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The role of Arc/Arg3.1 in hippocampal synaptic plasticity in adulthood and during early postnatal development

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1. Introduction

1.1. Learning and memory

“Like waking from a dream…every day is alone itself…” H.M.

The scientific community considers the early description of H.M. the inauguration of modern memory research. H.M. was a patient who, after a bicycle accident at the age of 9, developed minor seizures and at the age of 27 he could not work or live a normal life due to the severe epilepsy. Thus, he underwent a bilateral medial temporal lobe resection in an attempt to control the epileptic seizures. Besides an amelioration of the seizures, H.M. was no longer able to transfer short-term memory into long-term memory, but memories of childhood events, personality and general intelligence were mostly preserved. The bilateral lobotomy of the medial temporal lobes and consequently the appearance of specific memory deficits established the fundamental principle that memory involves distinct areas of the brain. The neurosurgeon W. Penfield and the psychologist B. Milner systematically studied the memory deficits developed by the patient H.M.[1] and this influenced memory research mainly for two reasons. First, they suggested that memory is a brain function which can be categorized in two main categories (fig. 1) Declarative memory (explicit and/or conscious) of facts and events, requires temporal lobe structures like hippocampus, subiculum, amygdala and entorhinal cortex. The other form of memory, known as procedural memory (implicit and/or unconscious), lies outside the province of the medial temporal lobe, in regions comprising the striatum, amygdala, neocortex, cerebellum[2]. Second, memories to be acquired and then retained might undergo different temporal steps, including an immediate memory which later on consolidates into stable long-term memory.

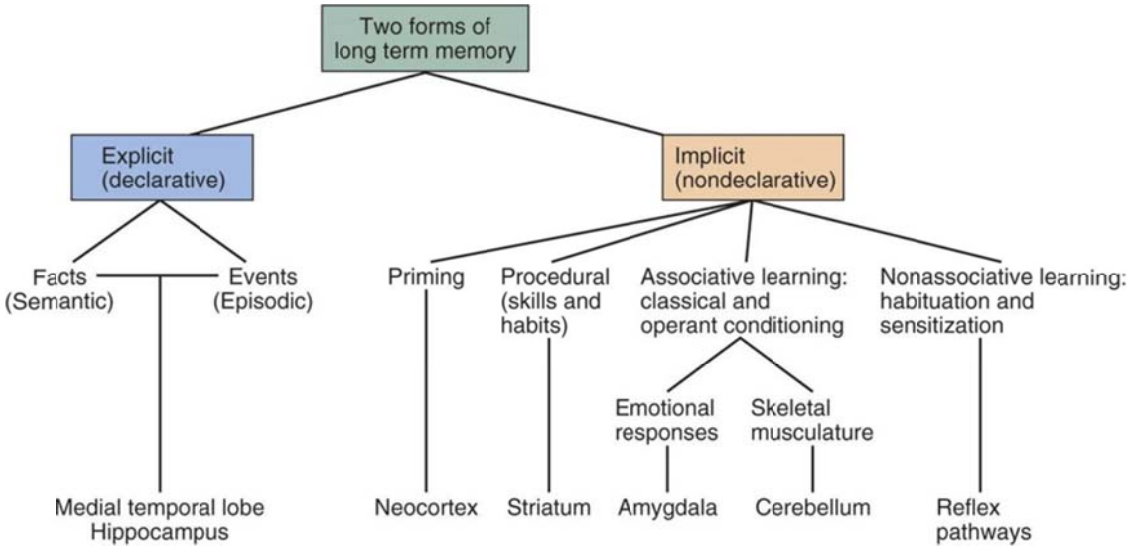

Figure 1. Classification of the two main categories of memory.
Explicit memory includes facts and events and relies on medial temporal lobe and hippocampus. Priming (neocortex), procedural (striatum), associative (amygdala and cerebellum) and non-associative learning (reflex pathways) are considered implicit memory (Principle of Neural Science, fourth Edition).

Years later, the group of Eric Kandel and colleagues, working on sensitization on *Aplysia,* [3] found a common feature of explicit and implicit memory: behavioral, cellular and structural changes require the expression of genes and proteins for long term memory only. Loss of memory function is associated with aging, and is a feature of a broad range of neurodegenerative diseases and psychiatric disorders, including Alzheimer Disease, Parkinson's disease and Schizophrenia.[4], [5], [6] Each of these pathological conditions embraces different types and stages of memory deficits which, thanks to the broadly acknowledged pioneering study of H.M. by Brenda Milner, are systematically investigated using a broad range of methods to find the proper treatment. Moreover, previous studies emphasized the similarities in memory function and structure across mammalian species,[7] propelling further research into memory.

1.2 The Hippocampus

The hippocampus, belonging to the medial portion of the anterior temporal lobe, is part of the limbic system, playing a key role in emotion, motivation, olfaction and memory formation.[8] In text-books, the hippocampus proper has three subdivisions: Cornu Ammonis (CA) 1-3. They, together with the dentate gyrus (DG), subiculum, presubiculum, parasubiculum and entorhinal cortex comprise a functional system called the hippocampal formation. Another common usage, and the one used in this thesis, is to let "hippocampus" refer to CA1-3, DG and subiculum.

The pyramidal cell is the primary excitatory neuron in the CA regions of the hippocampus, whereas the granule cells populate the dentate gyrus. A connecting circuit between these areas is as follows. First, axons from the entorhinal cortex (EC), known as the perforant path (PP), project onto the granule cells of the DG. Secondly, mossy fibers (MF) project from the DG to CA3. The axons of CA3 divide into two branches: one branch forms the commissural fibers projecting to the contralateral CA1 through the corpus callosum the other branch forms the Schaffer Collateral (SC) which extends to the apical dendrites of the hemilateral CA1 (fig. 2). CA1 then projects back to the EC. This connection system–EC-DG-CA3-CA1-EC is referred to as the trisynaptic circuit.

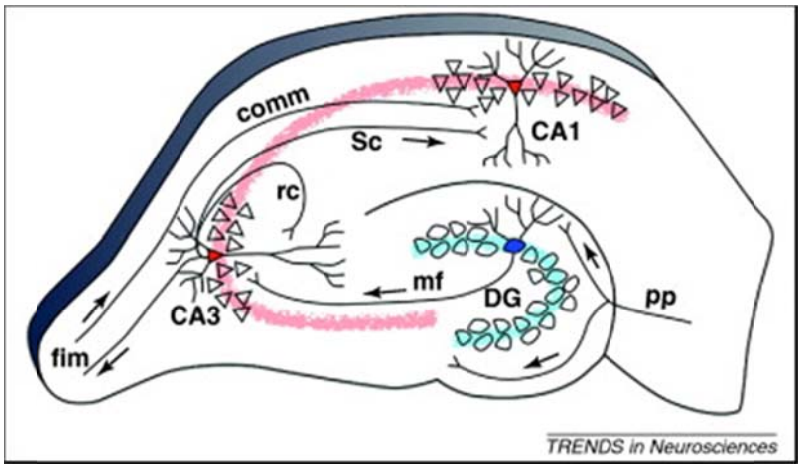

Figure 2. The hippocampal trisynaptic circuit.
Neurons in layer II of the EC project to the DG through the performant path (PP). The axons of the granule cells project to the CA3 field via the mossy fibers (MF). CA3 axons connect to the contralateral hippocampus and to the apical dendrites of CA1 through the Schaffer Collateral (SC) (Yassa and Stark, 2011).

CA1 is divided in stratum oriens (basal dendrites), stratum pyramidale (cell soma), stratum radiatum (proximal apical dendrites) and stratum lacunosum-moleculare (distal apical dendrites). CA1 stratum radiatum is where the majority of Schaffer collateral fibers project to. The relatively simple organization of its connectivity patterns coupled with the highly organized laminar distribution has allowed for extensively studying the hippocampal circuit.

1.3 Extracellular Field Recording

The development of slice preparation by the work of Henry McIlwain´s group allowed neurons to be studied in vitro.[9] This technique offered a new tool to investigate functional anatomy, brain physiology under pharmacological treatments and synaptic plasticity. Following electrical excitation of the schaffer collaterals, an extracellular electrode placed in stratum radiatum will first measure a small potential reflecting a current sink from the presynaptic axonal fibers, referred to as the fiber volley (FV), the amplitude of which indicates the excitability of the presynaptic fibers. Neurotransmitter released by the presynaptic fibers, evoke a transmembrane ionic flow through postsynaptic transmitter receptor channels. In the hippocampus, the parallel arrangements of apical dendrites gives rise to a summed current sink, that can be measured as potential difference (fEPSP) (fig. 3) through the neurotransmitter channels.

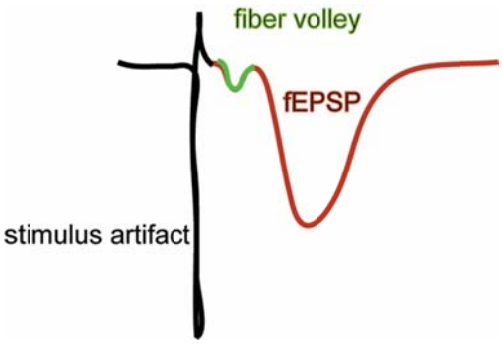

Figure 3. Extracellular field recording.
Schematic extracellular field recording showing a stimulus artifact, FV and fEPSP.

With increased excitation currents, the fEPSP amplitude increases as additional fibers are stimulated activating more synapses. At strong stimulation intensities, synaptic currents will eventually evoke synchronouse postsynaptic action potentials appearing as a sharp summed potential on top of the fEPSP and termed the population spike (pop-spike). The synaptic strength of the activated synapses is assessed by calculating the slope descending slope of the fEPSP, which serves as a proxy for the pure synaptic current (Fig. 4).

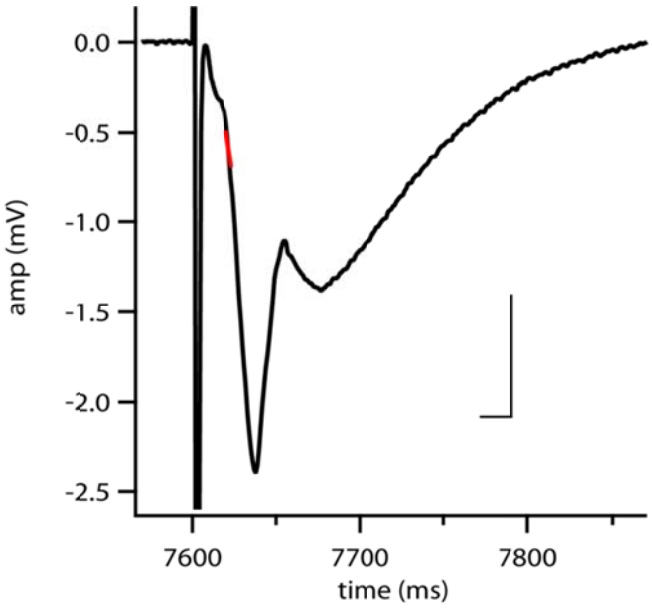

Figure 4 fEPSP slope. The slope of the fEPSP, marked in red, is measured at the initially linear portion of the fEPSP and provides a measure of the strength of excitatory synaptic transmission

1.4 Hippocampal synaptic plasticity

In the 18th century English philosopher David Hartley was the first to hypothesize that memories were encoded through hidden motions in the nervous system. Later on, Donald Hebb intuited that "neurons that fire together, wire together". He proposed that encoding of memories is the result of highly connected neurons and that this connection was established through repetitive and simultaneous firing between the same neurons.[10] It has been proposed that memories are encoded by modification of synaptic strength. Homeostatic scaling is a form of synaptic plasticity that tune the strength of a neuron's excitatory synapses in order to maintain stability and integrity of the underlying neuronal circuit.[11] The cellular models underlying a decrease and increase of synaptic strength are known as Long Term Depression (LTD) and Long Term Potentiation (LTP),[12] respectively. Three well-described characteristics of synaptic plasticity: cooperativity, associativity and input-specificity, are essential to support the hypothesis that it may be a biological substrate for some forms of memory.[13] Cooperativity occurs when a weak stimulation is associated with a strong stimulation. The associativity principle assumes that activating a few fibers is insufficient to induce LTP in either synapse, but simultaneous stimulation of neighboring synapses will trigger LTP at all of them. Input-specificity determines that upon stimulation only the fibers receiving that stimulation will undergo synaptic plasticity.

1.4.1 LTD

In LTD in, synaptic strength is reduced in an experience-dependent manner. There are several types of LTD: it can be homosynaptic (induced by a direct stimulation to a specific set of fibers) or heterosynaptic (as a secondary effect due to a stimulation of neighboring fibers) and can be *de novo* or following LTP (which case it is called

depotentiation). In this paragraph I will focus on describing homosynaptic *de novo* LTD. In CA1 synapses LTD can be induced electrically by a prolonged period of Low Frequency Stimulation (LFS) of the Schaffer Collaterals or by application of an appropriate receptor agonist, known as chemical LTD.[14],[15] Initial studies showed that the "electrical" LTD, induced by one train LFS (1 Hz stimulation for 15 min, 900 pulses), is homosynaptic, saturable and requires the activation of postsynaptic N-methyl-D-aspartate (NMDA) receptors.[16],[17] NMDARs are assembled from NMDAR subunit 1 (NR1) and at least one type of NR2 subunit, where NR2A and NR2B are the predominant NR2 subunits in the adult hippocampus[18]. It has been reported that distinct NMDAR subunits are critical factors that determine whether a stimulation paradigm will result in an LTP or LTD (Liu et al., 2004). Studies propose that LFS-induced LTD is age dependent, since LFS induces a robust and stable LTD only in slices from young mice (P6-P17), not from older animals.[20] Moreover, hippocampal LTD is facilitated by exposing an animal to mild stress.[21] On a molecular level, hippocampal NMDAR-dependent LTD requires the activation of the downstream protein phosphatases calcineurin (a calcium-CaM-regulated phosphatase, also termed PP2B) and PP1, both present in the postsynaptic density (PSD)[22] (fig. 5).

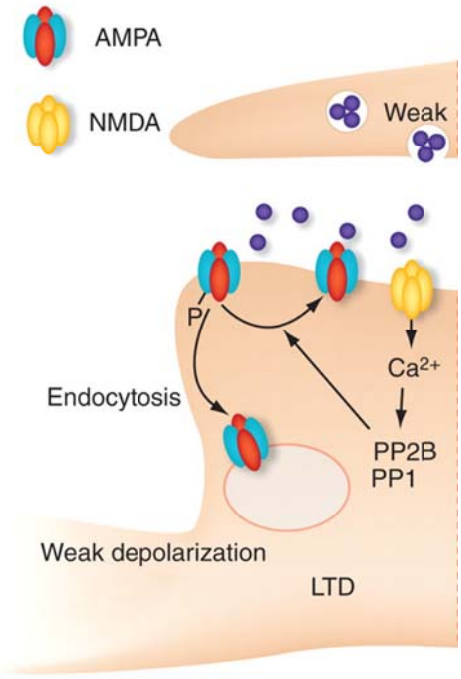

Figure 5 Postsynaptic expression mechanisms of LTD.
Weak activity of the presynaptic neuron leads to modest depolarization and calcium influx through NMDA receptors. This preferentially activates phosphatases that dephosphorylate AMPA receptors, thus promoting receptor endocytosis. (Modified Trend in Neuroscience, Lüscher and Malenka, 2012).

The proper targeting of PP1 to synapses is important for LTD. For instance, α-amino-3-hydroxy-5methylisoxasole-4-propionic-acid (AMPA) receptor internalization triggered by NMDAR activation required protein phosphatase which is correlated with dephosphorylation of the GluR1-containing AMPA subunit on Ser845, a protein kinase A (PKA) site[23]. Furthermore, it has been shown that the long lasting maintenance of this form of LTD does not require the synthesis of novel proteins[24]. Studies on the role of NMDAR-LTD on behavior suggest that this form of plasticity is required for behavioral flexibility[25].

A second major form of LTD in CA1 requires the activation of metabotropic glutamate (mGlu) receptors. It is usually induced by paired-pulse LFS or by application of the group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG)[15]. Group 1 mGluRs are

comprised of mGluR1 and mGluR5. In the hippocampus, mGluR5 is mainly expressed in dendritic fields of stratum radiatum, whereas mGluR1 is mostly found on cell bodies[26]. The first selective mGluR antagonist that was discovered, α-methyl-4-carboxyphenylglycine (MCPG), blocks *de novo* LTD[27]. Moreover, it has been shown that rapid dendritic protein synthesis is essential for mGluR-dependent LTD, whereas transcription inhibition has no effect [24]. Interestingly, the dependence of mGluR-LTD on novel protein synthesis has some exceptions. In *Fmr1* KO mice, a mouse model of Fragile X syndrome (FXS), mGluR-LTD does not require new protein synthesis, although *Fmr1* KO mice show the same postsynaptic LTD expression mechanism, e.g. a decreased AMPARs surface expression[28].

1.4.2 LTP

Discovered in 1973 by Bliss and Lomo, LTP was first induced by brief high frequency stimulation, resulting in a long lasting increase in synaptic strength[29]. HFS induces a persistent potentiation lasting for many hours and is converted to a decremental potentiation when a translational inhibitor is present during the repeated tetanization[30], Kläschen. Med Thesis, 2014). LTP is commonly divided into two distinct temporal phases: early phase (E-LTP) which is transient (1-2 hrs), sensitive to disruption and requires modification of preexisting proteins, whereas the late phase (L-LTP) is long lasting (>3 hrs) and requires gene expression and novel protein synthesis[3]. E-LTP is usually induced by one train of high frequency stimulation (HFS, 100 Hz for 1s), and is unaffected by transcriptional or translational inhibition. On the contrary, L-LTP is induced by repeated, intermittent trains of HFS and relies on gene transcription and mRNA translation[30]. Moreover, it has been recently shown that the stability of L-LTP is a balance between synthesis and

degradation of novel proteins: interfering with either protein synthesis or degradation abolishes L-LTP[31].

Another form of LTP can be induced by Theta Frequency Stimulation (TBS, 5 Hz, 30 sec) (Huang and Kandel, 2005). Hippocampal theta oscillations were originally described as the hippocampal “arousal rhythm” since it was correlated with a neocortical desynchronization characteristic of wake, attentive state[33]. Years later, it was considered as correlate of voluntary movement and REM sleep[34],[35]. The late phase of TBS-LTP is known to be transcription independent and specifically requires local protein synthesis[32]. Transcription, protein synthesis and degradation can function as mechanisms of maintenance, supporting the long lasting stability of L-LTP. Besides these mechanisms, there are also induction mechanisms, transient and very brief during stimulation that might involve both presynaptic and postsynaptic responses and are modulated primarily by ionotropic glutamate receptors and calcium channels. It is generally agreed that the influx of calcium through the NMDAR is required for LTP, producing a significant rise in the postsynaptic calcium concentration[36].

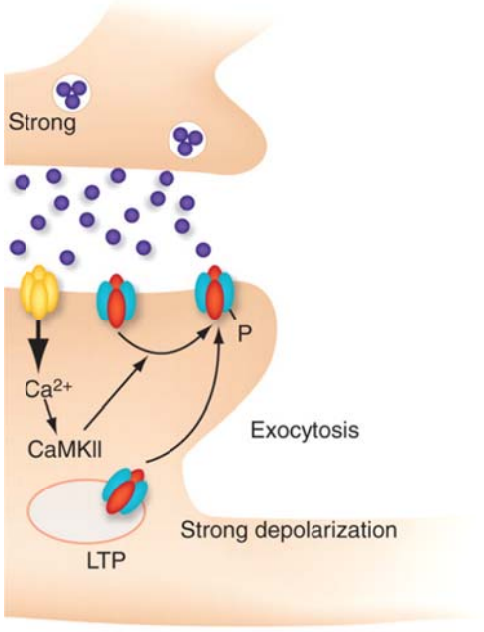

Figure 6 Postsynaptic expression mechanism of LTP.
Strong activity paired with strong depolarization triggers LTP in part via CaMKII, receptor phosphorylation, and exocytosis. (Modified Trend in Neuroscience,[37]).

The higher concentration of calcium leads to activation of calcium/calmodulin-dependent protein kinase II (CaMKII), which phosphorylates the GluA1 subunits of AMPARs and increases the levels of AMPARs at the synapses (Fig. 6) [38], [39], [40].

1.5 Arc/Arg 3.1

In 1995, an Immediate Early Gene (IEG) Arc/Arg3.1 was identified to be upregulated in neurons by synaptic activity such as LTP and LTD, in response to learning and novel experience, and following seizures[41],[42]. Arc/Arg3.1 has unique qualities among the IEGs: its gene is transcribed within 5 minutes of stimulation after which its mRNA is rapidly transported to dendrites[43], making it a good marker to map neuronal activity[44]. On a cellular level, Arc/Arg3.1 mRNA is also rapidly translated following stimulation and correlates with the localization of its mRNA in the nucleus, dendrites and post-synaptic density, where it directly affects synaptic function[45]. Arc/Arg3.1 is specifically required for long term memory formation. In fact, Arc/Arg3.1-deficient

mice exhibit a complete loss of memory in a variety of behavioral and learning paradigms [46](Xiaoyan Gao, 2016, Castro-Gomez, 2016).Several lines of evidence implicate Arc/Arg3.1 as a crucial element in homeostatic synaptic scaling, LTD and LTP.

An essential mechanism to regulate glutamatergic synaptic strength is to increase or decrease the accumulation of AMPA receptors in the postsynaptic membrane and Arc/Arg3.1 has been found to be directly involved in the endocytosis of these receptors[47], [48]. A schematic representation for Arc/Arg3.1 regulation of AMPARs trafficking is shown in figure 1.2. Arc/Arg3.1 directly interacts with components of the endocytic pathway, e.g. endophilin and dynamin, increasing the rate of AMPARs endocytosis[49] (fig. 7). This is corroborated by the observation that Arc/Arg3.1 KO mice show a significant increase in surface GluR1-containing AMPARs [47]. Furthermore, it has been demonstrated that Arc/Arg3.1 plays a key role in regulating visual experience-dependent homeostatic plasticity of excitatory synaptic transmission[50].

Alteration of Arc/Arg3.1 function has been shown to be related to a neurological disorder. A mutation of the E3 ubiquitin ligase Ube3A causes Angelman Syndrome (AS). Ube3A regulates excitatory synapse development by controlling Arc/Arg3.1 degradation. Disruption of this gene leads to elevated levels of Arc/Arg3.1 and consequently an excessive internalization of AMPARs. It has been proposed that impaired AMPARs trafficking may be the cause of the cognitive dysfunction that occurs in AS [51]. Rapid translational upregulation of Arc/Arg3.1 is required for rapid, mGluR-dependent AMPA receptor endocytosis [5253]. The increase in Arc/Arg3.1 translation requires eEF2K, a calcium/calmodulin-dependent kinase that binds to mGluR and dissociates upon mGluR activation. Phospho-eEF2K inhibits general

translation but simultaneously increases Arc/Arg3.1 translation. Genetic deletion of this translation mechanism impaired mGluR-LTD. FMRP is a translation-repressor protein and it represses also Arc/Arg3.1 translation. FMRP-deficient mice (Fmr1 KO) exhibit impaired eEF2K translational machinery. Taking together, it has been proposed that eEF2K-FMRP machinery coordinately controls Arc/Arg3.1 translation [52](fig. 7).

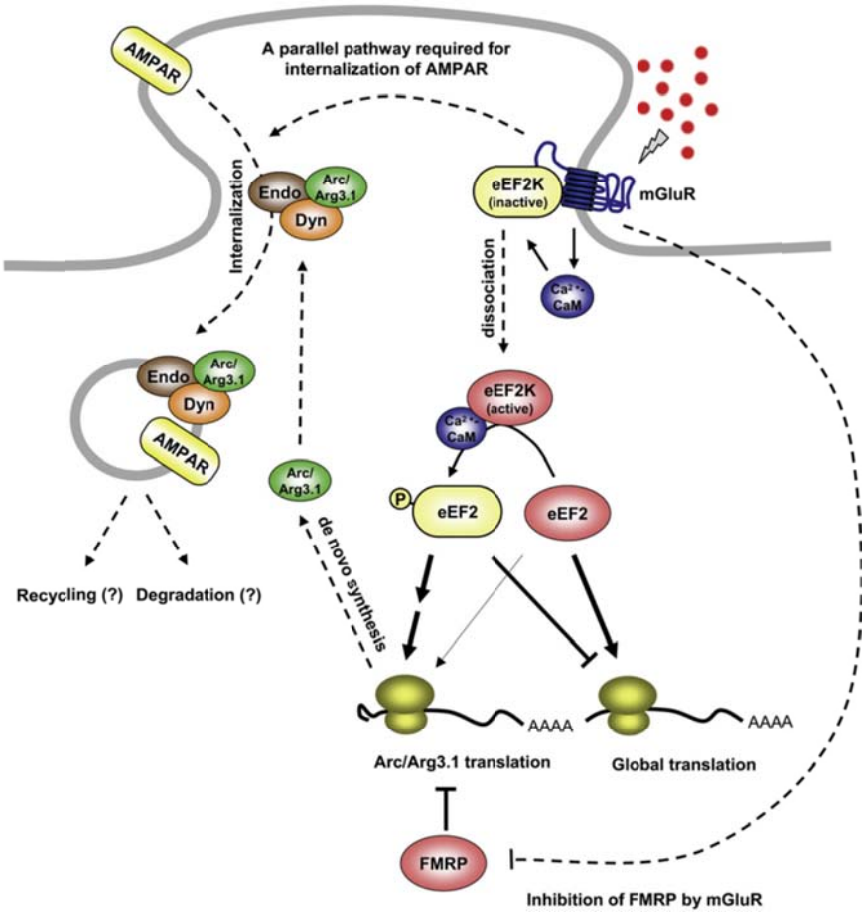

Figure 7 mGluR-dependent LTD and Arc/Arg3.1.
Arc/Arg3.1 forms a complex with endophilin2/3 (Endo) and dynamin (Dyn) and induces the internalization of AMPAR. Activation of mGluRs through DHPG leads to phosphor-eEF2 which inhibits general translation but increase Arc/Arg3.1 translation. mGluR activation results in dephosphorilation of FMRP and this reduces its inhibitory action on translation. FMRP inhibits the translation of Arc/Arg3.1 at the basal state (figure taken from [52]).

It has been previously shown that HFS of the PP induces Arc/Arg3.1 expression in the DG and causes the newly synthesized mRNA to localize selectively in the

molecular layer through NMDARs activation[54]. Moreover, studies on Arc/Arg3.1 function found that in Arc/Arg3.1 KO mice, E-LTP is enhanced while L-LTP is blocked in both DG *in vivo* and in CA1 *in vitro*[46].The role of Arc/Arg3.1 in the maintenance of long lasting synaptic plasticity is still not clear. However, it has been proposed that Arc/Arg3.1 might interact with the inactive form of CaMKIIβ in synapses with low activity or inactive synapses, promoting AMPARs endocytosis[55]. As a consequence, in synapses that receive strong inputs, CaMKIIβ might be more active and therefore the interaction with Arc/Arg3.1 weaker, leading to a redistribution of Arc/Arg3.1 to other sites[55]. This inverse synaptic tagging of Arc/Arg3.1 might explain how the only synapses previously potentiated can maintain their state over time, whereas the inactive synapses are weakened through Arc/Arg3.1 and AMPAR internalization.

1.6 Arc/Arg3.1 and development

Neuronal activity models the brain throughout the entire life. However, during specific time windows of early postnatal life this activity might considerably impact molecular mechanisms across brain regions and potential arousal in adulthood[56], [57]. Especially during early postnatal development, formation of neuronal connections is initiated by an excess of synaptogenesis. During the course of development, some synapses are selectively strengthened and other synapses are weakened and/or eliminated[58]. It has been already reported that Arc/Arg3.1 has a critical role in activity-dependent climbing fibers (CFs) synapse elimination during cerebellar development[59]. Moreover, studies on visual cortex plasticity during a time window particularly sensitive to changes in activity (P25-32) show that mice lacking Arc/Arg3.1 do not show depression of deprived eye response or shift in ocular dominance after brief monocular deprivation like control mice[60]. These data suggest primary key role of

Arc/Arg3.1 in experience-dependent synaptic regulation in visual cortex of excitatory synaptic transmission in vivo in juvenile mice.

Infantile amnesia is a phenomenon in which adults are unable to recall events from early childhood [61]. Recently it was found that long lasting changes taking place in the dorsal hippocampus during a developmental critical period through a BDNF and mGluR5-dependent switch in the ratio of GluN2B/GluN2A expression represent key processes to develop the ability to form explicit, associative long-term memories in adulthood [62]. The latent memory formed at P17 requires mGluR5 and GluN2B but not GluN2A, whereas at P24 the more strong memory requires GluN2A but not mGluR5. Since Arc/Arg3.1 is involved in juvenile forms of plasticity such as mGluR-dependent LTD[63],[64] and is crucial for the consolidation of long term memory[65], it seems likely that Arc/Arg3.1 plays a role here.

2 Aims of the PhD thesis

The overall aim of this thesis was to investigate forms of plasticity linked to memory consolidation and in particular to reveal their underlying mechanisms in WT and Arc/Arg3.1 deficient mice. Specific aims were:

I. Establish a novel form of LTD in mature hippocampal slices of WT mice and to study the mechanisms underlying induction and maintenance;

II. Induce LTD with the novel protocol in KO, in cKO and in dendritic Arc/Arg3.1 deficient mice and explore mechanisms of induction and maintenance;

III. Induce a form of LTD mediated by mGluRs in juvenile WT and KO mice and investigate the protein synthesis;

IV. Induce HFS-LTP and examine the protein synthesis in KO and late cKO;

V. Induce TBS-LTP and examine the protein synthesis in KO;

3 Material and methods

3.1 Animals care

The animal care, maintenance and experimental procedures were performed in accordance with the Ministery of Science and Public Health of the City State of Hamburg, Germany. Mice were kept in plastic cages under standard housing conditions (rodent provender and water ad libitum, nesting material provided). Light/dark cycles were not reversed. Adult mice aged 2-6 months and juvenile (P21-P23) were used in experiments. Network organization and plasticity in adult mice have reached a mature state[66]. Mice of both sexes are included in the experiments at balanced numbers.

3.2 Genotypes and breeding schemes

The aims of this study were to find how the ablation of Arc/Arg3.1 at different times during development affects adult hippocampal synaptic plasticity. We were also interested in investigating the role of dendritically translated Arc/Arg3.1 in adult plasticity. To answer those questions 4 mouse line have been generated:

Germline KO

This line represents the constitutive knockout mice, in which the full gene locus of Arc/Arg3.1 was deleted from the germ line (Plath et al, 2006). This mouse line was raised in a C57Bl/6J background, and comprises WT (Arc/Arg3.1 $^{+/+}$), heterozygous (Arc/Arg3.1 $^{+/-}$) and KO (Arc/Arg3.1 $^{-/-}$). In this study I used WT and KO mice.

Conditional KO mice

The following two Arc/Arg3.1 conditional KO mice lines have been generated in our laboratory with a Cre/LoxP recombination system (Xiaoyan Gao PhD thesis, Castro-Gomez PhD thesis) and used in this study.

Early cKO

Early-cKO, was generated in which the Tg(CaMkIIα-cre)1Gsc[67] started to ablate the Arc/Arg3.1 gene after P7 and completed before P14. Control mice were Arc/Arg3.1 early-cKO$^{+/+}$ with CamkIIα-cre, later referred as early WT.

Late cKO

The Arc/Arg3.1 late cKO mouse line, later on referred as late-cKO, was generated with the same Cre/LoxP recombination system as used for Early-cKO mice.Tg (CaMKIIα-cre)T29-1Stl was used to obtain the late-cKO mice [68]. Arc/Arg3.1 ablation started after P21 and was completed before P35. Also for the experiments performed with this mouse line, the control mice were Arc/Arg3.1 early-cKO$^{+/+}$ with cre, later referred as late-WT.

Tg(3´UTR) Arc/Arg3.1

One of the unique characteristic of Arc/Arg3.1 is that the mRNA is located in the dendrites; in our laboratory a specific mouse line has been generated in which the dendritic Arc/Arg3.1 mRNA is missing. The 3' UTR of the Arc/Arg3.1 gene that regulates Arc/Arg3.1 mRNA in the dendrites has been replaced with the one of Zif, another immediate early gene, leading to the generation of transgenic mice (tg) that lack dendritic Arg3.1/Arc mRNA. This mouse line includes also WT and KO mice. Figure 8 shows Arc/Arg3.1 mRNA and protein expression in WT and tg (3´UTR)Arc/Arg3.1 mice of CA1-hippocampal neuron.

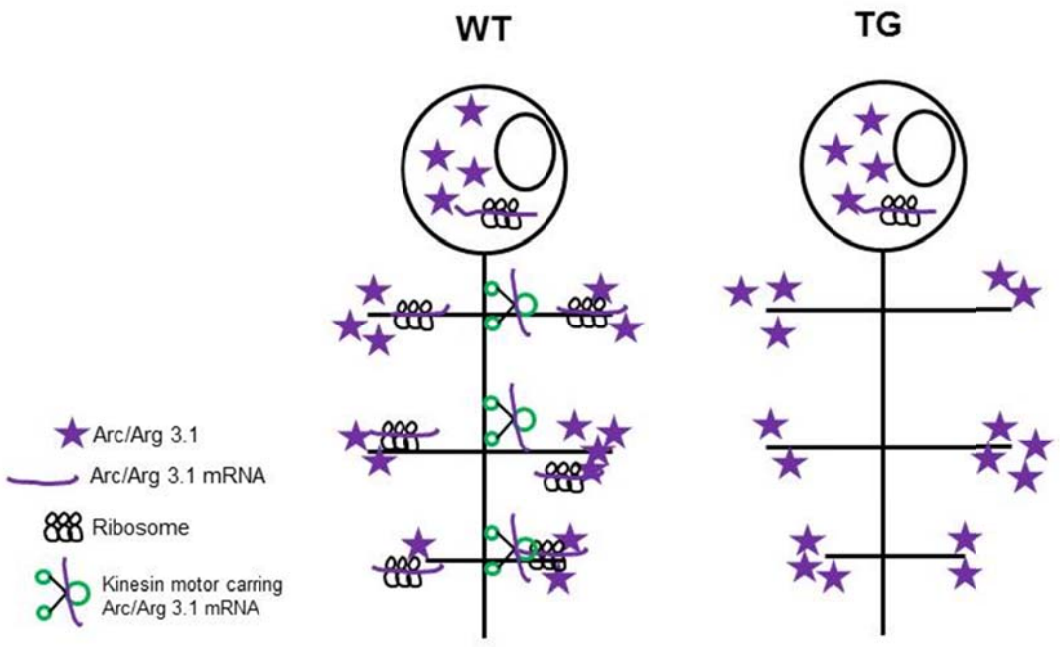

Figure 8 Expression of Arc/Arg3.1 mRNA and protein in WT and tg neurons
In WT neuron, Arc/Arg3.1 mRNA is transcript and transported to the dendrites by kinesin5 (KIF5) motors on microtubules[69]. In tg neuron, Arc/Arg3.1 mRNA is absent in the dendrites. Arc/Arg3.1 protein is normally distributed in the nucleus and at the dendrites in tg.

3.3 Electrophysiology

3.3.1 Slice preparation

Male and female mice aged 2-5 months were anesthetized by sedation with Isoflurane (100 µl) and transverse hippocampal slices, 350 µm thick, were prepared in iced, gassed aCSF (*LTD*, in mM: NaCl 125, KCl 4.4, NaHCO3 25, NaH2PO4 1.25, MgSO4 1, glucose 10 and CaCl2 2; *LTP*, in mM: NaCl 119, KCl 2.5, NaHCO3 26, NaH2PO4 1.25, MgSO4 1.3, glucose 10, CaCl 2.5). Slices were always prepared between 8:00 and 9:00 AM. Microm vibrato was used to perform the slicing. Slices were allowed to recover at 30°C for LTD and at 37°C for LTP experiments for 2 hours and then transferred into submerged recording chambers. Recordings started after about 1 h of resting period maintained at 30°C and 37°C for LTD and LTP, respectively. The temperature of the aCSF in the incubator was constantly monitored.

3.3.2 The multi-slice field recording system Synchroslice

All the experiments were performed in the Synchroslice system (synchroslice, Lohmann Research Equipment, Castrop-Rauxel). Synchroslice is a multi-slice field recording system containing four independent submerged chambers (fig. 9).

Figure 9 Synchroslice set-up
Synchroslice set-up and the four independent chambers used to measure fEPSPs in acute hippocampal slices.

The perfusion rate of gassed aCSF (95% O2, 5% CO2 and pH 7.4) was maintained at 3 ml/min, unless otherwise specified. Concentric, bipolar electrodes (stainless steel, contact diameter 0.35 mm, impedance 0.1 MΩ) and platinum/tungsten electrodes (impedance 0.5–0.8 MΩ) were used for excitation and recording, respectively. Two excitation electrodes were positioned in stratum radiatum of hippocampus CA1, at both sides of the recording electrode at equal horizontal distances. This set-up allows us to excite two independent sets of schaffer collateral fibers, evoking field excitatory postsynaptic potentials (fEPSP) from the same population of postsynaptic neurons, but separate sets of synapses (fig. 10).

