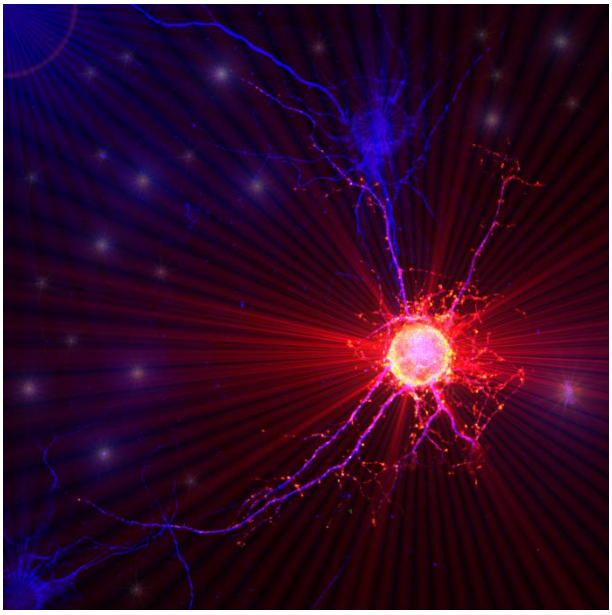
Developmentally Regulated Induction and Expression Mechanisms of Long-Term Potentiation at Hippocampal CA3–CA1 Synapses

Natalia V. Luchkina



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If our brains were simple enough for us to understand them, we'd be so simple that we couldn't.

— Jack Cohen and Ian Stewart.
The Collapse of Chaos: Discovering Simplicity in a Complex World (1994).

Table of Contents

List of original publications

This thesis is based on the following publications which are referred to in the text by their Roman numerals:

I. **Luchkina NV***, Sallert M*, Clarke VRJ, Taira T, Lauri SE. Mechanisms underlying induction of LTP-associated changes in short-term dynamics of transmission at immature synapses. *Neuropharmacol* 67: 494–502 (2013).

II. **Luchkina NV***, Huupponen J*, Clarke VRJ, Coleman SK, Keinänen K, Taira T, Lauri SE. Developmental switch in the kinase dependency of long-term potentiation depends on expression of GluA4 subunit-containing AMPA receptors. *PNAS* 111: 4321–26 (2014).

III. **Luchkina NV**, Coleman SK, Huupponen J, Kivistö A, Taira T, Keinänen K, Lauri SE. Molecular mechanisms controlling synaptic recruitment of GluA4 subunit-containing AMPA receptors critical for functional maturation of CA1 glutamatergic synapses. *Manuscript*.

* Equal contribution

Candidate's contribution:

I. The candidate performed the electrophysiological experiments addressing involvement of PKC and postsynaptic Ca^{2+} in neonatal presynaptic LTP (npLTP) and, together with M.S., studied the correlated pre- and postsynaptic activation, the role of L-type Ca^{2+} channels, NMDARs and PKA in npLTP. The candidate also designed some of the experiments, analysed the data and contributed to the writing process.

II. The candidate performed the following electrophysiological experiments: role of postsynaptic PKA and C-terminal interactions of AMPAR subunits in forskolin-induced potentiation of mESPCs and evoked EPSCs (together with S.E.L.), effect of forskolin on mEPSCs in GluA4$^{-/-}$ vs. WT mice and in lentivirally transduced cells (together with J.H.), LTP in GluA4$^{-/-}$ and WT mice, and field recordings (together with V.R.J.C.). The candidate also did most of the stereotactic injections and carried out the DAPI stainings. The candidate contributed to the design of experiments and the writing process.

III. The candidate contributed to the experimental design and performed the major part of the experimental work (experiments addressing the developmental profile of glutamatergic circuit maturation in WT and GluA4$^{-/-}$ mice, characterisation of the GluA4 C-terminal sequence motifs in activity-dependent synaptic insertion and in GluA4 trafficking). The candidate analysed the results and participated in the writing of the manuscript.

List of abbreviations and acronyms

ABP	AMPA-binding protein
AC	adenylyl cyclase; adenylate cyclase
ACSF	artificial cerebrospinal fluid
AM	acetoxymethyl ester
AMPA(R)	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (receptor); 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (receptor)
ANOVA	analysis of variance
ATD/NTD	amino/N-terminal domain
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CA1–3	areas of the hippocampus, from cornu ammonis
CaM	calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CKAMP44	cystine-knot AMPAR modulating protein
CNIH	cornichon homologue
CTD	carboxyl-terminal domain
DAG	diacylglycerol
D-AP5	D(–)-2-amino-5-phosphonopentanoic acid
DAPI	4',6-diamidino-2-phenylindole; 2-(4-amidinophenyl)-1H-indole-6-carboxamidine
DG	dentate gyrus
DIV	days *in vitro*
E(day)	embryonic (day)
EC_{50}	half maximal effective concentration
EGFP	enhanced GFP
EGTA	ethylene glycol tetraacetic acid
ELKS	a protein of active zone enriched with glutamic acid (E), leucine (L), lysine (K) and serine (S)
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
F-actin	filamentous actin
fEPSP	field EPSP
GABA	γ-aminobutyric acid; 4-aminobutanoic acid
GAD65	glutamic acid decarboxylase isoform of 65 kDa
GFP	green fluorescent protein
GluA1–4	AMPAR subunits 1–4
GluK1–5	KAR subunits 1–5
GluN1–3	NMDAR subunits 1–3 (GluN1, GluN2A–D, GluN3A–B)
GRIP	glutamate receptor-interacting protein
GST	glutathione S-transferase
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
IQGAP1	IQ motif-containing GTPase-activating protein 1
KAR	kainate receptor
LBD	ligand-binding domain
L-LTP	late phase of LTP
LTP	long-term potentiation

M1–4	transmembrane domains 1–4
MAP2	microtubule-associated protein 2
mEPSC	action potential-independent spontaneous (miniature) EPSC
MPR	membrane proximal region
munc13	mammalian uncoordinated 13
munc18	mammalian uncoordinated 18
NGS	normal goat serum
NMDA(R)	*N*-methyl-D-aspartate (receptor)
npLTP	neonatal presynaptic LTP
NSF	*N*-ethylmaleimide-sensitive factor
P(day)	postnatal (day)
PBS	phosphate buffered saline
PDZ	acronym from PSD95/SAP90, Drosophila discs large homolog 1 (Dlg1; = synapse-associated protein 97, SAP97), and zonula occludens (tight junction) protein 1 (ZO-1)
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PICK1	protein interacting with C-kinase 1
PKA	cAMP-dependent protein kinase, protein kinase A
PKC	protein kinase C
PKG	cGMP-dependent protein kinase, protein kinase G
PKI	PKA inhibitor fragment (6-22) amide
PPF	paired-pulse facilitation
Pr	probability of release
PSD	postsynaptic density
QX-314	*N*-ethyllidocaine; [2-(2,6-dimethylanilino)-2-oxoethyl]-triethylazanium
RIM	Rab3-interacting molecule
RIM-BP	RIM-binding protein
RMS	root mean square
ROI	region of interest
S1–2	glutamate-binding sites
SAP	synapse-associated protein
SC	Schaffer collateral
SM	sec1/munc18-like
SNAP-25	synaptosomal-associated protein 25
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
Src	from sarcoma, family of non-receptor tyrosine kinases
SynDIG1	synapse differentiation induced gene 1
TARP	transmembrane AMPAR regulatory protein
Trk	tropomyosin receptor kinase (e.g. TrkB)
tSNARE	target SNARE
VAMP	vesicle-associated membrane protein, synaptobrevin
vSNARE	vesicular SNARE
WT	wild type

Abstract

Activity-dependent synaptic plasticity, and long-term potentiation in particular, represents the predominant model of memory and learning at the cellular level. In addition, synaptic plasticity plays a critical role in the activity-dependent refinement and fine-tuning of neuronal circuits during development by maintaining and stabilising certain synaptic connections and eliminating others.

The main goal of this project was to increase our understanding of the molecular mechanisms underlying activity-dependent synaptic plasticity in the developing brain, with particular emphasis on the mechanisms that are specific to early postnatal development. First, we characterise in detail the properties of developmentally restricted neonatal presynaptic long-term potentiation (LTP) in CA1 area of the hippocampus and demonstrate its susceptibility to regulation via protein kinase C (PKC) signalling. Next, we explore the physiological functions of GluA4 subunit-containing AMPA type glutamate receptors, predominantly expressed at developing CA3–CA1 synapses. We show that GluA4 expression is necessary for protein kinase A (PKA)-dependent LTP at immature synapses. Further, the loss of GluA4 expression in parallel with circuit maturation explains the developmental switch in LTP signalling requirements from PKA- to Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-dependent. Further, we also explore the role of GluA4 C-terminal interaction partners in synaptic trafficking of GluA4-containing AMPA receptors and its importance for synapse maturation. We confirm a critical role for the membrane proximal region of GluA4 C-terminal domain in trafficking and identify a novel mechanism for activity-dependent synaptic delivery of GluA4 by the extreme C-terminal region. Finally, we show an important role of the GluA4 subunit in strengthening of AMPA receptor-mediated transmission, observed during early postnatal development.

In summary, we provide novel information on the pre- and postsynaptic plasticity mechanisms operating at hippocampal CA3–CA1 synapses during the critical period of activity-dependent maturation of glutamatergic neuronal circuitry in rodents. This expands our knowledge on the cellular mechanisms guiding development of synaptic connectivity in the brain. Dysfunction of such mechanisms may play fundamental roles in the underlying pathophysiological causes of various neurodevelopmental disorders.

1. Introduction

Activity-dependent synaptic plasticity, the ability of synapses to change their strength in response to prior activity, dominates present models of cellular and molecular mechanisms of memory and learning. The synaptic plasticity and memory hypothesis states: "Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed" (Martin et al., 2000). Exciting contemporary techniques such as multi-electrode array, optogenetics and advanced molecular genetics continue to uncover substantial evidence in support of the hypothesis (e.g. Whitlock et al., 2006; Ramirez et al., 2013; Nabavi et al., 2014; Takeuchi et al., 2014). One of the most studied forms of synaptic plasticity is LTP, which is characterised by a long-lasting increase in synaptic strength in response to brief (<1 s) periods of patterned electrical activity. Although LTP is observed at many synapses throughout the central nervous system and at different stages of development, it is extensively studied in the hippocampus, the region in which it was first observed (Bliss and Lømo, 1973).

In addition, synaptic plasticity plays an important role in early development, when neuronal circuitry is just forming. Synapse formation *per se* doesn't require the presence of synaptic activity; for instance, in the absence of neurotransmitter secretion (munc18-1-deficient mice), initial normal brain assembly (including morphologically defined synapses and functional postsynaptic receptors) is preserved (Verhage et al., 2000). However, plasticity-like processes are thought to be involved in the activity-dependent refinement and fine-tuning of neuronal circuits: maintaining and stabilising certain synaptic connections and eliminating others (Goda and Davis, 2003; Hanse et al., 2009). During the course of development, the ability to induce synaptic plasticity, its roles and underlying molecular mechanisms change considerably (Lohmann and Kessels, 2014). Explicitly, at glutamatergic synapses in the hippocampus, a number of key synaptic components in both pre- and postsynaptic compartments undergo age-dependent modifications (e.g. Groc et al., 2006a; Yashiro and Philpot, 2008; Hanse et al., 2009; Lohmann and Kessels, 2014).

Much evidence suggests that many nervous system diseases originate from perturbed development of the neuronal circuitry and/or in glutamate receptor function (e.g. Dingledine et al., 1999; Bowie, 2008; Fatemi and Folsom, 2009; Bozzi et al., 2012; Meredith, 2015). Basic research providing information on the mechanisms that guide the development of glutamatergic circuitry is expected to facilitate the development of novel therapeutic approaches to such diseases.

2. Review of the literature

2.1. Morphological and molecular determinants of synaptic transmission in the hippocampus and their developmental profile

Synaptic transmission at chemical synapses can be divided into general steps. Initially, neurotransmitter is released from a presynaptic compartment. Then, it diffuses across the synaptic cleft and binds postsynaptic receptors. These transmembrane proteins transform the extracellular chemical signal into changes in membrane potential and, in some cases, transfer it through second messengers systems (e.g. Ca^{2+}) to various downstream signal transduction pathways. One main component that influences neurotransmitter diffusion is synaptic structure, e.g. the presence or absence of specialised morphological compartments, called dendritic spines. Glutamatergic synaptic transmission and plasticity has been widely studied at synapses between CA3 (from cornu ammonis) and CA1 pyramidal neurons (CA3–CA1 synapses), the final part of the classical hippocampal trisynaptic circuit (Fig. 1).

In spite of the aforementioned consolidated general steps in chemical synaptic transmission, there is great degree of heterogeneity in details (presynaptic mechanisms, spine structure and size, postsynaptic receptor composition) at different developmental stages at glutamatergic CA3–CA1 synapses (e.g. Harris et al., 1992; Fiala et al., 1998; Groc et al., 2006a; Lauri et al., 2006; Yashiro and Philpot, 2008; Hanse et al., 2009; Bassani et al., 2013; Rose et al., 2013; Lohmann and Kessels, 2014) and even within the same neuron (e.g. Sobczyk et al., 2005).

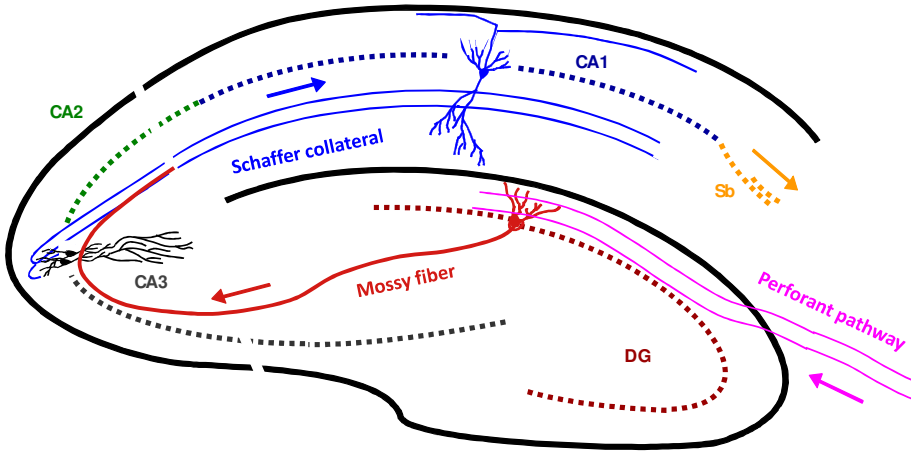

Figure 1. The classical trisynaptic circuit of the hippocampus. Granule cells of the dentate gyrus (DG) send glutamatergic mossy fibers to form synapses with CA3 pyramidal neurons, which then synapse onto CA1 pyramidal neurons via Schaffer collaterals. The major input into the hippocampus is via the perforant pathway from the entorhinal cortex, and CA1 pyramidal cells form its major output by sending excitatory glutamatergic fibers to the subiculum (Sb) and entorhinal cortex as well as subcortical targets.

2.1.1. Presynaptic nerve terminal

At the presynaptic nerve terminal, an action potential induces the opening of voltage-gated Ca^{2+} channels, and the resulting transient increase in Ca^{2+} concentration causes exocytosis of synaptic vesicles (Südhof, 2004). Vesicle fusion and neurotransmitter release occur in response to an action potential with a certain probability (probability of release, Pr). Transmission at hippocampal CA3–CA1 synapses is very unreliable, with more than 50% of action potentials failing to trigger release, due to probabilistic release mechanisms (Hessler et al., 1993; Allen and Stevens, 1994). The area where vesicles accumulate, dock, prime and get released is called the active zone and is tethered in opposition to the postsynaptic compartment via cell adhesion molecules (Südhof, 2004; Clarke et al., 2012). The active zone is an evolutionarily conserved complex with main proteins: RIM (Rab3-interacting molecules), munc13, RIM-BP (RIM-binding proteins), α-liprin, and ELKS (enriched with glutamic acid (E), leucine (L), lysine (K) and serine (S)). In particular, RIM and RIM-BP are necessary for the recruitment of Ca^{2+}-channels within the active zone and synchronise neurotransmitter release.

