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Short- and long-term neuronal plasticity in hippocampal CA1 region of rat

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To Science

Populärvetenskaplig sammanfattning

Hjärnan är ett komplicerat organ och filosofer har undrat om denna skapelse verkligen är kapabel att förstå sig själv. En svårlöst fråga är hur bräckliga byggstenar med begränsad tillförlitlighet (nervceller, gliaceller och kommunikationspunkter i form av synapser) kan kopplas samman till en tillförlitlig enhet (normalt i varje fall) med målinriktat beteende. En del av förklaringen ligger i aktivitetsberoende plasticitet, dvs hjärnans förmåga att förändras beroende på interna och externa signaler. Sådan plasticitet, som bland annat är viktig för minnesfunktionen, har studerats i hippocampus, en utvecklingshistoriskt gammal del av hjärnbarken. Synapser i hippocampus kan upp- och nedregleras i ett tidsperspektiv från mindre än en sekund till upp till kanske år. Synapserna kan förstärkas långsiktigt om de förbundna nervcellerna är aktiva tillsammans under en bråkdel av en sekund (Hebbs regel). Denna princip anses vara grunden för vårt associativa minne.

Mitt projekt har varit inriktat på att studera olika former av synaptisk plasticitet i hippocampus med hjälp av elektrisk registrering i hjärnvävnad från försöksdjur. En studie behandlar korttidsplasticitet (parpulsfacilitation och d:o depression) och har fokuserat på tillförlitlig jämfört med otillförlitlig aktivering av enstaka synapser. Hur påverkas den uppmätta korttidsplasticiteten under dessa förhållanden och vilken betydelse har variationer av nervimpulströskeln? I en andra studie har jag analyserat långvarig synaptisk plasticitet (långtidspotentiering och d:o depression). Vilka faktorer och signaler bestämmer om synapsernas styrka skall öka eller minska? Som försökspreparat används halvmillimetertunna skivor av råttans hippocampus som hålls vid liv i en näringslösning. Under experimentet kan synapserna förstärkas (minnas) eller försvagas (glömma) som svar på elektrisk stimulering.

Synaptisk transmission har en inneboende osäkerhet eftersom de ansvariga biologiska förloppen inte fungerar förutsägbart utan har en slumpmässig karaktär. Detta gäller både alstringen av nervimpulser utifrån en viss teststimulering och synapsens frisättning av signalsubstans när den väl har aktiverats av en nervimpuls. Mina metodologiska undersökningar har påvisat att spontana och systematiska variationer i retbarhet på synapsens sändarsida påverkar resultaten mer än vad man tidigare har trott. Detta kan vara en källa till fel vid studier av korttidsplasticitet med minimalstimulering, en metod för aktivering av enstaka synapser med användning av en mycket svag stimuleringsstyrka. En typ av glutamataktiverad receptor, NMDA-receptorn, spelar en viktig roll för induktion av långvarig aktivitetsberoende plasticitet. Mina undersökningar av långtidspotentiering och långtids-depression i hippocampus tyder på att NMDA-receptorns sammansättning i form av subenheter inte har någon avgörande betydelse för alstringen av plasticitet utan det viktiga är mängden av kalcium som släpps in i mottagarcellen (principen om likvärdigt kalcium). Mitt arbete visar att det inte bara är den för tillfället rådande kalciumkoncentrationen som inverkar utan att tidigare aktivitet i synapsen också har betydelse (så kallad metaplasticitet). En viss kalciumpuls kan till exempel förstärka synapsen till att börja med, men om samma stimulering upprepas under minuter eller timmar leder den till en försvagning. Dessutom har jag iakttagit en NMDA-kalcium-oberoende försvagning av synapserna (passiv glömska).

Plasticitet i hjärnans kopplingar, synapserna, är viktig för hjärnans utveckling samt för inlärning och minne. Rubbningar av synaptisk funktion och plasticitet förekommer vid många sjukdomar och skador som drabbar hjärnan. Mitt projekt bidrar till att klargöra mekanismer för långvarig och kortvarig synaptisk plasticitet och kan på sikt få betydelse för metoder att behandla minnesstörningar.

Abstract

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The brain is highly plastic, displaying both short- and long-term changes, resulting from developmental processes as well as learning and memory. Moreover, short-term plasticity such as paired pulse facilitation and depression (PPF, PPD) have long been used to monitor the presynaptic versus postsynaptic changes occurring during more lasting processes such as long-term potentiation and depression (LTP, LTD). Many issues remain unresolved, e.g. how PPF and PPD are related to the probabilistic features of synaptic transmission, an issue which has also methodological aspects. Regarding LTP and LTD, it is still uncertain how Ca^{2+} via NMDA receptors (NMDA-R) produces either increases or decreases of synaptic strength.

Experiments were performed on hippocampal slices from 1-21 day-old Sprague-Dawley rats. Intracellular recordings were obtained from visually identified CA1 pyramidal cells using whole-cell patch clamp technique. Extracellular recordings were obtained under low magnification optical resolution by assessing field potentials evoked in the synaptic layer. AMPA-R and NMDA-R mediated responses were assessed in parallel via early and late measurements of composite excitatory postsynaptic potentials (EPSPs).

I first examined short-term plasticity in the millisecond to second range, including PPF and PPD, using weak paired or multiple stimuli to presynaptic afferents (minimal stimulation). Excitatory synaptic currents (EPSCs) in CA1 cells revealed a strength dependence, which was hard to explain as an isolated synaptic phenomenon, and so suggesting a role for unreliable activation of afferents. This idea was supported by CA3 cell recording, either to monitor axonal activity or used as a model for near threshold spike generation. Action potential firing thresholds in CA3 cells/axons were significantly lower for the second pulses of the paired-pulse stimulation than for the first pulses. This has consequences for interpreting measurements of synaptic parameters under unreliable presynaptic activation; e.g. release probability, paired pulse ratio and coefficient of variation.

The subsequent work involved longer lasting plasticity. Subunit-specific NMDA-R antagonists were used to target NR2A- or NR2B-containing receptors and were tested on LTP and two forms of LTD. It was found that NR2A-containing receptors dominate, both with respect to plasticity induction and their contribution to isolated NMDA-R responses. Experiments using a lowered Mg^{2+} concentration to amplify Ca^{2+} entry demonstrated that both subunit types contributed to induction of LTP and LTD. The data suggest that Ca^{2+} influx into the postsynaptic spine via different types of NMDA-Rs makes up a "final common pathway", controlling synaptic plasticity by its magnitude and temporal pattern, regardless of the source. This issue was further interrogated by a protocol where NMDA-R activation was suddenly increased by switching from single-pulse stimulation (SPS) to paired-pulse stimulation (PPS). This led to an initial short-term potentiation of AMPA responses followed by a slowly developing LTD of both AMPA and NMDA. These results suggest that NMDA-dependent synaptic changes do not only depend on the instantaneous Ca^{2+} concentration in the postsynaptic spine but are also influenced by prior induction events. The results can be described by a modified BCM-model of metaplasticity with an activity-dependent sliding threshold. In addition to NMDA-R driven processes, passive relaxation contributes to the plasticity and in some cases can outbalance the active control.

Keywords: Glutamate, hippocampus, plasticity, synapse, LTP, LTD, AMPA, NMDA
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List of papers in this thesis

I: **Huang FS**, Meng K, Tang JS. Properties of paired-pulse firing thresholds and the relationship with paired-pulse plasticity in hippocampal CA3–CA1 synapses. *Eur J Neurosci. 2007 Jun; 25(11):3253-63.*

II: **Huang FS**, Tang JS. Variability of AMPA-EPSCs at CA3-CA1 synapses. *Manuscript.*

III: Li R, **Huang FS**, Abbas AK, Wigström H. Role of NMDA receptor subtypes in different forms of NMDA-dependent synaptic plasticity. *BMC Neurosci. 2007 Jul 26;8:55.*

IV: **Huang FS**, Abbas AK, Li R, Afanasenkau D, Wigström H. Bidirectional synaptic plasticity in response to single or paired pulse activation of NMDA receptors. *Neurosci Res. 2010 67: 108-116.*

List of other publications as co-author

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Contents

Abbreviations

AC: action current
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPA-R: AMPA receptor
AP: action potential
AP5: D (-)-2-amino-5-phosphonopentanoic acid
BCM: Bienenstock-Cooper-Munro model
BPAP: back-propagating action potentials
CA: cornu ammonis
CaM: calmodulin
CaMKII: calcium/calmodulin-dependent protein kinase II
CO: carbon monoxide
CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione
CNS: central nervous system
CV, coefficient of variation
DD: de-depression
DG: dentate gyrus
DP: de-potentiation
E-LTD: early long-term depression
E-LTP: early long-term potentiation
EPSC: excitatory postsynaptic current
EPSP: excitatory postsynaptic potential
fEPSPs: field excitatory postsynaptic potentials
GABA: γ-aminobutyric acid
HFS: high-frequency stimulation
Ife: Ifenprodil
KA: kainate
KA-R: kainate receptor
LFS: low-frequency stimulation
L-LTP: late long-term potentiation
LTD: long-term depression
LTP: long-term potentiation
MF: mossy fiber
iGluR: ionotropic glutamate receptor
mGluR: metabotropic glutamate receptor
NMDA: N-methyl-D-aspartate
NMDA-R: NMDA receptor
NO: nitric oxide

NVP: NVP-AAM077
PNS: peripheral nervous system
PP: perforant path
PPD: paired-pulse depression
PPP: paired-pulse plasticity
PPR: paired-pulse ratio
PPS, paired-pulse stimulation
PPF: paired-pulse facilitation
P_r: release probability
P_{res}: postsynaptic response probability
PTP: post-tetanic potentiation
Ro: Ro25-6981
SNARE: SNAP (Soluble NSF Attachment Protein) REceptor
SPS: single-pulse stimulation
STD: short-term depression
STDP: spike timing dependent plasticity
STP: short-term potentiation
TBS: theta-burst stimulation
TFS: test-frequency stimulation
Tr: reliable AP firing threshold
Tu: unreliable AP firing threshold
VDCC: voltage-dependent calcium channel

INTRODUCTION

The brain is highly plastic, displaying both short- and long-term changes, resulting from developmental processes as well as learning and memory. The underlying events, which can be biochemical, physiological as well as morphological, include changes at the neuronal level (neuronal plasticity) but can also involve other cells, such as the different types of glia (glial plasticity). Neurons are the prime computing and signaling elements of the brain. They are electrically excitable cells that process and transmit information by electrochemical processes and are connected to each other, or to other cells (muscle fibers, secretory cells), via specialized junctions called synapses. Of special concern in this thesis is synaptic plasticity, which implies changes in the neurons' capability to communicate with each other.

Neuronal plasticity

A typical neuron has several thousand synapses, and most synapses connect axons to dendrites, such as in the hippocampal CA3-CA1 synapses that are studied here. The output of the neuron, in terms of action potential (AP) firing, results from a sophisticated integration of the incoming excitatory, inhibitory and modulatory signals. It is not just the amount of incoming frequencies that play a role but membrane properties of the target cell as well as the synaptic properties play equally important roles. For instance, the threshold for triggering an action potential is not the same in all neurons and the synapses involved can differ with respect to their efficiency in affecting the target cell. These properties undergo various kinds of changes, both in the short and long term, and both kinds of neuronal plasticity play important functional roles. Neuronal plasticity can be diffusely generated by chemical signals or can be specifically triggered by current or prior activity in neurons or neuronal elements such as synapses. The latter form of plasticity, often referred to as activity dependent or use-dependent, is commonly considered to represent a cellular mechanism for learning and memory although it may have other functions as well. For instance, certain membrane conductance properties can change in a bistable manner, allowing the cell to function as an on/off switch, which might be essential for working memory. Synaptic changes, on the other hand, allow for large scale information storage and have been inferred in various forms of short-term and long-term memory.

Types of plasticity

Synaptic plasticity can be divided into short- and long-term forms. Short here refers to plasticity lasting less than 30 min, and some forms only last for milliseconds to seconds, such as the phenomena of paired-pulse facilitation (PPF) and paired-pulse depression (PPD). Long-term synaptic plasticity normally lasts longer than 30 min, and those forms include long-term potentiation (LTP) and long-term depression (LTD), as well as their reversals de-potentiation (DP) and de-depression (DD). Some forms of plasticity require coincident activity in pairs of neurons and are named associative (typical members are LTP and LTD) whereas others depend on a single source of activity and are named non-associative (PPF and PPD are two examples). A special case of associative plasticity is the Hebbian type, to be considered later in more detail. Another distinction for synaptic plasticity is between homosynaptic (depending on activity in the "own" presynaptic axon) and heterosynaptic (depending on activity in other axons). In their most common forms, the above-mentioned examples of plasticity are all of the homosynaptic type, a feature also referred to as input specificity. An essential functional characteristic of a synapse is its efficacy, or strength, defined as the size of the postsynaptic response (expressed as for instance electric charge) for each presynaptic AP. Synaptic plasticity implies a change of the synaptic efficacy. Depending on whether that change is an increase or a decrease, we talk about potentiation or depression, respectively.

How to study synaptic plasticity?

A common way to study synaptic transmission and plasticity experimentally is to stimulate presynaptic axons electrically by electrical test pulses via an electrode, and to measure the evoked postsynaptic electric response (voltage or current) via another electrode. The rate of test pulses is generally kept low, with stimuli separated by seconds, minutes or even longer (referred to as test-frequency stimulation, TFS). The size of the response obtained for a constant-sized test pulse is a measure of the synaptic efficacy and can thus be used to monitor the potentiation or depression that is associated with synaptic plasticity. Such experiments can be carried out in intact animals as well as in isolated tissue. The present thesis describes work carried out in transverse hippocampal slices from experimental animals (rats) as detailed in the Methodology section. Moreover, studies can be performed on a larger or smaller scale, from the multicellular multisynapse level down to the single-cell single-synapse level, both of which techniques are used in the present work. Single-synapse (and near single-synapse) recording has certain methodological difficulties which will be dealt

with, including how reliable versus unreliable stimulation of presynaptic axons will influence measurements of PPF and PPD. Another theme relates to LTP and LTD and how these long-term forms of plasticity are controlled. An essential question is how the same messenger system, using postsynaptic calcium as a signal, can induce LTP in some conditions and LTD in others.

Glutamatergic synaptic transmission

Types of glutamate receptors

Hippocampal CA3–CA1 synapses (Schaffer collateral pathway) have been used as a major model system for understanding basal synaptic transmission and synaptic plasticity in the brain. These synapses are excitatory and use glutamate as transmitter. Glutamate is the most abundant excitatory neurotransmitter in the vertebrate central nervous system (CNS), and is believed to play an essential role in learning and memory, brain development as well as in neurological disorders (Collingridge and Singer 1990; Danysz, Zajaczkowski et al. 1995). In the CNS, more than 80% of the neurons and 90% of synapses are glutamatergic. The targeted receptors are divided into ionotropic glutamate receptors (iGluRs), which are directly coupled to an ion channel, and metabotropic glutamate receptors (mGluRs), which are coupled to intracellular second messengers. Glutamate elicits fast synaptic responses by activation of iGluRs; these responses are mediated via combinations of Na^+, K^+, and Ca^{2+} ionic currents, depending on the type of receptor. The iGluRs include N-methyl-D-aspartate (NMDA), kainate (KA), and α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid (AMPA) receptor subtypes. Most glutamatergic synapses use a combination of AMPA/kainate receptors (AMPA-R, see below) and NMDA receptors (NMDA-R).

Basics of glutamatergic transmission

Let us consider the essential features of glutamatergic transmission by help of the cartoon in Figure 1, focusing on the simpler part A (left) where only AMPA-Rs contribute to the postsynaptic response. When the AP arrives at the presynaptic terminal, it activates voltage dependent calcium channels (VDCC) causing influx of Ca^{2+} ions. Glutamate is then released in a quantal (all-or-nothing) manner from presynaptic vesicles, generally with no more than one or a few quanta at a time (Hsia, Malenka et al. 1998; Xu-Friedman and Regehr 2004). The fusion of vesicles with the cell membrane (exocytosis) is regulated via specific, Ca^{2+} sensitive proteins, such as SNARE proteins (Jena 2009), calmodulin and calmodulin-binding proteins (Igarashi

and Watanabe 2007). On the postsynaptic side, the released glutamate activates iGluRs in the dendritic spine compartment, resulting in passage of ions such as Na^+ and Ca^{2+} (into the postsynaptic cell) and K^+ (out of the cell). Whereas some of the receptors (AMPA-Rs) open unconditionally in response to glutamate, others (NMDA-Rs) require additional conditions such as depolarization of the postsynaptic neuron. The latter ones, which are essential for triggering synaptic plasticity (part B of the figure), will be considered further in the section on LTP and LTD.