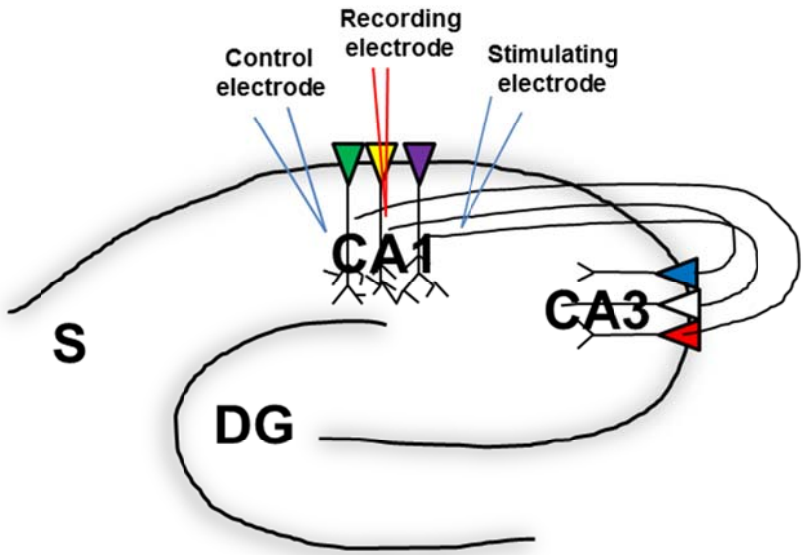

Figure 10 A schematic of an acute hippocampal slice with electrodes
Schematic rappresentation of an acute hippocampal slice with the placement of the stimulating electrode and the recording electrode.

For each experiment, one of these electrodes was chosen to induce LTP or LTD, henceforth referred to as the stimulating electrode, whereas the other, henceforth referred to as the control electrode, was used to monitor the stability of the recordings and for heterosynaptic effects of the stimulation protocol. For any given drug or drug combination, the experiments were interleaved with control experiments. Data acquisition, electrical excitation and perfusion were controlled via the Software "Synchrobrain" (Lohmann Research Equipment, Castrop-Rauxel). Signals were amplified 1000x and sampled at 1 KHz. Offline analysis of fEPSPs was performed with the same program and in custome made algorithms written in IgorPro (WaveMetrics) . Regular cleaning was performed with constant perfusion of acetic acid diluted in distillate water (DDW). Electrodes were cleaned with Protease K.

3.3.3 Input/Output curve

The Input/ Out curve was the test chosen to measure the basal synaptic transmission at the Schaffer collateral- CA1 synapses for each slice in both the to be stimulated and control pathway by exciting with step-wise increasing currents. Excitation pulse-duration was always set at 200 µs. The control electrode excited its respective fiber bundle 1 sec after the stimulating electrode. Figure 10 represents an example of I/O curve with the 16 point representing the evoke fEPSP and the fit-curve described by a sigmoidal function (fig. 11).

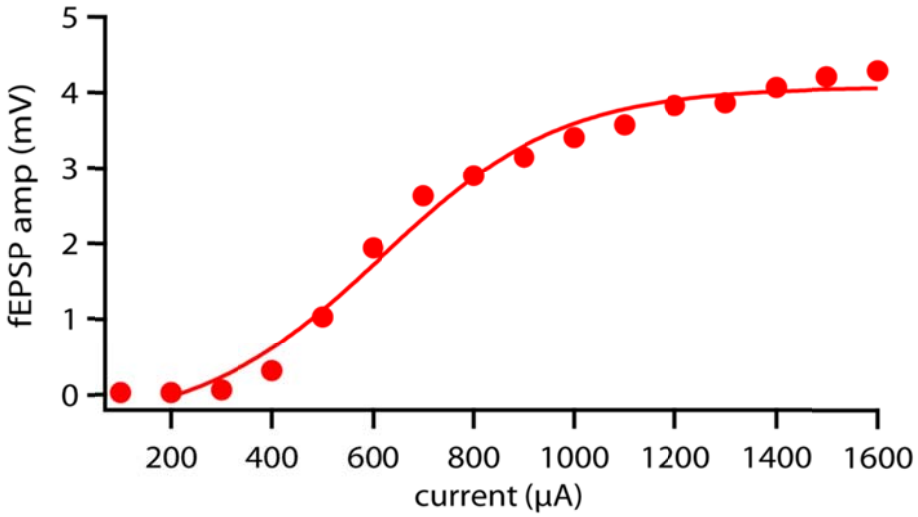

Figure 11. I/O curve.
Representative example of an I/O curve fitted with a sigmoidal function.

3.3.4 Stimulation paradigms

The stimulus intensity (SI) to evoke fEPSPs was different between LTD and LTP experiments. For LTD experiments, the stimulus intensity (SI_{LTD}) was chosen such that the fEPSP amplitude it evoked was 70% of the maximum of the fitted I/O curve. For LTP experiments, the weakest current step before a pop-spike became visible was noted. SI_{LTP} was then chosen as 50% of that current. Baseline responses were evoked by excitation at either SI_{LTD} or SI_{LTP} at a frequency of 0.0333 Hz for at least

30 min. Afterwards, either an LTD or LTP induction protocol (see below) was applied. In between and after an induction protocol, fEPSPs were again evoked as described for the baseline. Experiments were continued for 1 hour and 4 hours after an LTD and LTP protocol, respectively.

3.3.4.1 LTD induction protocol

The LTD-inducing stimulus consisted of two trains of 900 pulses given at a rate of 1 Hz for 15 min with inter-train interval of 10 min. The second form of LTD was a chemical-LTD and it was induced by bath application of DHPG for 5 min at specific concentration (25-50-100 μM).

3.3.4.2 LTP induction protocols

LTP was induced electrically by two different protocols.

HFS

LTP induction consisted of three train of HFS (100 Hz/s) with an inter-train interval of 5 min (fig. 12).

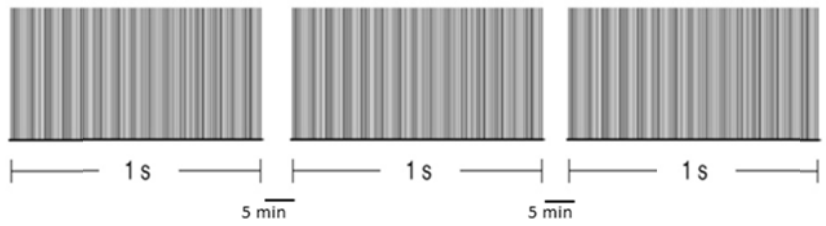

Figure 12 HFS protocol
Three train HFS composed of 100 Hz each and intertrain interval of 5 min.

TBS

The second protocol used to induce LTP was Theta Burst Stimulation (TBS). LTP was induced by 4 trains of TBS (L. Stanislawa Kucharczyk unpublished Dr. Med Thesis). TBS protocol consists of four trains, where each train is composed of 10 burst, each burst has 5 pulses at 100 Hz, with bursts repeated at 5 Hz (fig. 13). Recordings were continued for at least 4 hours following LTP induction in both protocols.

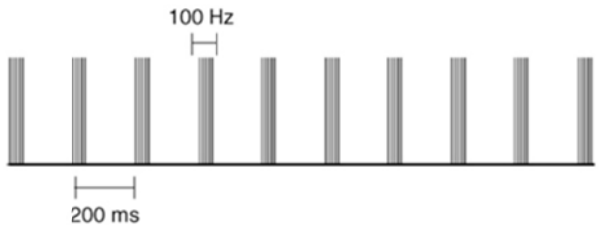

Figure 13 TBS train
One train of a TBS protocol, composed of five pulses at 100 Hz, repeated 10 times at 5 Hz. Each train is repeated 4 times, separated by 30 sec.

3.4 Rejection criteria

To be included in the final analysis the experiments had to satisfy specific criteria. The first criterion considered necessary was the state of the general recording, like perfusion and temperature stability and health of the slice. Experiments were rejected if baseline recordings were unstable or if the control pathways had changed more than 20% from the baseline.

3.5 Data Analysis and Statistics

All experiments were recorded and analyzed online with Synchrobrain software. Raw data were transferred and further organized in Microsoft Office Excel (Version 2007). Igor pro was used to analyze and visualize the data. The initial slope of the evoked fEPSPs was calculated and expressed as a percent change from the baseline mean. Error bars in figures denote SEM. Successful LTD was defined as a decrease of the fEPSP slope below 80% of the baseline and duration of at least half an hour. For LTD protocol, the time-window considered was from t=40 to t=115, unless otherwise specified. For LTP two time window were considered: E-LTP from t=4.5 to t=29.5, L-LTP from t=274.5 to t=299.5. Successful LTP was defined as an increase of the fEPSP slope above 120% compared to the baseline following HFS or TBS. Group results are plotted as means ± standard. For all experiments, both number of slices and number of animals are mentioned, where each slice was considered a single experiment. Summary of the I/O curves of each experiment for each group was plot in a graph as sigmoidal function, and compared the mean of the single fit curve per experiment per genotype. Statistical significance was evaluated using Prism GraphPad. Tests used ANOVA (analysis of variance) RM (Post-hoc) and Student t-test for two group comparison and Mann Whitney U-test if the data was not normally distributed.

3.6 Reagents

Name	Blocker of	Concentration	Solvent	Wash-out	Ref.
APV-5	NMDAR	50 µM	DDW	NO	[70]
RO25- 6981	NR2B- containing NMDAR	5 µM	DDW	NO	[71]
MCPG	group I/II mGluR receptors	500 µM	DDW	NO	[72]
Nifedipine	L-type VGCC	20 µM	DMSO	NO	[73]
CHX	Protein synthesis	120 µM	DDW	NO	[74]
Leupeptin	Lysosome/ Protease	20 µM	DMSO	NO	[75]
MG-132	Proteasome	20 µM	DDW	YES	[76]

Table 1
Blockers list.

All drugs were diluted in aCSF and bath applied for at least 45 minutes before LTD or LTP induction (table1). The drugs were made up as stock solutions in either double-distilled water (DDW) or 99% v/v DMSO and stored in -20°. DMSO at final concentration of 0.01-0.1% was added to the solution of the control group.

4 Results

4.1 A novel protocol to induce LTD in adult WT mice

In order to investigate the functional role of Arc/Arg3.1 in a form of plasticity where synaptic strength is reduced in an experience-dependent manner, I established a novel protocol to induce LTD. I aimed to find an LFS that could elicit LTD in adult acute hippocampal slices. Several published LFS protocols were attempted, but they were able to induce only transient LTD. I established a new stimulation paradigm that successfully induced a long lasting LTD, which consisted of two trains of 900 pulses delivered at 1 Hz, separated by a 10 minutes interval (fig. 14).

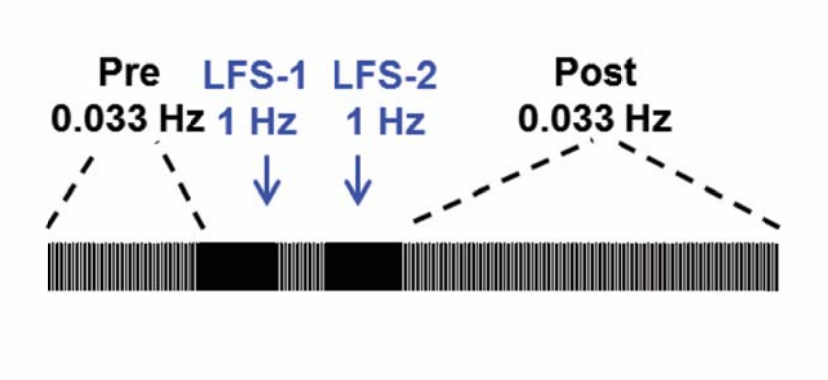

Figure 14 LFS protocol
fEPSPs were evoked at a low frequency (0.033 Hz) for 30 min followed by two trains of LFS (1 hz, 900 pulses each), applied to the stimulated pathway only. In parallel, in the control pathway fEPSPs were evoked at a low frequency (0.033 hz) and only during LFS it was paused.

SI_{LTD} was chosen such that the fEPSP amplitude it evoked was 70% of the maximum of the fitted I/O curve (fig. 15 a & b).

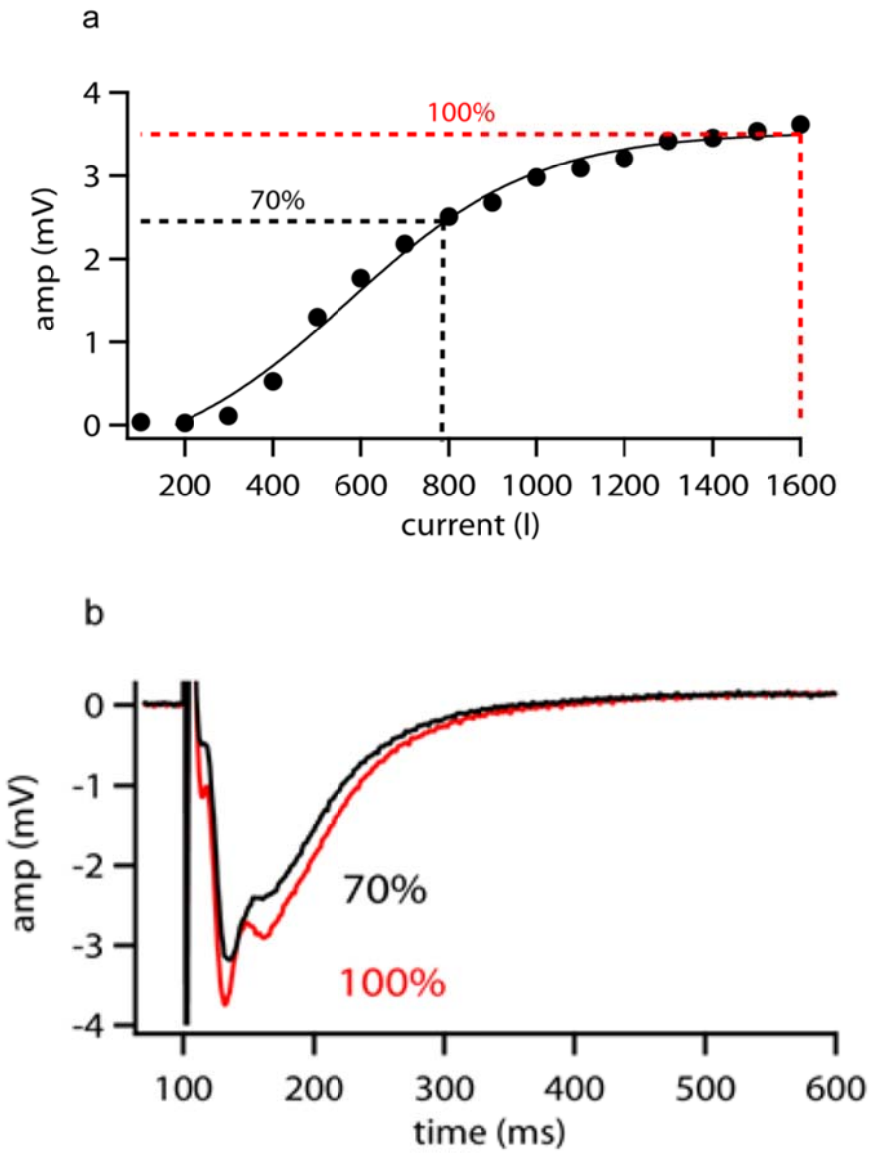

Figure 15 I/O curve analysis to choose the stimulation intensity threshold
Representative example of an I/O curve, from which 70% of the maximal amplitude was chosen to evoke fEPSPs.(b) fEPSP trace representing the maximal amplitude reached with 1600 µA (red) and fEPSP trace representing the 70% of the maximal amplitude (black).

The double-train LFS was essential for LTD induction, since other LFS protocols failed to induce LTD (data not shown). Two independent groups of fiber bundles, on the same population of postsynaptic cells were alternately excited at a low stimulation rate of 0.033 Hz for 30 minutes to establish a stable measurement of fEPSPs. Afterwards, LFS was applied to one of the pathways only. During LFS, the stimulated pathway showed a transient increase in fEPSP slope, but subsequently gradually

declined below baseline level and remained depressed throughout the entire recording (120 min). Figure 16a shows a representative experiment in which LTD was induced only in the synapses that received LFS. Summary data obtained is shown in figure 16b, where fEPSP slopes were normalized to baseline, show a stable LTD (81.30 ±3.15% N= 17 n= 28). Furthermore, control pathways confirmed that LTD was input specific (101.47 ±3.04% N= 17 n= 28, treatment p <0.0001 time p <0.0001 interaction p <0.0001, two-way ANOVA RM).

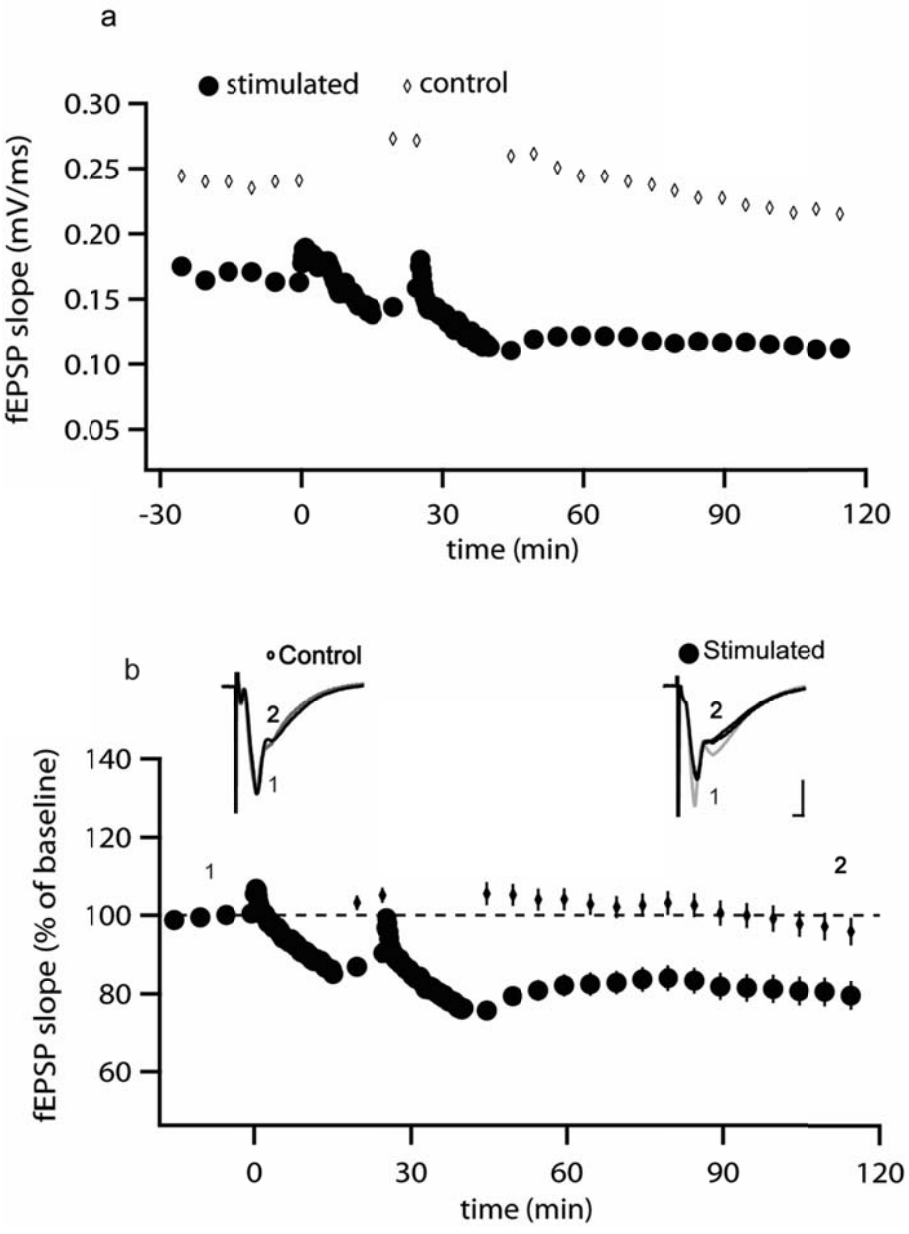

Figure 16 Examplary and averaged LTD experiments in WT slices.

(a) Exemplary LTD experiment in a WT slice. fEPSP slope rising phase was measured and plotted against time. fEPSPs in the stimulated pathway decreased by 23% following LFS stimulation, while control fEPSPs remained unchanged. (b) Averaged time course of LTD in 28 WT slices (n=28) obtained from 17 mice. Individual fEPSP slopes were normalized to their own baseline, presented as percentage and averaged across experiments. The averaged fEPSP slopes decreased during LFS stimulation to a minimal level of 75.71% (t= 45) and was maintained at 81.30% of baseline throughout the remainder of the recording.

4.2 Mechanisms underlying LTD- induction in WT mice

Because a novel form of LTD was established in adult hippocampal slices, the first aim was to investigate the mechanisms underlying its induction and maintenance. It has been already shown that in hippocampal CA1 pyramidal cells of juvenile (11-35 days old) rats two distinct forms of LTD coexist. One form of LTD depends on the activation of NMDA receptors, while the other form relies on the activation of mGluRs [77]. In addition, in adult a form of LTD was described, which was dependent on postsynaptic calcium ion entry through L-type voltage-gated calcium channels paired with the activation of mGlu receptors [78].

Therefore, I first investigated the role of NMDARs, mGluRs and VGCC on LTD induction.

4.2.1 LTD and mGluR receptors

Group I metabotropic glutamate receptors, including mGluR1 and mGluR5, are the most prevalent group of mGluRs present in the hippocampus and are believed to be involved in multiple forms of experience dependent synaptic plasticity events, including learning and memory[79]. In order to investigate the role of group I mGluR receptors, LTD was induced in either standard recording medium or in presence of 500 µM MCPG. The non-selective group I/group II metabotropic glutamate receptor antagonist[72]. As established, the LTD protocol in untreated slices caused a decreased fEPSP slope to 80.92 ±4.52% of baseline (N= 6 n= 11) (fig. 17). Likewise,

MCPG-treated slices expressed LTD (78.33 ±3.8% of baseline, N= 4 n= 7) (fig. 17). Direct comparison between LTD of untreated and MCPG–treated slices showed no statistical difference (genotype p= 0.6857 time p< 0.0001 interaction p= 0.0004; two-way ANOVA RM).

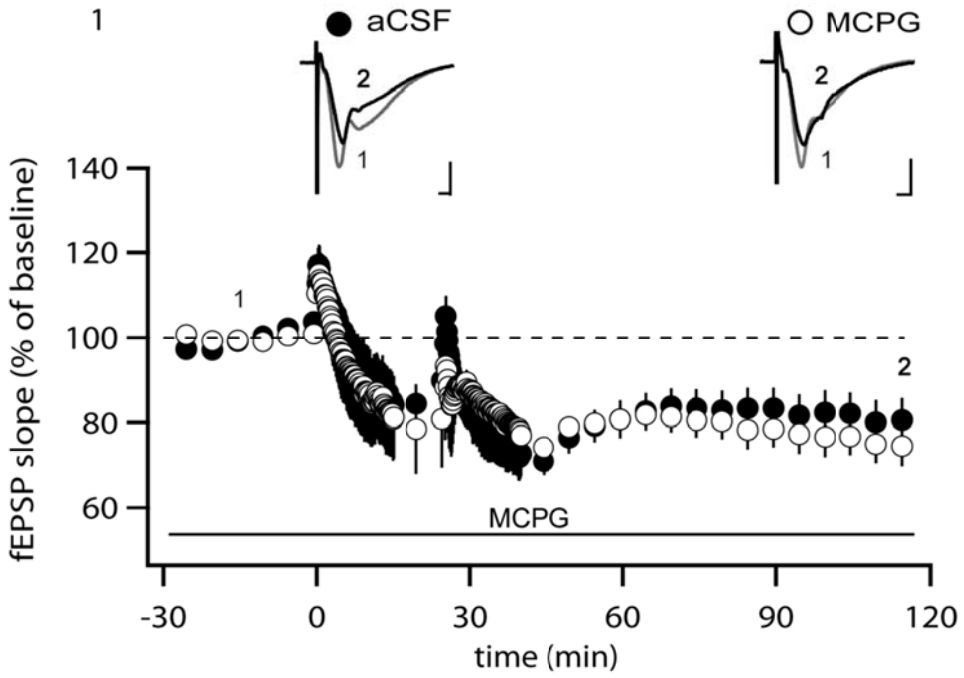

Figure 17 Summary LTD experiment in untreated and MCPG treated slices
LTD in MCPG- treated slices (500 µM) resulting in decreased fEPSP slopes to 78.33 ± 3.79% of baseline (empty circles; N= 4 n= 7) compared to untreated slices (filled black circles; 80.92 ±4.5% of baseline N= 6 n= 11).

fEPSPs of control pathways in both untreated and MCPG-treated slices were unaffected by the LTD protocol (untreated 101.72 ± 2.63%, N= 6 n= 11, p= 0.512 paired t-test; MCPG-treated 102.30 ± 7.37%, N= 4 n= 7; p= 0.7647 paired t-test). Thus, our results suggest that mGluR receptors are not essential for LTD induction.

4.2.2 LTD and NMDA receptors

In the adult brain the most common form of LTD is mediated by NMDA receptors and their downstream targets, under the condition that the activation remains below the threshold to induce potentiation[80]. To examine the role of the NMDAR on LTD induction I bath applied APV (50 μM), a well-known selective NMDAR antagonist. Untreated slices showed stable LTD following the LFS protocol (fig. 18a) (78.12 ±7.11%, N= 2 n= 6; averaged from t=40 to t=115). In contrast, in the presence of APV, LTD was blocked (fig. 18a) (98.74 ±5.06% N=8 n=18). The difference between untreated and APV-treated slices was significant (treatment p= 0.7061, time p < 0.0001, interaction p= 0.0049, two-way ANOVA RM). In conclusion, our LTD in CA1 of adult mice is mediated by NMDA receptors.

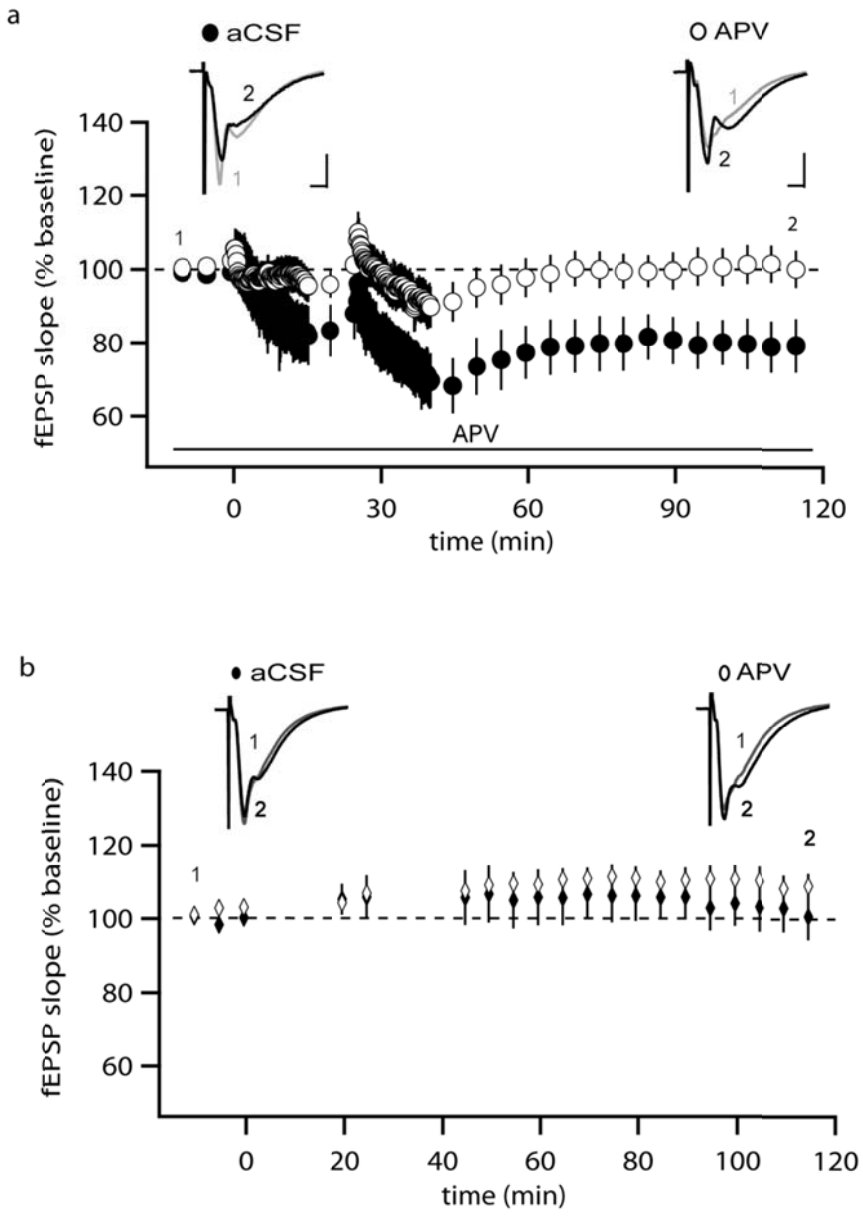

Figure 18 APV blocks LTD in adult WT slices

(a) LTD in APV-treated slices (50 µM) (empty circles; N= 8 n= 18), resulting in a fEPSP slopes of 119.51 ± 8.56% of baseline. Untreated slices (filled black circles; N= 2 n= 6) recorded in parallel to APV-traeted slices, decrease in fEPSP slope to 78.12 ±7.11% of baseline. Comparison of fEPSP traces, before and after LFS stimulation, in both untreated and APV-treated slices is shown above the graph. (b) Summary of control pathways of untreated (filled black circles, N= 2 n= 6) and APV- treated slices (empty circles; N= 8 n= 18). Comparison of fEPSP traces, before and after LFS stimulation, of both untreated and APV-treated slices were shown above the graph.

Next, control pathways were examined. In untreated slices mean fEPSP slope following LTD protocol was 104.88 ±6.81% (N=2 n=6), which was not significantly different from baseline slopes (p= 0.5015, paired t-test) (fig. 18b). In contrast, control pathways of APV-treated slices exhibited a persistent and significant increase in

fEPSP slopes following LFS stimulation (115.98 ±4.26% N=8, n=18; p= 0.0008, Paired t-test) (fig. 18b). In order to verify whether the enhanced control pathways in APV-treated slices following LFS were due to enhanced presynaptic excitability, fEPSP amplitudes of FVs were assessed. Comparison between FVs before and after LFS showed no significant differences (fig. 19) (104.03 ±2.51% of baseline average from t= 40 to t= 115, N=8 n= 14; p= 0.1019 paired t-test). Therefore, the persistent increase of fEPSP slopes of control pathways in APV- treated did not result from enhanced presynaptic fiber excitability.

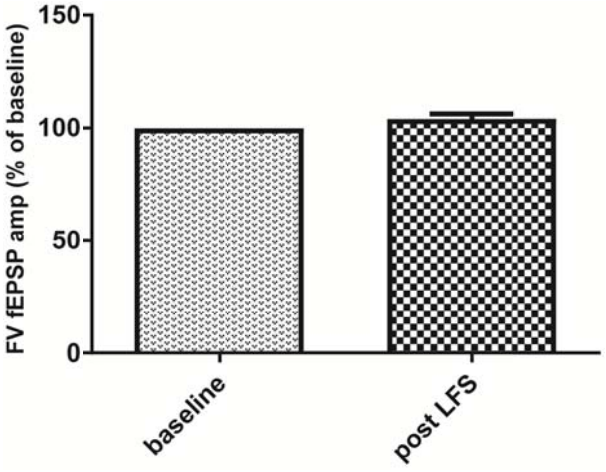

Figure 19 FV analysis of APV-treated slices.
The comparison between mean FV amplitude during baseline and post LFS of the stimulated pathway showed no significant difference (104.03 ±2.51% of baseline average from t= 40 to t= 115, N=8 n= 14; p= 0.1019 paired t-test).

Normalized fEPSPs amplitude (% of baseline) of FV were calculated in a subset of experiments in APV- treated slices (104.03 ±2.51% N= 8 n= 14)

4.2.3 LTD and NR2B-containing NMDAR

For a more comprehensive understanding of the role of NMDARs on LTD, we looked at the NMDAR-subunits. Among the six regulatory subunits of NMDARs, NR2A- and NR2B- containing NMDARs are broadly expressed in the postnatal hippocampus and

are believed to play important roles in synaptic plasticity[81]. Therefore, we decided to begin by investigating the role of NR2B- containing NMDAR. To test whether NR2B is required for LTD induction, I bath applied RO25-6981, an NR2B-selective antagonist (RO25). Untreated slices showed normal LTD (fig. 19) (85.5 ±8.55% of baseline N= 3 n= 6; average from t= 40 to t= 115). Likewise, RO25-treated slices presented normal LTD (fig. 20) (83.15 ± 3.47% of baseline N= 3 n= 6; average from t= 40 to t= 115). Direct comparison of LTD experiments induced in both untreated and RO-treated slices showed no significant difference (fig. 20) (treatment p= 0.727 time p < 0.0001 interaction p= 1, two-way ANOVA RM).

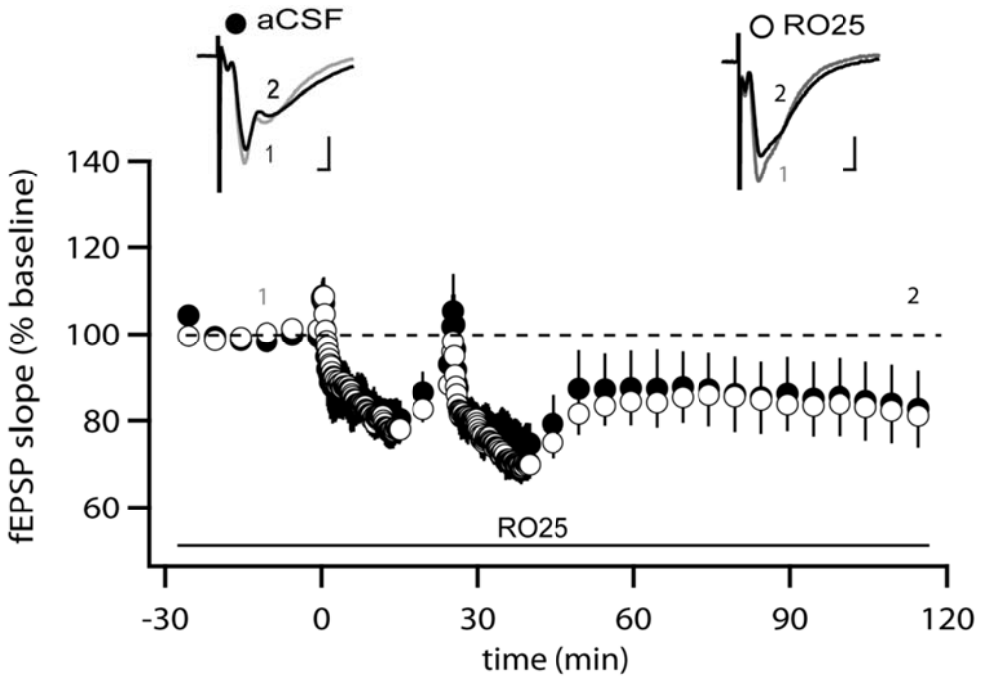

Figure 20 Unaffected LTD in RO25-6981 treated slices
LTD in WT RO25- treated slices (empty circles; N= 3 n= 6), resulting in a EPSP slopes of 83.15 ± 3.47% of baseline compared to untreated control slices (filled black circles; N= 3 n= 6), decrease in EPSP slope of 85.5 ±8.55% of baseline.

Control pathways were not affected by LFS stimulation (untreated 112.88 ± 8.98% of baseline N= 3 n= 6, p= 0.2061 paired t-test; RO25- treated 93.99 ±7.02% of baseline

N= 3 n= 6; p= 0.428 paired t-test). These results indicated that NR2B- containing NMDAR is not crucial for LTD induction in adult mice.

4.2.4 LTD and L- type VGCCs

L-type VGCCs are major sites of post-synaptic calcium influx for induction of some forms of synaptic plasticity in the hippocampus [82]. In order to test whether calcium influx through L-type VGCCs is necessary for this form of plasticity, I induced LTD in presence of nifedipine, the L-type VGCC blocker [83]. It has to be mentioned that in this set of experiments untreated slices were perfused with DMSO, since the preparation of nifedipine stock solution was made with DMSO. Remarkably, fEPSP slopes of slices “treated” with DMSO showed significantly smaller E-LTD compared to untreated slices (fig. 21a) (DMSO 89.32 ± 3.64% N= 4 n= 8, untreated 79.52 ± 2.57% N= 17 n= 28; p= 0.0458 Mann Whitney U-test).