Two other protein complexes are essential components in the process of vesicle fusion: SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) and SM (Sec1/munc18-like) proteins. SNARE is a complex of three proteins: syntaxin-1 and SNAP-25 (synaptosomal-associated protein 25), located on the presynaptic membrane, and vesicle-associated membrane protein (VAMP, also called synaptobrevin) in the synaptic vesicle. Vesicular (vSNARE) and target membrane-localised SNARE (tSNARE) proteins bind like a zipper into a four-helix trans-SNARE complex that pulls the two membranes tightly together. Formation of SNARE complex produces energy that catalyses membrane fusion. SM proteins (e.g. munc-18), organised in a "clasp" shape, organise SNARE complexes spatially and temporally; also they may be important for phospholipid mixing during actual fusion (Südhof and Rothman, 2009; Südhof and Rizo, 2011).

Complexin and synaptotagmin are the main regulatory proteins which keep the vesicular machinery in a primed and readily releasable state until the trigger comes; these proteins are responsible for precise timing of neurotransmitter release but not for fusion *per se*. Synaptotagmin binds both Ca^{2+} and SNARE proteins, and works as a Ca^{2+} sensor. Complexin promotes the action of synaptotagmin (Südhof and Rothman, 2009; Südhof and Rizo, 2011). After fusion vesicles endocytose, which can occur by three pathways: "kiss and stay" refilling without undocking, "kiss and run" undocking and local recycling, or full clathrin-dependent endosomal recycling (Südhof, 2004). Furthermore, additional forms of endocytosis were reported recently (e.g. Watanabe et al., 2013).

The protein complex of active zone mediates short- and long-term plasticity through its ability to change in response to extra- or intracellular signals. When a synapse is activated with two stimuli with a short interval in between (usually 10–200 ms), the second response of the pair at most synapses is increased, or facilitated. This phenomenon of paired-pulse facilitation (PPF) is considered to be a

good assay of presynaptic function (Manabe et al., 1993). According to the hypothesis of residual Ca^{2+}, introduced by Katz and Miledi in 1968, PPF results from an increased Pr due to summation with residual Ca^{2+} from the first stimulus. Since Pr cannot exceed 1, there is less scope for PPF when Pr increases (Bliss et al., 2007; Bliss and Collingridge, 2013). Therefore, the amount of PPF is inversely correlated with the initial Pr (Dobrunz and Stevens, 1997).

More recently, it has been estimated that the residual Ca^{2+} can only account for ~4% of observed facilitation at most synapses (Regehr, 2012). Due to high cooperativity of the Ca^{2+} sensors (e.g. five Ca^{2+} binding sites at synaptotagmin I; Ubach et al., 1998; Fernandez et al., 2001), the relationship between Ca^{2+} concentration and neurotransmitter release is nonlinear: release is restricted to a short period of time as the suprathreshold Ca^{2+} transient terminated rapidly, even with residual Ca^{2+} present (Südhof, 2004). Furthermore, presence of Ca^{2+} sensors/binding sites with different kinetics and affinity may add to this nonlinearity and affect facilitation (Regehr, 2012). Interestingly, recent data suggest that changes in Ca^{2+} influx affect synaptic transmission not only through Pr, but also by regulating the size of the readily releasable pool (Thanawala and Regehr, 2013). The presence of high-affinity rapid Ca^{2+} buffers (such as calbindin) in presynaptic terminals and their successive saturation during the subsequent impulse is another proposed mechanism of facilitation. Slow Ca^{2+} buffers such as parvalbumin can also contribute to facilitation (Regehr, 2012). In addition, the mechanisms of short-term plasticity include modulation of Ca^{2+}-channels (e.g. via G-protein-mediated mechanisms) or direct modifications of proteins within vesicular release machinery (Südhof, 2012). Regulation of protein interactions within the fusion machinery, for instance, by protein phosphorylation or dephosphorylation, may be one mechanism underlying fast modulation of synaptic transmission. Furthermore, activation of certain protein kinases in presynaptic locus correlates with increased transmitter release (Leenders and Sheng, 2005).

There are some age-dependent differences in presynaptic function. A subpopulation of immature presynaptic terminals in the CA1 area of the hippocampus (first week of development) has a low probability of glutamate release (Gasparini et al., 2000; Lauri et al., 2006). Low release probability is maintained by developmentally expressed, tonically active presynaptic kainate receptors (KARs; Lauri et al., 2006). Tonic endogenous activity of KARs is rapidly switched off by induction of Hebbian LTP under experimental conditions and during development (Lauri et al., 2006; Sallert et al., 2007; 2009). Furthermore, studies of developing hippocampal synapses in culture also suggest decreased number of docked vesicles (Renger et al., 2001; Mozhayeva et al., 2002; Rose et al., 2013) and lower rates of vesicle recycling (Rose et al., 2013). Others observe slow glutamate release in their experiments and suggest noncomplete opening of presynaptic fusion pore as a possible explanation (Choi et al., 2000; Renger et al., 2001; but Kerchner and Nicoll, 2008).

2.1.2. Postsynaptic nerve terminal

2.1.2.1. Dendritic spines

Dendritic spines are tiny protrusions from dendritic shafts, typically composed of a spine head (<1 µm in diameter) and a thin spine neck (<0.2 µm in diameter). Spines are the main gateway of excitatory synaptic transmission in the adult brain (Adrian et al., 2014; Araya, 2014). However, during the first weeks of the development, the number of synapses is low and most of them are located on dendritic shafts (~55%) or filopodia (~20%), long and thin dendritic processes often without visible heads (Fiala et al., 1998). The number of spine synapses is very low at postnatal day (P) 1 (~5%), reaching about 40% by P12 and more than 90% in adult (Harris et al., 1992; Fiala et al., 1998). During the second and third weeks of development, the number of synapses increases dramatically, as the rate of synaptogenesis reaches its peak, and the majority of newly formed synapses are spine synapses (Nimchinsky et al., 2002; Lohmann and Kessels, 2014). These complementary changes in the numbers of spine and shaft synapses led to the hypothesis of spines arising from shaft synapses by a process of outgrowth, possibly via a filopodium stage (Fiala et al., 1998). However, such transitions have never been observed under experimental conditions. Indeed, studies utilising new advanced techniques such as the combination of two-photon laser-scanning microscopy and two-photon laser uncaging of glutamate have demonstrated that the appearance of new spine synapses (functional within 30 min after growth) could happen at any place along the dendrite, with no need for preceding filopodia or shaft synapses (Zito et al., 2009; Kwon and Sabatini, 2011). Glutamate provides sufficient stimulus to induce spinogenesis in the developing brain and requires *N*-methyl-D-aspartate (NMDA) receptor activation. However, the downstream signalling cascades involved are still controversial (Kwon and Sabatini, 2011; Hamilton et al., 2012).

Why is it useful to have synapses located on spines? Morphologically, the presence of spines (as well as dendrites and axonal branching) makes the wiring of the brain efficient, with maximal interconnectivity for available tissue volume (Chklovskii, 2004). On the other hand, spines provide biochemical isolation which is important for input specificity and local activation of signalling cascades (Adrian et al., 2014; Araya, 2014). Narrow spine neck restricts diffusion; therefore, biochemical signals, especially Ca^{2+}, compartmentalise in the spine head for several milliseconds (Araya, 2014). However, biochemical isolation can occur in absence of spines, for instance, Ca^{2+} localisation (<1 µm) is observed in aspiny dendrites of fast-spiking interneurons and is dependent on fast kinetics of Ca^{2+} influx through Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type glutamate receptors (Goldberg et al., 2003). The morphology of dendritic spines can also affect both lateral diffusion of membrane-bound receptors and their active vesicular trafficking (Adrian et al., 2014). As a result of electrical compartmentalisation (due to passive filtering mechanism in the spine neck or presence of active conductances), spine heads may maintain higher depolarisation

compared to dendrites (Araya et al., 2006; but Popovic et al., 2014). Reduced synaptic potentials access dendritic shafts, preventing dendritic saturation and allowing input integration when many inputs are activated simultaneously (Yuste, 2013). Structural plasticity (e.g. head enlargement, spine neck shortening or widening) accompanies LTP in many studies (e.g. Yuste and Bonhoeffer, 2001; Matsuzaki et al., 2004; Bosch et al., 2014) with consequences for compartmentalisation; however, it's still unknown whether these structural changes are concurrent or causally linked to functional LTP (Straub and Sabatini, 2014).

Despite the proposed critical functions of spines in synaptic transmission and plasticity, there are limited numbers of spine synapses in developing brain. It is possible that the absence of compartmentalisation by itself represents a fundamental feature of developing brain, which promotes activity-dependent clustering of coactive synaptic inputs (Kleindienst et al., 2011; Lohmann and Kessels, 2014). Alternatively, biochemical and electrical compartmentalisation may not be critical during development or it may be accomplished by other ways (e.g. as in fast-spiking interneurons). Given the higher rates of plasticity observed in developing brain, it could be more cost-efficient to form and eliminate shaft as compared to spine synapses (Lohmann and Kessels, 2014).

2.1.2.2. AMPA receptors

The majority of fast excitatory neurotransmission is mediated by AMPA type of ionotropic glutamate receptors (AMPARs). AMPARs are tetramers (Rosenmund et al., 1998) of the subunits GluA1–GluA4 (Hollmann et al., 1989; Boulter et al., 1990; Keinänen et al., 1990; for nomenclature Collingridge et al., 2009). All subunits have a similar structure (Fig. 2) and consist of an extracellular amino-terminal domain (ATD or NTD), ligand-binding domain (LBD, including S1 and S2), three transmembrane domains (M1, M3 and M4), cytoplasmic re-entrant loop (M2) and intracellular carboxyl-terminal domain (CTD). The four subunits associate in different combinations to form receptor subtypes with distinctive properties, expressed in different brain areas and during specific stages of development (Boulter et al., 1990; Keinänen et al., 1990; Zhu et al., 2000). In addition, AMPAR subunits are subjected to different post-transcriptional modifications such as alternative splicing and RNA editing (Fig. 2), leading to even higher structural and functional variety. Complexity of AMPARs is further increased by the presence of non-pore-forming auxiliary subunits, directly interacting with AMPARs and affecting their surface expression and localisation, channel properties and pharmacology. The currently identified mammalian auxiliary subunits of AMPARs are transmembrane AMPAR regulatory proteins (TARPs), cornichon homologues (CNIHs), and the candidate auxiliary proteins such as synapse differentiation induced gene 1 (SynDIG1) and cystine-knot AMPAR modulating protein (CKAMP44) (Jackson and Nicoll, 2011; Yan and Tomita, 2012; Haering et al., 2014). For instance, in the adult hippocampus, AMPARs form a tripartite protein complex with TARP γ-8 and CNIH-2, synergistically modulating the properties of hippocampal AMPARs (Kato et al., 2010).

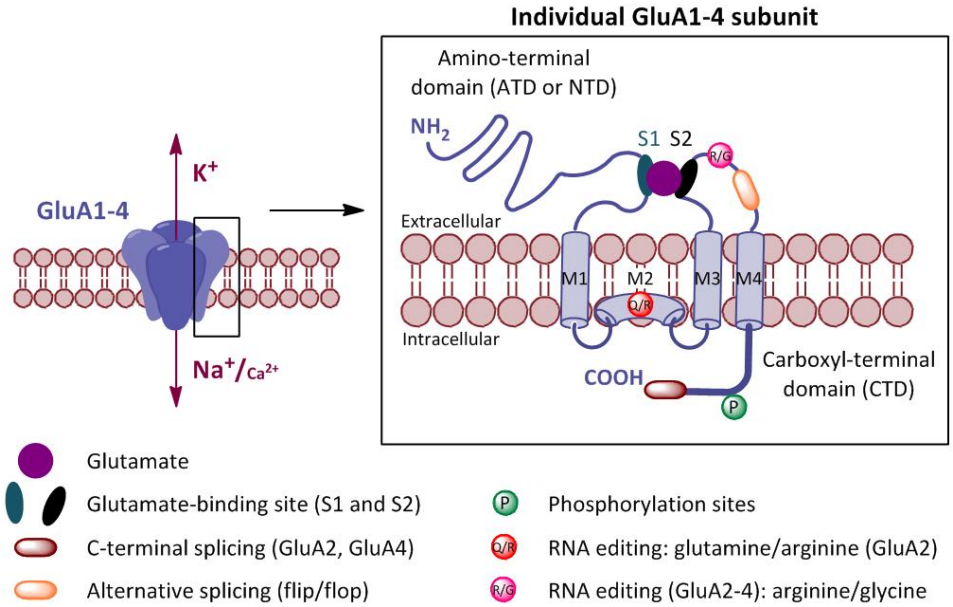

Figure 2. Schematic of AMPAR topology and subunit structure. Tetrameric AMPARs assemble as dimers of dimers. AMPAR subunits GluA1 and GluA4 can form both homo- and heteromers. All subunits consist of three transmembrane domains (M1, M3 and M4) and cytoplasm facing re-entrant membrane loop (M2). Thus, the N-terminal domain (ATD or NTD) is located extracellularly and C-terminal domain (CTD) intracellularly. Glutamate binds at the ligand-binding domain (LBD), formed by S1 and S2 segments.

All AMPAR subunits exist in two alternatively spliced versions, flip and flop, that are encoded by adjacent exons of the receptor genes and localised in the LBD just before the M4 region (Fig. 2; Sommer et al., 1990). While mRNAs encoding flip forms are expressed through embryonic and postnatal development (CA1, CA3 and DG in the hippocampus), expression of flop versions is very low prior to P8, but increases with age, reaching adult levels by P14 in the rat. This developmental profile varies depending on cell type and receptor subunit composition. In particular, whereas AMPARs at CA1 pyramidal cells undergo the developmental switch from flip to flop, adult CA3 pyramidal neurons express flip version throughout development (Monyer et al., 1991). Interestingly, flip forms generally desensitise more slowly and less profoundly in response to glutamate than flop forms. Thus, flip isoforms pass more current into the cell and, therefore, the cells expressing them would be more excitable (increased charge transfer leading to large depolarisation) (Sommer et al., 1990; Dingledine et al., 1999; Traynelis et al., 2010).

AMPAR subunits show distinct structural variability within their CTD, where, in the case of GluA2 and GluA4 subunits, another site of alternative splicing is localised. GluA1, GluA4, and $GluA2_L$ (alternative splice form of GluA2) have long cytoplasmic tails, while GluA2, GluA3, and $GluA4_S$ (alternative splice form of GluA4) have short CTDs (e.g. Malinow and Malenka, 2002). Furthermore, the number and

composition of receptors at the synapses constantly and dynamically changes during an active process of receptor relocation called trafficking. Receptor trafficking involves transport of receptors from sites of their synthesis to their location at the membrane, and then to sites of degradation. The receptors are inserted to the membrane by a process of exocytosis and removed by endocytosis. Moreover, they can move within the plasma membrane by lateral diffusion (Collingridge et al., 2004). AMPARs can be inserted directly into the synapses (Kennedy et al., 2010; Patterson et al., 2010) and/or exocytose extrasynaptically and then laterally diffuse to the synapses (Adesnik et al., 2005; Yudowski et al., 2007; Lin et al., 2009; Makino and Malinow, 2009). Recent data suggest that synaptic recruitment of AMPARs is largely due to the lateral diffusion and capture of pre-existing surface receptors to the postsynaptic density (70–90% vs. 10–30% of newly exocytosed receptors; Patterson et al., 2010). Different modes of trafficking have been proposed for AMPAR subunits with long (activity-dependent synaptic insertion) and short (basal synaptic delivery) CTDs (Hayashi et al., 2000; Shi et al., 2001; Malinow and Malenka, 2002). However, new data from Prof. Roger Nicoll's lab, using a single-cell molecular replacement strategy where all endogenous AMPA receptors are replaced with recombinant subunits, illustrate that GluA1 with long CTD can constitutively traffic to synapses under these conditions (Granger et al., 2013).

