requires activation of αCaMKII (Yasuda et al., 2003). Thus, inhibitors of αCaMKII did not affect the level of LTP at the end of the first postnatal week, whereas this LTP was abolished by blocking the activity of PKA. In line with this finding, αCaMKII activation is not required for synaptic delivery of the GluR4 subunit (Zhu et al., 2000). Moreover, at the end of the second postnatal week simultaneous application of αCaMKII blockers and blockers of protein kinase A or C is required to fully inhibit LTP, whereas, when applied alone, the inhibitors have no effect (Wikstrom et al., 2003). Hence, the importance of αCaMKII for the expression of LTP increases during development.

In addition to these differences regarding AMPAR trafficking and LTP at different developmental stages there is also evidence that developmental LTP rely on additional mechanisms. For example, there is evidence of developmental LTP expressed as an increase in release probability (Bolshakov and Siegelbaum, 1995; Palmer et al., 2004; Lauri et al., 2006). Lauri et al showed that LTP is associated with an increased release probability of low release probability synapses based on a removal of a tonic presynaptic inhibition mediated via presynaptic G-protein coupled kainate receptors. This form of presynaptic LTP was restricted to the first postnatal week. An additional mechanism present at P12 is an increased conductance of the AMPAR channel (Luthi et al., 2004; Palmer et al., 2004). The relative magnitude of these, and putative others, developmentally restricted manifestations of LTP versus AMPA unsilencing in developmental LTP is not clear. In my thesis work I address this question by comparing the amount of LTP that is possible to elicit with the amount of preceding AMPA silencing.

Although mature LTP is virtually abolished in the GluR1 -/- mice, these mice have an almost normal LTP at the end of the second postnatal week (Jensen et al., 2003). However, the initial decaying phase of LTP, the STP, was absent from the developmental LTP in these mice. Very little is known about developmental STP, and another main issue in my thesis work is to examine the characteristics of developmental STP to better understand its relationship to mature LTP and STP.

AIM

The AMPA silent synapse and its conversion into an AMPA signaling synapse has been critically implicated as a key mechanism for LTP, and thus as a basis for learning and memory as well as in the early maturation of the glutamate synapse. However, since AMPA silent synapses are preferentially expressed in the developing brain and since critical differences exist between developmental and mature LTP, the involvement of AMPA unsilencing as a key mechanism for LTP in general is questionable. The overall aim of this thesis is therefore to examine the relative importance of AMPA unsilencing as a mechanism for LTP.

Specific aims

I. To find out, using the perforant path-dentate granule cell synapse, whether AMPA silencing exists in synapses other than the CA3-CA1 synapse.

II. To examine, using the CA3-CA1 synapse, whether AMPA silent synapses require synaptic activity to maintain its AMPA silent state.

III. To establish to what extent developmental LTP can be explained by unsilencing of AMPA silent synapses.

IV. To examine the possible connection between AMPA silencing and the initial decaying phase of developing LTP, the so called STP.

METHODOLOGICAL CONSIDERATIONS

The hippocampus

For the past 30 years the rat hippocampus has been a popular research object for electrophysiological studies in general and synaptic plasticity in particular (Skrede and Westgaard, 1971). Since its structure and function has been examined so extensively, much is known about its cellular architecture, its connectivity and the properties of its neurons. It is also a simple structure, which enables the researcher to focus on defined monosynaptic connections. It has been proved to be a good model for common synaptic characteristics since it shares the general features of other cortical regions. Also, regarding the study of synaptic development and plasticity, the hippocampus offers a great advantage since most of its synaptogenesis in the rat occurs during the first postnatal month.

The hippocampus proper consists of the *cornu ammonis*, which can be subdivided into CA1, 2, 3 and 4, where CA1 and CA3 are the most prominent structures (*Figure 2*). In the CA1 region, the principal cells have their cell bodies located in a single pyramidal cell layer, and their apical and basal dendrites extend through the stratum radiatum -lacunose-moleculare and through the stratum oriens, respectively. The hippocampal formation includes besides the hippocampus proper, the dentate gyrus, the subiculum and the entorhinal cortex. The hippocampal circuitry consists of a trisynaptic circuit. Cells in the entorhinal cortex give rise to axons that project to the dentate gyrus via the medial and lateral perforant paths. The dentate gyrus consists of the granule cell layer, where the cell bodies of granule cells are located, the molecular layer into which their dendrites extend, and the hilus into which their axons project. These axons then project, as mossy fibers, into hippocampus proper, connecting to the pyramidal cells of the CA3 field. These CA3 cells, in turn, make up the major input, the Schaffer collateral axons, to the CA1 field. CA1 pyramidal cells, in their turn, project to the subiculum, and from there neurons project to the entorhinal cortex, closing the hippocampal loop. In the present study, slices from the dorsal hippocampus were used. There are interesting functional differences between the dorsal and ventral hippocampus where the dorsal hippocampus in required for memory formation, whereas the ventral part is involved in endocrine functions (Moser and Moser, 1998).

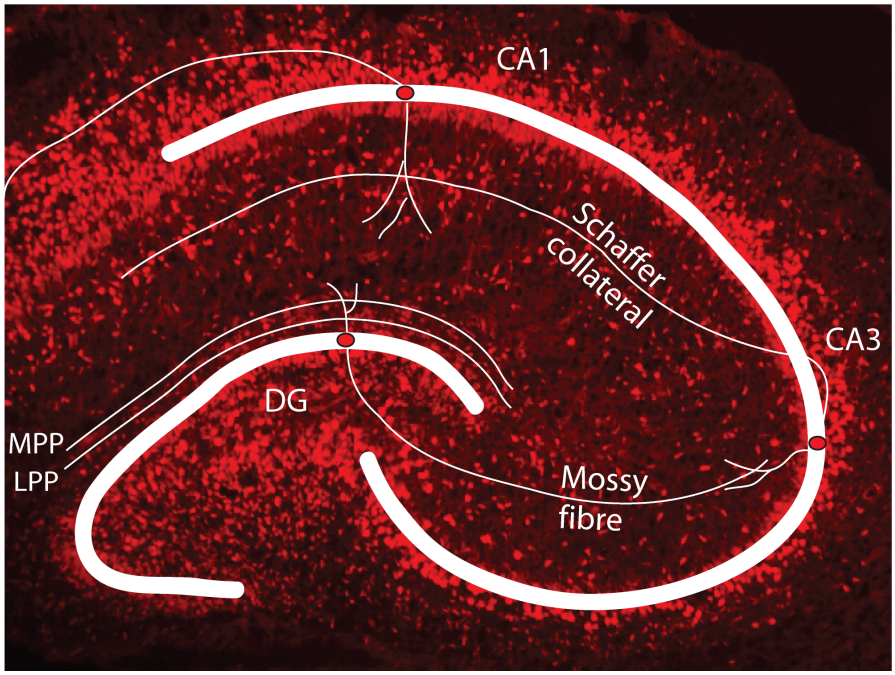

Figure 2. Schematic drawing of the hippocampal slice with the major regions and projections indicated. The background is a photo of a P2 hippocampal slice labeled with the neuronal marker NeuN. CA - Cornu Ammonis, DG – dentate gyrus, LPP – lateral perforant path, MPP – medial perforant path.

Acute hippocampal slices have several advantages compared to cultured preparations, e.g. their cells are likely to be much closer to their in vivo state than cells in culture or in organotypic tissue cultures. This is likely particularly true regarding developmental aspects since ex vivo development may take courses that are quite different from those taken in vivo. Furthermore, stimulation and recording electrodes can be placed with great precision and the lack of blood-brain barrier makes it easy to directly apply drugs or to change extracellular ion concentrations. However, although the acute hippocampal slice preparation offers some advantages there are drawbacks compared to the in vivo situation. For example, modulatory inputs are lost when preparing the slice. Moreover, the preparation of the brain slices results in a significant injury as a result of the ischemia prior to, and the trauma during, the slicing procedure. Generally, young tissue, i.e. from 1-4 week old rats, is less likely to suffer from damage from the slice preparation than tissue from older animals, possibly because of the more extensive dendritic branching in the adult animals. Cells that lose their dendrites during the slicing procedure are often in poor shape, characterized by swollen somata and condensed

chromatin. Finally, the preparation of slices results in spine withdrawal that is followed, within an hour, by spinogenesis and synaptogenesis, a problem that is increased by slicing in cold temperatures, and by using rats older than two weeks (Kirov et al., 2004).

Preparation of hippocampal slices

The experiments were performed on hippocampal slices from 5 to 47 days old Wistar rats, and in accordance with the guidelines of the Göteborg ethical committee for animal research. Rats enter puberty when they are about 30 days old. Up to this age rats of both sexes were used whereas after this date males were preferentially used to avoid hormonal influences from the estrous cycle. For example, the number of spines varies substantially during the estrous cycle, where estrogen induces spine formation (Gould et al., 1990; Woolley and McEwen, 1992). Before decapitation, the rats were anaesthetized with isoflurane (Abbott). The brain was removed and placed in ice-cold solution containing (in mM): 140 cholineCl, 2.5 KCl, 0.5 $CaCl_2$, 7 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1.3 ascorbic acid and 7 dextrose. The low temperature lowers the metabolism in the tissue and hence increases neuronal survival (Kataoka and Yanase, 1998). Choline chloride was used instead of NaCl because the Na^+ substitute, choline, is an impermeant ion. Choline therefore protects the cells from the Na^+ influx that makes the neurons depolarize and swell during the anoxia that occurs during dissection and slicing (cf. Hoffman and Johnston, 1998). NaCl can also be substituted by sucrose, which decreases the neurotoxic effect of passive chloride influx followed by cell swelling and disintegration during the preparation (Aghajanian and Rasmussen, 1989; Bischofberger et al., 2006). Transverse hippocampal slices (300-400 µm thick) were cut with a vibratome (Slicer HR 2, Sigmann Elektronik, Germany) in the same ice–cold solution and were subsequently stored in artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 2 $CaCl_2$, 4 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 0.5 ascorbic acid, 3 myo–inositol, 4 D,L–lactic acid, and 10 D–glucose at 25º C. Ascorbic acid, myo-inositol and D,L-lactic acid were added for their antioxidant effect to reduce oxidative cellular damage (Pellmar, 1995).