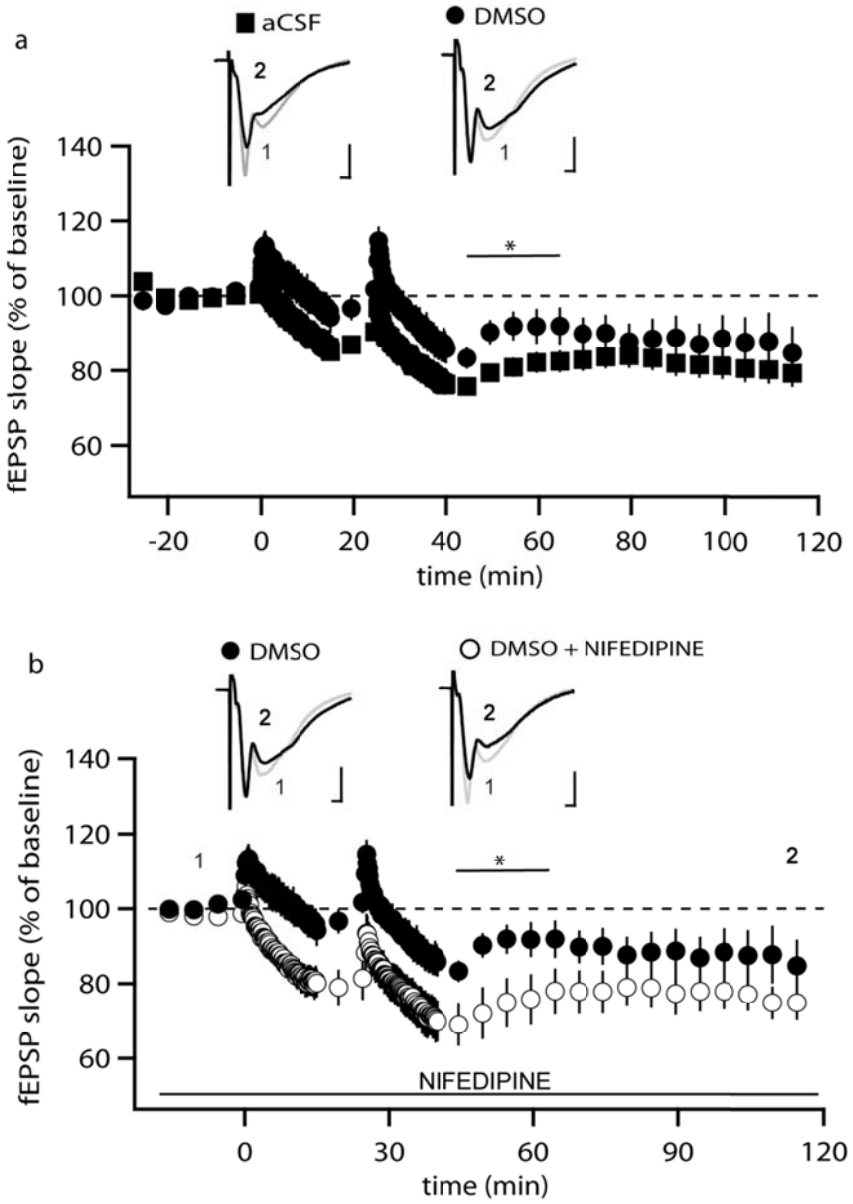

Figure 21 Summary of nifedipine experiments
(a) Summary experiments of slices in DMSO (filled black cicles, N= **4** n= 8) compared to experiments in aCSF (filled black squares, N=17 n=28). (b) LTD in nifedipine- treated (empty circles; N= 4 n= 8) compared to slices in DMSO (filled black circles; N= 4 n= 8).

Following LFS, statistical comparison showed an enhanced E-LTD in nifedipine treated relative to DMSO slices (fig. 21b) (DMSO 89.32 ±3.64% of baseline N=4 n=8, nifedipine-treated 73 ±6.46% of baseline N=4 n=8, average from t=40 to t=60, treatment p= 0.0414 time p< 0.0001 interaction p= 0.566, two-way ANOVA RM). Control pathways of both untreated and nifedipine-treated slices followed LFS

showed no significant difference from baseline values (untreated 114.48 ±8.19% N= 4 n= 8; average from t= 40 to t= 115; p= 0.1115 paired t-test, nifedipine-treated 100.39 ±4.89% N= 4 n= 8; average from t= 40 to t= 115; p= 0.9374). Taking together these results, first DMSO partially blocks LTD during and after LFS. Second, blocking L-type VGCCs enhances LTD induction.

4.2.5 LTD and the simultaneous inhibition of NMDAR and L-type VGCC

Previous studies showed that in hippocampal CA3-CA1 pathway specific patterns of stimulation differentially activate NMDARs and L-type VGCCs, resulting in distinct forms of LTP [83]. Since I assessed that LTD induction is NMDA receptors dependent and blocking L-type VGCC enhanced LTD, we were interested now to investigate the simultaneous cross talk between NMDAR and L-type VGCC on LTD induction. To test the simultaneous requirement of NMDARs and L-type VGCCs on LTD induction, APV (50 μM) alone or together with nifedipine (20 μM) was bath-applied and LTD experiments were performed. As previously reported, APV alone blocked LTD (see paragraph 4.2.2, fig.18). No significant differences were found between LTD of APV-treated and (APV + nifedipine)-treated slices (fig.22) (APV-treated 96.79 ±6.51% N= 3 n= 6, (APV + nifedipine)-treated N= 3 n= 6; treatment p= 0.9139, time $p < 0.0001$, interaction p= 0.6231 two-way ANOVA RM). Control pathways of APV-treated and (APV + nifedipine)-treated slices were stable throughout the entire recording (APV-treated 110.15 ±5.31% of baseline; average from t= 40 to t= 115, p= 0.1020 paired t-test; (APV+nifedipine)-treated 108.71 ±7.98% of baseline; average from t= 40 to t= 115, p= 0.3167 paired t-test).

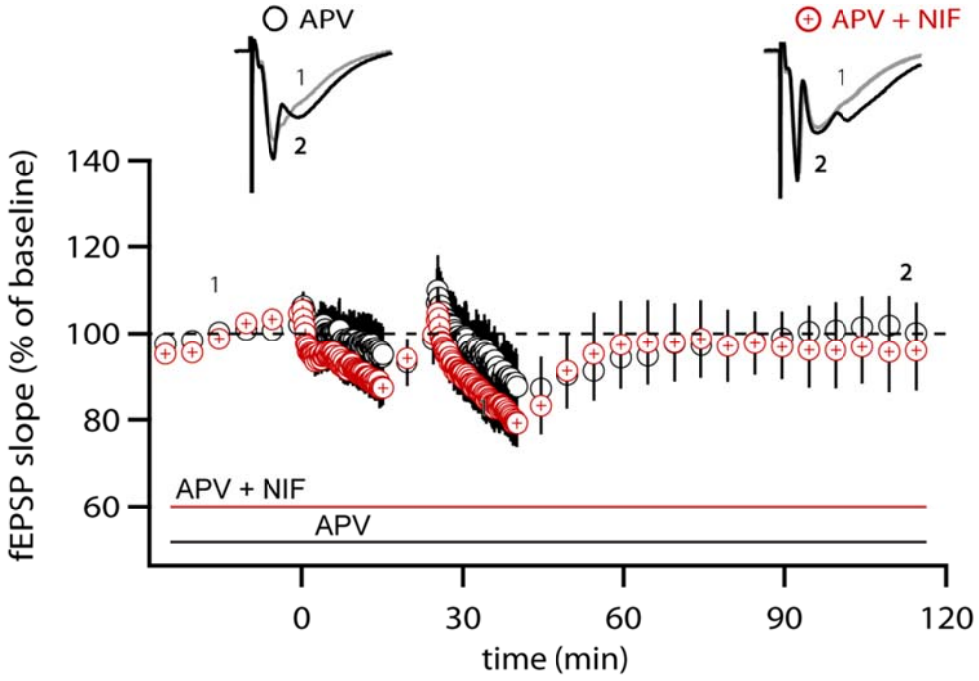

Figure 22 Summary graphs of APV + nifedipine experiments
LTD in APV + nifedipine-treated slices (empty red circles; 95.63 ±8.76% of baseline, N= 3 n= 6) compared to APV- treated slices (empty black circles; 96.79 ±6.51% of baseline, N= 3 n= 6). Direct comparison between both groups showed no statistical differences treatment p= 0.9139 time p< 0.0001 interaction p= 0.6231 two-way ANOVA RM).

As previously assessed, NMDA receptors are essential for LTD induction. Here, the simultaneous blocking of NMDARs and L-type VGCCs also prevented LTD induction. Interestingly, based on these results NMDARs might act upstream to the L-type VGCCs, which cannot sustain LTD independently. 4.3 Mechanisms underlying LTD maintenance.

4.3 Mechanisms underlying LTD-maintenance in WT mice

Long lasting models of synaptic plasticity, including LTD and LTP, are believed to be cellular basis of memory formation. The mechanisms required for their maintenance are thought to regulate and be regulated by activity dependent gene transcription and *de novo* protein synthesis [84]. Additionally, evidence suggests that protein degradation mediated by ubiquitin-proteasome system (UPS) and lysosomal pathways are critical regulators of LTD and LTP as well [85],[84]. In order to evaluate the mechanisms

underlying LTD maintenance, I investigated the role of protein synthesis and degradation pathways in LTD.

4.3.1 LTD and protein synthesis

First, we were interested in the role of novel protein synthesis in LTD maintenance. I applied cycloheximide (CHX 120 μM), a well-known protein synthesis blocker. In untreated slices, LFS stimulation induced a normal decrease in synaptic transmission to 71.15 ±4.12% of baseline (N= 4 n= 5) (fig. 23a). In contrast, LTD was blocked in CHX- treated slices (fig. 22a) (91.30 ±4.93% N= 4 n= 7). Statistical analysis showed significant differences between untreated and CHX-treated slices (fig. 23b) (average from t= 40 to t= 115, treatment p= 0.0138 time p < 0.0001 interaction p= 0.7108, two-way ANOVA RM). Control pathways were unaffected by LFS stimulation (untreated 92.69 ±4.31% of baseline, N= 4 n= 5, p= 0.1579 paired t-test; CHX-treated 100.68 ±8.17% of baseline, N= 4 n= 7, p= 0.9362 paired t-test). In conclusion, LTD maintenance is supported by the synthesis of novel proteins.

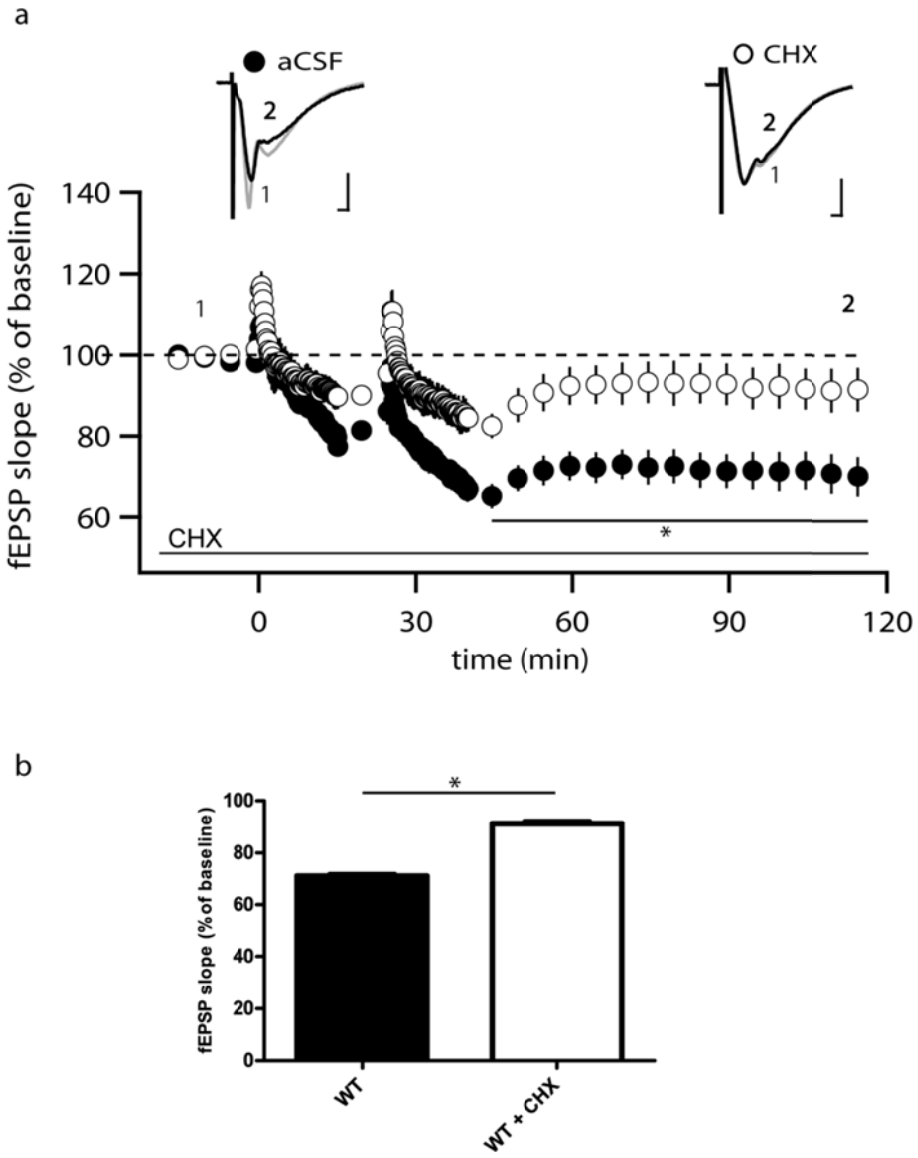

Figure 23 Protein synthesis inhibitor CHX blocks LTD maintenance
CHX-treated slices show LTD to 91.30 ± 4.93% of baseline (empty circles; N= 4 n= 7), in untreated slices LTD decreased to 71.15 ±4.12% of baseline (filled black circles; N= 4 n= 5). (b) Bar graph showing mean normalized fEPSP slope following LFS stimulation in untreated and CHX-treated slices.

4.3.2 LTD and proteasomal degradation

Evidence from the last decades suggests that protein degradation mediated by the ubiquitin-proteasome system (UPS) may also be a critical regulator of LTP[31]. However the role of proteasome in regulating LTD remains elusive. Here, I applied MG 132 (500 µM) to investigate the role of proteasome activity in LTD maintenance,

the results of which are shown in Fig 4.12. Mean fEPSP slopes of untreated slices following LFS stimulation decreased to 73.61 ±2.93% (N= 8 n= 20) (fig. 23). Direct comparison with mean fEPSP slopes of MG132-treated slices showed no significant difference in LTD maintenance (fig. 24) (75.88 ±5.86% of baseline N= 6 n= 13, treatment p= 0.9052 time p < 0.0001 interaction p= 0.9547, two-way ANOVA RM).

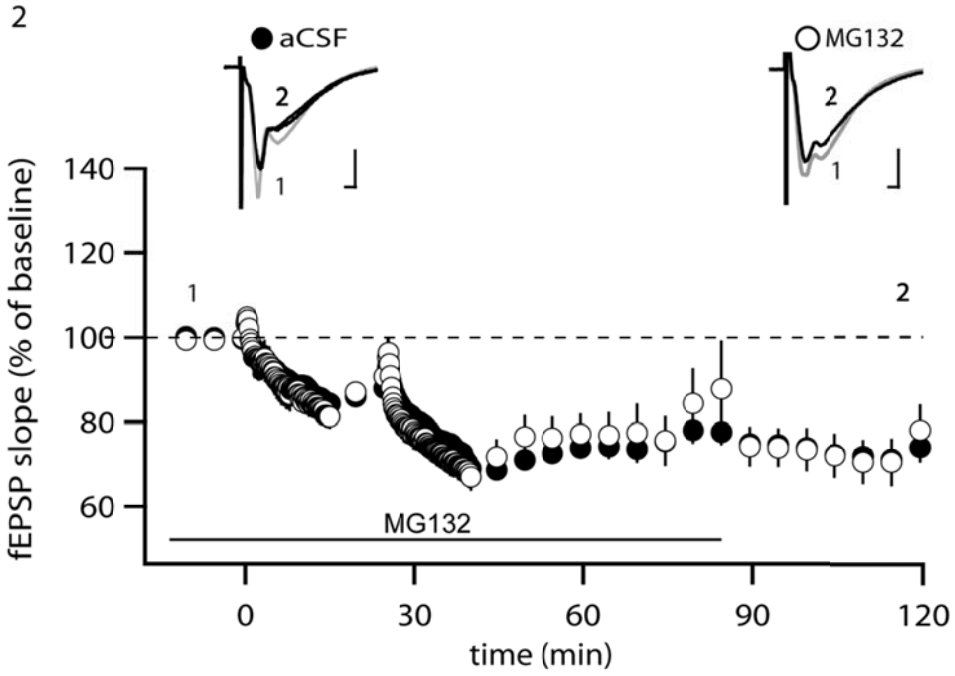

Figure 24. The proteasome inhibitor MG-132 does not affect LTD
LTD in MG132-treated slices decrease to 78.88 ± 5.86% of baseline (500 µM) (empty circles; N= 6 n= 13) compared to untreated slices, decreasing to 73.61 ±2.93% of baseline (filled black circles; N= 8 n= 20) (average from t= 40 to t= 115).

No significant differences were found in control pathways (untreated 91.66 ±5.19 % of baseline N= 6 n= 15, p= 0.1477 paired t-test; MG132- treated 88.74 ±6.63% of baseline N= 6 n= 12; p= 0.1860 paired t-test). In summary, under our experimental conditions, LTD is not dependent on ubiquitin proteasome degradation.

4.3.3 LTD and lysosomal degradation

Trafficking of AMPA receptors is a key component in modulating the strength of synaptic transmission, resulting in either LTP or LTD. The lysosomal distribution of AMPARs after LTD induction is well accepted[23],[86]. In order to examine the role of lysosomal degradation in LTD maintenance, I induced LTD in the presence of leupeptin, a lysosomal blocker. Untreated slices following LFS stimulation showed LTD of 84.38 ±3.53% of baseline (fig. 25) (N= 5 n= 6). LTD in leupeptin-treated slices was blocked (99.78 ±8.2% N= 5 n= 8; $p < 0.0001$ Mann Whitney test). Analysis of control pathways of both untreated and leupeptin-treated slices showed no significant changes in fEPSPs before and after LFS (untreated 105.39 ±4.77% of baseline N= 5 n= 6, p= 0.3055 paired t-test; leupeptin-treated 105.7 ±3.87% of baseline N= 5 n= 8, p= 0.2002 paired t-test). Based on these results, lysosomal degradation is essential for the maintenance of LTD.

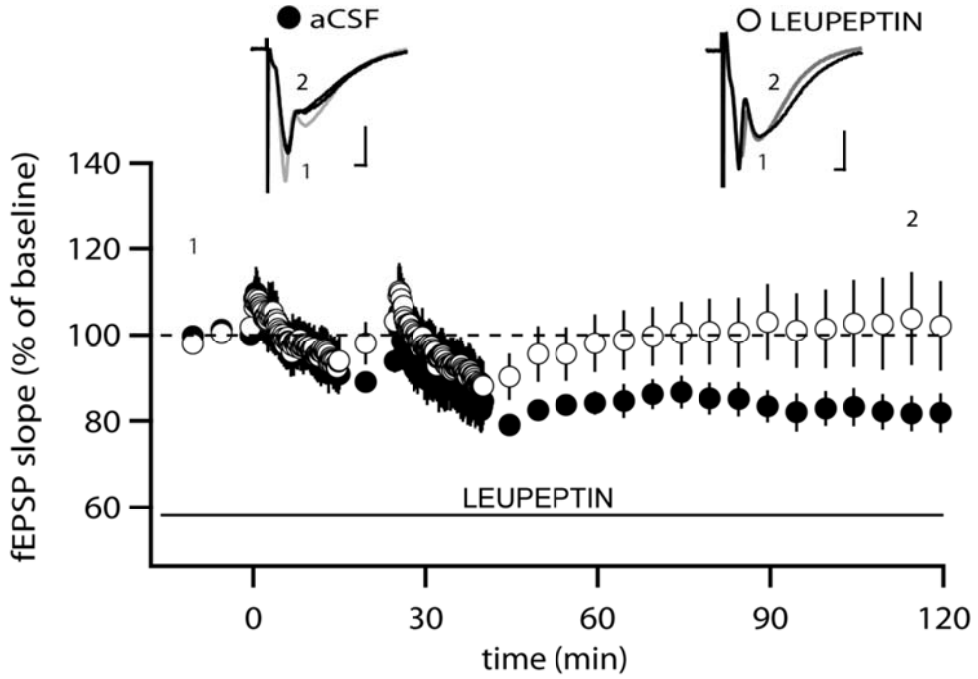

Figure 25 Summary of leupeptin experiments
LTD in LEU-treated slices (20 µM) (empty circles; N= 5 n= 8), resulting in EPSP slopes of 99.78 ± 8.2% of baseline compared to untreated slices (filled black circles; N= 5 n= 6), decrease in EPSP slopes of 83.38 ±3.53% of baseline ($p < 0.0001$ Mann Whitney test).

4.3.4 LTD is preserved by simultaneous blocking of protein synthesis and lysosomal degradation

Long lasting changes in neuronal networks rely on several steps which are based on a variety of signaling cascades at synapses. It has already been shown that maintenance of LTP is determinate by a balance of protein synthesis and proteasomal degradation[31]. In order to assess whether our LTD was a balance between synthesis of novel proteins and degradation pathways, we explored the effect of simultaneously blocking both protein synthesis with CHX and lysosomal degradation with leupeptin. LTD in (CHX + LEU)-treated slices was normal (fig. 25) (86.6 ±3.91% of baseline, N= 4 n= 7), and comparison with LTD of untreated slices found no significant differences (fig. 26) (84.10 ±4.38% of baseline, N= 5 n= 8; treatment p= 0.6753 time p < 0.0001 interaction p= 0.9462, two-way ANOVA RM). Synaptic specificity was confirmed in both groups of slices (untreated 101.64 ±3.03% of baseline N= 5 n= 8, p= 0.4819 paired t-test; (CHX + LEU)–treated 100.57 ±2.86% of baseline N= 4 n= 7, p= 0.849 paired t-test).

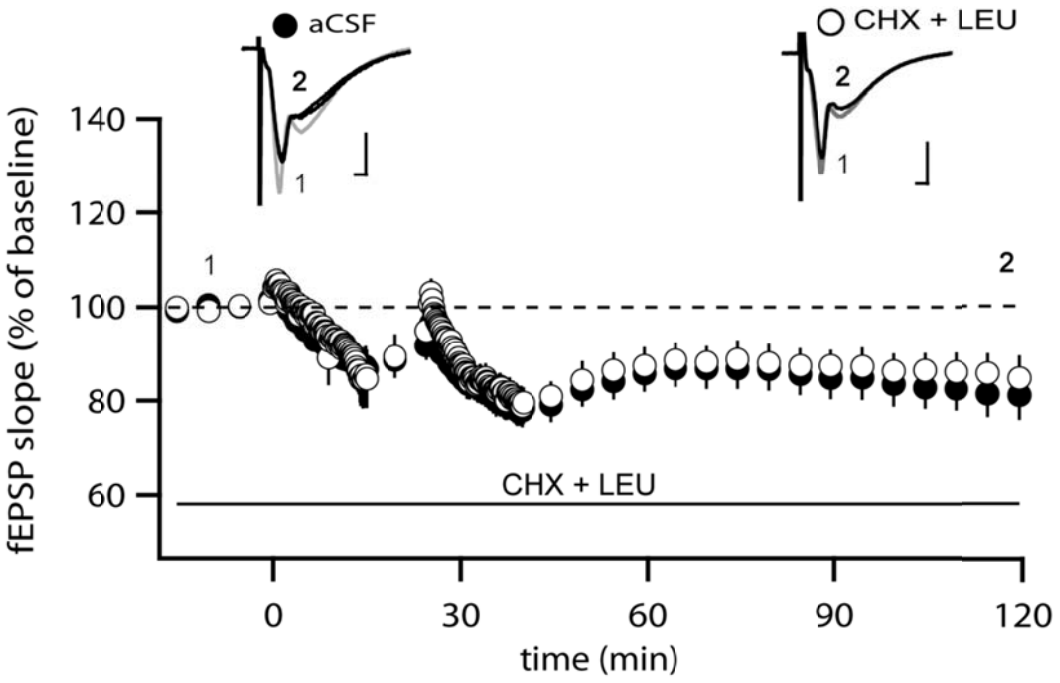

Figure 26. Combined blockade of protein synthesis and degradation by CHX and LEU restores LTD in WT slices
LTD in (CHX + LEU)-treated slices (empty circles; N= 4 n= 7), resulting in EPSP slopes of 86.6 ±3.91% of baseline compared to untreated slices (filled black circles; N= 5 n= 8), decrease in EPSP slopes of 84.10 ±4.38% of baseline. (Average from t= 40 to t= 115, treatment p= 0.6753 time p< 0.0001 interaction p= 0.9462).

These results show that blockade of either protein synthesis or lysosomal degradation separately antagonizes LTD maintenance. However, when protein synthesis and protein degradation are inhibited at the same time, LTD is restored to control levels. Thus, NMDAR-LTD is supported by a balanced cross-talk between novel protein synthesis and lysosomal degradation.

4.4 Synaptic plasticity in germline Arc/Arg3.1 KO mice

4.4.1 Evaluation of basal synaptic transmission

Initially, to estimate basal synaptic transmission in WT and KO slices, I measured maximum fEPSP amplitude reached with stimulation intensity from 0 to 1600 µA. Statistical analysis showed no significant difference between IO curves generated in WT and KO slices (fig. 27a) (WT N= 7 n= 12 KO N= 7 n= 14, (genotype p= 0.1618 time p < 0.0001 interaction p= 0.7749, two-way ANOVA RM). Since I/O curves include pop-spikes evoked at higher stimulation intensities, we performed a more detailed analysis to isolate the synaptic properties of CA1. Therefore, I examined the I/O results from individual experiments to see at what current a pop-spike occurred, noting the corresponding stimulation intensity ($SI_{threshold}$) and resulting fEPSP threshold amplitude at that current. Here, the fEPSP threshold amplitude was not significantly different between WT and KO slices (fig. 27c) (WT N= 8 n= 15 KO N= 8 n= 16, p= 0.6494, Mann Whitney t-test). However, KO slices showed significantly

increased $SI_{threshold}$ compared to WT slices (fig. 27b) (WT N= 8 n= 15 KO N= 8 n= 16, p= 0.0364, Mann Whitney U-test).

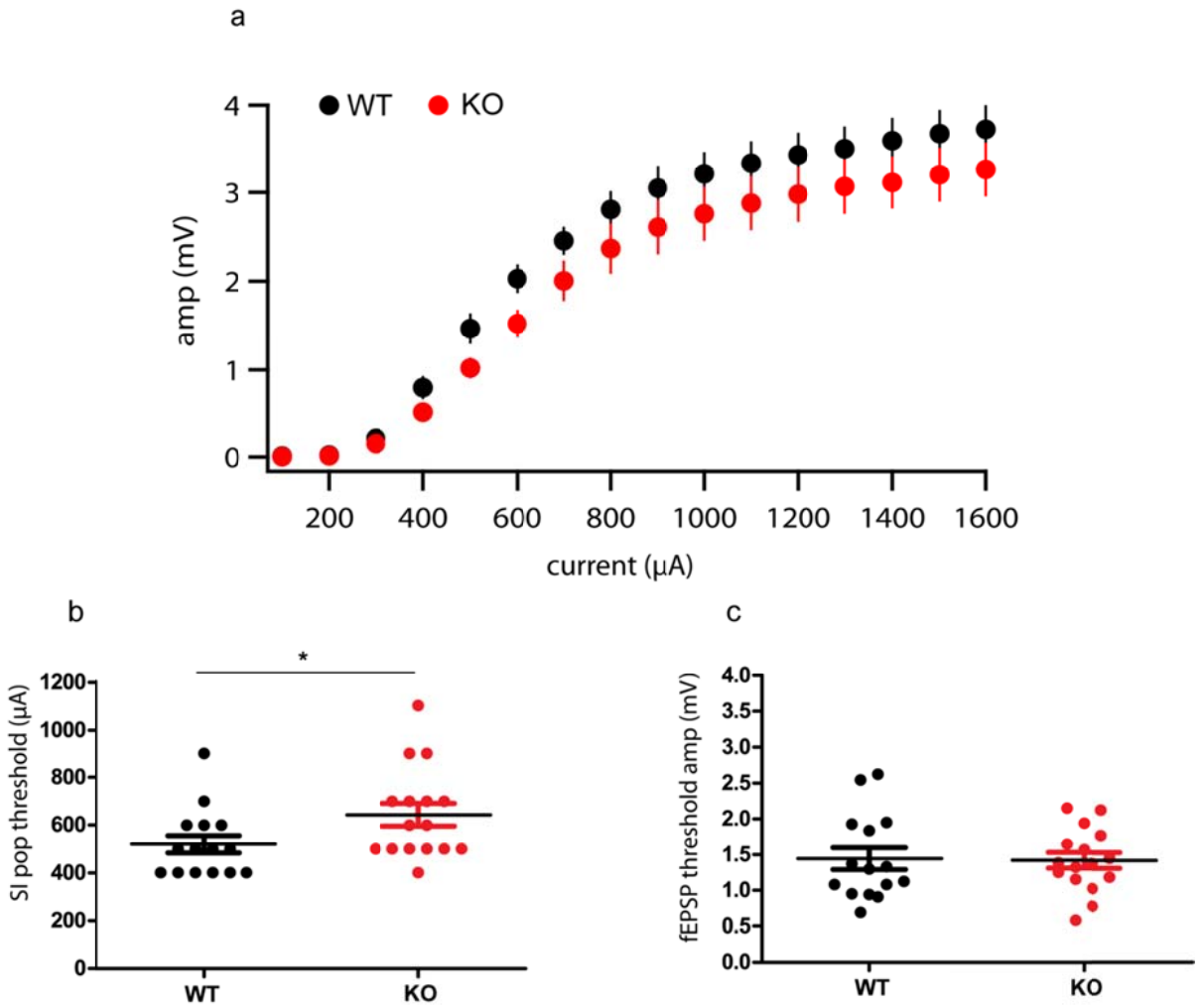

Figure 27 Analysis of I/O curves in WT and KO slices
(a) Summary I/O curves were generated over a range of stimulus intensities (0-1600 µA). Each point represents the mean of all slices tested (WT N= 7 n= 12 KO N= 7 n= 14). (b) Summary graph showing $SI_{threshold}$ calculated in WT and KO slices (WT N= 8 n= 15 KO N= 8 n= 16). KO slices showed significantly increased in $SI_{threshold}$ compared to WT slices (p= 0.0364, Mann Whitney t-test). (c) Summary graph showing fEPSP threshold amplitude in WT and KO slices (WT N= 8 n= 15 KO N= 8 n= 16). No significant difference was found among groups (p= 0.6494, Mann Whitney U-test).

In summary, initial I/O curve analysis showed no significant difference among genotypes. However, $SI_{threshold}$ in KO slices appeared significantly higher compared to $SI_{threshold}$ in WT slices, namely, KO slices need higher current to evoke pop-spike.

The increased amount of current needed to induce action potentials in KO slices might be due to the reduced membrane excitability.

4.4.2 LTD in KO

Arc/Arg3.1 is an activity regulated- immediate early gene implicated in several mechanisms underlying synaptic plasticity, i.e it plays a key role in determining synaptic strength through facilitation of AMPA receptor endocytosis [49,47]. Arc/Arg3.1-deficient mice show impairment in LTD [46,52,87]. Nonetheless, LTD protocols previously used differ substantially from the new established protocol. We were interested to explore the new LFS protocol in our adult KO slices. A representative example of LTD in a KO slice is shown in figure 28. Following LFS stimulation, fEPSPs decreased to 70.07% of baseline (average from t= 40 to t= 60) and remained at 74.93% of baseline (average from t= 100 to t= 120) until the end of the recording.

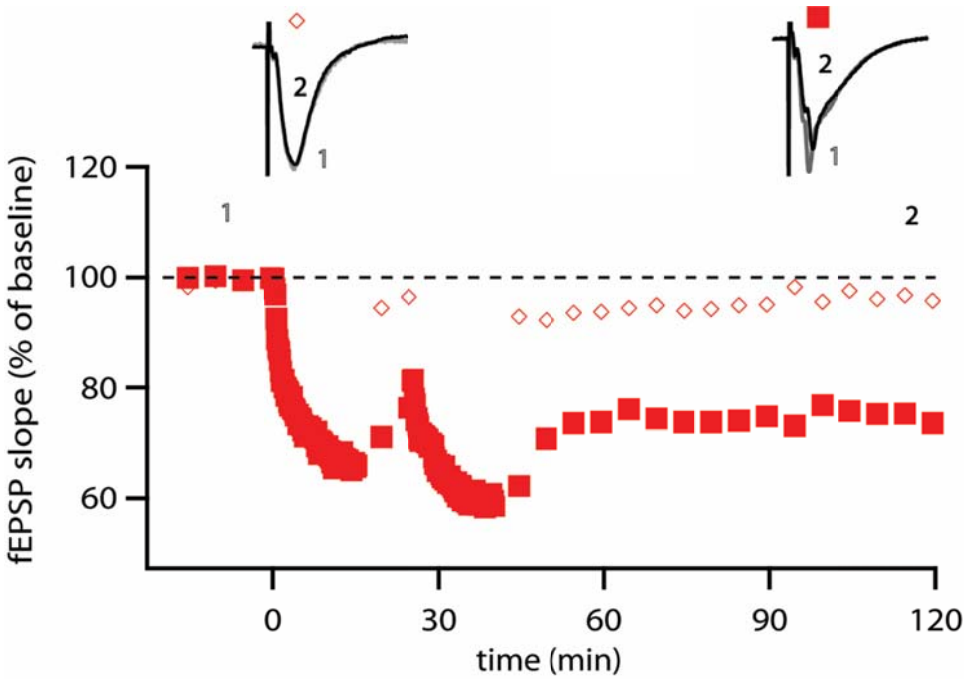

Figure 28 LTD is induced and persists in KO slices
Exemplary experiment in which LTD was induced in a KO slice.

I repeated the same experiment in 14 mice (n= 21 slices) and compared directly with WT LTD experiments (fig. 29a). E-LTD was significantly enhanced in KO slices compared to WT slices (fig. 29b) (WT 79.528 ± 2.567% of baseline N= 17 n= 28, KO slices 71.769 ±2.725% of baseline N=14 n= 21, average from t= 40 to t= 60; genotype p= 0.0441 time p < 0.0001 interaction p < 0.0001, two-way ANOVA RM). Nevertheless, L-LTD was normal (fig. 29c) (WT 79.528 ± 2.567% of baseline N= 17 n= 28, KO slices 71.769 ± 2.725% of baseline N= 14 n= 21, average from t= 100 to t= 120; genotype p= 0.9539 time p= 0.0595 interaction p= 0.162, two-way ANOVA RM).

