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SYNAPTIC MECHANISMS OF HEBBIAN AND HOMEOSTATIC PLASTICITY DRIVEN BY INTRINSIC ACTIVITY IN THE DEVELOPING HIPPOCAMPUS

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Huupponen J*, Molchanova SM*, Lauri SE, Taira T. 2013. Ongoing intrinsic synchronous activity is required for the functional maturation of CA3-CA1 glutamatergic synapses. Cerebral Cortex. 23:2754–2764.

II Huupponen J, Molchanova SM, Taira T, Lauri SE. 2007. Susceptibility for homeostatic plasticity is down-regulated in parallel with maturation of the rat hippocampal synaptic circuitry. The Journal of Physiology. 581:505–514.

III Luchkina N*, Huupponen J*, Clarke VR, Coleman S, Keinänen K, Taira T and Lauri SE. Developmental switch in the kinase dependency of LTP depends on expression of GluA4- subunit containing AMPA- receptors. Under revision.

* Authors contributed equally to this work

The publications are referred to in the text by their roman numerals.

Author´s contribution to the studies included in the thesis:

I The author did most of the electrophysiological experiments (Most experiments addressing the effect of CBX and 50 nM TTX on spontaneous activity, most of the AMPA and NMDA mEPSC recordings, some of the AMPA/NMDA EPSC recordings and all western blot experiments), analysed the data and participated in the writing of the manuscript.

II The author did most of the electrophysiological experiments, analysed the data and participated in the writing of the manuscript (mEPSC and mIPSC recordings from P0, P4 and P8 rats).

III The author contributed to the cloning and production of GluA1 and GluA4 viruses, tested constructs with HEK cells and contributed to the electrophysiological recordings from virus infected rats. The author contributed to the production of GST-

fusion peptides. The author did most of the mEPSC recordings from GluA4 knockout and wild-type mice, and was responsible for the mouse breeding and genotyping. The author analysed the data and participated in the writing of the manuscript.

Unpublished data presented in the text:

The author designed most of the experiments, did all the electrophysiological recordings and data analyses.

ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid
AP	action potential
Arc	activity-regulated cytoskeleton associated protein
BDNF	brain-derived neurotrophic factor
CBX	Carbenoxolone
CA	Cornu Ammonis area of the hippocampus
Ca^{2+}	calcium
CaMKII	calcium/ calmodulin- dependent protein kinase II
cAMP	cyclic adenosine monophosphate
Cl^{-}	cloride
CNS	central nervous system
CREB	cAMP response element-binding protein
CTD	C- terminal domain
DIV	days *in vitro*
E-LTP	early phase of long-term potentiation
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
HEK cells	human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
GABA	γ-aminobutyric acid
GDP	giant depolarizing potential
GFP	green fluorecent protein
GluA1-4	glutamate AMPA-receptor subunits 1-4
GST	glutathione S-transferase
iGluR	ionotropic glutamate receptor
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
IQGAP1	Ras GTPase-activating-like protein IQGAP1
KA	kainate
LTD	long-term depression
LTP	long-term potentiation
L-LTP	late phase of long-term potentiation
NMDA	N-methyl-D-aspartic acid
MF	mossy fiber
mEPSC	miniature excitatory postsynaptic current
mGluR	metabotropic glutamate receptor
mIPSC	miniature inhibitory postsynaptic current
mPSC	miniature postsynaptic current

P	postnatal day (P0 indicates the date of birth)
PKA	protein kinase A
PKC	protein kinase C
PICK1	AMPAR binding protein interacting with kinase 1
PP	perforanth pathway
Pr	neurotransmitter release probability
PSD	post-synaptic density
SC	Schaffer collateral
SPAs	synchronous plateau assemblies
TNF- α	tumor necrosis factor α
TTX	tetrodotoxin
VDCC	voltage-dependent calcium channel
VGLUT1	vesicular glutamate transporter 1
WT	wild type

ABSTRACT

The formation of synaptic connections in the brain is guided by genetic and activity-dependent mechanisms. The initial hard-wiring of the circuitry is followed by a phase during which connections are refined. During this process the genetic factors are less important and refinement is guided by electrical activity. However, the mechanisms underlying the activity-driven synaptic fine-tuning are still poorly understood.

The features of electrical activity and the mechanisms of synaptic transmission differ in the developing networks as compared to those of the adult. Electrical activity in the developing networks comprises of intermittent, highly synchronous bursts of action potentials interleaved by more silent, asynchronous neuronal firing. In the hippocampus this immature-type electrical patternity coincides temporally with the intense synaptic reorganization. Moreover, there is a parallel, developmentally-regulated expression GluA4 subunit of AMPA-type ionotropic glutamate receptors in the hippocampal neurons. Despite frequent speculation on the relative importance of synchronous vs. asynchronous neuronal activity on the synaptic development in the brain, there have been no direct experiments to study this issue.

In this thesis we have, for the first time, been able to experimentally dissect the roles of asynchronous vs. synchronous activity on synaptic refinement in the hippocampus. Specifically, we show that spontaneous synchronous activity is essential for the stabilization and maturation of immature CA3-CA1 synapses, and that network desynchronization leads to weakening of glutametergic transmission in the CA1 area. Plasticity changes caused by different endogenous activity patterns were strongly dependent on the synapse type (glutamatergic vs. GABAergic), the anatomical area (CA1 vs. CA3) and maturational stage of the neurons. In addition, the GluA4 was shown to be critical for both the Hebbian type and homeostatic plasticity mechanisms in developing glutamatergic synapses. In the absence of GluA4, the homeostatic regulation of the immature glutamatergic networks in response to manipulation of endogenous activity patterns was perturbed. Finally, GluA4 was shown to be necessary and sufficient for protein kinase A–dependent long-term potentiation (LTP), typical of immature CA3-CA1 synapses. These data demonstrate the instrumental role of spontaneous synchronous activity and GluA4 AMPAR subunit expression in the formation and refinement of hippocampal synaptic networks.

1 INTRODUCTION

The formation and elimination of synaptic connections during development primes the brain's neuronal circuitries for their later delicate functions, such as producing and processing consciousness, emotions and memories. In parallel with the formation of synaptic contacts, the nascent networks become electrically active. The early activity is mainly intrinsic in its origin, thus resulting from spontaneous firing of neurons (O'Donovan 1999; Ben-Ari 2001), and typically consists of intermittent, highly synchronous bursts seen both *in vitro* and *in vivo* (Ben-Ari et al., 1989; Lahtinen et al., 2002; Leinekugel et al., 2002; Karlsson et al., 2006). These bursts (also known as giant depolarizing potentials, GDP's) constitute the first synchronized activity in the hippocampus. This developmental activity pattern is seen in virtually all areas of the vertebrate central nervous system (CNS) and is presumably instrumental in the formation and refinement of initial synaptic connections when the redundant connections are removed and the retained contacts reinforced (Zhang and Poo 2001; Khazipov et al., 2004; Hanse et al., 2009; Kano and Hashimoto 2009).

The activity-dependent modulation can take place at individual synapses or at the level of the neuronal network (Bliss and Collinridge 1993; Zhang and Poo 2001; Goda et al., 2003; Turrigiano and Nelson 2004). Correlated or uncorrelated activity between neurons can lead to long-term input-specific alterations of the strength of synapses between these neurons. Well-characterized examples of these so-called Hebbian changes are long-term potentiation (LTP) and long-term depression (LTD). However, if the potentiation or depression of synaptic efficacy would operate without any balancing mechanisms, the network would become hyper- or hypoactive, leading to the saturation of synaptic strength. To avoid this, neuronal networks are able to detect the overall activity level of the circuitry and regulate their own excitability with homeostatic plasticity mechanisms. Thus, both the Hebbian type and homeostatic plasticity mechanisms are vital for the maintenance of stable function and information encoding in neuronal networks.

In general, regulation of synaptic strength in a global, non-synapse-specific manner (also known as synaptic scaling) is the central mechanism underlying homeostatic plasticity. However, the exact pre- and postsynaptic mechanisms involved vary a lot depending on the experimental model (Turrigiano et al., 1998; Lauri et al., 2003; Colin-Le Brun et al., 2004; Gonzales-Islas and Wenner 2006; Kim and Tsien 2008). Most of the studies on homeostatic plasticity have been done using neuronal cultures lacking the typical patterns of endogenous activity seen in ex vivo brain tissue, or *in vivo* (e.g., Turrigiano et al., 1998; Burrone et al., 2002; Kim and Tsien 2008). Hence, it is essential to study the roles of Hebbian vs. homeostatic plasticity

on synaptic development in a model system retaining the physiological activity patterns.

Modifications of neurotransmitter receptors are the main postsynaptic mechanisms for the regulation of synaptic efficacy in the brain (Malinow and Malenka 2002). The molecular composition of synaptic a-amino-3-hydroxy-5-methyl-4-isozazolepropionic acid-type ionotropic glutamate receptor (AMPAR), which mediate most of the fast glutamatergic neurotransmission in the brain, is developmentally regulated (Monyer et al., 1991; Zhu et al., 2000). In addition, the mechanisms regulating the AMPAR at developing glutamatergic synapses are in part distinct from those operating at mature synapses (Zhu et al., 2000; Yasuda et al., 2003). In particular, the GluA4 subunit of AMPAR's is transiently expressed at the pyramidal neurons in the neonatal hippocampus (Zhu et al., 2000). However, it has not been known what the role of GluA4 in Hebbian vs. homeostatic plasticity in the developing hippocampus is.

2 REVIEW OF THE LITERATURE

2.1 FAST SYNAPTIC SIGNALING IN THE HIPPOCAMPUS

2.1.1 MECHANISMS OF NEUROTRANSMISSION IN THE HIPPOCAMPUS

The communication between neurons is achieved via synapses in a process called neurotransmission. Neurotransmitter release to the synaptic cleft from the presynaptic terminal is triggered by an influx of calcium (Ca^{2+}) through voltage-gated channels, usually as a consequence of presynaptic action potential (AP). Neurotransmitters bind to neurotransmitter receptors at the postsynaptic membrane, which leads to depolarization (excitatory postsynaptic potential, EPSP) or hyperpolarization (inhibitory postsynaptic potential, IPSP) of target neurons and thus make them more or less likely to fire AP (Johnston and Wu 1995).

Neurotransmitter receptors can be grouped in two classes, ligand-gated ion channels and G-protein-coupled 'metabotropic' receptors. Fast excitatory neurotransmission in the hippocampus (Fig.1) is mediated by ligand-gated ionotropic glutamate receptors (iGluR's) that comprise a-amino-3-hydroxy-5-methyl-4-isozazolepropionic acid receptors (AMPAR's), kainate receptors (KAR's) and N-methyl-D-aspartate receptors (NMDAR's). The iGluR's are tetrameric glutamate-gated cation channels. All iGluR's are permeable to sodium (Na^{+}) and potassium (K^{+}), while NMDAR's are also permeable to calcium (Ca^{2+}). The Ca^{2+} permeability of other iGluR's depend on their subunit composition and mRNA editing. AMPAR's mediate most of the fast excitatory neurotransmission. While, NMDAR activation needs concurrent glutamate release and membrane depolarization, due to a voltage-dependent magnesium block of the ion channel. These functional characteristics of NMDAR's enable their well-known role as coincidence–detectors in the induction of synaptic plasticity (see section 2.2). KAR's, the third type of iGluR's, have a minor role in fast synaptic transmission and their main known function is the modulation of synaptic transmission in both pre- and postsynaptic sites (reviewed by Ozawa et al., 1998).

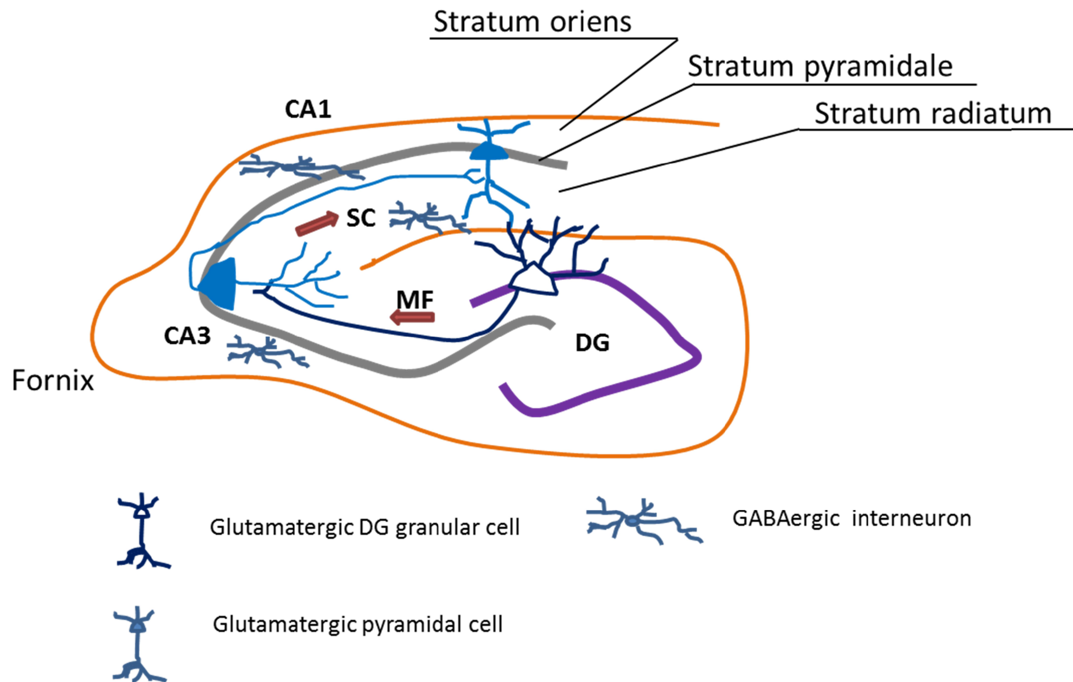


Figure 1 The functional anatomy of the hippocampus. The hippocampus is a part of the hippocampal formation that also includes the entorhinal cortex, the parasubiculum, the presubiculum, the subiculum (not shown) and the dentate gyrus (DG) . The hippocampus is devided in three subdivisions; *cornu ammonis* (CA) 1-3. The majority of cells in the CA pyramidal layer (stratum pyramidale) are glutamatergic pyramidal cells. γ-aminobutyric acid (GABA) mediated interneurons are mainly situated in the *stratum radiatum* and *stratum lacunosum-moleculare*, although some cell bodies are also located in the pyramidal layer. The entorinal cortex projects to the dentate gyrus, CA1 (from layer III) and CA3 (from layer II) via the perforant pathway (PP, not shown), which provides the major excitatory input to the hippocampus. The dentate gyrys granule cells innervate CA3 via mossy fibers (MF). CA3 cells are also heavily innervated by collaterals of their own and by contralateral axons that make the CA3 network highly excitable. Bursting activity (that lasts usually 30 to 50 ms with an action potential frequency of 100 to 300 Hz) is seen in both CA1 and CA3 areas but is much more prominent in the CA3 area *in vivo* (Traub et al., 1989). Connections from CA3 pyramidal cells to area CA1 are called the Schaffer collateral/commissural (SC) pathway. CA1 pyramidal cells project back to the entorinal cortex and the subiculum. In addition to the glutamatergic inputs, both the CA1 and CA3 pyramidal neurons receive GABAergic input from the interneurons. The hippocampus receives inputs also, e.g., from the amygdala, the hypothalamus, and noradnegic and serotonergic inputs from the brain stem (not shown). The subiculum (not shown) is the major output region of the hippocampal formation. (Brown and Zador 1990)

The metabotrobic glutamate receptors (mGluR's) are divided into three groups (group I-III). The main function of metabotrobic glutamate receptors is to modulate glutamatergic transmission. Group I mGluR's are coupled to phospholipase C whose activation leads to increase in the intracellular Ca^{2+} concentration (Yuzaki and Mikoshiba 1992), whereas activation of group II and III mGluR's inhibits the formation of cyclic adenosine monophosphate (cAMP) (Baba et al., 1993; Takahashi et al., 1996). All mGluR's have also been shown to modulate ionotropic channels, including inhibition of voltage-dependent Ca^{2+}-channels (VDCC; group I-III) and activation of inwardly rectifying K^{+}-channels (group II). In addition to the postsynaptic site, iGluR's

and mGluR's are located also in the presynaptic side of both excitatory and inhibitory synapses, where they may function as auto- and heteroreceptors that modulate transmitter release (Pinheiro and Mulle 2008).

Inhibitory GABAercig transmission is mediated via ionotropic $GABA_A$-receptors and metabotropic $GABA_B$-receptors. $GABA_A$-receptors are ion channels permeable to chloride (Cl^-) and bicarbonate ions and they mediate both fast and tonic inhibition (Farrant and Nusser 2005; Capogna and Pearce 2011). Activation of $GABA_B$-receptors leads to a G-protein mediated opening of postsynaptic inwardly rectifying potassium channels. At the presynaptic side $GABA_B$ receptor activation inhibits presynaptic Ca^{2+}-channels and reduces neurotransmitter release (Kohl and Paulsen 2010).