After 1-8 hours of storage, a single slice was transferred to a recording chamber where it was kept submerged in a constant flow (~2 ml min^{-1}) of ACSF at 30–32º C. The perfusion ACSF contained (in mM): 124 NaCl, 3 KCl, 4 $CaCl_2$, 4 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 10 D–glucose. All solutions were continuously bubbled with 95% O_2 and 5% CO_2, which kept

the slices oxygenated and the pH at about 7.4. A recovery time of at least one hour was chosen since cells that initially look unhealthy recover within this time period (Moyer and Brown, 1998). The temperature in the recording chamber was slightly below the physiological temperature of the immature rat brain, which has a temperature of approximately 35º C (Conradi et al., 1984). There are a few advantages in keeping the temperature slightly lower, e.g. it is presumed to be easier to obtain a gigaohm seal when performing patch-clamp experiments. However, it is important to be aware of the fact that various aspects of synaptic physiology are very sensitive to changes in temperature. For example, temperatures above and below the physiological temperature change the magnitude and kinetics of short-term plasticity (Takeya et al., 2002; Klyachko and Stevens, 2006; Kushmerick et al., 2006).

Picrotoxin (100 μM) was always present in the perfusion ACSF to block $GABA_A$ receptor–mediated activity. For the experiments carried out in the CA1 region a surgical cut between CA3 and CA1 regions was made to prevent epileptiform activity from the cells in the CA3 region from affecting the cells in the CA1 region. Also, higher than normal Ca^{2+} and Mg^{2+} concentrations were used to prevent spontaneous network activity (Fink et al., 2007). Under our experimental conditions the spontaneous activity in the slice preparation is very low, spontaneous action potential-dependent EPSCs occurring at a frequency of less than 0.5 Hz (Hsia et al., 1998; Groc et al., 2002a).

Electrophysiological recordings

The patch-clamp technique

The patch-clamp technique was developed by Sakmann and Neher in 1976 (Neher and Sakmann, 1976). In 1989 it was shown that it is possible to patch visually identified neurons in brain slices (Sakmann et al., 1989). The method is based on forming a high resistance seal (R>1 gigaohm) between the cell membrane and the tip of a fluid-filled glass pipette. Recordings using the patch-clamp technique can be performed in two different modes; voltage-clamp, or current-clamp mode. When performing a current clamp experiment, a known current is applied and the change in membrane potential caused by the current is measured. In a voltage clamp experiment, on the other hand, the membrane potential is controlled and the current required maintaining that potential is measured. Because of the high seal resistance patch-clamp recordings can be used to detect very small currents (pA).

Generally, there are four different types of patch-clamp configurations, the cell-attached, the whole-cell, the inside-out and the outside-out configurations. In addition, there is the perforated patch-clamp technique (see below). During a whole-cell experiment a thin glass pipette with a very small tip diameter (~1 μm) is filled with intracellular solution. The pipette is moved close to the surface of the somatic cell membrane and a tight seal between the cell membrane and the pipette is created through gentle suction. This is the so called cell-attached configuration. A stronger suction paired with a short voltage pulse ruptures the membrane patch under the pipette and the pipette solution can enter the cell, the so called whole-cell configuration. A significant advantage of this method is the ability to control the intracellular environment, which makes it simpler to study interactions between intracellular biochemistry and electrophysiology. However, changing the intracellular environment can also be a major disadvantage, since there is a risk of wash-out of important substances. For example, LTP induction in the whole-cell configuration is only possible during the first ten minutes after break-in, a phenomenon probably caused by wash-out (Malinow and Tsien, 1990).

In this study, whole–cell patch–clamp recordings were performed on visually identified CA1 pyramidal cells or dentate granule cells, using infrared–differential interference contrast videomicroscopy mounted on a Nikon E600FN microscope (Nikon, Japan). Patch pipette resistances were 2-6 MΩ. EPSCs were recorded at a sampling frequency of 10 kHz and filtered at 1 kHz, using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany).

For AMPA EPSC recordings cells were held in voltage-clamp mode at –70 or -80 mV. Only AMPA EPSCs are observed since the NMDAR-mediated currents are blocked by Mg^{2+} at these membrane potentials. To record NMDA responses the cell has to be depolarized to remove the Mg^{2+} blockade, hence these responses were recorded at +40 mV. At this holding potential also AMPA responses are present, but they are less prominent than the NMDA responses.

The pipette solution contained (in mM): 130 Cs–methanesulfonate, 2 NaCl, 10 HEPES, 0.6 EGTA, 5 QX–314, 4 Mg–ATP and 0.4 GTP (pH ~7.3 and osmolality 270–300 mOsm). Cs^{+} was used instead of K^{+} with the purpose of blocking K^{+}-channels, while QX-314 was added to block ion flux through primarily Na^{+}-channels (Talbot and Sayer, 1996), both procedures with the intention of reducing the noise level and improve the quality of the voltage-clamp. HEPES and EGTA were used to buffer pH and Ca^{2+}, respectively. For some experiments, high

concentrations of BAPTA, a more efficient Ca^{2+}-buffer, was used instead of EGTA to prevent induction of synaptic plasticity when high-frequency trains were used as stimulation. ATP and GTP were added to replace the loss of these molecules following wash-out of essential substances after break-in to the whole-cell configuration. A KCl based pipette solution was used in some experiments, containing (in mM): 130 KCl, 2 NaCl, 20 HEPES, 0.2 EGTA, 4 Mg–ATP and 0.4 GTP (pH ~7.3 and osmolality 270–300 mOsm). Liquid junction potential was both measured and calculated to be about 2 mV (K^+-based) or 8 mV (Cs^+-based) and it was not corrected for. Series resistance was monitored using 5-ms, 10-mV hyperpolarising pulses. The series resistance was not allowed to change more than 15% otherwise the experiment was discarded. Increases in the series resistance often occur during patch-clamp experiments and can be caused by, for example, that the membrane re-seal or that the cell nucleus blocks the opening of the pipette.

Perforated patch-clamp

One of the advantages using whole-cell patch-clamp recordings is the possibility to control the intracellular environment, since the soluble components in the cytoplasm of the cell is replaced by the pipette solution. However, this is also a problem since essential substances are removed from the cell, substances that might be important e.g. for the induction of synaptic plasticity (see above). One way to avoid this problem is to use the perforated patch-clamp method, in which only certain small substances, particular ions, are allowed to enter/leave the cell. This method was introduced about a decade following the introduction of the patch-clamp method (Lindau and Fernandez, 1986). The perforated patch-clamp method is based on addition of antifungal substances, which produce tiny holes in the cell membrane through which only ions can pass (Horn and Marty, 1988). Larger molecule, e.g. cAMP and other second messengers will therefore stay in the cell; hence, there is no need to add ATP or GTP to the pipette solution. Several different pore-forming antifungal agents exist; the most commonly used are nystatin, amphotericin B and gramicidin. The former two make pores where monovalent cations and chloride can pass through (but not multivalent cations such as Ca^{2+} or Mg^{2+}), while for gramicidin only cations can pass through. Using this method it is important to avoid applying the perforating solution to the cell membrane before a tight seal has developed, otherwise the membrane will be damaged. This is achieved by filling the tip of the pipette with normal intracellular solution before back-filling the pipette with the perforating solution. This allows for a gigaseal to be created before the antifungal solution has

diffused to the tip of the pipette and started to perforate the cell membrane. In my experiments amphotericin B was used with a concentration of 240 µg/ml in a K^+- or Cs^+-based intracellular solution. Series resistance was higher than in whole-cell experiments. A capacitive transient started to develop generally a few minutes after the seal was made, and when this transient was stable the recordings started. A fluorescent substance, Lucifer yellow, was always present in the pipette solution to detect possible membrane leakage using a fluorescence camera. If the dye entered the cell the experiment was discarded. Voltage-clamp was used even though it is clear that the clamp is severely compromised at the high series resistances encountered during perforated patch-clamp experiments, about 50 MΩ.

Extracellular field recordings and stimulation

The parallel orientation of its principal cells and the laminated structure of the hippocampus make it an ideal structure to study synaptic transmission using extracellular field recordings. Synaptic field potentials are generated by currents flowing in the extracellular space in response to activation of a spatially restricted population of synapses (Andersen et al., 2007). This type of recording measures synaptic responses from a large population of neurons resulting in a low trial-to-trial variability of the responses. There are several advantages in using this type of recording, such as its experimental simplicity, the intact intracellular milieu and the generation of stable recordings during a long period of time. Moreover, the field EPSP is preceded by a clear presynaptic volley, proportional to the number of stimulated axons, which provides for a measure of the stability of the number of presynaptic axons that are stimulated.

In this study extracellular field recordings were made in the stratum radiatum of the CA1 region or in the molecular layer of the dentate gyrus using a glass micropipette (~ 1 MΩ, filled with 1 M NaCl). For the CA1 recordings the stimulating electrodes were placed in the stratum radiatum to activate axons from CA3 pyramidal cells that make synapses onto the apical dendrites of the CA1 pyramidal cells. Electrical stimulation of the perforant path was carried out in the outer two-thirds of the molecular layer. The stimuli consisted of biphasic constant current pulses (200 + 200 µs, 5-60 µA) delivered through a glass pipette (resistance ~ 0.5-1 MΩ) or an insulated tungsten electrode (resistance ~ 0.3-0.5 MΩ). An advantage of using tungsten electrodes is their lower resistance and capacitance. For the extracellular field recordings, the stimulating electrodes were positioned on either side of the recording

electrode, whereas for the whole-cell recordings they were positioned close to the cell body layer where the recording electrode was placed. Test stimulation frequencies ranged between 0.033 and 0.2 Hz.