While capturing some basic features of glutamatergic transmission, Figure 1 leaves out several important details such as the existence of mGluRs and the fact that receptors can be situated both pre- and post-synaptically. Other issues not illustrated are the possible spillover of glutamate from neighboring synapses (Kullmann and Asztely 1998) as well as the action of neurotransmitters other than glutamate within the glutamatergic synapse. Such neurotransmitters can be released from either the own or other synapses, from other neurons, as well as from glia cells (Henneberger, Papouin et al. 2010). The figure is primarily intended as an illustration of a spine synapse, such as the ones on pyramidal cells and other principle-type neurons, whereas glutamatergic synapses on interneurons differ in certain respects, generally by the lack of dendritic spines.

Important types of iGluRs: AMPA and NMDA

The pharmacological agents such as iGluR antagonists used in the present work lack ability to distinguish between AMPA-Rs and KA-Rs. The mentioning of AMPA mediated responses in the thesis may therefore be read as "AMPA/KA mediated responses". It is generally believed that KA-Rs play a minor role in synaptic signaling and plasticity compared to AMPA-Rs (Song and Huganir 2002), and so we will only consider AMPA-Rs and NMDA-Rs in the following description of important iGluR types.

AMPA-Rs

AMPA-Rs are composed of four types of subunits (GluR1, GluR2, GluR3, and GluR4) each with a binding site for glutamate, which combine to form tetramers (Mayer 2005). Activation of AMPA-Rs by glutamate results in the opening of an ion channel which allows Na^+ ions to flow into the cell and K^+ ions to flow out. Certain less common combinations of subunits provide additional Ca^{2+} permeability (Jayakar and Dikshit 2004). AMPA-Rs open and close quickly and are responsible for most of the fast excitatory synaptic transmission of the CNS. A relevant issue for synaptic plasticity is the fact that phosphorylation can regulate AMPA-R localization as well as conductance and open probability. In addition to the glutamate binding site, the

receptor has a modulatory (allosteric) site by which certain drugs can influence the channel kinetics. Cognitive enhancers such as aniracetam act at this site by reducing AMPA-R desensitization, so prolonging the EPSP/EPSC and increasing charge transfer; this implies an increase of synaptic efficacy (Isaacson and Nicoll 1991).

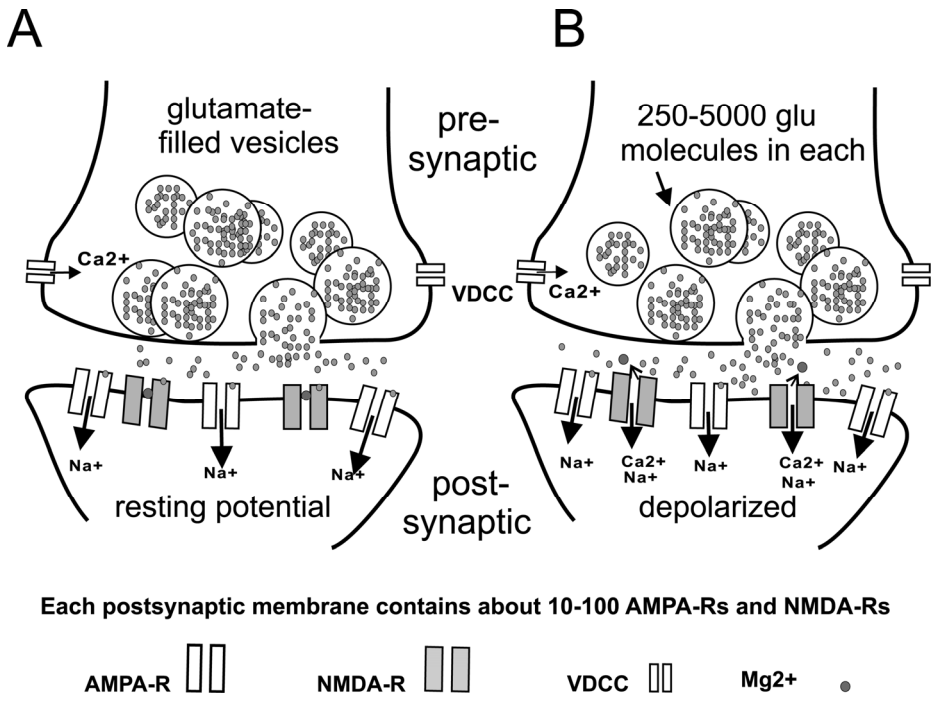

Figure 1. *Illustration of glutamatergic synaptic transmission such as in the CA3-CA1 synapse. The figure shows Ca^{2+} dependent release of glutamate from presynaptic vesicles and activation of postsynaptic ion channels AMPA-R and NMDA-R.* ***A****, demonstrates that under normal resting potential condition, glutamate only can activate AMPA-R. This results in the opening of an ion channel which allows flow of Na^{+} into the cell (shown) and K^{+} out of the cell (not shown).* ***B****, demonstrates that under depolarization of the postsynaptic neuron, glutamate can activate both AMPA-R and NMDA-R. Activation of NMDA-R results in the opening of an ion channel which allows flow of Na^{+} and small amounts of Ca^{2+} (shown) into the cell and K^{+} out of the cell (not shown).*

NMDA-Rs

NMDA-Rs have a more complicated subunit composition than AMPA-Rs and are also functionally more complex. They form a heterotetramer between two NR1 and two NR2 subunits (Mayer 2005); in addition, a related gene family of NR3A and 3B subunits has an inhibitory effect on NMDA-R activity. NMDA-R has eight variants of the NR1 subunits (1-4, a-b) and four variants of NR2 subunits (A-D), which contain the binding site for glutamate; NR3 subunits include two types (A-B). The NR2A and

NR2B are the predominant forms in the hippocampus and have been suggested to be differentially involved in LTP versus LTD (Liu, Wong et al. 2004; Massey, Johnson et al. 2004). NMDA-Rs have slower channel kinetics than AMPA-Rs, and the kinetics also differs among NR2A-containing and NR2B-containing receptors (Ewald, Van Keuren-Jensen et al. 2008).

There are many ways to regulate NMDA receptors in addition to their control by glutamate. The voltage-sensitivity is a key controlling factor related to Mg^{2+} ions, which cause a voltage-dependent block of the receptor (Nowak, Bregestovski et al. 1984). Under normal or hyperpolarized membrane potential, Mg^{2+} is attracted to the negative inside of the channel and so blocks transport of other ions. Depolarization weakens the attraction, increasing the probability that the Mg^{2+} ion leaves the channel. The activated (open) NMDA-R allows Na^{+} and small amounts of Ca^{2+} to flow into the cell and K^{+} out of the cell. In terms of permeability, the Ca^{2+} permeability of the open channel is actually higher for Ca^{2+} than for Na^{+}. The binding site for glycine is of special interest. Not only can NMDA-Rs be modulated by exogenous application of glycine but they are influenced by D-serine, an endogenous NMDA-glycine site agonist, which is believed to be released from glia cells such as oligodendrocytes (Schell, Brady et al. 1997). Polyamines is another class of modulators of the NMDA-R (Lu, Xiong et al. 1998).

Recording of AMPA-R and NMDA-R mediated responses

In experiments on synaptic plasticity, the AMPA-R mediated response is generally the one that is recorded, considering that NMDA-Rs are largely blocked due to the presence of Mg^{2+} in the extracellular tissue. Under certain conditions (depolarization of the postsynaptic cell membrane or using low extracellular Mg^{2+}) the NMDA component is also visible and can be studied in isolation under pharmacological blockade of AMPA-Rs. It is notable that comparing the changes of AMPA-R versus NMDA-R mediated responses during synaptic plasticity provides a clue to the underlying mechanism. Accordingly, an equal-sized change of the two responses is generally taken to indicate a presynaptic change involving altered transmitter release. On the other hand, if only the AMPA-R (or only the NMDA-R) mediated response is changed, this is considered evidence for a postsynaptic mechanism. The latter conclusion relies on the assumption that effects related to receptor saturation are not involved. Due to their different time course, AMPA and NMDA components of composite EPSPs can be measured in parallel during a series of trials using suitably positioned time windows for the two measurements (Xiao, Karpefors et al. 1995), a technique that was employed in Papers III-IV.

Hippocampus – a sensitive memory and orientation center

Hippocampus and its use as a model system

The term hippocampus is often used to mean the "hippocampal formation" which consists of the hippocampus proper or Cornu Ammonis (CA), the dentate gyrus (DG) and the subiculum. Hippocampus is an essential component of the limbic system of the mammalian brain, and it is considered to represent an evolutionary old type of cortex. The hippocampus has been shown to be deeply involved in functions such as long-term memory and spatial navigation. Analogous structures are found in other vertebrates such as ray-finned fishes and birds, where they play similar roles as the hippocampus in mammals (Colombo, Broadbent et al. 2001; Gomez, Vargas et al. 2006). Several forms of synaptic plasticity can be reliably induced in the hippocampus. Together with the fact that the hippocampus has a simple layered structure that makes it easy to work with, this has led to the hippocampus becoming a popular structure for the study of neuronal plasticity.

Some of the main excitatory pathways, forming the tri-synaptic circuit, are illustrated in Figure 2. The circuit comprises (1) the perforant path (PP) connection to dentate granule cells, (2) dentate granule cells via mossy fibers (MF) to CA3 pyramidal cells, and (3) CA3 pyramidal cells via Schaffer-collaterals to CA1 pyramidal cells; these connections are all glutamatergic. In addition to the illustrated cell types, more than a dozen interneuronal types have been demonstrated, mostly inhibitory GABA-ergic ones. Electrophysiology can be carried out in different types of hippocampal preparations: either *in vivo* using anesthetized or awake animals, or *in vitro* using isolated slices, mostly of the transverse type cut perpendicular to the longitudinal axis. Work in slices has demonstrated that important connections, such as the tri-synaptic circuit, are functionally well preserved. The hippocampal slices can be acutely prepared (as in the present thesis) or be grown as organotypic cultures for days or weeks. The duration of recordings ranges from hours (acute slices) to years (animals with implanted electrodes).

In Alzheimer's dementia, a disease characterized by early occurring memory problems and disorientation, the hippocampus is among the major regions of the brain that are subjected to damage (Chetelat and Baron 2003). The hippocampus can also be injured as a result of oxygen and glucose deprivation (ischemia), encephalitis, and epilepsy of the medial temporal lobe (Chetelat and Baron 2003; Jellinger and Attems 2007). Compared to many other brain regions, the hippocampus, especially the CA1 region appear to be especially sensitive to ischemic insults (Kirino 1982). The hippocampus is

therefore also suited as a model for synaptic plasticity in relation to diseases and trauma of the brain.

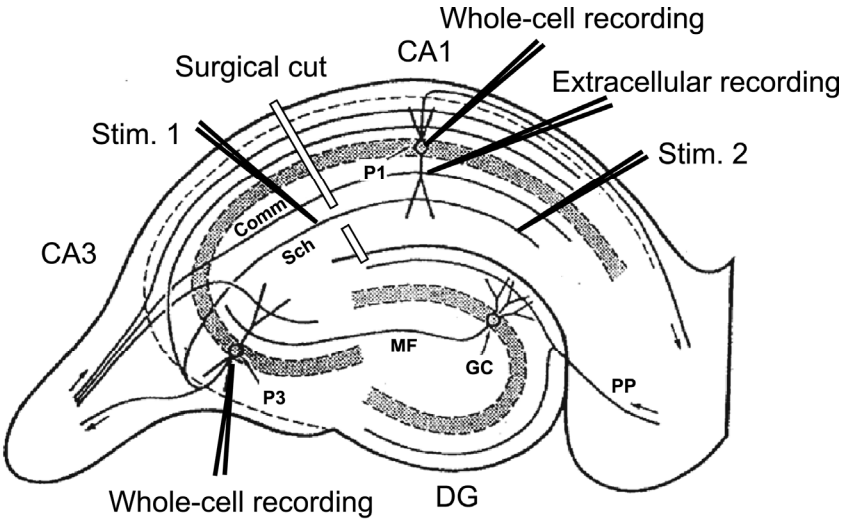

Figure 2. *Hippocampal slice with stimulation and recording positions. The figure illustrates the typical structure of the hippocampus with main areas CA1, CA3 and DG and the trisynaptic circuit (see text). Schaffer collateral fibers are stimulated with electrodes Stim. 1 and Stim. 2. Whole-cell recording is obtained from CA3 or CA1 pyramidal cells and extracellular recording from the CA1 dendritic layer. To limit the number of Schaffer collateral axons in some experiments, a surgical cut operation was done as demonstrated. PP, perforant path; MF, mossy fibers; Sch, Schaffer collaterals; Comm, commissural fibers; DG, dentate gyrus; CA1, CA3 areas of Cornu Ammonis; P1, P3 pyramidal cells; GC, granule cells.*

Neuronal plasticity in hippocampus

Short-term plasticity, such as PPF and PPD, appears to be a global phenomenon not specific for the hippocampus. Long-term plasticity, like LTP or LTD (see special paragraph) has been demonstrated in all parts of the hippocampal tri-synaptic circuit. The types of LTP/LTD involved are not all the same and, for instance, mossy fiber LTP differs from the LTP of the other two systems by its non-associative character and major dependence on presynaptic mechanisms (Weisskopf and Nicoll 1995). In contrast, LTP of the dentate and CA1 areas are associative with an induction that is critically dependent on activation of postsynaptic NMDA receptors (Bliss and Collingridge 1993). Beyond synaptic plasticity as a basis for memory it is generally assumed that neuronal level and network level plasticity also play a role, possibly in relation to working memory. With respect to such mechanisms in the hippocampus, it was recently considered that a special kind of dentate granule cell might function as an on-off switch cell (Walker, Pavlov et al. 2010). Recurrent connections of the CA3 area

remain a popular candidate for autoassociative stabilization of memory recall, and this mechanism was proposed to be coupled with gamma oscillations, 30-100 Hz (de Almeida, Idiart et al. 2007). Hippocampus is also relatively unique as it is one of a couple of brain regions where new neurons are born from progenitor/stem cells (Eriksson, Perfilieva et al. 1998).

Memory, LTP and NMDA receptors

A relation between hippocampus-dependent learning, LTP and NMDA-Rs is supported by the fact that blockade of NMDA-Rs by a locally applied antagonist is not only effective in preventing LTP induction but also significantly impairs encoding of new memories (Morris, Anderson et al. 1986). The latter was shown in a behavioral task, where rats were trained to find a hidden platform in a water-filled tank, known as water maze. Also other drugs have been shown to influence both hippocampal LTP and memory (Abraham and Williams 2008), and certain genetic manipulations in mice give parallel effects (Aiba, Chen et al. 1994). Recording from CA1 cells in behaving animals have demonstrated the existence of cells described as “place cells” (O'Keefe and Dostrovsky 1971), that fire in relation to the animal’s spatial location; these cells interact with “grid cells” of the entorhinal cortex (Hafting, Fyhn et al. 2005), and “head direction cells” of several other brain regions (Taube, Muller et al. 1990; Taube, Muller et al. 1990). Place cell learning was demonstrated in rats during a running task (Mehta, Barnes et al. 1997). The associated place fields were then shifted in the opposite direction of the path, suggesting the rat brain was able to anticipate the sequence of places encountered during the path. This effect was considered to be related to LTP. Hippocampus is still not the only “memory center” of the central nervous system. The neocortex as well as certain cerebellar structures are likely to be essential neural substrates for memory, and conditioned reflex learning and long-term synaptic plasticity have been demonstrated even in the spinal cord (Franzisket 1963; Gerber, Youn et al. 2000). It has been suggested that synaptic plasticity and memory exist and are linked together in all parts of the brain, even though NMDA receptors are not involved in all of the cases.

The phenomena of PPF and PPD

Definitions and basic mechanisms

As inferred above, the present thesis has a focus on certain forms of neuronal plasticity, including both short-term and long-term types. Among the short-term ones, PPF and PPD are millisecond to second long forms of synaptic plasticity which are

observed during presynaptic activation by pairs of pulses of equal strength (implying constant presynaptic activation), typically with an inter-stimulus interval of 50 ms. They are often referred to as paired-pulse plasticity (PPP). Using extracellular recording of field EPSPs, or whole-cell voltage-clamp recording of EPSCs, the phenomenon is manifested as a paired-pulse ratio (PPR = response 2 / response 1) that differs from unity. Generally, a PPR larger than 1 is called PPF, whereas a PPR smaller than 1 is called PPD. Early studies have demonstrated that PPF can occur in the peripheral nervous system (PNS), and was possible at the neuromuscular junction even if transmitter was not released on the first stimulus (Del Castillo and Katz 1954). In the central nervous system (CNS), either PPF or PPD is observed in practically all synapses where PPP has been tested. The mechanism of PPF is usually believed to involve the effects of residual presynaptic calcium, which alters the transmitter release probability by raising the peak level of the second calcium transient (Katz and Miledi 1968; Wu and Saggau 1994). PPD is mainly attributed to vesicle depletion (Liley and North 1953; Dobrunz and Stevens 1997; Wang and Kaczmarek 1998).