In addition to AP triggered release, neurotransmission may occur spontaneously as showed in classical studies of the neuromuscular junction by Bernard Katz and others in 1952 and 1953 (Castillo and Katz 1952; Fatt and Katz 1953). Katz and his co-workers assumed that the vesicle released from presynaptic terminal contains a fixed amount of neurotransmitter and that transmission is quantal. Although quantal analysis of synaptic transmission at CNS is complicated due to the large number and functional variability among the synapses into a given cell, the postsynaptic response to spontaneous release of vesicles is widely used for the estimation of synaptic strength and number. Usually these studies are made by preventing AP-dependent activity with voltage-gated Na^+-channel blocker tetrodotoxin (TTX) and by recording postsynaptic miniature currents (mPSCs) (e.g., Turrigiano et al., 1998). The changes in the average mEPSC amplitude reflect changes in postsynaptic glutamate receptors or presynaptically, changes in the amount of glutamate released by a synaptic vesicle. On the other hand, changes in mPSC frequency correspond to changes in the neurotransmitter release probability (Pr) or in the number of functional synapses.

2.1.2 MECHANISMS OF PLASTICITY AT GLUTAMATERGIC SYNAPSES

2.1.2.1 Long-term potentiation and long-term depression

Long-term potentiation (LTP) is by definition a long-term increase in the response of the postsynaptic cell to presynaptic stimulation, while long-term depression (LTD) refers to persistent reduction in the synaptic efficacy. Both LTP and LTD can be experimentally induced in hippocampal synapses and are commonly used models for studying the synapse-level mechanisms of learning and memory (Bliss and Gardner-Medvin 1973; Bliss and Lomo 1973; Lynch et al., 1977; Dundwiddie and Lynch 1978; Dudek and Bear 1992; Abraham 2003). At hippocampal CA3-CA1 synapses, LTP can be induced by a variety of protocols including brief high-frequency stimulation (e.g., 100

Hz, 1s), theta-stimulation (e.g., 4 pulses at 100 Hz at 200 ms intervals) or a pairing protocol (pairing of presynaptic stimulation with postsynaptic depolarization). By contrast, LTD is induced, e.g., with the prolonged trains of stimulation at lower frequencies (e.g., 900 stimuli at 1 Hz) (Lynch et al., 1977; Dundwiddie and Lynch 1978; Dudek and Bear 1992). LTD can be induced following LTP (called depotentiation) or *de novo*. Both LTP and LTD are usually input-specific and restricted to activated synapses.

The most studied form of LTP induction in the CNS is the LTP at CA3-CA1 synapses that depends on NMDAR activation (Collinridge et al., 1983), while the mGluR activation is commonly needed for the induction of *de novo* LTD in the adult CA1 (Kemp et al., 2000). However, NMDA-independent and mGluR- or KAR-dependent LTP forms (Nicoll and Malenka 1995) as well as NMDA- and Ca^{2+}-permeable AMPAR-dependent forms of LTD have also been found in certain synapses (Collingridge et al., 2010).

The activation of NMDAR's serves as a coincidence detector for presynaptic transmitter release and changes of postsynaptic membrane potential during plasticity induction, and leads to an increase in postsynaptic Ca^{2+}-concentration. The elevated Ca^{2+}-concentration activates various signalling cascades that control synaptic efficacy. The relative magnitude and duration of intracellular Ca^{2+}-concentration and temporal factors are essential determinants for LTP and LTD induction. In particular, LTP is usually triggered as a consequence of brief high-magnitude Ca^{2+}-concentration changes, while LTD induction needs modest and prolonged change in the intracellular Ca^{2+} level (Malenka et al., 1992; Yang et al., 1999). Further, Ca^{2+} celators such as EGTA and BAPTA prevent the LTP induction which further demonstrates the important role of Ca^{2+} and Ca^{2+}-permeable channels in LTP induction (Lynch et al., 1983; Malenka et al., 1992).

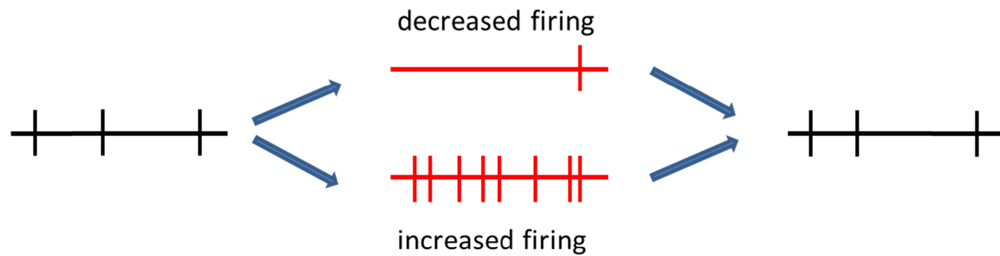
The expression of an early phase of LTP (E-LTP) is thought to be independent of protein synthesis, while maintenance of potentiation in the late phase of LTP (L-LTP), about 1h after induction, needs gene transcription and protein synthesis (Schuman et al., 2006; Frey et al., 1993; but see Abbas et al., 2009; Villers et al., 2012). Expression of LTP in the CA1 area is thought to be mainly postsynaptic and AMPAR-dependent, although possible presynaptic mechanisms can not be completely excluded (Lisman 2003). Presynaptic mechanisms for LTP expression exist in other synapses, a well-characterized example being the mossy fibre synapses in the area CA3 (e.g., Nicoll and Malenka 1995).

Several protein kinases, including protein kinase A (PKA), mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) take part in the LTP induction, and their contributions depend on the used stimulation protocol and developmental stage (see section 2.2.4). The most important kinase for LTP induction in adult hippocampal CA1 is CaMKII (Malenka and Nicoll 1999; Lisman et al., 2002). CaMKII is transiently activated in response to LTP-inducing stimuli and its activity reverts to baseline in about 1 min; thus

the processes that maintain LTP have to be downstream of CaMKII activation (Lee et al., 2009). The kinases can phosphorylate AMPAR's and/or their interacting proteins and thereby affect the conductance and synaptic localization of the receptors (Roche et al., 1996; Barria et al., 1997). Further, PKA-, CaMKII-, PKC- and MAPK-induced signalling pathways converge to regulate activation of transcription factor cAMP response element-binding protein (CREB) that regulates expression of numerous target genes. These include, for example, the brain-derived neurotrophin factor (BDNF), known to regulate both AMPAR delivery (see section 2.1.3.) and glutamate release (Caldeira et al., 2007; Li and Keifer 2008; Sallert et al., 2009).

2.1.2.2 Homeostatic plasticity

Homeostatic regulation, meaning "staying the same through change", is needed for the networks' ability to maintain their potential for plasticity and to keep neuronal firing rates highly stable over long time periods (Turrigiano and Nelson 2004; Pozo and Goda 2010; Turrigiano 2012, Fig.2). This regulation can take place on a scale of networks (e.g., Lauri et al., 2003), individual neurons (e.g., Burrone et al., 2002), subcellular dendritic regions (Yu and Goda 2009) or individual synapses (Hou et al., 2008; Beique et al., 2011; Makino and Malinow, 2011). Experimentally, homeostatic plasticity is induced by silencing the entire network with long-term (24-48 h) application of the the voltage-gated sodium channel blocker tetrodotoxin (TTX), or by hyperactivating the network by preventing GABAergic inhibition, e.g., with bicuculine (Turrigiano et al., 1998). More recently, local methods for regulation of activity levels have been developed, and include shunting of neuronal activity in individual neurons by overexpression of potassium channels (Burrone et al., 2002; Hou et al 2008; Beique et al., 2011) or activating single synapses with light-gated glutamate receptors (Hou et al., 2011). The changes in synaptic strength after activity manipulation are usually estimated by recording miniature postsynaptic currents (mPSC´s; see section 2.1.2).



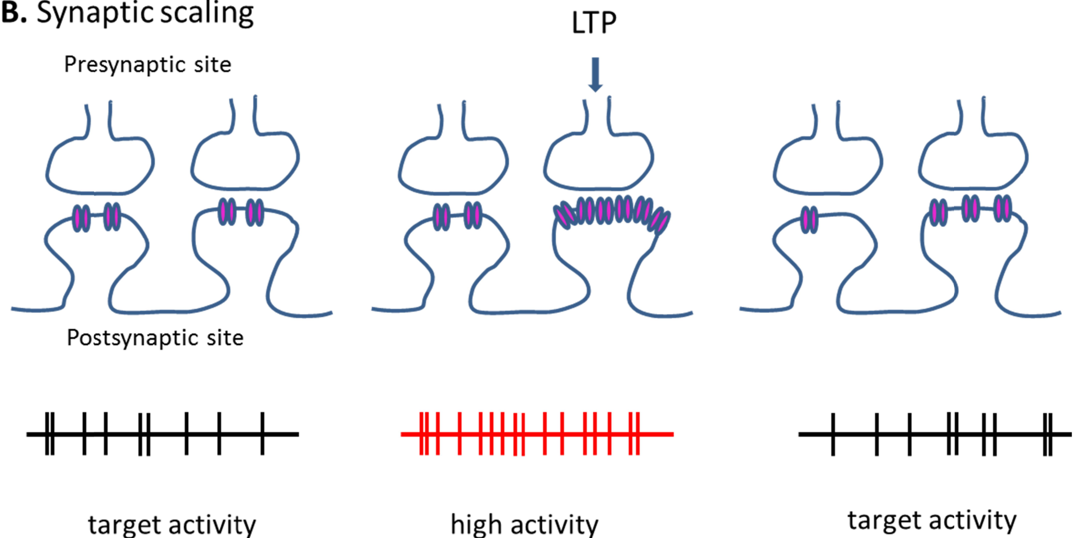


Figure 2 **Homeostatic plasticity.** A. Neurons tend to keep their firing rates constant over long time periods. Increase or decrease in firing rate leads to the homeostatic regulation of intrinsic and synaptic properties so that baseline firing rates are restored. B. One homeostatic mechanism is synaptic scaling. When some inputs are strengthened (e.g. via Hebbian type LTP) synaptic scaling reduces the strength of synapses so that the relative differences (caused by Hebbian LTP/LTD) between synapses are remained (adapted from Turrigiano 2012).

Several molecules have been implicated in mediating the induction of homeostatic plasticity. However, the detailed mechanisms for the ability of individual neurons or whole networks to sensor changes in activity levels are unknown. A pathway that involves changes in spike-mediated somatic calcium influx and activation of Ca^{2+}/calmodulin-dependent protein kinase type IV or CaMKII has been implicated in induction of synaptic scaling (Thiagarajan et al., 2002; Ibata et al., 2008). The suggested induction-mediating molecules for synaptic upregulation include BDNF (Rutherford et al., 1998), cytocine tumor necrosis factor α (TNF- α) (Beattie et al., 2002; Stellwagen and Malenka 2006; Steinmez and Turrigiano 2010), b3 integrins (Cingolani et al., 2008), AMPAR-binding protein interacting with C kinase 1 (PICK1) (Anggno et al., 2011) and activity-regulated cytoskeleton associated protein (Arc) (see section 2.1.3) (Shepherd et al., 2006). By comparison, decreasing synaptic transmission in response to elevated activity level involves, e.g., the polo-like kinase 2 (Plk2)- CDK5 pathway (Seeburg et al., 2008; Evers et al., 2010) and the Homer 1a gene-expression (Hu et al., 2010;

Fu et al., 2011). Similarly to Hebbian plasticity, up– and downregulation of transmission via homeostatic mechanisms involves common signalling elements, such as the CaM-kinase pathway (Goold and Nicoll 2010), which in turn diverge to more specific mechanisms of regulation (Sun and Turrigiano 2011).

A total activity block in the hippocampus or in neuronal cultures results in upregulation of glutamatergic transmission, which involves both pre- and postsynaptic mechanisms (Turrigiano et al., 1998; Murthy et al., 2001; Burrone et al., 2002; Lauri et al., 2003; Ju et al., 2004). The reported presynaptic mechanisms include the changes in Pr (Murthy et al., 2001; Thiagarajan et al., 2005) and quantal content (De Gois et al., 2005; Wilson et al., 2005), while demonstrated postsynaptic mechanisms involve mainly changes in AMPAR's (O´Brien et al., 1998; Turrigiano et al., 1998; Lauri et al., 2003). In parallel with an increase in glutamatergic transmission, a decrease in GABAergic transmission has been observed (Rutherford et al., 1998; Kilman 2002; Gonzalezislas and Wenner 2006; Hartman et al., 2006; Karmarkar and Buonomano, 2006; Swanwick et al., 2006; Chang 2010). Downregulation of GABAergic transmission in the hippocampus involves presynaptic changes in GABA quantal content (Hartman et al., 2006) and postsynaptic changes in pyramidal cell GABAR's and interneuronal AMPAR's (Kilman et al., 2002; Swanwick et al., 2006; Chang et al., 2010). Other homeostatic plasticity mechanisms include regulation of neuronal excitability (Marder and Prinz 2003; Aptowicz et al., 2004; Marder and Goaillard 2006; Karmarkar and Buonomano , 2006) and synapse number (Kirov et al., 1999; Lauri et al., 2003; Wierenga et al., 2006). Further, the homeostatic processes influence the threshold for LTP and LTD induction (Bienenstock et al., 1982).

2.1.3 AMPA-RECEPTORS AND THEIR ROLE IN SYNAPTIC PLASTICITY

2.1.3.1 Structure and biosynthesis of AMPA-receptors

AMPAR's are composed of GluA1-4 subunits (also known as GluRA-D or GluR1-4) (Fig.3). Receptor subunit composition varies in different brain regions and developmental stages. Most receptors in the adult rat hippocampal pyramidal cells are tetramers of GluA1/2 or GluA2/3. In addition, a small proportion of GluA1 homomers also exist (Lu et al., 2009). Interneurons express mainly GluA1 and GluA4 subunits (Geiger et al., 1995; Catania et al., 1998). The amount of AMPAR's that express GluA2 is increased from about 67% to 96% during the first two postnatal weeks (Pickard et al., 2000). The GluA4 is expressed in the pyramidal cells only during the first two postnatal weeks, and it's expression is replaced later with other subunits, including GluA1 (Monyer et al., 1991; Zhu et al., 2000)

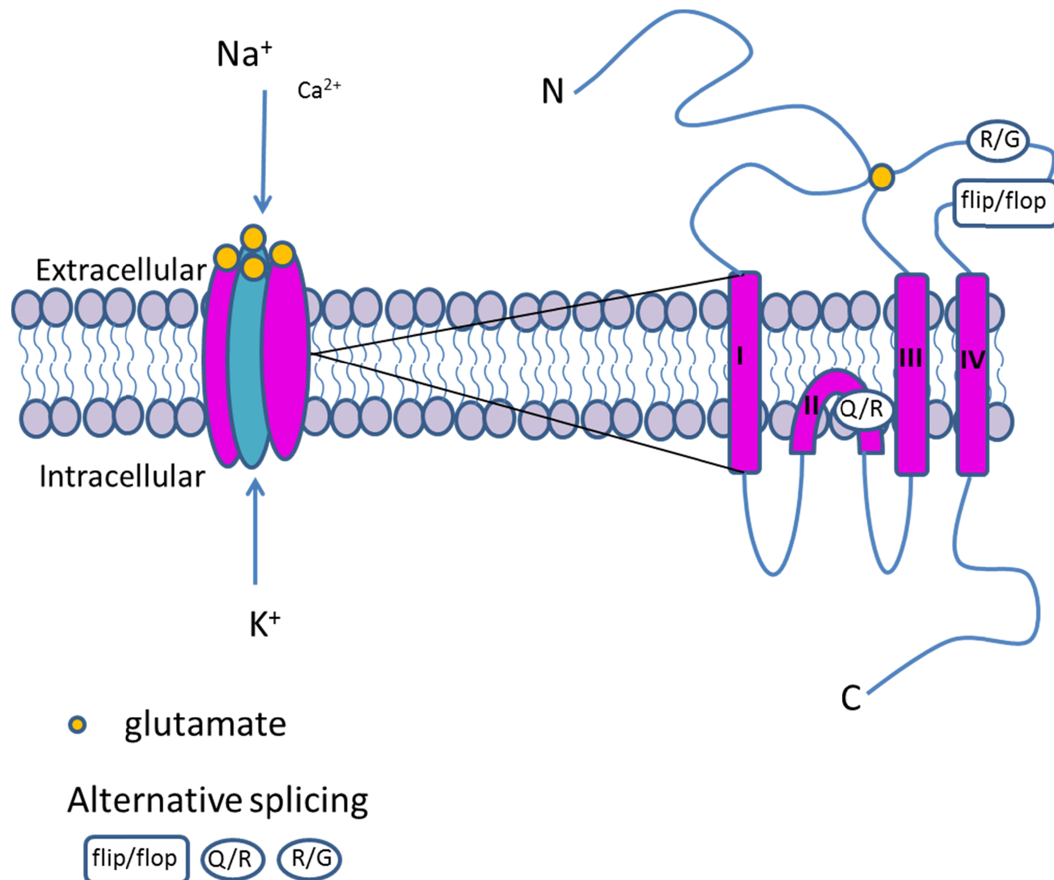


Figure 3 **The AMPAR structure.** AMPAR subunits are about 900 amino acids long glycosylated membrane-spanning polypeptides, composed of ligand binding amino-terminal (N)-sites, transmembrane segments (I-IV) and carboxy-termininal sites (C) that involve many protein binding motifs. Tetrameric AMPAR's are permeable for monovalent cations and at resting membrane potential Na^{+} influx accounts for most of the current. Subunits are regulated by RNA editing. Receptors that lack GluA2 or have unedited (R) form of that subunit are permeable to Ca^{2+}. The GluA1 and -4 and an alternative splice form of GluA2 (GluA2L) have long C-terminal tails, whereas the predominant splice variant of GluA2, GluA3 and an alternative splice form of GluA4 (GluA4S) have short cytoplasmic tails. All subunits have also differentially spliced flip and flop forms (extracellular ligand-binding domain, LBD) that influence the pharmacologic and kinetic properties of the receptors (Monyer et al., 1991).