Data analysis

AMPA EPSCs were measured as the difference between the baseline level immediately preceding the stimulation artefact, and the mean amplitude during a 2-ms time window around the negative peak between 3 and 8 ms after the stimulation artefact. To avoid contamination from AMPA responses, NMDA EPSCs were measured as the difference between the baseline level immediately preceding the stimulation artefact, and the mean amplitude during a fixed time window 14-19 ms after the stimulation artefact.

Field EPSPs amplitudes were measured as the difference between the baseline level immediately preceding the stimulation artefact, and the mean amplitude during a 2 ms time window around the negative peak between 3 and 8 ms after the stimulation artefact. Field EPSPs were also measured as initial slope, which is the common, and more correct, method to estimate the size of the EPSP, because of the potential effect of cellular firing on the EPSP amplitude. Since the experimental design precluded adjustment of stimulation intensity, experiments in which the field EPSPs exhibited signs of population spike activity were discarded. For the EPSPs not excluded by this criterion initial slope and amplitude measurements gave the same results. Since the amplitude measurements were less noisy these measurements were used for further analysis. Synaptic depression in the individual experiments was calculated as the percentage decrease between the 1st evoked EPSP/EPSC and the average EPSP/EPSC after 80-120 stimuli. Recovery from the depression following stimulus interruption was calculated as percentage of the synaptic depression itself (i.e., depression as 100%). To reduce the noise inherent in constructing such a ratio the average of the first three EPSPs at the onset of stimulation and after stimulus interruption, respectively, was used. The presynaptic volley was measured as the peak–to–peak amplitude of the initial positive–negative deflection and was not allowed to change more than 10%, otherwise the experiment was discarded.

Field EPSPs and EPSCs were analyzed off–line using custom–made IGOR Pro (WaveMetrics, Lake Oswego, OR) software.

Statistics

Values are given as averages +/- standard error of the mean (SEM). Statistical significance for paired and independent samples was evaluated using Student's *t* test.

RESULTS

Synaptic fatigue in the dentate gyrus

AMPA silencing in response to very low frequency activation (e.g. 0.05 Hz) has been described for the neonatal CA3-CA1 synapse (Xiao et al., 2004). Whether this silencing is a phenomenon exclusive to these synapses or a more general feature is not known. To examine whether such AMPA silencing exists in other synapses as well the perforant path-dentate granule cell synapse was examined. This synapse is also distinct from the CA3-CA1 synapse in particular with an ongoing neurogenesis into adulthood (Altman and Das, 1965; Schlessinger et al., 1975). This adult neurogenesis might have an over-all effect on the ability for AMPA silencing, i.e. do newly generated synapses in the adult contribute to AMPA silencing in adulthood? To examine the effect of low-frequency activation on synaptic transmission previously non-stimulated synapses were used, and the very first response was used as a reference. Using a 0.2 Hz stimulation frequency a substantial depression of the AMPA EPSCs was observed, which reached approximately 50% of the initial synaptic strength within 40-60 stimuli (Paper I, figure 1).

AMPA silencing

To verify that the AMPA EPSC depression observed was indeed AMPA silencing several experiments were performed. When paired-pulses were used instead of single stimuli the paired-pulse ratio (PPR) was unaffected by the depression. Furthermore, the depression was not affected by NMDAR or mGluR antagonists, which separates it from conventional LTD, which generally requires either activation of NMDARs or mGluRs. To assess possible changes in quantal parameters, the change in AMPA EPSC variance ($1/CV^2$) was estimated demonstrating that the depression was associated with a proportional decrease in this value. This result indicates either a decrease in the presynaptic release probability or in the number of functional synapses. Since PPR did not change the latter option seems more probable, these results then being consistent with a silencing of AMPA signaling in a subset of the synapses. Furthermore, if all perforant path – granule cell synapses signal via both AMPARs and NMDARs before depression, the quantal content for AMPA and NMDA EPSCs after depression should differ with an amount corresponding to the depression. This was in fact

also observed since the $1/CV^2$ value for AMPA EPSCs after depression was about 60 % of that of the NMDA EPSCs (Paper I, figure 6), comparable to the amount of depression (to 54% of the naïve AMPA EPSC value). If only one or a few synapses are activated it should be possible to find, after depression, a complete disappearance of the AMPA signaling, while NMDA responses still exist, i.e. to find an AMPA silent synapse. In a few experiments, such complete AMPA silencing was seen in a unitary input (Paper I, figure 3).

If the depression in the dentate gyrus is caused by an AMPA silencing similar to that in the CA1 region it should also be observed using lower stimulation frequencies (Xiao et al., 2004), such as 0.033 Hz. A depression of the AMPA EPSCs was indeed also observed using this lower frequency but smaller (30%) compared to that using 0.2 Hz. When switching to the higher frequency (0.2 Hz), a further depression was, as expected, observed. This latter depression was rapidly reversible when the stimulation frequency was returned to 0.033 Hz. This readily reversible component of depression thus seems distinct from AMPA silencing described for the CA3-CA1 synapse (see further below).

When examining depression of NMDA EPSCs (at a holding potential of +40 mV) using the same protocol as for the AMPA EPSCs a depression of about 40% was observed (Paper I, figure 4). In common with the AMPA EPSC there was a readily reversible component (about 25 %), indicating a non-readily reversible component for the NMDA EPSC as well. However, this component appeared distinct from that of the AMPA EPSC since it required NMDAR activation for its induction.

When examined in the adult rat depression (at 0.2 Hz) was smaller (about 25 %) and totally reversible when lowering the frequency to 0.033 Hz. This developmental profile indicates that adult neurogenesis have no effect on AMPA silencing since no non-readily reversible component of depression was observed. Possibly, the level of cell proliferation in the adult dentate gyrus is too low to have any impact on the over-all level of AMPA silencing.

Low-frequency depression

The readily-reversible component of the synaptic fatigue, hereafter referred to as low-frequency depression, was first described in the dentate gyrus by White et al. who observed a decrease of the synaptic responses when stimulation frequencies above 0.05 Hz were used

(White et al., 1979). In the present study, a switch from 0.033 to 0.2 Hz decreased the EPSC amplitude with approximately 25% (Paper I, figure 2 and 4). The depression was expressed to the same extent by AMPA and NMDA EPSCs and was found, as noted above, with a similar magnitude in the adult rat .These features distinguish the low-frequency depression from the depression caused by AMPA silencing. The level of low-frequency depression did not differ between the lateral and medial perforant paths, despite their differences in release probability, consistent with previous results (Harris et al., 1979). Interestingly, the low-frequency depression was not associated with any changes in the PPR (Paper I, figure 8) but with an decrease in the $1/CV^2$.

A decrease in the number of readily releasable vesicles can occur without any change in the PPR (Hanse and Gustafsson, 2001a). To estimate the relative number of readily releasable vesicles brief high-frequency stimulations were used (Schneggenburger et al., 1999). The EPSCs during the later part of the train are relatively stable compared to the first ones, indicating equilibrium between release and recruitment of the vesicles. The cumulative EPSC response was plotted against stimulus number in the train, and a linear regression for the linear part of the curve was calculated. When this line is extrapolated to the first stimulus in the train this intersection with the y-axis represents the relative size of the readily releasable vesicle pool while the slope of the curve is proportional to the recruitment rate of new vesicles. Using trains evoked at 0.2 and 0.033 Hz, respectively, resulted in a 50% smaller recruitment rate when the higher frequency was used, while the size of readily releasable vesicle pool was 60% smaller (Paper I, figure 9). Hence, the higher stimulation frequency only allows for partial recovery of vesicles to the readily releasable pool.

Reversibility of AMPA silencing

When AMPA and NMDA miniature EPSCs were recorded from developing CA1 neurons they exhibit the same frequency, suggesting that the synapses express both AMPAR and NMDAR-mediated signaling, i.e. there are no AMPA silent synapses (Groc et al., 2002a). This is in contrast to evoked responses where the AMPA signaling component can easily be silenced, i.e. AMPA silence is a consequence of the applied evoked stimulation (Xiao et al., 2004). When a stimulus interruption of eight minutes was executed after AMPA silencing had been elicited no recovery of the AMPA signaling was observed. Several interpretations can be applied for this lack of recovery. First, AMPA silencing might be specific for the whole-cell

configuration per se, i.e. substances essential for proper AMPA signaling are removed. Second, even if AMPA silencing does occur in intact cells, the ability to restore the AMPA signaling during interruption of evoked activity can be sensitive to the whole-cell dialysis. Third, the time required for the initiation of recovery of the AMPA signaling might exceed eight minutes. To test for the potential negative effect of whole-cell dialysis AMPA silencing in the CA1 region was examined using extracellular field recordings. When a naïve synaptic input was stimulated at 0.2 Hz a field EPSP depression of approximately 40% was observed, indicating the potential presence of AMPA silencing, albeit somewhat less than in the whole-cell configuration, also when examining intact cells. The presynaptic volley was continuously measured in these experiments and was stable.