Comparison with other forms of short-term plasticity

Augmentation (also under the name of frequency facilitation) and posttetanic potentiation (PTP) are two other forms of plasticity that are likely related to presynaptic residual Ca^{2+} but with durations of usually a few seconds and minutes, respectively (Magleby and Zengel 1976; Zucker and Regehr 2002). This can be compared to PPF (or PPD) which lasts from tens to hundreds of milliseconds. Augmentation refers to the build-up of responses during a stimulus train, whereas PTP is the increase of synaptic efficacy after that train, as detected by subsequent, sparsely distributed (single) test stimuli. During the stimulus train there is generally not only a successive increase of response amplitudes but also depression of the responses in the later part of the train. Despite basic similarities among the mentioned forms of short-term plasticity, the underlying mechanisms appear to be separated in some cases. It was thus reported that augmentation and PTP "arise from Ca^{2+} acting at a separate site from facilitation" (Kamiya and Zucker 1994). There are also differences among glutamatergic synapses for "the same" type of plasticity. A comparative study revealed that frequency facilitation of mossy-fiber CA3 synapses differed from that of CA3-CA1 synapses due to a dependence on adenosine-mediated presynaptic depression (Salin, Scanziani et al. 1996). A form of presynaptic short-term depression was recently described, which was heterosynaptic in contrast to "standard PPD" which is known to be homosynaptic (Andersson, Blomstrand et al. 2007). The heterosynaptic depression was found to be mediated via astrocytes and involved mGluRs. Another form of plasticity, with a time dependency similar to that of PPF, was related to spike after-potentials in the presynaptic axon and so involving changes in axonal

excitability; this plasticity is therefore not of the synaptic type (Wigström and Gustafsson 1981; Soleng, Baginskas et al. 2004).

PPP as an indicator of presynaptic changes

Although PPF (as well as PPD) is by itself quite shortlasting, it can undergo stable changes as a correlate of other, more stable alterations of the synaptic efficacy, especially presynaptic ones. Consider, for instance, a change in the extracellular Ca^{2+} / Mg^{2+} ratio. The resulting increase or decrease in transmitter release is associated with a concomitant change of PPR in the reverse direction, a result which has been demonstrated in both PNS and CNS (Del Castillo and Katz 1954; Zucker and Regehr 2002; Thomson 2003). Basically, this holds true also for other manipulations that influence the (probability of) transmitter release. A high versus low release probability is thus associated with a low versus high PPR, and vice versa. Even a switch from PPF to PPD is possible under a large increase in transmitter release (Thomson 2000; Zucker and Regehr 2002).

The sensitivity of PPR to changes in transmitter release has led to its use as an index of presynaptic effects, comprising a research tool to detect whether or not long-term synaptic plasticity, such as LTP or LTD, is expressed presynaptically (Asztely, Xiao et al. 1996; Debanne, Guerineau et al. 1996). Still, the PPF test is not "fool-proof". It has been considered that a selective change in the number of immediately releasable vesicles will not substantially influence PPR though it does influence the probability of transmitter release (Hanse and Gustafsson 2001; Abrahamsson, Gustafsson et al. 2005). In contrast, if the change of transmitter release is due to a corresponding change of release probability per vesicle (under a constant pool of releasable vesicles), this will lead to a change of PPR in the other direction (see also Andersson, Blomstrand et al. 2007). A change in PPF is therefore likely to signify an altered transmitter release presynaptically whereas a lack of change could be due to either a presynaptic or a postsynaptic modification. However, postsynaptic factors influencing PPF have also been reported (Clark, Randall et al. 1994; Zinebi, Russell et al. 2001), suggesting that an observed change in PPR could in fact also indicate a postsynaptic expression mechanism. As a further complexity, even a seemingly obvious case of presynaptic change can be (re-)interpreted as a postsynaptic one in terms of an altered weighting of PPF among a population of synapses (see Paper IV). At least in extracellular studies, nonlinear behavior due to spiking on the second response makes up still another complicating factor for the interpretation of changes in PPR.

Relation between PPF/PPD and long-term plasticity

Regardless of the mentioned difficulties, the PPF test has remained a popular tool for detecting changes of presynaptic transmitter release (Asztely, Xiao et al. 1996; Debanne, Guerineau et al. 1996), possible reflecting its simplicity together with the fact that the alternatives are equally problematic. In particular, the test was used in several prior investigations to probe the expression mechanism of LTP and LTD. A change of PPR was only observed, however, for an early or decaying part of LTP (Kleschevnikov, Sokolov et al. 1997; Volianskis and Jensen 2003). Most of the available studies reported that PPR was not affected by LTP (McNaughton 1982; Gustafsson, Huang et al. 1988; Manabe, Wyllie et al. 1993), and a similar lack of effect was found for LTD (Mulkey and Malenka 1992; Xiao, Karpefors et al. 1995). A PPF increase was nevertheless observed for striatal LTD, implying a presynaptic mechanism; the LTD in this case was induced postsynaptically via VDCCs but not involving NMDA-Rs (Choi and Lovinger 1997). Mixed effects with changes in opposite directions were observed for both LTP (Schulz, Cook et al. 1995) and LTD (Santschi and Stanton 2003) in the hippocampus, and those effects were correlated with the magnitude of the long-term change.

A generalized form of the PPF test was performed using long stimulus trains rather than activation via PPS; the idea was to find out whether LTP preserves the "temporal fidelity" of synaptic transmission. It was found that LTP resulted in a uniform potentiation of individual responses throughout the burst rather than a redistribution of synaptic strength (Selig, Nicoll et al. 1999), implying that fidelity was preserved, a result most likely explained via a postsynaptic mechanism.

In addition to those works testing a relation between PPF and the expression of LTP (or LTD), there are studies that link PPF to the induction of LTP. Thus, the degree of PPF during baseline transmission was found to be correlated with the level of LTP that was induced later on (Kleschevnikov, Sokolov et al. 1997; Volianskis and Jensen 2003), whereas no such effect was found in another study (Asztely, Xiao et al. 1996).

Measurements of PPF/PPD and transmitter release at single synapses

The relation between PPR and the probability of transmitter release has been confirmed in many synaptic systems using different transmitter substances in both CNS and PNS, in vertebrates as well as invertebrates, and so appears to be a more or less universal feature. Assessing the release probability generally requires access to single synapses. It is also possible, in principle, to work with a few synapses/release

sites using quantal analysis; however, such experimental data obtained in hippocampal synapses are not easy to work with due to a large variability of the quantal amplitude. Much work on single synapse transmission in CNS has focused on CA3-CA1 synapses, a possible reflection of their important role in synaptic plasticity as well as their relatively simple release mechanism with only a single release site in most cases (Harris and Stevens 1989; Xu-Friedman and Regehr 2004). To activate single synapses in this and other synaptic systems generally requires special experimental protocols.

Minimal stimulation is a popular method to isolate the response of a putative single synapse, using a weak stimulus strength which is meant to activate only one axon (Raastad, Storm et al. 1992; Allen and Stevens 1994; Dobrunz and Stevens 1997). The method obviates the need for paired cell recording, albeit at the expense of lesser control over the presynaptic activation. Some work with minimal stimulation has reported a large range of release probability and PPR at CA3–CA1 synapses (Dobrunz and Stevens 1997; Hanse and Gustafsson 2001). For the interpretation of the evoked responses, understanding of the reliability of presynaptic activation from the individual pulses is essential. However, since the presynaptic APs were usually inaccessible for monitoring, it may be hard to judge whether the observed successes versus failures of transmission were due to a probabilistic transmitter release or due to unreliable activation of the particular axon. Certain protocols have been developed to handle minimal stimulation safely but provide no guarantee for successful use. These technical problems are enhanced when PPS or train stimulation is used. Upon application of such stimulation, it has been reported that the neuronal excitability on the second and later stimuli was modulated by the first stimulation pulse (Wigström and Gustafsson 1981; Soleng, Baginskas et al. 2004). This indicates the possibility that PPS or multiple stimulations could activate different presynaptic axons even under stable conditions. Moreover, the firing threshold of the axon is not necessarily the same for the first and the second pulse even when such activity-dependent conditioning is absent. This is the general background for the analysis of PPR and release probability by minimal stimulation in Papers I-II, where a critical perspective is adopted in interpreting the results with respect to measurements of transmitter release probability (P_r), paired pulse plasticity (PPP) and variability of synaptic responses.

The phenomena of LTP and LTD

LTP and LTD are activity-dependent changes of synaptic efficacy, which have been considered as prime candidates for the processes underlying memory and learning. These forms of plasticity are found in glutamatergic pathways of hippocampus as well as neocortex, and specific types of glutamate receptors are involved in their induction

and expression. Induction here refers to the initial events triggering the onset of synaptic plasticity whereas expression refers to the synaptic modification that is responsible for the increase (or decrease) of synaptic efficacy. A related term is maintenance, which refers to the mechanism considered responsible for the longevity of LTP/LTP.

Essentials of LTP

The mostly studied of the two phenomena is LTP, first observed in the mid 1960's by Terje Lømo (Lømo 1966, 2003). Early experiments on LTP were performed in the hippocampus of rabbits, either anesthetized (Bliss and Lømo 1973) or unanesthetized (Bliss and Gardner-Medwin 1973). Later, LTP has been demonstrated in many other brain regions including the neocortex, and the experiments are generally performed on rats or mice, using either whole animals or in vitro brain slices. It was soon realized that LTP had many of the necessary requirements for a memory mechanism. The phenomenon is easily induced, within seconds, and it can last for a long time, up to weeks or longer (Bliss and Gardner-Medwin 1973). Moreover, its induction was shown to be associative (Levy and Steward 1979) in a manner similar to conditioned reflex learning, implying that a weak afferent pathway (few synapses) is potentiated if its activation is combined with activation of another, strong pathway (many synapses). The need for activating a sufficient number of synapses was named cooperativity (McNaughton, Douglas et al. 1978). It has been shown that the associative property is related to the need for simultaneous pre- and postsynaptic activity to induce LTP (Wigström, Gustafsson et al. 1986); this implies that LTP obeys Hebb's learning rule (to be dealt with later). The ability of the synapse to detect coincidence between pre- and postsynaptic events arises from the unique properties of glutamate receptors of the NMDA type, requiring both transmitter and voltage to be activated.

Figure 1 illustrates how LTP is induced in a typical glutamatergic synapse of the CA3-CA1 connection. We have previously considered the case of standard synaptic transmission illustrated in part A of the figure, where presynaptic APs are translated into postsynaptic charge transfer; the current was here considered to pass only via AMPA-Rs because of the Mg^{2+} dependent blockade of NMDA-R in the normal case. Hebb's condition implies the presence of both (1) presynaptic glutamate release and (2) a sufficient level of postsynaptic depolarization, a situation depicted in part B of the figure. The depolarization of the postsynaptic neuron, leads to the Mg^{2+} ion being expelled into the extracellular space. When this happens, the glutamate can activate (open) the NMDA-Rs; these are permeable to Ca^{2+} ions in addition to their basic permeability to Na^{+} and K^{+}, leading to influx of Ca^{2+}. The increased Ca^{2+} concentration is believed to trigger specific enzymes leading to the expression of LTP

in terms of a persistent increase of the synaptic efficiency (Lisman 1994). Whether this synaptic modification only involves AMPA-Rs or whether responses via NMDA-Rs are also potentiated is still controversial. It can be noted that the depolarization from the own synapse is generally insufficient to remove the blockade by Mg^{2+} but cooperation of many synapses is needed (the basis for associativity and cooperativity).

There are a few major hypotheses for the expression of LTP. In principle, the same mechanisms but working in the other direction are valid for LTD, to be dealt with in the next paragraph. The evidence derives from extensive research on LTP (and LTD) during many years (Bliss and Collingridge 1993; MacDonald, Jackson et al. 2006) .

1. An increase in the probability of glutamate release. It has been postulated that this will require a retrograde messenger. Among proposed messenger candidates are arachidonic acid as well as diffusible gases such as nitric oxide (NO) and carbon monoxide (CO).
2. Phosphorylation-induced changes in the properties (e.g conductance) of postsynaptic AMPARs. Persistent activation of CaMKII by autophosphorylation has been considered to be involved as a switching mechanism to explain the maintenance process.
3. Trafficking of postsynaptic AMPA receptors, implying that these receptors (and possibly also NMDARs) are moved into (or out of) the synaptic membrane. New receptors may be derived from the extrasynaptic membrane or by adding receptors/membrane via exocytosis from intracellular vesicles.
4. Morphological changes. These could involve re-shaping of entire synapses or addition (or removal) of new ones via splitting or in some other way.

As a special case, alternatives 2-3 may involve a transformation of synapses totally lacking AMPAR mediated responses (silent synapses, see Durand, Kovalchuk et al. 1996) into AMPA-responsive ones (silent synapses become speaking). It can be noted that AMPA-silent synapses still have the NMDA-Rs needed for induction of LTP. Whether totally silent synapses exist, perhaps dependent on VDCCs for plasticity induction, is unclear.

LTD and depotentiation

Types of LTD

In addition to LTP, the opposite mechanism LTD has been described, representing a long-lasting weakening of synaptic strength. Several types of LTD exist, with different properties regarding both induction and expression. The LTD of prime interest in

relation to the present work is induced via postsynaptic NMDA-dependent Ca^{2+} influx (Bear and Abraham 1996). Other forms may require Ca^{2+} via VDCCs, or depend on activation of mGluRs (Kemp and Bashir 2001). In the cerebellar cortex, a special form of associative LTD depends on coincident activation of two types of glutamatergic synapses, activated via climbing fibers and parallel fibers, respectively (Ito 1986). LTD in the hippocampus was first described by the Gary Lynch group in the 1970's. This LTD was heterosynaptic and regarded as a correlate of LTP as it was observed to accompany LTP in an untreated control pathway (Lynch, Dunwiddie et al. 1977). However, other work suggested that this depression was unrelated to LTP (Dunwiddie and Lynch 1978; Abraham and Goddard 1983). It was not until fifteen years later that homosynaptic LTD was discovered by Bear and associates. This LTD occurred under weak, prolonged activation of NMDARs by low-frequency stimulation (LFS) and was considered to represent a mechanism mirroring LTP (Dudek and Bear 1992; Mulkey and Malenka 1992; Dudek and Bear 1993). It has later been argued that the mirroring is not complete, considering that separate CaMKII phosphorylation sites appeared to be involved in the two forms of plasticity (Lee, Barbarosie et al. 2000). Whether the homosynaptic LTD operates in an associative manner is not entirely clear. The dependence on NMDARs, with their coincident "pre-post control", suggests that an element of associativity may be involved. Even so, a form of LTD induced by "asynchronous pairing" was explicitly described as "associative" but was found to depend on mGluRs and VDCCs but not NMDA-Rs (Stanton and Sejnowski 1989; Normann, Peckys et al. 2000). In the following, the term LTD will be used to denote the homosynaptic NMDA-dependent form.

With respect to the expression of NMDA-dependent LTD as well as other forms, the possible candidate mechanisms are analogous to those involved in LTP, though obviously with changes in the other direction. The underlying modification could thus be a presynaptic decrease of transmitter release, a decrease in the efficacy or number of postsynaptic receptors, or a structural change involving all or part of the synapse. It can be noted that AMPA-Rs play a less central role for LTD than for LTP, as LTD is generally associated with near equal changes of AMPA and NMDA components of the synaptic response (Xiao, Karpefors et al. 1995a). This is in contrast with LTP, which is characterized by a predominant change of AMPA (Xiao, Karpefors et al. 1995b). Accordingly, LTD could be due to a decrease of transmitter release presynaptically. To the extent that LTD is due to postsynaptic receptor changes, there appears to be a parallel change of the two receptor types, perhaps coordinated in some way. Some prior works argue that the observed equal involvement of AMPA and NMDA is a chance effect and, depending on experimental conditions for LTD induction, the depression of either receptor type can dominate (Selig, Hjelmstad et al. 1995).