The half- life of AMPAR's in the synapses is about 20-30 h (Mammen et al., 1997) and the total receptor abundance is determined by the balance between receptor synthesis and degradation. Trafficking and synaptic targeting of AMPAR's (Fig. 4) depend on their subunit specific interactions with a variety of intracellular and transmembrane proteins, located in post-synaptic density (PSD). In addition, native AMPAR's can contain transmembrane AMPAR Regulatory Proteins (TARPs) and cornichons (CNIHs) as auxiliary subunits that modulate both the trafficking and the channel properties of AMPARs (Jackson and Nicoll 2011; Straub and Tomita 2012).

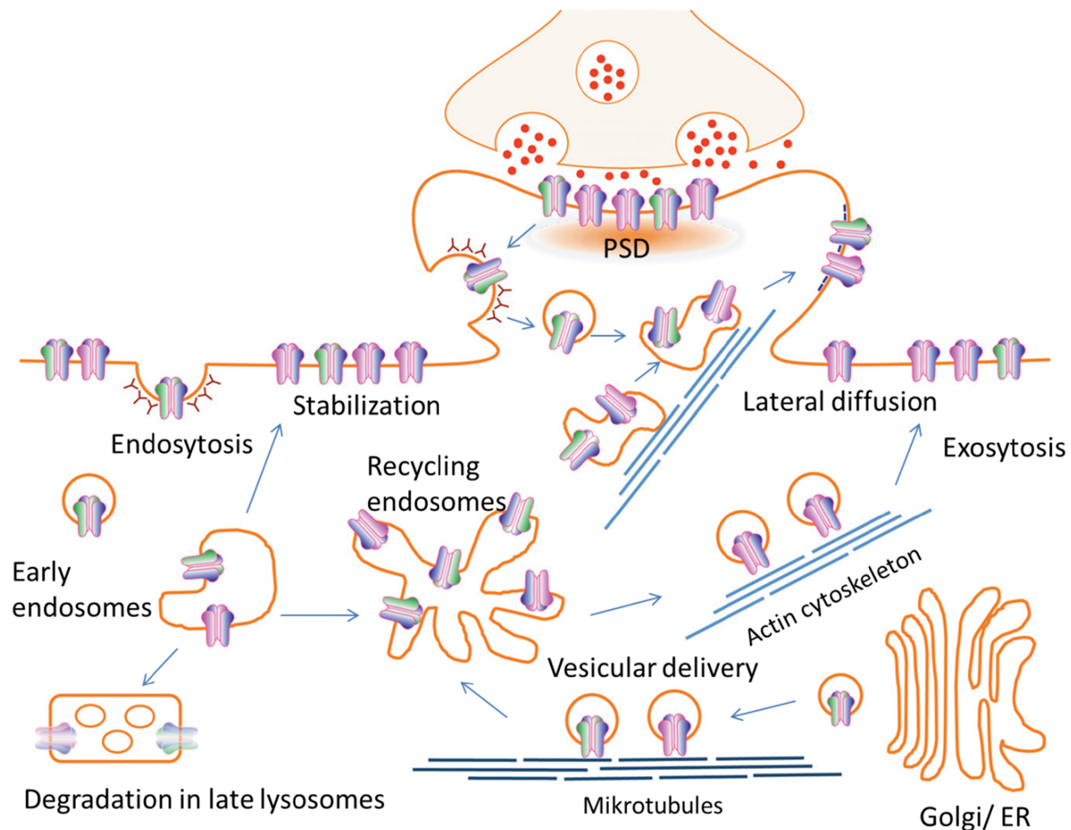


Figure 4. Phases of AMPAR trafficking. Like other transmembrane proteins, AMPAR's are first assembled in the endoplasmic reticulum (ER). Glycosylation and lipid modification of proteins such as palmitoylation take place in the Golgi apparatus and receptors are then exported to the cell surface. The C-terminal domain (CTD) of AMPAR's regulates ER retention and exit form ER and the Golgi apparatus. It is not known if AMPAR's are directly inserted to synapses (Park et al., 2006; Kopec et al., 2006; Kennedy et al., 2010) or extrasynaptic sites where they are transported to synapses via lateral diffusion (Borgdorff et al., 2002; Ehlers et al., 2007; Yang et al., 2008; Makino et al., 2009). AMPAR insertion may also occur via SNARE-dependent exocytosis, since insertion is prevented by tetanus toxin (Lu et al., 2001). AMPAR endocytosis occurs via clathrin-coated pits and requires dynamin. After internalization receptors are sorted to either early or late endosomes and lysosomes and recycled to extracellular membrane or degraded (Ehlers 2000, Bredt 2003; Lee et al., 2004; figure modified from Aggono and Huganir, 2012).

2.1.3.2 Molecular mechanisms for activity-dependent regulation of AMPA-receptors

The activation of various kinases or phosphatases is a key mechanism involved in the induction of activity-dependent plasticity, and may regulate synaptic AMPAR's directly or indirectly via C-terminal domain (CTD)-interacting proteins (Table 1). LTP and LTD reversibly modify the phosphorylation of the AMPAR GluA1 subunit (Lee et al., 2000) to directly influence their electrophysiological properties. On the other hand, covalent modifications modulate the C-terminal protein interactions of AMPARs (Lin et al., 2009), which enables activity-dependent regulation of synaptic localization of the receptors. GluA1 and GluA4 (as well as GluA2L) AMPAR

subunits have a long CTD that has been strongly implicated in the activity-dependent trafficking of AMPAR's (Malinow and Malenka, 2002; Bredt and Nicoll, 2003).

The phosphorylation sites of GluA1 subunit include four serine residues (Ser831, Ser 845, Ser818 and Ser816) and one threonine residue (Thr840). Phosphorylation of Ser831 by CaMKII and PKC and Ser845 by PKA controls the GluA1 AMPAR insertion to synapses, whereas Ser818 and Ser816 phosphorylation by PKC enhances GluA1 binding to 4.1N. Binding to 4.1N regulates the surface expression of GluA1 and the insertion to extrasynaptic sites (Lin et al., 2009). Knockdown of 4.1N impairs the maintenance of LTP without affecting E-LTP (Lin et al., 2009). On the other hand, phosphorylation of Thr840 regulates the removal of the GluA1 subunit containing AMPAR's in LTD (Lee et al., 2006; Delgado et al., 2007). The extreme CTD PDZ site of GluA1 binds directly to synapse-associated protein of 97kD (SAP97). SAP97 is believed to have a critical role in AMPAR trafficking and LTP as it is targeted into spines upon CaMKII phosphorylation (Aggono and Huganir 2012). Indeed, overexpression of SAP97 induces LTP (Rumbaugh et al., 2003; Nakagawa et al., 2004). Yet, the mutated GluA1 PDZ (class I) motif that binds to SAP97 prevents the GluA1 transport to synaptic membranes whereas complete deletion of this motif has no effect of GluA1 plasticity (Passafaro et al., 2001; Kim et al., 2005; Boehm et al., 2006).

The GluA4 subunit can be phosphorylated by PKA, PKC and CaMKII on Ser-842 and PKC on Thr-830 (Carvalho et al., 1999; Correia et al., 2003). The delivery of GluA4 to synaptic membranes is controlled by PKA (Zhu et al., 2000; Esteban et al., 2003). In addition, it has been shown that phosphorylation of Ser842 is sufficient for synaptic incorporation of GluA4 (Gomes et al., 2007). Similar to GluA1, GluA4 directly interacts with protein 4.1 (Coleman et al., 2003), although the role of this interaction in controlling GluA4 surface expression is somewhat controversial (Coleman et al., 2003, Gomes et al., 2007). GluA4 lacks the extreme C-terminal PDZ interaction motif present in the GluA1, but has been reported to bind α-actinin-1 and Ras GTPase-activating-like protein IQGAP1 (IQGAP1) (Coleman et al., 2003; Correia et al., 2003; Nuriya et al., 2005). The direct interaction with α-actinin-1 has been suggested to retain the GluA4 in the intracellular pool. This interaction is disturbed after PKA phosphorylation of Ser842, which further preserves the GluA4 interaction with IQGAP1 (Nuriya et al., 2005).

In contrast to GluA1 and GluA4, the subunits with the short CTD (GluA2 and 3) are constitutively recycled in and outside the synapses. The tyrosine-rich CTD of the GluA2 subunit is also subject to phosphorylation and its interaction with several proteins, including N-ethylmaleimide-sensitive fusion protein (NSF) and PICK1, regulates the GluA2 abundance in synapses (Luthi et al., 1999; Beretta et al., 2005, Evers et al 2010).

Apparently, the regulation of AMPAR trafficking during LTP, LTD and homeostatic scaling are fundamentally different. The expression of LTP requires mainly the GluA1 subunit (Zamanillo et al., 1999; Hayashi 2000; Malinow and Malenka 2002; Reisel et al., 2002; Lee et al., 2003; Boehm et al., 2006; Shepherd and Huganir 2007; Lin et al 2009), while GluA2 has been implicated in LTD and homeostatic scaling (O´Brien et al., 1998; Chung et al., 2000; Kim et al., 2001; Chung et al., 2003; Ashby et al., 2004; Malenka and Bear 2004; Gainey et al., 2009). However, the detailed mechanisms underlying AMPAR trafficking in different forms of synaptic plasticity are not fully understood and remain, in part, controversial. For example, in addition to GluA2, GluA1 subunit and Ca^{2+}- permeable (GluA2 lacking) AMPAR's have been implicated to have role in homeostatic scaling (O´Brien et al., 1998; Gainey et al., 2009; Beique et al., 2011). Furthermore, a recent report by Granger and co-workers (2013) showed that LTP can occur in the absence of GluA1. In this study the authors deleted genes for GluA1-3 receptors and showed that re-expression of any AMPAR subunit or even KAR's were able to restore LTP (Granger et al., 2013). However, deleting genes for all important AMPAR subunits may lead to various compensatory mechanisms, complicating the interpretation of these results.

It is not known in detail whether AMPAR accumulation to synapses after induction of LTP or homeostatic plasticity is due to lateral diffusion of AMPAR's (Borgdorff et al., 2002; Ehlers et al., 2007; Makino et al., 2009) or exocytosis of receptors (Kopec et al., 2006; Park et al., 2006; Kennedy et al., 2010). In both cases the receptors need to be released from putative extra- or intrasynaptic retention sites and captured in the PSD. As exocytosis is a rather slow process it is suggested that lateral movement of extrasynaptic AMPAR's occurs in E-LTP (Ashby et al., 2006). It is also thought that during LTP induction, PKA activation leads to insertion of AMPAR's to perisynaptic sites, which provides a pool of extrasynaptic receptors available for synaptic incorporation (Yang et al., 2008). In LTD, the GluA2 containing AMPAR's are endocytosed from extrasynaptic sites where receptors diffuse laterally from PSD (Carroll et al., 1999; Blanpied et al., 2002; Lee et al., 2002).

Table 1 The list of the CTD-interacting proteins. *Additional interacting or auxiliary proteins such as cornichons and myosin V (Wang et al., 2008) have been implicated in regulation of AMPARs during their dendritic trafficking and in the ER.*

Interacting protein	AMPAR subunit	Proposed function	references
α- Actinin-1	GluA4	Function unknown, proposed to anchor AMPARs to cytoskeleton	Nurriya et al., 2005
4.1N and **4.1G**	GluA1 GluA4	Anchors AMPARs to spectrin/actin cytoskeleton , proposed to regulate AMPAR surface expression	Song and Huganir 2002; Coleman et al., 2003; Schulz et al., 2004
ABP (GRIP2), AMPAR binding protein	GluA2 GluA3	Anchors AMPAR at synapses, AMPAR trafficking in LTD, forms a complex with GRIP	Osten et al., 2000; Daw et al., 2000
AP- 2, clathrin adaptor complex	GluA2 GluA3	Regulates AMPAR internalization	Kastning et al., 2007
BRAG2, brefeldin-resistant ADP ribosylation factor GEF1	GluA2	AMPAR internalization during LTD	Scholz et al., 2010
GRIP, glutamate receptor interacting proteins	GluA2 GluA3	Anchors AMPAR at synapses, AMPAR trafficking in LTD	Osten et al., 2000 ; Daw et al., 2000
IQGAP1, Ras GTPase-activating-like protein 1	GluA4	Function unknown	Nurriya et al., 2005
NSF, N-methylmaleimide-sensitive fusion protein	GluA2	Stabilizes AMPARs at synaptic membrane , regulates basal AMPAR trafficking , implicated in synaptic scaling	Luthi et al., 1999; Beretta et al., 2005, Evers et al 2010
PICK1, protein interacting with C kinase 1	GluA2 GluA3	Stabilizes intracellular pools of AMPARs, AMPAR regulation in LTD , LTP and in synaptic scaling	Dev et al., 1999; Xia et al., 1999; Daw et al., 2000; Anggono et al., 2011
SAP97, synapse associated protein 97	GluA1	Regulates AMPAR transport through ER and Golgi, AMPAR trafficking to synapses, AMPAR regulation in LTP	Leonard et al., 1998; Lee et al., 2003 Coleman et al., 2010
Shank3	GluA1	AMPAR regulation in LTP	Uchino et al., 2006; Rayanud et al., 2013

2.2 MECHANISMS OF SYNAPTIC TRANSMISSION AND PLASTICITY IN THE DEVELOPING HIPPOCAMPUS

2.2.1 SPONTANEOUS ELECTRICAL ACTIVITY IN THE DEVELOPING CA3-CA1 CIRCUITRY

The developing nervous system generates endogenous spontaneous activity even before the first synapses are formed (O´Donovan 1999; Ben-Ari 2001). The earliest form of activity consists of synchronous Ca^{2+} transients between neurons that are coupled with gap junctions (Allene et al., 2008, Yang et al., 2009). After the first synapses are formed the activity emerges as a network-driven depolarizing event during which the neurons in the network are synchronously activated. These synchronous depolarizing events are widely seen in many parts of the developing CNS, such as the retina, the spinal cord and several forebrain structures (Blankenship et al., 2010).

In the rodent hippocampus the immature pattern of electrical activity is restricted to the first postnatal weeks which correspond to a period of intense synaptogenesis and neuronal growth (Khazipov et al., 1997; Garaschuk et al., 1998; Tyzio et al., 1999). It is seen in two distinct forms; synchronous, mainly Ca^{2+}-mediated, plateau assemblies (SPA's) and giant depolarizing potentials (GDP's). SPA's are seen a few days before and after birth and these bursts of plateau potentials are correlated across 3-7 neurons. GDP's emerge a few days after birth, overlap briefly with SPA's and disappear during the second postnatal week (Ben-Ari et al., 1989; Garaschuk et al., 1998; Crepel et al., 2007; Allene et al., 2008). In neonatal networks the synchronous events occur at frequencies ranging from 20 to 200 Hz (Palva et al., 2000; Lamsa and Taira 2003; Crepel et al., 2007). It has been shown that the CA1 area is able to generate GDP's even if it is isolated from CA3. However, the frequency is lower compared to control conditions, implicating the role of CA3 area in burst initiation (Ben-Ari et al., 2007; Juuri et al., 2010).

Although the existence of spontaneous activity has been known for decades its physiological implications are still insufficiently known. Spontaneous activity in spinal cord controls neuronal path finding and synaptic maturation (O´Donovan et al., 1999; Gonzales-Islas and Wenner, 2006; Hanson et al., 2008) while activity seen in the retina can affect the remodeling of the synaptic connections in the visual cortex (Mooney et al., 1996; Torborg et al., 2005). Furthermore, spontaneous activity bursts have been shown to guide the development of the primary somatosensory cortex (Khazipov et al., 2004).

High-frequency activity in neonate hippocampus co-incides with a behavioral phenotype called active sleep (Lahtinen et al., 2002). In *in vivo* experiments hippocampal sharp waves (SPWs, local hippocampal field potentials recorded, e.g., during slow wave sleep; Buzsaki et al., 1983) occur simultaneously with contractions of skeletal muscles (startles) that are the early behavioral events observed in infant mammals (Gramsbergen et al.,

1970; Karlsson et al., 2006). These events have been implicated in the development of both the glutamatergic and GABAergic circuitry in the hippocampus (Lauri et al., 2003; Colin-Le Brun et al., 2004). However, it is not known in detail how different activity patterns (i.e., asynchronous vs. synchronous activity) affect the development of synaptic networks.