Using field recordings it is not possible to directly test for AMPA silencing by examining e.g. EPSC variance, failure rate and the stability of the NMDAR-mediated responses. Instead, I examined to what extent the field EPSP depression shared other characteristics with the AMPA silencing observed in the whole-cell experiments. In common with AMPA silencing the field EPSP depression was found not to depend on stimulation frequency since it was elicited to the same degree when using either 0.2 or 0.05 Hz as stimulation frequency (Paper II, figure 2). It was also input specific because a second input, activated ten minutes after the first one, exhibited the same level of depression. The field EPSP depression was also independent of NMDAR or mGluR activation, further establishing the correlation between the AMPA silencing found in the whole-cell recordings and the field EPSP depression. In the whole-cell recordings, no depression was observed when using animals above the age of one month. When this was examined using extracellular field recordings a slight depression was observed in the older animals (8%) but it was significantly reduced compared to that seen in the younger ones.

As a final test to rule out that AMPA silencing is an artefact produced by the whole-cell configuration 120 stimuli were given while a patch-clamp recording electrode was kept in a cell-attached position. Immediately after the 120 stimuli, the cell membrane was ruptured to establish the whole-cell configuration after which stimulation was resumed within 2-3 minutes. In these pre-stimulated cells virtually no decay was observed, indicating that the EPSC depression was already elicited in the intact cell (Paper II, figure 2), confirming that AMPA silencing does take place in intact cells.

To study whether recovery of the field EPSP depression occurred when using intact cells and to investigate if the depression required continued synaptic activation, stimulus interruption was performed. The stimulation was interrupted for different time periods, in this case for 1, 2, 5, 20 and 40 minutes. The field EPSP responses showed an increased amount of recovery with increasing time of inactivity but once the stimulation was resumed the field EPSP depression occurred again, i.e. the recovered responses were labile (Paper II, figure 3). After 20-40 minutes of inactivity a recovery of about 80% was observed, and the calculated time constant for the recovery from the depression was found to be about 7 minutes.

Recovery of AMPA signaling was previously investigated using a time period of eight minutes for the stimulus interruption (Xiao et al., 2004). When the recovery properties was re-examined in the present study using whole-cell recordings, a stimulus interruption period of 20 minutes was used. The use of this longer time of inactivity did not result in any recovery of the AMPA signaling (Paper II, figure 4). One likely culprit to the absence of recovery in the whole-cell configuration is the wash-out of substances that are important for the induction of plasticity, e.g. LTP has proved to be difficult to induce using whole-cell patch-clamp recordings (Malinow and Tsien, 1990). To examine this possibility perforated patch-clamp recordings with which the interior milieu of the cell is kept intact were performed. When the stimulation was interrupted for 15 minutes a significant recovery of the AMPA signaling was observed (Paper II, figure 5). Using perforated patch-clamp recordings the magnitude of depression also resembled that seen in the extracellular field recordings, i.e. about 40% depression. Furthermore, no change in PPR was observed during the depression, an additional correlation between the depressions observed using patch-clamp recordings and extracellular field recordings, respectively.

Developmental LTP and AMPA silencing

An often proposed expression mechanism for LTP is the conversion of AMPA silent synapses into AMPA signaling ones (Malinow and Malenka, 2002; Isaac, 2003). Even though silent synapses are common during development, it is not clear to what extent developmental LTP is explained by AMPA unsilencing. To investigate the potential role of AMPA silencing and AMPA silent synapses in developmental LTP, field EPSPs were depressed by 120 stimuli using a naïve input. The synapses were then subjected to an LTP-induction protocol, consisting of three 20-impulse trains at 50 Hz given at 20 seconds interval. This tetanization

was also simultaneously applied to a second input to increase cooperativity, which otherwise may be difficult to achieve in the developing hippocampus because of its sparse innervation (Liao and Malinow, 1996; Hsia et al., 1998). After an STP (see below), the subsequent potentiation stabilized close to the naïve signaling strength when measured after 20 minutes (Paper III, figure 1). When a lower test frequency was used, 0.05 Hz, such stabilization at the naïve level was recorded for an hour.

These results indicate that during the second postnatal week, LTP is unable to stabilize beyond the naïve synaptic strength. To establish whether this result was caused by the particular LTP induction protocol used, the effect of other protocols was also investigated. Even when using a considerable weaker induction protocol, a single 10 impulse train at 50 Hz, the potentiation almost reached the naïve synaptic strength after 20 minutes (80 % recovery). On the other hand, when a significantly stronger protocol was given, 6 x 20 impulses, the potentiation nevertheless did not reach beyond the naïve synaptic strength. A common LTP induction protocol is theta burst stimulation (ten 4-impulse, 100 Hz, bursts at 5 Hz), a protocol which is believed to resemble a more physiological activation pattern (Larson et al., 1986). This protocol did not result in any potentiation beyond the naïve synaptic strength. As a final test the original LTP-induction protocol (3 x 20 impulses) was given three times, 20 minutes apart. Neither in this case did the potentiation, 20 minutes after the final tetanization, reach beyond the naïve synaptic strength. These results demonstrate that developmental LTP is merely a restoration of the naive signaling strength.

Given that the naïve synaptic strength represents the upper limit for the developmental LTP, no LTP should be induced if the field EPSP depression could somehow be prevented, i.e., the initial AMPA silencing is a pre-requisite for the developmental LTP to occur. Since there does not exist any method to block the field EPSP depression without interfering with LTP induction this prediction was tested by directly exposing naive synapses to high-frequency stimulation (only preceded by two stimuli at test stimulation frequency to establish the naïve synaptic strength). In this case, when no initial field EPSP depression was allowed to take place, the LTP induction did not produce any potentiation above the naïve synaptic strength when measured after 20 minutes (Paper III, figure 3). When the same experiment was performed in the presence of the NMDAR and mGluR antagonists D-AP5 and LY 341495, respectively, no potentiation occurred but an immediate depression of approximately 40% was observed. Pairing experiments were also performed, in which the naïve test synapses

were stimulated with ten single activations at 0.2 Hz while these stimulations were paired with short trains (3 impulses at 50 Hz) to a different set of synapses. The field EPSP response reached 80% of the naïve synaptic strength after 20 minutes. Thus, even though these test synapses were subjected to concurrent pre- and postsynaptic activity this activity was not sufficient to fully counter the AMPA silencing, only to substantially reduce it.

Since AMPA silent synapses are believed to be scarce in adult animals (Liao et al., 1995; Durand et al., 1996; Hsia et al., 1998) LTP expression in these animals is unlikely to be caused by unsilencing of silent synapses. Given that LTP obviously exists in adult animals, a prediction is that it must be expressed as a potentiation beyond the naïve synaptic strength. To test this prediction the silencing/LTP protocol was repeated using animals of a more mature age, in this case P13-25 and >P30. While the initial field EPSP depression in these two age groups gradually diminished with age, the LTP increased throughout development and stabilized well beyond the naïve synaptic strength (Paper III, figure 4).

Even though developmental and mature LTP are similar in some respects, e.g. their induction relies on NMDA receptor activation, some differences have been established, an important one being that developmental LTP is dependent on PKA activation while mature LTP is dependent on αCaMKII activation (Yasuda et al., 2003). To examine whether PKA activation alone can unsilence AMPA silent synapses and whether this effect is also limited by the naïve synaptic strength, the PKA activator forskolin was used. When forskolin was added after the field EPSP depression had occurred the synaptic signaling returned to the naive synaptic strength (paper III, figure 5). The same result was achieved when the experiment was performed in the presence of D-AP5, indicating that the potentiation produced by forskolin was not secondary to activation of NMDARs (Otmakhov et al., 2004). In adult animals forskolin produced no potentiation of the field EPSP consistent with previous results (Yasuda et al., 2003). The effect of forskolin using whole-cell patch-clamp recording was also tested. In this case only a very limited effect on the AMPA EPSC amplitude could be observed, which is consistent with the absence of AMPA unsilencing during inactivity (Paper II) and the difficulties of inducing LTP when using the whole-cell configuration (Malinow and Tsien, 1990). Despite the very limited effect of forskolin on evoked EPSCs in whole-cell recordings, a 2-fold increase of the spontaneous EPSC frequency was found. This can however be explained by the fact that PKA-dependent phosphorylation of synaptotagmin-12 increases spontaneous vesicle release but does not affect evoked release (Maximov et al., 2007).

Forskolin did not completely occlude LTP when an LTP-inducing protocol was applied but the potentiation observed was significantly smaller when forskolin was present than in control experiments.

Short-term potentiation and AMPA silencing

When LTP is induced using high-frequency train stimulation there is an initial transient potentiation that decays within 5-15 min, the so called short-term potentiation (STP). When examined in the CA3 – CA1 synapse during the second postnatal week STP emerged as an elevation above the naïve synaptic signaling level, lasting for 5-10 minutes. Like LTP it was found to require NMDAR activity for its induction. Also, the developmental STP was found to be established within half a minute, similar to mature STP (Gustafsson et al., 1989; Hanse and Gustafsson, 1992).

The STP decay exhibited a remarkable similarity to that of the AMPA silencing of a naïve input. For example, when scaled to the same level of depression both exhibited a similar time course, suggesting that the expression mechanism might be the same in these two cases. STP was also found to decay in a stimulation dependent manner and its decay was not affected by blockade of NMDA or mGlu receptors (Paper IV, figure 2 and 3). Furthermore, STP was found to recover after stimulus interruption (Paper IV, figure 4). Moreover, when the LTP induction protocol was repeated once the STP had decayed, STP reoccurred with the same magnitude. This result is in contrast to that in mature synapses where repeated LTP induction leads to a progressively smaller STP (Gustafsson et al., 1989).