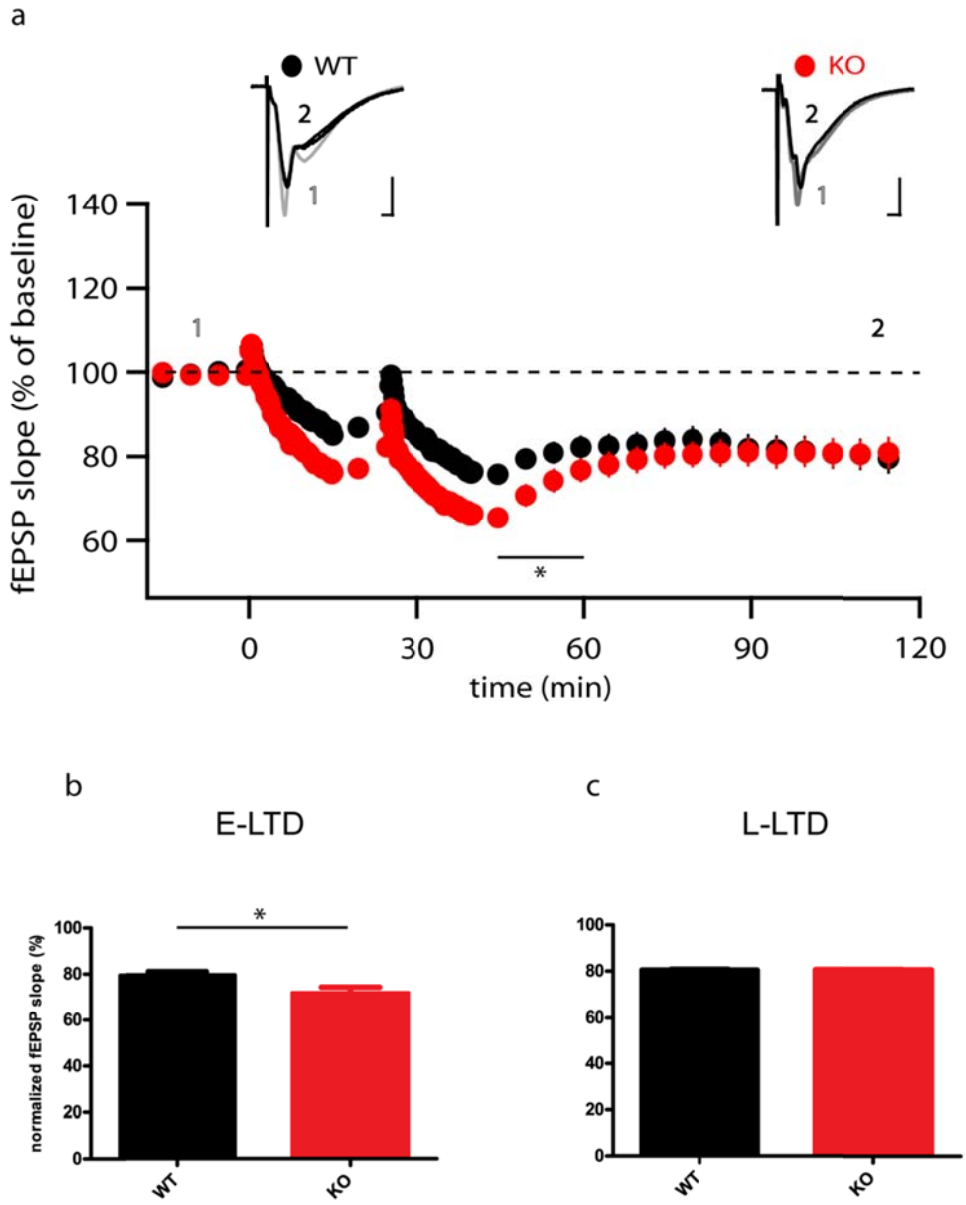

Figure 29 Summary of LTD experiments in WT and KO slices
(a) Summary graph comparing LTD of WT slices (black circles N=17 n= 28) and KO slices (red circles N= 14 n= 21). Insets fEPSP traces of baseline (grey line) and L-LTD (black line). (b) Bar graph comparing normalized mean fEPSP slopes of E-LTD in WT and KO slices (average from t= 40 to t= 60) (c) Bar graph comparing normalized mean fEPSP slopes of L-LTD in WT and KO slices (average from t= 100 to t= 120).

In summary, KO mice showed an increased E-LTD compared to WT mice and a normal L-LTD. These counterintuitive results are not consistent with literature which states that Arc/Arg3.1 is required for enduring forms of plasticity in mammalian brain (Guzowski et al., 2000, Plath et al., 2006, Linden et al., 2010).

4.5 Mechanisms underlying LTD induction in germline Arc/Arg3.1 KO mice

The previously shown long lasting LTD exhibited in our KO slices is not in line with the widely believed hypothesis that memory formation requires consolidation of synaptic connectivity changes, initiated at the time of learning[88]. It also differs from previous reports on LTD deficits in Arc/Arg3.1 KO mice[46], evoked by different stimuli. To better understand the relevance of this LTD to memory and of the importance of Arc/Arg3.1, I investigated the mechanisms underlying the aberrant plasticity in KO mice.

4.5.1 LTD is NMDAR dependent in KO

NMDAR activation has been linked to Arc/Arg3.1, targeting Arc/Arg3.1 mRNA to active synapses[89]. NMDAR- dependent synaptic plasticity events have been shown to be directly related to memory storage (Frey and Morris, 1997)[91]. However, the coupling of NMDAR Arc/Arg3.1 and memory in LTD is still elusive. In order to determine whether NMDA receptors mediate LTD induction in KO slices, I induced LTD in presence of APV (50 μM). Untreated slices, following LFS stimulation, underwent LTD (fig. 30a) (70.69 ±2.18% of baseline N= 4 n= 7). In contrast, LTD in APV-treated slices was completely blocked (fig. 30a) (109.78 ±7.94% of baseline N= 4 n= 8), the statistical difference between the two groups being highly significant (treatment p= 0.0009 time p < 0.0001 interaction p= 0.0237, two-way ANOVA RM). Control pathways of both groups were unaffected by LFS stimulation (untreated 95.73 ±2.4% of baseline N= 4 n= 7, p=0.2472 paired t-test; APV treated 105.08 ±2.5% of baseline N= 4 n= 8, p= 0.0606 paired t-test). The effect of APV on LTD in KO slices was comparable to LTD in WT slices treated with APV (fig. 30b) (APV treated- WT slices 98.74 ±5.07% of baseline N= 6 n= 10; APV treated- KO slices

109.78 ±7.94% of baseline N= 4 n= 8; average from t= 40 to t= 115; genotype p= 0.1384 time p <0.0001 interaction p= 0.2904, two-way ANOVA RM). Taken together, these results suggest that NMDAR signaling is required for LTD induction in both the Arc/Arg3.1 KO and WT.

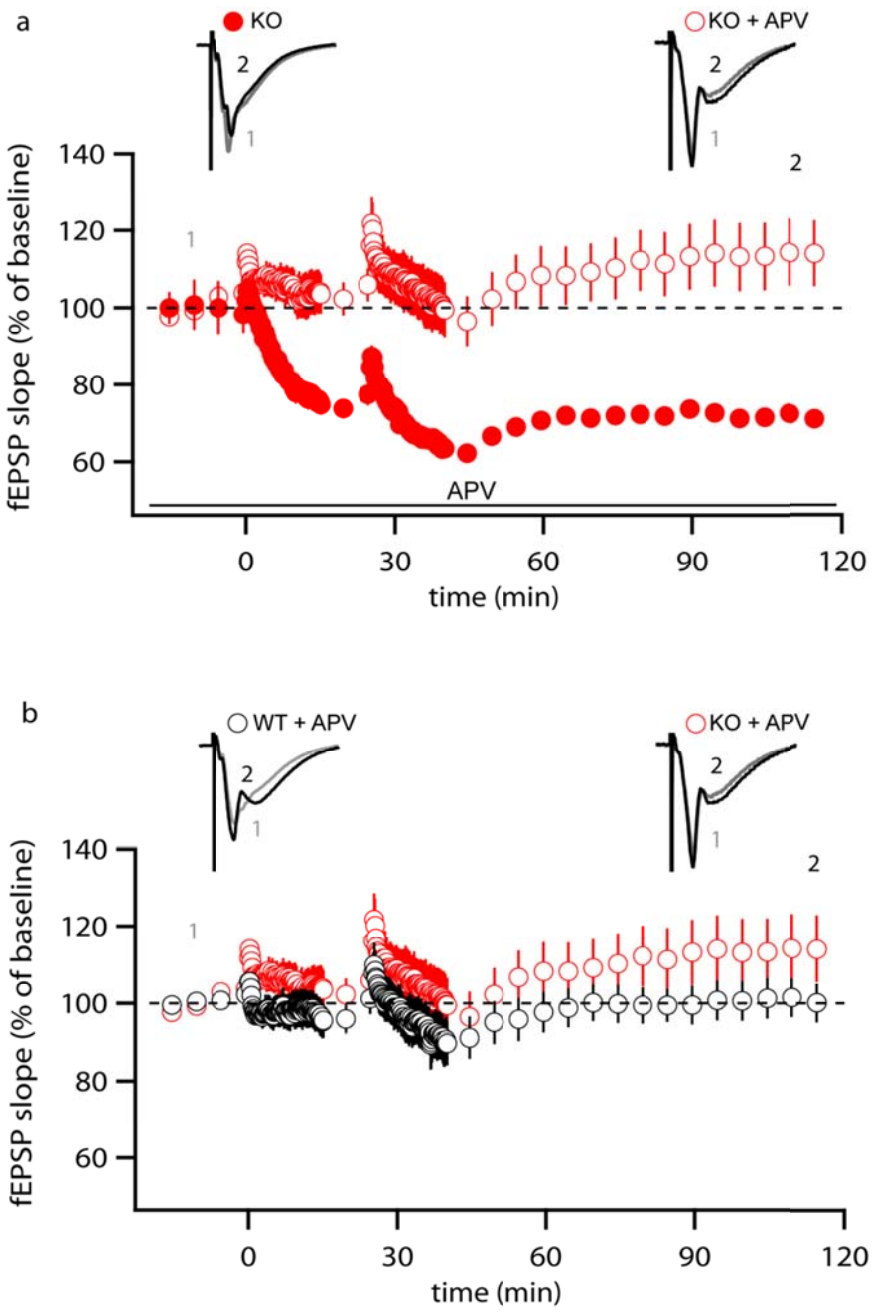

Figure 30 Summary of LTD experiments in APV-treated KO slices.
(a) Summary graph comparing LTD of KO (red filled circles N=4 n= 7) and KO APV-treated slices (red empty circles N= 4 n= 8). Above the graph, fEPSP trace of baseline (grey line) compared to the fEPSP trace of L-LTD (black line) for each group. (b) Summary graph representing the mean of LTD experiments in APV- treated WT (N= 6 n= 10) and KO slices

(N= 4 n= 8) (average from t= 0 to t= 120; genotype p= 0.2212 time p < 0.0001 interaction p= 1, two-way ANOVA RM).

4.5.2 LTD is independent of GluN2B-containing NMDAR subunit

To assess whether blocking NR2B-containing NMDARs affects LTD induction in KO slice, I bath applied RO 25. Following LFS stimulation, LTD remained unaffected when blocking NR2B-containing NMDARs (fig. 31) (untreated KO 75.35 ±8.58% of baseline N= 3 n= 6, RO25- treated KO 70.77 ±3.11% of baseline N=3 n=6, treatment p= 0.62, time p < 0.0001, interaction p= 0.2855, two-way ANOVA RM).

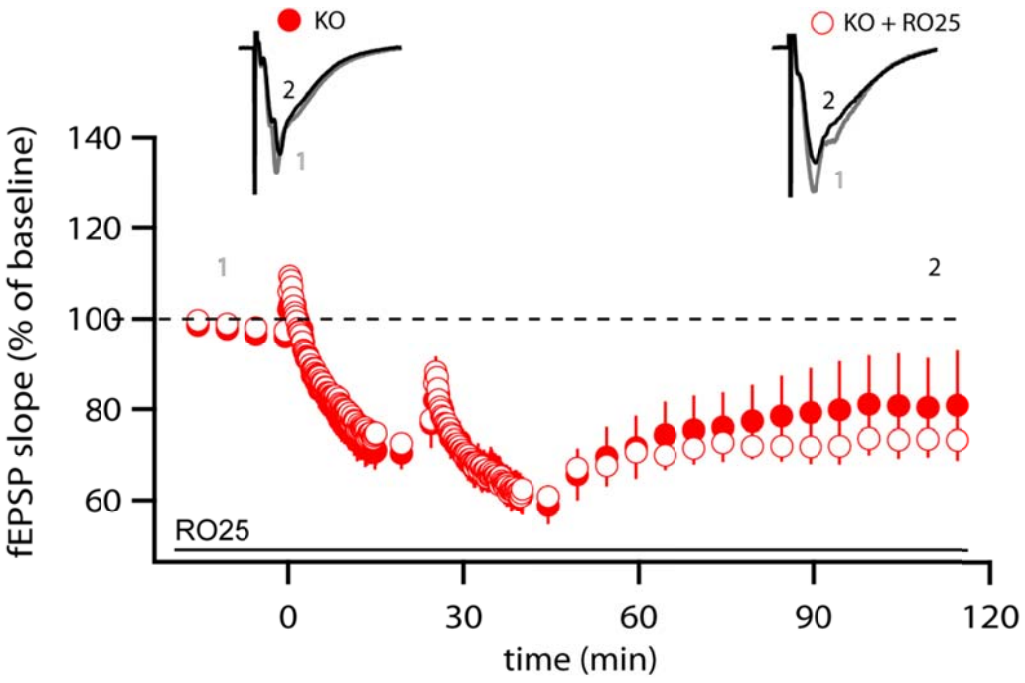

Figure 31 Summary of LTD in untreated and RO-treated KO slices
Summary graph comparing LTD of untreated (red filled circles N=3 n= 6) and RO 25- treated KO slices (red empty circles N= 3 n= 6). Above the graph, fEPSP trace of baseline (grey line) compared to the fEPSP trace of L-LTD (black line) for each group.

Control pathways remained stable throughout the entire recordings in both untreated and Ro-25 treated slices (untreated 91.164 ± 5.958% of baseline, N= 3 n= 6, p= 0.1952 paired t-test; RO 25-treated 95.294 ± 10.561% of baseline, N=3 n= 6; p=

0.6727, paired t-test). In summary, KO slices showed NMDAR-dependent LTD which is not dependent on NR2B-subunit.

4.6 Mechanisms underlying LTD-maintenance in germline Arc/Arg3.1 KO mice

4.6.1 LTD is protein synthesis independent

Activity-dependent long lasting changes in synaptic function are believed to be mediated by the synthesis of novel proteins, eventually leading to memory consolidation[92]. I previously reported that the novel form of LTD in WT is supported by the synthesis of novel proteins (see paragraph 4.3.1, fig. 23). In order to assess whether maintenance of the LTD in KO slices is similarly mediated by the synthesis of novel proteins, I repeated the same LTD experiments in presence of CHX. Application of LFS stimulation to KO slices reliably induced LTD of evoked excitatory synaptic responses to 77.15 ±8.17% of baseline (fig. 34) (N= 4 n= 7). Surprisingly, CHX had no effect on LTD in KO slices (fig. 34) (77.30 ±4.74% of baseline, N= 4 n= 8; treatment p= 0.828 time p < 0.0001 interaction p= 0.9924, two-way ANOVA RM).

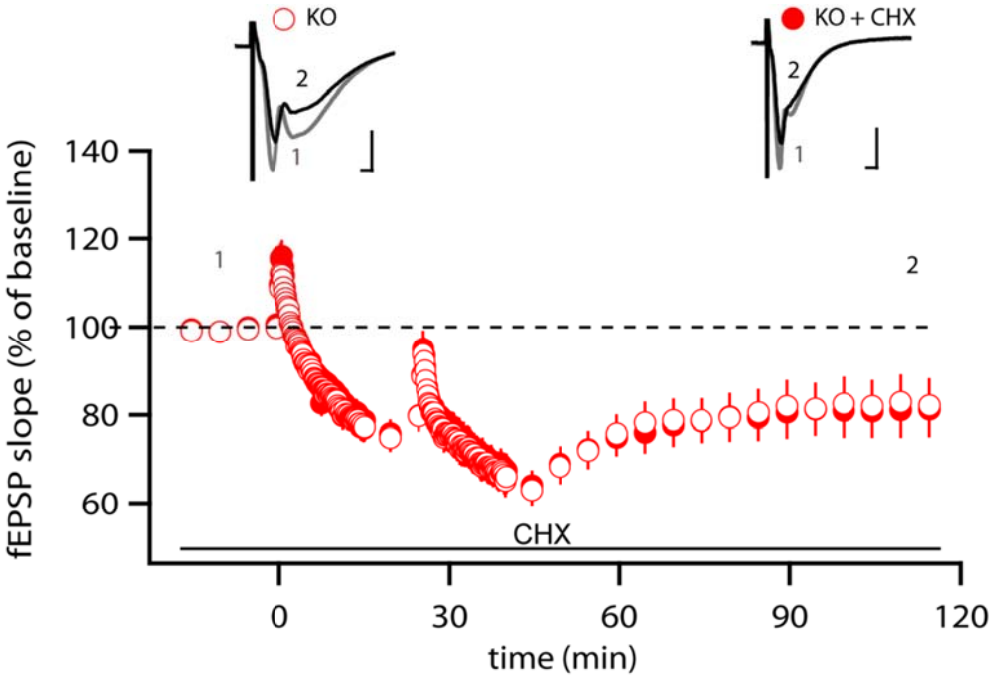

Figure 32. Summary graph of LTD experiments in untreated and CHX-treated KO slices
LTD in CHX- treated KO slices (120 µM) (empty circles; N= 4 n= 8), resulting in a decrease in fEPSP slopes to 77.30 ± 4.74% of baseline compared to untreated slices (filled circles; N= 4 n= 7), decrease in EPSP slope to 77.15 ±8.17% of baseline.

Control pathways remained stable throughout the entire recordings (untreated slices 100.59 ±7.72% of baseline, N= 4 n= 7, p= 0.9415 paired t-test; CHX- treated slices 94.92 ±3.79% of baseline, N= 4 n= 8, p= 0.218 paired t-test). Our data shows that although LTD in KO slices is preserved, it is not mediated by novel protein synthesis.

4.6.2 Basal synaptic transmission and protein synthesis

Novel protein synthesis is a cellular mechanism involved in consolidation of synaptic plasticity and memory storage[93]. In accordance, protein synthesis blocker affected LTD in WT slices but had no effect on LTD in KO slices. Since synapse maintenance may also require ongoing protein synthesis, we aimed to check whether basal synaptic properties were altered by incubation with the protein synthesis blocker. Here, statistical comparison of the I/O curve of WT CHX-treated and untreated slices show that CHX tends to decrease the fEPSP amplitude at higher stimulation

intensities (fig. 32a) (untreated N= 4 n= 8 CHX-treated N= 4 n= 8, treatment p= 0.1585 current p < 0.0001 interaction p= 0.0417, two-way ANOVA RM). Conversely, CHX treatment caused an increase in the upper part of the I/O curve of KO slices, however, this was not statistically significant (fig. 32b) (untreated N= 4 n= 8 CHX-treated N= 4 n= 8, treatment p= 0.2088 current p < 0.0001 interaction p = 0.0861, Two-way ANOVA RM).

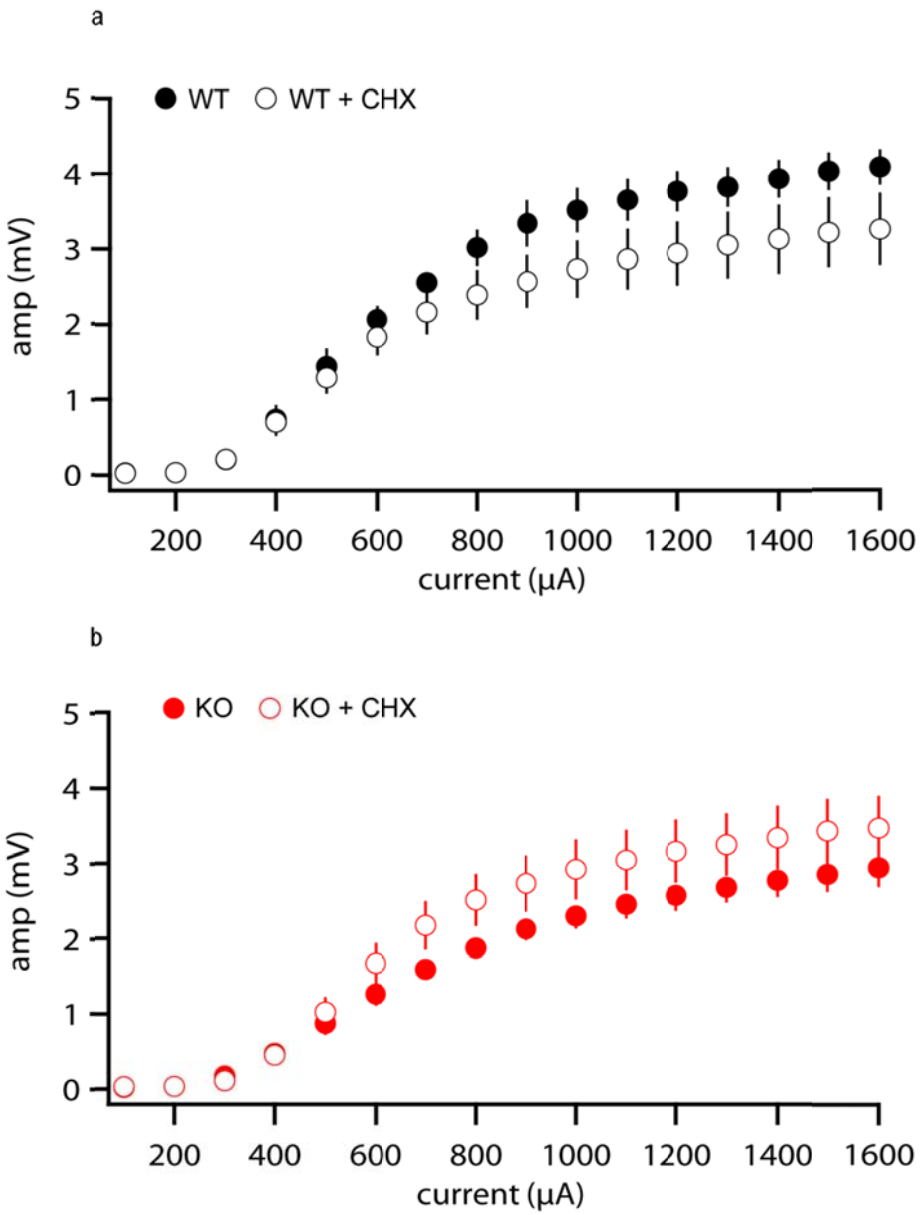

Figure 33 Comparison of I/O curves of WT and KO untreated and CHX-treated slices. (a) Summary I/O curves of WT untreated and CHX-treated slices were generated over a range of stimulus intensities (0-1600 µA). Each point represents the mean of all slices tested

(untreated N= 4 n= 8 CHX-treated slices N= 4 n= 8). (b) Summary I/O curves of KO untreated and CHX-treated slices were generated with the same experimental conditions used in WT slices.

These I/O curves represent a mixture of synaptic and somato-dendritic currents that separately reflect synaptic transmission and membrane excitability. To dissect the effect of CHX on either current, I assessed fEPSP threshold amplitude and the $SI_{threshold}$ in untreated and CHX-treated slices. In WT CHX-treated slices $SI_{threshold}$ was enhanced, but no statistically significant difference in fEPSP threshold amplitude was found compared to untreated slices, meaning that the basal synaptic transmission remains intact combined with modulation of pop-spike threshold (fig. 33a) ($SI_{threshold}$: untreated N= 4 n= 7 CHX-treated N= 4 n= 8, p= 0.0105 Mann Whitney U-test; fEPSP threshold amplitude: untreated N= 4 n= 7 CHX-treated N= 4 n= 8, p= 0.4178 Mann Whitney U-test). Interestingly, KO CHX-treated slices showed increased values in both $SI_{thrshold}$ and fEPSP threshold amplitude (fig.33b) ($SI_{threshold}$: untreated N= 4 n= 8 CHX-treated N= 4 n= 8, p= 0.0389 Mann Whitney t-test; fEPSP threshold amplitude: untreated N= 4 n= 8 CHX-treated N= 4 n= 8, p= 0.0348 Mann Whitney U- test). Thus, the basal synaptic transmission was significantly affected by the CHX-treatment.

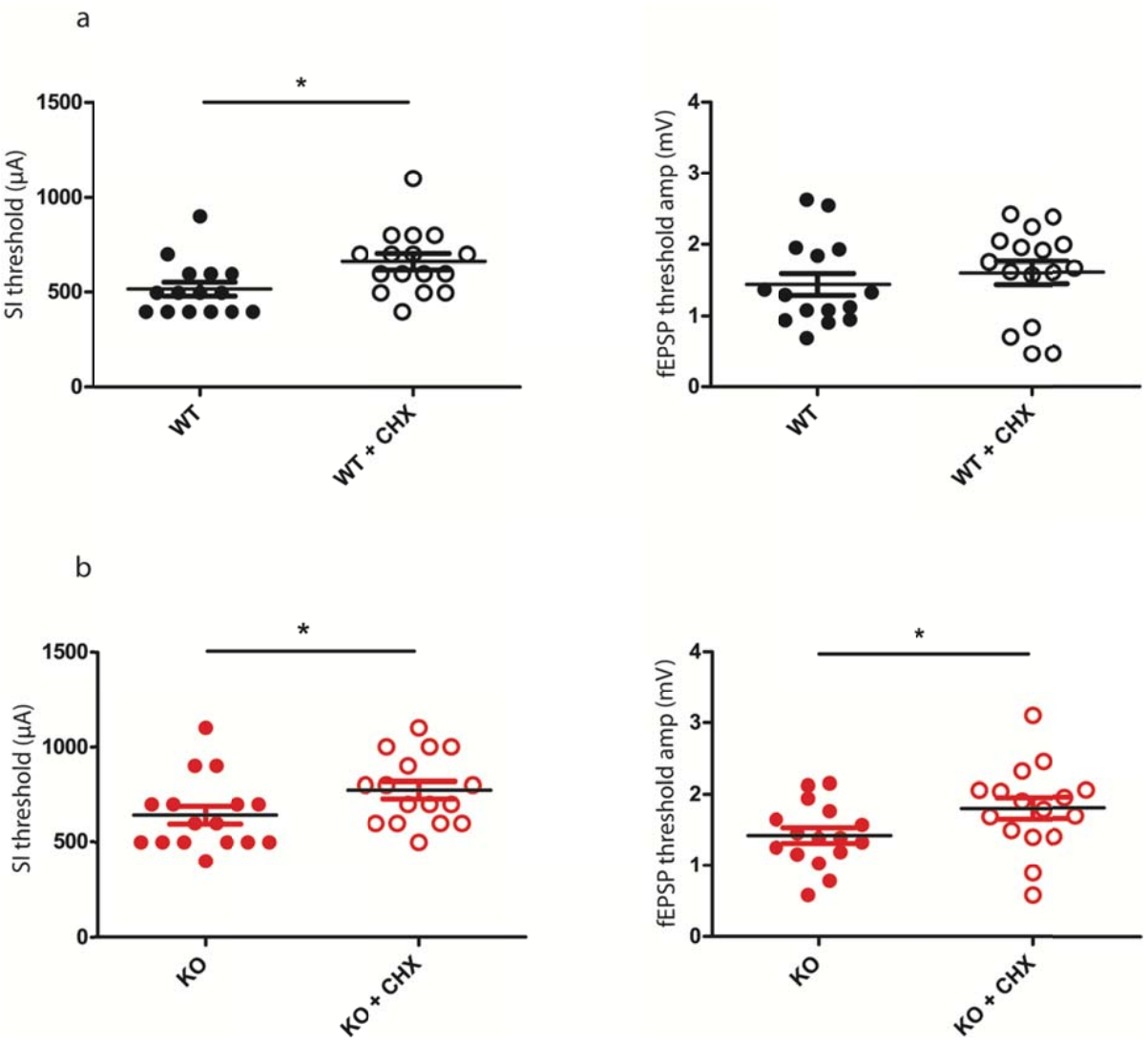

Figure 34 Effects of CHX on SIthreshold and maximum fEPSP amplitudes in WT and KO slices.
(a) WT CHX- treated slices showed increased $SI_{threshold}$ (untreated N= 4 n= 7 CHX-treated N= 4 n= 8, p= 0.0105 Mann Whitney t-test), however, no significant difference was noted in fEPSP threshold amplitude (untreated N= 4 n= 7 CHX-treated N= 4 n= 8, p= 0.4178 Mann Whitney t- test). (b) In KO slices, CHX caused significantly increased $SI_{threshold}$ and fEPSP threshold amplitude compared to untreated slices ($SI_{threshold}$: untreated N= 4 n= 8 CHX-treated slices N= 4 n= 8, p= 0.0389 Mann Whitney t-test; fEPSP threshold amplitude: untreated N= 4 n= 8 CHX-treated slices N= 4 n= 8, p= 0.0348 Mann Whitney t- test).

Taking together these results, CHX does not affect basal synaptic transmission in the WT mice, but increases the threshold to induce action potentials, therefore decreasing the excitability of the neurons. In KO slices, al synaptic transmission was significantly affected by CHX-treatment. CHX increased both the current-threshold to

induce action potentials and the fEPSP amplitude at the threshold, although it did not affect fEPSP amplitudes at lower stimulus intensities.

4.6.3 LTD is lysosomal degradation independent

Activity dependent regulation of AMPA receptors is fundamental for synaptic plasticity, in particular for LTD[94]. Endosomal trafficking is important for the sorting of ionotropic glutamate receptors, and because Arc/Arg3.1 is directly involved in endocytosis of AMPA receptors[49], we were interested to assess the lysosomal function on KO mice blocking the lysosome activity with leupeptin. Figure 36 showed the summary of the LTD experiments comparing untreated and leupeptin–treated KO slices. LTD in leupeptin- treated slices was indistinguishable from LTD in untreated KO slices (untreated 80.69 ±2.74% of baseline N= 7 n= 10; leupeptin-treated 82.94 ±4.28% of baseline N= 7 n= 10; treatment p= 0.6493 time p <0.0001 interaction p= 0.0378, two-way ANOVA RM).

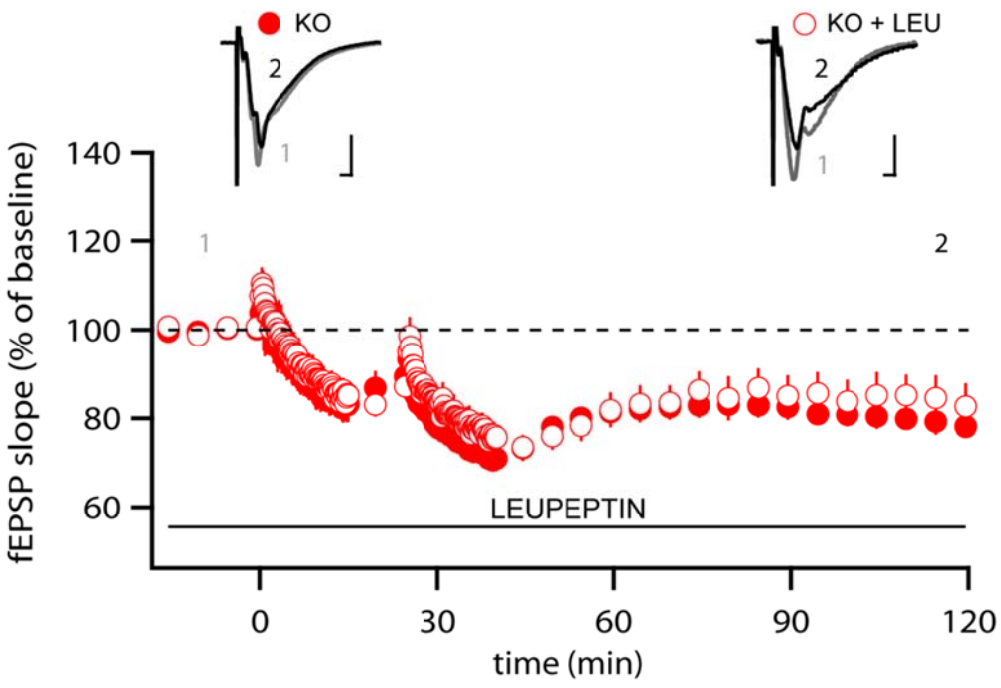

Figure 35 Summary graph of LTD experiments in KO untreated and leupeptin-treated slices.

LTD in WT leupeptin- treated slices (20 µM) (empty circles; N= 7 n= 10), resulting in a decrease in EPSP slopes to 82.94 ± 4.28% of baseline compared to untreated slices (filled circles; N= 7 n= 10), decrease in EPSP slope to 80.69 ±2.74% of baseline.

Control pathways of untreated and leupeptin-treated slices were unaffected by LFS stimulation (untreated 101.36 ±2.29 % of baseline N= 7 n= 10, p= 0.541 paired t-test; leupeptin-treated 103.34 ±3.93% of baseline N= 7 n= 10; p= 0.3978 paired t-test). The block of lysosomal degradation had no effect on LTD in KO slices, and this result leads to the conclusion that although KO slices show stable long-lasting plasticity, it is not coupled to typical expression mechanisms.

4.6.4 Basal synaptic transmission and lysosomal degradation

In order to figure out whether basal synaptic transmission in WT and KO slices was affected by the lysosomal blocker, I performed I/O curves in presence of leupeptin. WT slices were not affected by the lysosomal blocker (fig. 35a) (untreated N=5 n= 6, LEU-treated n= 5 n= 8, treatment p= 0.6949 current p < 0.0001 interaction p= 0.9977, two-way ANOVA RM). On the contrary, KO LEU-treated slices showed a significant reduction in fEPSP amplitude (fig. 35b) (untreated N=6 n= 9, LEU-treated N= 6 n= 10, treatment p= 0.0053 current p < 0.0001 interaction p < 0.0001).

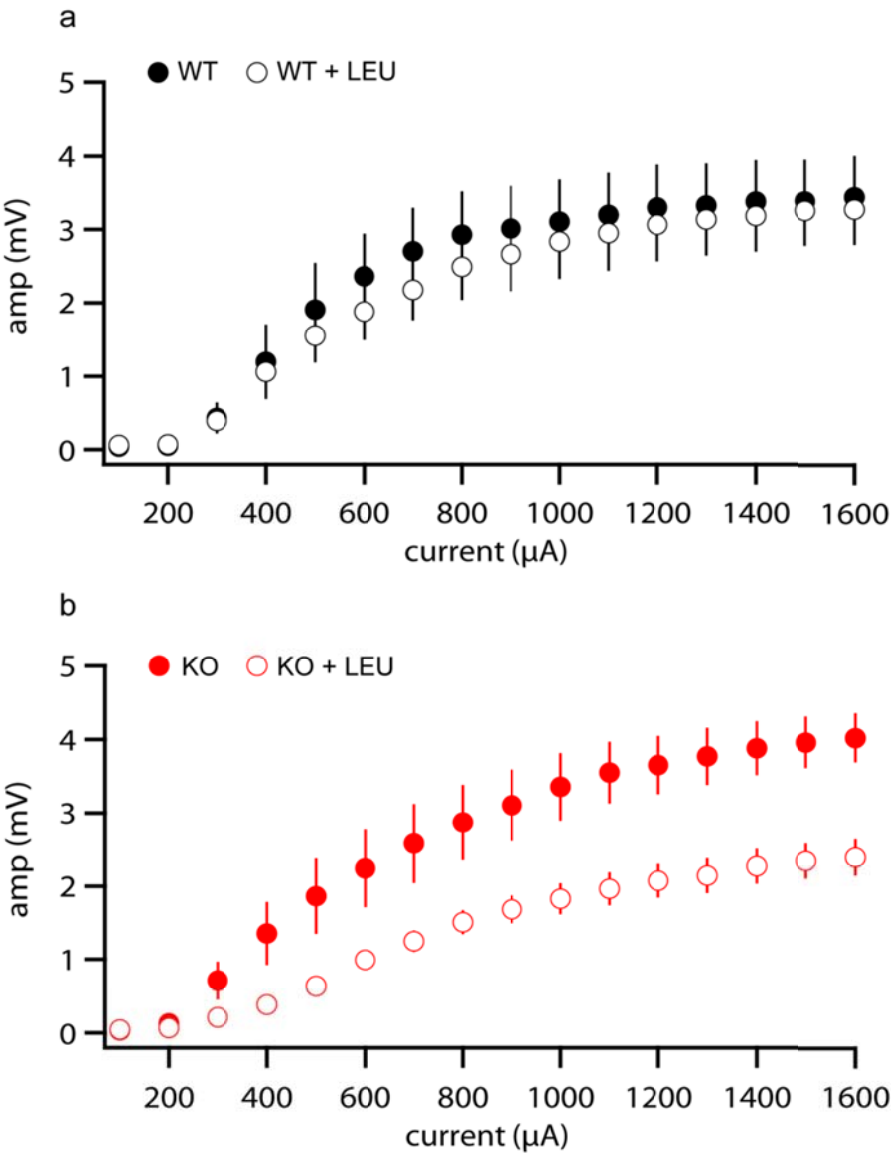

Figure 36 Summary graphs of I/O curves in untreated and leupeptin-treated WT and KO slices.
(a) Summary I/O curves of WT untreated and LEU-treated slices were generated over a range of stimulus intensities (0-1600 µA). Each point represents the mean of all slices tested (black filled circles, untreated N= 5 n= 6, black empty circles LEU- treated slices N= 5 n= 8). (b) Summary I/O curves of KO untreated (red filled circles, N= 6 n= 9) and CHX- treated slices (red empty circles, N= 6 n= 10).

4.6.5 LTD is preserved by simultaneous blocking of protein synthesis and lysosomal degradation

Protein synthesis and lysosomal degradation are activity dependent processes that regulate turnover of synaptic membrane proteins involved in plasticity events [95,96]. Here, LTD in KO slices seems to be unaffected by blocking either novel protein synthesis or lysosomal degradation. Previously I reported that the simultaneous blocking of protein synthesis and lysosomal degradation restored LTD in WT slices (see paragraph 4.3.4, fig. 26). Despite the persistency of LTD in KO slices, we wanted to test whether simultaneous application of CHX and leupeptin alters LTD maintenance in KO slices. Here, co-application of CHX and leupeptin had no effect on LTD and no significant changes were found compared to LTD in untreated slices (fig. 37) (untreated 83.62 ±4.46% of baseline, N=7 n= 10; (CHX+LEU)-treated 81.02 ±5.1% of baseline, N= 4 n= 7; treatment $p=0.7178$ time $p < 0.0001$ interaction $p= 0.4123$, two-way ANOVA RM). No significant changes were found in the control pathways (untreated 101.33 ±3.77% of baseline, N= 4 n= 5, $p= 0.7346$ paired t-test; (CHX + LEU)-treated 97.06 ±3.33% of baseline, N= 4 n= 7, $p= 0.3964$ paired t-test).