2.2.1.1 Mechanisms that generate spontaneous activity in the hippocampus and the role of gap-junctions

The precise mechanisms that underlie spontaneous activity patterns in the developing hippocampus are still partly unknown and have been recently under intense debate (Rheims et al., 2009; Holmgren et al., 2010; Ruusuvuori et al., 2010; Tyzio et al., 2011). It is thought that interneuronal GABAergic transmission, pacemaker-like neurons and gap-junctions have an essential role (Lenekugel et al., 1997; Sipilä et al., 2006, 2008; Ben-Ari et al., 2007; Crepel et al., 2007). However, the pacemaker type of activity has shorter intervals than GDP's and it is likely that generation and modulation of spontaneous activity depends on interplay between intrinsic membrane properties and various types of synaptic interactions that may vary between different areas and developmental stages. A critical feature regulating the network excitability is the balance between glutamategic and GABAergic transmission (Khazipov et al., 1997; Bolea et al., 1999; Lamsa et al., 2000) that depends on various factors, for example the activity of KAR's (Lauri et al., 2005; Juuri et al. 2010).

Gap-junctions form electrical synapses and allow a low resistance pathway for current spreading between neurons. By this virtue, the gap-junctions play a central role in the synchronization of neurona activity both in the developing and adult networks (Strata et al., 1997 ; Draghun et al., 1998 ; Lamsa and Taira 2003). The neuronal expression level of gap junctions is higher in developing nervous system than in the adult brain (Peinado et al., 1993; Kandler and Katz 1995). In the hippocampus, gap-junctions are found mainly between interneuronal dendrites, but also axo-axonal and dendritic-axonal coupling of CA3 pyramidal cells have been found (Fukuda and Kosaka, 2000; Schmitz et al., 2001; Traub et al., 2002). Gap-junctions are hydrophilic intracellular channels formed by the docking of two hexameric connexins. Although most electrical synapses are bidirectional, rectification of electrical transmission has been found (Pereda et al., 2013). Blocking of gap-junctions prevents the generation of spontaneous activity in the hippocampus, the spinal cord and the retina (Hanson et al., 2003; Lamsa and Taira 2003 ; Syed et al., 2004; Crepel et al., 2007). Moreover, mice lacking connexins have altered spontaneous activity (Chang et al., 1999; Personius et al., 2007). A limitation that has significantly complicated studies on the physiological roles of gap-junctions is that gap-junction blockers have several non-specific actions including blocking of VDCC, activation of

calcium-activated potassium channels, and inhibition of synaptic release (Vessey et al., 2004; Takeda et al., 2005; Tovar et al., 2009).

2.2.2 DEVELOPMENT OF GABAERGIC AND GLUTAMATERGIC CONNECTIVITY IN THE AREAS CA1 AND CA3

The formation of neuronal connections starts with axonal pathfinding followed by target recognition and synapse formation (Waites et al., 2005; Shepherd and Huganir 2007). Glutamatergic synapses are initially formed on filopodia that develop over time to dendritic spines (Fiala et al., 1998). In the rat hippocampus GABAergic synapses are formed before glutamatergic ones (Tyzio et al., 1999; Khazipov et al 2001; Hennou et al., 2002). At birth (postnatal day 0, P0), 10% of the rat pyramidal cells have a small apical dendrite and only GABAergic currents. While, 10% of cells have more complex dendritic trees reaching to *stratum lacunosum-moleculare* and have both GABA and glutamate receptors. The majority of cells (80%) have a small cell soma and no spontaneous currents (Tyzio et al., 1999). During the first two postnatal weeks about half of the excitatory synapses at CA1 area occur on dendritic shafts and 20% on filopodia (Fiala et al., 1998).

At P0, the rat pyramidal cell layer is composed of 6 to 10 cell rows while in adult rats, there are only 2 to 3 cell layers. Dendritic length and morphology of the pyramidal neurons is more variable in area CA3 as compared to CA1, where dendritic trees are more homogenous and cells are smaller (Ishizuka et al., 1995; Pyapali et al., 1998). The CA3 area develops before the CA1 (Bayer 1980). The first synapses are made into interneurons and both interneurons and pyramidal cells follow the same GABA-glutamate sequence of synapse formation (Hennou et al., 2002). Previous findings have also showed that nicotinic cholinergic signaling develops before the glutamatergic one and nicotinic acetylcholine receptor activation promotes glutamate receptor formation but does not affect GABAergic connections (Lozada et al., 2012).

2.2.3 FUNCTIONAL PROPERTIES OF IMMATURE GLUTAMATERGIC SYNAPSES

Immature glutamatergic synapses are functionally silent, meaning that they are not conductive at resting membrane potential (Isaac et al., 1995; Liao et al., 1995). These synapses can be postsynaptically silent, i.e., they lack functional postsynaptic AMPAR´s, while NMDAR-mediated transmission is observed upon membrane depolarization (Durand et al., 1996). However, other explanations for silent synapses are plausible (Friedman et al., 2000). One hypothesis is that NMDA-only responses may be due to activation of high-affinity NMDAR's (Patneau and Mayer 1990) by glutamate diffusion from adjacent synapses (spillover) (Asztely et al., 1997). There is also evidence that silent synapses can be explained by presynaptic mechanisms,

and that NMDA-only responses are due to release of a low amount of glutamate, e.g., via incomplete fusion of presynaptic vesicles, failing to activate AMPAR's (whispering synapses) (Choi et al., 2000; Renger et al., 2001). Indeed, the presynaptic properties at glutamatergic synapses change considerably during development (Bolshakov and Siegelbaum 1995; Chavis and Westerbrook 2001; Wasling et at., 2004; Lauri et al., 2006; Sallert et al., 2009; Luchkina et al., 2013).

At the developmental stage where a functional contact exists, AMPA transmission is typically labile and highly sensitive to synaptic stimulation (Xiao et al., 2004; Hanse et al., 2009, Riebe at al., 2009). This lability is observed both at glutamatergic inputs to pyramidal neurons and interneurons during the early postnatal development, after which adult – type, stabile AMPA-mediated transmission is predominant.

2.2.4 PROPERTIES OF NEONATAL LTP AND LTD

The induction and expression mechanisms of LTP and LTD vary during development (Dudeck and Bear 1992; Bashir et al., 1993; Olliet et al., 1997; Palmer et al., 1997; Sallert et al., 2009; Luchkina et al., 2013). For instance, studies on neonatal hippocampal slices have shown that both NMDAR and mGluR-dependent forms of LTD exist in area CA1 and their expression depends on the used induction protocol, while later on in development, it is more difficult to induce de novo NMDAR-LTD (Domenici et al., 1998; Kemp et al., 2000; Wasling et al., 2002; Pavlov et al., 2004; Nosyreva and Huber 2005). In contrast to LTD, the activation of NMDAR's is the predominant mechanisms for induction of LTP at CA3-CA1 synapses in both adults and neonates (Collingridge et al., 1983; Luchkina et al., 2013).

Activation of CaMKII is required for LTP at adult hippocampal CA3-CA1 synapses (see section 2.1.3.1). However, the expression level of CaMKII is lower during the first postnatal week (Kelly et al., 1987) and its activity is not needed for the LTP induction at neonatal CA3-CA1 synapses (Yasuda et al., 2003). Over the first postnatal days (before P13) PKA activation is crucial for LTP induction (Yasuda et al., 2003). Later on in development (after P13) LTP requires two kinase cascades, one involving CaMKII and the other PKA and PKC (Wikström et al., 2003).

The most important AMPAR subunit for the expression of adult CA3-CA1 hippocampal LTP is GluA1. Its delivery to synaptic membranes requires CaMKII activity (Malenka and Nicoll 1999; Zamanillo et al., 1999; Hayashi et al., 2000; Malinow and Malenka 2002; Lisman et al., 2002; Esteban et al., 2003; Lee et al., 2003; Boehm et al., 2006; Shepherd and Huganir 2007; Lin et al 2009). Furthermore, the LTP is impaired in adult GluA1-/-mice, whereas young animals show LTP (Jensen et al., 2003), suggesting a GluA1-independent LTP expression during early development. However, the detailed mechanisms of GluA1 independent and PKA dependent developmental CA3-CA1 LTP are still unknown. Interestingly, the activation

of PKA leads to the phosphorylation and synaptic incorporation of GluA4 containing AMPAR's in organotypic hippocampal slices, suggesting the possible role of the GluA4 subunit in the early PKA-dependent LTP (Yasuda et al., 2003).

2.2.5 PROPERTIES OF NEONATAL HOMEOSTATIC PLASTICITY

Recent studies have revealed a wide range of signaling processes that contribute to the homeostatic regulation of synaptic strength (see section 2.1.2.2). However, different plasticity mechanisms are likely to operate over various temporal and spatial scales depending on the used activity manipulation, experimental model, cell type and developmental stage (Burrone et al., 2002; Sutton et al., 2006; Wierenga et al., 2006; Echegoyen et al., 2007; Ibata et al., 2008). For example, the TTX injection to an individual neuron scales up synaptic strength in the same way that manipulation of the overall activity of the network does (Ibata et al., 2008), whereas hyperpolarizing the individual neuron by overexpressing an inwardly rectifying K^+-channel (Kir) does not induce postsynaptic scaling (Burrone et al., 2002; Hou et al., 2008). In addition, previous studies in dissociated neocortical or hippocampal cultures and retina have revealed that the response to the activity manipulation strongly depends on the developmental stage of the network (Burrone et al., 2002; Hartman et al., 2006; Wierenga et al., 2006 Maffei and Turrigiano; 2008) Furthermore, TTX application increases the spine number in the adult but not the neonatal hippocampus (Kirov et al., 1999).

Moreover, one important but less-studied issue is the mechanisms that developing networks use for balancing activity levels at the time of intense synapse formation, characterized by spontaneously generated electrical activity (see section 2.2.3). Most of the experiments considering homeostatic plasticity mechanisms are performed with cultures (Turrigiano et al., 1998; Burrone et al., 2002; Kim and Tsien 2008) where developmental factors such as intrinsic electrical activity and depolarizing GABAergic transmission (Ben-Ari, 2001), silent synapses (Isaac et al., 1995; Liao et al., 1995) and the immature –type mechanisms for glutamate release (e.g., Lauri et al., 2006) are perturbed; these are, without a doubt, critical factors for neuronal maturation in more physiological conditions. Yet, only a few previous studies have considered the homeostatic plasticity in developing hippocampal circuitry. In these studies both glutamatergic and GABAergic transmission has been shown to respond to a total activity deprivation (Lauri et al., 2003; Colin-Le Brun et al., 2004). However, the relative contributions of different intrinsic activity patterns and detailed plasticity mechanisms involved have not been studied before.

3 AIMS OF THE STUDY

The specific aims of this study were:

1. To study the relative contributions of asynchronous vs. synchronous electrical activity in synapse formation and maintenance in developing hippocampal neurons (I, II).

   It is evident that electrical activity is important for the formation and maturation of developing neuronal circuits. However, our understanding of how exactly the different components of the spontaneous activity (synchronous and asynchronous) guide the synaptic development is still limited. To study this, we selectively blocked either synchronous or global neuronal activity and investigated the consequences on the development of functional synapses in the area CA3-CA1.

2. To explore homeostatic plasticity mechanisms in distinct cell types and at different stages of development (I, II).

   Although many studies have established that the neuronal cultures maintain activity levels constant via homeostatic regulation, less is known about the corresponding mechanisms in the intact developing networks, which display immature-type network activity and mechanisms of synaptic transmission. The aim was to study the homeostatic regulation of glutamatergic and GABAergic synapses in parallel and at different stages of hippocampal development.

3. To study the role of the AMPA-receptor subunit GluA4 in activity-dependent synaptic plasticity during early development (I, III, unpublished data).

   The physiological significance of transient expression of GluA4 in the hippocampal pyramidal neurons during early development is not known. The goal here was to understand the role of GluA4 in activity-dependent plasticity of CA3-CA1 synapses, including both the Hebbian LTP and homeostatic plasticity mechanisms.

4 MATERIALS AND METHODS

The detailed description of methods used in this thesis can be found in the original publications (I- III). A list of the used methods is presented in Table 2. Only those procedures are listed in the table where the author was personally involved.

Table 2. *The methods used in this thesis in publications I-III and unpublished data.*

Method	Publication
Acute hippocampal slice preparation	I-III, unpublished
Cell culture	II, III
Whole-cell patch-clamp	I-III, unpublished
Western blot	I, III
PCR	III, unpublished
Agarose gel electrophoresis	III, unpublished
SDS- PAGE	I, unpublished
Cloning of lentiviral constructs	III
Production of lentiviral vectors	III
Fusion-peptide production	III
Mouse breeding and genotyping	III, unpublished

4.1 EXPERIMENTAL ANIMALS

Experiments were performed on Wistar rats (4- to 20-day-old) and GluA4-/-mice (4- to 5-day-old) kindly provided by Hannah Monyer (Fuchs et al., 2007). All experiments with animals were done in accordance with the University of Helsinki Animal Welfare Guidelines.

4.2 HIPPOCAMPAL SLICE PREPARATION

Postnatal day (P)0-P14 Wistar rats (P0 = the day of birth) or P4-P11 mice were rapidly killed by decapitation with or without (animals P0-6) anesthesia. Hippocampal slices (400 µm) were cut with a vibratome using standard methods (e.g. Lauri et al., 2006). The slices were prepared for overnight incubation (see section 5.1) or used for electrophysiological recordings 1-5 hours after dissection.

4.3 ELECTROPHYSIOLOGY

Whole-cell patch clamp recordings were used to study the spontaneous network activity and for recordings of AMPAR-, NMDAR- and $GABA_AR$-mediated synaptic currents. All the recordings were done in a submerged recording chamber, at 32°C. The slices were constantly perfused with and artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 1 MgSO4, 26 NaHCO3, 15 D-glucose, and 2 CaCl2; 5% CO2–95% O2, at a rate of 1–2 ml/min.

Spontaneous activity was recorded from CA3 pyramidal cells with microelectrodes (3-5 MΩ) containing low Cl^- (2 mM) filling solution (See publication I and II). Cells were voltage clamped at – 58 mV. Under these conditions, the calculated $GABA_AR$ reversal potential is -109 mV (excluding the contribution of HCO_3^-) and AMPAR and NMDAR around 0 mV (see e.g. Hestrin et al., 1990) and the GABAergic synaptic events are seen as outward currents and glutamatergic synaptic events as inward currents.

Synaptic glutamatergic (AMPAR- and NMDAR-mediated) currents (evoked and spontaneous) were recorded from CA3 or CA1 pyramidal cells with patch pipets (3-5 MΩ) filled with Cesium (Cs)-methanosulfate based solution (See publication I-III). Cs blocks K^+- and $GABA_BR$'s which improves 'space clamp', i.e. the electrical control of distant dendritic structures. Fast synaptic currents mediated by different types of receptors were isolated using specific pharmacological tools and the voltage-dependent activation profile of NMDARs. For the recordings of AMPAR-mediated mEPSCs 1 µM TTX and 100 µM $GABA_AR$ antagonist picrotoxin (PiTX) were added to ACSF and cells were clamped at -70 mV. Under these conditions AMPAR-mediated synaptic events were seen as inward currents, while NMDAR mediated activity was inhibited due to the voltage-dependent block. For recording of NMDAR-mediated mEPSCs 10 mM BAPTA (Ca^{2+} chelator) was included in the filling solution and cells were voltage clamped at + 40 mV in the presence of 1 µM TTX, 100 µM PiTX and 10 µM AMPAR and KAR antagonist 2.3-dihydroxy-6-nitro-7-sulfamoylbenzo (f) quinoxaline (NBQX). Under these conditions NMDAR-mediated mEPSCs were seen as outward currents. For recordings of $GABA_A$-mediated mIPSCs the filling solution Cl^- concentration was increased to 30 mM (see publication II) and cells clamped at -70 mV. mIPSCs were recorded in the presence of 1 µM TTX, 10 µM 2.3-dihydroxy-6-nitro-7-sulfamoylbenzo(f) quinoxaline (NBQX) and 1 µm $GABA_B$-R antagonist 3-N[1-(S)-(3,4-dichlorophenyl)ethyl,]amino-2-(S)-hydroxypropyl-P-benzyl-phosphinic acid (CGP55845). Under these conditions the $GABA_AR$ reversal potential was approximately – 38 mV and at holding potential – 70 mV the $GABA_AR$-mediated synaptic events were seen as inward currents.