The expression profile of AMPAR subunits in CA1 pyramidal cells changes during development. During the first postnatal week, GluA4 subunits are predominantly expressed, while the levels of the other subunits are low. A significant fraction of GluA4-containing AMPARs lack GluA2: ~70% at P2, ~25% at P6 (Zhu et al., 2000). As GluA4 levels decrease, $GluA2_L$ increases with an expression peak between P7 and P14 (Kolleker et al., 2003; Lohmann and Kessels, 2014). Subsequently, levels of other AMPAR subunits GluA1, GluA2 and GluA3 rise and reach adult levels during the third postnatal week of development (Zhu et al., 2000; Lohmann and Kessels, 2014). According to current studies, at mature stages, GluA1/GluA2 heteromers are the dominant receptor subtype present in CA1 pyramidal cells (~80% of AMPARs at the synapses, ~95% of extrasynaptic receptors). The remaining synaptic component (~16%) comprises GluA2/GluA3 heteromers. Notably, all receptors are thought to contain the GluA2 subunit in adult CA1 principal cells (Lu et al., 2009; in contrast to Wenthold et al., 1996).

The additional heterogeneity in AMPARs comes from RNA editing, a process of post-transcriptional alteration of the mRNA nucleotide sequence (Seeburg, 1996). There are two sites for RNA editing: the Q/R site (Sommer et al., 1991) in the pore-lining M2 segment and the R/G site (Lomeli et al., 1994) located in the extracellular part between M3 and M4 (Fig. 2). Editing of the former occurs only in GluA2 subunits (Sommer et al., 1991), while the latter is edited in GluA2, 3 and 4 (Lomeli et al., 1994). While unedited GluA2(Q) subunits coexist with edited GluA2(R) during embryogenesis [embryonic day (E) 14 and P0], no adult expression of unedited form is observed (Burnashev et al., 1992). The presence of positive arginine (R) instead of neutral glutamine (Q) strongly reduces Ca^{2+}-permeability of GluA2-containing

receptors, forming AMPARs with relatively low conductance and linear current-voltage characteristics. By contrast, AMPARs lacking edited GluA2 are Ca^{2+}-permeable and show strong inward rectification (Burnashev et al., 1992), the latter due to intracellular polyamines, such as spermine, which preferentially block the pore at depolarising membrane potentials (Bowie and Mayer, 1995; Dingledine et al., 1999). R/G editing, resulting in coding of glycine (G) instead of arginine (R), plays an important role in recovery from desensitisation, with faster recovery rate for edited forms. During rodent brain development, the percentage of editing at R/G site increases with age, but in a way that appears specific for each subunit and its underlying flip/flop splice form (Lomeli et al., 1994).

2.1.2.3. NMDA receptors

NMDA receptors (NMDARs), another group of ionotropic glutamate receptors, play an important role in multiple forms of synaptic plasticity due to their fundamental biophysical properties (e.g. Malenka and Nicoll, 1993). The NMDAR channel is effectively blocked by Mg^{2+} in a voltage-dependent manner. Therefore, for NMDAR to be activated, two simultaneous events should take place: sufficient membrane depolarisation to remove Mg^{2+} block and glutamate binding. Thus, NMDAR can act as a molecular coincidence detector (Bliss and Collingridge, 1993; Dingledine et al., 1999). NMDARs may also play a role in the stabilisation of excitatory synapses and spines through non-ionotropic physical signalling via the CTD of this receptor (Alvarez et al., 2007).

NMDARs are heterotetrameric assemblies of GluN1/GluN2 subunits or GluN1/GluN2/GluN3 subunits. To date, seven different subunits have been described: GluN1, GluN2A–D, and GluN3A–B (Paoletti et al., 2013). GluN1 subunit has eight different isoforms due to presence of three alternatively spliced exons in the NTD (N1 cassette) and CTD (C1 and C2 and C2' cassettes). Presence of the N1 cassette affects gating and pharmacological properties. The C1 cassette is involved in receptor clustering and contains PKC phosphorylation sites, while the C2' cassette is responsible for the interaction with postsynaptic density protein 95 (PSD95; Dingledine et al., 1999; Paoletti et al., 2013).

GluN1 expression is ubiquitous from embryonic stages to adulthood. The four GluN2 subunits, the main source of NMDAR heterogeneity, demonstrate differential spatial and temporal expression. Early in the development, GluN2B and GluN2D are expressed, with predominant GluN2B expression in the hippocampus. GluN2B level is maintained, being highly expressed before birth and during the first weeks of development. GluN2A and GluN2C expression appears later in the development and gradually increase during the first three weeks, coinciding with a decline to adult levels in GluN2B. In particular, GluN2A mRNA is detectable in pyramidal cells of hippocampus around P7 (Monyer et al, 1994; Paoletti et al., 2013; Lohmann and Kessels, 2014). Thus, in adult hippocampus, GluN1, GluN2A and GluN2B are prominent in pyramidal neurons and granule cells (Monyer et al, 1994; Paoletti et al., 2013). By P14, these subunits completely account for NMDA currents in CA1 pyramidal cells (Gray et al., 2011). In contrast,

GluN2C and GluN2D appear restricted to interneurons (Monyer et al., 1994). The isoforms of the GluN3 subunit also show a differential developmental profile: GluN3A expression is high in early development and then decreases with 50% expression in adult compare to P0 (Wong et al., 2002). GluN3B is absent or weakly expressed in neonatal brain and peaks in adulthood in many brain structures, including hippocampus (Wee et al., 2008; Pachernegg et al., 2012). GluN3A is expressed heavily in the subiculum and retrohippocampal cortex and moderately in some hippocampal cells (Wong et al., 2002).

The specific expression of GluN2B, GluN2D and GluN3A subunits during development suggests they play an important role in synaptogenesis and synapse maturation. In particular, in the hippocampus, the switch from predominant GluN2B to a GluN2A/GluN2B mixture (65% GluN2A and 35% GluN2B subunits in adult CA1 area; Gray et al., 2011) may underlie age-dependent changes in the ability to induce plasticity (Yashiro and Philpot, 2008; Gray et al., 2011; Lohmann and Kessels, 2014). In mature synapses, where both GluN2A and GluN2B are present, the majority of GluN2B-containing receptors are peri- or extrasynaptic according to some studies (Groc et al., 2006b; Lohmann and Kessels, 2014), though others suggest the presence of both subunits in both synaptic and extrasynaptic compartments (Harris and Pettit, 2007; Yashiro and Philpot, 2008).

2.1.2.4. Intracellular signalling pathways: synaptic serine/threonine protein kinases

Several signal transduction pathways have been characterised to regulate synaptic transmission and plasticity. These include cascades involving tyrosine kinases (such as tropomyosin receptor kinase or Trk, activated by neurotrophin family of growth factors, or Src family non-receptor tyrosine kinases, downstream of receptor tyrosine kinases, G-protein-coupled receptors and Ca^{2+}), serine/threonine kinases (such as mitogen-activated protein kinases or MAPKs, activated via small G-proteins; cGMP-dependent protein kinase G or PKG; Ca^{2+}/calmodulin-dependent protein kinases or CaMKs; cAMP-dependent protein kinase or PKA; and protein kinase C or PKC) and the lipid kinase, phosphatidylinositol 3-kinase (PI3K; e.g. regulated by TrkB) (Purcell and Carew, 2003; Bliss et al., 2007; Giese and Mizuno; 2013). The most relevant to this study are serine/threonine protein kinases CaMKII, PKA and PKC. These kinases can alter properties of different synaptic proteins and their interactions by phosphorylation. In particular, with regards to glutamate receptors, phosphorylation is a key mechanism regulating both their synaptic trafficking and channel function (Dingledine et al., 1999; Traynelis et al., 2010).

Protein kinase A (PKA)

The PKA holoenzyme consists of two regulatory and two catalytic subunits. In the absence of cAMP, the catalytic subunits are inhibited by the regulatory ones. PKA is activated by binding of cAMP to its regulatory dimer (Taylor et al., 2012). PKA is expressed during early development. Large developmental increases in PKA activity are observed from P5 to P15, whereupon it reaches adult levels (Kelly, 1982). This period corresponds to the time when the process of active

synaptogenesis begins (Nimchinsky et al., 2002; Lohmann and Kessels, 2014). The PKA activator cAMP is produced from ATP by the enzyme adenylyl cyclase (AC). Amongst the many isoforms of ACs with diverse activation mechanisms, AC1 and AC8 can be activated by Ca^{2+}/calmodulin (CaM), for example, in response to NMDAR activation (Wong et al., 1999). Both AC1 and AC8 are expressed in the hippocampus, but AC8 is limited to the CA1 area (Nicol et al., 2005). AC1 expression in hippocampus as well as its Ca^{2+}-stimulated activity increases dramatically (7-fold) during the first two weeks of postnatal development, with only 2-fold increase for AC8 (Villacres et al., 1995). Interestingly, when detailed cellular distribution is assessed, the expression of AC1 in CA1 area is maximal during embryonic development and during the first two postnatal weeks (when compared to 2-months old adults), whereas a gradual increase of AC1 gene expression is observed in other areas such as the DG and CA2 area (Nicol et al., 2005). In the CA1 area, AC1 is already expressed at E15, whereas AC8 becomes visible around P1 (Nicol et al., 2005).

Among AMPA and NMDA receptor subunits, the targets of PKA phosphorylation include GluA1, GluA4, GluN1, and GluN2C (Traynelis et al., 2010). During development, spontaneous synaptic activity leads to activity-dependent phosphorylation of GluA4 subunits at Ser862[1] and subsequent incorporation of GluA4 into synapses (Zhu et al., 2000; Esteban et al., 2003) possibly by disrupting its interaction with α-actinin-1 (Nuriya et al., 2005). PKA phosphorylation of GluA1 at Ser863[2] increases its surface expression by promoting insertion and reducing endocytosis (Man et al., 2007), as well as increasing channel open probability (Banke et al., 2000). PKA phosphorylation is only one of the requirements for synaptic GluA1 incorporation (Blitzer et al., 1998; Esteban et al., 2003).

Protein kinase C (PKC)

PKC is a family of Ca^{2+}- and phospholipid-dependent kinases. Activation of certain G-protein-coupled receptors (e.g. group I metabotropic glutamate receptors) causes phospholipase C to hydrolyse a phospholipid component of plasma membranes, phosphatidylinositol-4,5-bisphosphate, into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). The latter activates the Ca^{2+} release from the endoplasmic reticulum (Sun and Alkon, 2014). In turn, PKC is classically stimulated by increase in concentration of DAG and Ca^{2+}. However, genetic screening identified at least twelve members of the family sorted into three groups based on their structural and biochemical characteristics: classical (α, βI, βII and γ) activated by DAG and Ca^{2+}, novel (δ, ε, η and θ), which do not require Ca^{2+}, and Ca^{2+}- and DAG-insensitive atypical (ζ and λ/ι) (Zeng et al., 2012; Wu-Zhang and Newton, 2013). Many different PKC isoforms are expressed in the hippocampus

[1] Specifies the position of the residue as in UniProt database with numbering starting with the initiating methionine, including the N-terminal signal peptide (20 amino acids in GluA4). Corresponds to Ser842 in the literature where the numbering starts from the first residue of the presumed mature peptide (when the signal peptide is cleaved).

[2] The signal peptide of GluA1 is 18 amino acids. Thus, Ser863 corresponds to Ser845 in some publications.

with different developmental profiles. For example, classical PKCs, PKCε and PKCη are expressed at low levels at birth and increase during development (Jiang et al., 1994; Roisin and Barbin, 1997), while others (e.g. PKCδ and PKCζ) either do not show any changes or decrease during development (Jiang et al., 1994). Interestingly, high levels of PKCζ expression are observed already during embryonic development (at E18; Jiang et al., 1994). Notably, PKCγ is highly specific to neuronal tissue and, within brain, is the most abundant in the hippocampus, cerebellum and cerebral cortex (Saito and Shirai, 2002). The development of this isoform is delayed: the expression levels are very low before P7 (Jiang et al., 1994; Roisin and Barbin, 1997). GluA1, $GluA2_s$ (short), GluA4, GluN1, GluN2A–C are targets for PKC phosphorylation (Traynelis et al., 2010). PKC phosphorylation of GluA1 at Ser834 and Ser836[3] facilitates GluA1 insertion into the extrasynaptic plasma membrane via enhancement of its interaction with actin-binding protein 4.1N (Boehm et al., 2006; Lin et al., 2009). Phosphorylation of GluA4 by PKCγ at Ser862 increases its membrane expression (Gomes et al., 2007). However, PKC phosphorylation of GluA2 has an opposite effect: it slows recycling of GluA2-containing AMPARs after internalisation via disruption of GluA2 interaction with glutamate receptor-interacting protein/AMPAR-binding protein (GRIP/ABP) and enhances its binding to protein interacting with C-kinase 1 (PICK1; Matsuda et al., 1999; Chung et al., 2000; Seidenman et al., 2003; Lin and Huganir, 2007). PKMζ (brain-specific truncated isoform of PKCζ) promotes diffusion of GluA2-containing AMPARs from the extrasynaptic pool by releasing from PICK1 and interacting with *N*-ethylmaleimide-sensitive factor (NSF; Yao et al., 2008).

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)

CaMKII is a holoenzyme that consists of twelve functional subunits. Each subunit contains a catalytic, an autoregulatory (autoinhibitory), and an association domain. CaMKII is maintained in an inhibited state by its autoregulatory domain which acts as a pseudosubstrate for the catalytic domain. Upon binding of Ca^{2+}/CaM, the autoregulatory domain is displaced, disinhibiting the kinase, which now autophosphorylates at Thr286 (or Thr287). This autophosphorylation allows CaMKII to sustain its activity, even in the absence of Ca^{2+}/CaM. It also increases the affinity of CaMKII for Ca^{2+}/CaM by more than 1000-fold. Another unique property of CaMKII is its ability to be activated in a frequency-dependent manner due to independent activation of the subunits, making CaMKII activation dependent on both the amplitude and frequency of Ca^{2+} oscillations in the cell (Hudmon and Schulman, 2002; Lisman et al., 2012). Once active, CaMKII moves from an F-actin-bound state in the cytosol to a PSD-bound at the synapses (called “translocation”; Shen and Meyer, 1999), localising the kinase near its targets, e.g. GluA1 and GluN2B (Traynelis et al., 2010). The mechanisms behind this translocation are simple diffusion and direct binding of CaMKII to NMDA receptors, most notably to GluN2B. This interaction keeps the kinase in an active state even in absence of Ca^{2+}/CaM for >30 min (Bayer et al., 2001; Bayer and Schulman, 2001; Lisman et al.,

[3] Corresponds to Ser816 and Ser818, respectively.