Ways to induce LTD and DP

LTD is generally induced by LFS (1-5 Hz) of presynaptic axons for several minutes, as compared to LTP which is induced by one or a few bursts of HFS (e.g. 100 Hz for 1 s). An alternative way to induce LTD is chemically by direct application of the agonist NMDA for a few minutes (see Paper III). Saturation experiments as well as other tests have shown that such chemically induced LTD is equivalent to the stimulus-induced variant (Lee, Kameyama et al. 1998; Li, Dozmorov et al. 2004). When applied to a pathway where LTP has been already induced, these LTD induction protocols (via stimulation or chemically) generally lead to a decrease of the potentiated responses, often back to the original baseline level (Dudek and Bear 1993; Lee, Kameyama et al. 1998). This process is referred to as DP rather than LTD to signify that the synaptic transmission is reset back to initial conditions and so allows a another round of LTP induction (repotentiation, see Paper III). Selective activation of AMPA-Rs has also been reported to induce DP in some cases (Staubli and Chun 1996) but these protocols were never successful in our hands. Still another variant of inducing NMDA-dependent LTD is to use long-term TFS (e.g. 0.1 Hz) under conditions of facilitated NMDA-R activation (Dozmorov, Niu et al. 2003). The facilitation of NMDA-Rs is achieved by perfusing the slices with a low (0.1 mM) Mg^{2+} solution leading to partial removal of the Mg^{2+} dependent block of NMDA-Rs that is otherwise present (at millimolar concentration). Baseline responses are recorded in the presence of the NMDA-R antagonist AP5, which can subsequently be washed out to initiate the induction of plasticity. This protocol has an advantage that it obviates any direct presynaptic effects. Since both the presynaptic stimulation and unblocking of NMDA-Rs are essential, the protocol was referred to as pharmacological pairing (Dozmorov, Niu et al. 2003). Interestingly, not only was LTD induced but also an initial transient potentiation, possibly a form of short-term potentiation (STP). The pharmacological pairing protocol is useful e.g. when a large "test LTD" is needed (Paper III). A further development of the protocol by combining it with PPS allowed the study of temporal factors controlling bidirectional plasticity (Paper IV).

From induction to expression: how to control bidirectional plasticity

Hebb's rule

The induction of LTP depends on coincident activity in pre- and postsynaptic cells (Wigström and Gustafsson 1985, 1986; see also Baranyi and Feher 1981). This was demonstrated in intracellular studies using a "pairing protocol" with concurrent delivery of (single) presynaptic stimuli and postsynaptic depolarization via the intracellular electrode. During extracellular recording, LTP is generally induced by

HFS applied to the presynaptic axons, which is sufficient to also generate the necessary postsynaptic activity.

The induction of LTP via pre- and postsynaptic coincident activity is in line with Ivan Pavlov's idea about cortical associators/analyzers more than a century ago, and it agrees with the theoretical proposals of Donald Hebb and Jerzy Konorski in the late 1940's (Konorski 1948; Hebb 1949). The original formulation by Hebb states: "When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased". Synapses that change their efficiency in this manner are called Hebbian. Studies on artificial neural networks with Hebbian synapses have demonstrated that stable states (recalled memories) can be formed in line with Hebb's idea of cell assemblies (Sakurai 1996; de Almeida, Idiart et al. 2007). In the original formulation (see above), the important thing for inducing synaptic strengthening is that the sending (presynaptic) cell is involved in generating APs in the receiving (postsynaptic) cell. The successful promotion of APs is thus reinforced, a way of thinking that might be lost when simplifying the reasoning in terms of concurrent firing, as in the popular saying "cells that fire together wire together". While spiking is not needed for LTP under certain artificial experimental treatments (Gustafsson, Wigström et al. 1987), it might well be important under more natural conditions. It has been considered that backpropagating dendritic spikes play a role in forwarding the message about AP generation back to the responsible synapses that generated the original depolarization (Markram, Lubke et al. 1997).

Bidirectional control via the induction strength

The biological signal corresponding to the successful fulfillment of Hebb's condition is Ca^{2+} influx via NMDA-Rs, leading to LTP via activation of Ca^{2+} dependent enzymes (Lynch, Larson et al. 1983; Lisman 1989; Malenka, Kauer et al. 1989). Among several types of kinases implied, CaMKII is of special interest and constitutive activation of this enzyme via autophosphorylation has been considered to play a key role (Lisman 1994). As we have seen, LTP is not the only form of plasticity which is induced by activation of NMDA-Rs, another important form being LTD. An essential question is how activation of the same type of receptor can lead to opposite changes in synaptic strength. Since the original Hebb rule dealt with conditions leading to only potentiation, supplementary rules are needed to explain the induction of bidirectional plasticity. From a theoretical point of view, one could argue that synaptic activation leading to just a weak Ca^{2+} signal would be suitable for LTD induction. This idea was examined experimentally by comparison between different induction frequencies

(Dudek and Bear 1992). Whereas HFS (50 Hz) resulted in LTP, LFS (1 or 3 Hz) resulted in LTD. No change occurred at an intermediate frequency (10 Hz). These results were taken as evidence that LTD is induced by a small induction strength whereas LTP is due to stronger induction. As a consensus of this and other works, a small (or moderate) increase in Ca^{2+} concentration of the postsynaptic cell leads to LTD whereas a larger concentration increase leads to LTP (Lisman 1989; Dudek and Bear 1992; Mulkey and Malenka 1992). It is believed that enzymes with different sensitivities to Ca^{2+}, involving both kinases and phosphatases, are responsible for LTP and LTD (MacDonald, Jackson et al. 2006). The over-all result of the biochemical chain is phosphorylation or dephosphorylation of certain target proteins, including receptors.

Dependence on the calcium source

In a variant of the described scenario with frequency/intensity controlling NMDA-dependent potentiation or depression, it has been considered that activation of different types of NMDA-Rs might be involved and selectively triggered by certain activation patterns. Of special interest is the composition of NMDA-Rs in terms of NR2A and NR2B subunits. The genetic expression of these subunits is developmentally regulated with NR2B dominating in the early postnatal brain and NR2A taking over later (Liu, Murray et al. 2004). The corresponding receptor types containing these subunits also differ through several functional characteristics (Ewald, Van Keuren-Jensen et al. 2008). One can imagine a scenario where spatially segregated NMDA-R types are specifically linked to plasticity-inducing enzymes in different parts of the postsynaptic spine membrane. In line with this idea, it was previously found that the induction of LTP by HFS was mediated via NR2A-containing NMDA-Rs whereas the induction of LTD by LFS was mediated via NR2B-containing NMDA-Rs, possibly located extrasynaptically (Liu, Wong et al. 2004; Massey, Johnson et al. 2004). Contrasting results have also been reported where both subunit types contribute to LTP or LTD (Zhao, Toyoda et al. 2005; Berberich, Jensen et al. 2006). The issue about subunit involvement is further examined in Paper III of this thesis.

Spike-timing-dependent plasticity (STDP)

The factors determining the magnitude of influxed Ca^{2+} are (1) presynaptic and (2) postsynaptic activity and (3) the degree of coincidence between them. According to STDP theory, coincidence is particularly important and the induction of LTP or LTD is controlled by precise, millisecond timing between pre- and postsynaptic spiking. The mechanism is aided by back-propagating action potentials (BPAPs) travelling from the initiation site in the soma/axon and out into the dendritic tree. Several experimental

studies support the existence of STDP (Markram, Lubke et al. 1997; Dan and Poo 2004), but the idea has also received criticism (Lisman and Spruston 2005). STDP can result from presynaptic spikes preceding postsynaptic ones (pre-post spiking) or postsynaptic spikes preceding presynaptic ones (post-pre spiking). Usually, it is considered that pre-post spiking causes LTP of the synapse because of activation of NMDA-Rs leading to a large influx of Ca^{2+}. Post-pre spiking causes LTD due to limited activation of NMDA-Rs and resulting smaller increase of Ca^{2+} influx; in this case the NMDA-R activation is under control of spike after-potentials rather than by the spike itself. STDP can be understood in terms of Hebb's postulate in its original asymmetric form, where the presynaptic cell A is required to take part in firing the postsynaptic cell B. Such active contribution to cell firing will only occur with pre-post spiking. In the post-pre case the prespike occurs too late to assist in firing because the postspike has already occurred.

Dependence on slow time factors and prior activation

Both the size and millisecond timing of the induction signal are thus possible determinants of the directionality of the synaptic change. It has also been considered that the temporal character of signals is also important in a longer time perspective. This is in line with the commonly used induction protocols, whereby LTP is induced by seconds-long HFS (Bliss and Collingridge 1993) whereas LTD uses LFS for a much longer time, often up to 10-15 min (Kemp and Bashir 2001). The previously cited evidence for frequency-dependent induction used a constant number of impulses (Dudek and Bear 1992); it can not therefore be excluded that stimulus duration was important in controlling the direction of the synaptic change. The role of a slow time factor was supported by previous work in our lab using TFS (0.1 Hz) under low Mg^{2+} conditions to activate NMDA-Rs. It was found that longer times of induction favored LTD over LTP (Dozmorov, Niu et al. 2003). Another study using Mg^{2+}-free solution to unblock NMDA-Rs reported that LTD was only induced with stimulation longer than about 3 min (Mizuno, Kanazawa et al. 2001).

Still other works have questioned the need for longer times to induce LTD, arguing that the size of the induction signal is the key parameter. It has thus been demonstrated that LTD could be induced by a relatively short HFS (20 Hz for 30 s) in combination with procedures that reduce Ca^{2+} influx such as partial NMDA-R blockade or low extracellular calcium (Mulkey and Malenka 1992; Cummings, Mulkey et al. 1996). Notably, LTP was induced when the partial blockade was absent. Different levels of depolarization were also used to modulate the induction strength, and either LTP (extracellularly) or LTD (intracellularly in a hyperpolarized cell) could be induced by a 10 Hz train applied for only 2 seconds.

In conclusion, the role of slow time factors in LTP/LTD induction is still a controversial issue. We have further examined this issue in Paper IV using a novel experimental model in which single-pulse stimulation (SPS) and paired-pulse stimulation (PPS) were applied under low Mg^{2+} conditions. It was found that the directionality of synaptic plasticity depended on prior activation for tens of minutes or longer, and these results were related to ideas about metaplasticity, the plasticity of plasticity (Abraham and Bear 1996).

MAJOR AIMS OF THE THESIS

The present study makes use of the hippocampal slice technique combined with electric recording and pharmacological manipulation to investigate the complex biological events involved in activity-dependent neuronal plasticity, including short-term synaptic plasticity such as PPF/PPD and STP, and long-term synaptic plasticity such as LTP, DP and LTD. The work also explores methodological issues related to the stimulation of presynaptic fibers at weak intensity, focusing on the reliability of stimulation, properties of neuronal excitability and their relationship with transmitter release and synaptic plasticity.

Specific goals

1. To explore the variability of synaptic responses evoked in CA1 pyramidal cells by weak afferent stimulation, considering both unreliable presynaptic activation and the probabilistic nature of transmitter release; and using CA3 cell activation to elucidate important features of unreliable activation.

2. To examine how measurements of synaptic parameters in the CA3-CA1 connection, including release probability (Pr), paired pulse ratio (PPR) and coefficient of variation (CV), are influenced by unreliable activation.

3. To investigate the role of NMDA-R subunit composition, specifically in terms of NR2A and NR2B subunits, for the induction of LTP, LTD and DP; with consequences for the role of Ca^{2+}.

4. To study how the induction of NMDA-dependent plasticity is influenced by a sudden increase of NMDA-R activation; with emphasis on the way that temporal factors control the induction of bidirectional plasticity.

5. To determine how different temporal components of bidirectional synaptic plasticity are related to each other and to NMDA-R activation.

METHODOLOGY

The brain-slice technique is a popular method for studying synaptic transmission between neurons, providing for well controlled stimulation and recording as well as easy exchange of extracellular solutions. We used submerged hippocampal slices from neonatal or young rats in combination with electrophysiological techniques that allowed us to study two independent pathways in each slice. Synaptic responses were recorded extracellularly as field potentials in the CA1 apical dendritic layer, or intracellularly as synaptic potentials or currents from visually identified CA1 or CA3 pyramidal neurons. In this study we recorded either isolated AMPA-R or NMDA-R mediated responses, or composite ones containing both AMPA and NMDA components that were assessed in parallel.

Preparation and maintenance of brain slices

All animal handling and subsequent procedures to prepare brain slices conformed with the guidelines of the Swedish Council for Laboratory Animals and were approved by the Local Ethics Committee of the University of Gothenburg. The animals were housed in a special core facility and were transferred, one at a time, to the electrophysiological laboratory at the day of the experiment. After decapitation under initial isoflurane (Forene) anesthesia, the brain was removed and placed in an ice-cold artificial cerebrospinal fluid (ACSF) composed of (mM): NaCl 119, KCl 2.5, $CaCl_2$ 0.5, $MgCl_2$ 6, $NaHCO_3$ 26, NaH_2PO_4 1 and glucose 10, oxygenated by 95% O_2 and 5% CO_2. The hippocampus of one or both sides was dissected out and transverse slices (400 μm) were cut using a vibrating tissue slicer (Campden Instruments). The slices were allowed to recover for 1-2 h in a holding chamber where they were stored at 22-24 °C (room temperature) in an ACSF solution similar to that above but with $CaCl_2$ 2 mM and $MgCl_2$ 6 mM.

For the electrophysiological experiments, slices were transferred as needed to one or several submerged-type recording chambers, perfused (1.5-2 ml/min) at 30-32 °C by ACSF similar to that above but (generally) containing 2.5 mM Ca^{2+}, 1.3 mM Mg^{2+} (for recording of isolated AMPA responses) or 2 mM Ca^{2+}, 0.1 mM Mg^{2+} (for recording of composite AMPA-NMDA-responses). The usage of low Mg^{2+} allowed for

expression of an NMDA-R mediated component of the synaptic response. In this case, the ACSF also contained a low concentration (0.5-1 μM) of the AMPA-R antagonist CNQX to partially block AMPA-R mediated responses, leading to a balanced mixture of AMPA and NMDA components. Recording of excitatory postsynaptic potentials (EPSPs) generally commenced with isolated AMPA EPSPs in the presence of 50 μM of the specific NMDA-R antagonist AP5; the antagonist was later washed out to allow additional expression of the NMDA component. The solutions were generally re-circulated by means of a multi-channel peristaltic pump. Different chemicals could be applied in the solution to determine the possible effects on synaptic transmission and/or plasticity. The subsequent procedures differed between extracellular and intracellular recording, and are described separately.

Extracellular experiments

Electric stimulation and recording

Stimulating and recording electrodes were positioned via micromanipulators in the slice under visual guidance by a microscope. Synapses were activated by stimulation of the Schaffer collateral commissural pathway from CA3 to CA1 of the hippocampus. Field EPSPs (fEPSPs) were recorded from the CA1 apical dendritic layer by a glass micropipette filled with ACSF or 1-3 M NaCl (2-5 MΩ resistance). Normally, AMPA-receptor mediated fEPSPs were recorded but, if needed, NMDA-receptor mediated responses could be obtained in a modified perfusion solution containing a lowered concentration of magnesium. Two monopolar tungsten stimulating electrodes were placed on either side of the recording electrode to provide for stimulation of two separate synaptic pathways. Negative, constant current pulses, 100 μs, 10-50 μA, were alternately delivered to the two stimulating electrodes, the interval between successive stimuli generally being 5 s (10 s for each pathway). Paired pulse facilitation was studied by means of double pulses, generally with a 50 ms interstimulus interval. LTP was induced by either high frequency stimulation (HFS), typically consisting of 1-3 stimulus trains, each containing 100 pulses at 100 Hz, or by theta burst stimulation (TBS), typically consisting of 1-3 sequences of 10 brief 100 Hz trains, 4 impulses each, repeated at 5 Hz. If required, the non-synaptic response consisting of stimulus artifact and presynaptic volley was recorded under pharmacological blockade of both AMPA and NMDA receptors; the resulting potential was then subtracted from the previously recorded fEPSP. LTD was induced by test frequency stimulation (TFS, 0.1 Hz) for one or a few hours in a in low Mg^{2+} (0.1 mM) solution (stimulus-induced LTD), or by application of the agonist NMDA (20-30 μM) under perfusion with normal Mg^{2+} (1.3 mM).

Data acquisition and analysis

The recorded signals were amplified, filtered, digitized and transferred to a "PC clone" computer for on-line and off-line analysis by specially designed electronic equipment and own-developed computer software. The fEPSPs were measured using an early time window of 1-2 ms duration positioned just after the presynaptic volley, thereby avoiding contamination by postsynaptic firing. Measurements were calculated by integrating the fEPSP curve along the specified time window after subtraction of the prestimulus baseline. Similar results were obtained with slope measurements. PPF was quantified as PPR, defined as the size of the second pulse response relative to the first. In experiments with composite EPSPs the AMPA component was assessed by the just-mentioned early measurement whereas the NMDA component was assessed by a late measurement, using a time window at about 30-45 ms after the stimulus artifact (Figure 3). Potentiation or depression was generally quantified as fEPSP size relative to the initial baseline; an exception concerns PPR, where the percentage change relative to the baseline was used. Statistical comparisons were generally made by help of Student's *t*-tests. Data were presented as mean ± SEM.