4.4 WESTERN BLOT

Western blot technique allows the separation and identification of proteins based on their molecular weight. Proteins are separated with SDS-PAGE and transferred to a membrane. The relative amounts of the protein present in different samples can be measured with labeled antibodies specific to the protein of interest. The antibody complexes can be detected e.g. with ECL (enhanced chemiluminescence) technique (see publication I). Western blot assay was used for quantification of the changes in total protein levels of synaptophysin, vesicular glutamate transporter 1 (VGLUT1), GluA2 and GluA4 in the area CA1 after long-term (15-20h) activity manipulation or incubation in control conditions (see publication I). Western blot assay was also used for the verification of the appropriate size of recombinant proteins GluA1 and GluA4 as well as GST-fusion proteins (lentiviral and fusion-peptide production, see publication III).

4.5 PRODUCTION OF LENTIVIRAL VECTORS

Lentiviral vectors are used as an effective tool for gene delivery to virtually all mammalian cells both *in vitro* and *in vivo*. This technique was used in this study for overexpression of GluA1 and GluA4 in the hippocampus *in vivo*. To achieve the strong fluorescent signal that helped the localization of infected pyramidal cells, the cDNA encoding rat GluA1 and GluA4 (both as flip isoform and both with green fluorecent protein (GFP) fused to the extracellular N-terminus after the signal peptide, Coleman et al., 2006) were cloned into double promoter lentiviral transfer vectors. In these vectors GFP-tagged AMPAR subunits and enhanced green fluorecent protein (EGFP) are expressed under separate neuronal specific synapsin 1 promoter. The cloned constructs were verified by restriction mapping and the appropriate size of the encoded recombinant proteins was confirmed by western blot of transfected human embryonic kidney 293t cells (HEK cells) cells using primary antibodies anti-GluA2/4ctdlong (1:2000, rabbit, Coleman et al., 2006) and anti-GluA1 (1:1000, rabbit polyclonal, Synaptic Systems). Production of lentiviral particles were made by transfecting HEK293t cells with Fugene6 (Roche Applied Science) using 0,75 μg envelope- coding plasmid pMD.G, 2,25 μg packaging plasmid psPAX2 and 3 μg of the relevant pLen transfer vector. Viral particles were harvested 48 h after transfection using PEG-itTM virus precipitation solution (System Biosciences) and suspended in phosphate buffered saline (PBS).

4.6 GST FUSION-PEPTIDES

Glutathione S-transferase (GST) fusion proteins are widely used for antibody production and studies of protein-protein interaction since the GST

binding to gluthatione provides basis of simple purification without denaturation. In this thesis we wanted to study the role of AMPAR subunit C- terminal protein interactions in PKA induced synaptic potentiation. After verifying that the GST infusion to the neuron cytoplasm had no effect on glutamatergic transmission, the GST-CTD-fusion proteins could be used as tools to scavenge and thereby spefically inhibit protein interactions to the corresponding endogenous protein sequence. The relevant purified GST-fusion proteins or GST were prepared as described (Coleman et al., 2010) Briefly, the plasmid encoding for the GST fusion protein (constructs provided from Kari Keinänen's lab) was expressed in E.coli BL21 according to manufacturer's instructions (GE Healthcare). The protein expression was induced with 1 mM IPTG for 3 h at 37°C in cultures with OD600 between 0.4-0.6. Bacteria were pelleted, frozen and resuspended in PBS buffer containing 1 mM serine protease inhibitor PMSF and sonicated. After centrifugation, the supernatant was incubated with glutathione-sepharose (GE Healthcare) for 2 h at 4°C. After washing the proteins were eluted in 10 mM glutathione in 50 mM Tris-HCl, pH 8 and dialyzed against slice puffer (10 mM HEPES, 0.5 mM EGTA, 8 mM NaCl) for 48 h at 4°C with mixing.The GST-fusion proteins were included in the intracellular filling solution at concentration of 0.5 μM.

4.7 DATA ANALYSIS

Axoscope 9.2 (Axon instruments), WinEDR (version 2.3.3, Strathclyde Electrophysiology Software) or WinLTP (0.95b or 0.96, www.winltp.com, Anderson and Collingridge, 2007) was used for data acquisition. Offline analysis was done using MiniAnalysis 6.0.3 program (Synaptosoft Inc.) or WinLTP.

Uncompensated series resistance was monitored (< 40 MΩ) and cells were discarded if this parameter varied by more than 20%. Membrane properties were estimated from the current response to a 5 mV pulse step. Series resistance ($R_s = V_{step}/I_{peak}$) and membrane resistance ($R_m = V_{step}/I_{ss}$) were calculated according to Ohm's law from the peak amplitude of the current transient (Ipeak) and from the steady state current (Iss). Membrane capacitances Cm were estimated using the equation $C_p = \tau\ (1/R_s - 1/R_p,\ R_p = R_m - R_s)$ where decay time constant (τ) was obtained by exponential fitting of the decay of the transient current in response to a 5 mV pulse step using pCLAMP software (Clampfit 9.2, Axon Instruments).

Student's two-tailed t- test (I-III), two-way ANOVA (III) and ANOVA followed by the post hoc Tukey test and Pearson's correlation test (II) was used for statistical analysis. The level of significance was set as $P < 0{,}05$.

5 RESULTS

5.1 DEVELOPMENT OF A NOVEL METHOD TO STUDY HOMEOSTATIC PLASTICITY IN THE DEVELOPING HIPPOCAMPAL CIRCUITRY (I, II AND UNPUBLISHED)

The aim of this work was to understand how endogenous activity regulates synapse maturation. The starting point was to develop an experimental model where the three-dimensional tissue structure and endogenous network activity is preserved. Neuronal cultures are commonly used for studies of plasticity mechanisms (e.g., Turrigiano et al., 1998; Burrone et al., Kim and Tsien 2008), but due to the absence of immature network activity in these preparations, they were not suitable for our studies. The whole hippocampal preparations have been used in a few previous studies (Lauri et al., 2003; Colin-Le Brun et al., 2004). This preparation meets the requirement of endogenous electrical activity patterns, but the electrophysiological recordings and drug applications with this preparation are complicated. To this end, we developed a method for long-term (up to 48 h) *in vitro* incubation of acutely isolated neonatal brain slices.

For the incubation procedure, hippocampal acute slices were placed on semi-permeable filters with a modified and buffered ACSF including HEPES for pH control, but no growth factors (Fig. 5). For the validation of the preparation, baseline spontaneous electrical activity was recorded from pyramidal neurons in the area CA3 after incubation. The typical patterns of intrinsic network activity were preserved in the incubated slices and no significant effects on the baseline glutamatergic (mEPSC's or EPSCs) or GABAergic (mIPSCs or IPSCs) transmission compared to acute slices were found (online supplemetary material, publication I). Furthermore, incubation had no significant effects on passive membrane properties of the pyramidal neurons (input resistance, capacitance and holding current under voltage clamp, online supplementary material, publication II).

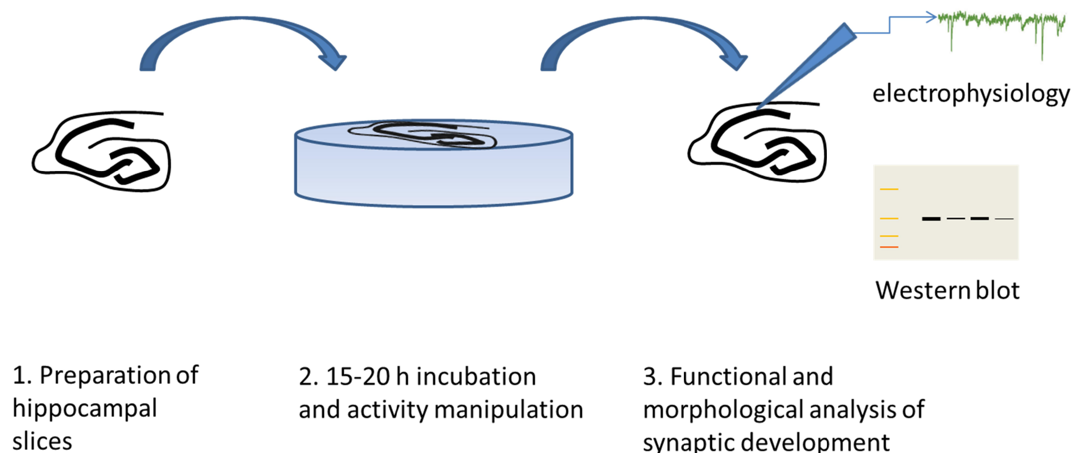


Figure 5. Scheme of the experimental design. In order to study the long-term effects of different electrical activity patterns on synaptic development, the acute hippocampal slices were prepared for 15-20 h incubation (35 ºC, 5% CO2 in air). In these experiments the slices were washed with 1 ml incubation solution containing (in mM): 105 NaCl, 3 KCl, 1 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, 2 CaCl2, 15 D-glucose and 25 HEPES, and placed into Millicell CM 0.4 µm membrane inserts (Millipore, Bedford) with 1 ml of the above solution. The drugs used were added to incubation solution. After incubation the slices were used for electrophysiological recordings or as samples for western blot.

5.2 ROLE OF SYNCHRONOUS SPONTANEOUS ACTIVITY IN THE DEVELOPMENT OF GLUTAMATERGIC SYNAPSES AT CA1 AND CA3 (I)

5.2.1 PHARMACOLOGICAL TOOLS FOR DESYNCHRONIZATION OF THE NETWORK

To selectively block the synchronous hippocampal activity without affecting the unitary neuronal firing, the gap-junction blocker carbenoxolone (CBX, 50 µM; Placantonakis et al., 2006; Bissiere et al., 2011) was used. CBX has been reported to have unspecific effects, for example to reduce activation of VDCCs at retinal photoreceptors (Vessey et al., 2004), increase of the AP threshold in cultured neurons (Rouach et al., 2003) and block NMDAR's in area CA1 (Chepkova et al., 2008). Even though we could not detect any significant effect of acute 50 µM CBX application on the mEPSC's or evoked EPSCs in the neonate CA3 area, all key experiments were repeated with 50 nM TTX. Nanomolar TTX concentration blocks the persistent sodium current and thus uses a different mechanism for network desynchronisation (Stasheff et al., 1993; Gasparini et al., 2000). Both treatments blocked the intrinsic activity bursts without significantly affecting unitary synaptic activity (Fig. 1 and 2 in publication I).

For long-term desynchronization of the network, hippocampal slices were treated for 15-20 h with CBX or 50 nM TTX. In parallel, a 1µM TTX

concentration, which prevents all AP-dependent activity, was used for total activity block (e.g., Lauri et al., 2003). Propidium iodide staining for the identification of dead cells was performed to evaluate neuronal viability of slices after the CBX incubation procedure and no toxic effect was found (online supplementary material in publication I). Furthermore, the block of synchronous activity was persistent and no bursting activity was seen after 15-20 h incubation with CBX.These results suggest that CBX is a suitable tool for long-term network desynchronization.

5.2.2 15-20 H NETWORK DESYNCHRONIZATION LEADS TO DOWNREGULATION OF TRANSMISSION AT CA1 GLUTAMATERGIC SYNAPSES BUT HAS NO EFFECT IN THE AREA CA3

The functional consequences of the chronic (15-20 h) manipulation of the network activity were studied electrophysiologically. The changes in synaptic transmission were estimated by recording spontaneous miniature currents (mPSCs) that generally correspond to the synaptic response to a release of a single transmitter vesicle and are widely used to predict the changes in synaptic strength and number (Castillo and Katz 1952; Fatt and Katz 1953, see section 2.1.1).

Network desynchronization and inhibition of all action-potential dependent activity had different effects on glutamatergic transmission in the areas CA1 and CA3 (Fig. 6, see also Fig. 3 in publication I). Desynchronization lead to weakening of glutamatergic transmission in the area CA1, observed as a decrease in both the amplitude and frequency of mEPSC's, whereas total activity block had no net effect on mEPSC's. In contrast, the desynchronization had no net effect on mEPSC's in CA3 while a total activity block caused homeostatic upregulation of glutamatergic transmission, manifest as an increase in mEPSC frequency and amplitude (see also Lauri et al., 2003). The properties of AMPAR-mediated transmission were further studied by recording EPSCs evoked by stimulation of the SC pathway. The maximum EPSC amplitude and the mean slope of the input-output curve were decreased after the network desynchronization in the CA1 area, which further confirmed the downregulation of glutamatergic transmission as a response to chronic network desynchronization (Fig.4 in publication I). These results together suggest a key role of synchronous activity in establishing the nascent CA3-CA1 circuitry.

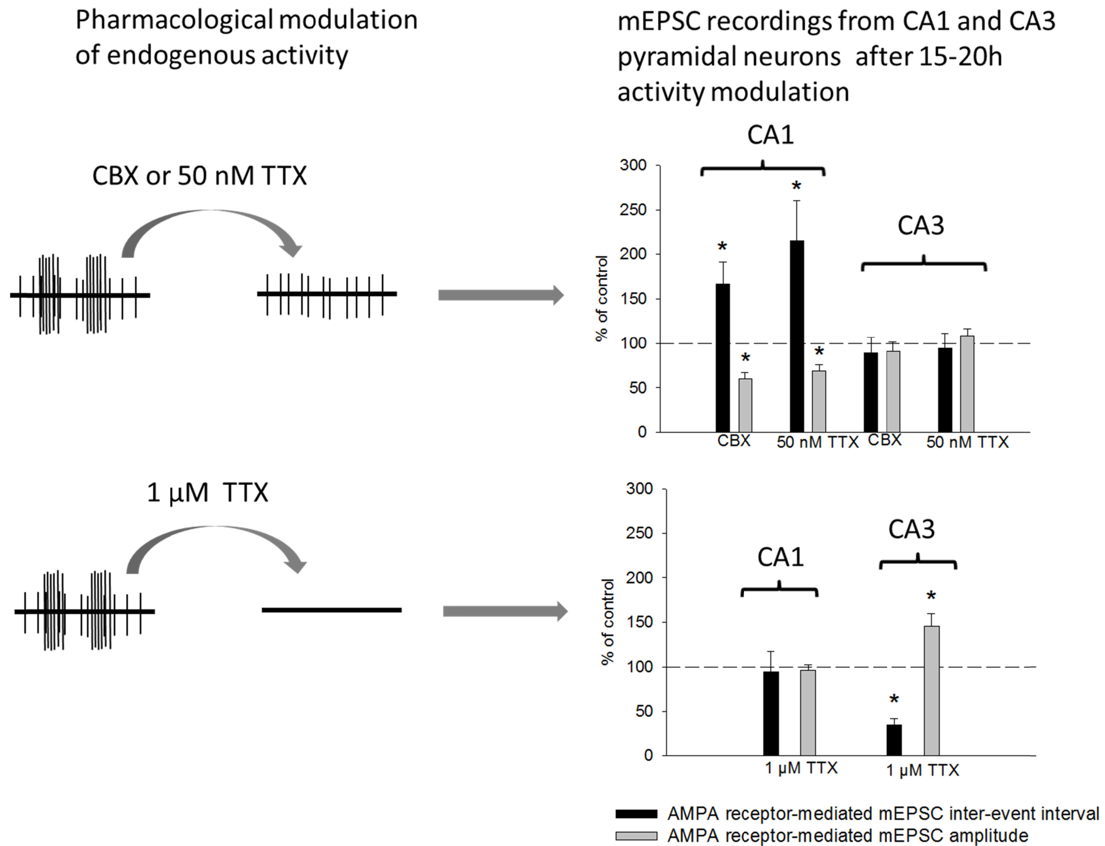


Figure 6. Long-term CA3-CA1 network desynchronization and total activity blockade have different effects on glutamatergic transmission in areas CA1 and CA3. A. Network desynchronization (50 µM CBX or 50 nM TTX) leads to downregulation of the amplitude and frequency of AMPAR-mediated mEPSC in CA1 pyramidal neurons and has no net effect on glutamatergic transmission in CA3. B. Inhibition of all action potential-dependent activity (1 µM TTX) has no net effect to AMPAR mEPSC's in CA1 but leads to upregulation of mEPSC frequency and amplitude in CA3 pyramidal neurons.

5.2.3 NETWORK DESYNCHRONIZATION INCREASES THE NUMBER OF SILENT SYNAPSES IN THE AREA CA1

We next studied the mechanisms behind the downregulation of glutamatergic transmission in more detail by assessing the number of silent synapses after CBX treatment. The amplitude ratio of evoked AMPA- and NMDA-mediated EPSCs (AMPA/NMDA ratio) was calculated and used as an index of the number of silent synapses (Isaac et al., 1995; Durand et al., 1996). Indeed, AMPA/NMDA ratio was found to be lower after network desynchronization as compared to control conditions (Fig.6 in publication I). These data suggest that the long-term absence of synchronous activity increases the number of silent synapses in the CA1 area.