Since forskolin was found to mimic developmental LTP (see also Yasuda et al., 2003) the question arose whether it also induces a transient STP. When forskolin restored AMPA signaling after field EPSP depression had been induced no STP was seen, but this result might be caused by an active removal of the STP by the test stimulation. To test for this possibility, the stimulation was interrupted while forskolin was applied to avoid a stimulation-induced depression of the STP. When the stimulation was resumed an STP, exceeding the naïve synaptic strength, was observed. Finally, to test whether the forskolin-induced STP was associated with a change in the $1/CV^2$ value, slices were pre-incubated in forskolin after which a whole-cell patch-clamp experiment was performed. Similar to the AMPA silencing

experiments, a previously non-stimulated input was activated. The stimulation resulted in an EPSC depression that was associated with a decrease in the $1/CV^2$ value.

To explore possible changes in quantal parameters during STP perforated patch-clamp recording was used. STP was induced using the same high-frequency stimulation protocol previously described, and was associated with an increased $1/CV^2$ value while PPR was unaltered. These results suggest an increase in the number of functional release sites, which are eventually lost during the decay of the STP.

DISCUSSION

Most synapses in the rat hippocampus are generated during the first postnatal month. During the first one and a half postnatal week the glutamatergic synaptic connection between a CA3 and a CA1 pyramidal neuron consists of at most a single release site that is either AMPA silent or AMPA signaling. The AMPA silent connection thereafter disappears and connections with multiple release sites appear. This latter expansion of connectivity between CA3 and CA1 neurons is commonly attributed to an increased number of independent boutons linking the two neurons (Hsia et al., 1998). The present thesis presents a model for how this maturation of the synaptic connectivity instead, at least partly, may develop within a single bouton, promoted by synaptic plasticity (*Figure 3*).

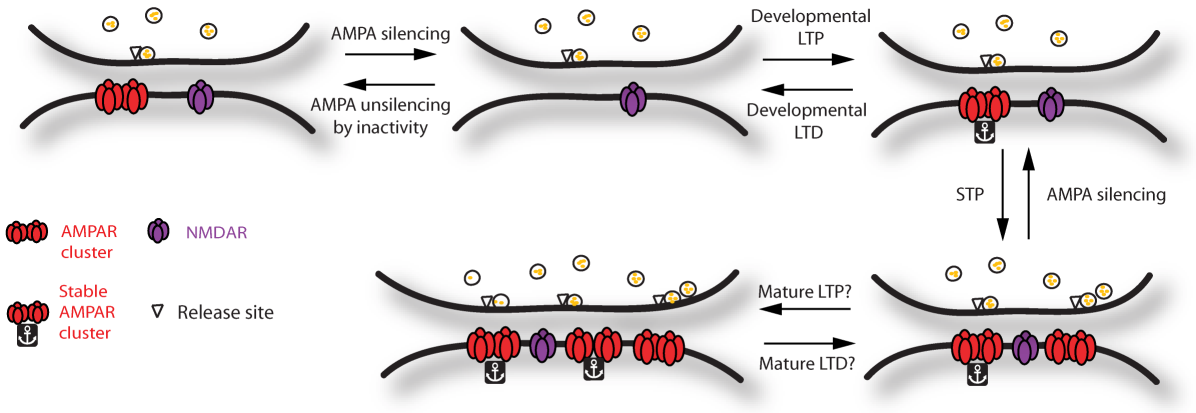

Figure 3. Model for how synaptic plasticity can promote functional maturation of the glutamate synapse. The synapse is schematically illustrated with its apposing pre- and postsynaptic membrane including release sites, vesicles and postsynaptic AMPARs and NMDARs.

According to this model, the glutamate synapse is born with a single module of AMPARs. This module of AMPARs is labile since it is easily lost by synaptic activation, resulting in an AMPA silent synapse. In the absence of synaptic activation the labile AMPAR module is reinstalled. Thus, at very low levels of synaptic activation the nascent glutamate synapse cycles between an AMPA signaling and an AMPA silent state. By correlated pre- and postsynaptic activity, i.e. by LTP induction, this AMPA labile synapse acquires a more stable

AMPAR cluster and the synapse thereby becomes a more permanent and reliable component of the growing synaptic network. Developmental LTP thus stabilizes, rather than potentiates, nascent synaptic contacts. During the first two postnatal weeks when an already stable AMPA synapse is subjected to correlated pre- and postsynaptic activity, another AMPAR module, together with its independent release site, is inserted into the synapse. This added AMPAR module is however labile and is silenced by synaptic activity, which explains the decay of the developmental STP. At the end of the second postnatal week however, an important shift occurs in the ability of LTP to promote functional synaptic growth. At that time a second stable AMPAR module, with its independent release site, can be inserted into the synapse by correlated pre- and postsynaptic activity. Thus, according to this model, mature LTP, which gradually emerges during the third and fourth postnatal week, is the addition of new functional and stable AMPAR modules, at existing synapses. The model thus suggests that developmental LTP promotes synaptic stabilization during the early formation of functional synaptic networks and that mature LTP promotes the subsequent growth of the glutamate synapse that may occur during learning.

The general question at the outset of this thesis was whether synaptic plasticity underlying learning in the adult brain is fundamentally different from, or instead a remnant of, the synaptic plasticity that promotes the activity-dependent formation of functional neural networks early in brain development. The model proposed above would imply that developmental and mature synaptic plasticity are based on the same principle, namely the addition and removal of synaptic modules. However, experimental data suggests a decisive restriction on developmental synaptic plasticity; more than a single stable synaptic module per synapse is not allowed, no matter the degree of exposure to correlated pre- and postsynaptic activity. Developmental plasticity thus seems adapted to refine the activity-independent formation of synaptic contacts by selective stabilization of appropriate connections, resulting in a naïve network of uniform connectivity. Mature plasticity, on the other hand, by producing strong connections between cells with correlated activity creates functionally tightly connected cell assemblies within in this network.

The various steps and states in the proposed model are discussed below.

AMPA silent synapses

Ever since the AMPA silent synapse was discovered (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996) it has been debated whether the lack of AMPAR-mediated currents derive from a pre- or postsynaptic locus or not. Some confusion has arisen due to a lack of distinction between presynaptically silent synapses and AMPA silent synapses. Presynaptically silent synapses, or "mute" synapses with very low release probability have been described in the hippocampus (Gasparini et al., 2000; Hanse and Gustafsson, 2001c). A "mute" synapse has neither AMPA nor NMDA responses when activated at low frequency, but at high frequency activation both AMPA and NMDA responses are observed. The hallmark of an AMPA silent synapse, on the other hand, is the lack of AMPA responses and the presence of NMDA responses, irrespective of which frequency is used.

Regarding presynaptic explanations for AMPA silent synapses, two alternatives have emerged; the restricted fusion model ("kiss-and-run") and the spill-over model. According to the restricted fusion model, the lack of AMPA responses is caused by a restricted opening of the presynaptic fusion pore rather than a full fusion, which results in a lower glutamate concentration in the synaptic cleft (Choi et al., 2000). The lower concentration of glutamate can only activate the high-affinity NMDARs, thus no AMPA responses are elicited. However, the mere existence of kiss-and-run exocytosis has been questioned (Granseth et al., 2006; Wienisch and Klingauf, 2006). The other alternative is the spill-over model. In this model, glutamate escapes from the synaptic cleft and activates the high-affinity NMDARs at neighboring inactive synapses, leaving those synapses with only NMDAR-mediated responses (Kullmann and Asztely, 1998). Even though these two models have the capacity to explain AMPA silent synapses and their conversion to AMPA signaling synapses, most of the evidence indicate a postsynaptic explanation where AMPA silent synapses lack functional postsynaptic AMPARs (Lledo et al., 1998; Malinow and Malenka, 2002; Ward et al., 2006). Nevertheless, it is possible that pre- and postsynaptic mechanisms can coexist, e.g. it has been put forward that the presence of postsynaptic AMPARs is a prerequisite for full fusion of the transmitter vesicle (Lisman and Raghavachari, 2006).

AMPA silencing

Xiao et al was the first to describe that AMPA silent synapses are created from AMPA labile synapses by sparse synaptic activation (Xiao et al., 2004). That study used whole-cell patch-clamp recordings from CA1 cells, and AMPA silencing was only observed in rats younger than about one month. The present study, using extracellular field recordings and perforated patch-clamp recordings, demonstrates that AMPA silencing can be elicited in intact cells (Paper II). It also provides a more detailed developmental profile for AMPA silencing, showing a steady reduction between the second and the fifth postnatal week (Paper III). Moreover, it demonstrates that AMPA silencing exists in synapses other than the CA3-CA1 synapse, in this case the perforant path-dentate granule cell synapse (Paper I). AMPA silent synapses and AMPA silencing has also been found at glutamate synapses on GABAergic interneurons (Riebe, Gustafsson and Hanse, *in preparation*). At these synapses, however, AMPA silencing is not restricted to the developmental period. Taken together, these findings suggest that AMPA silencing may be a widespread form of synaptic plasticity.

As described for the CA3-CA1 synapse, AMPA silencing in the perforant path-granule cell synapse was restricted to the developmental period (Paper I). Thus, the ongoing neurogenesis in the adult dentate gyrus (Altman and Das, 1965; Schlessinger et al., 1975) does not seem to promote AMPA silencing. However, since the newborn neurons constitute only a minor fraction of the granule cells in the mature dentate gyrus, it cannot be excluded that AMPA silencing occurs at synapses on these cells. Newborn neurons in the adult dentate gyrus exhibit depolarizing GABA responses (Overstreet Wadiche et al., 2005) which indicates that newborn cells can exhibit similar characteristics irrespective of the age of the animal. To examine whether AMPA silencing is also so preserved would likely require labeling (e.g. by using fluorescent proteins expressed under the promoter for doublecortin) of newborn granule cell specifically examining their synaptic responses.