2012; but Lee et al., 2009). Locked at the synapse, CaMKII can phosphorylate different synaptic proteins (Lisman et al., 2012). CaMKII phosphorylates GluN2B at Ser1303 (Omkumar et al., 1996), though the functional consequences of this phosphorylation are not clear (Traynelis et al., 2010). Phosphorylation of GluA1 at Ser849[4] increases conductance of homomeric GluA1 channels (Derkach et al., 1999), but not of GluA1/GluA2 heteromers (Oh and Derkach, 2005). Furthermore, increased CaMKII activity delivers recombinant GluA1 tagged with green fluorescent protein (GFP) into synapses (Hayashi et al., 2000). CaMKII also enhances lateral diffusion of AMPARs from extrasynaptic pools to synapses by phosphorylating the TARP, stargazin (Tomita et al., 2005).

Interestingly, the developmental expression profile of CaMKII and its accumulation in synaptic fraction is different from PKA which reaches its adult levels around P15 (Kelly, 1982). Expression of CaMKII in forebrain is comparatively low during the first two weeks of development with a large age-dependent increment occurring during the third and fourth postnatal weeks (Kelly and Vernon, 1985). Later studies have identified a more complicated developmental profile, when different CaMKII isoforms are considered (Bayer et al., 1999). There are four related and highly conserved isoforms of CaMKII: brain-specific α and β, expressed later in the development, and ubiquitous, prenatally present γ and δ (Bayer et al., 1999; Hudmon and Schulman, 2002). In the rodent forebrain, CaMKII is mainly represented by CaMKIIα homomers and CaMKIIα/β heteromers (Lisman et al., 2012).

2.2. Long-term potentiation at hippocampal CA3–CA1 synapses

Long-term potentiation at synapses between CA3 and CA1 pyramidal neurons is the most studied, classical example of Hebbian LTP (derived from Hebb's rule often summarised as "Cells that fire together, wire together"). Induction of LTP by patterned stimulation at these synapses leads to summation of fast AMPAR-mediated component of the excitatory response and causes strong depolarisation of the postsynaptic cell. This depolarisation alleviates Mg^{2+} block of NMDARs, resulting in an increase in intracellular Ca^{2+} concentration (Bliss and Collingridge, 1993; Dingledine et al., 1999). It can cause further Ca^{2+}-induced Ca^{2+} release from intracellular stores and subsequent activation of different downstream signals. This, in turn, can lead to persistent postsynaptic changes (larger postsynaptic responses to the same amount of glutamate) such as an increase in the number of AMPARs or modulation of existing receptors. Presynaptic increase in glutamate release (e.g. increase of release probability) is also a mechanism of LTP expression, however it is observed only under some conditions [e.g. to strong induction protocols in adults (e.g. Zakharenko et al., 2001; Bayazitov et al., 2007; Padamsey and Emptage, 2014) or early in the development (Palmer et al., 2004; Lauri et al., 2006; 2007)]. Several different modifications of AMPARs may underlie changes in synaptic efficacy following LTP induction. These include an increase in the number

[4] Corresponds to Ser831.

of postsynaptic AMPARs (Shi et al., 1999; Hayashi et al., 2000; Lu et al., 2001b; Pickard et al., 2001; Malinow and Malenka, 2002; Poncer et al., 2002; Andrásfalvy and Magee, 2004) either through direct AMPAR insertion (Kennedy et al., 2010; Patterson et al., 2010) or/and extrasynaptic insertion and subsequent lateral diffusion from extrasynaptic compartments (Adesnik et al., 2005; Yudowski et al., 2007; Lin et al., 2009; Makino and Malinow, 2009), an increase in single-channel conductance of existing synaptic AMPARs (Benke et al., 1998; Derkach et al., 1999; Poncer et al., 2002), increased open probability (Banke et al., 2000), increased glutamate affinity, and changes in channel kinetics (Malinow and Malenka, 2002; Shepherd and Huganir, 2007).

Interestingly, many of these possible substrates of synaptic plasticity change gradually in parallel with development of neuronal circuitry (Fig. 3; Crair and Malenka, 1995; Wikström et al., 2003; Yasuda et al., 2003; Palmer et al., 2004; Lauri et al., 2006; Yashiro and Philpot, 2008; Lohmann and Kessels, 2014).

2.2.1. Developmental changes in induction and early expression mechanisms of LTP

2.2.1.1. LTP in CA1 area of hippocampus in adult brain

The majority of excitatory synapses in adult brain are located on spines (Harris et al., 1992; Adrian et al., 2014; Araya, 2014; Lohmann and Kessels, 2014), providing sufficient biochemical (e.g. compartmentalisation of Ca^{2+} signal in spine for 1–10 s; Sabatini et al., 2002) and electrical compartmentalisation (Yuste, 2013), and, therefore, input specificity of LTP. GluA1/GluA2 heteromers are the most abundant AMPARs in hippocampal CA1 pyramidal neurons at this time (Lu et al., 2009). In mature synapses, where both GluN2A and GluN2B are present, GluN2A is dominant (~65%; Gray et al., 2011).

It is generally accepted that LTP at CA1 synapses requires activation of NMDARs (Collingridge et al., 1983), allowing Ca^{2+} influx into the dendritic spine. Ca^{2+} activates a number of signalling pathways at mature synapses, with CaMKII playing a key role (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992a; Pettit et al., 1994; Lledo et al., 1995; Otmakhov et al., 1997; Giese et al., 1998). CaMKII has been shown to be both necessary (Silva et al., 1992a; Giese et al., 1998) and sufficient (Pettit et al., 1994; Lledo et al., 1995) for LTP at hippocampal CA3–CA1 synapses. Mice with mutated CaMKIIα also demonstrate impaired spatial learning (Silva et al., 1992b; Giese et al., 1998). However, postsynaptic application of CaMKII inhibitor peptides after LTP induction doesn't affect the maintenance of LTP (Otmakhov et al., 1997). Studies of synaptic plasticity in the hippocampus of AC1 and AC8 double knockout mice (Wong et al., 1999) as well as transgenic mice expressing an inhibitory form of the PKA regulatory subunit (Abel et al., 1997) have shown impaired late phase of LTP (L-LTP) and long-term memory defects, suggesting the importance of cAMP-PKA signalling pathway for LTP maintenance and long-term memory consolidation. PKA phosphorylation of GluA1 makes AMPARs available for synaptic incorporation thereby working as a gate for synaptic plasticity (Blitzer et al., 1998; Esteban et al., 2003). In addition, PKMζ is thought to

be important for LTP maintenance in the hippocampus and spatial memory (Pastalkova et al., 2006) by promoting diffusion of GluA2-containing AMPARs from the extrasynaptic pool (Yao et al., 2008). However, both normal LTP at SC–CA1 synapses and hippocampus-dependent learning and memory can be induced in PKCζ and PKMζ knockout mice (Volk et al., 2013). The possibility remains that other PKCs could compensate for the loss of PKCζ and PKMζ in these studies, e.g. a closely related kinase PKMι/λ (Bliss and Collingridge, 2013). However, given that both conditional and conventional knockouts were used, these compensatory mechanisms should be very effective (emerging within 2–3 weeks; Volk et al., 2013). Interestingly, in PKCγ knockout studies, LTP in hippocampus is abnormal when induced by tetanic stimulation, but normal LTP is observed if low frequency stimulation is given prior to the tetanus, leading authors to the suggestion that PKC plays a regulatory role but is not part of the LTP signalling cascade (Abeliovich et al., 1993).

Upon activation, CaMKII translocates to the synapses via direct interaction with NMDARs, specifically with GluN2B (see section 2.1.2.4). The CaMKII-GluN2B interaction is important for LTP: transgenic mice expressing GluN2B CTD, with specifically disrupted interaction of CaMKII with GluN2B, show reduced LTP and impaired spatial learning (Zhou et al., 2007), while studies with GluN2 chimeras demonstrate a requirement of GluN2B CTD for LTP that is independent of GluN2B-containing NMDAR channel function (Foster et al., 2010). Recently a mouse with two point mutations that impair CaMKII binding to GluN2B was generated (Halt et al., 2012). Interestingly, while translocation of CaMKII to the synapses is prevented in these mice, NMDAR- and CaMKII-dependent LTP could still be induced, though reduced by 50%. Locked at the synapse, CaMKII can phosphorylate other synaptic proteins, including GluA1 and TARPs (see section 2.1.2.4; Barria et al., 1997; Tomita et al., 2005; Lisman et al., 2012).

There are several modifications of AMPARs that might lead to an increased synaptic efficacy. In adult CA1 pyramidal cells, AMPAR number increases (probably GluA1/GluA2), but their biophysical characteristics remain unchanged following LTP induction (Andrásfalvy and Magee, 2004), suggesting a critical role for AMPAR trafficking in LTP expression.

Activity-dependent synaptic incorporation of AMPARs has been proposed to involve long CTD forms, therefore postulating the requirement of long CTD for LTP, while AMPARs with short CTDs undergo constitutive trafficking (see section 2.1.2.2; Shi et al., 2001; Malinow and Malenka, 2002; Bredt and Nicoll, 2003). The CTDs of AMPARs contain multiple regulatory motifs which are subject to post-translational modifications, including palmitoylation, glycosylation, ubiquitination and especially phosphorylation, all of which have functional consequences on AMPAR trafficking. The CTDs also interact with multiple scaffold proteins and through them with different cytoplasmic signalling molecules and cytoskeletal proteins. Therefore, the CTDs were thought be important for the regulation of AMPAR function (e.g. Malinow and Malenka, 2002; Henley et al., 2011; Anggono and Huganir, 2012). However, recent data illustrate that the GluA1 CTD is not absolutely necessary for LTP, and, in principle, any glutamate receptor (AMPARs with short CTD or even KAR

subtypes) is sufficient and can be inserted into synapses to mediate LTP (Granger et al., 2013; Granger and Nicoll, 2014). Though, when long and short CTD-containing AMPARs are present, long CTD-containing AMPARs seem to be preferentially inserted into synapses following synaptic potentiation (e.g. Tanaka and Hirano, 2012; Sheng et al., 2013).

LTP-associated changes in the presynaptic locus of expression in mature SC-CA1 synapses are observed in some studies. However, this LTP requires strong but not prolonged postsynaptic depolarisation (e.g. 200 Hz or theta-burst) and activation of L-type voltage-gated Ca^{2+} channels, and therefore represent a mechanistically distinct form of LTP (e.g. Zakharenko et al., 2001; Bayazitov et al., 2007; Padamsey and Emptage, 2014).

2.2.1.2. LTP in CA1 area of hippocampus in developing brain

During the first weeks of postnatal development, the number of synapses is low, when compared with adult hippocampus, and the majority of them are situated on dendritic shafts and filopodia (Fiala et al., 1998; see section 2.1.2.1).

During this stage of development, the contribution of AMPARs compared with NMDARs to postsynaptic currents is relatively low and an age-dependent increase in AMPA/NMDA ratio is observed in many brain regions (Crair and Malenka, 1995; Hsia et al., 1998; Lu et al., 2001a; Ye et al., 2005). Postsynaptically, this may be explained by the presence of postsynaptically silent synapses ("deaf" synapses) at this age i.e. synapses with functional NMDARs but not AMPARs and, therefore, silent at the resting membrane potential (Liao et al., 1995; Isaac et al., 1995; Durand et al., 1996). Hanse and colleagues proposed a model suggesting functional lability of developing synapses which could be vanquished by Hebbian activity. Both pre- and postsynaptic mechanisms contribute to this functional lability, which is necessary for activity-dependent tuning of immature contacts (Hanse et al., 2009). Postsynaptically, the signalling can change between different states, i.e. AMPA-stable (upon Hebbian activity), AMPA-labile and AMPA-silent, depending on the overall level of synaptic activity (Xiao et al., 2004; Hanse et al., 2009). Interestingly, early in development, activity of NMDARs, composed of GluN1 and GluN2B subunits, limits the number of functional synapses by suppressing AMPAR trafficking (Adesnik et al., 2008; Gray et al., 2011). During this period, external sensory information is absent or limited and synchronous bursts of neuronal activity are spontaneously generated by the cooperative action of glutamate and depolarising γ-aminobutyric acid (GABA) within the developing networks (Ben-Ari et al., 1989; Ben-Ari, 2001). GluA4-containing AMPARs are dominant in pyramidal neurons at this stage (Zhu et al., 2000; see section 2.1.2.2). Trafficking of GluA4 homomers into synapses is activity-dependent, and spontaneous activity is sufficient to drive recombinant GluA4 into the synapses in a PKA- and NMDAR-dependent manner (Zhu et al., 2000; Esteban et al., 2003). Thus, during synapse maturation and establishment of glutamatergic synaptic connectivity, spontaneous activity-dependent trafficking of GluA4 is thought to be one of the dominant mechanisms of synaptic strengthening. When presynaptic

activation correlates with high-frequency synchronous bursts, even silent synapses experience Hebbian co-incidence leading to an enhancement of synaptic efficacy (Kasyanov et al., 2004; Mohajerani et al., 2007) and stabilisation of AMPAR signalling (Hanse et al., 2009). The correlated pre- and postsynaptic activity leads to both insertion of AMPARs and appearance of AMPAR currents, i.e. unsilencing (Liao et al., 1995; Isaac et al., 1995; Durand et al., 1996), and switches the composition of synaptic NMDA receptors from GluN2B- to GluN2A-containing (Bellone and Nicoll, 2007). Such a switch thus raises the threshold for further LTP induction (Yashiro and Philpot, 2008; Gray et al., 2011).

GluN1/GluN2B NMDA receptors have a higher affinity for glutamate, slower deactivation and desensitisation, higher charge transfer, and Ca^{2+}-permeability as compared to the GluN2A-containing receptors expressed in adult (Monyer et al., 1994; Dingledine et al., 1999; Yashiro and Philpot, 2008; Gray et al., 2011; Lohmann and Kessels, 2014). So, due to these functional properties, GluN2B-containing NMDARs in the developing brain may be more sensitive to the changes in neurotransmitter concentration and require less precise temporal coupling between pre- and postsynaptic activity (Yashiro and Philpot, 2008). Furthermore, at certain synapses in the cortex, GluN2B/GluN2A switch correlates with the end of the critical window of synaptic plasticity (e.g. Crair and Malenka, 1995; Quinlan et al., 1999), suggesting that GluN2B expression may also increase the ability to evoke LTP in hippocampus during development (Yashiro and Philpot, 2008; Gray et al., 2011; Lohmann and Kessels, 2014). Interestingly, GluN2A CTD may directly inhibit LTP in organotypic hippocampal slice cultures (Foster et al., 2010), while overexpression of GluN2B in adult mice forebrain facilitates potentiation and improves learning and memory (Tang et al., 1999).

In addition to increase in AMPAR number, an increase in single-channel conductance is observed in young animals (P13–P15) and expression systems (Benke et al., 1998; Poncer et al., 2002), but not in adults (Andrásfalvy and Magee, 2004).

In contrast to CaMKII-dependent LTP in adult hippocampus, LTP in neonatal hippocampus (P7–P8) requires PKA, and activation of PKA by forskolin occludes LTP (Yasuda et al., 2003). Interestingly, at later stages of development (P14), LTP can only be fully blocked following coapplication of a CaMKII inhibitor with either a PKC inhibitor or a PKA inhibitor. This suggests that parallel CaMKII and PKA/PKC pathways are involved in LTP induction and, furthermore, one can compensate for the other (Wikström et al., 2003). Interestingly, several Ser/Thr phosphorylation sites for these kinases have been identified on the GluA1 subunit and phosphorylation of at least two of these is required for LTP (e.g. Esteban et al., 2003; Boehm et al., 2006; Lee et al., 2007; Lee et al., 2010). In contrast, PKA phosphorylation is sufficient to drive surface expression of recombinant GluA4 receptors, which may contribute to the lower threshold and lack of input specificity for plasticity in the developing brain (Esteban et al., 2003).