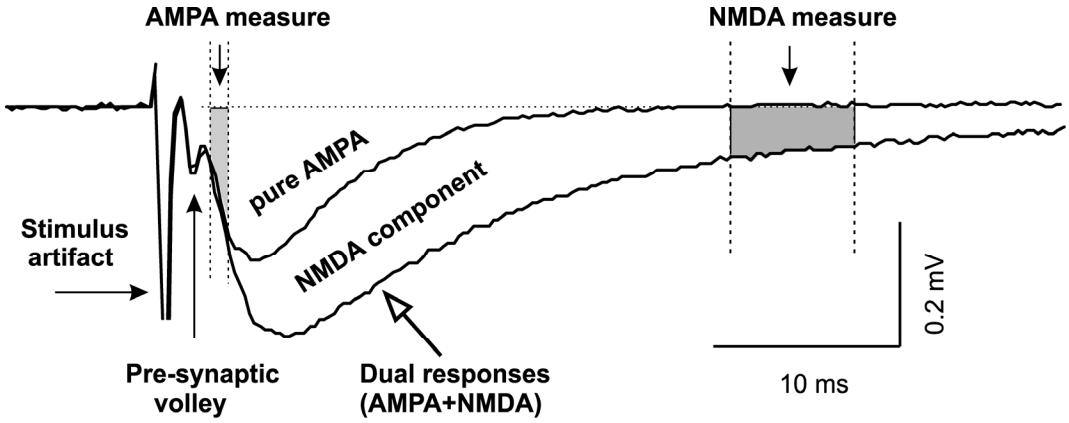

Figure 3. *Illustration of the measurements of AMPA and NMDA components. The dual component EPSP was obtained in low Mg^{2+} solution (lower trace) whereas the isolated AMPA EPSP was obtained after application 50 μM of the specific NMDA-R antagonist AP5 (upper trace). AMPA EPSPs could also be obtained in normal magnesium without AP5. The initial part of EPSPs was used to measure the AMPA component (AMPA measure) and the later part (after 30 ms) was used to measure the NMDA component (NMDA measure).*

Equipment for running parallel experiments

Four slices could be run in parallel in a "multichamber setup" with 4 individual chambers. Each of the chambers provided two stimulating electrodes and one recording electrode. Comparison between pathways in the same slice gives higher and better experimental yield. Thus, plasticity induced in one pathway can be compared with plasticity in another pathway, or the responses of an untreated control pathway can be used to compensate for unspecific decline of responses during long experiments. Electrode positioning was controlled by mechanical 3D micromanipulators operated by hand. The multi-chamber system was equipped with a pinch valve operated perfusion multiplexer, making it possible to select one bottle of solution out of 4 alternative ones. Special measures were taken to prevent cross-contamination between bottles. A computer system was interfaced with a specially designed hardware controller, allowing electric stimulation as well as solution exchange in an automated manner. A script language was used to define all events during an experiment, including commands for controlling stimulators, valves and heaters with respect to amplitude as well as timing. Experiments could thus be run automatically once the initial baselines were achieved under manual control. Additionally, the system was able to handle current injection through the microelectrodes as well as automatic DC-offset for all amplifiers. The automatic offset prevents loss of recordings in long-term experiments.

Whole-cell electrophysiology

Whole-cell recordings were obtained from visually identified CA3 and CA1 pyramidal cells, performed with an Axopatch 1D or 700B amplifier (Axon Instruments Inc., CA, USA). Patch pipettes (1.5 mm / 0.86 mm, borosilicate glass; Clark Electromedical Instruments, UK) were pulled using a horizontal puller (Model P-97; Sutter Instruments, CA, USA). They had a resistance of 2–5 MΩ (when filled) and were neither polished nor coated.

Activation and recording of single (or small numbers of) synapses

Whole-cell recording was performed from visually identified CA1 pyramidal cells in neonatal rats (1–6 days). The properties of the postsynaptic response in the assumed single (or small numbers of) synapse(s) were examined by the application of 10 impulses of 50-Hz burst stimulation, repeated at 0.1 Hz to Schaffer collaterals under voltage clamp (-80 mV). For recordings of AMPA-R mediated excitatory postsynaptic

currents (EPSCs), the pipette solution contained (in mM): CsCl, 135; QX-314, 5; Mg-ATP, 4; Na-GTP, 0.4; EGTA, 0.2; HEPES, 10 (pH 7.3, adjusted with CsOH). The series resistance was continuously monitored during the voltage clamp experiments, and resistances lower than 30 MΩ were accepted. The EPSCs that were evoked by the initial two pulses were analyzed for the study of PPP. Similar patch pipettes were used for stimulation electrodes, with a tip diameter of about 10 μm and a resistance of about 0.5 MΩ after they were filled with extracellular solution. Recordings were collected for analysis during periods of stable series resistance (changes less than 10%).

Activation and recording of limited synapses

PPS (pulse duration 0.1 ms, pulse interval 20–50 ms, repeated at 0.1 Hz) of the Schaffer collaterals bridge and voltage-clamp (-80 mV) whole-cell recording from 12- to 28-day-old CA1 hippocampal pyramidal cells were applied to study the properties of paired pulse plasticity in limited synapses (surgically limited Schaffer collateral bridge). Extracellularly, fEPSPs were recorded via a glass micropipette (filled with 3 M NaCl or extracellular solution) in the apical dendritic layer of the CA1 region. PPS was applied to the Schaffer collaterals or surgically isolated bridge of Schaffer collaterals with tungsten stimulating electrodes.

AP firing thresholds

To study the properties of the AP firing thresholds of PPS from visually identified CA3 pyramidal cells in young animal slices (12–21 days), the PPS was injected via the recording pipette (pulse duration 1 ms and variable pulse interval, repeated at 0.1–0.33 Hz) or applied to Schaffer collaterals using tungsten stimulating electrodes (pulse duration 0.1-0.4 ms and variable pulse interval, repeated at 0.1–0.33 Hz). Current-clamp whole-cell recordings were obtained with Axopatch 1D or 700B amplifier. To determine the AP firing thresholds and input/output characteristics, 10 trials were repeated at each test current level. An unreliable AP firing threshold (Tu) was defined as the value of the stimulation at which 20–50% of the stimuli elicited APs. A reliable AP firing threshold (Tr) was defined as the minimal value of stimulation for 100% firing APs. The pipette solution contained (in mM): K-gluconate, 130; NaCl, 5; KCl, 15; Mg-ATP, 4; Tris-GTP, 0.2; EGTA, 0.4; HEPES, 10 (pH 7.25, adjusted with 2 M KOH).

AC firing thresholds

To study the properties of the action current (AC) firing thresholds of PPS from visually identified CA3 pyramidal cells in young animal slices (12–21 days), PPS (pulse duration 1 ms, pulse interval 30 ms, repeated at 0.1–0.33 Hz) was injected in a stepwise manner via the recording pipette, and voltage-clamp (-70 mV) whole-cell recordings were obtained with a 700B amplifier.

Data analysis

All responses, such as APs, EPSCs and (f)EPSPs, were filtered at 2 kHz and sampled at 10 kHz. The peak amplitudes of EPSCs and EPSPs were measured. All data are presented as mean ± SD. A paired *t*-test was used to determine statistical significance. The correlation between facilitation of AP firing threshold and depolarization level was determined by use of the correlation coefficient (r). The analysis was performed using custom software written in Igor Pro (Wavemetrics, Lake Oswego, OR, USA). Detection of miniature synaptic events was done automatically with Mini Analysis Program (Synaptosoft, NJ, USA), and confirmed manually in doubtful cases.

RESULTS AND DISCUSSION

Reliability of stimulation, properties of neuronal excitability and their relationship with transmitter release and postsynaptic response probability (Papers I, II)

Studies of transmitter release at hippocampal synapses often make use of an experimental protocol where a low stimulus strength (minimal stimulation) is used to activate only one or a few synapses. In the present study, EPSCs were recorded in CA1 cells under such weak stimulation using paired pulses and/or short stimulus trains. As detailed below, the measurements of several key synaptic parameters did not only depend on synaptic properties but also on the fact that the applied electrical stimulation did not reliably induce axonal firing. The features of such unreliable activation were examined using different protocols, including CA3 cell stimulation and recording, and were compared to the observed synaptic response patterns in CA1 cells.

Paired-pulse firing thresholds and relation with paired-pulse plasticity (Paper I)

The stimulus strength was set to elicit EPSCs in putative single (or a few) synapses and repeated 10-impulse 50 Hz trains or PPS were delivered. The notation P_{res} is used to describe the postsynaptic response probability and as such will include variability at the stimulus site as well as variability due to the transmitter release process. P_{res} was found to vary considerably among recorded cells/pathways, from less than 0.1 to above 0.9. Notably, in cases with low values of P_{res}, increased stimulation generally led to significant increases of P_{res} although the apparent size of the unitary response was not much altered. The response pattern during train stimulation (or PPS) underwent substantial changes as a result of this strength increase. In cases where responses mainly occurred late during the train, increasing the stimulus strength caused the response distribution to be shifted to earlier times. As a consequence, there was an associated change of PPR. The PPR resulting from the lower strength PPS often had a substantial magnitude but changed to below one with the higher strength PPS, implying a conversion from PPF to PPD. The most plausible explanation of these

results is that the afferent stimulation was not reliably evoking action potentials for the first pulses at lower strength, leading to a right-shifted response distribution and associated overestimation of PPR. At higher strengths, causing a more reliable activation, the PPR value is dominated by the synaptic properties. Thus, the release probability P_r can be represented by P_{res} at reliable activation, and was found to be high (near one), in conformity with previously published estimates (Bolshakov and Siegelbaum 1995). It should be noted that this part of the study was carried out in neonatal rats (1-6 days), which have a sparse distribution of functional CA3-CA1 connections (Durand, Kovalchuk et al. 1996; Hsia, Malenka et al. 1998), making reliable stimulation possible at the higher strengths with a relatively small risk of activating multiple synapses.

Variability of burst-induced AMPA responses in relation to reliability of stimulation (Paper II)

Among synaptic parameters that can be derived from EPSCs under minimal stimulation is the coefficient of variation (CV), defined as the standard deviation over the mean of the EPSC during a set of trials. In the present study, CV was measured for successful transmitter release events, the latter being judged by computer aided detection in combination with manual approval (see Methodology). The CV defined in this manner depends on both the heterogeneity of synaptic vesicle size and the degree of saturation of postsynaptic AMPA receptors (McAllister and Stevens 2000). Receptor desensitization is likely to play a minor role since such effects were found to be absent between successive responses in CA1 synapses (Hjelmstad, Isaac et al. 1999). Regardless, the interpretation of CV is not critical for the present methodologically oriented approach. It can be noted that CV is expected to be less dependent on the reliability of stimulation than some other measures such as the response probability (P_{res}), considering that only successful events counted for the calculation of CV as defined here. It was therefore of interest to see whether experimental factors influencing the reliability of stimulation would have any influence on CV. Such factors involve setting of the stimulus strength as well as selecting first versus later responses for measurement. Values of CV were not constant among these possibilities, and especially the CV of first responses during reliable activation was smaller than the CV of later responses during unreliable stimulation. A possible explanation is that the train stimulation can induce multiple release from either the same or different synapses. An alternative scenario is that during sparse temporal activation, such as during unreliable train stimulation, vesicles near the release site have time to reorganize, and so influencing the EPSC distribution during the train compared to the case with reliable stimulation. These experiments illustrate the difficulties associated with the use of the

minimal stimulation method as a means to stimulate single synapses. In conclusion, using the CV of responses evoked by multiple stimuli to reveal essential synaptic mechanisms (including saturation of postsynaptic receptors) therefore needs to take into account both the reliability of stimulation in setting up presynaptic APs as well as the possible contributions from multiple sites of release.

Using CA3 cells as models for paired pulse activation

In addition to neonatal rats, aged 1-6 days (see above), experiments were carried out with 12- to 21-day-old rats, referred to as "young" in the following. The observed left-shift of the temporal response distribution with increasing stimulus strength suggested a lower AP threshold for the second and later pulses as compared to the first one. To directly study this inferred characteristic is problematic because the CA3 cell axons (Schaffer collaterals) are not amenable to analysis of excitability at the unitary level. In an attempted model system, intracellular activation of single CA3 cells was tested during whole cell soma recording in both voltage and current clamp mode. The results showed that the AP firing threshold in the same CA3 pyramidal cell was significantly lower for the second pulse of PPS than for the first one. This effect was dependent on the interval between stimuli and closely related to the degree of depolarization remaining from the first pulse at the time of the second. Using higher strength, firing occurred in response to both pulses. No threshold difference between first and second firing event was seen in recordings carried out in voltage clamp mode, supporting the idea that depolarization remaining from the first stimulation played a critical role in the current clamp experiments. Additionally, it was found that AP firing thresholds varied on successive trials (in the same CA3 pyramidal cells) and it was possible to define a reliable as well as an unreliable threshold.

As a methodological note, it can be added that the observation of firing under voltage clamp implies that clamping was not fully effective, as is often the case in experiments dealing with neurons. This limitation is partly due to the presence of an electrode resistance that can not be fully eliminated by electronic compensation. The resistance prevents voltage clamping when large voltage-dependent currents are involved, such as the Na^+ current. The elongated shape of CA3 cells, as well as other neurons, is also a factor that negatively influences proper clamping. As a result, all or none events (probable action potentials) were still possible under the applied voltage clamp, the recorded current being referred to as action current (AC).

It can be appreciated that the CA3 cell firing model displayed results in line with the observed strength dependency of synaptic response patterns in CA1 cells during Schaffer collateral stimulation. Our results indicate that the first pulse can be relatively

unreliable for inducing APs as compared to the second one. Hence, the PPF observed for synaptic responses in CA1 cells might be caused by the intensity of the first pulse being subthreshold or unreliable for the activation of some presynaptic axons. In the same situation, the second pulse may reliably evoke APs of the same presynaptic axons, leading to a high value of PPR.

Despite the striking analogy between minimal-stimulation-evoked EPSCs in CA1 cells and the excitability testing carried out in CA3 cells, it is motivated to question the explanatory power of the CA3 cell model. To what extent can it account for events at the axon level beyond a simplistic description? Certainly, the ion channels and conductances involved in soma firing versus axonal firing are not all the same. According to a dominating theory, action potential initiation occurs in the axon hillock, assumed to have a high density of Na^+ channels (Wollner and Catterall 1986). Early studies on hippocampal neurons suggested, however, that active conduction may start in the dendrites (Andersen, Storm et al. 1987). It was later reported in patch clamp studies that APs originate in the axon hillock or even in the axon beyond it (Spruston, Schiller et al. 1995; Colbert and Johnston 1996). The presently studied firing threshold may therefore indeed represent the firing threshold of the axon (or axon hillock/initial segment). Even so, the initiation of firing patterns by repetitive activation is not only a threshold matter but depends on various conductances/potentials, including spike after-potentials, that are different in soma and axon. A previous study reported that, if the first pulse of PPS evokes an AP in axons, this AP facilitates the response to a second pulse and makes it easier to activate an AP on the second one (Soleng, Baginskas et al. 2004). This result was suggested to be due to spike after-potentials. Our results showed, however, that the first sub-threshold pulse had the ability to facilitate the second pulse of PPS for firing an AP even in the absence of firing on the first one. This implies that spike after-potentials will not affect the threshold measurements in this case, although they will certainly contribute to the firing pattern under stronger stimulation.

A remaining concern is the cell membrane time constant, which will influence temporal summation properties in the sub-threshold region. Does it differ between soma and axon? This question remains to be answered. Another relevant issue is the filtering effect of the soma capacitance with respect to electric events in the axon. Considering that the varying threshold on consecutive trials may be due to ion channel fluctuations in the axonal membrane (se General Discussion), the soma experiment would tend to underestimate these fluctuations. As a consequence, the unreliability (variation) of the axonal firing threshold will tend to be underestimated.