What is the functional relevance of AMPA silencing? AMPA silencing can been regarded as an extreme variant of a novelty detector mechanism, only providing for at most a few discrete activations before a several minutes long silence. It is however more plausible that it reflects the behavior of a glutamate synapse not yet stabilized within a neural network. A tempting explanation is that the developing synapse has to receive the "correct" type of activity pattern to be stabilized and mature. If the activity pattern is inappropriate, e.g. sparse uncorrelated

activity, the AMPA signaling is removed since it serves no functional purpose. Why then is the glutamate synapse born with a seemingly useless AMPA signaling capacity and not simply born AMPA silent? An explanation may be that the synapse is then already equipped with the full machinery for AMPA signaling which quickly can be taken into use once the synapse receives a correlated pre- and postsynaptic activity. The present results suggest that this unsilencing is established within half a minute, or so (Paper III).

Induction mechanisms for AMPA silencing

Although there seem to be minimal requirements for the induction of AMPA silencing they are nevertheless still elusive. Xiao et al showed that postsynaptic Ca^{2+} is required since AMPA silencing was blocked by high concentrations of BAPTA, a fast Ca^{2+} chelator, in the intracellular solution (Xiao et al., 2004). There are several potential Ca^{2+} sources in the postsynaptic neuron, including NMDAR channels, mGluR-activated Ca^{2+} release from internal stores, voltage-gated calcium channels and Ca^{2+} permeable AMPARs. NMDARs and mGluRs can be rejected as Ca^{2+} sources since AMPA silencing is not blocked by the addition of D-AP5 or LY341495, antagonists of NMDARs and mGluRs respectively (Paper I, Paper II Xiao et al., 2004). Postsynaptically, several types of voltage-gated calcium channels exist, L, N, P/Q, R and T-types. All, except for the L-types, are largely inactivated at depolarized potentials and are therefore not likely to be critically involved in AMPA silencing since AMPA silencing is observed when the postsynaptic cell is depolarized to +40 mV (Paper I, Xiao et al., 2004). It is however possible that such voltage-gated calcium channels could nonetheless be involved in the induction of AMPA silencing if they are critical for providing a certain level of resting Ca^{2+} concentration, which in turn is necessary for the induction of AMPA silencing. L-type channels, on the other hand, are generally not active at resting potentials and can thus not be responsible for AMPA silencing observed at those potentials. However, there is one interesting exception, namely L-type channels based on $Ca_v1.3$, that may act as "leak channels" (Magee et al., 1996; Clark et al., 2003; Lipscombe et al., 2004). Preliminary results using extracellular field recordings showed that nifedipine, an L-type channel blocker, had a weak inhibiting effect on AMPA silencing. However, this drug is unreliable since it can increase release probability in a Ca^{2+}-independent manner (Hirasawa and Pittman, 2003). Nimodipine, a similar L-type channel blocker, which does not exhibit this unrelated effect, did not have any effect on AMPA silencing, and Bay-K, an L-type channel agonist, did not increase the level of AMPA silencing. Furthermore, using whole-cell

recordings, D-890, an inhibitor of voltage-gated calcium channels, had no effect on AMPA silencing when applied intracellularly. These findings, albeit preliminary, point towards a non-existing role for voltage-gated calcium channels in AMPA silencing. It should however be noted that there may exist compensatory mechanisms such that different Ca^{2+} sources can compensate for each other for the induction of AMPA silencing. Thus, it remains to be tested whether combinations of blockers of voltage-gated calcium channels and antagonists of NMDARs and/or mGluRs are effective. Finally, the GluR2-lacking Ca^{2+}-permeable AMPAR appeared to be a good candidate since it is more abundant on principal neurons early in development than in the adult (Isaac et al., 2007). Moreover, these channels are expressed in hippocampal GABAergic interneurons irrespective of age (Isaac et al., 2007), and glutamate synapses on these interneurons exhibit AMPA silencing both during development and in adulthood (Riebe, Gustafsson and Hanse, *in preparation*). However, preliminary findings do not support an important role for Ca^{2+}-permeable AMPARs. AMPA silencing was not different from control when the postsynaptic cell was depolarized to +40 mV and spermine (that blocks Ca^{2+}-permeable AMPARs at depolarized potentials) was added to the intracellular solution. Moreover, extracellular application of philanthotoxin, a blocker of Ca^{2+}-permeable AMPARs, had no effect on AMPA silencing. However, these results may not conclusively role out a critical role for Ca^{2+}-permeable AMPARs for the induction of AMPA silencing. This is because philanthotoxin blocks these AMPARs in a use-dependent manner and such a putative use-dependent blockade of AMPARs may give the appearance of AMPA silencing. In summary, the inhibition of AMPA silencing with high concentration of intracellular BAPTA shows that postsynaptic Ca^{2+} is somehow necessary for the induction of AMPA silencing, but the Ca^{2+} source remains elusive.

Apart from postsynaptic Ca^{2+}, glutamate binding to the AMPARs seems a necessary requirement for AMPA silencing to occur. For example, the time course of AMPA silencing is consistent with the release of glutamate from a population of synapses with low and variable release probability since it resembles the time course of inhibition of NMDA EPSCs using the use-dependent, non-competitive NMDAR antagonist MK-801 (cf. Wasling et al., 2004). A firm test for the requirement of glutamate binding would require experiments in which the AMPA silencing protocol is applied in the presence as well as in the absence of AMPAR antagonists. This experiment is however difficult since the wash-out of the antagonist requires a certain amount of time and AMPA silencing itself recovers relatively quickly (Paper II). Provided that glutamate binding to the AMPARs is a necessary

requirement for AMPA silencing to occur, a possibility is that the AMPARs would need to desensitize before being removed, e.g. by internalization, from the synapses. Preliminary experiments in the presence of cyclothiazide, a drug that blocks desensitization of AMPARs, have however shown a normal AMPA silencing. Taken together, our tentative conclusion is that AMPA silencing is induced at labile AMPA synapses as a consequence of glutamate binding provided there is a sufficient permissive level of postsynaptic Ca^{2+}.

Expression mechanisms for AMPA silencing

Most experimental evidence points to a postsynaptic explanation for the AMPA silent synapse and for AMPA unsilencing during LTP (Malinow and Malenka, 2002; Isaac, 2003; Ward et al., 2006). It then follows that AMPA silencing likely reflects a removal of the AMPAR cluster from the synapse. Such a postsynaptic mechanism for AMPA silencing is supported by the finding that chelating postsynaptic Ca^{2+} blocks AMPA silencing (Xiao et al., 2004) and that the inactivity-induced recovery from AMPA silencing is prevented by wash-out of essential substances from the postsynaptic cell (Paper II). AMPARs can be removed from the synapse either by diffusing away laterally from the PSD or by being internalized. These two mechanisms are not exclusive since internalization of AMPARs occurs laterally to the PSD (Ehlers, 2000). An important restriction to putative AMPAR trafficking mechanisms explaining AMPA silencing (and unsilencing) is that they should cause a complete removal (or installation) of all the AMPARs at the synapse.

Glutamate receptors, both AMPARs and NMDARs, are mobile in the synapse and they can diffuse laterally between the synaptic and extrasynaptic membrane (Choquet and Triller, 2003; Tardin et al., 2003; Groc et al., 2004). In general, AMPARs are more mobile than NMDARs and AMPARs at immature synapses are more mobile than AMPARs at mature synapses, findings consistent with a role for lateral diffusion of AMPARs in AMPA silencing (Groc et al., 2006).

AMPARs also cycle between an extracellular and an intracellular pool by endocytosis/exocytosis. Internalization and recycling of receptors in response to exogenous agonist exposure is a well established phenomenon. It has been shown morphologically that a brief application of glutamate to hippocampal cultures causes a significant loss, and internalization, of synaptic AMPARs (Lissin et al., 1998), while NMDARs are more or less

unaffected. Even though pharmacological activation of both NMDARs and mGluRs has been shown to cause removal of synaptic AMPARs (Carroll et al., 1999; Beattie et al., 2000; Ehlers, 2000) the activation of AMPARs alone can be a sufficient trigger (Lissin et al., 1998; Carroll et al., 1999; Beattie et al., 2000; Ehlers, 2000). It is possible that AMPA silencing represents the physiological correlate to such a pharmacologically induced removal of AMPARs triggered by activating only AMPARs. Ligand-induced AMPAR internalization is inhibited by blocking dynamin (Carroll et al., 1999). It would thus be interesting to test the possible effect of dynamin blockade on AMPA silencing in future studies.

The constitutive, activity-independent, cycling of AMPARs depends on GluR2/3 containing receptors. A disruption of the GluR2 and GluR3 genes results in an increased LTP and LTD, but a strongly reduced basal synaptic transmission (Meng et al., 2003). One of the most important proteins for trafficking of GluR2-containing receptors is the *N*-ethylmaleimide-sensitive fusion protein (NSF). NSF is an ATPase that binds to GluR2 and is involved in membrane fusion (cf. Nishimune et al., 1998). When CA1 pyramidal cells were loaded with a peptide that disrupted the NSF-GluR2 interaction the amplitude of AMPA EPSCs rapidly decreased in an activity-dependent manner, indicating that NSF has a role in regulating membrane insertion, or stabilisation, of synaptic AMPARs (Nishimune et al., 1998; Luscher et al., 1999; Luthi et al., 1999; Noel et al., 1999). This depression of AMPAR-mediated signaling resembles AMPA silencing, pointing to the possibility that NSF, or a molecule with similar function, is lacking in AMPA labile synapses. Other molecules that are important for the stability of GluR2-containing AMPARs are GRIP (glutamate receptor interacting protein), ABP (AMPA receptor binding protein), PICK1 (protein interacting with C-kinase 1) and AP2 (clathrin adaptor protein complex). ABP and GRIP anchor the AMPAR to synaptic sites (Daw et al., 2000; Osten et al., 2000), while PICK1 probably has the opposite function, i.e. to mobilize AMPARs from ABP and GRIP and hence to internalize the AMPARs during LTD (Kim et al., 2001). The AP2 protein has a role in clathrin-dependent endocytosis and, similar to PICK1, appears to be necessary for activity-dependent internalization of GluR2-containing AMPARs during LTD (Lee et al., 2002).