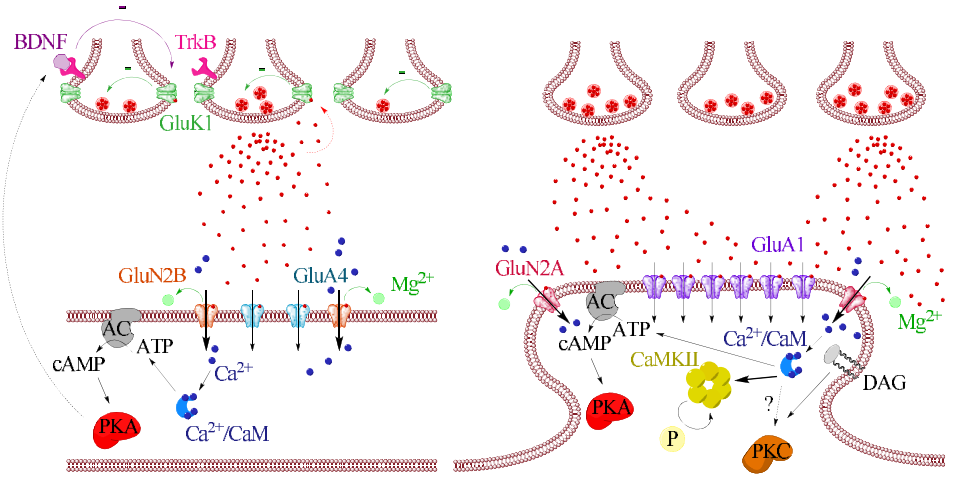

Figure 3. Induction of LTP at SC-CA1 synapses of hippocampus in developing (left) vs. adult brain (right). **LTP in developing brain (left):** Most synapses are located on dendritic shafts (Fiala et al., 1998; Lohmann and Kessels, 2014). Presynaptic terminals show lower probability of release and decreased number of docked vesicles compared to adult (Gasparini et al., 2000; Renger et al., 2001; Mozhayeva et al., 2002; Lauri et al., 2006). Tonically active presynaptic GluK1-containing KARs maintain low Pr (Lauri et al., 2006). Release of glutamate activates GluA4-containing AMPA receptors, the dominantly expressed AMPARs at this age (Zhu et al., 2000), releases Mg^{2+} block of NMDARs and triggers Ca^{2+} influx through GluN1/GluN2B NMDARs which are prevalent at this time (Monyer et al, 1994; Dingledine et al., 1999; Lohmann and Kessels, 2014). Likely candidates for activation by Ca^{2+}/CaM are adenylyl cyclases AC1 and/or AC8, both abundant in CA1 area of hippocampus during development (Nicol et al., 2005), and consequently PKA (Yasuda et al., 2003). Downstream this may lead to the release of brain-derived neurotrophic factor (BDNF). This, in turn, binds to presynaptic TrkB receptors and downregulates KARs controlling glutamate release via G-protein signalling (Lauri et al., 2006; Sallert et al., 2009). **LTP in adult brain (right):** The majority of excitatory synapses in adult brain are located on spines (Harris et al., 1992; Lohmann and Kessels, 2014), providing biochemical and electrical compartmentalisation (Yuste, 2013; Adrian et al., 2014; Araya, 2014). GluA1/GluA2 heteromers are the most abundant AMPARs at that time (Lu et al., 2009). In mature synapses, where both GluN2A and GluN2B are present, GluN2A is dominant (~65%; Gray et al., 2011). GluN2B may be located extrasynaptically (Groc et al., 2006b; Lohmann and Kessels, 2014; but Harris and Pettit, 2007). Ca^{2+} influx through NMDARs activates a number of signalling pathways, with CaMKII playing a key role (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992a; Pettit et al., 1994; Lledo et al., 1995; Otmakhov et al., 1997; Giese et al., 1998). PKA is proposed to gate LTP induction (Blitzer et al., 1998) by phosphorylating GluA1 and making it available for synaptic insertion (Esteban et al., 2003). PKC may help to maintain LTP expression (Pastalkova et al., 2006, but Volk et al., 2013) in addition to a regulatory role during induction (Abeliovich et al., 1993).

In addition to postsynaptic modifications (e.g. see section 2.2), rapid activity-dependent changes in presynaptic efficacy contribute to mechanisms of LTP expression in immature synapses (Choi et al., 2000; Palmer et al., 2004; Lauri et al., 2006; Sallert et al., 2009). Presynaptically labile synapses (see section 2.1.1) may contribute to the phenomenon of silent synapses (presynaptically silent "mute" or "whispering" synapses) observed early in development (Gasparini et al., 2000; Voronin and Cherubini, 2004; although this is controversial Kerchner and Nicoll, 2008). Whether they are presynaptically silent or not, a population of synapses exist in neonate CA1 with initially low probability of release, and characterised by large facilitation in response to brief high-frequency stimulation (Hanse and Gustafsson, 2001; Lauri et al., 2006). In response to LTP induction these synapses demonstrate large increases in Pr (Palmer et al., 2004; Lauri et al., 2006; 2007). These presynaptic changes during LTP are associated with downregulation of tonically active presynaptic high-affinity KARs, sensing ambient levels of glutamate and involving G-protein signalling, via BDNF-TrkB receptor signalling (Lauri et al., 2006; Sallert et al., 2009).

3. Aims

The major aim of this Thesis was to investigate in detail the molecular mechanisms underlying induction and early expression of LTP at CA3–CA1 synapses in the developing rodent hippocampus. More specifically, the aims were:

1. To characterise in detail the signalling mechanisms required for induction and expression of neonatal presynaptic LTP (I).
2. To study the physiological significance of the developmentally restricted expression of GluA4 in CA1 pyramidal cells, focusing on its possible causal link to the differences in LTP induction mechanisms in developing vs. adult hippocampus (II).
3. To examine the postsynaptic expression mechanisms of neonatal LTP and, in particular, the molecular mechanisms underlying activity-dependent synaptic recruitment of GluA4-containing AMPARs (II, III, unpublished).
4. To assess the role of GluA4 in activity-dependent maturation of AMPAR-mediated transmission at CA3–CA1 synapses (II, III, unpublished).

4. Materials and Methods

An overview of the materials and methods is provided in this chapter; for a detailed description see corresponding sections of the original publications indicated by Roman numerals. All experimental procedures were approved by the Research Ethics Committee on Animal Experiments at the University of Helsinki. The methods used in this study are listed in the table 1.

Table 1. List of experimental methods used. Only those procedures in which the author was personally involved are listed here.

Method	Publication
Isolation of hippocampus and protein extraction	II
Preparation of acute hippocampal slices	I–III
Cell culture	III
Transfection	III
Immunohistochemistry	II
Immunocytochemistry	III
Confocal microscopy	III
Whole-cell patch-clamp	I–III
Perforated patch-clamp	I, II
Field potential recordings	II
Stereotactic surgery	II

4.1. Animals (I–III)

Experiments were performed on 4- to 55-day-old Wistar rats (I–III) and 4- to 34-day-old WT or GluA4$^{-/-}$ mice (II, III). GluA4$^{-/-}$ mice were generously provided by Hannah Monyer (University of Heidelberg, Heidelberg; Fuchs et al., 2007).

4.2. Slice preparation (I–III)

Animals were decapitated, and the brains were rapidly removed from the skull and submerged in ice-cold dissection artificial cerebrospinal fluid (ACSF) containing high Mg^{2+} (10 mM) and equilibrated with 95% O_2/5% CO_2. Parasagittal hippocampal slices [250 µm for immunohistochemistry (II), 350–400 µm for electrophysiology] were cut with a vibratome (Leica Microsystems, Wetzlar, Germany) in the dissection solution and placed in a recovery chamber, submerged in solution containing the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 3 $MgSO_4$, 26 $NaHCO_3$, 15 D-glucose, and 2 $CaCl_2$ (bubbled with 95% O_2/5% CO_2; 45 min at 32°C, then at room temperature). To prevent recurrent excitation, the CA3 region of the slices was cut in experiments where evoked excitatory postsynaptic currents (EPSCs) were recorded.

4.3. Electrophysiology (I–III)

After 1–5 hour storage in a recovery chamber, an individual slice was transferred to the recording chamber where it was constantly perfused with oxygenated ACSF containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 26

$NaHCO_3$, 15 D-glucose, and 2 $CaCl_2$ (flow rate 1–1.5 ml/min, at 30°C). Electrophysiological experiments were performed on CA1 pyramidal cells under visual guidance using a MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA, USA) in voltage-clamp mode. Fluorescent neurons (in case of transfected neurons) were identified under UV illumination with parallel differential interference contrast or bright-field optics.

For perforated patch recordings, electrodes with high resistance (6–12 MΩ) were used and amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) was added to the filling solution at 300 µg/ml. Amphotericin B diffuses into the membrane and forms ion-permeable pores (permeable to monovalent cations), allowing electrical access without washout of important intracellular components, e.g. those critical for LTP induction/expression. For whole-cell patch-clamp, electrodes (3–5 MΩ) contained the following (in mM): 130 $CsMeSO_4$, 10 HEPES, 0.5 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 5 QX-314, and 8 NaCl (278±5 mOsm, pH 7.2–7.4). Uncompensated series resistance (for whole-cell recordings <30 MΩ, for perforated patch <150 MΩ) was monitored throughout recordings by measuring the instantaneous whole-cell capacitance current in response to a –5 mV voltage step command applied during each sweep [every 15–20 s for recordings of evoked responses, every 2 minutes for recordings of action potential-independent spontaneous miniature EPSCs (mEPSCs)], and recordings were discarded from analysis if this parameter changed by >20%. Evoked EPSCs were elicited by Schaffer collateral pathway stimulation with a bipolar electrode in the presence of picrotoxin (table 2). For mEPSC recordings, tetrodotoxin was also included in the perfusing solution (table 2). Baseline stimulation frequency was 1/20 s^{-1} or 1/15 s^{-1}, and the intensity was adjusted to the minimum strength eliciting a stable response and, for experiments estimating AMPA/NMDA ratio (III), with average amplitude in the range 20–50 pA. Synaptic facilitation was tested before and 20–30 min after the pairing protocol by brief bursts of high-frequency stimulation (5 pulses at 50 Hz) given at 60 s intervals (I). LTP was induced by pairing postsynaptic depolarisation (–10 mV) to 10 short bursts (five pulses at 50 Hz at 5 s intervals) of afferent stimulation (I, II).

To activate a few presynaptic fibres, a minimal stimulation protocol was employed. Briefly, the stimulus intensity was set so that 25% change didn't affect response amplitude or failure rate, and failures were observed about 50% of the time (Stevens and Wang, 1995; Isaac et al., 1996). Synaptic transmission was elicited at low frequency (1/15 s^{-1}) to avoid frequency-dependent synaptic depression (Saviane et al., 2002; Voronin and Cherubini, 2004).

Field excitatory postsynaptic potentials (fEPSPs; II) were recorded in an interface chamber, using ACSF-filled electrodes (2–4 MΩ) positioned within the CA1 stratum radiatum. Synaptic responses were evoked every 15 s and the slope of the initial rising phase of fEPSPs (20–80%) was used as a measure of the efficacy of synaptic transmission. Stimulation intensity was adjusted such that baseline fEPSP slope was 20–40% of the slope at maximal intensity that resulted in the appearance of a population spike. LTP was induced by tetanic stimulation (100 Hz for 1s).

Input specificity of LTP was confirmed by activating two independent pathways in the recordings (field and some perforated patch recordings). Independence was assessed by comparing paired-pulse facilitation at 50 ms interval between and within inputs during the baseline period (e.g. Bliss et al., 2007).

For manipulation of ion currents and signalling cascades different pharmacological compounds were used (table 2). The relevant purified glutathione S-transferase (GST)-fusion proteins or GST were prepared by Drs. S. K. Coleman or J. Huupponen as described (Coleman et al., 2010) and included in the intracellular solution at a concentration of 0.5 µM. Encoded residues were GluA1(827–907) (Uni-Prot KB no. P19490); $GluA2_{Long}$(834–901) (UniProtKB no. P19491-3); $GluA2_{Short}$(834–883) (UniProtKB no. P19491-1); and GluA4(835–902) for complete GluA4 CTD (Uni-ProtKB no. P19493); all based on numbering of the full polypeptide sequence. The GluA4 CTD mutations encoded the residues GluA4(870–902), GluA4(835–869), GluA4(835–896), GluA4(835–902; S862A), GluA4(835–902; S862D), GluA4(835–902; R841S, K845S, R846S).

Table 2. Pharmacological tools used in electrophysiological experiments. The pharmacological compounds were obtained from Tocris Bioscience (Bristol, UK), Sigma-Aldrich (St. Louis, MO, USA) and Abcam (Cambridge, UK).

Drug	Concentration	Action	Application	Publication
Picrotoxin	100 µM	Noncompetitive $GABA_A$ antagonist	Bath	I–III
Tetrodotoxin	1 µM	Voltage-gated Na^+ channel blocker	Bath	II, III
Forskolin	50 µM	AC activator	Bath	II, III
PKI 14-22 amide	1 µM	PKA inhibitor	Bath	I
KT 5720	1–2 µM	PKA inhibitor	Bath	II
BAPTA-AM	5 mM	Cell-permeant Ca^{2+} buffer	Filling solution	I
Nitrendipine	10 µM	L-type Ca^{2+} channel blocker	Bath	I
D-AP5	50 µM	Competitive NMDAR antagonist	Bath	I
MK801	40 µM	Noncompetitive NMDAR antagonist	Bath	I
Bisindolylamide XI	0.5 µM	PKC inhibitor	Bath	I
PKA inhibitor fragment (6-22) amide (PKI)	100 µM	PKA inhibitor	Filling solution	II
KN-62	3 µM	CaMKII inhibitor	Bath	II

4.4. Stereotactic surgery (II)

Lentiviral vectors encoding enhanced GFP (EGFP)-GluA1, EGFP-GluA4 or EGFP (produced by Dr. J. Huupponen) were injected into area CA1 of 0- to 5-day-old rat pups under isoflurane anaesthesia. The animals were injected subcutaneously with 0.05 ml of Rimadyl 1 mg/ml (1:50 dilution in PBS; Pfizer, Helsinki, Finland) on the day of operation and the following 2 days. The pups were fixed onto a stereotactic

frame and injected subcutaneously into the skull with 0.02–0.03 ml of 20 mg/ml lidocaine (Orion Pharma, Espoo, Finland). The skull was exposed and small holes were created in each hemisphere using a dental drill. Three injections of 0.7 µl of lentiviral suspension were made into the hippocampus of each hemisphere. The stereotactic coordinates for CA1 were recalculated with the respect to bregma–lambda distance and varied in the following range: anteroposterior 1.2–1.6, mediolateral 1.2–1.6, dorsoventral 1.6–2.0. The wound was treated with Bacibact gel (Orion Pharma, Espoo, Finland) and sutured, and the pup was left to recover on a heat pad. As soon as fully recovered, it was returned to its mother.

4.5. Immunohistochemistry (II)

To estimate the rate of EGFP-Glu4 lentiviral infections, 250 µm thick slices from P37–P39 (n=4) rats were fixed with 4% paraformaldehyde (PFA) overnight at +4°C, stained with 300 nM DAPI in 0.3% Triton X-100/PBS for 1 h at room temperature, and then mounted with Fluoromount mounting medium (Sigma-Aldrich, St. Louis, MO, USA).