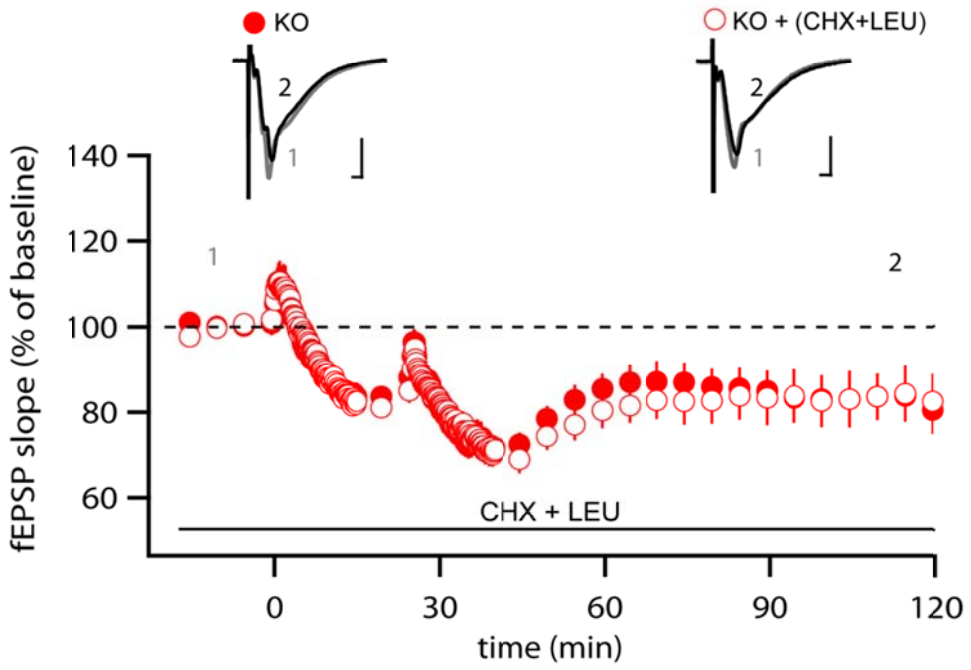

Figure 37 Summary graph of LTD experiments in untreated and (CHX+leupeptin)-treated slices.
Following LFS stimulation, LTD in (CHX + leupeptin)-treated slices, decreased to 81.02 ± 5.1% of baseline (empty circles; N= 4 n= 7) and compared to LTD in untreated slices (filled circles) no significant difference was found (83.63 ±4.46% of baseline N= 4 n= 7, treatment p=0.7178 time p< 0.0001 interaction p= 0.4123, two-way ANOVA RM).

4.7 LTD in adult conditional Arc/Arg3.1 KO mice

In order to assess whether the changes observed in LTD in adult KO slices were the result of Arc/Arg3.1 expression during early-development, we decided to apply this stimulation in slices derived from early and late cKO mice. Expression of Arc/Arg3.1 was normal in Early cKO mice and Late cKO mice, up to the first and third postnatal weeks, respectively. Cre-recombinase dependent removal of Arc/Arg3.1 was completed by the end of the second (Early cKO) or fifth (Late cKO) postnatal weeks. With these mice lines, we aimed to investigate a possible role of Arc/Arg3.1 in plasticity Arc/Arg3.1prior to eye-opening[97] (Early cKO) or after completion of

synaptogenesis Arc/Arg3.1[98] (Late cKO), in setting the capacity for adult synaptic plasticity.

4.7.1 Basal synaptic transmission in early cKO mice

I recorded I/O curves of early cKO slices. I/O curves showed no significant difference among early WT and cKO slices (fig.38a) (early WT N= 4 n= 16 early cKO N= 5 n= 20, genotype p= 0.9939 current p <0.0001 interaction p= 0.0946, two-way ANOVA RM). Further analysis showed no significant differences in $SI_{threshold}$ between genotypes (fig.38b) ($SI_{threshold}$: early WT N= 4 n= 16 early KO N= 5 n= 20, p= 0.0853 Mann Whitney U-test) or in the fEPSP threshold amplitude (fig.38c) (fEPSP threshold amplitude: early WT N= 4 n= 16 early cKO N= 5 n= 20, p= 0.185 Mann Whitney U-test). Thus, the effect of Arc/Arg3.1 is determined prior to the first postnatal week.

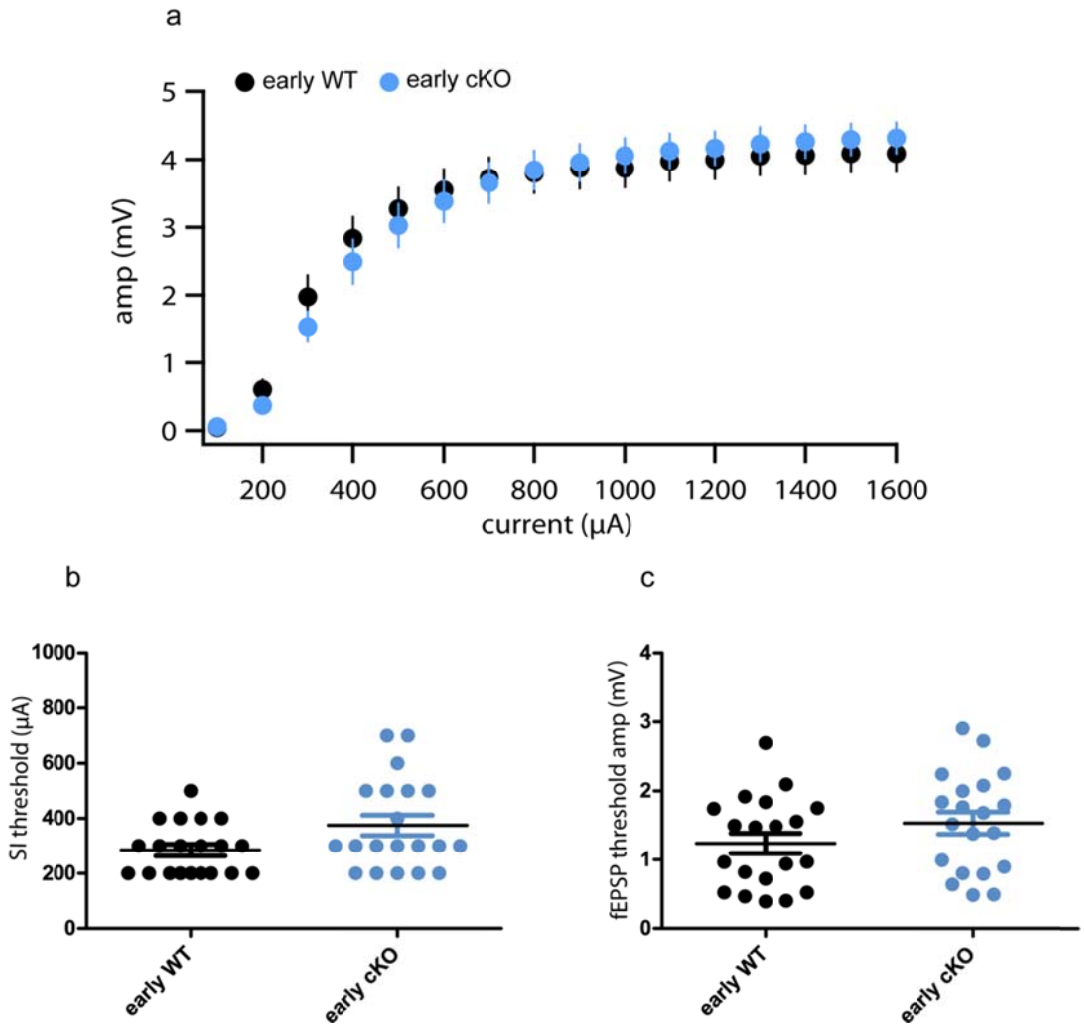

Figure 38 Analysis of I/O curves in early WT and cKO slices
(a) Summary I/O curves of early WT slices (N= 4 n= 16) and cKO slices (N= 5 n= 20). NO significant difference was found among genotypes (genotype p= 0.9894 time $p < 0.0001$ interaction $p < 0.0001$, two-way ANOVA RM). (b) (c) Graphs showing $SI_{threshold}$ and fEPSP threshold amplitude calculated in early WT and early cKO slices (early WT N= 4 n= 16 early cKO N= 5 n= 20). No significant differences in both parameters were found among genotypes ($SI_{threshold:}$ p= 0.0853, Mann Whitney t-test; fEPSP threshold amplitude p= 0.185, Mann Whitney t-test).

4.7.2 LTD in early cKO mice

Adult cortical plasticity depends on sensory events occurring during distinct phases of postnatal development[56]. I first proceeded with investigating LTD in early cKO mice. In early cKO slices, LFS induced LTD which was significantly enhanced within 20 min post stimulation relative to early WT slices (fig.39) (early WT 79.84 ±2.51% of

baseline N= 5 n= 19, early cKO 73.1 ±3.43% of baseline N= 5 n= 17, average from t=40 to t= 60; p= 0.0266 Mann Whitney U-test).

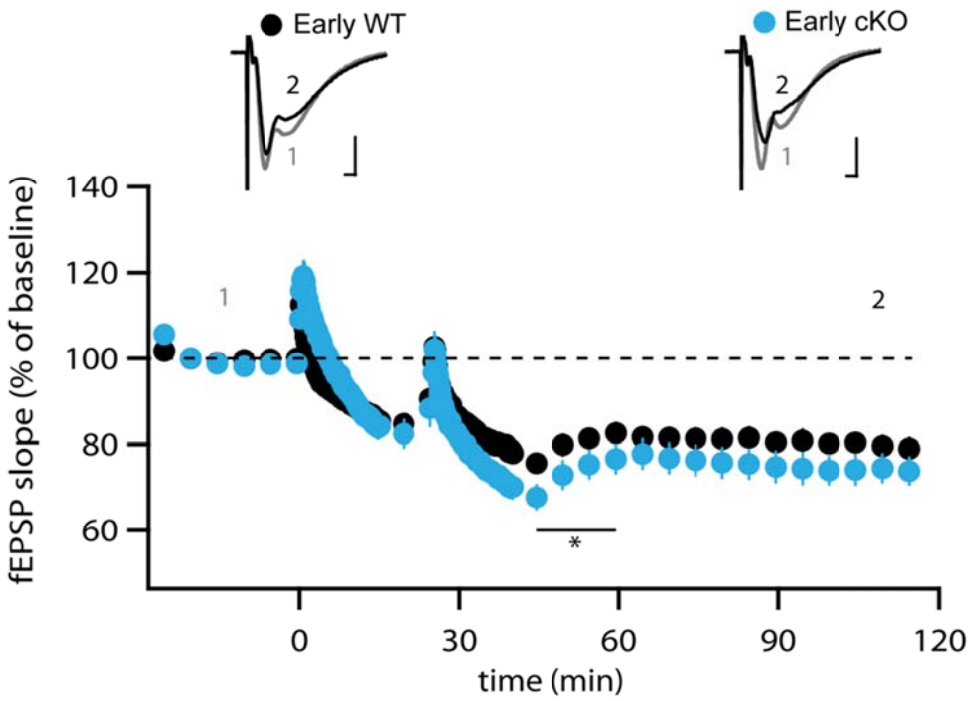

Figure 39 Summary graph of LTD in early cKO slices.
E-LTD in early cKO slices was significantly enhanced compare to e-LTD in early WT (early WT (black circles) 79.84 ±2.51% of baseline N= 5 n= 19, early cKO (light blue circles) 73.1 ±3.43% of baseline N= 5 n= 17, average from t=40 to t= 60; p= 0.0266 Mann Whitney U-test).

Control pathways of early WT and cKO slices remained stable throughout the entire recordings (early WT 97.77 ±2.55% of baseline N= 5 n= 19, p= 0.3857 paired t-test, early cKO 97.68 ±2.85% of baseline N= 5 n= 17, p= 0.4056 paired t-test). In summary, these results suggest that the presence of Arc/Arg3.1 until the second postnatal week was not sufficient to normalize LTD amplitudes in the Early cKO mice. The absence of Arc/Arg3.1 afetr the second postnatal week resulted in persistent increase in eLTD, that was also observed in germline KO slices (see 4.4.2).

4.7.3 Basal synaptic transmission in late cKO

No significant difference was found among I/O curves of late WT and cKO slices (fig. 40a) (late WT N= 7 n= 18 late cKO N= 9 n= 26, genotype p= 0.3311 time p <0.0001 interaction p= 0.9235, two-way ANOVA RM). No statistical differences were found in $SI_{threshold}$ (p= 0.2291, Mann Whitney t-test) (fig. 40b) and fEPSP threshold amplitude (p= 0.9744, Mann Whitney U-test) (fig. 40c).

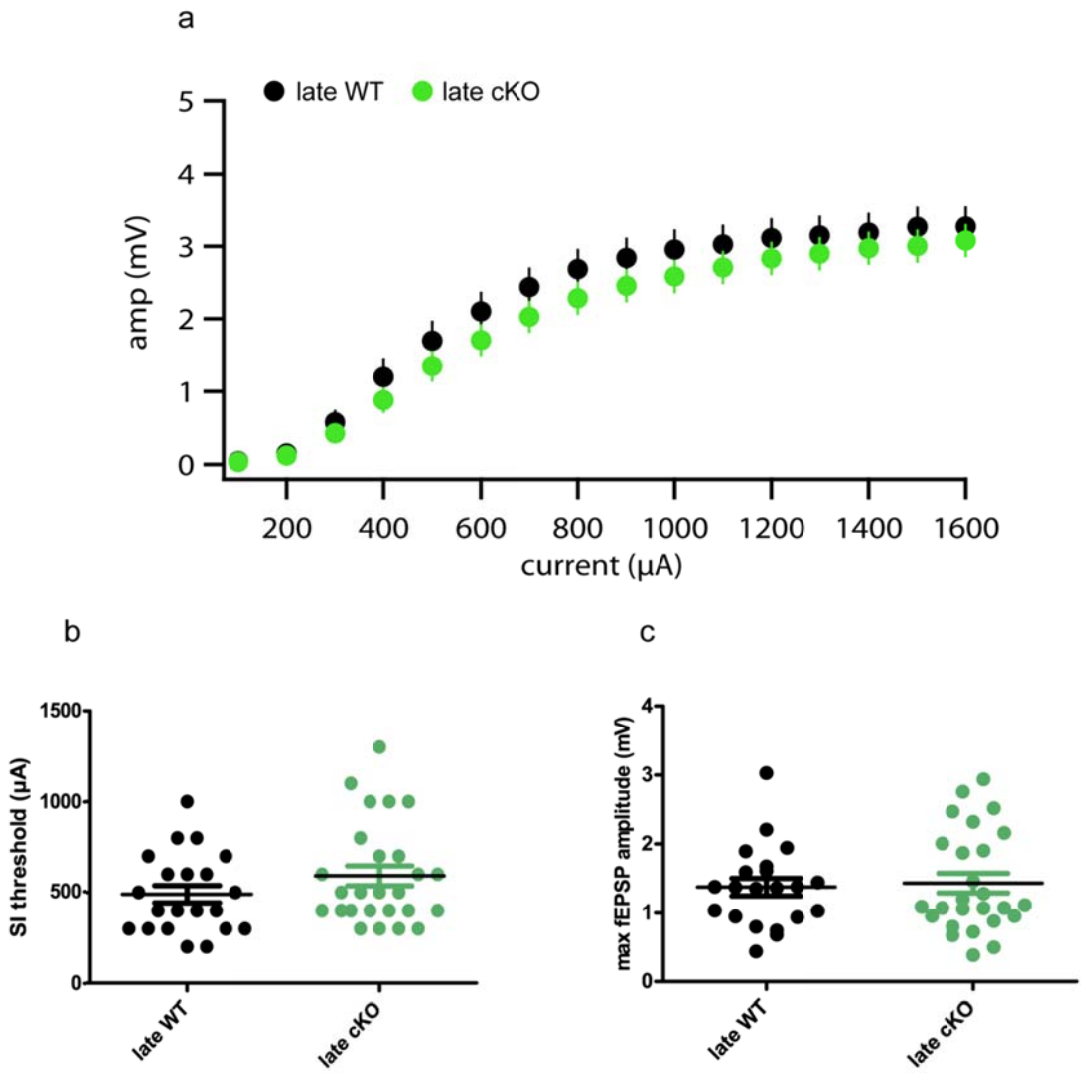

Figure 40 Analysis of I/O curves in late WT and cKO slices
(a) Summary I/O curves of late WT (N= 7 n= 18) and late cKO slices (N= 9 n= 26). No significant difference was found among genotypes (genotype p= 0.3311 time p< 0.0001 interaction p< 0.9235, two-way ANOVA RM). (b) (c) Graphs showing $SI_{threshold}$ and fEPSP threshold amplitude calculated in early WT and early cKO slices (early WT N= 4 n= 16 early cKO N= 5 n= 20). No significant differences in both parameters were found among genotypes

($SI_{threshold:}$ p= 0.0853, Mann Whitney t-test; fEPSP threshold amplitude p= 0.185, Mann Whitney U-test).

4.7.4 LTD in late cKO mice

Next, I was interested to investigate whether the ablation of Arc/Arg3.1 after P21 influences adult LTD. Following LFS stimulation, late WT slices showed decreased fEPSP slopes to 85.5 ±2.52% of baseline (fig. 41) (N= 8 n= 13). LTD was successfully induced in late cKO slices and no statistical differences were found compared to WT littermate slices (fig. 41) (82.72 ±3.14% of baseline N= 9 n= 16; genotype p= 0.4912 time p<0.0001 interaction p= 0.0106 two-way ANOVA RM).

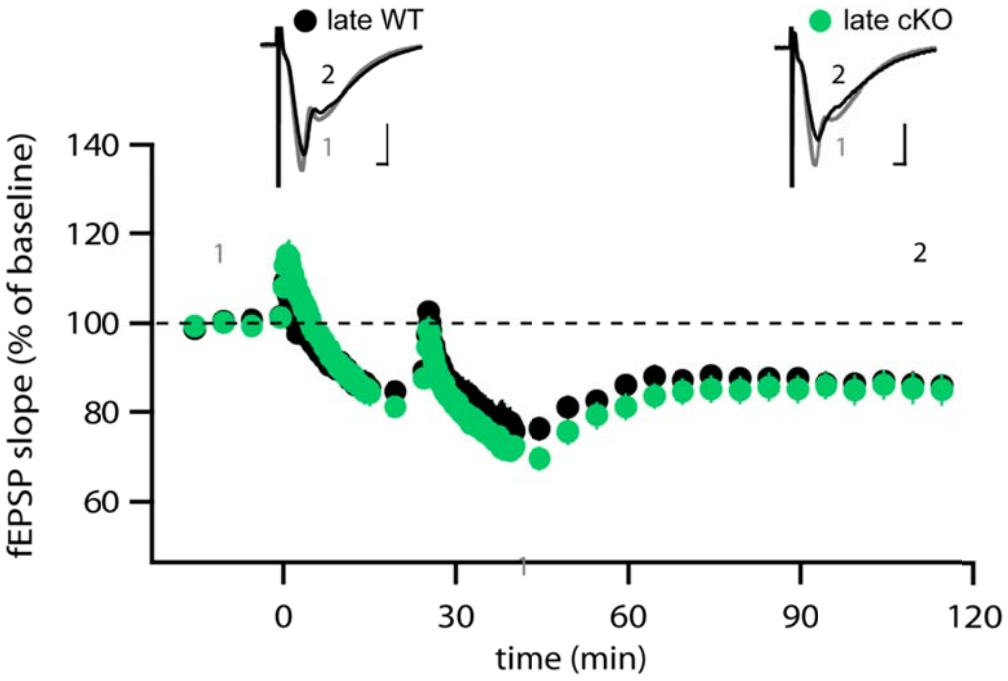

Figure 41 Summary graph of LTD in late cKO slices.
In late WT slices, LTD decreased to 85.50 ±2.52% of baseline (N= 8 n= 13). Late cKO slices showed normal LTD (82.72 ±3.14% of baseline N= 9 n= 16; genotype p= 0.4912 time p< 0.0001 interaction p= 0.0106 two-way ANOVA RM).

Synaptic specificity, examined by evaluation of fEPSPs of control pathways, was also confirmed (late WT 105.95 ± 5.08% of baseline N= 8 n= 13, p= 0.2612 paired t-test, late cKO 111.3 ± 5.41% of baseline N= 9 n= 16, p= 0.0522 paired t-test). These

results suggest that the ablation of Arc/Arg3.1 after P21 does not affect the induced LTD.

4.7.5 Basal synaptic transmission and protein synthesis in late cKO

We were interested to investigate whether CHX differentially affected synaptic properties of late WT and cKO slices. Therefore I compared untreated and CHX-treated slices among genotypes. I/O curves of late WT slices showed no significant differences between untreated and CHX-treated slices (fig. 42a) (untreated N= 7 n= 13 CHX-treated N= 7 n= 13, treatment p= 0.5381 time $p < 0.0001$ interaction p= 1, two-way ANOVA RM). As measured in late WT slices, I/O curves of late cKO CHX-treated slices were not significantly different from untreated slices (fig. 42b) (untreated N= 6 n= 12 CHX-treated N= 6 n= 12, treatment p= 0.708 time $p < 0.0001$ interaction p= 1, two-way ANOVA RM). $SI_{threshold}$ and fEPSP threshold amplitude were estimated among genotypes in presence of CHX. In late WT CHX-treated slices, $SI_{threshold}$ was enhanced (fig. 43a) (untreated N= 7 n= 13 CHX-treated N= 7 n= 13, p= 0.0006 Mann Whitney U-test). However, no significant difference was found in fEPSP threshold amplitude (untreated N= 7 n= 13 CHX-treated N= 7 n= 13, p= 0.3569 Mann Whitney U-test). In late cKO slices both $SI_{threshold}$ and fEPSP threshold amplitude were unaffected by CHX application (fig. 43b) ($SI_{threshold}$: untreated N= 6 n= 12 CHX-treated N= 6 n= 12, p= 0.5344 Mann Whitney U-test; fEPSP threshold amplitude: untreated N= 6 n= 12 CHX-treated N= 6 n= 12, p= 0.7645 Mann Whitney U-test). In summary, late WT and cKO slices showed no significant difference

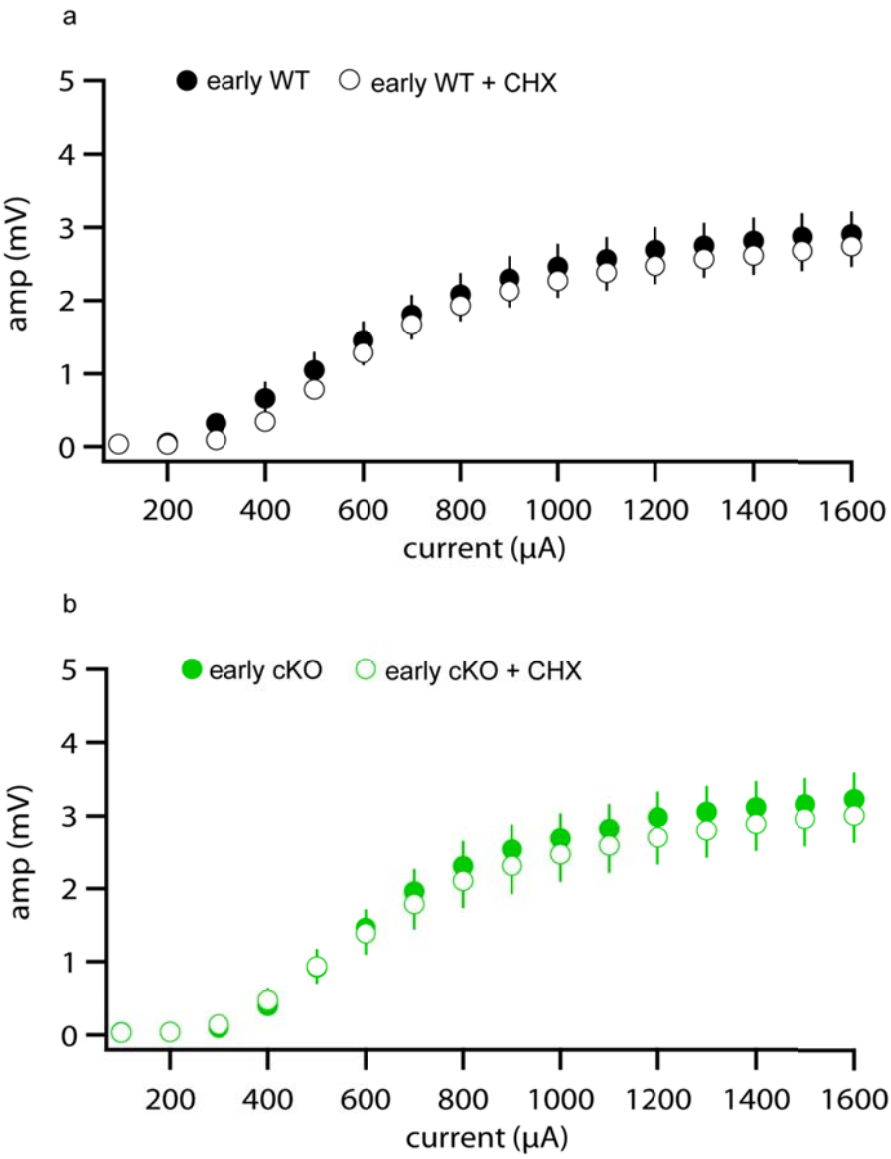

Figure 42 Comparison of I/O curves in WT and KO untreated and CHX-treated slices. (a) Summary I/O curves of late late WT untreated and CHX- treated slices. No significant difference was found among the two groups of slices (untreated N= 7 n= 13 CHX-treated N= 7 n= 13, treatment p= 0.5381 time p< 0.0001 interaction p= 1, two-way ANOVA RM) (b) Summary I/O curves of late cKO untreated and CHX- treated slices. No significant difference was found among the two groups of slices (untreated N= 6 n= 12 CHX-treated N= 6 n= 12, treatment p= 0.708 time p< 0.0001 interaction p= 1, two-way ANOVA RM)

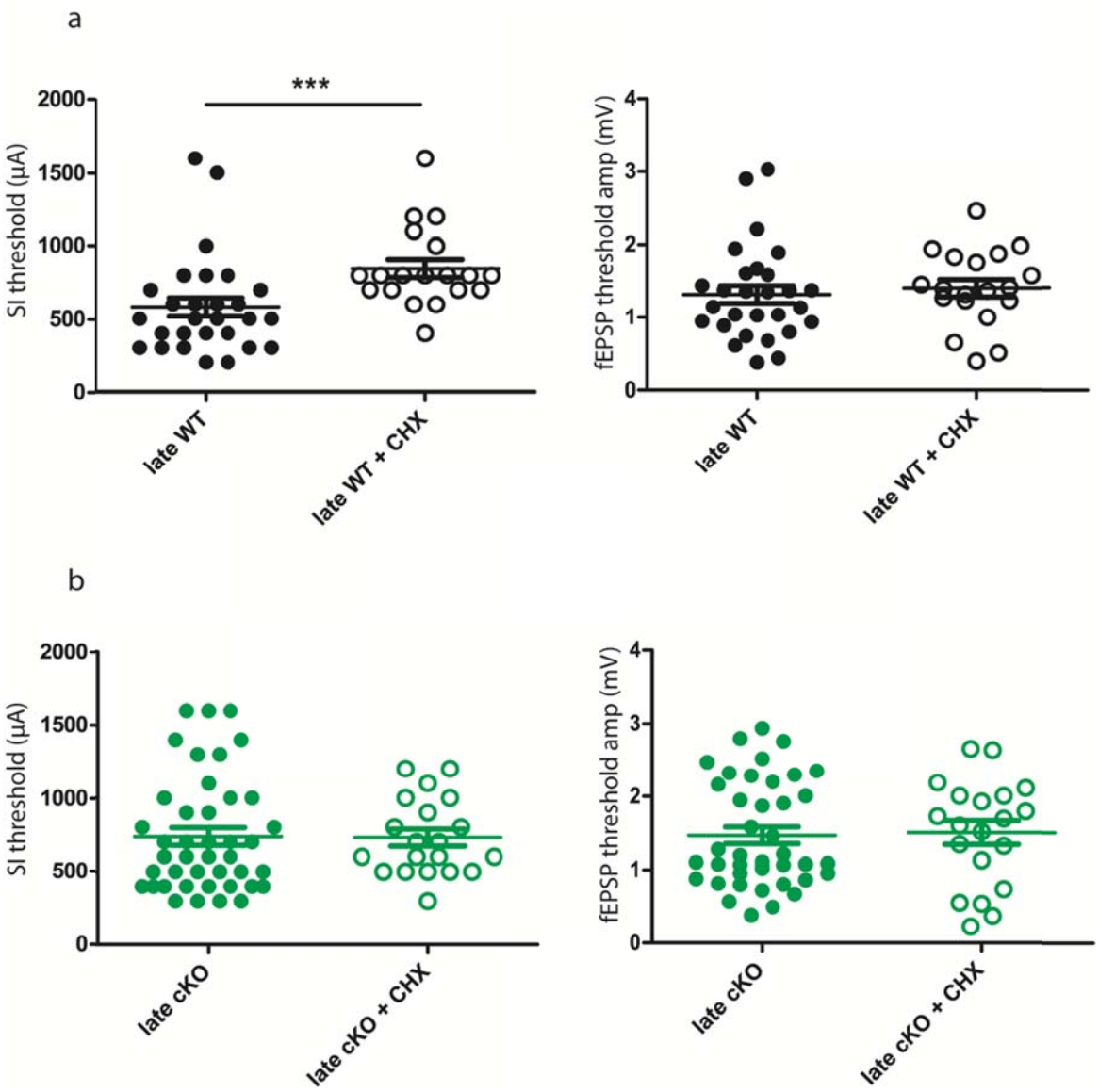

Figure 43 $SI_{threshold}$ and fEPSP threshold amplitude were calculated among genotypes in presence of CHX

(a) Late WT CHX- treated slices showed increased $SI_{threshold}$ (untreated N= 7 n= 13 CHX-treated N= 7 n= 13, p= 0.0006 Mann Whitney U-test), however no significant difference was noted in fEPSP threshold amplitude (untreated N= 7 n= 13 CHX-treated N= 7 n= 13, p= 0.3569 Mann Whitney U- test). (b) In KO slices, CHX caused significantly increased $SI_{threshold}$ and fEPSP threshold amplitude compared to untreated slices ($SI_{thnreshold}$: untreated N= 4 n= 8 CHX-treated N= 6 n= 12, p= 0.5344 Mann Whitney t-test; fEPSP threshold amplitude: untreated N= 6 n= 12 CHX-treated N= 4 n= 8, p= 0.7645 Mann Whitney U- test).LTD and protein synthesis in late cKO mice

I previously reported that LTD in KO mice was not impaired in the absence of novel protein synthesis (see paragraph 4.6.2, fig. 34). Next question was whether the seemingly normal LTD in the Late cKO mice was dependent on protein synthesis, as it was in WT slices. I performed LTD experiments in late cKO slices in the presence

of CHX. In late WT slices CHX blocked LTD (fig. 44a) (untreated 85.47 ±2.18% of baseline N= 8 n= 12; CHX- treated 97.1 ±7.14% of baseline N= 7 n= 14; $p < 0.0001$ Mann Whitney U-test), demonstrating the expected protein-synthesis dependence. However, in late cKO slices CHX had no impact on the LTD, which was indistinguishable from late cKO untreated slices (fig. 44b) (untreated 83.77 ±3.13% of baseline N= 10 n= 17; CHX-treated 85.68 ±4.11% of baseline N= 6 n= 11; treatment $p= 0.703$ time $p<0.0001$ interaction $p= 0.8751$ two-way ANOVA RM).

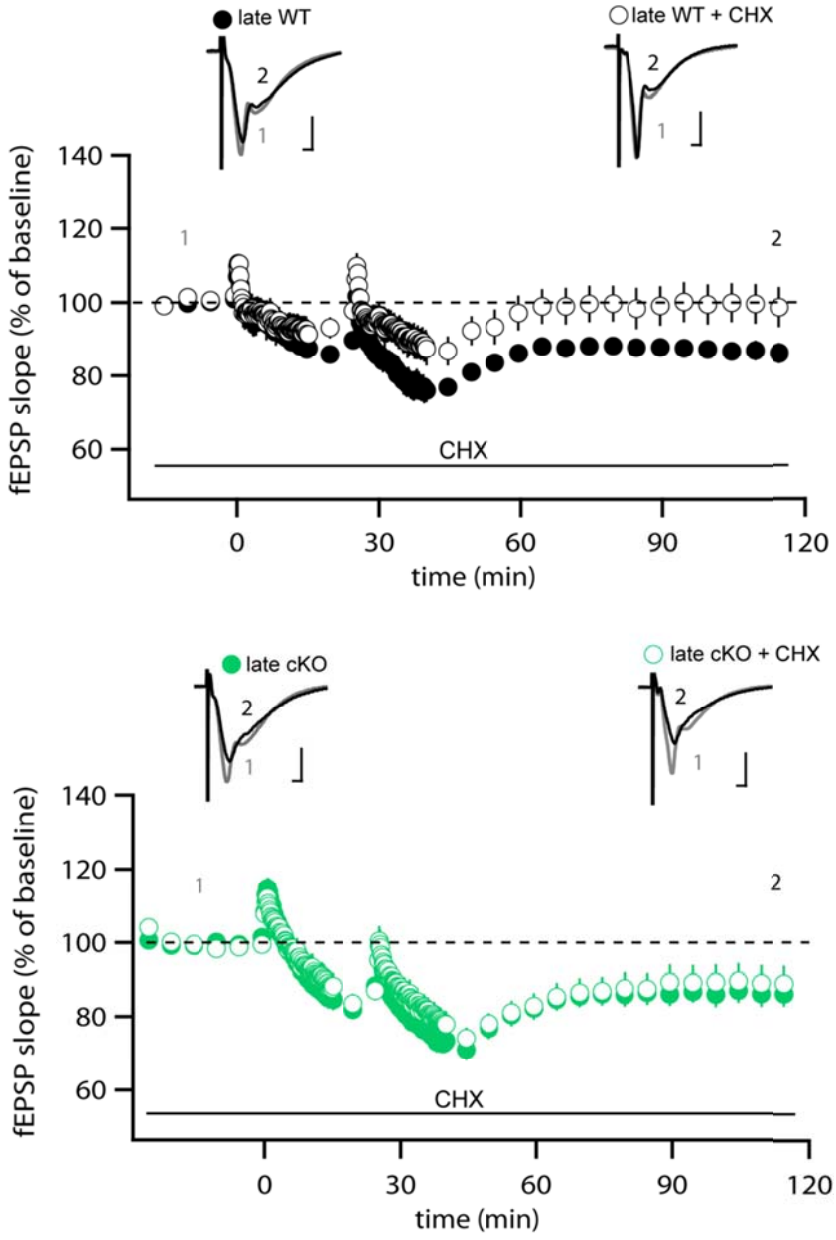

Figure 44 Summary graph of LTD in late WT and cKO slices in presence of CHX

(a) In late WT slices, LTD was blocked in presence of CHX (untreated 85.47 ±2.18% of baseline N= 8 n= 12, CHX- treated 97.1 ±6.18% N= 7 n= 14; p= <0.0001 Mann Whitney U-test). (b) Following LFS stimulation fEPSPs of late cKO CHX- treated slices did not show any statistical differences compared to late cKO untreated slices (late cKO untreated 83.77 ±3.13% .of baseline N= 10 n= 17, late cKO CHX treated 85.68 ±4.11% of baseline N= 6 n= 11; treatment p= 0.703 time p= < 0.0001 interaction p= 0.8751, two-way ANOVA RM).

Interestingly, control pathways of late WT CHX-treated slices increased significantly compared to baseline (fig. 45a) (late WT CHX- treated 108.25 ±2.42% of baseline N=7 n= 14, p= 0.0036 paired t-test). This effect was not seen in late WT untreated slices (fig. 45a) (104.5 ±2.60% of baseline N= 8 n= 12, p= 0.104 paired t-test). Yet, control pathways of the late cKO, both untreated and CHX- treated, showed increased fEPSP slopes following LFS stimulation (fig. 45b) (late cKO untreated 111.81 ±5.11% of baseline N=10 n= 17, p= 0.0329 paired t-test; late cKO CHX-treated 110.49 ±3.72% of baseline N= 6 n= 11, p= 0.0169 paired t-test).