Using CA3 cell recording to monitor the result of extra-cellular stimulation of axons

In an attempt to study unreliable versus reliable stimulation in more detail, intracellular recording was performed from CA3 cells during extracellular stimulation of the Schaffer collaterals. The idea was that the pattern of CA3 cell activity would mimic that of single synapse activation of CA1 cells. It was found that, during weak PPS, APs occurred preferentially in response to the second stimulation, thus indicating a lower activation threshold. With increasing stimulus strength, APs also occurred on the first pulse until there was reliable firing in response to both stimulus pulses. Ideally, the employed experimental model should use a situation where the studied CA3 cell fires antidromically in response the activation of its axon (the Schaffer collateral). The success rate of finding the suitable arrangements of stimulation and recording was relatively low, however, leading us to use a stimulus strength higher than normal. The possibility can not be excluded that the higher stimulus strength will activate adjacent axons and causing EPSPs via CA3 cell recurrent connections (de Almeida, Idiart et al. 2007). Nevertheless, in many cases (see illustration in Paper I, Fig. 4A), the activation mode was likely of the antidromic type as revealed by the short latencies. Despite the mixed activation types, the model can still be useful in predicting synaptic activation of CA1 cells; after all, the CA3 area maps onto CA1.

Experiments with a "limited Schaffer collaterals bridge"

Whereas the neonatal rats have a weakly developed network of functional CA1-CA3 connections, the corresponding network in the young ones is much denser (Durand, Kovalchuk et al. 1996; Hsia, Malenka et al. 1998). This makes it difficult to obtain an experimental situation where a shift between unreliable and reliable activation can be achieved by a stimulus strength increase without risking to activate multiple axons. A modified experimental strategy was therefore used in the young rats by adding a surgical cut between CA3 and CA1 areas, leaving only a bridge of unsevered fibers, about 50-100 μm wide. Unfortunately, this led to poor experimental yield and only 4 out of 28 cells exhibited a verifiable synaptic connection, even though a number of stimulus electrode tracks were tested. It was found that the bridge-stimulated CA1 cells in young rats had similar properties as the previously studied cells in neonatal rats. Thus, applying low and high stimulations to the limited Schaffer collaterals bridge caused a shift between PPF and PPD, respectively. This result was also observed with extracellular recording used in combination with the bridge-stimulation technique; however, the intracellular method should be the preferred one to use. It remains to verify the suggestion that the shell of unreliably stimulated fibers remains a constant proportion of the sphere of reliably stimulated ones, independently of the stimulus

strength (see Fig. 7 of Paper I). Regardless, the present data strongly suggest that changes in reliability associated with the first pulse of stimulation is an important source of the heterogeneity of P_{res} (of the first response) and the pattern of PPP observed in CA3–CA1 synapses of hippocampal slices.

Role of NMDA-R subunit composition in the induction of synaptic plasticity (Paper III)

A previously held conception is that specific types of NR2 subunits are responsible for different forms of NMDA-dependent synaptic plasticity (Liu, Wong et al. 2004; Massey, Johnson et al. 2004). We have investigated this issue in our experimental situation, using 2-3 week old rats, allowing consistent induction of both LTP and LTD. As subunit-specific blockers we used NVP-AAM077 (for NR2A) and Ro 25-6981 or Ifenprodil (for NR2B), simply referred to as NVP, Ro and Ife, respectively. Ro and Ife have been shown to be quite selective in blocking NR2B subunits (Carter, Rivy et al. 1989; Fischer, Mutel et al. 1997). For comparison, the selectivity of NVP is not so good, and in addition to its main effect on NR2A this compound has a significant effect on NR2B (Berberich, Punnakkal et al. 2005; Bartlett, Bannister et al. 2007). NVP and Ro/Ife were here tested on several forms of long-term synaptic plasticity: stimulus-induced LTP and LTD, NMDA-induced LTD and depotentiation, as well as repotentiation after prior depotentiation. To further clarify the roles of NR2A and NR2B subunits in plasticity induction we studied the individual contributions of NR2A and NR2B to isolated responses of NMDA-Rs.

Responses of NR2A- and NR2B-containing NMDA-Rs

Isolated NMDA-R mediated EPSPs were recorded in a low Mg^{2+} solution supplemented with CNQX to block AMPA-Rs and the effects of NR2A or NR2B blockers were tested. The results showed that EPSPs were reduced to about 70% of baseline by Ro/Ife (NR2B blocker), implying a reduction by 30% that was most likely accounted for by NR2B-containing receptors. Application of both Ro/Ife and NVP (NR2A blocker), almost completely blocked the NMDAR-EPSPs, indicating that the remaining 70% after Ro/Ife was entirely mediated via NR2A. When the drugs were applied in the opposite order, the part remaining after initial NVP application was about 20%, the reduction thus being as large as 80% in this case. Our interpretation of the results is that, in 12-18 days old rats, most of the NMDA-R current (70-80%) was contributed by NR2A-type receptors whereas the remaining current (20-30% of the fEPSPs) was mainly due to NR2B. The contribution from other NR2 subunits was

apparently quite small. According to our estimates, the current mediated by NR2A was thus about three times larger than that by NR2B.

It can be noted that the concentrations of blockers used by us (0.4 μM NVP in case of NR2A, 0.5μM Ro or 3 μM Ife in case of NR2B) have previously been shown to be effective in blocking the respective NMDA-R mediated currents. However, in contrast to the case with Ro and Ife, which specifically act on NR2B, NVP appears to have limited selectivity. Thus, in a study on subunits expressed selectively in transfected HEK293 cells, it was found that NVP at "our concentration" had a cross-blocking effect (on NR2B) amounting to as much as 67% (Berberich, Punnakkal et al. 2005; see also Weitlauf, Honse et al. 2005). A later study in the same type of cells gave a value of around 40% (Bartlett, Bannister et al. 2007). In our case, the large reduction of the NMDA-R mediated response by NVP, down to 20% as compared to the 30% blocking effect of Ro/Ife, can be taken to suggest a cross-blocking effect of NVP on NR2B amounting to about one third (actually 42% if using unrounded values; not discussed in the paper).

LTD and its relationship with NR2A and NR2B

Due to the limited magnitude of standard LFS-induced LTD, we studied two related forms of LTD that are generally associated with larger amounts of depression. One form was induced by transient application of the antagonist NMDA (chemically induced LTD), the other by test-frequency stimulation under low Mg^{2+} perfusion (stimulus-induced LTD). Our results showed that blockage of NR2A-containing receptors by NVP, prevented the induction of both forms of LTD. In contrast, blockage of NR2B-receptors by NVP Ro/Ife had no effect on either form of LTD. A simple interpretation of the results is that NR2A subunits are responsible for LTD, however, contrasting prior work showing that these subunits are exclusively involved in LTP (Liu, Wong et al. 2004). An alternative explanation would be that the blockade of LTD by NVP is an unspecific effect due to a reduction of the total NMDA current below a critical level. As we showed in the NMDA EPSP blocking experiments, NR2A units provide most of the NMDA-R current in our situation, but this does not rule that the remaining NR2B may also contribute to LTD, although to a lesser extent.

A relevant question is whether, under favorable conditions, the NR2B-mediated current alone may give rise to LTD. This issue was addressed in experiments aiming to increase the amount of influxed Ca^{2+} in a situation where the NR2A subunits were blocked. Considering that Mg^{2+} is a blocker of NMDA-Rs, we further decreased the Mg^{2+} concentration, from the already low 0.1 mM to 0.01 mM, in order to enhance the unblocking effect. Our experiments, carried out with the stimulus-induced variant of

LTD, revealed an obvious depression induced under blockade of NR2A subunits. Taken together, our results imply that both NR2A and NR2B subunits contribute to LTD with NR2A and NR2B as major and minor contributors, respectively. Interestingly, either subunit type could mediate LTD induction without the help of the other. In conclusion, the present results support previous findings that both types of subunits contribute to LTD (Berberich, Punnakkal et al. 2005; Weitlauf, Honse et al. 2005; Zhao, Toyoda et al. 2005).

Testing NR2 subunit involvement in LTP, depotentiation and repotentiation

LTP

We also examined LTP induced in the presence of subunit-specific antagonists. The results showed that LTP was fully prevented by blockade of NR2A-containing NMDA-Rs. This is in partial agreement with previous work that suggested specific involvement of NR2A subunits in LTP induction (Liu, Wong et al. 2004; Massey, Johnson et al. 2004). However, blocking of NR2B subunits was also potent in our case in that it partially prevented LTP, leading to a "residual potentiation" amounting to about half the normal size. Despite the small size, this LTP was stable throughout the recording period of about one hour. The results suggest a major involvement of NR2A subunits in LTP induction as well as a partial involvement of NR2B, similar to the situation with LTD. This idea is consistent with several previous works, evidencing that both types of subunits contribute to LTP (Berberich, Punnakkal et al. 2005; Weitlauf, Honse et al. 2005; Zhao, Toyoda et al. 2005) but is inconsistent with others (Liu, Wong et al. 2004; Massey, Johnson et al. 2004).

As with LTD, the induction of LTP could be supported by NR2A subunits in isolation, though at a reduced magnitude. A remaining issue of interest is whether NR2B subunits alone can give rise to LTP. In analogy with our strategy to study LTD, we performed facilitation experiments, in which a lowering of the Mg^{2+} concentration was used to facilitate the NMDA current and thereby amplify the associated Ca^{2+} influx. The Mg^{2+} concentration was here lowered from the standard value of 2.5 mM to 0.1 mM (compare the LTD experiments where Mg^{2+} was decreased from an already low Mg^{2+}value, 0.1 mM, to an even lower one, 0.01 mM). Indeed, the low-magnesium experiments revealed a small but significant LTP even under NR2A block by NVP, suggesting that NR2B subunits were actually sufficient to induce LTP. Our results imply that both NR2A- and NR2B-containing NMDA-Rs contribute to LTP induction and that, under the proper circumstances, either type can by itself support the induction.

Depotentiation and repotentiation

In another set of experiments, initial LTP was followed by depotentiation under both normal conditions and in the presence of subunit-specific blockers. As a means for depotentiation, we used the same NMDA application protocol previously used for LTD. Since two pathways were used, one with prior induction of LTP and the other naive, we could in fact induce depotentiation and LTD in the same slice in parallel. As evidence that NMDA application actually led to depotentiation, we can consider the fact that (in the drugfree case) the potentiated level was reduced to near the original baseline. Moreover, after responses returned to baseline, further LTP could be induced; this will be referred to as repotentiation. These results and ideas are consistent with a previous study on NMDA-induced LTD and depotentiation (Lee, Kameyama et al. 1998).

In similar experiments, either NR2A or NR2B blocker was applied just after induction of the first LTP. It was found that the subsequent NMDA-induced depotentiation was substantially prevented by blocking of NR2A but not NR2B subunits. In parallel, there was a similar pattern of effects on the NMDA-induced LTD observed in the control pathway (lacking prior LTP induction), supporting our previous LTD-only experiments. Blocking of NR2A as compared to NR2B also had a predominant effect on repotentiation; however this result is hard to evaluate and might be due to differences in the preceding depotentiation.

In conclusion, our results indicate that NR2A subunits are the main contributors of the synaptic responses of NMDA-Rs, whereas NR2B receptors are minor contributors. Similar to this distribution pattern, NR2A subunits were also found to be main contributors of both LTP and LTD, as well as depotentiation and possibly repotentiation. We believe that the role of subunits in plasticity is likely a reflection of their role in NMDA-R mediated synaptic responses. The underlying cause of these differences is therefore probably the age-dependent expression of subunits in combination with their individual electrical properties. Our results thus suggest that the induction of LTP, LTD and other NMDA-dependent plasticities mainly depends on the amount of influxed postsynaptic Ca^{2+} but not specifically on NR2A- or NR2B-containing receptors.

Features of bidirectional control of synaptic plasticity (Papers III, IV)

NMDA-dependent plasticity, such as LTP and LTD, is believed to depend on Ca^{2+} influx into the postsynaptic, dendritic spines. Our study on the role of different NR2 subunits (Paper III) further advanced the idea of Ca^{2+} as a key factor, which operates without regard to how the concentration changes are brought about. Even so, it is still uncertain how Ca^{2+} determines the direction of the synaptic change. While the magnitude of the Ca^{2+} concentration is generally considered to be important, other results have suggested a role for a temporal factor (Mizuno, Kanazawa et al. 2001). We have examined how temporal factors influence the induction of potentiation and depression in an experimental model where slices were subjected to an increased level of NMDA-R activation from a previously lower level.

Potentiation and depression following slow NMDA-R activation (Papers III, IV)

A relatively slow activation of NMDA-Rs was achieved by washing out the receptor antagonist AP5 during perfusion with a low-Mg^{2+} solution. Under physiological conditions, Mg^{2+} causes a voltage-dependent block of the NMDA-Rs, and using a low Mg^{2+} concentration will largely remove this blocking effect. The idea of unblocking NMDA-Rs by washing out AP5 under already low Mg^{2+} is that direct presynaptic effects are avoided, considering that changes of extracellular Mg^{2+} can influence transmitter release. Moreover, the use of test frequency stimulation (TFS, 0.1 Hz) throughout the experiment further tends to eliminate presynaptic effects.

During the period of solution exchange of about 15-20 min, there was also an effect on the AMPA-R mediated component of the synaptic response. Typically, there was a biphasic change with an initial potentiation followed by a slowly developing depression of substantial magnitude. The latter involved both AMPA and NMDA components. In a prior study the slowly developing depression was found to share several important features with standard LFS-induced LTD, such as near equal involvement of AMPA-Rs and NMDA-Rs (Dozmorov, Niu et al. 2003). In addition, the slow LTD was partially occluded by standard LTD, suggesting that it represents a closely related form of plasticity, although with a significantly larger magnitude. This large magnitude was a major reason for using the slowly developing depression as a model for stimulus-induced LTD in Paper III. The plasticity induced by slow NMDA-R activation is further confirmed by the control pathway recordings of Paper IV. As can be seen in the time course plots of Papers III and IV, reapplying AP5 stopped the

progression of the LTD leaving a stable level of responses, consistent with previous results (Dozmorov, Niu et al. 2003).

To further explore the effect of an increase of NMDA-R activation we developed a protocol, described in the following, in which a fast step of activation was possible. Due to a balanced, two-pathway experimental design, the effects of fast and slow NMDA-R activation could be compared and related to each other.

Potentiation and depression following a fast rise of NMDA-R activation (Paper IV)

Unblocking of NMDA-Rs by washing out AP5 generally took 15 min or more before reaching full effect. Since we were interested in obtaining a much faster activation, we had to look for other options. Switching from single pulse stimulation (SPS) to paired pulse stimulation (PPS), all under low Mg^{2+} perfusion, was used as a means to transiently increase the rate of NMDA-R activation. We found that the PPS induced activation of NMDA-Rs (considering both responses together) was about three times higher than for SPS; this large value was due to the increase of the second response by PPF. Prior to the onset of SPS, these experiments generally commenced by washing out of AP5 while using SPS in both pathways, giving rise to an initial potentiation as described above. On top of this pre-potentiation, further plasticity was induced by switching from SPS to PPS in the test pathway, leaving the other pathway with SPS as a control for the remainder of the experiment.

It was found that PPS led to a quickly onsetting potentiation, reaching a peak within about 1 min and followed by a decay. The initial potentiation resembled LTP in the sense that it had a similar time-to-peak value (Gustafsson, Asztely et al. 1989) and preferentially involved the AMPA component (Xiao, Karpefors et al. 1995). However, it was only short-lasting and decayed within 10-15 min regardless of the duration of PPS, which was varied from a few minutes to more than an hour. The initial potentiation was therefore classified as STP. Following the STP, test and control pathways followed similar decaying time courses, leading to gradually increasing levels of LTD. This occurred in a similar manner regardless of whether SPS or PPS was being applied. Moreover, the responses of the two pathways became more or less the same size within 15 min after PPS onset despite the substantial difference early on. From a comparison of time courses, it appears plausible that this symmetrization is a correlate of the decaying part of the STP.

The slowly developing depression could be halted (stabilized) by application of the NMDA-R antagonist AP5 after 1-2 h (or any other time), evidencing that it was actually a form of LTD, not just a temporary depressive effect due to the ongoing stimulation (see also Paper III and Dozmorov, Niu et al. 2003). However, when carrying out experiments entirely under blockade of NMDA-Rs, most of the PPS-induced plasticity was absent, leaving only a slight depression that returned to baseline when SPS was resumed, and thus representing a form of STD. The biphasic plasticity under the standard protocol, including the STP as well as LTD, was thus dependent on NMDA-Rs. We suggested that the small STD remaining under blockage of NMDA-Rs might be induced via mGluRs, although we did not explicitly test for this possibility.