4.6. Cell culture (III)

4.6.1. Culture and transfections

Primary hippocampal neurons were obtained from E17 mouse embryos provided weekly by the university core facilities. Dissociated cells were plated at a density of 50 000 cells/cm^2 on poly-D-lysine-coated ∅ 12 mm round glass coverslips on 24-well plates in glial cell-conditioned B27-supplemented Neurobasal medium (Life Technologies, Carlsbad, CA, USA). The cells were transfected on day 10 *in vitro* (DIV10) by using the calcium phosphate method (Li et al., 2007). The medium was changed to prewarmed Neurobasal (no supplements) containing 10 mM $MgCl_2$ (transfection medium) 1 h before the transfection. For each well, a total of 35 µl contained: 2 µg of plasmid DNA, 0.25 M $CaCl_2$ mixed with 17.5 µl of 2×HEPES-buffered saline (pH 7.06; 42 mM HEPES, 274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 15 mM D-glucose), and the transfection mixture was added dropwise to the cells. Cells were incubated at 37°C in 5% CO_2 for 4 hours. After a fine precipitate was formed, cells were washed 2–3 times with prewarmed Neurobasal (no supplements) containing 10 mM $MgCl_2$. Thereafter, the transfection medium was replaced with the original glial-conditioned culture medium. The cells were analysed 5–7 days later (DIV 15–17).

Constructs used for transfection (provided by Dr. S. K. Coleman) were based on the rat GluA4 (UniProt P19493; flip isoform) containing full-length construct encoded residues 22–902 (residues 1–21 encode the signal peptide) with N-terminal EGFP tag. The encoded residues in EGFP-GluA4 mutants were: EGFP-A4(22–896), EGFP-A4(22–837, 870–902), EGFP-A4(22–902; R841S, K845S, R846S).

4.6.2. Immunofluorescence staining and confocal microscopy

For surface immunostaining of EGFP-constructs, anti-GFP antibodies (table 3) were added into each well and incubated for 30 min at room temperature. Then hippocampal neurons were washed in PBS (2 × 10 min) and fixed in 4% PFA in PBS for 20 min, rinsed with PBS (2 × 10 min), and permeabilised in 0.2 % Triton X-100 in PBS for 10 min. The cells were then incubated for 2 h with 4% bovine serum albumin (BSA), 3% normal goat serum (NGS), 0.05% gelatine and 0.2% Triton X-100 (blocking solution; in PBS) and left overnight at +4°C with the primary antibodies/antisera in the blocking solution. For colocalization studies, anti-PSD95 monoclonal antibody was used, and in some cases anti-MAP2 mouse monoclonal antibody was used as a dendritic marker and anti-GAD65 mouse monoclonal antibody for identification of GABAergic hippocampal neurons (table 3). The stained cells were washed with 0.2% Triton X-100/PBS (1 × 10 min) and with PBS/1%BSA/1%NGS (2 × 10min) and incubated with appropriate Alexa Fluor 405 (blue)- and Alexa Fluor 568 (red)-conjugated secondary antibodies (1:500; Life Technologies, Carlsbad, CA, USA). After the cells were mounted in Fluoromount, images were acquired as z-stacks using the 63× oil immersion objective and 0.7× mechanical zoom at optimal resolution using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Table 3. List of antibodies used.

Primary antibody	Host	Dilution	Provider	Publication
GFP	Rabbit polyclonal	1:1000	Abcam (Cambridge, UK)	III
PSD95	Mouse monoclonal	1:1000	NeuroMab (Davis, CA, USA)	III
MAP2	Mouse monoclonal	1:500	Sigma-Aldrich (St. Louis, MO, USA)	III
GAD65	Mouse monoclonal	1:1000	EMD Millipore (Billerica, MA, USA)	unpublished

4.7. Data analysis (I–III)

4.7.1. Electrophysiology (I–III)

The WinLTP 2.01 program (Bristol, UK; Anderson and Collingridge, 2001) was used for data acquisition. Prior to analysis, recordings were low-pass filtered to 1 kHz with ClampFit 9.2 (Molecular Devices, Sunnyvale, CA, USA). Spontaneous events were detected using the MiniAnalysis Program 6.0.7. (Synaptosoft, Decatur, GA, USA). The amplitude threshold was set to 4–5 times of the baseline RMS (root mean square) noise level and all detected events were verified visually. Evoked synaptic responses were analysed using WinLTP. The amplitude of AMPA currents at –70 mV was measured as the peak relative to the average baseline level before the stimulation, for NMDA currents at +40 mV 50–60 ms after stimulation (when AMPA component is fully decayed), and fEPSP slope was calculated between 20–80% of the peak amplitude. In experiments, where minimal stimulation was used, the amplitude threshold for identification of responses vs. failures at both –70 and +40 mV was set

to 2 times of the RMS noise level at +40 mV due to the higher noise level at this potential. All responses were verified visually and were invariant in shape.

For time course plots, detected events were calculated in 60 s or 120 s bins and normalised to the baseline level. For bar charts, data are presented as percentage of the last 10 min of the relevant dataset after drug application or LTP induction relative to the baseline level.

4.7.2. Image analysis (III)

Images were collapsed to maximal projection and analysed in Matlab with SynD (Schmitz et al., 2011). Background fluorescence was measured in a region without cells and subtracted prior to analysis in ImageJ (Schneider et al., 2012). Synapses were detected based on the staining for PSD95. Detected regions were subsequently used to measure the synaptic intensity of surface expression of EGFP-constructs. Mean fluorescent intensity of the soma was calculated by averaging the intensity from 10 regions of interest (ROIs) placed in the soma. Synaptic recruitment was estimated as the ratio of the mean intensity at synapses to mean somatic intensity, while dendritic delivery was calculated as mean dendritic intensity (including synaptic and extrasynaptic regions) to mean somatic intensity.

4.7.3. Statistical analysis (I–III)

All the error bars represent the standard error of the mean (SEM). Statistical significance has been assessed using ANOVA or Student's two tailed t-test in SigmaPlot 11 (Systat Software, San Jose, CA, USA) or IBM SPSS Statistics (IBM Corporation, North Castle, NY, USA). If the assumption of normal distribution of the residuals failed, random permutation tests for ANOVA were performed in R software (R Core Team, 2014). The age-dependence was tested using simple linear regression analysis (method of least squares). $p<0.05$ was considered as statistically significant.

5. Results and discussion

5.1. Properties of LTP at the developing CA3–CA1 synapses (I, II)

In order to study the mechanisms of LTP at developing synapses, we chose to concentrate on NMDAR-dependent LTP in CA1 area of hippocampus where LTP mechanisms at mature synapses are well characterised. Stable input-specific LTP at neonate (P5–P8) CA1 synapses was induced by pairing postsynaptic depolarisation (–10 mV) with 10 short bursts (5 pulses at 50 Hz with 5 s intervals) of afferent stimulation (II: Fig. S2). The pairing protocol (with Cs^+ added to the filling solution) allows experimenters to accurately control postsynaptic depolarisation and, therefore, to determine the LTP requirements downstream of NMDAR activation (Nicoll and Roche, 2013; Granger and Nicoll, 2014). We first confirmed that this LTP was induced postsynaptically and depended on NMDAR activation and subsequent Ca^{2+} rise in the postsynaptic cell [I: Fig. 4A (a), Fig. 4C (a)], similar to plasticity at mature CA3–CA1 synapses. Consistent with previous studies (Yasuda et al., 2003), LTP at CA3–CA1 synapses during the first week of development required PKA (I: Fig. 5A (a); II: Fig. 3B), but not CaMKII activation (II: Fig. 3C), which is the pivotal player in LTP induction later in the development (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992a; Pettit et al., 1994; Lledo et al., 1995; Otmakhov et al., 1997; Giese et al., 1998).

Neonate CA1 synapses are highly heterogeneous in short-term synaptic dynamics (Hanse and Gustafsson, 2001; Palmer et al., 2004; Lauri et al., 2006), that most likely represents heterogeneity in glutamate release probability (Hanse and Gustafsson, 2001). Therefore, we divided our inputs in two groups according to their initial Pr by measuring the response to a train of 5 stimuli at 50 Hz: facilitatory (ratio of $5^{th}/1^{st}$ EPSC >2) and non-facilitatory (ratio of $5^{th}/1^{st}$ EPSC <2) (I: Fig. 1D, 1E). In our experiments we observed significantly bigger LTP as well as a profound decrease in facilitation after LTP induction at facilitatory synapses (Fig. 4; I: Fig. 1), which is in agreement with previous studies of our group (Lauri et al., 2006) and others (Palmer et al., 2004).

Change in synaptic facilitation is generally considered as a sensitive measure for variation in Pr (Manabe et al., 1993), though postsynaptically mediated changes in PPF are also theoretically possible, and have been observed in some conditions. For example, AMPARs inserted upon LTP induction may have different subunit distribution (higher proportion of GluA2-containing AMPARs) which can explain decrease in facilitation (Bagal et al., 2005). Lateral diffusion, desensitisation or saturation of AMPARs may lead to underestimation of presynaptic facilitation. Theoretically, a decrease in facilitation would be observed if LTP resulted from unsilencing of a population of synapses with higher Pr, when compared to Pr of synapses activated before LTP (Bliss et al., 2007). Previous studies (Palmer et al., 2004; Lauri et al., 2006) suggest that one of the expression mechanisms of neonatal LTP is an increase in Pr that may fully account for the observed decrease in synaptic facilitation. Therefore, LTP at neonatal facilitatory synapses most likely involves both pre- (increase in Pr) and postsynaptic expression mechanisms. In contrast,

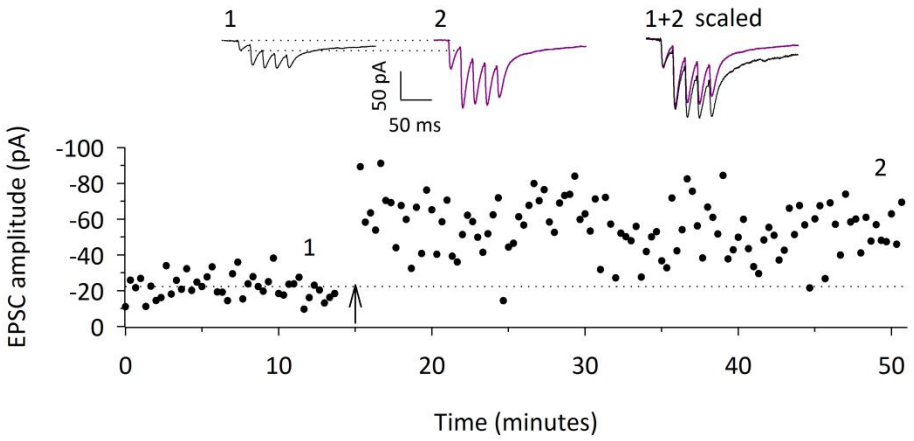

Figure 4. Single example of typical LTP experiment at facilitatory CA3–CA1 synapse with traces of facilitation before (1) and after (2) LTP induction (example of an experiment from I). The arrow indicates the time when pairing protocol was applied.

non-facilitatory synapses showed no change in facilitation in response to LTP induction, consistent with mechanisms relying solely on postsynaptic modifications. Consistent with others (Palmer et al., 2004; Lauri et al., 2006), we found that the presynaptic mechanisms of LTP were developmentally downregulated and were only observed during the first week of development (I: Fig. 2). Thus, pre- and postsynaptic mechanisms for expression of LTP coexist in the neonatal CA1 in a population of synapses with initially low Pr.

5.2. Mechanisms underlying presynaptic LTP at immature synapses (I, II)

Having established that pre- and postsynaptic mechanisms of LTP expression coexist in some CA3–CA1 synapses during the first week of development, we examined the signalling cascades required for pre- vs. postsynaptic LTP and whether they can be differentiated mechanistically.

At mature CA1 synapses presynaptic changes can be induced in an NMDAR-independent and an L-type Ca^{2+} channel-dependent manner in response to stronger stimulation protocols (e.g. Zakharenko et al., 2001; Padamsey and Emptage, 2014; see section 2.2.1.1). In contrast, we found that presynaptic and postsynaptic expression mechanisms of LTP in immature CA3–CA1 synapses were tightly coupled together (I).

Induction of presynaptic as well as classic postsynaptic changes at immature synapses required correlated pre- and postsynaptic activation, NMDAR activity, an increase in postsynaptic Ca^{2+}, and activation of PKA, but not L-type Ca^{2+} channels (I: Fig. 3–4, 5A). Previous studies support the role of postsynaptic Ca^{2+} for activity-dependent control of presynaptic function at immature CA3–CA1 synapses and cultured hippocampal neurons (Shen et al., 2006; Mohajerani et al., 2007). However, the role of NMDA receptors vs. L-type Ca^{2+} channels is more controversial (Shen et

al., 2006; Mohajerani et al., 2007). In our experiments the presence of an L-type Ca^{2+} channel blocker (nitrendipine) had no effect on pairing-induced increase in synaptic efficacy or the associated decrease in high-frequency facilitation (I: Fig. 4B). It is possible that the pairing protocol is not very efficient in activating L-type Ca^{2+} channels due to prolonged depolarisation which might cause desensitisation (Padamsey and Emptage, 2014). Alternatively, any role for L-type Ca^{2+} channels may only manifest under conditions when the NMDAR-dependent route is inaccessible. Inhibition of PKA blocked both pre- and postsynaptic components of LTP (I: Fig. 5A). Therefore, in these experiments it is not possible to distinguish whether PKA plays a role solely in a shared postsynaptic induction mechanism as shown before (Yasuda et al., 2003) or there is an additional function of PKA in the control of release probability subsequent to LTP induction. However, our other data suggested that a small (~15%), presumably presynaptic component of forskolin-induced PKA-dependent potentiation of EPSCs did exist at this developmental stage in the presence of PKA inhibitor in the postsynaptic cell (via the patch pipette) (II: Fig. 1A). PKA can directly phosphorylate presynaptic proteins, including SNAP-25, RIMs, and synaptotagmin, and, therefore, regulate properties of fusion machinery as well as the process of vesicle priming and recycling (Leenders and Sheng, 2005; Maximov et al., 2007; Kaeser et al., 2008; Park et al., 2014).

In a further attempt to discriminate between signalling cascades responsible for pre- vs. postsynaptic components of the neonatal LTP, we studied the role of PKC. Whereas the inhibition of PKA suppressed both pre- and postsynaptic components of LTP, inhibition of PKC selectively blocked the LTP-associated decrease in facilitation whilst having no effect on the level of (presumably postsynaptically expressed) LTP *per se*. Presynaptically expressed changes of synaptic efficacy that are dependent on postsynaptic induction mechanisms require the generation of some retrograde signal. Diffusible retrograde messengers BDNF and nitric oxide have been shown to regulate Pr via depression or enhancement of presynaptic KAR activity, respectively (Sallert et al., 2009; Clarke et al., 2014). Interestingly, the increase in Pr associated with NMDAR-independent LTP at mature synapses is also dependent on BDNF signalling (Zakharenko et al., 2003). BDNF-TrkB signalling activates parallel signal transduction cascades with various functions, including downstream activation of PKC (Minichiello, 2009). Moreover, activation of protein kinases (including PKC) in the presynaptic terminal is associated with increased transmitter release (Leenders and Sheng, 2005). Phosphorylation by PKC promotes endocytosis of KARs (Martin and Henley, 2004; Rivera et al., 2007; Konopacki et al., 2011; Chamberlain et al., 2012) which may underlie the switch from (KAR-dependent) high to low facilitation. PKC activation has also been shown to increase the size of the readily releasable pool of vesicles and the rate of pool replenishment at glutamatergic synapses in hippocampal cell culture (Stevens and Sullivan, 1998). Among the proteins of vesicle release machinery, one candidate for a downstream target in PKC pathway is munc18-1 which is rapidly phosphorylated upon depolarisation (de Vries et al., 2000; Craig et al., 2003). Phosphorylation of munc18-1 increases vesicle fusion efficiency and induces the redistribution of

vesicles towards the active zone. Furthermore, the efficacy of release during paired-pulse and repetitive stimulation depends on munc18-1 phosphorylation by PKC in autaptic hippocampal neurons (Wierda et al., 2007).