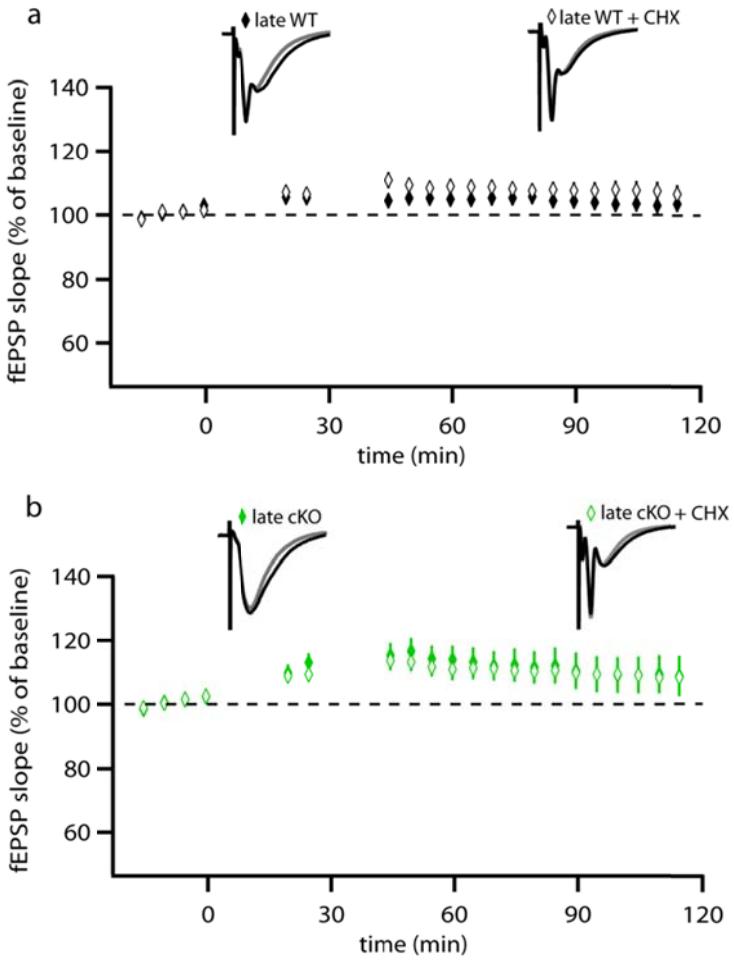

Figure 45 Control pathways of late WT and cKO slices

(a) fEPSPs of control pathways in late WT CHX- treated slices increased significantly following LFS stimulation late (108.25 ±2.42% of baseline N=7 n= 14, p= 0.0036 paired t-test). This effect was not detected in late WT untreated slices (104.5 ±2.60% of baseline N= 8 n= 12, p= 0.104 paired t-test). (b) control pathways of late cKO both untreated and CHX-treated slices showed increased fEPSP slopes following LFS stimulation (late cKO untreated 111.81 ±5.11% of baseline N=10 n= 17, p= 0.0329 paired t-test; late cKO CHX-treated 110.49 ±3.72% of baseline N= 6 n= 11, p= 0.0169 paired t-test).

Summarizing, late cKO mice showed normal LTD magnitude. However this form of plasticity was not supported by the synthesis of novel proteins. These results are similar with the results obtained in KO slices (see 4.4.2 and 4.6.2). Thus, the mechanisms involved in the consolidation of this form of plasticity do not seem to be associated with the developmental role of Arc/Arg3.1 early after birth.

4.8 Tg (3'UTR) Arc/Arg3.1 mice

4.8.1 Basal synaptic transmission in tg(3´UTR) Arc/Arg 3.1 mice

In response to regular neuronal activity, Arc/Arg3.1 mRNA is rapidly transcribed and traffics into dendrites, where it undergoes local translation[99]. Transgenic mice carrying a modified Arc/Arg3.1 allele that is not targeted to the dendrites were generated to investigate the importance of local Arc/Arg3.1 translation in dendrites. Here, I asked whether dendritic Arc/Arg3.1 translation is needed to support basal synaptic transmission at CA1-hippocampal region. I/O curves of tg(3´UTR)Arc/Arg3.1 mice were performed and compared to relative WT and KO. I/O curves of tg(3´UTR)Arc/Arg3.1 slices were significantly reduced compare to I/O curves of WT and KO slices (fig. 46a) (WT N= 3 n= 12 tg N= 3 n= 10 KO N= 3 n= 10, genotype p= 0.0009 time p< 0.0001 interaction p< 0.0001, two-way ANOVA RM). Furthermore, analysis of $SI_{threshold}$ among genotypes found a significantly enhanced threshold in tg(3´UTR)Arc/Arg3.1 slices compared to WT slices (fig.46b) (WT N= 3 n= 12 tg N= 3 n= 12, p= 0.0465 one-way ANOVA). On the other hand, fEPSP threshold amp was

comparable among genotypes (fig.46c) (WT N= 3 n= 12 tg N= 3 n= 12 KO N= 3 n= 10, p= 0.3394 one-way ANOVA). In summary, basal synaptic transmission in tg(3´UTR) Arc/Arg3.1 mice is reduced.

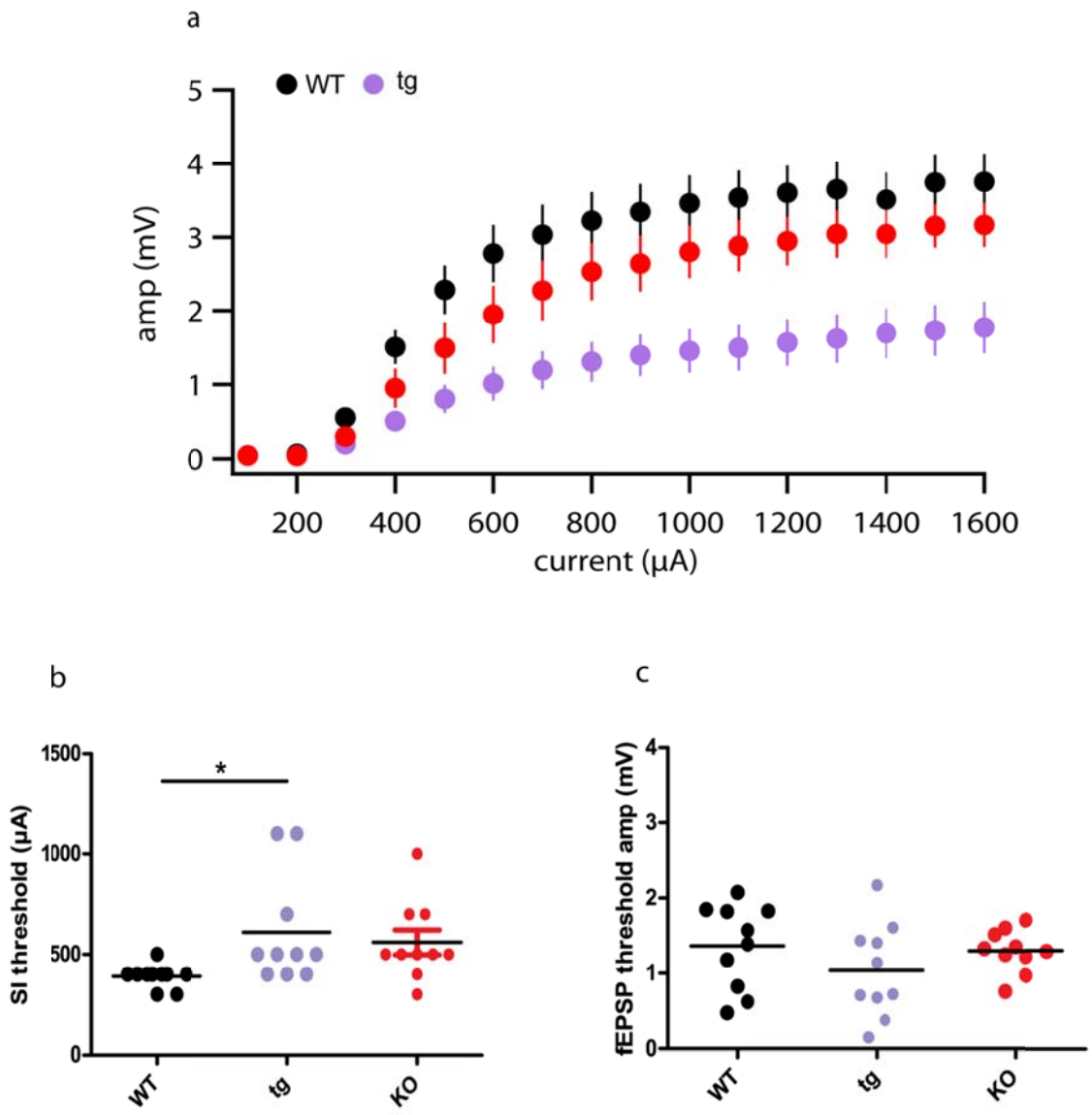

Figure 46 Analysis of I/O curves in WT, KO and tg(3´UTR)Arc/Arg3.1.
(a) Summary I/O curves of WT slices (N= 3 n= 12), KO slices (N= 3 n= 10) and tg slices (N= 3 n= 12). I/O curves in tg slices showed significant differences compared I/O curves in WT and in KO slices (tg N= 3 n= 12 WT N= 3 n= 12, genotype p= 0.0009 time p< 0.0001 interaction p< 0.0001, two-way ANOVA RM; KO N= 3 n= 10, genotype p= 0.0161 time p< 0.0001 interaction p< 0.0001, two-way ANOVA RM). No significant changes were found between I/O curves in WT and KO slices (genotype p= 0.142 time p < 0.0001 interaction p < 0.0001, two-way ANOVA RM). (b) $SI_{threshold}$ was significantly increased in tg slices compared to $SI_{threshold}$ in WT slices, but no statistical differences were found with $SI_{threshold}$ in KO slices (tg N= 3 n= 10, WT N= 3 n= 12, KO N= 3 n= 10, p= 0.0465 one-way ANOVA) (c) fEPSP

threshold amp (mV) was not significantly different among genotypes (tg N= 3 n= 10, WT N= 3 n= 12 KO N= 3 n= 10, p= 0.3394 one-way ANOVA).

4.8.2 LTD in Tg(3'UTR)Arc/Arg3.1 mice

Next, I investigated the contribution of local Arc/Arg3.1 translation to LTD. Since tg mice came from a new mouse line, including WT and KO mice, I initially investigated LTD on WT and KO slices. Both WT and KO slices showed LTD following LFS stimulation. At no time point was there a statistically significant difference between these groups (fig. 47a) (WT 83.2 ±2.99% of baseline N=3 n= 11, KO 80.1 ±4.19% of baseline N= 3 n= 8, genotype p= 0.5324 time p < 0.0001 interaction p < 0.0001, two-way ANOVA RM). On the contrary, LTD was completely blocked in tg slices (fig. 47a) (98.87 ±6.45% of baseline, N= 3 n= 8). Statistical comparison between LTD of tg slices with WT and KO slices resulted in a significant LTD reduction (WT 83.2 ±2.99% of baseline N=3 n= 11, tg 98.87 ±6.45% of baseline, N= 3 n= 8, genotype p= 0.0241 time p= 0.0001 interaction p= 0.0002, two-way ANOVA RM; KO 80.1 ±4.19% of baseline N= 3 n= 8, genotype 0.0258 time p <0.0001 interaction p= 0.2563, two-way ANOVA RM). Concerning control pathways, WT and KO slices did not show any significant changes in fEPSPs post- LFS stimulation compared to baseline fEPSPs (fig. 47b) (WT 103.31 ±3.24% of baseline N= 3 n= 11 average from t= 40 to t= 115, p=0.3117 paired t-test; KO 105.17 ±4.91% of baseline N=3 n= 8 average from t= 40 to t= 115, p= 0.3171 paired t-test). In contrast, tg slices presented slightly increased fEPSPs following LFS stimulation (fig. 47b) (112.36 ±4.49% of baseline N= 3 n= 8 average from t= 40 to t= 115, p= 0.0242 paired t-test).

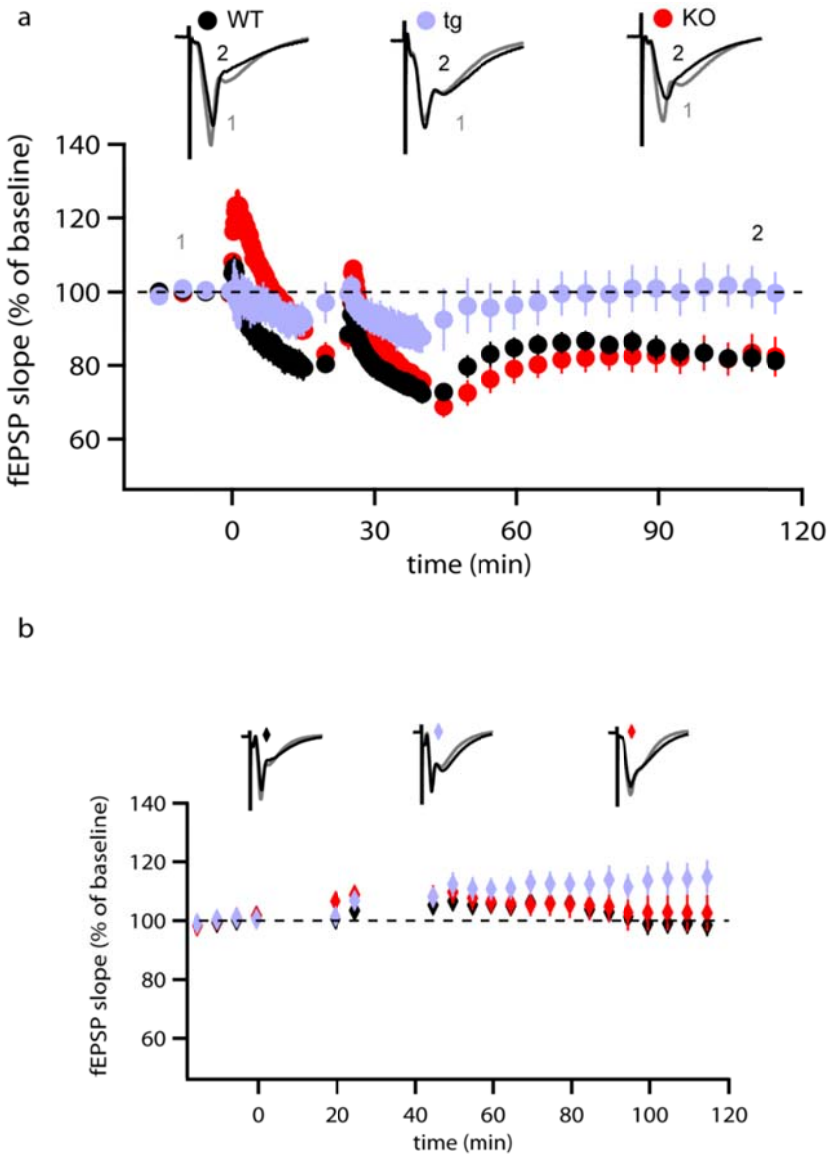

Figure 47 Summary graph of LTD in Tg(3´UTR) Arc/Arg3.1 slices
(a) In WT and KO slices LTD was induced to 83.2 ±2.99% of baseline N= 3 n= 11 and to 80.1 ±4.19% of baseline N= 3 n= 8, respectively. On the contrary, LTD was blocked in tg slices (98.87 ±6.45% of baseline N= 3 n= 8) (b) Graph showing control pathways of WT, KO and tg slices. In WT and KO no significant changes in control were found (WT 103.31 ±3.24% of baseline N= 3 n= 11 average from t= 40 to t= 115, p=0.3117 paired t-test; KO 105.17 ±4.91% of baseline N=3 n= 8 average from t= 40 to t= 115, p= 0.3171 paired t-test). On the contrary tg showed increased fEPSP slopes following LFS stimulation (112.36 ±4.49% of baseline N= 3 n= 8 average from t= 40 to t= 115, p= 0.0242 paired t-test)

These results demonstrate that when Arc/Arg3.1 protein is present, as in the tg mouse, it is the local Arc/Arg3.1 translation in dendrites which is essential for maintenance of LTD Arc/Arg3.1. In contrast, complete loss of Arc/Arg3.1 translation

and protein in the soma and dendrites of germline KO mice, does not reduce the LTD magnitude. Arc/Arg3.1The link between local Arc/Arg3.1 translation and NMDAR-mediated LTD remains to be explored.

4.9 Metabotropic GluR-LTD in juvenile WT and KO

The second form of LTD present in CA1 hippocampal region is dependent on mGluR activation and relies on rapid Arc/Arg3.1 translation [775352]. Although it has been already published that lack of Arc/Arg3.1 leads to mGluR-mediated LTD impairment, we aimed to confirm it under our experimental conditions and for our mouse lines.

4.9.1 DHPG- induced LTD in WT and KO mice

Beside LTD induced by electrical stimulation, long lasting decreases in synaptic transmission can be evoked by pharmacological activation of mGlu receptors[52].In order to investigate mGluR-dependent LTD in WT and KO slices, I initially induced LTD by application of 25μM DHPG, a group I mGluR agonist. In WT slices, 25 μM DHPG produced a stable reduction of synaptic strength (fig. 48a) (76.3 ±7.02% of baseline N= 3 n= 6, average from t= 4.5 to t= 119.5). In KO slices, treatment with 25 μM DHPG (fig. 48a) (73.54 ±10.03% of baseline N= 2 n= 4, average from t= 4.5 to t= 119.5), surprisingly, evoked an LTD indistinguishable from WT slices (genotype p= 0.8024 time p <0.0001 interaction p= 0.3226, two-way ANOVA RM). DHPG concentration may affect neuronal excitability and consequently electrophysiological properties of CA1 pyramidal cells[100]. Therefore, in order to assess whether the long lasting LTD in KO slices was dependent on DHPG concentration, we decided to double the amount to 50 μM. Again, 50 μM DHPG induced a LTD in WT slices which was comparable to KO slices (fig. 48b) (WT 72.55 ±4.93% of baseline N= 9 n= 18 KO 67.98 ±5.28% of baseline, average from t= 4.5 to t= 119.5, genotype p= 0.5068 time p < 0.0001 interaction p= 0.365, two-way ANOVA RM). Even after doubling the

concentration again 100 µM DHPG induced LTD in WT slices was not statistically different from the LTD in KO slices (fig.48c) (WT 69.63 ±8.18% of baseline N= 4 n= 7 KO 68.95 ±4.63% of baseline N= 3 n= 6, average from t= 4.5 to t= 119.5, genotype p= 0.9427 time p < 0.0001 interaction p= 0.0001, two-way ANOVA RM).

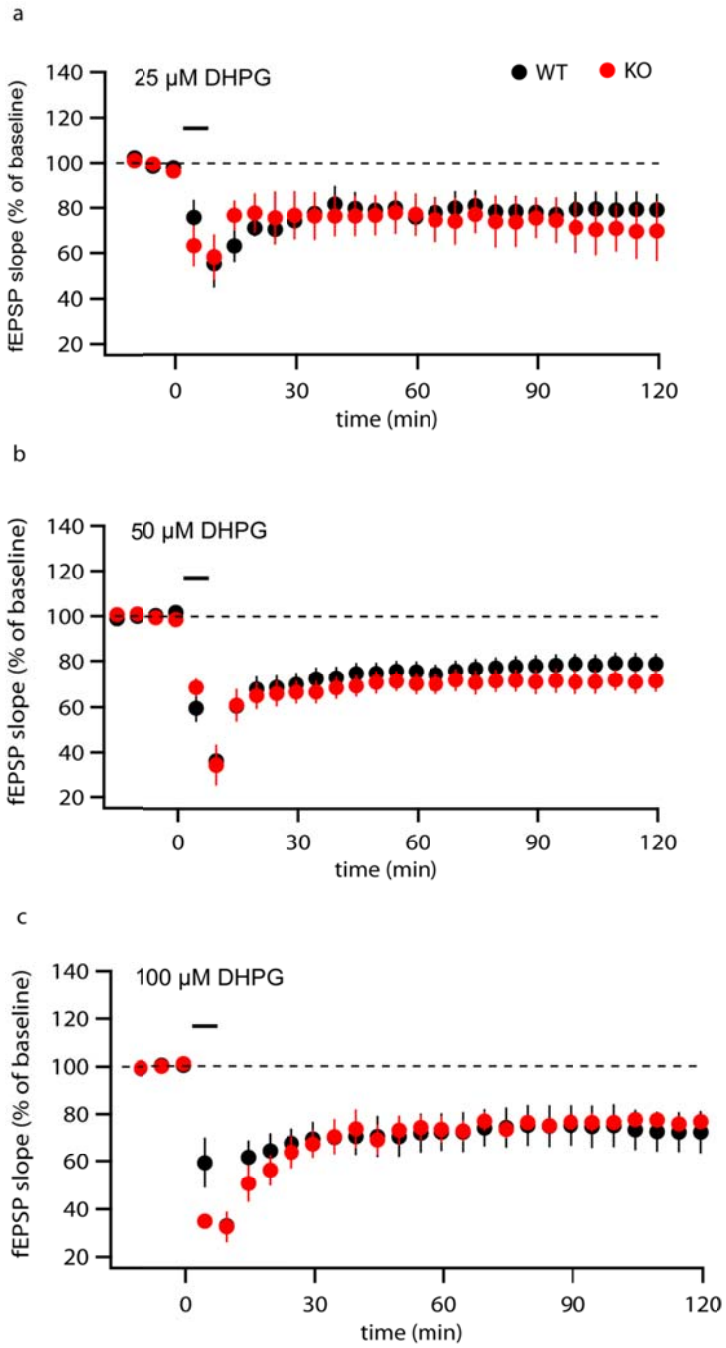

Figure 48 LTD induced with 25, 50, 100 µM DHPG in WT and KO slices
(a) 25 µM DHPG induced LTD in WT slices to 75.32 ±8.65% of baseline (N= 3 n= 6, average from t= 4.5 to t= 214.5) and in KO slices to 70.09 ±11.61% of baseline N= 2 n= 4. No statistical differences were found between WT and KO slices (genotype p= 0.8024 time p

<0.0001 interaction p= 0.3226, two-way ANOVA RM). (b) In WT slices, 50 μM DHPG induced LTD to 74.61 ±4.94% of baseline (N= 9 n= 18, average from t= 4.4 to t= 214.5), whereas in KO to 68.82 ±5.12% of baseline (N= 6 n= 11, average from t= 40 to t= 214.5). Again, no statistical difference on LTD in WT and KO slices was found (genotype p= 0.5068 time p <0.0001 interaction p= 0.365, two-way ANOVA RM). (c) 100 μM DHPG- induced LTD in WT and KO slices was comparable in magnitude (WT 68.82 ±7.72% of baseline N= 4 n= 7, KO 69.32 ±4.07% of baseline N= 3 n= 6, average from t= 4.5 to t= 214.5, genotype p= 0.9427 time p= 0.0001 interaction < 0.0001).

In conclusion, surprisingly KO slices show normal mGluR- mediated LTD with each applied DHPG concentration. These results are in contrast with literature, which claims mGluR-LTD relies on Arc/Arg3.1 translation[101].

4.9.2 DHPG- induced LTD and protein synthesis

It is widely claimed that mGluR-mediated LTD in hippocampal area CA1 is protein synthesis dependent and relies on Arc/Arg3.1 translation[28],[52]. First, we were interested to investigate the mechanism of protein synthesis on LTD in juvenile WT slices at 25, 50 and 100 μM DHPG. Surprisingly, bath application of CHX, the protein synthesis blocker, did not affect any LTD induced by 25, 50 or 100 μM DHPG and no significant differences were found compared to LTD in untreated slices (fig. 49 e, f, g) (25 μM DHPG: untreated N= 3 n= 6 CHX-treated N= 3 n= 6 , average from t= 4.5 to t= 119.5, treatment p= 0.7014 time p= < 0.0001 interaction p= 0.9994 two- way ANOVA RM; 50 μM DHPG: untreated N= 9 n= 18 CHX- treated N= 7 n= 13, average from t= 4.5 to t= 119.5, treatment p= 0.4609 time p < 0.0001 interaction p= 0.3253 two-way ANOVA RM; 100 μM DHPG: untreated N= 4 n= 7, average from t= 4.5 to t= 119.5, treatment p= 0.3879 time p= < 0.0001 interaction p= 0.9903 two-way ANOVA RM). Next, the protein synthesis blocker was applied in KO slices and LTD was induced by 25, 50 and 100 μM DHPG. CHX did not block LTD induced by any DHPG concentration (fig. 49 h, i, l) (25 μM: CHX- treated 77.60 ±2.37% of baseline N= 3 n=

5, average from t= 4.5 to t= 119.5; 50 μM: CHX treated 67.33 ±2.56% N= 5 n= 9, average from t= 4.5 to t= 119.5; 100 μM CHX-treated 70.93 ±6.78% of baseline N= 3 n= 5, average from t= 4.5 to t= 119.5). In conclusion, under our experimental conditions, mGluR-mediated LTD is protein synthesis independent in WT slices. The results may appear counterintuitive since it is well established that this form of plasticity is sustained by novel protein synthesis. However, experimental conditions might play a considerable role in this form of plasticity[102].

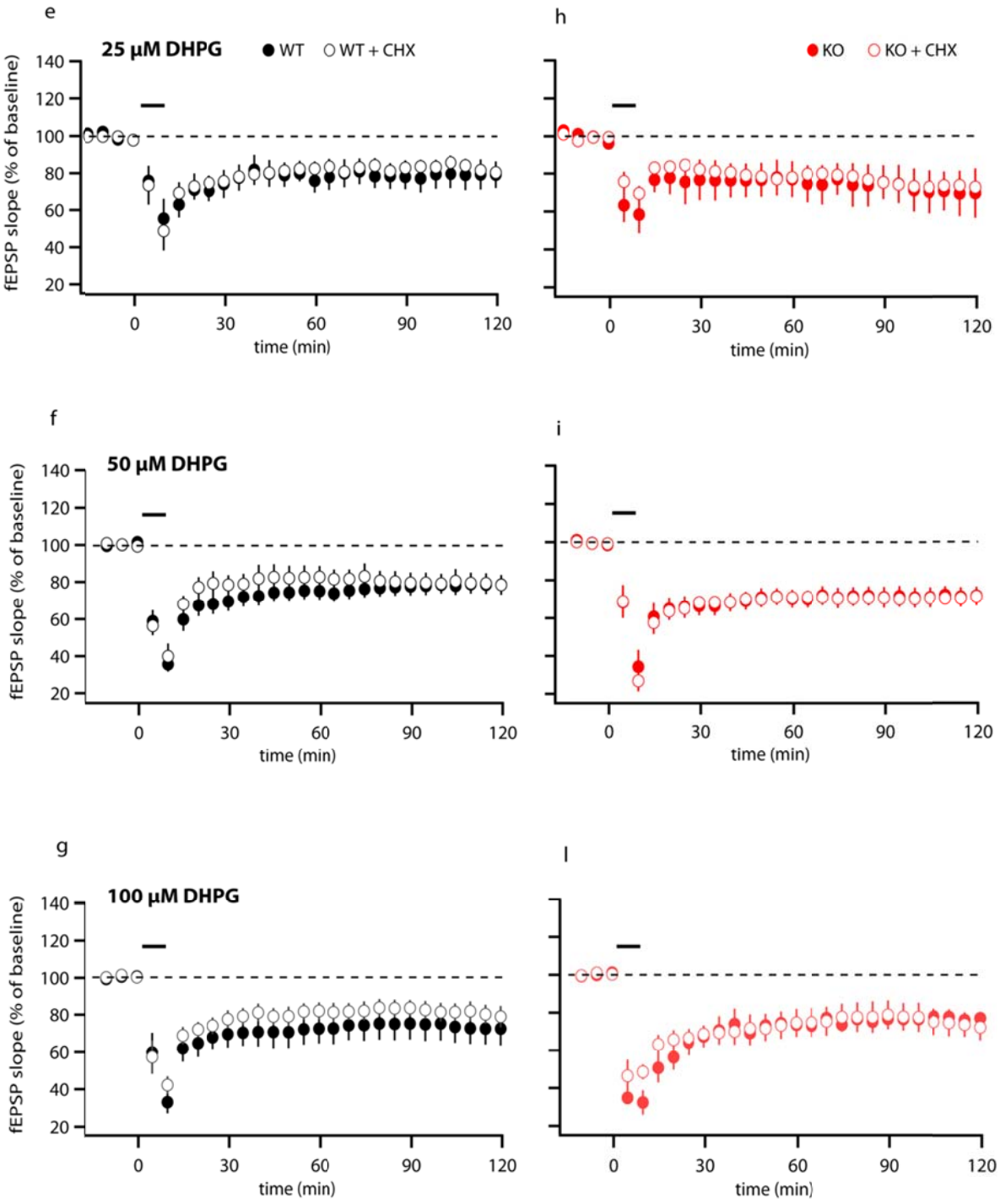

Figure 49 LTD induced at 25, 50, 100 µM DHPG in WT and KO slices in the presence of CHX

(e) 25 µM DHPG induced LTD in WT and KO slices, which was independent on protein synthesis (WT untreated N=3 n= 6 WT CHX-treated N= 3 n= 6, average from t= 4.5 to t= 119.5, treatment p= 0.7014 time p= 0.0001 interaction p= 0.9994 two-way ANOVA RM; KO treated N= 2 n= 4 KO CHX-treated N= 5 n= 9 , average from t= 4.5 to t= 119.5, treatment p= 0.6524 time p= <0.0001 interaction p= 0.7404 two-way ANOVA RM). (f) In WT and KO slices 50 µM DHPG induced a LTD comparable in terms of magnitude and in both protein synthesis independent (WT untreated N= 9 n= 18 WT- CHX treated N= 7 n= 13, average from t= 4.5 to t= 119.5, treatment p= 0.4609 time p= <0.0001 interaction p= 0.3253; KO

untreated N= 6 n= 11 KO CHX- treated N= 5 n= 9, average from t= 4.5 to t= 119.5, treatment p= 0.9042 time p= < 0.0001 interaction p= 1). (g) 100 µM DHPG was applied to induce LTD in WT and KO slices and CHX did not affect LTD in both groups of slices (WT untreated N= 4 n= 7 WT CHX treated N= 4 n= 8, average from t= 4.5 to t= 119.5, treatment p= 0.3879 time p < 0.0001 interaction p= 0.9903 two-way ANOVA RM; KO untreated N= 3 n= 6 KO CHX-treated N= 3 n= 5, average from t= 4.5 to t= 119.5, treatment p= 0.7877 time p <0.0001 interaction p= 0.0916 two-way ANOVA RM)

4.10 Correlation between early field EPSPs reduction and DHPG concentration

During DHPG (100 µM) application, fEPSPs of KO slices showed a transient decrease in synaptic response significantly different from the decrease in synaptic response of WT slices (fig. 50) (WT 40.37 ±10.556% of baseline N= 4 n= 7, t= 4.5, KO 65.22 ±1.55% of baseline N= 3 n= 6, t= 4.5, p= 0.035 Mann Whitney U-test). The difference in the reduction in fEPSP amplitude was not observed at 25 µM and 50 µM DHPG (fig. 50) (25 µM: WT 25 ±7.783% of baseline N= 3 n= 6, t= 4.5, KO 36.77 ±8.9% of baseline N= 2 n= 4, p= 0.2571 Mann Whitney U-test; 50 µM: WT ±5.988% N= 9 n= 18, t= 4.5, KO 31.57 ±4.05% of baseline N= 6 n= 11, t= 4.5, p= 0.3808 Mann Whitney U-test).

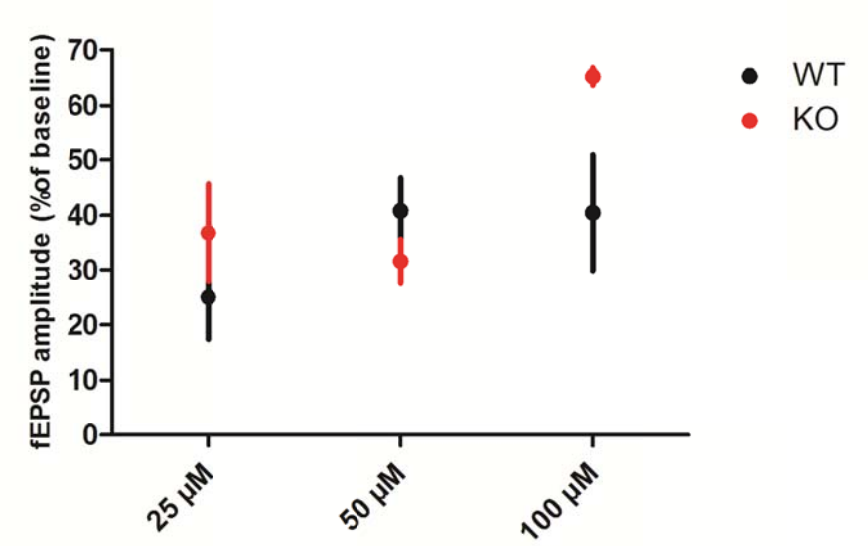

Figure 50 Reduced fEPSP amplitudes during DHPG application in WT and KO slices

LTD induction following 100 µM DHPG in KO slices decreased significantly more than in WT slices (WT 40.37 ±10.556% of baseline N= 4 n= 7, t= 4.5, KO 65.22 ±1.55% of baseline N= 3 n= 6, t= 4.5, p= 0.035 Mann Whitney t-test). No significant differences were found in LTD induction among genotypes at 25 µM and 50 µM DHPG (25 µM: WT 25 ±7.783% of baseline N= 3 n= 6, t= 4.5, KO 36.77 ±8.9% of baseline N= 2 n= 4, p= 0.2571 Mann Whitney t-test; 50 µM: WT ±5.988% N= 9 n= 18, t= 4.5, KO 31.57 ±4.05% of baseline N= 6 n= 11, t= 4.5, p= 0.3808 Mann Whitney t-test).

The stronger reduction of the KO fEPSP amplitude in 100 µM DHPG could indicate a higher sensitivity to mGluRs activation, for example, due to a greater abundance of the mGlu receptors. Alternatively, the high concentration of DHPG has unspecific effects that are stronger in KO slices.

4.11 HFS-induced LTP

LTP can be induced at Schaffer Collaterals-CA1 synapses by high frequency stimulation (HFS) [103]. Increasing numbers of HFS episodes result in larger and longer LTP. I applied 3 HFS episodes separated by 10 minutes to elicit LTP in WT and KO slices.

4.11.1 LTP in KO slices

First, LTP was induced in WT slices. Following 30 minutes baseline, 3xHFS was applied resulting in E-LTP of 173.10 ±17.2% of baseline (fig. 51) (N= 7 n= 9, average from t= 4.5 to t= 19.5) and, as expected, maintained stability until the end of the recordings (L-LTP 151.72 ±18.5% of baseline N= 7 n= 9, average from t= 274.5 to t= 299.5). Surprisingly, KO slices did not show any significant differences neither in the E-LTP (174.22 ±7.73% of baseline N= 4 n= 5 average from t= 4.5 to t= 19.5, genotype p= 0.9624 time p < 0.0001 interaction p= 0.939, two-way ANOVA RM), nor in the L-LTP (154.44 ±16.85% of baseline N= 4 n= 5, average from t= 274.5 to t= 299.5, genotype p= 0.9243 time p= 0.7175 interaction p= 0.6066, two-way ANOVA RM) (fig. 51). Control pathways of both WT and KO slices preserved stability throughout the entire recording (WT 99.11 ±5.56% of baseline N= 7 n= 9, average from t= 4.5 to t= 299.5, p= 0.3466 paired t-test; KO 97.93 ±10.56% of baseline N= 4 n= 5, average from t= 4.5 to t= 299.5, p= 0.8479 paired t-test). These results suggest that, under our experimental conditions, LTP in KO slices can be induced of a similar magnitude and duration to WT LTP. However, it is still unknown whether the mechanisms underlying this form of LTP are the same in WT and KO slices.