Changes of PPF during PPS-induced bidirectional plasticity (Paper IV)

Interestingly, both the initial STP and the following LTD were associated with corresponding changes of PPF, an often used indicator of presynaptic function. Taken at face value, the observed decrease of PPF during STP suggests an increase of transmitter release whereas the increase of PPF during the following LTD suggests a decrease of transmitter release. Several kinds of control experiments were carried out to exclude that these results were artifactual. Thus, the PPF effect was found to be NMDA-dependent and was unrelated to the fact that measurements were made on AMPA components of composite AMPA-NMDA responses. Moreover, the effect was not a correlate of the EPSP size change, dependent on system nonlinearities. Finally, a "calibration experiment" using adenosine to induce presynaptic changes revealed that the increase of PPF during LTD was nearly identical to the corresponding increase associated with an equally-sized reduction of the EPSP caused by adenosine. However, the simple conclusion of these results – that both the STP and LTD are expressed presynaptically – is not consistent with our data regarding the relative contributions of AMPA and NMDA components. STP was thus selectively AMPA-R mediated whereas LTD involved AMPA-Rs and NMDA-Rs to a similar extent, suggesting a mixed expression mechanism with an initial postsynaptic modification followed by a later presynaptic one. Regardless of these arguments, an entirely postsynaptic scenario is also possible. As pointed out in the paper, changes in over-all PPF can result from postsynaptic changes via an altered weighting of PPF contributions from low-release versus high-release synapses.

Blockage of NMDA-Rs after STP reveals two types of decay (Paper IV)

As described, the STP was followed by a gradually developing LTD and symmetrization of responses, the latter implying a fading of the early difference between pathways. The LTD as well as the symmetrization process could be blocked by AP5 applied after STP induction, leaving a persistent difference between pathways. It is tempting to use the term LTP to describe this remaining increase of test versus control responses. After all it seems to represent an extended survival of the STP for several hours after induction and so might be best described as LTP. This idea is especially attractive if, as suggested above, the symmetrization is considered to be a reflection of STP decay. The observed blockade of symmetrization/decay may then be interpreted as a conversion of STP to LTP. On the other hand, since both test and control pathways were stabilized at below the initial baseline, this is not a true LTP in terms of net potentiation. It exists merely as an interpathway relation and could, in principle, be due to different levels of LTD for the test and control inputs. Inspired by the journal reviewers, we performed experiments in which stimulation was stopped during the time of AP5 application, but without succeeding to convert the plasticity to net potentiation. How to deal with this issue is not obvious, and it may partly be a matter of definition. Still another conceivable alternative would be that STP was converted to "true LTP" but that this effect was masked by a superimposed depressive effect related to LTD/depotentiation. However, as will be explained in the following, the situation has further complexities due to the fact that the early part of the decay contained a component with aberrant properties, which was unrelated to NMDA-R activation.

Comparing the curves obtained with and without AP5 (Figure 6 of Paper IV) revealed that the PPS-induced STP actually decayed faster under blockade of NMDA-R by AP5 than it did under maintained NMDA-R activation. However, this conclusion is confounded by the presence of a possible crosstalk between AMPA and NMDA component measurements (Xiao, Karpefors et al. 1995); thus the blockade of the NMDA component may cause a small decrease of the AMPA measurement, leading to an overestimation of the plasticity-related decrease of (real) AMPA. We therefore converted to another experimental design, less sensitive to crosstalk. Here, peak STP was first induced in both pathways followed by a within-slice comparison between the decay obtained under PPS (standard stimulation pathway) and under SPS (minimal stimulation pathway). Notably, these new data supported the above idea of a faster decay under lesser NMDA-R activation and, conversely, of a slower decay under larger (standard) NMDA-R activation. The results suggest that the NMDA-R activation during the first 5-10 min after peak STP actually led to a potentiating effect,

however being masked due to the presence of a decay. The latter may represent a counter-reaction to the initial potentiation in terms of a passive, NMDA-independent relaxation that outbalanced the drive for further potentiation. Our naming of the initial potentiation as STP therefore seems justified, being not merely a descriptive term but reflecting the intrinsic properties of a truly short-term potentiation.

GENERAL DISCUSSION

This paragraph complements the "Results and Discussion" part of this thesis by focusing on some issues of great general interest. The first theme, on the unreliable character of synaptic transmission, relates to Papers I and II and has largely, but not exclusively, a methodological flavor. The following theme, on the up- versus down-regulation of synaptic strength, relates to Papers III and IV and thereby concerns an issue which is fundamental to our understanding of how synaptic memory is shaped.

On the unreliability of (studying) synaptic transmission

Possible causes of unreliable transmission

Synaptic transmission at many central synapses is variable in the sense that the postsynaptic response to a single presynaptic event fluctuates substantially on consecutive trials. Since it was often found that more than half of the presynaptic stimuli failed to evoke a postsynaptic response, such synaptic transmission was considered to be unreliable. The conclusion about unreliability of synaptic transmission in CA1-CA3 synapses is frequently based on applying the method of "minimal stimulation" to the Schaffer collaterals. This method is aimed to activate only one or a few synapses, which is a necessary condition for studying single release events. However, the observed variability of synaptic responses could have several reasons, some of which may be related to experimental conditions. In a previous study, four sources of variability were examined: (i) a fluctuating AP threshold in axons near the site of stimulation, (ii) deficient AP propagation due to conduction failure at axonal branch points, (iii) synaptic variability related to the unphysiological character of the slice preparation, and (iv) "true synaptic variability" representing a physiologic process of probabilistic transmitter release (Allen and Stevens 1994). By examining these possibilities one by one, the first three were judged insufficient to explain the observed unreliable transmission, leaving probabilistic transmitter release as the likely main determinator. In this manner, the mean failure rate of transmitter release in CA1-CA3 synapses was judged to be about 0.7, that is, with a successful release in only a minority of the events. However, this analysis is not unproblematic and is critically

dependent on the adequacy of the minimal stimulation method. In the first part of my thesis (Papers I and II), I argue that fluctuations of action potential threshold is an underestimated source of response variability that needs to be considered.

Firing threshold variability

Previous studies have indicated that the axon threshold is not fixed but has a random distribution (Peche 1939). Although the cause of these fluctuations can be multifold, simple biophysical events such as the stochastic opening and closing of voltage-dependent Na^+ channels were considered sufficient to explain the observed threshold variability in axons (Rubinstein 1995). In a computational model for stellate cells of the entorhinal cortex, such ion channel noise was concluded to have influences at both cellular and network levels (White, Klink et al. 1998). Moreover, in an experimental study on small cultured hippocampal neurons, the sodium channel based fluctuations were found to be big enough to cause even spontaneous firing (Johansson and Arhem 1994). It has also been considered that both Na^+ and K^+ channel fluctuations together contribute to variations of axonal excitability (Adair 2003). In the CA1-CA3 system, the threshold fluctuations were previously estimated to be 2.8% of the average level with a range of 2.0-3.5% (Allen and Stevens 1994). For a comparison, an estimated value of 6.6% was obtained in Paper I for the relative degree of threshold fluctuation. To what extent the observed threshold fluctuations of the Shaffer collaterals are due to ion channel noise and/or other sources of variability remains to be determined.

What is minimal stimulation?

The degree of axonal threshold fluctuation is one out of several factors that is critical for successful application of the minimal stimulation method. Another important issue concerns the procedure for properly setting the stimulus strength in relation to the axonal threshold. For the following discussion, let us consider in more detail how the method works. In short, the basic idea of minimal stimulation is to keep activation of a (putative) presynaptic axon as reliable as possible without causing activation of multiple axons. For this reason, the stimulus intensity is normally increased by a small amount of 5-10% after first establishing responses at a level of just above threshold (Allen and Stevens 1994; Hanse and Gustafsson 2001). The original criteria for minimal stimulation include the following two conditions: (1) the postsynaptic responses fail on about half of the stimulation trials or more, (2) the average size of the postsynaptic response, such as an EPSC, should remain constant as the stimulus strength is increased over a range of intensities (Raastad, Storm et al. 1992). In a later study, the second point was reformulated to state that (2) there should be no change in

average response amplitude and release failure rate over ± 5% alterations in stimulus intensity (Stevens and Wang 1995). Additionally, a third point was added implying that (3) the EPSC latency and shape (but not size) should be invariant for those stimulation trials on which a release occurs.

High demands and small margins

To accurately determine the relationship between average EPSC size (or failure rate) and stimulus intensity – a preparatory step for establishing minimal stimulation – can be quite time-consuming. Measuring random events of binary character has an inbuilt lack of precision as described by the binomial statistical distribution. For instance, if the probability of successful responses is $P_{res} = 0.5$, then presentation of say 100 trials yields a theoretical average of 50 responses with a standard deviation of 5, the latter calculated as the square root of 100 * P_{res} * (1 - P_{res}). Assuming for example a stimulus frequency of 0.2 Hz implies a time of over 8 min to get a single measurement of reliability, and still the accuracy is not better than 10%. A similar reasoning can be applied for the average amplitude. Considering that stimulation and recording conditions may be drifting with time, it seems that successful use of minimal stimulation is a demanding task. Even if it is possible to set a strength that is sufficient to reliably activate a certain axon, it may be hard to determine whether this stimulation will also activate other axons, in the short-term as well as long-term. In Paper II, the EPSC amplitude was analyzed in terms of coefficient of variation (CV) for successful release events. The study showed that this measure depended on the stimulus strength, a result that was interpreted as evidence for a proportion of multiple release events occurring at the higher strengths. However, it is generally not possible to judge from the individual traces when stimulation leads to multiple release due to the naturally occurring signal noise. As suggested by my experiments (see also Paper I), small stimulus strength adjustments do not only affect the reliability of AP generation in a single axon but also determine whether one or more axons are activated.

Minimal stimulation versus other methods

Since its introduction, minimal stimulation has been a popular method to isolate putative single synaptic responses (Konnerth, Keller et al. 1990; Raastad, Storm et al. 1992; Allen and Stevens 1994; Hanse and Gustafsson 2001). As discussed, the margins for implementing the method are relatively small. Nonetheless, in neonatal animals (1-7 days) the conditions are favorable due to a sparse pattern of functional neuronal connections as compared to older animals (Durand, Kovalchuk et al. 1996; Hsia, Malenka et al. 1998). As a consequence, it is easier to reliably activate a certain

presynaptic axon, a fact that was taken advantage of in some prior studies (Hanse and Gustafsson 2001) as well as in the present one (Papers I and II). In fact, by suitably changing the stimulus strength it was possible to switch between a situation with unreliable stimulation and one that was mostly reliable (Paper I). My recordings obtained under reliable conditions suggested a high release probability in the neonatal animals, partly in line with data by others (see the discussion below). There are also alternative methods for single axon activation, generally based on paired cell recording. While solving some of the problems associated with minimal stimulation, the alternative(s) may have drawbacks such as low experimental yield. In the most straightforward case, whole cell recording is established for a connected pair of CA3 and CA1 cells. Using this method on organotypic hippocampal slices frequently resulted in successful paired recordings (Debanne, Gahwiler et al. 1996), probably related to the fact that many of the connections available in this preparation develop in situ after the preparation of slices. However, it is uncertain whether the obtained data from such long-term cultures (several weeks) can be generalized to normal tissue. Optical techniques is another possibility to monitor the activation of specific synapses (Zhang and Oertner 2007; Zhang, Holbro et al. 2008). Still another method was used in Paper I, based on making a (partial) surgical cut between CA1 and CA3, saving only a tiny bridge of Schaffer collaterals connecting the two areas. This allowed reliable activation of axons even in rats above neonatal age.

Comparing previous literature reveals a discrepancy between the results obtained with different methods. Accordingly, minimal stimulation studies in neonatal rats described that the release probability had a large numerical range, from below 0.1 to more than 0.9 (Dobrunz and Stevens 1997; Hanse and Gustafsson 2001). Studies based on paired cell recording, however, reported a single high value in the order of 0.9 (Bolshakov and Siegelbaum 1995; Feldmeyer and Radnikow 2009). The reason for these discrepancies is not clear. A possibility is that unreliable activation contributed to the reported low P_r values, in line with the ideas presented in Papers I-II. On the other hand, due to the technical difficulties associated with paired cell recording, there might be a sampling problem with this kind of technique. One could imagine that with synaptic reliability as low as a few percent, such connections may not be easily detected, leading to a risk of discarding the associated cell pair. Regardless of which method is used, it is clear that very low capacity synapses are elusive to analysis, difficult to grasp regardless of the applied method. To thoroughly resolve the issue of transmitter release at neonatal synapses may have to await further results from optical recording. With respect to older animals (above one week) there seems to be a consensus on the existence of probabilistic transmitter release, even though the technical difficulties are substantial – at least for the case of using minimal stimulation – due to the high density of functional connections.

Pairs and trains of stimuli

An extra complication with regard to the minimal stimulation method appears when stimulation is performed as pairs or trains of stimuli. In fact, such multiple stimuli were often applied and the evoked multiple responses were analyzed in the minimal stimulation approach (Dobrunz, Huang et al. 1997; Hanse and Gustafsson 2001). A critical issue is that during multiple stimuli, the neuronal excitability is modulated by the first stimulation. The first and the second (or later) pulses therefore may activate different presynaptic axons (Wigström and Gustafsson 1981; Soleng, Baginskas et al. 2004), an effect that was considered to depend on spike after-potentials. Whereas this effect is thus contingent on the generation of an AP by the first stimulus, a similar effect can occur without AP generation on the first pulse. As described in Paper I, firing occurred on the second pulse without associated firing on the first one, indicating a stimulus-dependent lowering of the threshold. It was concluded that this effect was due to a remaining depolarization from the first stimulation.

Threshold variations during a train can have profound effects on secondary measures of synaptic transmission, such as PPF, unless reliable stimulation is secured by a high enough stimulus strength. Thus, whereas unreliable stimulation in neonatal rats generally resulted in PPF, reliable activation in the same situation (except for the stimulation) was associated with PPD (Paper I). The latter result agrees with work based on dual whole-cell recording (Bolshakov and Siegelbaum 1995; Feldmeyer and Radnikow 2009), a method that mostly eliminates the risk of unreliable activation. In fact, a high P_r in combination with a pronounced PPD appears to be a significant feature of neonatal synapses in many brain regions. This seems to be related to the fact that very few vesicles are available for release (Feldmeyer and Radnikow 2009). Stimulation at high frequencies can even irreversibly damage synaptic transmission at such immature synapses. With increasing age, the immediate release probability becomes smaller and PPF rules over PPD.

Final notes about unreliable transmission

Knowing whether fluctuating synaptic responses are due to unreliable stimulation or due to probabilistic transmitter release is an important issue but generally not so easy to answer. Each of these processes obeys binomial statistics and the combined effect will do so as well, hence providing no clue as to the individual contributions. The minimal stimulation method has been a popular tool for studying synaptic transmission at putative single synapses; however, my thesis work suggests that it should be used

with care. Even some of the early pioneers of the method later declared that "it is not an ideal and reliable method for the studies of the response of a putative single synapse, including the reliability of release probability" (Regehr and Stevens 2001). Minimal stimulation can still be safe to use under certain experimental conditions such as in neonatal rats, especially under reliable single pulse activation, taking advantage of the fact that the sparse innervation allows a bigger stimulus strength margin. Another way to improve conditions for reliable stimulation is to combine the minimal stimulation method with a "bridge cut", leading to an even sparser mesh of axons for possible stimulation. In addition to the factors of unreliability at the beginning and end of the transmission chain, distortion at intermediate levels has been observed in some invertebrate systems. In the leech nervous system, axonal branch points may cause (activity dependent) failure of AP propagation resulting in impaired transmission, or even reflection of APs which leads to synaptic transmission at more than twice the normal level (Baccus 1998; Baccus, Burrell et al. 2000). To what extent similar mechanisms operate in the vertebrate nervous system is not clear.

Control of bidirectional plasticity by NMDA-dependent calcium influx

Both potentiation and depression are believed to be essential components of synaptic plasticity, being able to refine the pattern of functional connections between neurons in the brain in order to achieve certain physiological goals. A key issue concerns the way that these processes are controlled. Papers III and IV add significant knowledge to our understanding of how postsynaptic calcium via NMDA-Rs can trigger bidirectional plasticity, considering NMDA-R subunit contributions as well as the control of the direction of the synaptic changes via a common signal.

Sources of Ca^{2+}

Involvement of NR2A versus NR2B

Our results revealed a similar pattern of contributions by different NR2 subunits to (1) NMDA-R mediated synaptic current, (2) LTP and (3) LTD, thus forming a coherent picture. Nevertheless, they do not fit well with some other works that demonstrated a specific coupling of subunits versus type of plasticity, generally implying a preferential involvement of NR2A subunits in LTP and of NR2B subunits in LTD (Liu, Wong et al. 2004; Massey, Johnson et al. 2004). However, still other works concluded that both types of NR2 subunits contributed to LTP as well as LTD (Berberich, Punnakkal et al. 2005; Weitlauf, Honse et al. 2005; Zhao, Toyoda et al. 2005), in general similarity

with our results. The reason for these discrepancies among studies remains to be explained. It can be noted that some of the studies differed in methodological details, especially with regards LTD, which can be difficult to induce by standard LFS stimulation and so may require special induction conditions. Whereas in our case we studied LTD induced under low-Mg^{2+} conditions, as well as a chemically induced variant, another study made use of a glutamate uptake inhibitor to obtain an analyzable LTD (Massey, Johnson et al. 2004).