The actin cytoskeleton has been shown to be involved in receptor trafficking, actin-dependent processes underlying the delivery of AMPARs during both basal transmission and LTP (Kim and Lisman, 1999). Phalloidin, a drug that inhibits depolymerisation of actin, affects the same pool of receptors that is affected by blocking the interaction between NSF and GluR2 (Kim

and Lisman, 2001). These findings prompted me to test the possible effect on AMPA silencing of blockers of actin depolymerisation. Although not included in this study, neither jasplakinolid nor phalloidin, when added to the intracellular solution, were found to affect AMPA silencing, suggesting that depolymerisation of actin filaments has no role in the presumed removal of the AMPARs from the synapse during AMPA silencing. This lack of effect when inhibiting depolymerisation of actin filaments appears consistent with the result that phalloidin blocked the NMDA component of the LTD, while it had no effect on the AMPA component (Morishita et al., 2005).

Inactivity-induced unsilencing

It is well established that AMPA silent synapses gain AMPAR-mediated signaling following exposure to correlated pre- and postsynaptic activity (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996). In this study I describe the surprising finding that the AMPA silent synapses can also gain their signaling when exposed to an absence of synaptic activity (Paper II). However, in contrast to the AMPA signaling gained by correlated activity, the AMPA signaling gained by the absence of activity was not stable in that the synapse was again silenced when activated. To my knowledge, this is a new cycle of AMPA silencing/unsilencing occuring within tens of minutes, where very modest physiologically relevant stimuli evokes cycling of ionotropic receptors. An interesting finding was that the inactivity-induced AMPA unsilencing was found to occur only in intact cells. Thus, using the whole-cell configuration there seemed to be a wash-out of substances essential for the recovery of AMPA signaling. A potentiation of synaptic transmission by the absence of synaptic activation has previously been described in a few instances. Niu et al. found recovery of AMPAR- and NMDAR-mediated responses by inactivity following NMDAR-mediated LTD (Niu et al., 1999). However, this recovery seems distinct from that presently found since it was blocked by NMDAR antagonists and the experiments were performed in the presence of a very low concentration of extracellular Mg^{2+} to unblock the NMDARs. I have recently found that the inactivity-induced AMPA unsilencing is clearly present in the presence of the NMDAR antagonist D-AP5 (unpublished observation). Another example of inactivity-induced potentiation is that following the AMPA EPSC depression induced by the interruption of the interaction between GluR2 and NSF (Duprat et al., 2003). Under these conditions synaptic activation causes AMPA EPSC depression that recovered back towards initial levels when the stimulation was stopped for a time period of approximately 30 minutes.

This apparent similarity with the presently described inactivity-induced AMPA silencing may further suggest that AMPA labile synapses lack critical molecules that stabilize AMPARs in the PSD.

The cycling of the developing, AMPA labile, glutamate synapse between an AMPA signaling and an AMPA silent state explains the apparently contradictory result that no, or few, AMPA silent synapses are observed when spontaneous, rather than evoked, EPSCs are examined (Groc et al., 2002a). Another consequence of this bidirectional AMPA silent/AMPA labile switching is that the proportion of AMPA signaling and AMPA silent synapses should depend on the type and level of activity in the network. During the first postnatal week the dominating activity in the intact rat hippocampus consists of GDPs (Ben-Ari, 2001). In the hippocampal slice preparation many of the pyramidal neurons participate in this synchronized network activity (Garaschuk et al., 1998) and synapses connecting participating CA3 and CA1 pyramidal cells are expected to become stabilized (Hanse et al., 1997; Kasyanov et al., 2004). Synapses connecting pyramidal cells where either the pre- or the postsynaptic cell is not participating are, on the other hand, expected to remain AMPA labile and perhaps eventually become eliminated. In the adult anesthetized rat the average firing rate of hippocampal neurons *in vivo* is in the order of a few Hz (Abraham et al., 2007). If this firing rate is representative for the developmental period after the GDPs have disappeared, but when AMPA labile/silent synapses still can be found in acute slice preparations, it is likely that most of these synapses should reside in their silent state *in vivo*. It should be noted that under our experimental conditions with acute hippocampal slices the spontaneous activity is very low, the spontaneous EPSC frequency generally being below 1 Hz. I routinely used a high concentration (4 mM) of Ca^{2+} and Mg^{2+} to further reduce spontaneous and recurrent activity in the slice. Thus, it is not surprising that almost all AMPA labile synapses reside in their AMPA signaling state when not electrically stimulated under our experimental conditions.

AMPA stable synapses

This thesis shows that when examined in a population of synapses approximately 30 – 60% (depending on the recording conditions and age) of the synapses in the developing CA1 and dentate gyrus undergo AMPA silencing in response to test frequency stimulation. Conversely, about 40-70% of the synapses are AMPA stable in the sense that they are not silenced by low test frequency stimulation. Such AMPA stable synapses can also be silenced, but by LTD-

inducing stimulation (Montgomery and Madison, 2004). The stable synapses increase in number with development, supposedly due to the fact that the immature AMPA labile synapses are stabilized by correlated pre- and postsynaptic activity. In fact, the present thesis proposes that this stabilization is the major function for developmental LTP.

Developmental LTP

The present results indicate that during the second postnatal week, all developmental LTP can be explained by unsilencing of AMPA silent synapses (Paper III). No LTP was observed unless its induction was preceded by AMPA silencing. It is thus concluded that developmental LTP is a restoration and stabilization of the naïve AMPA signaling strength, rather than a true potentiation of AMPA signaling.

Available evidence indicate that AMPA unsilencing during developmental LTP is mediated via synaptic delivery of AMPARs containing GluR4 or $GluR2_{long}$ (Zhu et al., 2000; Kolleker et al., 2003). It is less likely that synaptic incorporation of GluR4 homomers explains LTP during the second postnatal week, suggested by the preliminary results showing that this LTP was not affected by the presence of philantotoxin and that this drug should block GluR4 homomers. We also found no effect of philantotoxin on LTP later in development (Plant et al., 2006; Adesnik and Nicoll, 2007; Gray et al., 2007). In future studies it would be interesting to test whether AMPA unsilencing as a consequence of developmental LTP (or inactivity) can be inhibited by interfering with the synaptic delivery of GluR4 and $GluR2_{long}$ containing AMPARs. This could be done by introducing peptides to the postsynaptic cell that prevent, or mimic, crucial protein-protein interactions. However, with respect to AMPA unsilencing there is a "catch-22" situation with such experiments since AMPA unsilencing is quickly washed-out during whole-cell recordings and such peptides need to be washed-in to the cell via the patch pipette.

Towards the end of the second postnatal week there is a significant switch in the biochemical requirements for LTP. Prior to that age activation of PKA is both necessary and sufficient for developmental LTP (Yasuda et al., 2003). Accordingly, it should be possible to restore AMPA signaling after AMPA silencing by applying a PKA activator. In line with this prediction, when the PKA activator forskolin was added the synaptic signaling was potentiated, but limited to the naïve synaptic strength (Paper III). Interestingly, in common

with AMPA unsilencing by LTP or by inactivity, this effect of forskolin was severely compromised in whole-cell experiments. Despite almost no potentiation of the evoked synaptic transmission by forskolin the frequency of spontaneous EPSCs was increased. This apparent contradiction could be explained by the recent finding that PKA phosphorylation of synaptotagmin-12 increases spontaneous fusion of vesicles without affecting action-potential dependent release of vesicles (Maximov et al., 2007). PKA activation affects AMPAR trafficking such that PKA phosphorylation of GluR4 at ser842 is sufficient for synaptic incorporation of GluR4-containing AMPARs whereas PKA phosphorylation of GluR1 at ser845 is necessary but not sufficient for synaptic incorporation of GluR1-containing AMPARs (Esteban et al., 2003). This result indicates that PKA activation per se can unsilence AMPA silenced synapses.

Although other manifestations of developmental LTP than AMPA unsilencing (see Introduction) have been described, they appeared to make little, or no, contribution under my experimental conditions. The increase in release probability described by Lauri et al (Lauri et al., 2006) was restricted to low release probability synapses and to the first postnatal week. My LTP experiments were mainly performed during the second postnatal week. From the end of the second postnatal week an increased conductance of the existing AMPARs should contribute to LTP (Wikstrom et al., 2003; Palmer et al., 2004). It is possible that this LTP mechanism contributes to my finding that LTP starts to potentiate beyond the initial naïve level of synaptic strength during the third postnatal week. Another possibility is that the high concentration of extracellular Ca^{2+} in our experiments precludes such a manifestation of LTP. It has been found for neocortical synapses that raising extracellular Ca^{2+} concentration increases the quantal size (in addition to increasing release probability) (Hardingham et al., 2006). The increase in extracellular Ca^{2+} increases the affinity of the mGluRs for glutamate such that mGluRs can become tonically activated by background levels of extracellular glutamate. Such group I mGluR activation results in a phosphorylation of the AMPARs such that their conductance increases. It is thus possible that the conductance of the AMPARs is maximally increased when the extracellular Ca^{2+} is high, precluding further increases by LTP induction.