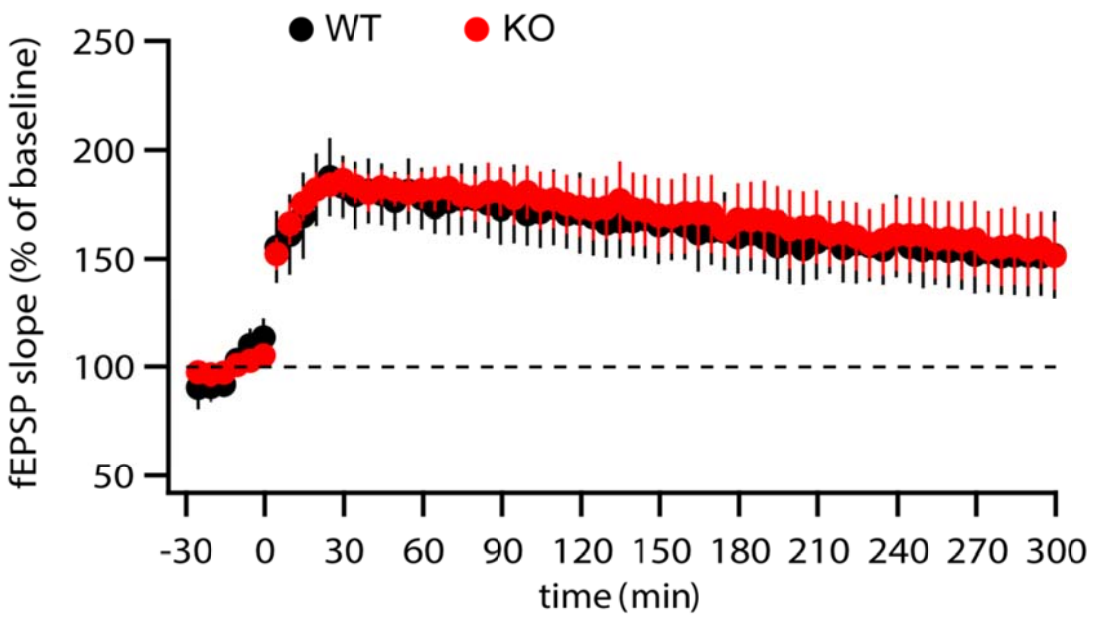

Figure 51 Summary of HFS-inducing LTP experiments in WT and KO slices
Comparison of LTP experiments between WT and KO slices showed no significant differences neither in the E-LTP (WT slices 173.1 ±17.2% of baseline N= 7 n= 9, average from t= 4.5 to t=29.5 KO slices 174.22 ±7.73% of baseline N= 4 n= 5, average from t= 4.5 to t= 29.5, genotype p= 0.9624 time p= < 0.0001 interaction p= 0.939, two-way ANOVA RM), nor in the L-LTP (WT slices 151.72 ±18.5% of baseline N= 7 n= 9, average from t= 274.5 to t= 299.5 KO slices 154.44 ±16.85% of baseline N= 4 n= 5, average from t= 274.5 to t= 299.5, genotype p= 0.9243 time p= 0.7175 interaction p= 0.6066, two-way ANOVA RM)

4.11.2 HFS-induced LTP and protein synthesis

It is widely believed that new proteins are required to stabilize some forms of LTP and usually these forms of plasticity induced by high frequency stimulation require activation of transcription and translation[32]. Therefore, first we were interested to investigate whether HFS-induced LTP requires novel protein synthesis in WT slices and second, whether LTP in KO slices is supported by the same mechanism. In WT slices, bath application of the protein synthesis inhibitor CHX blocked L-LTP (fig. 52 a and c) (untreated 151.72 ±18.5% of baseline N= 7 n= 9, average from t= 274.5 to t= 299.5, CHX-treated 113.96 ±7.27% of baseline N= 10 n= 15, average from t= 274.5 to t= 299.5, treatment p< 0.0001 time p= 0.999 interaction p > 0.999, two-way

ANOVA RM). In contrast, CHX had no effect on L-LTP in KO slices (fig. 52 b and d) (untreated 154.44 ±16.85% of baseline N= 4 n= 5, average from t= 274.5 to t= 299.5 CHX- treated 143.78 ±9.55% of baseline N= 4 n= 6, average from t= 274.5 to t= 299.5, genotype p= 0.1996 time p= 0.9989 interaction p> 0.999). Control pathways were assessed among groups of slices and only control pathways of WT CHX-treated slices showed significant decreased fEPSPs following LTP protocol (90.88 ±4.35% N= 10 n= 15, average from t= 274.5 to t= 299.5, p= 0.0385 paired t-test). In conclusion, LTP is protein synthesis dependent in WT slices. Moreover, although LTP size in KO slices did not differ significantly from WT LTP, it was different by not being maintained through novel protein synthesis. It is still unknown which mechanisms underlie LTP maintenance in KO slices.

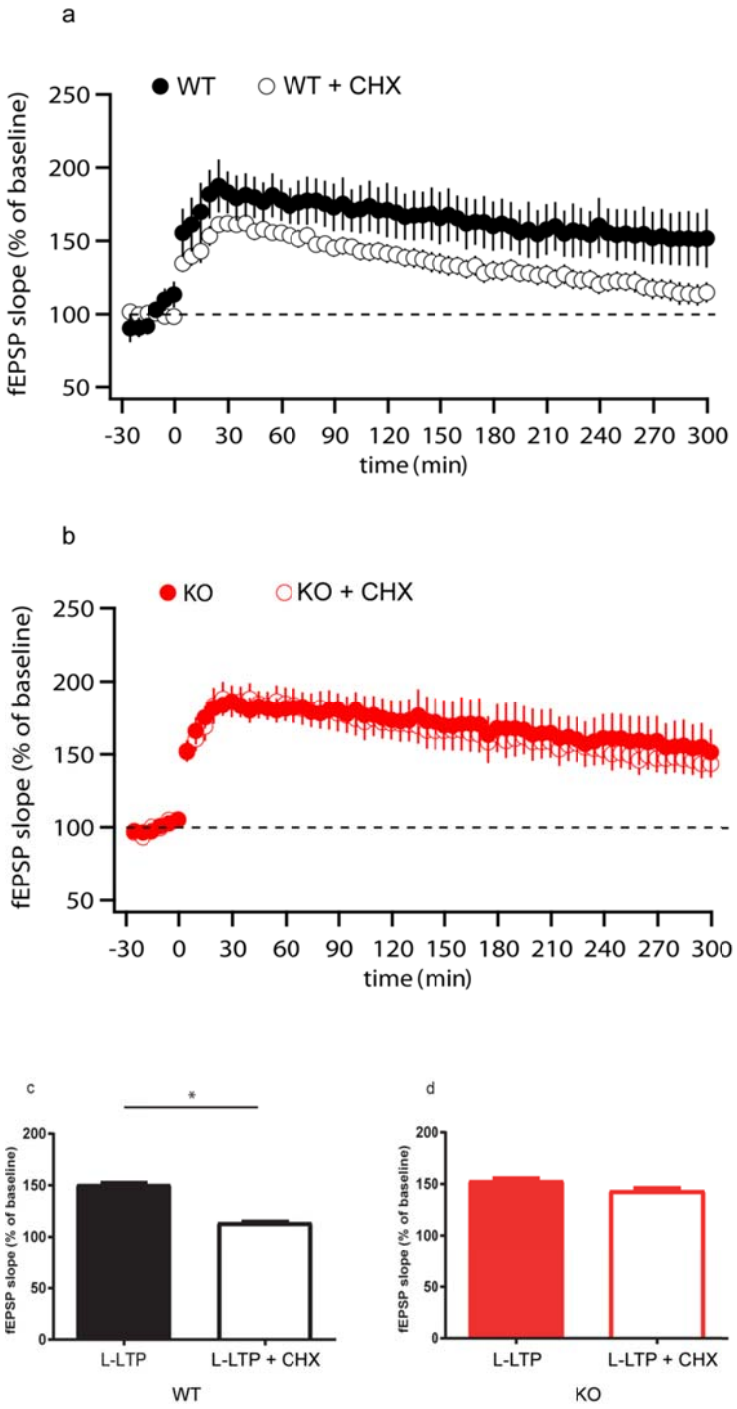

Figure 52 Summary of LTP experiments in CHX-treated WT and KO slices
(a) Following HFS, LTP was blocked in WT CHX- treated slices (untreated 151.72 ±18.5% of baseline N= 7 n= 9, average from t= 274.5 to t= 299.5; CHX- treated 113.96 ±7.27% of baseline N= 10 n= 15, average from t= 274.5 to t= 299.5, treatment $p < 0.0001$ time p= 0.999 interaction p> 0.999, two-way ANOVA RM); (b) No significant difference was found in LTP between KO untreated and CHX- treated slices (untreated 154.44 ±16.85% of baseline N= 4 n= 5, average from t= 274.5 to t= 299.5, CHX- treated 143.78 ±9.55% of baseline, treatment p= 0.1996 time p= 0.9989 interaction p= > 0.999, two-way ANOVA

RM). (c) (d) Bar graphs showing L-LTP of untreated and CHX- treated slices among WT and KO slices

4.11.3 LTP in early cKO mice

Several lines of evidence suggest that experience-dependent changes occurring in mature cortical circuitry built their fundaments during specific time window of development[56], [104]. In order to assess the developmental role of Arc/Arg3.1 in adult plasticity, I induced LTP in early cKO mice. In early WT slices, E-LTP was induced to 166.55 ±14.68% of baseline (N= 4 n= 7, average from t= 4.5 to t= 29.5) and resulted in a long lasting L-LTP (148.84 ±15.73% of baseline N= 4 n= 10, average from t= 274.5 to t= 299.5) (fig. 53). Early cKO slices showed a non-significant increase in E-LTP compared to early WT slices (207.28 ±20.07% of baseline N= 4 n= 10, average from t= 274.5 to t= 299.5, genotype p= 0.1383 time p < 0.0001 interaction p= 0.2031, two-way ANOVA RM). L-LTP was comparable in early cKO slices (184.59 ±21.16% of baseline N= 4 n= 10, average from t= 274.5 to t= 299.5, genotype p= 0.2287 time p= 0.3506 interaction p= 0.902, two-way ANOVA RM) (fig. 53). No significant differences were found in fEPSPs of control pathways, before and after LTP protocol, among genotypes (early WT 96.52 ±12.93% of baseline N= 3 n= 6 average from t= 4.5 to t= 299.5, p= 0.776 paired t-test; early cKO 99.37 ±8.05% of baseline N= 4 n= 10, average from t= 4.5 to t= 299.5, p=0.9382 paired t-test). Taking together these results, in early cKO slices the LTP protocol induced a form of plasticity not substantially different from early WT slices. However, it is still unknown whether the mechanisms required are the same.

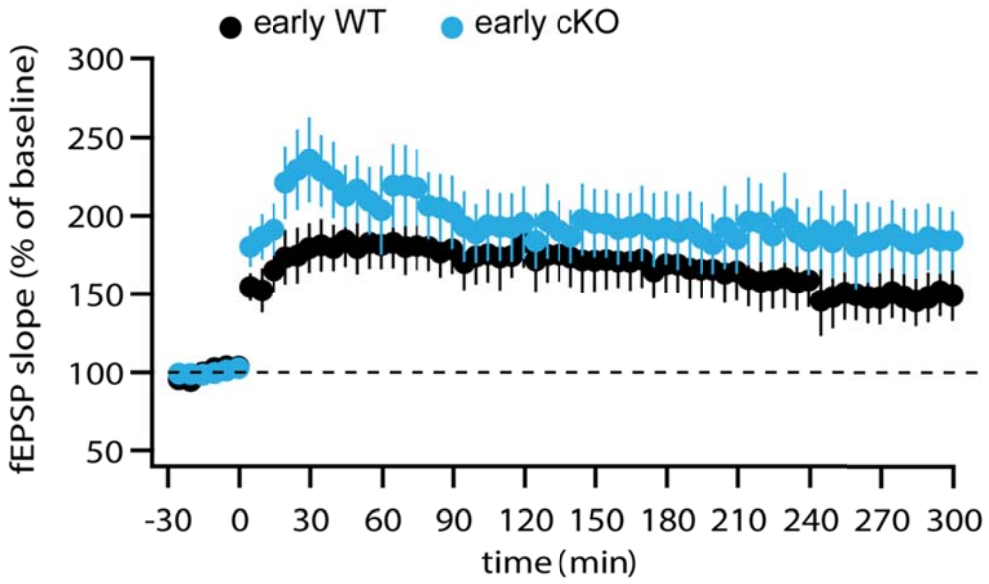

Figure 53 Summary of HFS-inducing LTP experiments in early WT and cKO slices. Early cKO slices showed a slightly although no significant enhanced E-LTP compared to early WT slices (early WT 166.55 ±14.68% of baseline N= 4 n= 7, average from t= 4.5 to t= 29.5 early cKO 207.28 ±20.07% of baseline N=4 n= 10, average from t= 4.5 to t= 29.5, genotype p=0.1383 time p < 0.0001 interaction p= 0.2031, two-way ANOVA RM). In early cKO slices, L-LTP was normal (early WT 148.84 ±15.73% of baseline N= 4 n= 7, average from t= 274.5 to t= 299.5 early cKO 184.59 ±21.16% of baseline N= 4 n= 10, average from t= 274.5 to t= 299.5, genotype p= 0.2287 time p= 0.3506 interaction p= 0.902, two-way ANOVA RM)

4.11.4 LTP in late cKO mice

Sensory and cortical circuits in the mouse brain are mostly modeled within 3 weeks after birth [105]. In order to test the cellular role of Arc/Arg3.1 early postnatally on mature hippocampal circuits, I induced LTP in late cKO slices. First LTP was induced in late WT slices. HFS induced an E-LTP to 178.82 ±12.45% of baseline (N= 10 n= 22, average from t= 4.5 to t= 29.5), which was not significantly different from E-LTP of late cKO slices (189.03 ±9.66% of baseline, N= 11 n= 22, average from t= 4.5 to t= 29.5, genotype p= 0.5084 time p < 0.0001 interaction p= 0.0025, two-way ANOVA RM) (fig. 54). Similarly, L-LTP in late cKO slices did not show any significant distinction with L-LTP in late WT slices (late WT 141.81 ±6.44% of baseline N=10 n= 21, average from t= 274.5 to t= 299.5; late cKO 154.02 ±6.98% of baseline N= 10 n=

22, average from t= 274.5 to t= 299.5, genotype p= 0.2008 time p= 0.7768 interaction p= 0.722, two-way ANOVA RM) (fig. 54). Control pathways of both genotypes remained stable throughout the entire recording (late WT 99.82 ±3.49% of baseline N= 10 n= 21, average from t= 4.5 to t= 299.5, p= 0.9592 paired t-test; late cKO 97.48 ±3.64% of baseline N= 10 n= 22, average from t= 4.5 to t= 299.5, p= 0.442 paired t-test). In summary, HFS in late cKO slices triggered similar levels of LTP. However, whether the apparent normal plasticity showed by late cKO mice is be supported by the same cellular mechanisms of late WT slices remains to be clarified.

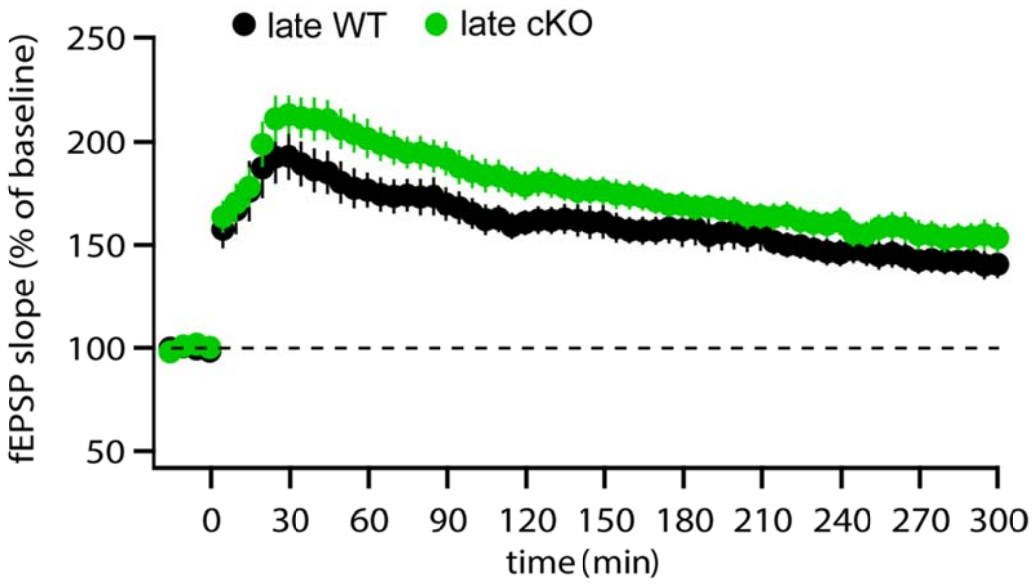

Figure 54 Summary of HFS-inducing LTP experiments in late WT and cKO slices.
In late WT slices, E-LTP was induced to 178.82 ±12.45% of baseline (N= 10 n= 22, average from t= 4.5 to t= 29.5), and compared to E-LTP of late cKO slices, no significant difference was found (189.03 ± 9.66% of baseline N= 11 n= 22, average from t= 4.5 to t= 29.5, genotype p= 0.5084 time p < 0.0001 interaction p= 0.025, two-way ANOVA RM). L-LTP of late cKO slices was compared to L-LTP of late WT slices and no statistical difference was found (late WT 141.81 ±6.44% of baseline N= 10 n= 22, average from t= 274.5 to 299.5; late cKO 154.02 ±6.98% of baseline N= 11 n= 22, average from t= 274.5 to t= 299.5, genotype p= 0.2008 time p= 0.7768 interaction p= 0.722, two-way ANOVA RM).

4.11.5 HFS- induced LTP and protein synthesis in late cKO mice

Next, we were interested in investigating LTP maintenance in late cKO slices. I initially bath applied CHX during LTP recordings in late WT slices and similarly to before, the protein synthesis inhibitor blocked L-LTP (fig. 55a) (untreated 136.35 ±5.68% of baseline N= 6 n= 6, average from t= 274.5 to t= 299.5 CHX- treated 109.69 ±8.09% of baseline N= 9 n= 12, average from t= 274.5 to t= 299.5, treatment p= 0.0381 time p= 0.7463 interaction p= 0.6156, two-way ANOVA RM).On the other hand, L-LTP was not affected by CHX in late cKO slices (fig. 55b) (untreated 137.96 ±7.09% of baseline N=7 n= 12, average from t= 274.5 to t= 299.5 CHX- treated 143.67 ±10.29% of baseline N= 6 n= 10, average from t= 274.5 to t= 299.5, treatment p= 0.6402 time p= 0.2268 interaction p= 0.8891, two-way ANOVA RM). In summary, late cKO slices exhibited a protein synthesis independent LTP. LTP in KO slices was also independent of novel proteins synthesis. Taking together, our results suggested that the expression of Arc/Arg3.1 in mature synapses is essential to link synaptic consolidation with memory.

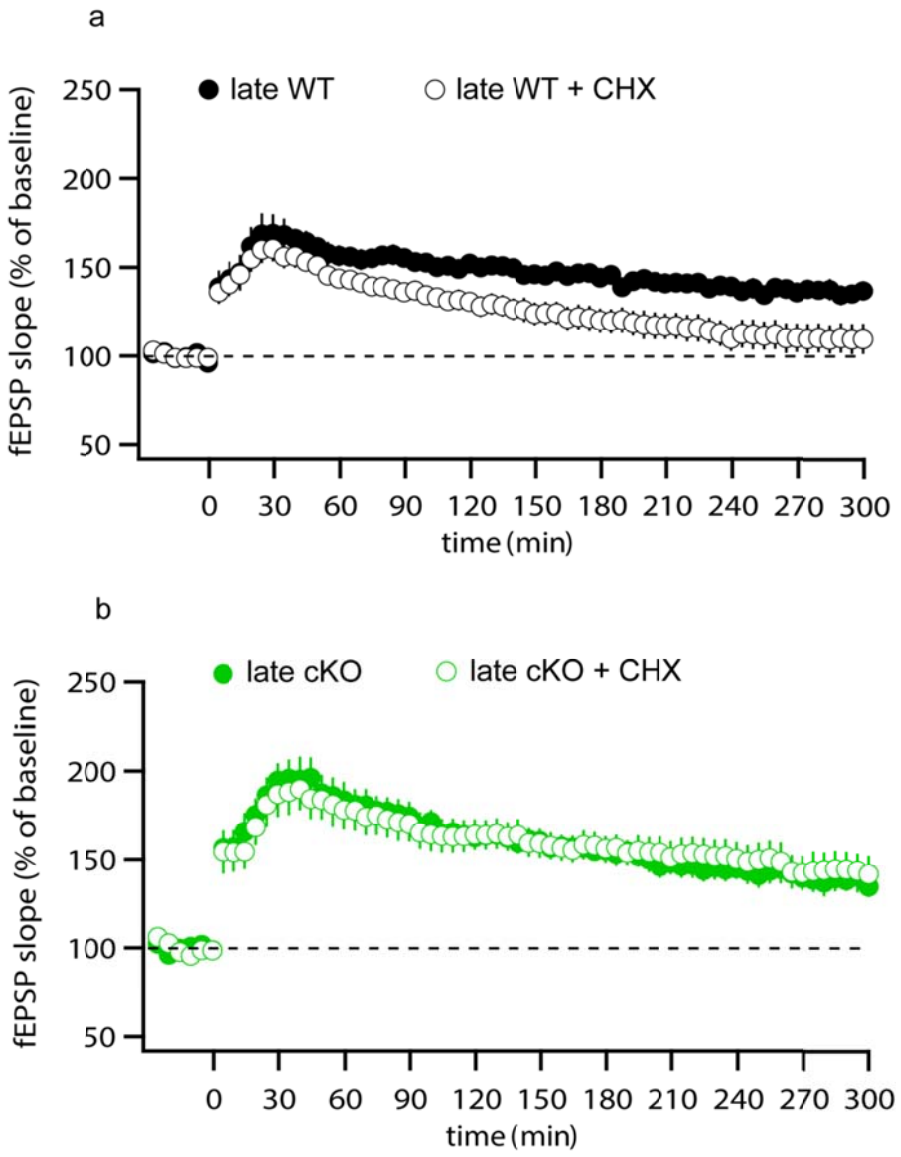

Figure 55 Summary of LTP experiments in late WT and cKO slices
(a) LTP was blocked in late WT CHX- treated slices (untreated 136.35 ±5.69% of baseline N= 6 n= 6, average from t= 274.5 to t= 299.5; CHX-treated 109.69 ±8.09% of baseline N= 9 n= 12, average from t= 274.5 to t= 299.5, treatment p= 0.0381 time p= 0.7463 interaction p= 0.6156, two-way ANOVA RM); (b) No significant difference was found in LTP between late cKO untreated and CHX- treated slices (untreated 137.96 ±7.09% of baseline N= 7 n= 12, average from t= 274.5 to t= 299.5, CHX-treated 143.67 ±10.29% of baseline, treatment p= 0.6402 time p= 0.2268 interaction p= 0.8891, two-way ANOVA RM). Concerning control pathways, only fEPSPs of WT CHX- treated showed a significant increased following LTP protocol (107.93 ±2.81% of baseline N=9 n= 12, average from t= 274.5 to t= 299.5 p= 0.0089 paired t- test).

4.12 TBS- induced LTP

Another form of LTP reported in the hippocampus is induced by Theta Burst Stimulation (TBS). KO mice show an exaggerated E-LTP but lack the ability to consolidate L-LTP mediated by TBS [46]. However, little is known about the developmental role of Arc/Arg3.1 on this form of plasticity at the hippocampal circuit. Here, I investigated TBS- inducing LTP in KO and late cKO mice.

4.12.1 TBS-induced LTP in KO mice

It has been shown that KO mice failed to consolidate L-LTP triggered by TBS, both in vivo and in vitro[46]. In order to evaluate the developmental role of Arc/Arg3.1 in adult TBS-induced plasticity, I first tested TBS on WT and KO slices. In WT slices, TBS induced E- LTP to 161.19 ±9.71% of baseline (fig. 55a) (N= 4 n= 8, average from t= 4.5 to t= 29.5) and maintained L-LTP to 131.88 ±4.77% of baseline (N= 4 n= 8, average from t= 269.5 to t= 294.5). As expected, KO slices generated an enhanced E-LTP compared to WT slices (fig. 56a) (191.25 ±15.87% of baseline N= 5 n= 16, average from t= 4.5 to t= 29.5, genotype p= 0.1898 time p <0.0001 interaction p= 0.0165, two-way ANOVA RM). But surprisingly, L-LTP in KO slices was similar to WT (133.16 ±7.98% of baseline N= 5 n= 16, average from t= 269.5 to t= 294.5, genotype p= 0.915 time p= 0.0035 interaction p= 0.6915, two-way ANOVA RM). WT control pathways were unchanged, whereas KO slices showed reduced fEPSP slopes following TBS (fig. 56b) (WT 103.01 ±4.88 N= 4 n= 8, average from t= 4.5 to t= 294.5, p= 0.5033 paired t-test; KO 88.24 ±2.4% of baseline N= 5 n= 16 average from t= 4.5 to t= 294.5, p < 0.0001 paired t-test).

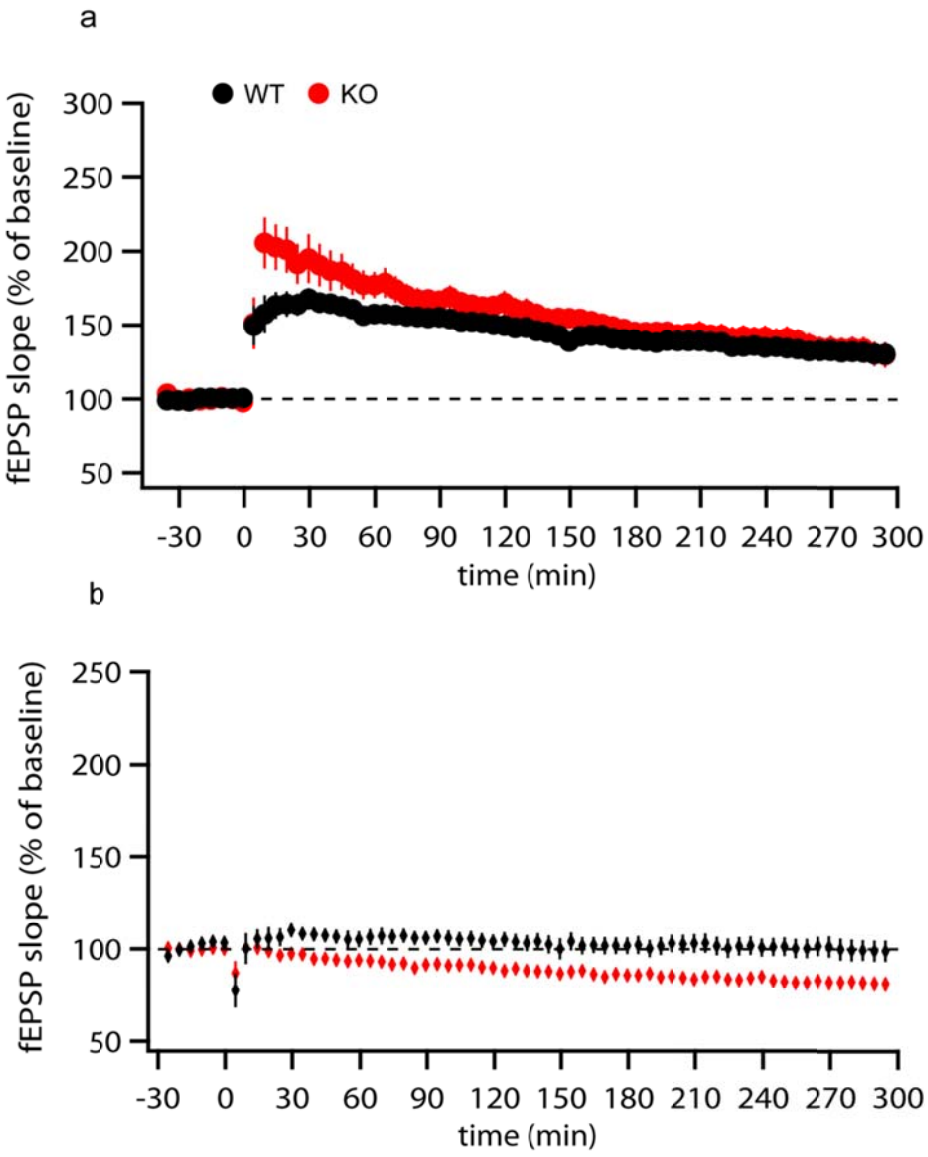

Figure 56 Summary of TBS-inducing LTP experiments in WT and KO slices
(a) In KO slices, E-LTP was slightly increased compared to E-LTP in WT slices (WT 161.19 ±9.71% of baseline N= 4 n= 8, average from t= 4.5 to t= 29.5 KO 191.25 ±15.87% of baseline N= 5 n= 16, average from t= 4.5 to t= 29.5, genotype p= 0.1309 time p= 0.6169 interaction p= 0.0273, two-way ANOVA RM). However L-LTP was comparable among genotypes (WT 131.88 ±4.77% of baseline N= 4 n= 8, average from t= 269.5 to t= 294.5 KO 133.16 ±7.98% of baseline N= 5 n= 16, average from T= 269.5 to t= 294.5, genotype p= 0.915 time p= 0.0035 interaction p= 0.6915, two-way ANOVA RM) (b) WT control pathways were unchanged, whereas KO slices showed reduced fEPSP slopes following TBS.

Next, we were wondering whether the magnitude of E-LTP and L-LTP was influenced by the fEPSPs amplitude measured before TBS stimulation. Therefore I divided LTP experiments of WT and KO slices in two groups: one group of slices having baseline

fEPSPs with amplitude below 1 mV and the second group of slices having baseline fEPSP with amplitude above 1 mV (fig. 57).

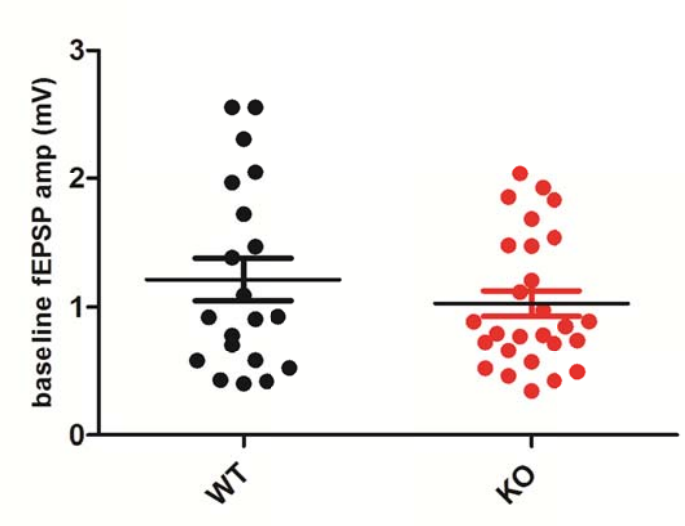

Figure 57 Means amplitude fEPSP baseline.
Summary of mean baseline fEPSPs amplitude in WT (black dots) and KO (red dots) slices.

Moreover, in order to collect an appropriate amount of data for this analysis, I combined together my LTP experiments with a previous PhD student's LTP experiments (Lilianna, Dr. Med. Thesis). Here, a comparison of LTP experiments between WT and KO slices having baseline fEPSPs below 1 mV revealed a non-significantly enhanced E-LTP in KO slices (fig.58a) (WT 160.17 ±6.13% of baseline N= 7 n= 10, average from t= 4.5 to t= 29.5, KO 191.12 ±16.04% of baseline N= 9 n= 11, average from t= 4.5 to t= 29.5, genotype p= 0.0802 time p <0.0001 interaction p= 0.1095, two-way ANOVA RM).

Remarkably, L-LTP was blocked in these KO slices (fig. 58a) (114.23 ±7.69% of baseline N= 9 n= 11, average from t= 269.5 to t= 294.5), whereas WT slices showed normal LTP (fig. 57a) (133.86 ±4.1% of baseline N= 7 n= 10, average from t= 269.5 to t= 294.5, genotype p= 0.0469 time p < 0.0001 interaction p= 0.0275, two-way ANOVA RM). In the group of slices having baseline fEPSPs above 1 mV, a non-

significant increase was seen in both E-LTP (fig.58b) (WT 159.71 ±10.22% of baseline N= 8 n= 11, average from t= 4.5 to t= 29.5 KO 184.06 ±10.7% of baseline N= 4 n= 9, average from t= 4.5 to t= 29.5, genotype p= 0.0839 time p < 0.0001 interaction p= 0.2785, two-way ANOVA RM), whereas and L-LTP (fig. 58b) (WT 120.5 ±8.29% of baseline N= 8 n= 11, average from t= 269.5 to t= 294.5 KO 145.84 ±11.3% of baseline average from t= 269.5 to t= 294.5, genotype p= 0.0797 time p= 0.1854 interaction p= 0.9813, two-way ANOVA RM).

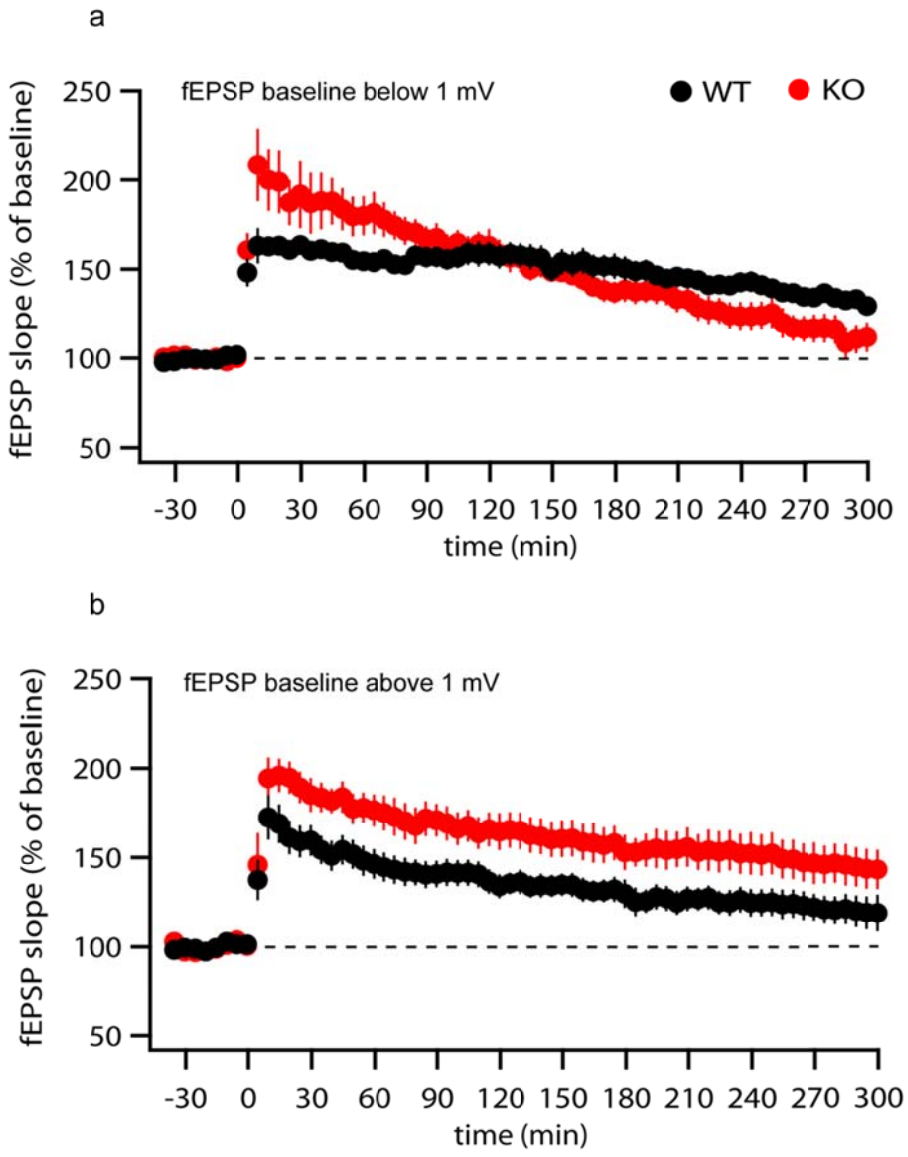

Figure 58 Summary LTP experiments of WT and KO slices having baseline below and above 1 mV.

(a) Comparison of LTP experiment of WT and KO slices having baseline fEPSP amplitude below 1 mV showed a slightly enhanced E-LTP in KO slices (WT 160.17 ±6.13% of baseline N= 7 n= 10, average from t= 4.5 to t= 29.5, KO 191.12 ±16.04% of baseline N= 9 n= 11, average from t= 4.5 to t= 29.5, genotype p= 0.0802 time p < 0.0001 interaction p= 0.1095, two-way ANOVA RM). L-LTP was blocked in KO slices (114.23 ±7.69% of baseline N= 9 n= 11, average from t= 269.5 to t= 294.5), WT slices showed normal LTP (133.86 ±4.1% of baseline N= 7 n= 10, average from t= 269.5 to t= 294.5, genotype p= 0.0469 time p < 0.0001 interaction p= 0.0275, two-way ANOVA RM). (b) In the group of slices having baseline fEPSPs amplitude above 1 mV, E-LTP was slightly enhanced in KO slices compared to WT slices (WT 159.71 ±10.22% of baseline N= 8 n= 11, average from t= 4.5 to t= 29.5 KO 184.06 ±10.7% of baseline N= 4 n= 9, average from t= 4.5 to t= 29.5, genotype p= 0.0839 time p= < 0.0001 interaction p= 0.2785, two-way ANOVA RM). KO slices exhibited an enhanced L-LTP, although not significantly different from L-LTP in WT slices (WT 120.5 ±8.29% of baseline N= 8 n= 11, average from t= 269.5 to t= 294.5 KO 145.84 ±11.3% of baseline average from t= 269.5 to t= 294.5, genotype p= 0.0797 time p= 0.1854 interaction p= 0.9813, two-way ANOVA RM).