5.2.4 THE GLUTAMATE RELEASE PROBABILITY AT CA3-CA1 SYNAPSES IS NOT CHANGED AFTER NETWORK DESYNCHRONIZATION

Downregulation of glutamatergic transmission and increase in the number of silent synapses could be due to alteration in presynaptic release (Pr) or postsynapstic regulation of AMPAR's. Thus, we determined the possible presynaptic mechanisms. Network desynchronization did not change the paired-pulse ratio of evoked EPSCs in area CA1 (Fig.4 in publication I). Immature glutamatergic connections in the hippocampus demonstrate facilitatory responses after high-frequency stimulation, and the rate of facilitation is determined by a developmentally regulated presynaptic mechanism (Lauri et al., 2006). Thus, the effect of the inhibition of synchronous activity to the described mechanism was determined and it was found that the ratio of the facilitatory responses in high frequency trains (50 Hz) was not affected by the long-term treatment with CBX (Fig.4 in publication I). Change in Pr should also affect the NMDA-transmission. However, there were no changes in NMDA-mediated mEPSC's after network desynchronization (Fig.5 in publication I) These results together suggest that network desynchronization did not change the release probability at CA3-CA1 synapses.

5.2.5 DESYNCHRONIZATION-INDUCED DOWNREGULATION OF GLUTAMATERGIC TRANSMISSION AT CA1 SYNAPSES REQUIRES PROTEIN PHOSPHATASES AND MGLUR ACTIVITY

The above data suggest that the downregulation of glutamatergic transmission in response to desynchronization of network activity is predominantly mediated by regulation of functional AMPAR's at the postsynaptic membrane. Next we determined the possible induction mechanisms for the synaptic depression. NMDAR activation can induce LTD in neonatal hippocampal slices (Dudek and Bear 1992; Oliet et al., 1997). However, the blockade of NMDAR's with antagonists (2R)-amino-5-phosphonopentanoate (AP- V, 50µM) or (+)-5-methyl-10,11-dihydroxy-5H-dibenzo(a,d)cyclohepten-5,10-imine (MK801, 40 µM) together with depression of synchronous activity with CBX or 50 nM TTX did not affect the depression of mEPSC's, suggesting that the downregulation was independent on NMDAR activity (Fig.7 in publication I). Further, antagonist treatment alone had no effect to mEPSC's. Next we tested if the depression involved the activity of protein phosphatases, implicated in the activity-dependent depression of glutamatergic transmission (Mulkey et al., 1993; Carroll et al., 1999; Ehlers et al., 2000; Chung et al., 2000; Kim et al., 2001; Chung et al., 2003; Ashby et al., 2004; Lee et al., 2004). To this end, we used fostriecin that inhibits the action of protein phosphatase types 2A (PP2A) and 4 (PP4) and sodium orthovanadate, an inhibitor of protein tyrosine phosphatases. Long-term treatment with fostriecin (140 nM) or orthovanadate (300µM)

together with CBX prevented the depression of synaptic strength in the CA1 area (Fig.7 in publication I).

As the depression of glutamatergic transmission was independent of NMDA- receptors and needed phosphatase activity we next tested the role of group I-II mGluR's, previously implicated in LTD induction in the neonate CA1 (Bashir et al., 1993; Oliet et al., 1997; Palmer et al., 1997). The depression of glutamatergic transmission was prevented with an mGluR antagonist (groups I and II) (S)--methyl-4- carboxyphenylglycine (MCGP) and (2S)-2-Amino-2-(1S, 2S)-2-carboxycy¬cloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY 341495) reasserting the role of mGluR activity in the depression of glutamatergic transmission after network desynchronization (Fig. 7 in publication I). Together these results suggest that network desynchronization down-regulates CA1 glutamatergic synapses via mechanisms involving protein phosphatases and mGluR activity.

5.2.6 CHRONIC DESYNCHRONIZATION IS ASSOCIATED WITH A CHANGE IN THE EXPRESSION OF AMPAR'S

In order to have a closer look at the possible changes in AMPAR subunit expression and presynaptic marker proteins induced by network desynchronization, we quantified the protein levels of synaptophysin, VGLUT1, GluA2 and GluA4 in the area CA1 isolated from incubated slices. The amount of synaptophysin (marker for synaptic density) or VGLUT1 was not altered after network desynchronization with CBX or 50 nM TTX (Fig. 8 in publication I). However, the amount of GluA4 was significantly decreased in both CBX and 50 nM TTX treated slices, while the amount of GluA2 subunit increased after CBX treatment. These data suggest that the network desynchronization directly leads to changes in AMPAR subunit expression.

5.3 THE DEVELOPMENTAL TIME FRAME OF THE INDUCTION OF HOMEOSTATIC PLASTICITY IN THE CA3 GLUTAMATERGIC AND GABAERGIC SYNAPSES (II)

5.3.1 THE THRESHOLD FOR HOMEOSTATIC PLASTICITY IN GLUTAMATERGIC AND GABAERGIC SYNAPSES IS INCREASED IN PARALLEL WITH THE MATURATION OF SYNAPTIC NETWORK

In the neonate hippocampus, 15-20 h total activities block lead to homeostatic upregulation of glutamatergic synaptic transmission in CA3 pyramidal cells (see also Lauri et al., 2003). In neuronal cultures, the upscaling of mEPSC's typically requires longer (40 h) TTX treatment (Murthy et al., 2001; Burrone et al., 2002; Nakayama et al., 2005; Hartman

et al., 2006; Stellwagen and Malenka 2006), which lead us to investigate in more detail the induction requirements of homeostatic scaling at different stages of development. Thus, in our experiments the scaling of mEPSC's after 15-20 h TTX treatment was seen at P4 but not at P8 (Fig.1 in publication II). We also determined the minimum time needed for mEPSC scaling at P4 and found that frequency was increased already after 7-10 h whereas upregulation of AMPAR mediated mEPSC amplitude needed longer 15-20 h activity deprivation.

As the fast (after 15-20 h activity deprivation) homeostatic upregulation was not present in the glutamatergic synapses after the first postnatal week (at P8) we next tested if the upregulation of glutamatergic transmission uses different mechanisms in more mature synapses that need longer time periods to be activated. However, in our slice preparation the viability of intact slices can not be maintained that long. Therefore, we studied the scaling in hippocampal organotypic cultures. We found that during the time of intense formation of glutamatergic connections (7-8 days *in vitro*, DIV) the time used (15- 20 h or 40 h) for activity blockade made no difference to the outcome and the upregulation of mEPSC frequency and amplitude was observed after both time points. However, in more mature conditions, after most synaptic connections have been established (14-6 DIV), the brief TTX treatment had no effect on mEPSC's while a 40 h inactivity caused scaling of both amplitude and frequency (Fig.3 in publication II).

For the GABAergic circuitry, the threshold for homeostatic upregulation changed earlier than the glutamatergic one. We managed to see the upregulation of miniature inhibitory postsynaptic current (mIPSC) frequency after 15-20 h or 40 h activity deprivation only at most immature neurons with capacitances below 30 pF (Fig.2 and 4 in publication II). Regulation of mIPSC amplitude needed longer 40 h activity deprivation and was independent of the neuron's capacitance and maturational stage, suggesting a different underlying mechanism. In conclusion, the threshold for homeostatic scaling at CA3 area increased in parallel with the maturation of synaptic circuitry and the induction of homeostatic plasticity at more mature synapses needed longer periods of activity deprivation.

5.4 ROLE OF GLUA4 AMPAR SUBUNIT IN THE SYNAPTIC PLASTICITY DURING EARLY DEVELOPMENT (I, III AND UNPUBLISHED)

5.4.1 WEAKENING OF GLUTAMATERGIC TRANSMISSION IN AREA CA1 AFTER NETWORK DESYNCHRONIZATION IS DEPENDENT ON GLUA4

Having established that downregulation of glutamatergic transmission in response to desynchronization of network activity is predominantly mediated

by regulation of synaptic AMPAR's (sections 5.2.3 and 5.2.6), we went on to study the mechanisms in more detail. The GluA4 subunit of AMPAR's is transiently expressed in CA1 pyramidal neurons during the time of synaptic refinement, suggesting a role in the immature–type mechanisms of transmission and plasticity (Zhu et al., 2000). Indeed, quantification of AMPAR subunit levels from CA1 showed that the expression of GluA4 subunit was significantly decreased after long-term network desynchronization with CBX or 50 nM TTX (section 5.2.6, III; Fig.8 in publication I).

To further understand the role of GluA4 in the activity dependent maturation of glutamatergic synapses, we used genetically modified mice lacking GluA4 (Fuchs et al., 2007). Basic characterization of GluA4-/-mice strain showed no differences in the frequency of spontaneous synchronous network burst or in baseline mEPSC's as compared to WT mice (Fig. 7; recorded from CA3 pyramidal cells with the same methods than in publication I), providing an ideal starting point for the study. However, the decay time constant of mEPSC's was significantly smaller in GluA4 mice (decay time constant 90-37%: 6,78 ± 0,74 ms WT, 4,5 ± 0,3 ms GluA4-/-, $p = 0,01$) suggesting subunit-specific changes in mEPSC kinetics.

The network desynchronization with CBX (15-20h) led to the typical downregulation of glutamatergic transmission in the slices from WT mice, observed as a decrease in mEPSC frequency and amplitude in CA1 pyramidal neurons. In contrast, there were no changes in mEPSC's in the GluA4-/- slices after network desynchronization with CBX (Fig. 8). This suggests an essential role of GluA4 in the activity-dependent maturation of glutamatergic synapses (the role of network desynchronization on mEPSC's at CA1 pyramidal cells was studied as previously in publication I, Fig. 7).

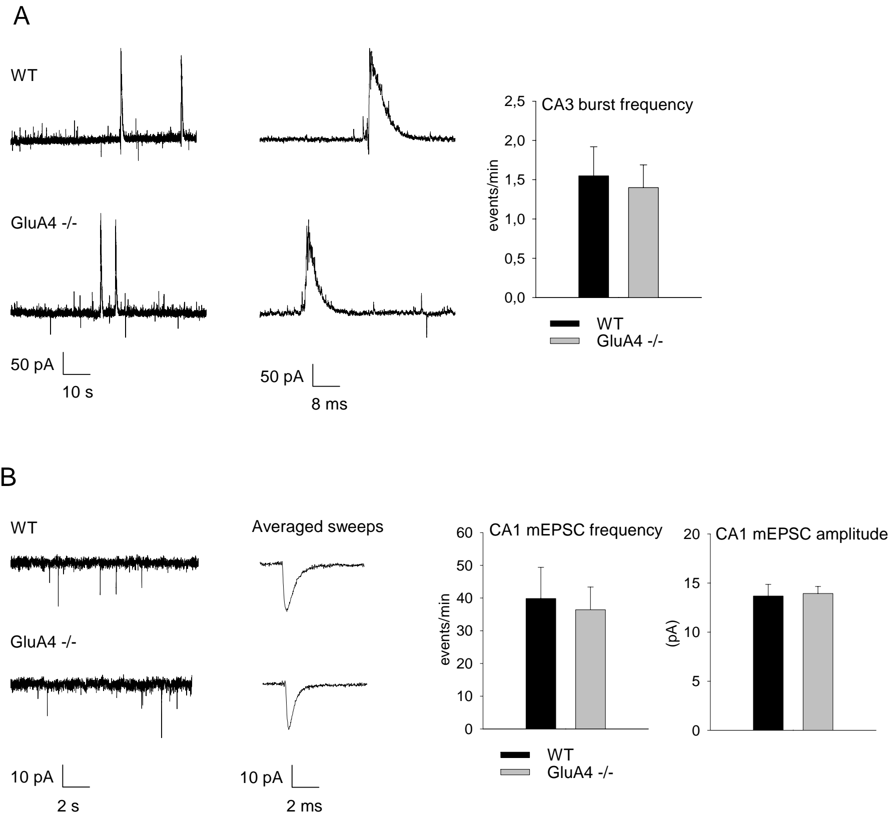


Figure 7. Spontaneous endogenous network bursts or baseline mEPSC activity is not altered in GluA4 -/- mice. A. Sample recordings (left) of spontaneous activity in CA3 pyramidal neurons from WT and GluA4-/-mice (P4-5) and averaged CA3 burst frequency (right) from WT (1,54 ± 0,37 events/min, n = 6) and GluA4-/- (1,40 ± 0,29 events/min, n = 10) mice. B. Sample recordings and averaged sweeps (left) of AMPAR-mediated mEPSC's in CA1 pyramidal cells from WT and GluA4-/-mice (P4-5) and averaged mEPSC frequency and amplitude (right) from WT (36,4 ± 6,9 events/min, 13,68 ± 1,18 pA, n = 11) and GluA4 -/- mice (34,9 ± 9,5 events/min, 13,68 ± 1,18 pA, n = 12).

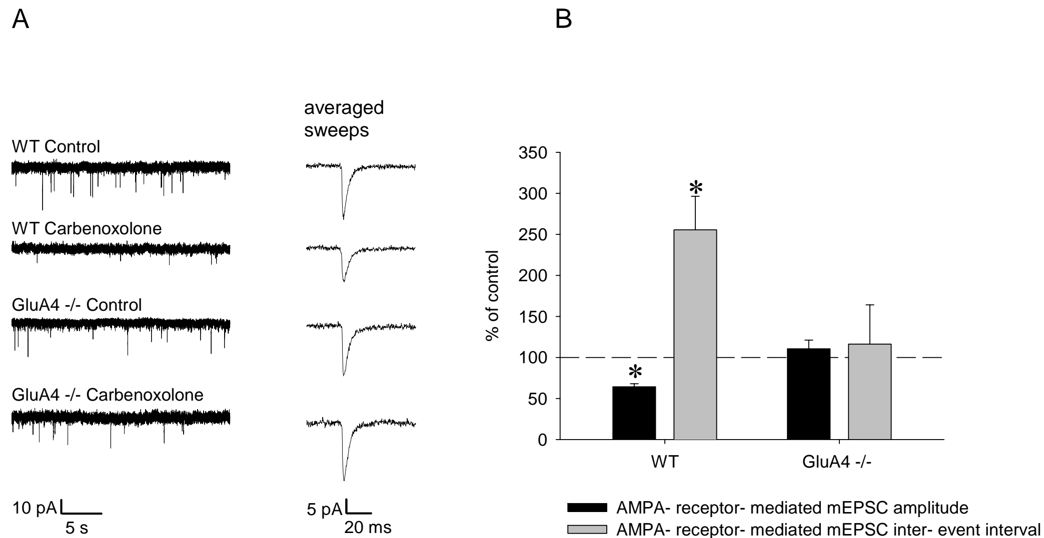


Figure 8. Network desynchronization does not induce any changes in the mEPSC frequency or amplitude in GluA4-/-mice. Sample recordings (A) and pooled data (B) of mEPSC's from CA1 pyramidal cells (at least 8 min of recording in each cell) from WT (n= 5-6) and GluA4-/- (n= 8) mice after 15-20 h treatment with 50 µM carbenoxolone. The traces in expanded time scale depict average of 8 events from each recording. *P < 0.05.

5.4.2 GLUA4 SUBUNIT IS INVOLVED IN THE EXPRESSION OF FAST HOMEOSTATIC PLASTICITY CHANGES IN RESPONSE TO TOTAL ACTIVITY BLOCK IN BOTH AREAS CA1 AND CA3 OF THE NEONATE HIPPOCAMPUS

After finding the critical role of GluA4 in downregulation of AMPA-mediated responses after network desynchronization, we studied how developing networks respond to a total activity block in the absence of GluA4. For that purpose, all action potential-dependent transmission was blocked by incubating acute slices from WT or GluA4-/-mice for 15-20 h with 1µM TTX. The efficacy of glutamatergic transmission was estimated by recording mEPSC's from areas CA1 and CA3 (mEPSC's after 15-20 h activity treatment were recorded with same methods as in publications I and II).

In area CA1, the total activity deprivation had no effect on mEPSC's in WT slices, similar to that previously observed in rat slices (Fig.3 in publication I). However, in the absence of GluA4 in CA1, the same treatment led to a decrease in the mEPSC frequency whereas amplitude remained at the same level as in control conditions. Interestingly, in the area CA3, the homeostatic upregulation of AMPAR-mediated mEPSC amplitude and frequency in response to activity deprivation was totally prevented in GluA4-/-slices, while the same treatment resulted in a significant increase in transmission in the WT, as expected (Fig. 9).

These data suggest that GluA4 is critical for the quick homeostatic (15-20 h) upregulation of glutamatergic transmission that is observed during a short

period of neuronal development, corresponding to the time of GluA4 expression at pyramidal cells.