Unified versus diversified triggering

In Paper III, we considered two possible, alternative scenarios for the way that different Ca^{2+} sources might involve in triggering synaptic plasticity. In the case of "unified triggering" it was conceived that different sources of Ca^{2+} interact in a cooperative way with contributions that sum together in making up a final common pathway of induction. Such integrative action could easily be brought about in terms of total Ca^{2+}concentration in the spine. In another scenario, "diversified triggering", NMDA receptor subtypes and associated Ca^{2+} sensitive enzymes near the site of Ca^{2+} entry, were considered to have a certain spatial distribution in the spine membrane, allowing local activation of receptor-enzyme complexes. In this manner, specific NR2 subunits would become functionally linked, via the proper enzymes, to specific forms of plasticity, such as potentiation or depression. The induction pattern and possibly other factors may then decide which subregion of the spine, characterized by a special receptor-enzyme combination, is activated. In line with this idea, a previous study suggested that extrasynaptic NMDA-Rs were specifically involved in LTD (Massey, Johnson et al. 2004) whereas the synaptic ones were assumed to be responsible for LTP; however, the study did not explicitly consider the idea of a receptor-enzyme linkage.

Evidence for unified triggering

Despite quantitative differences, the similar involvement of NR2 subunit types in plasticity and NMDA responses seems most easily explained by the unified triggering hypothesis. This choice of model is further supported by our finding that the two receptor types could take over each others' roles. Thus, both LTP and LTD were induced via either NR2A or NR2B subunits, although in the case of NR2A with the aid of (further) lowering the Mg^{2+} concentration to facilitate Ca^{2+} influx. It is therefore reasonable to consider total Ca^{2+} in the spine as a main controller of plasticity. Still, one may ask why blocking NR2B by Ro/Ife caused a 50% reduction of LTP but a reduction of the NMDA EPSP by only one third, whereas LTD was hardly affected at all. We therefore postulate that the different forms of plasticity are differentially

sensitive to blocking of NMDA-dependent Ca^{2+} influx, depending on the actual linkage between Ca^{2+} concentration and plasticity-specific enzymes. This idea is supported by previous work showing that LTP was more sensitive than LTD to partial, unspecific blockade of NMDA-Rs by AP5 (Liu, Wong et al. 2004).

The unified calcium model

Prior publications on NR2 subunit involvement did not explicitly discuss the two distinct models presented here. Nevertheless, the idea of NMDA-dependent calcium as a controlling factor is common in the LTP/LTD literature. In an extended version of this scheme, referred to as the unified calcium model, it was conceived that not only NMDA-calcium, but all kinds of sources of Ca^{2+} contribute to synaptic plasticity on equal grounds (Artola and Singer 1993; Shouval, Bear et al. 2002). Our data are compatible with this model, although we did not perform any experiments on other Ca^{2+} sources than via NMDA-Rs, such as via VDCCs or due to spillover between dendritic spines.

Directional control

Concentration magnitude

An important issue concerns how the calcium signal in the dendritic spine is translated into either an increase or a decrease of synaptic efficacy. As a possible solution to this question, several prior works revealed a dependency on concentration magnitude, implying that a large increase of Ca^{2+} concentration caused LTP whereas a smaller one led to LTD (Lisman 1989; Dudek and Bear 1992; Mulkey and Malenka 1992). Such a control via the magnitude of Ca^{2+} concentration might be related to different activation thresholds for LTP-triggering versus LTD-triggering enzymes. The idea is partly supported by our present low Mg^{2+} experiments, considering that under conditions of previously stable or slightly declining AMPA-R mediated responses, an increase of NMDA-R activation led to potentiation of the AMPA responses, albeit only in the short term. However, after a few minutes of continued stimulation, depression generally took over. As can be easily appreciated from our slow NMDA-R activation experiments, AMPA responses increased early on and became depressed later on, even under conditions of equally sized NMDA-R activation (Figure 4A). Similarly, in the experiments with fast NMDA-R activation due to SPS-PPS switching, equal induction strengths (total NMDA response for both pulses) gave rise to either potentiation or decay (Figure 4B). It is evident from these considerations that the magnitude of NMDA-dependent Ca^{2+} alone can not predict the direction of the synaptic change, and additional involvement of temporal factors (related to activation history) is implied.

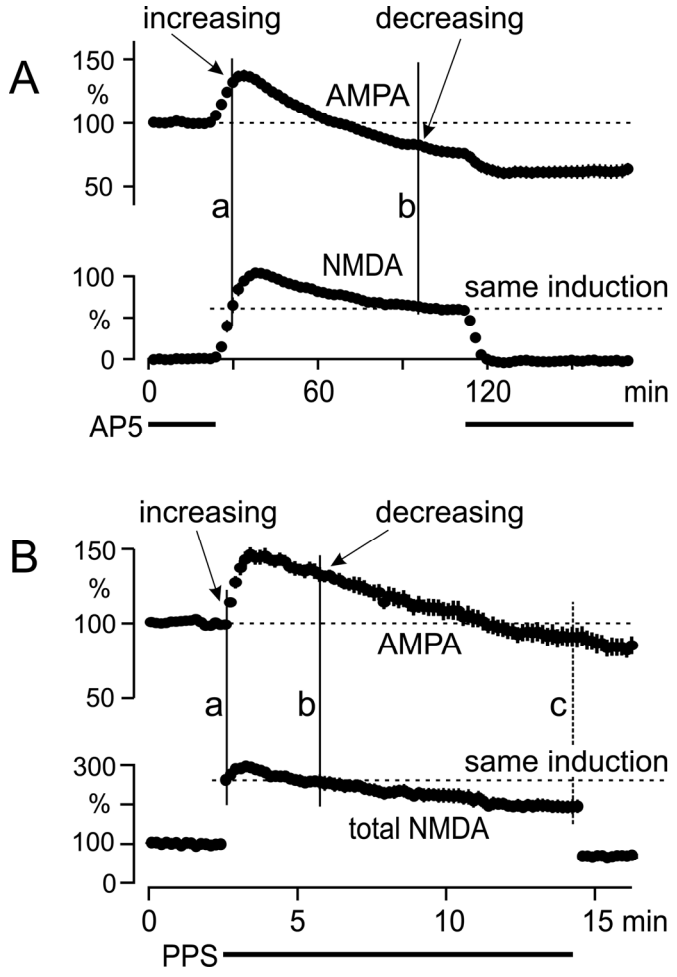

Figure 4. *Bidirectional synaptic changes by the same level of NMDA-R activation.* ***A****, experiment with SPS under conditions of unblocking (AP5 washout) and reblocking (AP5 washin) of NMDA-Rs. The same level of activation of NMDA-Rs initially (a) led to increasing AMPA responses but was later (b) associated with decreasing AMPA responses.* ***B****, illustrates that with PPS (under unblocked NMDA-Rs) the initial few stimuli (a) caused increasing AMPA responses whereas a few minutes later (b), the same level of activation of NMDA-Rs caused decreasing AMPA responses. Net depression occurred at (c) even though NMDA-R activation was still above the initial baseline. Adapted from Papers III, IV.*

Time factor and metaplasticity

Our finding that stimulation lasting over time preferentially induced depression is consistent with standard protocols for LTD induction, generally employing LFS at one or a few Hertz for 3-15 min. It is also in accord with previous work that practised induction via LFS under Mg^{2+}-free conditions, showing that LTD was only induced by long (a few minutes) trains at a relatively weak intensity whereas LTP could be induced by both short and long trains by using sufficiently strong activation (Mizuno, Kanazawa et al. 2001). A key result of our present work is that the same induction strength was able to produce either potentiation or depression, depending on the time during the experiment. In other words, the link between Ca^{2+} concentration and the directionality of synaptic plasticity was not fixed but changed as a function of prior induction events. Such plasticity of plasticity, generally referred to as metaplasticity (Abraham and Bear 1996), is believed to contribute to the adaptive behavior of the nervous system, besides normal plasticity. Prior studies have shown that synaptic activation of one pathway can influence the ability for subsequent LTP (or LTD) in the same or another pathway (Huang, Colino et al. 1992). The development of receptive

fields in cortical cells is a prominent example where metaplasticity has been suggested to play a role (Bienenstock, Cooper et al. 1982). The idea was to limit excessive potentiation in a cell-wide perspective, a feature also referred to as synaptic scaling. The temporal perspective was days-to-weeks in that case whereas the metaplasticity studied here was found to operate on a much faster time scale with synaptic changes that switched direction within a few minutes.

The BCM model and sliding of LTP threshold

A popular theoretical framework for describing metaplasticity is the BCM model, named after the initials of the inventors, and originally intended to simulate receptive field formation in the visual cortex (Bienenstock, Cooper and Munro 1982). Basically, the relation between induction strength and plasticity is assumed to follow the same kind of U-shaped relation as described above, where low levels of induction lead to negative changes (depression) and larger ones to positive changes (potentiation). Of interest is the LTP threshold, which defines the border between LTD induction and LTP induction. The key issue with the BCM and related models is that the so defined relation between induction and plasticity is modifiable, for instance, in terms of a sliding of the LTP threshold towards higher or lower values. In a variant of this model, there is also an LTD threshold but this complication does not need to be considered here. Assuming that a certain induction strength initially induces potentiation, an increase of the LTP threshold may then imply that the same induction strength will cause depression instead. On the basis of this idea, a BCM-like model was introduced in Paper IV to explain the results obtained with SPS and PPS stimulation under NMDA-R unblocked conditions. It was conceived that the LTP threshold successively increased during the persistent, weak NMDA-R activation. In an attempt to apply a functional perspective, one might think of the LTP threshold as reflecting the average NMDA-R activation prior to a certain time. As a consequence, the induced plasticity would be sensitive to a sudden increase of the induction strength, as actually observed. However, this scenario does not explain why, later on, depression just continued to develop.

Underlying biochemistry

Previous work suggests that LTD induction is basically mediated by phosphatase activity (dephosphorylation) whereas LTP is mediated by kinase activity (phosphorylation) (Lisman 1989; Mulkey, Herron et al. 1993; Lee, Barbarosie et al. 2000), a generally agreed upon issue. Substantially less is known about metaplasticity and the mechanisms underlying the sliding of LTP threshold. Despite its descriptive power, the model proposed above does not disclose the underlying biology. As

discussed in paper IV, several possibilities exist with respect to the biochemical control of the sliding of LTP induction threshold. The possible candidates include (1) processes affecting Ca^{2+} entry, (2) modulation of Ca^{2+} concentration and/or dynamics in the spine, and a number of different mechanisms downstream of Ca^{2+} activation such as (3) redistribution of certain molecules or (4) morphological changes. Unfortunately, the present work does not pinpoint any particular of these mechanisms and further work will be needed to find the responsible one. However, even if we knew the critical parameter(s) being modulated in the synapse, it still remains to find the critical inducer of that modulation. In our illustration of the BCM model in the final figure of Paper IV, it was suggested that the signal that determines the threshold-sliding is related to the Ca^{2+} influx. In retrospect, we realize that we could also have chosen some other parameter, for instance at a later stage of the signaling pathway. Notably, in the original BCM model, the controlling signal was not even synaptic but was related to over-all cell depolarization (Bienenstock, Cooper et al. 1982).

Unstable versus stable synaptic plasticity

To summarize, NMDA-dependent Ca^{2+} is an intracellular messenger that can trigger several forms of synaptic plasticity, being able to control both up and down regulation of the synaptic strength. Papers III and IV describe how bidirectional changes are generated as a result of this basic induction signal, depending on its strength and temporal features. Once plasticity is induced it remains more or less stable, provided that NMDA receptors are not further activated. A condition for long-term stability of synaptic plasticity is thus the absence of NMDA-R activation. This was established in the present thesis by demonstrating that the NMDA-antagonist AP5 prevented the slowly developing LTD and converted it into a stable change (Paper IV). Although, we did not manage to stabilize the early PPS-induced potentiation in a similar manner, other works have demonstrated that blockage of NMDA-Rs improves the stability of LTP by eliminating an NMDA-dependent decay, or depotentiation (Xiao, Niu et al. 1996; Villarreal, Do et al. 2002). Even so, LTP (and LTD) are not necessarily fully stable in the absence of NMDA-R activation but can decay due to other, inherent factors. One such issue relates to the fact that the long-term stability of LTP depends on synthesis of new proteins. Failure to activate this synthesis, due to physiological deficiencies or via pharmacological blockade, leads to a plasticity that generally decays within a few hours (Krug, Lossner et al. 1984; Stanton and Sarvey 1984; but see Abbas, Dozmorov et al. 2009). This remaining, unstable LTP is referred to as early LTP (E-LTP) in contrast to the more stable late LTP (L-LTP) that can persist for many hours. It can be noted that the conservation of asymmetry between PPS-SPS-treated pathways under NMDA-R blockade was maintained for at least 2 hours with virtually no change and so might represent a case of late plasticity, related to L-LTP and/or L-

LTD. The reason why E-LTP and E-LTD gradually decay is unclear, although it can be imagined that this may be related to recycling of proteins, causing a return of synaptic transmission to the baseline level.

In the present study, we are concerned about another example of an intrinsically decaying potentiation, however operating within a relatively short time span and therefore referred to as STP. This plasticity peaked at a time of one minute after the onset of PPS and was found to decay within about 10 min independent of NMDA-R activation (also referred to as passive decay or relaxation, see Paper IV). The term STP has previously been used by others to describe an initial, decaying part of LTP that can be seen before the stable part takes over (Hanse and Gustafsson 1992; Malenka, Lancaster et al. 1992). STP was even observed in isolation after weak NMDA-dependent induction. In our case of PPS-induced plasticity, the initial STP was not followed by LTP but rather by LTD. One might speculate that the lack of proper LTP in this case could be due to the sparse temporal distribution of relatively weak induction events (Colino, Huang et al. 1992). Our phenomenon had several similarities with regular STP in that its induction was dependent on NMDA-R activation whereas its decay was not. For this reason, we have kept the name STP, using it not only as a descriptive term but implicating a mechanistic similarity with the STP studied in prior work.

An NMDA-independent early decay of LTP was described in still another study, albeit using the term transient LTP (t-LTP) to characterize the decayable fraction of LTP and so leaving stable LTP (s-LTP) (Volianskis and Jensen 2003). The t-LTP could be arbitrarily postponed be delaying the delivery of test pulses for various times after LTP induction. Despite of its lack of NMDA-dependence, t-LTP was found to be stimulus-dependent, possibly mediated via a presynaptic mechanism. To what extent the decay of STP in our case was stimulus-dependent is not clear, although we did experiments with stopping of stimulation for a limited time. Finally, a stimulus-dependent and NMDA-independent decay of LTP was described under the notion of AMPA-silencing, a term referring to the presumed underlying mechanism (Abrahamsson, Gustafsson et al. 2008). In line with this idea, the currently observed STP and its decay were mainly expressed as changes of the AMPA component. However, the decay described in the cited study was related to the application of novel stimulation (to previously unstimulated synapses) and so appears to differ from the present one. Moreover, those previous results were contingent on the use of quite young animals (<12 day-old rats) whereas in our case (Paper IV) experiments were carried out in animals aged 13-22 days. Further research will be needed to clarify the LTP-STP relationship and the underlying molecular switch(es) deciding between unstable and stable synaptic plasticity.

CONCLUSIONS

- The AP firing threshold of CA3 cells/axons is lower for the second pulse of PPS than for the first one; under conditions of unreliable activation this is an essential factor that will influence measurements of PPP in CA3-CA1 synapses.

- The observed large range of CV of AMPA EPSCs during train stimulation can be due to differences in AP firing threshold of successive stimuli; with possible contributions from multiple vesicle release and/or activity-dependent vesicle reorganization.

- The differential actions on LTP/LTD when specifically blocking NMDA-R subunits can be explained by an unspecific reduction of Ca^{2+} entry, supporting the idea of a unified triggering of LTP/LTD via a common Ca^{2+} level in the dendritic spine.

- Activity-dependent synaptic plasticity does not only depend on the instantaneous NMDA-dependent Ca^{2+} concentration but is also influenced by prior synaptic events.

- Activity-dependent synaptic plasticity induced by NMDA-R activation decays in two ways; one is NMDA-dependent (active) and another is NMDA-independent (passive).

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