Interestingly, intracellular signalling underlying KAR-dependent depression of Pr at CA3–CA1 synapses has been shown to involve G-protein- (Lauri et al., 2006) and PKC-dependent mechanisms (Sallert et al., 2007). If tonic KAR-dependent depression of release is mediated via PKC signalling, we would not expect synapses to display high facilitation in presence of a PKC inhibitor. Indeed, the average level of synaptic facilitation in the presence of a PKC inhibitor was lower than in control slices (I: Fig. 5B). Therefore, the blockade of the LTP-associated decrease in facilitation in the presence of a PKC inhibitor could be, in part, due to occlusion of this tonic inhibition. However, we could still find synapses with facilitation considered high according to the chosen criteria (ratio of 5th/1st EPSC >2), suggesting that while PKC may play some modulatory role, it is not the only mechanism by which KARs depress release of glutamate or there are other KAR-independent mechanisms mediating high facilitation.

5.3. GluA4 subunit defines the induction mechanism of neonatal LTP (II)

Developmental changes in LTP induction mechanisms (from PKA- to CaMKII-dependent, see section 2.2.1) occur simultaneously with the switch in subunit composition of AMPARs (from GluA4- to GluA1-containing, see section 2.1.2.2). A role for GluA4 in PKA-dependent neonatal LTP has been suggested based on findings that spontaneous neuronal activity or PKA phosphorylation is sufficient to deliver AMPA receptors containing recombinant GluA4, but not GluA1, to synapses (Zhu et al., 2000; Esteban et al., 2003). However, the exact role of the developmentally confined expression of GluA4 and its possible causal link to the differences in LTP mechanisms are still not clear. Therefore, to test whether GluA4 expression can sufficiently explain the developmental switch in kinase dependency of LTP induction we compared pairing-induced LTP in P5–P8 wild type (WT) and GluA4$^{-/-}$ mice (II: Fig. 3A). Though the level of potentiation after LTP induction in WT and GluA4$^{-/-}$ mice was similar under normal conditions, a PKA inhibitor added into bath solution fully blocked LTP in WT mice but had no effect on the potentiation level in knockout animals (II: Fig. 3B). This indicates that 1) GluA4 is necessary for the PKA-dependence of the neonatal LTP and 2) the PKA/GluA4-dependent mechanism is not crucial for activity-dependent plasticity and the development of immature contacts and that the loss of GluA4 can be compensated for by other mechanisms. Indeed, the genetic loss of GluA4 increased the levels of the GluA1 subunit in the hippocampus (II: Fig. 3D), which is the dominant subunit in mature CA1 pyramidal cells (Lu et al., 2009) when LTP is CaMKII-dependent (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992a; Pettit et al., 1994; Lledo et al., 1995; Otmakhov et al., 1997; Giese et al., 1998). Furthermore, LTP induction at CA3–CA1 synapses of neonatal GluA4$^{-/-}$ mice was fully blocked by a CaMKII inhibitor (II: Fig. 3C), implying that CaMKII/GluA1-dependent mechanism compensates for the loss of PKA-dependent LTP in absence of GluA4.

Next, to test whether GluA4 expression at mature CA1 pyramidal cells would be sufficient to change induction mechanisms of LTP, we used lentiviral constructs to produce the stable expression of either EGFP-GluA4 or EGFP-GluA1 (as a control) in CA1 pyramidal neurons *in vivo* (II: Fig. 4A). LTP was analysed using field potential recordings, which allowed us to perform longer experiments required to test the reversibility of different kinase inhibitors. LTP was induced in the presence of a CaMKII inhibitor in GluA4-transfected slices (>P27; II: Fig. 4C, F), but was completely blocked in GluA1-transfected slices (II: Fig. 4B, F) as shown previously for WT animals at this developmental stage (e.g. Yasuda et al., 2003; Wikström et al., 2003). When a combination of CaMKII and PKA inhibitors was applied, LTP in GluA4-transfected slices was completely blocked (II: Fig. 4E, F). Therefore, GluA4 expression is sufficient to modify the signalling requirements of LTP induction (Fig. 5). Furthermore, the expression of GluA4 can fully explain the developmental switch in the kinase-dependency of LTP at hippocampal CA3–CA1 synapses. Interestingly, our data suggest that if PKA/GluA4-dependent mechanisms are available, they may override CaMKII/GluA1-dependent LTP. In our experiments, on average ~50% of the CA1 pyramidal neurons expressed EGFP-GluA4 in lentivirally infected adult slices (II: Fig. 4Aii). Field LTP in these slices was partially blocked by PKA antagonism (II: Fig. 4D, F), suggesting that LTP was PKA-dependent in the cells where GluA4 was lentivirally expressed. Furthermore, the CaMKII/GluA1-dependent mechanism was unmasked early in development in the absence of GluA4.

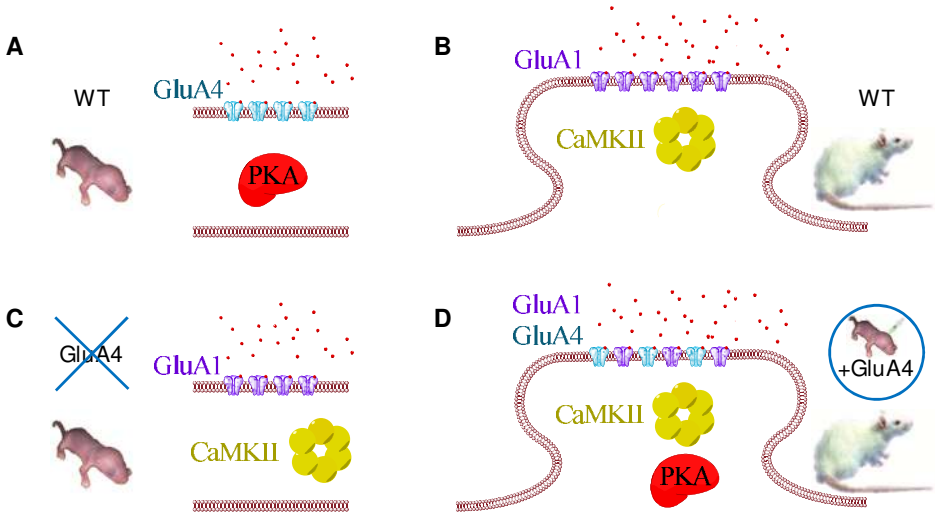

Figure 5. Expression of GluA4 alters the signalling requirements of LTP. A) Neonatal LTP (P5–P8) involves activation of PKA when GluA4 is expressed. B) LTP in adult CA1 pyramidal cells (>P27) requires CaMKII when GluA1 is dominantly expressed. C) In neonatal GluA4 knockout mice, GluA1 is upregulated as an apparent compensatory mechanism and LTP now depends on CaMKII. D) When both endogenous GluA1 and lentivirally transduced EGFP-GluA4 are present in CA1 area of adult hippocampus, LTP induction involves activation of both PKA and CaMKII. However, whether this occurs at the level of a single synapse or within separate populations of GluA1- and GluA4-expressing synapses, cannot be resolved from our current data.

In neonate, the PKA pathway may be more advantageous to use in the absence of biochemical and electrical compartmentalisation when Ca^{2+} transients are spatially more diffuse: AC1 with its higher Ca^{2+} affinity is ideally suited to sensing low Ca^{2+} transients (EC_{50}-values for Ca^{2+} of AC1 vs. CaMKII are 150 nM and 500–1000 nM, respectively) (Hudmon and Schulman, 2002; Yasuda et al., 2003; Ferguson and Storm, 2004). In contrast, in adults, the limited spine-neck diffusion may compartmentalise the Ca^{2+} signal in spines for up to 1–10 s, easily achieving the required synapse specificity (Sabatini et al., 2002) even with a low-affinity Ca^{2+} trigger such as CaMKII.

5.4. Postsynaptic LTP expression: synaptic recruitment of GluA4-containing AMPA receptors and underlying molecular mechanisms (II, III, unpublished)

Postsynaptically, different modifications of AMPARs underlie LTP expression (see section 2.2). At immature synapses, addition of functional AMPARs to previously silent synapses provided the first evidence to suggest synaptic insertion of AMPARs upon LTP. This mechanism provides a postsynaptic explanation for some experimental observations such as changes in failure rate, coefficient of variation or frequency of mEPSCs, previously interpreted as presynaptic modification during LTP (Kullmann, 1994; Liao et al., 1995; Isaac et al., 1995; Kullmann and Siegelbaum, 1995; Durand et al., 1996; see section 2.2.1.2). To date, a large amount of evidence supports the idea that AMPA receptor number increases at both mature and immature synapses following the induction of LTP (Shi et al., 1999; Hayashi et al., 2000; Lu et al., 2001b; Pickard et al., 2001; Malinow and Malenka, 2002; Poncer et al., 2002; Andrásfalvy and Magee, 2004). A suggested model for activity-dependent recruitment of AMPARs involves the insertion of receptors to extrasynaptic areas followed by lateral diffusion to synaptic sites and subsequent capture within the PSD (Opazo and Choquet, 2011; see section 2.1.2.2). However, in early development, spines which would limit lateral diffusion are mostly absent (Fiala et al., 1998; see section 2.1.2.1). Thus, mechanisms of AMPAR recruitment to immature synapses and therefore LTP expression are not fully understood.

To study the molecular mechanisms underlying expression of neonatal LTP, we used forskolin, a selective activator of AC and consequently PKA. Application of forskolin produces significant potentiation of glutamatergic transmission that occludes LTP at P7–P8 (Yasuda et al., 2003) and can thus be considered as a model for chemically induced neonatal LTP. In our experiments we observed robust forskolin-induced potentiation of evoked EPSCs (II: Fig. 1A) as well as an increase in frequency and amplitude of mEPSCs (II: Fig. 1B, 2A). Increase in mEPSC amplitude suggests either an increase in AMPAR number or their conductance or both, whereas a change in frequency corresponds to either an increase in Pr or number of release sites/synapses. PKI as well as a GST-fusion protein containing the full-length CTD of GluA4 (GST-A4 CTD) in the filling solution fully blocked or significantly reduced the forskolin-induced increase in mEPSC amplitude and mEPSC frequency, respectively (II: Fig. 1B). This suggests that PKA activation leads to an increase in

the number of synapses via postsynaptic unsilencing (see section 2.2.1.2), in addition to influencing AMPAR function at existing synapses.

To study the pre- vs. postsynaptic effect of forskolin in more detail, we used minimal stimulation techniques (Stevens and Wang, 1995; Isaac et al., 1996) to record both AMPA (at –70 mV) and NMDA (slow component at +40 mV) currents in neonate WT and GluA4$^{-/-}$ mice (III: Fig. 1). In WT mice, application of forskolin led to a significant increase in the amplitude of AMPAR-mediated EPSCs (average of all responses including failures), due to an increase in both potency (average size of successful responses) and success rate. We observed no significant changes in potency or success rate of NMDAR-mediated EPSCs. The most parsimonious explanation for these results is that PKA activation increases AMPA receptor number or conductance at previously undetected release sites (i.e. postsynaptically silent or below threshold of detection). However, the lack of any effect on NMDAR-mediated EPSC success rate is not consistent with our previous data suggesting an additional small component of PKA-dependent presynaptically expressed LTP (II: Fig. 1). One explanation may be that a modest presynaptic effect, even if present, may not be observed. The switch in the NMDARs composition upon LTP induction in neonates (Bellone and Nicoll, 2007) from GluN2B- to GluN2A-containing receptors, with lower affinity to glutamate and faster decay/desensitisation, may mask any presynaptic effect on NMDA currents. Furthermore, PKA-dependent increases in Pr may only be observed in a subpopulation of synapses (e.g. with initially low Pr, see sections 5.1; 5.2). In contrast to the WT mice, forskolin had no effect on the potency of AMPAR-mediated EPSCs in GluA4$^{-/-}$ mice, indicating a critical role for GluA4 in PKA-dependent potentiation of AMPARs. Notably, the success rate for AMPA was lower than for NMDA at ~67% of recorded synapses in WT mice, but there were no such differences in GluA4$^{-/-}$ mice. This suggests that a fraction of postsynaptically silent synapses exists within the population of activated synapses only in WT, and lack of GluA4 affects the proportion of silent inputs in the synapse population.

In conclusion, PKA-dependent unsilencing increases the number of active synapses and, therefore, increases the observed AMPA success rate in WT but not GluA4$^{-/-}$ mice. Some labile synapses under baseline conditions with low number of AMPARs may have small and therefore subthreshold AMPA-mediated postsynaptic currents. PKA-dependent insertion and/or an increase in single-channel conductance of GluA4 subunit-containing AMPARs produce suprathreshold AMPA currents at these synapses and thereby raise the AMPA success rate. Insertion of homomeric GluA4 receptors with higher single channel conductance (Swanson et al., 1997) to silent and/or labile synapses may also explain the increase in AMPA potency (for instance, about 25% of GluA4-containing AMPARs lack GluA2 at P6; Zhu et al., 2000). Also, if more than one fibre is activated with minimal stimulation, an increase in Pr would be expected to increase potency *per se*. However, based on the absence of significant change in NMDA potency in both WT and GluA4$^{-/-}$ mice, we feel that this is unlikely.

NMDAR activity is proposed to suppress the GluA2-dependent synaptic delivery of AMPARs and, therefore, maintain silent synapses in developing brain (2–

3-weeks-old animals; Adesnik et al., 2008; Gray et al., 2011; Lu et al., 2011). In our experiments, in the absence of GluA4, the proportion of silent inputs in the synapse population is decreased, suggesting that NMDARs may silence synapses specifically by controlling trafficking of GluA4-containing receptors at P4–P8. Therefore, the lack of GluA4 may lead to premature stabilisation of AMPAR-mediated transmission. Furthermore, this premature stabilisation in GluA4$^{-/-}$ mice may be CaMKII-dependent (data not shown). Application of the CaMKII inhibitor KN-62 led to significant depression of the amplitude of AMPAR-, but not NMDAR-mediated currents in GluA4$^{-/-}$ mice at P4–P8 ($p<0.01$, n=4; unpublished preliminary data). Such depression was not observed in either WT mice of this age (n=7, p=0.53) or juvenile WT and GluA4$^{-/-}$ mice (P14–P18; n=8, p=0.91 and n=6, p=0.94, respectively). Therefore, the proposed functional lability of developing synapses, which is necessary for activity-dependent refinement of immature contacts (Hanse et al., 2009), may actually rely on GluA4/PKA-dependent mechanisms.

Synaptic trafficking of GluA4-containing AMPARs is largely regulated by interactions mediated by its CTD. Overexpression of GFP-A4 CTD prevents GluA4 synaptic delivery by scavenging the endogenous CTD interactions (Zhu et al., 2000). In agreement, forskolin-induced potentiation of EPSCs was fully blocked in the presence of a GST-fusion protein containing the full-length CTD of GluA4 (GST-A4 CTD) in the filling solution (II: Fig. 1). To determine which GluA4 CTD interactions and interacting partners are important for its synaptic delivery, we used GST-A4 CTD fusion proteins with different mutations, affecting previously described interaction sites (III: Fig. 3A). Our data suggest that two CTD sites are important for PKA-dependent synaptic delivery of GluA4-containg AMPARs at neonatal CA3–CA1 synapses: membrane proximal region (MPR; III: Fig. 3B) and extreme C-terminal sequence (III: Fig. 3D). The MPR has been previously shown to be critical for spontaneous activity-dependent synaptic trafficking of the receptors (Boehm et al., 2006). It incorporates the established interaction sites for protein 4.1N (Coleman et al., 2003), PKCγ (Correia et al., 2003), α-actinin-1 and IQGAP1 (IQ motif-containing GTPase-activating protein 1; Nuriya et al., 2005). GluA4 binding to the cytoskeletal protein 4.1 has been proposed to promote receptor surface expression in heterologous cells (Coleman et al., 2003). IQGAP1 and α-actinin-1 bind GluA4 at the same region, but PKA phosphorylation of GluA4 at Ser862 differentially regulates these interactions: it disrupts the binding to α-actinin-1, but preserves the interaction with IQGAP1. Therefore, the authors suggest that α-actinin-1 keeps GluA4 in the intracellular pool and, upon synaptic activity and GluA4 phosphorylation, the binding to α-actinin-1 is disrupted to allow GluA4 incorporation into synapses (Nuriya et al., 2005). In contrast, Esteban and colleagues show that mutated GluA4, mimicking the dephosphorylated state of Ser862, is not restrained in the intracellular pool and is delivered to the synapses in the absence of neuronal activity (Esteban et al., 2003), questioning the proposed model.