STP

Induction of LTP in CA3-CA1 synapses not only leads to LTP but also to a transient potentiation, STP, lasting 5-15 minutes. STP requires correlated pre-and postsynaptic activity for its induction and can be seen either in isolation or as an initial decaying phase of LTP (Gustafsson et al., 1987). The present results suggest that developmental STP can be explained by the addition of synaptic modules containing a cluster of labile AMPARs and a new presynaptic release site at existing AMPA stable synapses (Paper IV). This proposal is based on two considerations. The first is that STP causes the AMPA signaling strength to exceed the naïve signaling strength and available evidence indicates that the naïve signaling strength at this early developmental stage corresponds to a single release site per CA3-CA1 connection (Hsia et al., 1998; Hanse and Gustafsson, 2001b; Groc et al., 2002a; Xiao et al., 2004). Since my results indicate that STP is expressed as an increase in the quantal content ($1/CV^2$) with no change in PPR (Paper IV) the likely explanation for potentiation beyond the naïve signaling strength is the addition of a new synaptic module. The second consideration is that this putative new synaptic module is AMPA labile. The decay of developmental STP was found to behave strikingly similar to AMPA silencing. Thus, developmental STP was stored in the absence of evoked activity, and, after having decayed, it could be restored by the absence of synaptic activation. Furthermore, it decayed in a stimulation-dependent manner with a time course similar to that of AMPA silencing and its decay did not require NMDAR or mGluR activation (Paper IV). The proposed mechanism for developmental STP is thus that developing synapses after the exposure to correlated pre- and postsynaptic activity, and in the absence of further sparse synaptic activation, can contain two independent synaptic AMPAR modules. It should however be noted that there is presently only limited evidence that synapses actually can contain two independent modules, each module with its own release site (Raghavachari and Lisman, 2004; Lisman and Raghavachari, 2006; Masugi-Tokita et al., 2007).

In agreement with mature STP (Volianskis and Jensen, 2003), developmental STP decays in an activity-dependent manner (Paper IV). This feature of STP may explain why it has been consistently difficult to elicit an STP phase on LTP induced using the pairing protocol, whereas STP is consistently observed when LTP is induced using a brief high-frequency stimulation. The pairing protocol often consists of postsynaptic depolarization coupled with rather high frequency of presynaptic activation (around 1 Hz) for one, or a few, minutes. It is

thus likely that the pairing protocol also elicits STP and that it is removed by synaptic activation before it can be detected by the test stimulation at resting membrane potentials.

We found no change in PPR during developmental STP (Paper IV). It is presently uncertain whether that feature is in common, or not, with mature STP. Volianskis and Jensen (Volianskis and Jensen, 2003) reported that PPR was decreased during STP measured using extracellular field recordings whereas others have reported no change in PPR (Manabe et al., 1993; Schulz and Fitzgibbons, 1997). It should be noted in this context that it is very easy to underestimate the magnitude of the second (facilitated) field EPSP in a pair, particularly following the induction of LTP, due to spike activity curtailing the peak of the field EPSP. Such underestimations would lead to false overestimations of decreases in PPR. There seems however to be at least one important difference between developmental and adult STP. As reported presently, developmental STP can be observed despite repeated tetanizations that saturate LTP (Paper IV), whereas the mature STP is saturated along with LTP (Gustafsson et al., 1989; Hanse and Gustafsson, 1992). Furthermore, developmental STP recovered when the stimulation was interrupted for a period of time (Paper IV), whereas mature STP does not seem to exhibit such inactivity-induced recovery (unpublished observation).

It is possible that the GluR1 subunit is critical for the establishment of both mature and developmental STP. Using the GluR1-/- mice not only was the mature STP/LTP almost abolished in the adult animals, but there was also no sign of STP in the developing (around P14) animals, although developmental LTP was normal in these mice (Jensen et al., 2003). It is however not likely that incorporation of GluR1 homomers explains developmental STP in the present study since preliminary results show that this STP was normal in the presence of philantotoxin. Moreover, our results indicate that application of the PKA activator forskolin is sufficient to induce developmental STP (Paper IV) and PKA-dependent phosphorylation of GluR1 should not be sufficient to drive GluR1-containing AMPARs to the synapse (Esteban et al., 2003).

Developmental transition to mature LTP

My STP results (Paper IV) suggest that a new synaptic AMPAR module can be incorporated into existing synapses. It is thus tempting to speculate that mature LTP can be explained by addition of new synaptic modules to existing synapses. The important difference with respect to STP is that for mature LTP these synaptic modules should be stable, not labile. It has

recently been proposed that addition of synaptic modules to existing synapses would reconcile many apparently contradictory results regarding the expression of LTP (Lisman and Raghavachari, 2006). Assuming that the addition of stable AMPAR modules explains mature LTP, my results suggest that synapses start to acquire the capacity to incorporate more than one stable AMPAR module at the end of the second postnatal week (Paper III). They further suggest that developmental LTP (AMPA unsilencing) and mature LTP coexist in the synaptic population during a developmental transition period between the third and the fifth postnatal week. Taking this developmental scenario for LTP at face value, an interesting question arising is; what are the developmental changes that enable synapses to grow and incorporate more than one stable AMPAR module?

Two such possible developmental changes, already discussed above, that might be important for the transition to mature LTP, and that occur at about the right time, is the developmentally delayed expression of GluR1 and αCaMKII. Another potentially interesting candidate in this context is the transmembrane AMPA receptor regulatory protein (TARP) that binds AMPARs without selectivity for the different subunits. This protein is stably associated with the AMPARs, as a so called auxillary subunit which anchors the receptors to PSD-95 and other MAGUK (membrane associated guanylate kinase) proteins in the PSD (Chen et al., 2000). One of its roles is to recruit AMPARs from extrasynaptic sites into the synapse during LTP. TARPs are four-transmembrane proteins that can form several isoforms; γ2, γ 3, γ 4, γ 7 and γ 8 (Nicoll et al., 2006). In the hippocampus the γ8 variant is the most dominant, but also 2, 3 and 4 can be found. When TARP γ8 is overexpressed the number of extrasynaptic receptors increases, while the AMPAR currents are unchanged. A deletion of this protein, on the other hand, causes the synaptic AMPAR-mediated currents to decrease and leads to a reduced level of LTP (Rouach et al., 2005). Thus, TARPs appear to stabilize AMPARs at the PSD, likely by their interaction to PSD-95 (Tomita et al., 2004). Interestingly, TARP γ8 is not present in the hippocampus during the first two postnatal weeks (Tomita et al., 2003) and its expression may then speculatively contribute to the development of mature LTP. It is unknown in what manner AMPARs are connected to the scaffolding proteins before the expression of TARP γ8. A possible candidate is Narp (neuronal activity-regulated pentraxin), which is a secreted protein that binds to the extracellular part of the GluR1/2/3 subunits and induces AMPAR clustering during synaptogenesis (O'Brien et al., 1999).

PSD-95 might also have an important role in the development of mature LTP. When PSD-95 is over-expressed the AMPA responses are increased and LTP is occluded, but there is no change in the number of AMPARs in the dendritic membrane (Schnell et al., 2002). When PSD-95 is deleted the AMPAR responses are decreased and the number of silent synapses as well as the level of LTP is increased (Beique et al., 2006). PSD-95 also exhibits a delayed developmental expression. Before postnatal day 10 SAP-102 is the dominating MAGUK and between postnatal day 10 and 35 there is a dramatic increase in the expression of PSD-95 (Sans et al., 2000; Elias et al., 2006). It has also been suggested that PSD-95 can drive synaptic maturation and stabilization (El-Husseini et al., 2000; Beique et al., 2006; Elias et al., 2006; Ehrlich et al., 2007). Thus, the expression of PSD-95 is likely to contribute to the ability of synapses to express mature LTP.

An important reason for proposing that mature LTP creates new stable synaptic modules at existing CA3–CA1 synapses is the temporal correspondence between the growth in connectivity between CA3-CA1 pyramidal neurons (Hsia et al., 1998) and the ability of LTP to potentiate synaptic strength beyond the level of a single release site (Paper III). Both starts at the end of the second postnatal week and they increase during the following month. In this context it is also interesting to note that hippocampus-dependent learning seems to evolve during this transition period. Stable hippocampus-dependent learning is not evident until the animal is relatively mature; about three weeks in the rat (Dumas, 2005) and about three years in the human (Nelson, 1998). For humans this lack of stable hippocampus-dependent memories has been called infantile amnesia and it manifests itself by our lack of declarative memories from the time before the age of 3-4 years.

CONCLUSIONS

The aim of this study was to investigate the role of AMPA silent synapses in long-term synaptic plasticity at different developmental stages.

AMPA silencing can be elicited in the developing perforant path-dentate granule cell synapse, indicating that AMPA silencing is a general phenomenon among developing glutamate synapses. It does not occur in the adult dentate gyrus, despite adult neurogenesis (Paper I).

The AMPA silent state requires synaptic activity to be maintained since in the absence of synaptic activity the AMPA signaling properties can be recovered within tens of minutes. Interestingly, the recovery of AMPA signaling only occurs in intact cells, indicating that this process is affected by the wash-out of substances essential for unsilencing (Paper II).

The expression of developmental LTP requires preceding AMPA silencing and is fully explained by unsilencing of AMPA silent synapses (Paper III).

The initial decaying phase of developing LTP, the STP, exhibits the properties of AMPA silencing (Paper IV).

These results, together with previous findings, suggest a model for synaptic maturation promoted by synaptic plasticity in which the glutamate synapse is born with a single AMPA labile module, which can easily be removed by sparse synaptic activation. Developmental LTP acts to stabilize this AMPA labile module rather than to potentiate the synapse. It can, however, transiently potentiate the synapse by the addition of a labile AMPA module to an existing synapse with a single stable AMPA module. It is proposed that mature LTP adds stable AMPA modules to existing synapses explaining the increasing synaptic connectivity between pre- and postsynaptic neurons which is observed after the initial period of synaptic stabilization. This thesis thus proposes that, using the same principle mechanism, namely the addition of stable AMPA modules, developmental LTP promotes initial synaptic stabilization while mature LTP promotes synaptic growth.

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