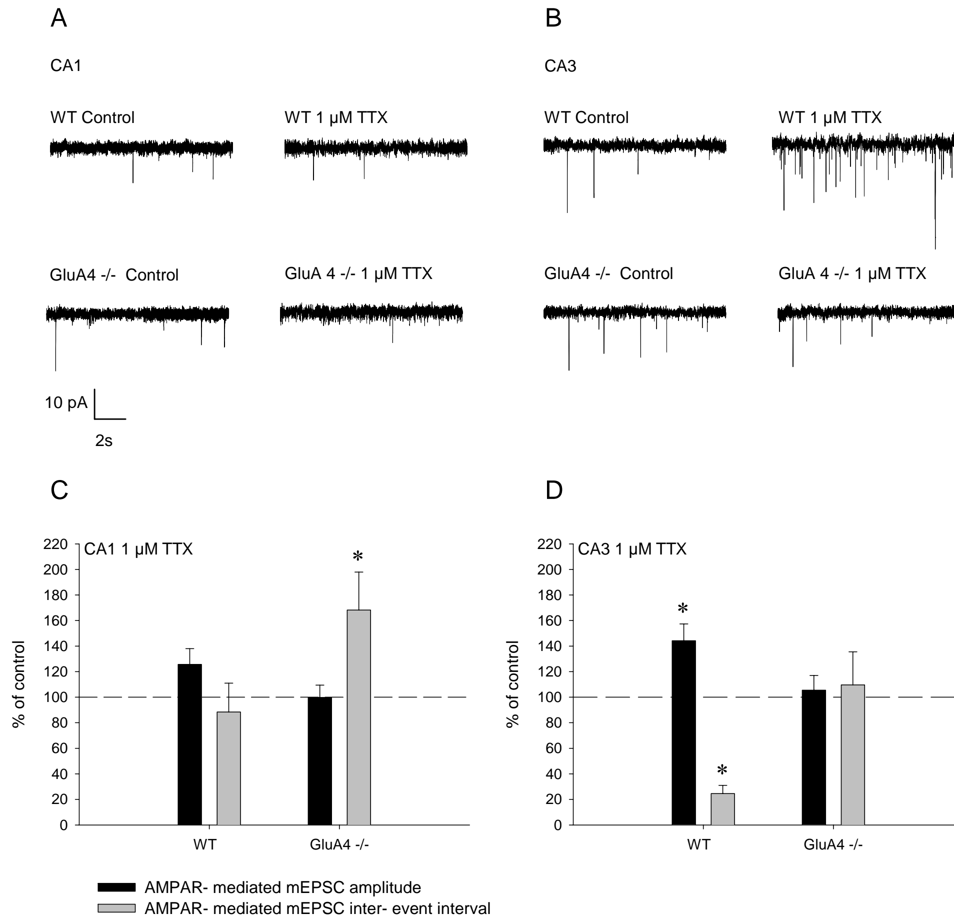


Figure 9. Total activity deprivation in slices from GluA4-/-mice leads to downregulation of mEPSC frequency at CA1 pyramidal cells and has no effect of mEPSC's at CA3 pyramidal cells (A and B). The example traces of mEPSC's after long-term treatment with 1 µM TTX from CA1 (A) and CA3 (B) pyramidal cells from WT and GluA4-/-mice. (C and D) Pooled data illustrating mean AMPAR- mediated mEPSC amplitude and interval from CA1 (n= 5-11) and CA3 (n=4-6) pyramidal cells after long-term activity deprivation with 1 µM TTX. *P < 0.05.

5.4.3 NEONATAL LTP MECHANISMS AND ROLE OF GLUA4

After establishing the critical role of GluA4 in synchronous activity-guided synapse maturation, we explored the mechanisms of synaptic targeting of GluA4 in more detail. The synaptic insertion of GluA4 as well as LTP induction during early development is independent of CaMKII activity and requires the activity of PKA (Zhu et al., 2000; Yasuda et al., 2003). The shift in the LTP induction mechanisms from PKA- to CaMKII-dependent parallels the loss of GluA4 expression at CA1 pyramidal cells. However, the direct link between these phenomena has not been shown before.

Forskolin raises the level of cAMP and activate PKA (Seamond and Daly 1981), and can thus be used as a pharmacological tool to mimic LTP induction in the neonate (Yasuda et al., 2003). Consistently, in the immature CA3-CA1 synapses, forskolin induced a robust potentiation of synaptic transmission that was dependent on activation of postsynaptic PKA, since this potentiation was prevented by selective PKA inhibitor (0,1 mM fragment (6-22) amide (PKI)) in the postsynaptic cell (Fig.1 in publication III). To study if the GluA4 subunit was responsible for PKA-induced potentiation of AMPA- responses, postsynaptic neurons were filled with fusion peptide containing the c-terminal domain (CTD) of GluA1 (ct-GluA1) or GluA4 (ct-GluA4). Peptides were injected to cells via a patch electrode and are expected to scavenge proteins interacting with endogenous CTDs and perturb processes dependent on these interactions. Recombinant peptides did not affect the baseline EPSC amplitude. However, forskolin-induced potentiation of transmission was blocked with ct-GluA4 but not with ct-GluA1, suggesting that the PKA-dependent synaptic potentiation depends selectively on proteins interacting with CTD of GluA4 (Fig.1 in publication III).

To further study the role of GluA4 in forskolin-induced increase in glutamatergic transmission we made experiments with GluA4-/-mice. In the absence of GluA4, forskolin-induced increase in mEPSC amplitude and frequency in the immature synapses (at P4-P5) were strongly reduced as compared to WT (Fig.2 in publication III). We were also able to show that overexpression of GluA4 at P13-18 using lentiviral vectors restored the forskolin-induced increase in mEPSC amplitude and frequency that is not seen in control conditions at this developmental stage when the endogenous GluA4 expression is not present at hippocampal pyramidal cells. Neurons overexpressing of GluA1 or enhanced green fluorescent protein (EGFP) were indistinguishable from uninfected control neurons (Fig.2 in publication III).

Finally, we confirmed that neonatal PKA-dependent LTP in the CA1 area is dependent on GluA4 subunit and that PKA dependency could be restored at mature synapses with viral GluA4 overexpression (Fig. 3 and 4 in publication III). These data together suggest that GluA4 is responsible for the PKA dependency of LTP at the immature synapses.

6 DISCUSSION

6.1 ROLE OF EARLY SPONTANEOUS ACTIVITY IN THE DEVELOPMENT OF SYNAPTIC NETWORKS (I)

6.1.1 IMMATURE CA3-CA1 SYNAPSES THAT ARE NOT INVOLVED IN THE CORRELATED ACTIVITY ARE SILENCED DURING EARLY DEVELOPMENT

The mechanisms of Hebbian and homeostatic plasticity have been widely studied (Zhang and Poo 2001; Turrigiano and Nelson 2004). However, not much is known about how they actually contribute to the activity-driven establishment of synaptic circuitries. The temporal patterns and in particular, the degree of synchrony of the neuronal activity in the network are important determinants of the use-dependent changes in synapses (Katz 1993). Coherent bursts of activity supposedly remodel synapses with mechanisms similar to LTP and LTD, whereas changes in overall activity level in the network are balanced with homeostatic plasticity mechanisms. Transiently expressed endogenous electrical activity is a characteristic feature of the developing neuronal networks during the first weeks of life, and it temporally parallels the developmental stage when the establishment of neuronal networks is most intensive (Ben- Ari et al., 2001). It has been previously shown that blockade of the endogenous spontaneous activity regulates the strength and number of glutamatergic and GABAergic synapses in the developing spinal cord (Gonzalez Islas et al., 2006) and the hippocampus (Lauri et al., 2003; Colin Le Brun et al., 2004) and alters the neuronal refinemet of visual retinal projections (Torborg et al., 2005 and Hanson et al., 2008). However, despite the frequent speculations (Zhang and Poo 2001) none of the previous studies have directly addressed the question on the relative roles of synchronous vs. asynchronous activity in the refinenment of the CA3-CA1 circuitry during development (Yet see Kasyanov et al., 2004).

Here, in order to study the effects of distinct patterns of endogenous activity on synaptic maturation, acute hippocampal preparations were used. It has been previously shown that neonatal hippocampus *in vitro* diplays spontaneous activity patterns similar to those seen *in vivo* (Palva et al. 2000; Lahtinen et al. 2002). This provided us with a model with a clear advantage over the neuronal cultures or organotypic slices devoid of natural-type network activity. We find the use of standard pharmacological tools a bit problematic, since they tend to change the overall neuronal activity in a quantitative manner, e.g. they either increase or decrease both the unitary firing and the summed-up network activation. Gap- junctional contacts between nascent hippocampal neurons critically contribute to neuronal

synchronization and thus, gap-junctional decoupling effectively attenuates synchronous firing of the neuronal networks (Fukuda and Kosaka, 2000; Schmitz et al., 2001; Traub et al., 2002; Hanson et al., 2003). Thus, here we used the gap-junction blocker CBX for network desynchronization in the developing hippocampus. By virtue of having its main effects on the electrical coupling between the neurons, CBX quite selectively blocked the synchronous network bursts but left most of the unitary neuronal firing intact. However, although unlikely, we cannot exclude the possibility that the slight decrease in the total excitatory drive after network desynchronization contribute to some of the seen effects.

Desynchronization of the ongoing network activity lead to a depression of AMPAR- mediated mEPSC's and to decreased AMPA/NMDA ratio in CA1 pyramidal neurons without affecting glutamate release probability. This strongly suggests that the synaptic depression was due to the loss of postsynaptic AMPAR's. These results support the idea of Hebbian type synapse induction via synchronous activity in the developing hippocampus and corroborate the previous findings of AMPAR silencing caused by uncorrelated low frequency activity (Xiao et al., 2004; Abrahamsson et al., 2008; Hanse et al., 2009).

Strikingly, our results showed, for the first time, that the network desynchronization led to a different outcome in the CA1 versus CA3 area. In the CA1 area desynchronization led to a weakening of glutamatergic transmission as indicated by the decrease in the frequency and amplitude of mEPSC's in pyramidal neurons, whereas in the CA3 area no change in the mEPSC's was seen. What physiological mechanism could then underlie this rather intriguing finding? Here, it should be first considered that due to the strong recurrent excitation typical for the CA3 the intrinsic activity in this area is much more intense than in the CA1 (see section 2.1.1). The CA3 area has also inputs from MF synapses that have four times more AMPARs than other hippocampal synapses (Nusser., et al 1998). In contrast, the CA1 pyramidal neurons receive most of their excitatory inputs via afferents from CA3 (see Fig. 1). Thus, it is plausible that the mere desynchronization still retains a sufficient level of activity in the area CA3 to sustain synaptic induction (see also 6.2.1). In contrast, in the area CA1 where the activity almost solely consists of excitatory barrages invading from CA3, the desynchronization results in a dramatic loss of coherent excitatory input and therefore, in attenuation of Hebbian-type synaptic induction. Moreover, the CA3 develops before the CA1 (Bayer 1980). So the CA1 cells are likely to be more immature and thus more vulnerable to changes in activity than the cells in the CA3. The effect of total activity block (1 μM TTX) is discussed more in detail in the section 6.2.1.

6.1.2 THE SYNAPTIC MECHANISMS RESPONSIBLE FOR THE DOWNREGULATION OF GLUTAMATERGIC TRANSMISSION IN THE CA1 AREA IN RESPONSE TO THE NETWORK DESYNCHRONIZATION

Induction of synaptic plasticity typically requires the activation of Ca^{2+}-dependent signalling cascades. In general, intracellular Ca^{2+} -levels in neurons can be modified by changing the Ca^{2+} membrane permeability (e.g. via NMDAR or VDCC) or via releasing Ca^{2+} from intracellular stores (e.g. mGluR's). Here, the desynchronization-induced downregulation of excitatory transmission at the CA3-CA1 synapses was found to be independent of NMDAR's but dependent on mGluR activity. In agreement, activation of the group I -II mGluR's has previously been shown to mediate induction of LTD in several synapses, including CA3- CA1 synapses (Bashir et al., 1993; Palmer et al., 1997; Oliet et al., 1997; reviewed by Collingridge et al., 2010). Further, our results suggest that the downregulation of transmission in response to network desynchronization is due to the loss of AMPAR function, which could be due to the receptor internalization or prevention of the insertion of new receptors to the cell membrane. For instance, the mGluR-dependent LTD is shown to regulate the expression of Arc which in turn regulates the AMPAR endocytosis (Shepherd et al., 2006; Chowdhury et al., 2006; Rial Verde et al., 2006; Park et al., 2008). One plausible link between mGluR-dependent downregulation of glutamatergic transmission can also be the mGluR-activated decrease in the level of cAMP (Baba et al., 1993; Takahaski et al., 1996) that decreases the PKA activity and PKA-dependent GluA4 delivery to synapses (Yasuda et al., 2003; see section 6.3 and Fig. 10).

The observed decrease in the level of GluA4 subunit after network desynchronization together with the findings from GluA4 -/- mice (discussed more in detail in section 6.3) strongly suggest the critical role for the GluA4 subunit in the downregulation of glutamatergic transmission. The attenuated GluA4 expression might be a result of internalization and degradation of the GluA4 subunit containing AMPAR's, but also involve slower transcriptional regulation of GluA4 expression. Previously, a total activity block with 2 µM TTX together with local glutamate receptor antagonism has been shown to increase the amount of GluA1 containing AMPAR's whereas blocking the action potentials alone (TTX) leads to accumulation of GluA2 (Sutton et al., 2006). In our experiments, network desynchronization increased the expression of the GluA2 subunit in the area CA1, but it was seen only after treatment with CBX and not with low concentrations of TTX (50nM). One possibility is that after gap- junction blockade with CBX the activity level is diminished more than with low concentration of TTX (50nM) and hence there is already some GluA2 –dependent homeostatic upregulation activated in CBX treated slices (O´Brien et al., 1998; Malenka and Bear 2004; Gayney et al., 2009). However, this upregulation apparently had a lower impact to

synaptic strength compared to the weakening of transmission via a GluA4-dependent mechanism.

In addition, we found that the downregulation of glutamatergic transmission after the network desynchronization involves activity of protein phosphatases, which have previously been strongly implicated in the mechanisms of LTD and AMPAR internalization (Mulkey et al., 1993; Carroll et al., 1999; Ehlers et al., 2000; Chung et al., 2000; Kim et al., 2001; Chung et al., 2003; Ashby et al., 2004; Lee et al., 2004). Further, phosphorylation of GluA1 ser831 has been shown to regulate AMPAR conductance (Derkach et al., 1999; Kristensen et al., 2011) and thus dephosphorylation may directly lead to decrease in AMPAR conductance. However, the effects of these inhibitors may be quite wide-ranging since they block many phosphatases that have various targets and can regulate several signal cascades and protein interactions inside the cell.

6.2 DEVELOPMENTAL MECHANISMS AND REGULATION OF HOMEOSTATIC PLASTICITY: THE INFLUENCE OF HIPPOCAMPAL AREA, NEURONAL TYPE, AND DEVELOPMENTAL STAGE, (I- III, UNPUBLISHED)

6.2.1 THE TOTAL ACTIVITY BLOCK HAS DIFFERENT EFFECTS ON GLUTAMATERGIC TRANSMISSION IN THE AREAS CA3 AND CA1

Although many studies have established that the networks keep neuronal activity levels constant via homeostatic regulation (Turrigiano et al., 1998; Burrone et al., 2002; Kim and Tsien 2008), less is known about the corresponding mechanisms in the developing networks that display the patterned intrinsic activity. Previously, Lauri et al. (2003) showed that total activity block in the immature tissue causes homeostatic upregulation of transmission, manifested as an increase in the mEPSC amplitude and frequency in CA3 pyramidal neurons. Similarly, we found here a prominent homeostatic upregulation of mEPSC's in the CA3 pyramidal neurons after total activity block by 1μM TTX. In contrast, the total activity block did not cause any net changes in mEPSC's in the CA1 pyramidal cells.

Total activity block causes the homeostatic upregulation of transmission, likely involving both pre- and postsynaptic mechanims (Turrigiano et al., 1998; Murthy et al., 2001; Burrone et al., 2002; Lauri et al., 2003; Ju et al., 2004). According to our results, the absence of ongoing synchronous activity leads to destabilization of postsynaptic AMPAR's and prevents synapse induction (see section 6.1). It is plausible that the total activity block also prevented the Hebbian-type synapse induction both in the CA3 and CA1 pyramidal neurons. However, it is likely that the homeostatic compensation in the area CA3 is more powerful than in the CA1 because of the more intense

intrinsic activity in the CA3 (see section 2.1.1). Thus, in the CA3 the activity loss would, via homeostatic induction, rapidly lead to abundance of functional glutamatergic synapses. In CA1, however, the synaptic scaling even in the total absence of activity would be more sluggish yet still able to restore but not overshoot the mEPSC's after a 15-20 h activity block. Further, the differences in CA1 and CA3 profile of maturation, morphology, excitability and activity (Bayer 1980; Traub and Miles 1989; Ishizuka et al., 1995; Pyapali et al., 1998, see section 2.1.1) most likely account for the distinct thresholds for homeostatic regulation in these areas.

Our results after total activity block with 1μM TTX from CA1 and CA3 areas are to some extent contradictory to findings reported by Kim and Tsien (2008). They showed that total activity block with 1μM TTX decreased mEPSC frequency at the CA3-CA3 synapses and had no effect on mEPSC's when both MF and collateral connections were intact. They also saw an increase in mEPSC's in CA1 pyramidal cells after the activity block. However, these studies were done by using organotypic slices (21-25 days *in vitro* DIV) where the network activity and maturational stages of the neurons are different than in our neonatal acute hippocampal slices. The activity deprivation time was also much longer (3-4 days) compared to our 15-20 h and may activate completely different plasticity mechanisms (see also sections 6.1.2 and 6.2.2; Beatie et al., 2002; Stellwagen and Malenka 2006; Steinmetz and Turrigiano 2010). It might be that in our conditions the CA1 area could also scale up mEPSC's if longer (e.g., over 40 h) activity deprivation would be used.