The triple mutant GST-A4 CTD R841S/K845S/R846S (RKR/SSS mutant), in which three positively charged side-chains were neutralised, such that protein 4.1

(Coleman et al., 2003) and presumably PKCγ (Gomes et al., 2007) sites were disrupted, fully blocked the forskolin-induced enhancement of EPSC amplitude (III: Fig. 3C), suggesting that these interactions are not important for PKA-dependent synaptic insertion of GluA4. Both dephospho- and phosphomimetic mutations of GST–A4 CTD at Ser862 also blocked the effect of forskolin (III: Fig. 3C), pointing to a role of IQGAP1 rather than α-actinin-1. However, an interaction between GluA4 and IQGAP1 has not been repeated in any other publication and was not detected *in vivo*, raising the possibility that other yet unidentified mechanisms might be involved. Interestingly, the GST-A4 CTD with six C-terminal amino acids deleted had no effect on forskolin-induced potentiation (III: Fig. 3D). No functional role for the extreme C-terminal region of GluA4 has yet been proposed, while in GluA1 this region contains the motif for PDZ domain[5] interactions (Leonard et al., 1998; Cai et al., 2002) critically involved in trafficking (Hayashi et al., 2000; Malinow and Malenka, 2002). The extreme C-terminal sequence alone was not sufficient to regulate GluA4 trafficking, as GST-A4 CTD with MPR deleted (but the extreme C-terminal sequence present) had no effect (III: Fig. 3B). Thus, it is likely that this region interacts with the MPR, which is involved in the mechanisms regulating GluA4 trafficking.

To further understand the importance of the identified sites for GluA4 trafficking, we studied the distribution of EGFP-tagged GluA4 with different CTD mutations in hippocampal cell culture. As reported earlier (e.g. Zhu et al., 2000; Coleman et al., 2003; Esteban et al., 2003; Coleman et al., 2006), EGFP-GluA4 was delivered to dendrites and expressed on the surface of glutamatergic hippocampal neurons in culture. Furthermore, we also observed that EGFP-GluA4 was readily distributed within dendrites and on the cell surface of GABAergic neurons, identified by staining against the 65 kDa isoform of glutamic acid decarboxylase (GAD65; Fig. 6). EGFP-GluA4 lacking MPR was completely restricted to the soma, most likely trapped in the endoplasmic reticulum (III: Fig. 4A). Both dendritic delivery and synaptic recruitment of EGFP-GluA4 with deletion of the extreme C-terminal sequence were significantly diminished as compared to wild type EGFP-GluA4, while the RKR/SSS mutant behaved similar to WT (III: Fig. 4). Therefore, our data confirm a critical role for the MPR in trafficking of GluA4 and identify a novel mechanism for activity-dependent synaptic delivery of GluA4 by the extreme C-terminal region. The molecular identity of the proteins interacting with these regions to regulate PKA-dependent trafficking of GluA4 cannot be resolved based on our data. While the role of protein 4.1 can be excluded, the data suggest that a yet unidentified interacting protein, by itself or together with IQGAP1, regulates PKA-dependent trafficking of GluA4 at immature hippocampal CA3–CA1 synapses.

[5] A common modular protein interaction domain (~90 amino acids) found in many proteins; acronym from PSD95/synapse-associated protein 90 (SAP90), *Drosophila* discs large homolog 1 (Dlg1; =SAP97), and zonula occludens (tight junction) protein 1 (ZO-1), where PDZ domains have been originally discovered (Sheng and Sala, 2001).

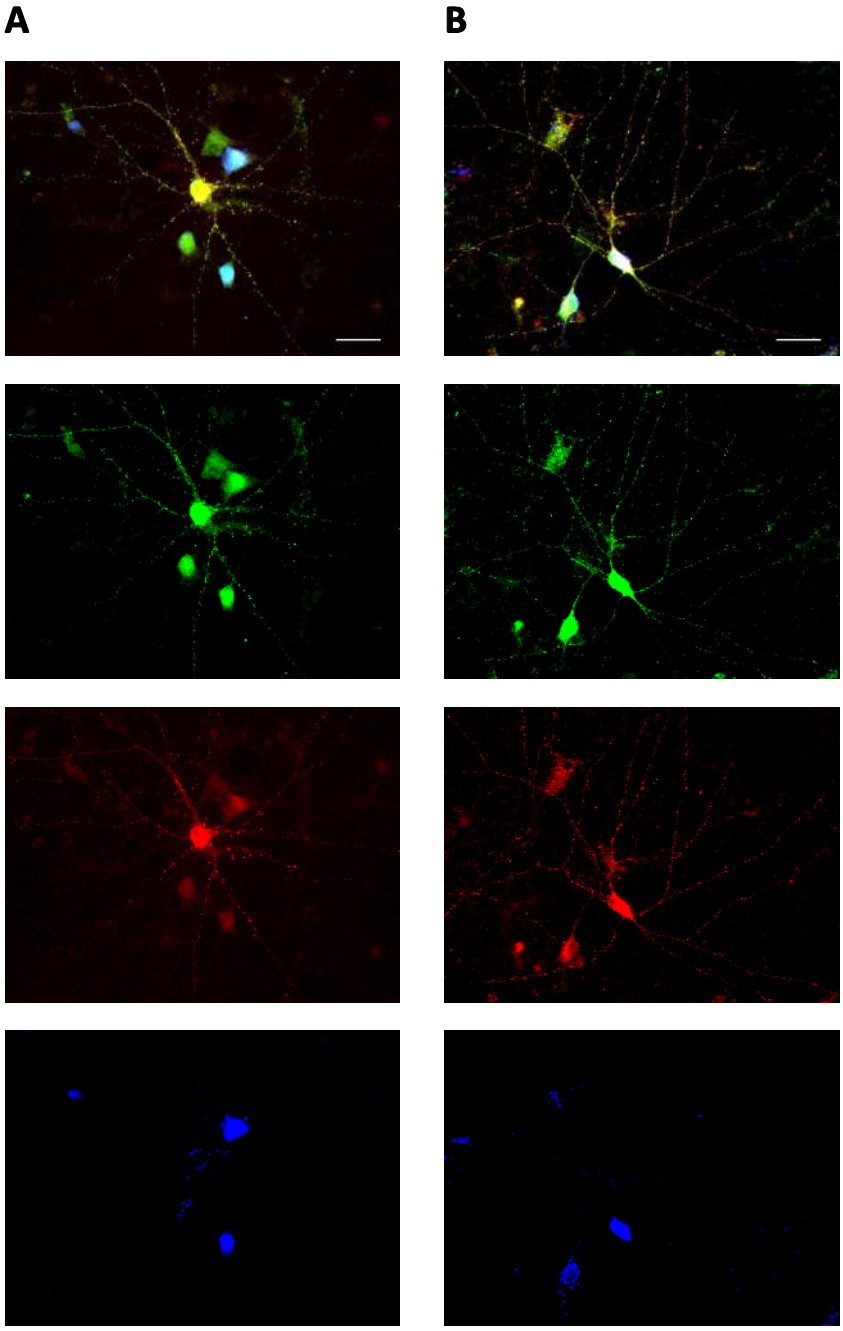

Figure 6. Fluorescent visualisation of EGFP-GluA4 expression (green) in pyramidal cell (A) and GAD65-positive (blue) interneuron (B) in hippocampal cell culture. EGFP-GluA4 is delivered to dendrites and expressed on the surface (red) in both cell types. The neurons have been surface stained for GFP (red) and, after permeabilisation, for GAD65 (blue). Scale bar 25 µm.

5.5. Role of GluA4 in activity-dependent maturation of AMPAR-mediated transmission (II, III, unpublished)

During the first two weeks of development, the relative contribution of AMPARs and NMDARs to postsynaptic currents changes in many brain regions (Crair and Malenka, 1995; Hsia et al., 1998; Lu et al., 2001a; Ye et al., 2005). To study the role of GluA4 in this process, we compared the AMPA/NMDA ratio in WT vs. GluA4$^{-/-}$ mice at different ages. A change in AMPA/NMDA ratio was significantly correlated with age in WT mice during the first two weeks of development (r^2=0.29, p<0.001, n=41), but not in GluA4$^{-/-}$ mice (r^2=0.03, p=0.24, n=45; Fig. 7). In GluA4$^{-/-}$ mice, an abrupt increase in AMPA/NMDA ratio was observed during the third week of development (III: Fig. 2A), corresponding to time when the ratio in WT mice had already stabilised and reached adult levels. Finally, no difference in the AMPA/NMDA ratio was detected between genotypes in adult (P27–P34; 2.0 ± 0.2, n=12 in WT; 2.4 ± 0.2, n=16 in GluA4$^{-/-}$ mice; p=0.21).

To study whether the delay in synaptic AMPAfication (synaptic delivery of AMPARs) during the first two weeks of development in GluA4$^{-/-}$ mice was reflected in the overall development of glutamatergic input to CA1 neurons, we assessed mEPSCs at P4–P6 and P10–P11. No significant differences in either mEPSC amplitude or frequency were detected between genotypes at P4–P6 (II: Fig. 2A) or P10–P11 (III: Fig. 2C). Together, these data suggest that glutamatergic CA3–CA1 circuitry develops in the absence of GluA4, possibly by employing compensatory mechanisms (see section 5.3). However, GluA4-driven AMPA strengthening appears to play a crucial role in the maturation of functional excitatory connections.

Interestingly, GluA4$^{-/-}$ mice exhibit some aspects of schizophrenia-related phenotypes (Sagata et al., 2010). Indeed, much evidence points to a neurodevelopmental model in the origin of schizophrenia (e.g. Fatemi and Folsom, 2009). However, whether GluA4 plays a crucial role in this phenomenon is still uncertain: for example, whilst some polymorphisms located within or very close to the human GluA4 gene are associated with schizophrenia in certain populations (namely Japanese; Makino et al., 2003), no association has been found in others (namely Chinese and Korean; Guo et al., 2004; Crisafulli et al., 2012). Furthermore, whilst GluA4 is expressed in parvalbumin-positive fast-spiking interneurons in adult hippocampus, it is undetectable in these neurons during development (e.g. Pelkey et al., 2015). Knockout of the GluA4 subunit in parvalbumin-positive interneurons results in reduced excitatory drive onto these neurons and disrupts hippocampal population gamma rhythms (Fuchs et al., 2007), and therefore, may also contribute to the observed GluA4$^{-/-}$ mice phenotype.

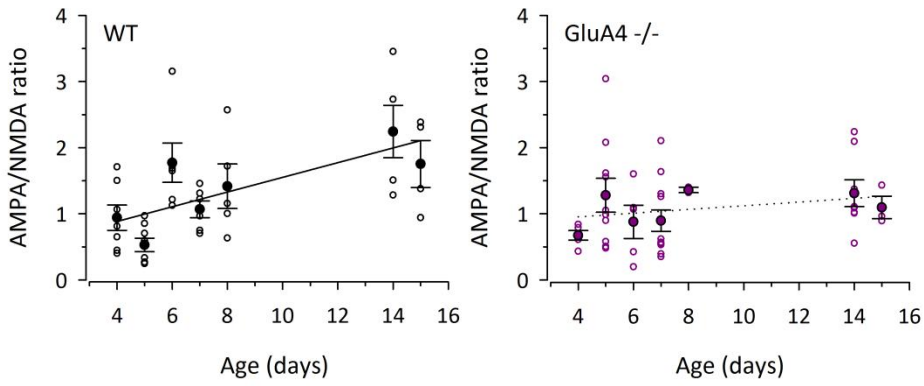

Figure 7. Change in AMPA/NMDA ratio at CA3–CA1 synapses in WT vs. GluA4$^{-/-}$ mice during the first two weeks of development. The association of AMPA/NMDA ratio and age was tested using linear regression analysis. There is a positive relationship between AMPA/NMDA ratio and age in WT mice (r^2=0.29, p<0.001, n=41), but not in GluA4$^{-/-}$ mice (r^2=0.03, p=0.24, n=45).

6. Conclusions

In the present work we have identified several novel plasticity mechanisms that underlie the appropriate refinement and maturation of hippocampal CA3–CA1 circuitry during early postnatal development.

Presynaptically, we show that the population of CA3–CA1 synapses with initially low Pr switch to more reliable transmission (increase in Pr) upon Hebbian-like activity during the first two weeks of development and that this process is specifically regulated by PKC. This rapid, developmentally restricted and PKC-dependent modulation of presynaptic function contributes to the additional presynaptic component of neonatal LTP. In the developing hippocampus, low Pr synapses are effectively tuned to respond only to high frequency natural like bursts of activity. Therefore, neonatal presynaptic LTP mechanisms may play an important role in an initial labelling process that leads to subsequent stabilisation and the formation of mature glutamatergic synaptic connections. Furthermore, PKC-dependent regulation of presynaptic function may adjust the threshold for synaptic plasticity imparting greater capacity to respond to a wider range of LTP-inducing paradigms.

Postsynaptically, we show that the PKA-dependency of LTP is selective to immature synapses due to the developmentally restricted expression of the AMPA receptor subunit GluA4. PKA-dependent insertion of GluA4 is critical for silent synapse activation and strengthening of AMPAR-mediated transmission at immature synapses during network development and requires a previously unidentified molecular mechanism involving interaction between the membrane proximal region and the extreme C-terminal sequence of the GluA4 CTD. The high responsiveness of the GluA4/PKA-dependent mechanism to patterned neuronal activity, characteristic for the developing neuronal circuitry, may provide enhanced capacity for plastic changes during the critical period of development, when synaptic reorganisation takes place. We further demonstrate that developing CA3–CA1 circuits are partially resistant to genetic removal of GluA4. In the absence of GluA4, compensatory mechanisms are expressed to generate LTP, qualitatively similar to that observed in WT circuitry. Intriguingly, we also observed that in the absence of GluA4, the proportion of silent inputs in the synapse population is decreased, implying that the lack of GluA4 may lead to premature stabilisation of AMPAR-mediated transmission. This suggests that either neonatal GluA4/PKA-dependent mechanisms have the ability to restrain the adult phenotype, or redundancy is built into neuronal networks to ensure that the activity is maintained during nascent synapse formation.

In summary, PKA-dependent unsilencing of GluA4-containing receptors, coupled to a PKC-regulated switch from low to high Pr, profoundly alters the dynamics of excitatory synaptic transmission in the developing circuitry. The activity-dependent fine-tuning, underlined by pre- and postsynaptic changes at GluA4-containing synapses, plays an instrumental role in the refinement of

synaptic connections during this critical period of network maturation. This knowledge contributes to our understanding of the mechanisms of brain development at the level of single synapses which may help to resolve how perturbations during development increase the risk of neurological disorders in later life.

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