6.2.2 DEVELOPMENTAL STAGE AND SUSCEPTIBILITY TO HOMEOSTATIC PLASTICITY

Today, it is well established that the mechanisms of synaptic scaling are dependent on the developmental stage of the neurons and on the duration of used activity manipulation (Burrone et al., 2002; Hartman et al., 2006; Sutton et al., 2006; Wierenga et al., 2006; Echegoyen et al., 2007; Ibata et al., 2008; Hou et al., 2008). However, at the time this study was conducted, practically nothing was known on the developmental profile of homeostatic plasticity mechanisms in the brain. Our data indicate that synaptic changes induced by activity deprivation are developmentally down-regulated in a temporally and mechanistically distinct manner in glutamatergic vs. GABAergic synapses. At immature glutamatergic synapses in P4 CA3 pyramidal neurons the mEPSC frequency was already upregulated after a 7-10 h activity block, whereas increase in the mEPSC amplitude was seen only after a 15 h activity block. Further, even though a 15-20 h activity deprivation caused homeostatic upregulation of mEPSC's at P4, it was not sufficient to induce plasticity at P8. However, we assumed that homeostatic scaling could be induced using longer activity deprivation times (up to 40 h) also in mature networks, and we were able to verify this hypothesis by using organotypic

cultures. Together, these data support the idea that the threshold for induction of homeostatic plasticity at glutamatergic synapses is increased in parallel with the maturation of the circuitry.

According to our results from GluA4 -/- mice (discussed more in detail in section 6.3), the quick homeostatic upregulation in nascent synapses is GluA4-dependent. Thus, downregulation of the GluA4 expression in pyramidal cells during development could explain the loss of synaptic scaling induced with 15-20 h activity deprivation.

The loss of GluA4-dependent plasticity mechanisms may be replaced later in development e.g. with longer induction times requiring TNF-α-dependent homeostatic mechanisms (Beattie et al., 2002; Stellwagen and Malenka 2006; Steinmez and Turrigiano 2010). Also, the morphological differentiation of the postsynaptic dendritic structures, i.e. the dendritic shaft, filopodia and spines, occurs during the first postnatal weeks (Tyzio et al., 1999; Fiala et al., 1998) and may affect to the activated plasticity induction and expression mechanisms e.g., via the regulation of the amount of Ca^{2+} in different microenvironments.

Previously, activity deprivation has been shown to affect excitation-inhibition balance by decreasing the efficacy of GABAergic inhibition, which in turn accentuates the network excitability (Rutherford et al., 1998; GonzalezIslas and Wenner 2006; Hartman et al., 2006; Swanwick et al., 2006, see section 2.1.2). During early development, however, $GABA_AR$-mediated postsynaptic responses can be depolarizing and thus contribute directly to excitatory drive (Ben-Ari, 2001). Thus, it was of interest to characterize the effects of activity deprivation on the properties of glutamatergic and GABAergic synapses at different stages of development in parallel. GABAergic synapses develop before glutamatergic ones, and at P0 the majority of hippocampal pyramidal cells have no spontaneous currents and 20 % of cells have only GABAergic currents (Tyzio et al., 1999). In our studies the 15-20 h activity block at P0 led to downregulation of mIPSC frequency in CA3 pyramidal neurons only at the most immature neurons having a capacitance of less than 30 pF. Further, increasing the time of activity manipulation (15-20h vs. 37-42 h) was not able to restore this regulation in mature neurons. However, long activity deprivation caused downregulation of the mIPSC amplitude, independent of the neurons' developmental stage. Hence, our result suggests that GABAeric synapses respond to brief activity deprivation (15-20 h) only at the most immature stages, an effect which may be related to the proposed constitutive role in GABAergic synapse formation (Colin Le Brun et al., 2004), whereas longer activity deprivation causes homeostatic downregulation of mIPSC amplitude irrespective of maturational stage.

6.3 ROLE OF GLUA4 IN ACTIVITY-DEPENDENT PLASTICITY AT NASCENT SYNAPSES (I, III AND UNPUBLISHED)

6.3.1 THE DOWNREGULATION OF GLUTAMATERGIC TRANSMISSION AFTER NETWORK DESYNCHRONIZATION IS DEPENDENT ON GLUA4

The GluA4 subunit of AMPAR's is incorporated to synapses by spontaneous activity, which has been suggested to be an important step in the formation of initial glutamatergic contacts in CA3-CA1 synapses (Zhu et al., 2000). The transient expression of GluA4 in hippocampal pyramidal cells also supports this idea, since the developmental downregulation of GluA4 takes place at the same time when the intrinsic activity is lost, temporally coinciding with the end of the period of intense synaptogenesis (Monyer et al., 1991; Zhu et al., 2000; Esteban et al., 2003).

Electrophysiological characterization of GluA4-/- mice showed that absence of GluA4 had no significant effect on the frequency of synchronous network bursts or on the spontaneous glutamatergic transmission in the developing hippocampus. Interestingly, in contrast to the WT animals, desynchronization of the network activity had no effect on glutamatergic transmission in the area CA1 in the GluA4-/- mice. Furthermore, homeostatic scaling in CA3 pyramidal neurons in response to total activity block (1µM TTX) was perturbed. These data suggest that GluA4 subunit has a significant role in homeostatic regulation of glutamatergic transmission in the developing hippocampus.

As the GluA4 -/- mice had normal spontaneous burst activity and there were no changes in baseline mEPSC frequency or amplitude compared to WT mice, compensatory GluA4-independent mechanisms for synapse maturation exist and account for the synapse development in these mice. Accordingly, results from our group suggest that the expression of GluA1 subunit is strongly enhanced in the GluA4-/- mice as compared to controls (data not shown). It might be that in the absence of quick GluA4 dependent homeostatic upregulation, other mechanisms are uncovered. In fact we observed that in the area CA1 of the GluA4-/- mice, total activity block caused a decrease in mEPSC frequency, a phenomenon that is not observed in the WT animals

Furthermore, the regulation of GluA4 may serve as a quick homeostatic plasticity mechanism to facilitate activity-dependent plasticity at nascent synapses. In more mature networks this GluA4-dependent mechanism is replaced by other homeostatic mechanisms that require a longer time to be activated (see section 6.2.2). For instance, the glial release of TNF-α after activity block takes about 40 h and has been shown to regulate homeostatic plasticity in many experiments (Beattie et al., 2002; Stellwagen and Malenka

2006; Steinmez and Turrigiano 2010). However, it would be of interest to overexpress GluA4 with lentiviral constructs in more mature network and to see if the rapid upregulation of mEPSC's at CA3 would be recovered. These data would further clarify the role of GluA4 in homeostatic plasticity.

6.3.2 KEY ROLE OF GLUA4 IN PKA-DEPENDENT LTP MECHANISMS

CaMKII activation and regulation of GluA1 AMPAR trafficking are critical for induction and expression of LTP at the adult Schaffer Collateral-CA1 synapses (Malenka and Nicoll 1999; Zamanillo et al., 1999; Hayashi et al., 2000; Lisman et al., 2002; Malinow and Malenka 2002; Esteban et al., 2003; Lee et al., 2003; Boehm et al., 2006; Shepherd and Huganir 2007; Lin et al 2009). However, in neonates the LTP induction is CaMKII-independent and dependent on the activity of PKA (Yasuda et al., 2003; Wikström et al., 2003). The downstream mechanisms that are responsible for the developmental switch in the LTP kinase dependency have not been demonstrated before. The previous findings suggest that PKA activation is the key mechanism that leads to synaptic insertion of GluA4 while PKA activity alone is not sufficient for synaptic incorporation of GluA1 (Zhu et al., 2000; Esteban et al., 2003). These data together suggest correlation between the transient developmental GluA4 expression and PKA-dependent LTP mechanisms during early development.

Our data using intracellularly applied peptides corresponding to the CTD of GluA4 as well as the characterization of GluA4 -/- mice showed that the increase in synaptic transmission in response to postsynaptic PKA activation was dependent on GluA4. Further, the GluA4 expression was necessary and sufficient for PKA-dependent LTP. Thus, CA3-CA1 synapses in the neonatal GluA4-/- mice expressed LTP which, however, was not dependent on PKA. In addition, lentiviral GluA4 overexpression *in vivo*, at the developmental stage where there is no endogenous GluA4 expression in pyramidal cells, restored the immature-type PKA-dependent LTP.

The increase in AMPAR accumulation to synapses e.g., after LTP induction or homeostatic plasticity may be due to the increase of AMPAR exocytosis (Park et al., 2006; Kopec et al., 2006; Kennedy et al., 2012), lateral diffusion (Borgdorff et al., 2002; Ehlers et al., 2007; Makino et al., 2009) or increased capture in PSD, all dependent on the CTD interactions (see section 2.2.2). The GluA4 CTD has been reported to interact with protein 4.1, PKC and α-actinin-1 and IQGAP1 (Coleman et al., 2003; Correia et al., 2003 Nuriya et al., 2005). Protein 4.1 is known to anchor AMPAR's to the spectrum/actin cytoskeleton and regulate the GluA1 insertion to extrasynaptic sites (Song and Huganir 2002; Coleman et al., 2003, Schultz et al., 2004; Lin et al., 2009). Further, the blocking of CTD interaction with 4.1N has been shown to stabilize the AMPAR surface expression (Shen et al., 2000; Hayashi et al., 2005). However, GluA4 incorporation to synapses has

also been shown to be independent of 4.1 interaction (Gomes et al., 2007). Our unpublished data using a GluA4-CTD peptide, where the interaction sequence of 4.1 has been destroyed by specific point mutation, indicate that this peptide is equally potent in inhibiting forskolin-induced increase in EPSC amplitude at immature CA3-CA1 synapses as the WT peptide. This indicates that protein 4.1 interaction is not involved in PKA-dependent potentiation of glutamatergic transmission, mediated by mobilization of GluA4 (data not shown). Rather, the use of truncated GluA4 CTD peptides in similar experiments point to a role of α-actinin-1/ IQGAP interaction (data not shown). Previously, these proteins have been suggeseted to act as an intracellular anchors to trap GluA4 in the intracellular cytoskeleton and release it in a PKA-dependent manner (Correia et al., 2003; Nuriya et al., 2005) providing a putative molecular mechanism for the activity-dependent insertion of GluA4 to immature synapses.

6.4 PHYSIOLOGICAL SIGNIFICANCE OF THE GLUA4-DEPENDENT PLASTICITY IN THE DEVELOPING NETWORKS

The results presented in this thesis suggest that endogenous synchronous activity in the developing hippocampal networks is required for strengthening and stabilization of nascent glutamatergic contacts. Expression of GluA4 was shown to facilitate both Hebbian and homeostatic plasticity during a developmental time window that is critical for synaptic maturation and for fine-tuning of the circuitry. First, the developing network expressed a quick, developmentally restricted and GluA4-dependent mechanism for homeostatic regulation of glutamatergic transmission. This may serve as an important safety mechanism for maintaining proper activity levels during the time of intense synaptic reorganization, in order to construct properly working neuronal networks. Second, the mechanism for input-specific plasticity, proposed to mediate the strengthening of the nascent glutamatergic connections in response to synchronous endogenous activity, was dependent on the expression of GluA4. GluA4 provides a minimal, PKA- dependent mechanism for LTP induction during development. After the developmental period of synaptogenesis, the expression of this subunit of AMPAR is lost, leading to the requirement of more complex signaling involving activation of several kinases for LTP induction. We hypothetize, that expression of GluA4 renders the developing networks susceptible for regulation by endogenous network activity. An outline of the most important findings in this thesis is presented in Figure 10.

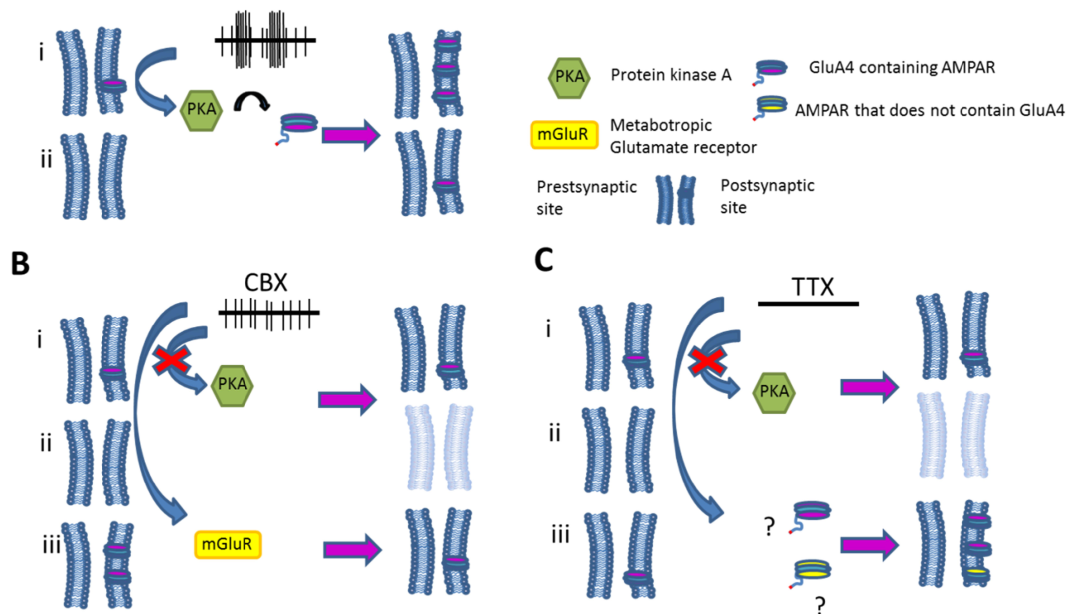


Figure 10. Endogenous synchronous activity is needed for the GluA4-dependent synapse induction and reinforcement in the area CA1. A. Synchronous activity leads to PKA-dependent delivery of GluA4-subunit containing AMPAR to the postsynaptic membrane and reinforces the existing contacts (i) as well as leads to synaptic induction (ii). The molecular mechanisms underlying PKA-dependent AMPAR trafficking to the postsynaptic membrane very likely involves interaction of GluA4 with IQGAP and α-actinin. B. Desynchronization leads to downregulation of AMPAR-mediated mEPSCs. We hypothetize that in the absence of synchronous activity, the PKA-dependent delivery of GluA4-containing AMPAR's is prevented and synapse reinforcement (i) and/or induction (ii) is obstructed. Since the downregulation of mEPSCs is prevented in the presence of the mGluR antagonist, another contributing mechanism is activation of LTD-like processes in response to asynchronous activity, where mGluR activation leads to removal of synaptic GluA4-containing AMPAR's (iii). Alternatively, mGluR-dependent downregulation might also be linked to mGluR-dependent decrease in cAMP level and inhibition of PKA activity (Baba et al., 1993; Takahashi et al., 1996). C. Total activity block causes no net effect to synaptic strength in the CA1 area. One plausible explanation is that in the absence of synchronous activity, the synaptic induction is prevented (i) via the same GluA4-dependent mechanisms explained in section B (i-iii). Concomitantly, the prevention of all action potential-dependent activity induces homeostatic plasticity mechanisms (iii) that leads to GluA4-dependent upregulation of mEPSCs, therefore compensating for the loss of synaptic induction (i). These mechanisms are at least partly dependent on the GluA4-subunit, since quick 15-20 h homeostatic upregulation is not present in GluA4 -/- mice.

The synchronous intrinsic activity is detected in primates including man during the third trimester of pregnancy (Khazipov et al., 2001; Vanhatalo and Kaila 2006; Warland et al., 2006). When considering the developmental stage of the CNS this time period corresponds to the first postnatal weeks in rats and mice. The results presented in this thesis demonstrate that even brief perturbances in the neuronal network activity during this critical period may result in dramatic changes in the synaptic function. It is becoming evident that a number of widely used drugs and substances can affect the synchronous activity of the brain during development. Since many of the diseases of the brain are of developmental origin (e.g. Fetal Alcohol Syndrome, certain mental illnesses), our findings will help in elucidating the mechanisms underlying these disorders.

7 CONCLUSIONS

I. Synchronous neuronal activity characteristic of the developing hippocampus is needed for the maturation and refinement of the glutamatergic synaptic connections. Immature synapses that are not involved in the correlated activity are weakened or silenced during early development.

II. The threshold for the induction of homeostatic plasticity is increased in parallel with the maturation of the neurons. The number and strength of synapses onto a neuron change during development, as a result of activity-dependent mechanisms. Thus, greater sensitivity to homeostatic regulation may protect the developing networks from instability during the time of intense activity-dependent remodeling.

III. The transient developmental expression of GluA4 in the glutamatergic neurons of the hippocampus has an instrumental role in both the Hebbian and homeostatic plasticity mechanisms typical for the immature synapses. The facilitated plasticity provided by GluA4 may promote synchronous activity-dependent maturation of synapses in the hippocampal CA3 and CA1 areas.

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Sincerely,

Johanna

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