Taking together, KO mice revealed a surprisingly long lasting LTP. However, further analysis shows that the magnitude of fEPSPs at baseline correlates with the outcome of LTP. fEPSPs of baseline above 1 mV, lLTP was preserved, whereas baseline fEPSPs amplitude below 1 mV lLTP was blocked. On the contrary, WT slices did not show any differences in LTP concerning baseline fEPSPs.

4.12.2 LTP and protein synthesis

It has been reported that Theta Burst stimulation applied at the Schaffer Collateral induces a form of LTP dependent on local translation but independent on transcription[32]. Therefore, we were interested to assess whether the dependence of translation was occurring in TBS-inducing LTP in WT and KO mice. In WT slices, CHX-treated slices showed a slightly enhanced E-LTP compared to untreated slices (fig. 59a) (untreated 134.64 ±9.62% of baseline N= 7 n= 8, average from t= 4.5 to t= 29.5 CHX-treated 155.93 ±6.64% of baseline N= 8 n= 10, average from t= 4.5 to t= 29.5, treatment p= 0.0532 time p < 0.0001 interaction p= 0.9321, two-way ANOVA RM). No significant difference was found in L-LTP (fig. 59a) (untreated 117.12 ±10.5% of baseline N= 7 n= 8, average from t= 269.5 to t= 294.5 CHX- treated 115.7

±6.18% of baseline N= 8 n= 10, average from t= 269.5 to t= 294.5, treatment p= 0.904 time p= 0.6532 interaction p= 0.1274, two-way ANOVA RM). On the other hand, KO untreated and CHX-treated slices did not show any significant difference neither in E-LTP (fig. 59b) (untreated 179.91 ±13.84% of baseline N= 6 n= 7, average from t= 4.5 to t= 29.5 CHX-treated 179.3 ±13.36% of baseline N= 6 n= 12, average from t= 4.5 to t= 29.5, treatment p= 0.9741 time p < 0.0001 interaction p= 0.6659, two- way ANOVA RM) nor in L-LTP (fig. 59b) (untreated 133.59 ±9.56% of baseline N= 6 n= 7, average from t= 269.5 to t= 294.5 CHX- treated 138.4 ±8.54% of baseline N= 6 n= 12, average from t= 269.5 to t= 294.5, treatment p= 0.7121 time p= 0.0578 interaction p= 0.3433, two-way ANOVA RM). In conclusion, LTP induced by TBS is not mediated by the synthesis of novel proteins in WT slices. Similarly, LTP in KO slices did not show any alteration by CHX administration.

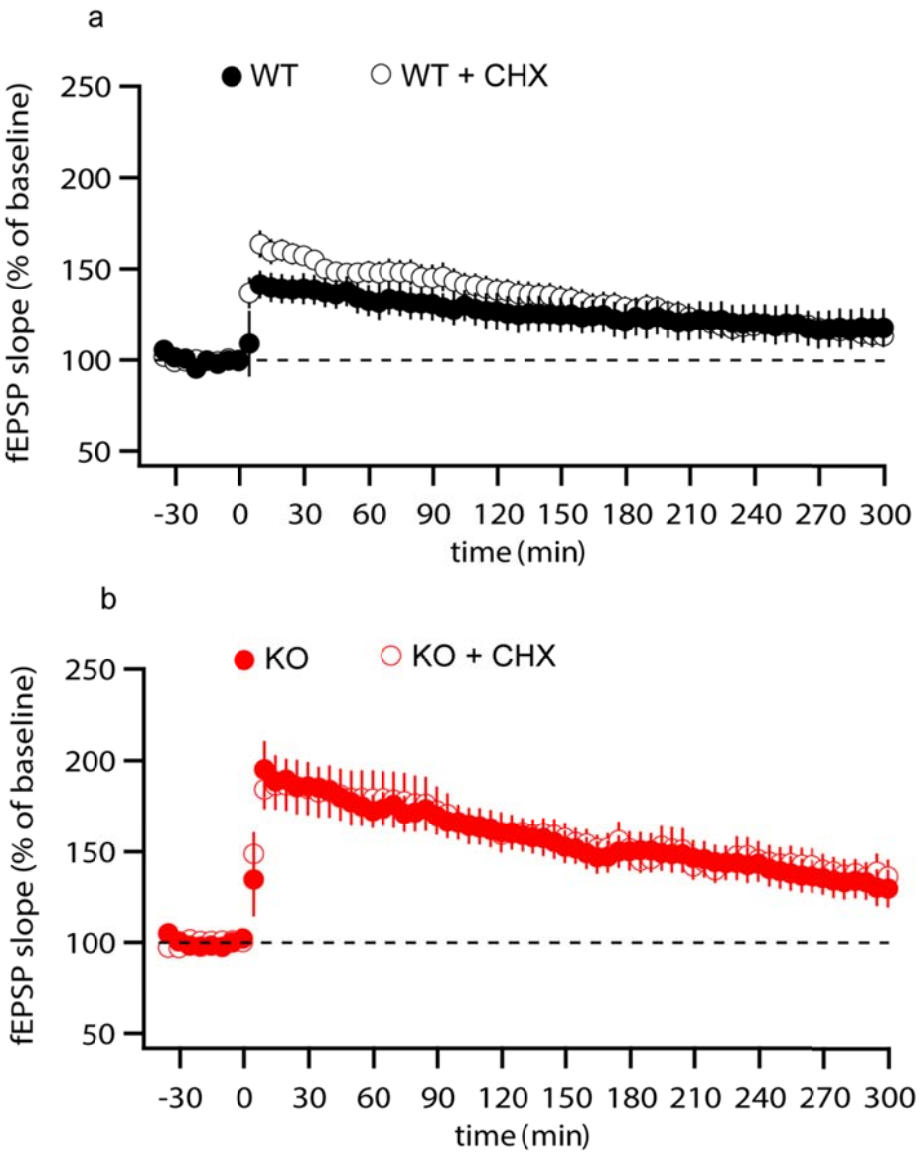

Figure 59 TBS-inducing LTP experiments in WT and KO untreated and CHX-treated slices

(a) In WT slices, CHX- treated slices showed a slightly enhanced E-LTP compared to untreated slices (untreated 134.64 ±9.62% of baseline N= 7 n= 8, average from t= 4.5 to t= 29.5 CHX- treated slices 155.93 ±6.64% of baseline N= 8 n= 10, average from t= 4.5 to t= 29.5, treatment p= 0.0532 time p < 0.0001 interaction p= 0.9321, two-way ANOVA RM). No significant difference was found in L-LTP (untreated 117.12 ±10.5% of baseline N= 7 n= 8, average from t= 269.5 to t= 294.5 CHX- treated 115.7 ±6.18% of baseline N= 8 n= 10, average from t= 269.5 to t= 294.5, treatment p= 0.904 time p= 0.6532 interaction p= 0.1274, two-way ANOVA RM). (b) KO untreated and CHX- treated slices did not show any significant difference neither in E-LTP (untreated 179.91 ±13.84% of baseline N= 6 n= 7, average from t= 4.5 to t= 29.5 CHX- treated 179.3 ±13.36% of baseline N= 6 n= 12, average from t= 4.5 to t= 29.5, treatment p= 0.9741 time p < 0.0001 interaction p= 0.6659, two- way ANOVA RM) nor in L-LTP (untreated 133.59 ±9.56% of baseline N= 6 n= 7, average from t= 269.5 to t= 294.5 CHX- treated 138.4 ±8.54% of baseline N= 6 n= 12, average from t= 269.5 to t= 294.5, treatment p= 0.7121 time p= 0.0578 interaction p= 0.3433, two-way ANOVA RM).

4.12.3 TBS-LTP in late cKO mice

Next, we aimed to determine whether LTP induced by TBS was dependent on the presence of Arc/Arg3.1 during development. Therefore, I induced TBS on late cKO slices. In late WT slices, TBS induced E-LTP to 159.78 ±7.93% of baseline (N= 5 n= 11, average from t= 4.5 to t= 29.5) and comparison with E-LTP in late cKO slices didn't show a significant difference (fig. 60) (164.57 ±12.67% of baseline N= 6 n= 16, average from t= 4.5 to t= 29.5, genotype p= 0.7693 time p < 0.0001 interaction p= 0.0297, two-way ANOVA RM). Late WT and cKO slices showed a similar L-LTP (fig. 60) (late WT 132.97 ±3.42% of baseline N= 5 n= 11, average from t= 274.5 to t= 299.5 late cKO 123.02 ±6.33% of baseline N= 6 n= 16, average from t= 274.5 to t= 299.5, genotype p= 0.2303 time p= 0.0577 interaction p= 0.1763, two-way ANOVA RM).

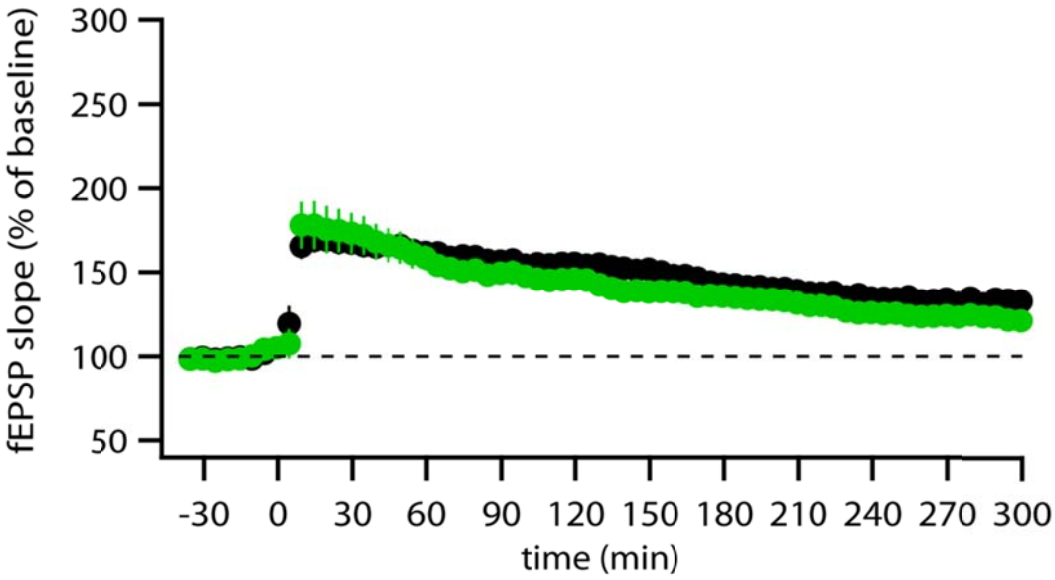

Figure 60 Summary TBS experiments in late WT and cKO slices
In late WT slices, TBS induced E-LTP to 159.78 ±7.93% of baseline (N= 5 n= 11, average from t= 4.5 to t= 29.5) and comparison with E-LTP in late cKO slices showed no significant difference (164.57 ±12.67% of baseline N= 6 n= 16, average from t= 4.5 to t= 29.5, genotype p= 0.7693 time p < 0.0001 interaction p= 0.0297, two-way ANOVA RM). Late WT and cKO slices showed an identical L-LTP (late WT slices 132.97 ±3.42% of baseline N= 5 n= 11, average from t= 274.5 to t= 299.5 late cKO slices 123.02 ±6.33% of baseline N= 6 n= 16,

average from t= 274.5 to t= 299.5, genotype p= 0.2303 time p= 0.0577 interaction p= 0.1763, two-way ANOVA RM).

Thus, late cKO slices showed normal LTP. This form of plasticity is sustained by mechanisms shaped during early stage of development in which Arc/Arg3.1 was still present. Therefore, TBS-induced LTP in late cKO slices is comparable to relative WT slices.

5 Discussion

5.1 A novel form of LTD in mature hippocampal synapses

Although LTP remains the most widely accepted model for learning and memory, over the last decades LTD has also been implicated in some forms of memory. For instance, studies showed that LTD might be involved in weakening synapses irrelevant for newly learned information and, using optogenetic stimulation, LTD has been linked to memory inactivation[25],[12]. The cellular mechanisms underlying LTD within the hippocampus change with development and stimulation protocols [106]. Two forms of LTD are known to coexist in the hippocampus: one is mediated by activation of NMDA receptors and the other by activation of mGluRs[77]. The present study identifies a novel form of LTD induced in adult hippocampal slices by multiple LFS and is mediated by NMDA receptors. Indeed, APV blocked the LTD, whereas MCPG the group I mGluRs antagonist, did not have any effect on this form of plasticity. In adult hippocampus, NR2A and NR2B are the two predominant NMDAR-subunits [18]. Using hippocampal slice preparation, it has been shown that selectively blocking NR2B-containing NMDARs abolished the induction of LTD but not LTP, whereas inhibition of NR2A-containing NMDARs prevents the induction of LTP without affecting LTD[19],[107]. Moreover, NR2A and NR2B subunits undergo a particularly well-characterized developmental shift in the cortex. NR2B subunits are abundant in the early postnatal brain, whereas NR2A increases with development and the recombination of these subunits is highly regulated by sensory experiences[108],[109]. In addition to changes in subunit-composition, in the cortex the localization of NR2A- and NR2B-containing NMDARs has a marked impact on LTD and LTP. For instance, NR2B-containing NMDARs might be targeted to extrasynaptic sites[107], [110]. We found that our LTD is not dependent on NR2B-containing NMDARs. Therefore, we presume

that this form of NMDAR-mediated LTD involves NR2A-subunits, which are sufficient to trigger the LTD. It is not yet known whether the NR2B antagonist Ro25-6981 blocks NR1/2A/2B heteromers.

L-type VGCCs are involved in numerous processes in the neurons, including gene expression, synaptic efficacy and cell survival[111]. Previous studies have shown that LTD in neonatal CA1 pyramidal cells depends on postsynaptic depolarization that evokes Ca^{2+} entry through L-type channels and on simultaneous activation of mGluRs[78]. Application of nifedipine, the L-type VGCC antagonist, induced a stronger LTD compared to the relative DMSO-control group. Interestingly, the simultaneous inhibition of NMDARs and L-type VGCCs activation during LFS blocks LTD to the same extent as does blockade of NMDARs only. Therefore, we suggest that L-type VGCCs are coupled downstream of NMDAR activation, and L-type channels alone are not able to sustain LTD without pairing with NMDARs. It was recently published that NMDARs and L-type VGCC cooperatively regulate the transfer of AMPARs to the dendritic plasma membrane (PM)[112]. This publication supports the statement that upon stimulation, AMPAR-recycling endosome (RE) docking and fusion to the plasma membrane requires NMDARs activation, and coincident L-type VGCCs activation is required to transfer for delivering the AMPARs to the PM. Interestingly, blocking NMDARs completely prevents all forms of activity-triggered RE fusion. On the other side, blocking L-type VGCCs has little effect on mobilization and fusion of REs with the PM; it drastically increases the incidence of display-mode events that transfer less RE cargo to the PM in dendritic shafts. Based on these results, we presume that a similar mechanism is involved in our NMDAR-LTD. However, the function of L-type VGCCs on this form of plasticity is still not clear. Figure 61 shows schematically the receptors and channels involved in LTD induction.

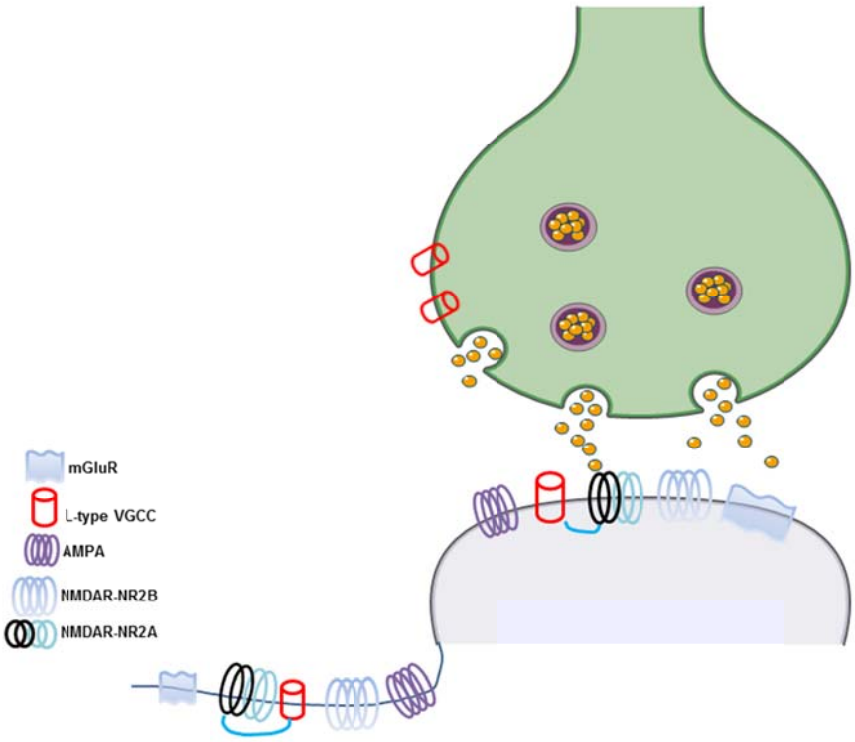

Figure 61 NMDAR-LTD in adult WT mice
Upon stimulation, Ca^{2+} enters into the presynaptic compartment and promotes the docking and the release of vesicles containing glutamate. Glutamate binds to AMPARs, which are the the major receptors mediating fast excitatory synaptic transmission and depolarize the cells. Activation of NMDARs depends on glutamate, D-serine or glycine binding and AMPARs-mediated depolarization of the postsynaptic membrane which relieves the voltage-dependent channel block by Mg^{2+}. Activation and opening of these receptors triggers LTD. The blocking of mGluRs has no effect on LTD-induction, meaning that they are not primarily involved in this form of plasticity. L-type VGCCs act downstream of NMDAR activation, alone these channels cannot trigger LTD.

5.2 NMDAR-LTD maintenance is a balance between protein synthesis and protein degradation

Previous studies have shown that the balance between novel protein synthesis and protein degradation is essential for long lasting hippocampal LTP. Indeed, the blocking of translation and of proteasomal pathway separately disrupts LTP expression[31],[113]. In line with this idea, we found that the novel form of LTD in WT mice is mediated by the simultaneous translation of novel proteins and lysosomal degradation of proteins (fig. 61). It is widely believed that novel protein synthesis is required for some forms of LTD, since inhibitors of translation cause a recovery of synaptic transmission within few hours of induction[114]. In our hands, application of CHX blocks LTD already during the induction phase, meaning that CHX might affect the production of transmembrane receptors involved in this form of plasticity, such as AMPARs. Interestingly, our LTD is regulated by lysosomes rather than by the UPS, even though it has been observed elsewhere that blocking lysosomal degradation did not have functional consequences on LTD maintenance[115]. This observation is also at odds with several recent studies suggesting a role for the proteolytic activity of the UPS in activity-dependent synaptic plasticity[84]. A role for lysosomes however is supported by other studies[116], [117]. The endocytosis of AMPA receptors is known to be important for the expression of NMDAR-dependent LTD[118]. Recently, studies in dissociated hippocampal neurons suggested that lysosomes move to the dendrites in an activity-dependent manner and directly correlate with the distribution of internalized membrane proteins and AMPARs[96] (fig. 62).

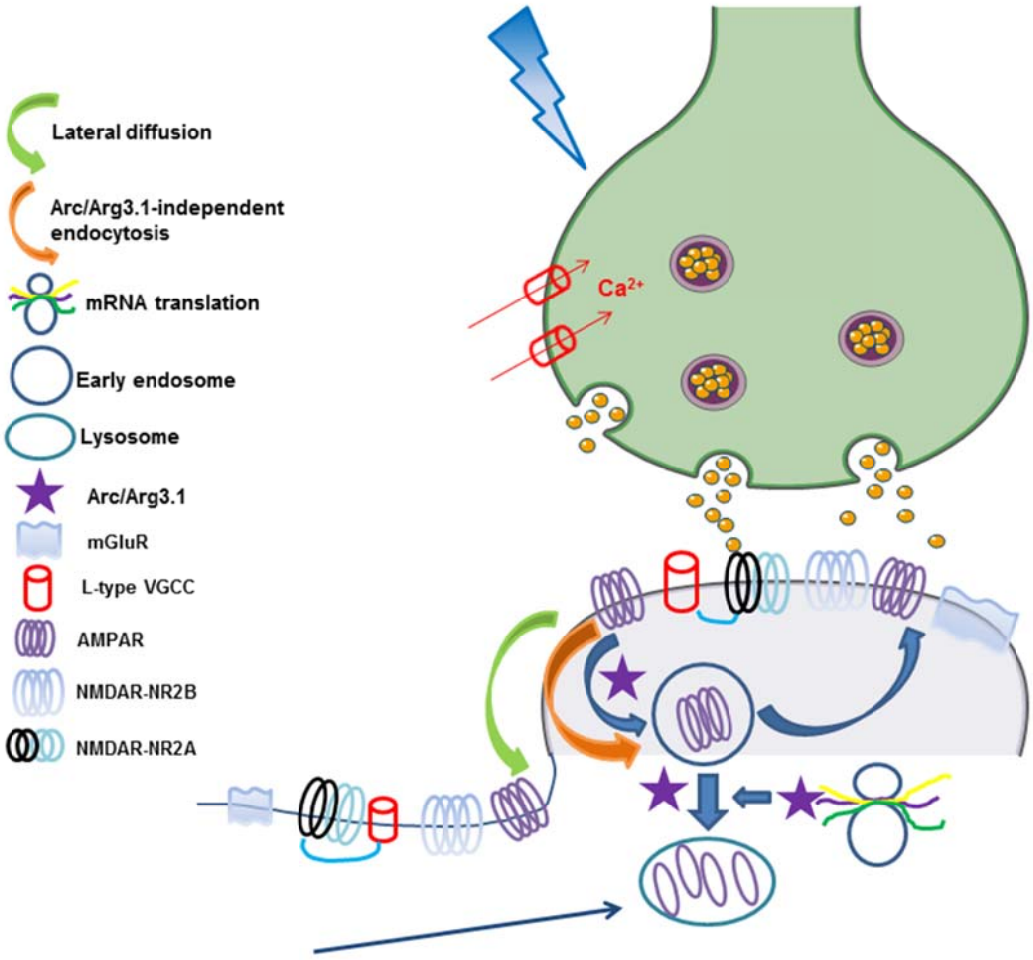

Figure 62 NMDAR-dependent LTD is supported by novel protein synthesis and by lysosomal degradation.
Following the removal of AMPARs from the membrane by Arc/Arg3.1-dependent and independent endocytosis, the novel synthesis of Arc/Arg3.1 seems to become essential in order to allow the transition of AMPARs from the endosomes to the lysosome for the degradation. The blocking of novel Arg3.1 synthesis or lysosomal degradation permit AMPARs to recycle back to the membrane without undergoing lysosomal degradation.

A fraction of internalized AMPARs might therefore be effectively degraded at the lysosomes[96]. Indeed, the transport of AMPARs via Rab7, a protein involved in membrane trafficking, ensures receptor removal from the synaptic membrane following LTD induction[115]. A reason, which can explain the discrepancy between our results and the results of previous publication, might be related to the phosphorylation state of AMPARs, a type of post translational modification (PTM)

that modulates the electrophysiological, morphological and biochemical properties of AMPA receptors in regulating synaptic plasticity[119]. For instance, phosphorylation sites at serine 845 (S845) of AMPARs is critical for LTD expression since mice lacking S845 lack LTD[120]. S845 is phosphorylated by PKA and is a key site controlling AMPARs trafficking. It traffics receptors to extrasynaptic membranes and primes extrasynaptic receptors for synaptic insertion[121] In contrast, phosphorylation of S880 of GluA2-containing AMPARs reduces GluA2 surface expression, which eventually promotes LTD[122]. It remains still unknown whether our LTD protocol is modulated by the same AMPAR-phosphorylation sites. Ubiquitination, another PTM, has emerged as an important regulator of AMPARs trafficking and function[123]. Depending on the chain length and topology of the targeted proteins, ubiquitination can target them to proteasomal or lysosomal degradation[124]. Therefore, it might be fundamental to check whether the same modifications in phosphorylation and ubiquitination of AMPARs are occurring in our experiments at the baseline level and upon stimulation. In summary, we hypothesize that in WT mice the translation of Arc/Arg3.1 might be fundamental in trafficking AMPARs to the lysosome. Yet, blocking Arc/Arg3.1 translation AMPARs recycle back to the transmembrane (fig.62).

5.3 Enhanced E-LTD in KO mice

Arc/Arg3.1 is a well-known activity-regulated immediate early gene, which is being intensively studied due to its crucial role in linking neuronal activity to long lasting synaptic plasticity and, ultimately, memory formation[46], [125]. Arc/Arg3.1-deficient mice show impaired hippocampal LTD and deficits in memory consolidation[46], [53]. However, in our hands KO mice exhibit enhanced E-LTD and sustained L-LTD and reveal a similar dependency on NMDAR activation as WT. Both pre and postsynaptic mechanisms have been proposed to explain NMDAR-dependent LTD[126]. Although Arc/Arg3.1 is known to be expressed only at postsynaptic structures[54], the stronger E-LTD showed by KO mice might be related to presynaptic alteration, such as for instance a more severe decrease of transmitter release due to less neurotransmitter available for the release or transient decrease of local Ca^{2+} concentration at the presynaptic active zone. To regulate synaptic strength postsynaptic neurons release substances from the cell bodies and dendrites, these messengers act in a retrograde manner to modulate neurotransmitter release from presynaptic terminals[127]. Recently, it was found that in drosophila Arc/Arg3.1 (dArc1) protein forms capsid-like structures that bind darc1 mRNA in neurons and is loaded into extracellular vesicles that are transferred from motorneurons to muscles[128]. It has been also reported that this retroviral-like mechanism of transfer is required for dArc1 function in Drosophila. We hypothesize that these EVs loaded with Arc/Arg3.1 mRNAs might also be present in mice hippocampus and act as a retrograde messenger from the postsynaptic cell to the presynaptic terminal following stimulation in order to regulate neurotransmitter release by moderating VGCCs kinetics. Therefore, lack of Arc/Arg3.1 might occlude this phenomenon, leading to an enhanced E-LTD. However, there is still no evidence of such a mechanism in mammalians. It is interesting to note that at the baseline level, KO slices showed an enhanced current threshold to evoke action potentials

compared to WT slices, although the resulting fEPSP amplitude reached by that current remains comparable in both genotypes. This analysis suggests that KO slices might be less excitable, due to enhanced inhibition, synapses or spines number.

One of the best characterized functions of Arc/Arg3.1 is scaling neuronal output through internalization of AMPA receptors[47]. It has been shown that loss of Arc/Arg3.1 leads to an increase in the steady state level of surface GluR1, relative to WT control[49]. One alternative to the direct internalization of AMPARs from the synaptic site includes the lateral diffusion of these receptors where they cycle with intracellular pools[129],[130]. Since KO mice show an enhanced E-LTD, this alteration might be linked to a temporary increased lateral diffusion of AMPARs to extra synaptic membrane and, eventually, their internalization (fig. 63).

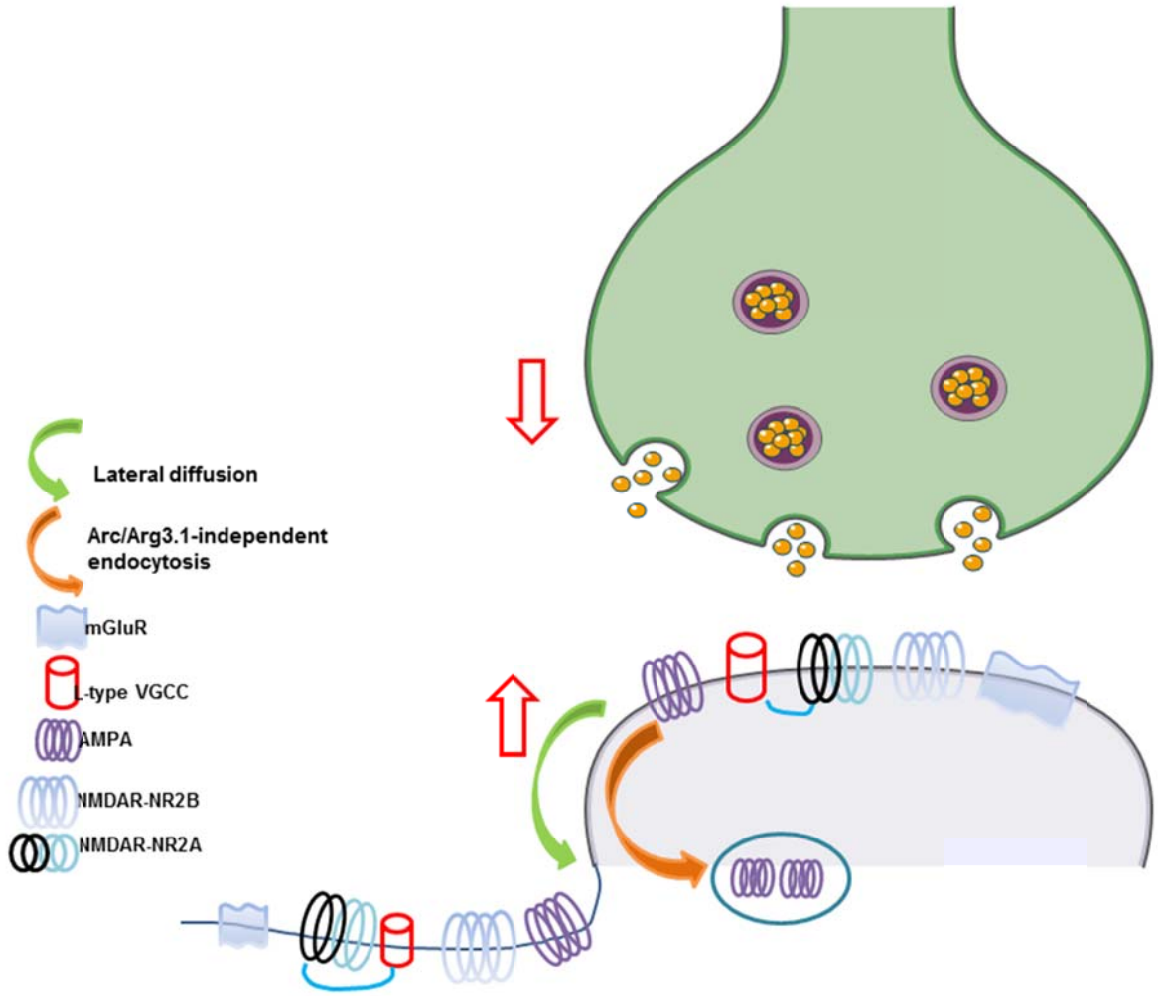

Figure 63 Enhanced E-LTD in KO.
In KO slices, the enhanced E-LTD might be due to a more severe decrease of transmitter release. Since Arc/Arg3.1 is involved in endocytosis of AMPARs and KO mice show more GluR1-containing AMPARs[49], an increased rate of endocytosis might happen directly for the synaptic receptors. Endocytosis of AMPARs occurs also following lateral diffusion to extrasynaptic site[129]. In KO mice, an increased rate of lateral diffusion of AMPARs might precede their endocytosis.

5.4 Novel protein synthesis and lysosomal degradation in LTD of KO mice

Memory consolidation is assumed to require specific patterns of activity that lead to stable modifications of synaptic structure and function. Those modifications are in part dependent on synthesis and degradation of proteins[131], [84]. Arc/Arg3.1 is an IEG, first identified as activity-dependent gene, fundamental for memory and synaptic consolidation[132],[46]. Surprisingly, our findings show that KO mice exhibit sustained LTD, although these mice still show severe deficits in memory consolidation[46],[133],[134]. Despite the similarity of LTD magnitude in WT and KO mice, in WT slices the inhibition of protein synthesis and lysosomal degradation separately prevents LTD maintenance. In contrast, KO slices do not show any LTD impairment either by blocking of protein synthesis and of lysosomal degradation, separately or simultaneously. It has been already shown that LTD induced *in-vitro*, under certain conditions, might endure several hours without synthesis of novel proteins[74]. Protein synthesis independent forms of LTP and LTD might rely on a pool of previously synthesized proteins, which will eventually be depleted. Thus, *in-vivo*, high protein turnover at the synapse renders memory persistence and plasticity dependent on novel protein synthesis. Indeed, in living animals, experience rapidly invokes short term memory that consolidates into long term memory via a process which also requires protein synthesis. Arc/Arg3.1 KO mice exhibit normal short-term memory but entirely lack all forms of long-term memory. My current findings, show that large LTD can be evoked and persist for 2 hours in the KO mice, similar to the duration of the short term memory. The absence of protein synthesis may however, prevent this LTD from consolidating into changes underlying long-term memory.

However, my findings differ from previous reports of transient LTD in the Arc/Arg3.1 KO mice[46]. One explanantion is that LTD protocols previously used in KO slices were different from our LTD protocol[46],[52]. LTD induced by a conditioning stimulation composed of one LFS train is prevented by application of NMDA receptors antagonist[80]. The LTD protocol used in our study was composed of two trains of LFS, with an inter-train interval of 10 minutes. We suggest that a mechanism of metaplasticity[135] might differently prime NMDA receptors function during the first LFS and consequently alter the cellular mechanism following the second LFS. Essentially, metaplasticity allows neurons to change their physiological and biochemical state in order to generate synaptic plasticity. Notably, metaplasticity is not supposed to alter the synaptic strength, but it modulates only the capability of the synapses to undergo LTP or LTD through, for instance, regulation of biochemical processes[135], [136]. Experience-dependent plasticity is modulated by changes in sensory input occurring over the course of the day[137],[138]. However, studies on circadian regulation of hippocampal plasticity found that endogenous circadian oscillator modulates synaptic plasticity in the hippocampus[139]. During sleep an activity dependent down-selection of synapses is supposed to renormalize net synaptic strength and restores cellular homeostasis, a crucial mechanism for memory consolidation[140]. All the LTD recordings were in the sleeping-state of the mice. LTD in WT slices seems to be sensitive to the pharmacological blocking of the mechanisms essential to its maintenance. Since LTD recordings in KO slices were not affected by any pharmacological treatments, we suggest that KO mice exhibit an inflexible shrinkage of synapses which underlies the persistent LTD and as a consequence a lead to deficits in memory consolidation.

On a basal synaptic transmission level WT slices treated with CHX or leupeptin did not exhibit any changes in I/O curve. In contrast, leupeptin-treated KO slices showed a significantly lower I/O curve compared to untreated KO slices, but no significant alteration was detected in CHX-treated slices. The reduced synaptic response seen by blocking the lysosome in KO slices might be the result of decrease membrane excitability due to less AMPARs at the PM or diminished Ca^{2+} influx pre or postsynaptically[116]. Further analysis by detecting lysosomal localization before and after stimulation could be useful to understand its function on LTD.

5.5 The role of dendritically-translated Arc/Arg3.1 in LTD

Previously, it has been shown that upon NMDAR activation newly synthetized Arc/Arg3.1 mRNA is specifically targeted to active synapses, where it undergoes local translation[89]. It was also reported that the targeting of Arc/Arg3.1 mRNA was not confounded by blocking of local translation, meaning that the signal for the localization at the dendrites is in Arc/Arg3.1 mRNA itself[54]. The present study shows that NMDAR-dependent LTD is completely blocked in adult slices of tg(3'UTR)Arc/Arg3.1 mice. Although lacking of local translation of dendritic Arc/Arg3.1 mRNA, the somatic expression of Arc/Arg3.1 protein and its delivery to the dendrites are intact in these mice. Additionally, It was also proven that Arc/Arg3.1 is specifically localized at the synapses of Tg(3'UTR)Arc/Arg3.1 mice. The resistance of tg(3'UTR)Arc/Arg3.1 mice to undergo LTD following LFS is caused by the lack of local Arc/Arg3.1 translation, which might result in insufficient activation of NMDARS activation, since LTD could not be even induced in tg(3´UTR)Arc/Arg3.1 mice. The impaired NMDAR-LTD induction can be generated by reduced AMPARs internalization and/or decrease in NMDARs number or conductance.

In addition, the basal synaptic transmission was significantly decreased in tg(3'UTR)Arc/Arg3.1 mice as indicated by significantly smaller fEPSPs compared to that of WT mice. This might be due to the significantly enhanced current threshold to induce action potentials, suggesting reduced membrane excitability in Tg(3'UTR)Arc/Arg3.1 slices. In order to better understand this phenomenon, it would be worthy to investigate voltage-gated channels activation, inactivation, and synaptic conductance on spike threshold. Additionally, decreased synaptic transmission can also be caused by reduced presynaptic neurotransmitter release which is modulated in part by retrograde messenger that travels from the postsynaptic to the presynaptic cells and directly impact synaptic plasticity[141]. Therefore, in Tg(3'UTR)Arc/Arg3.1 mice retrograde signaling mediated by, for instance, nitric oxide (NO)[142] might be attenuated. This could result in reduced neurotransmitter release, underlying the observed decrease in basal synaptic transmission.

5.6 mGluR-LTD is protein synthesis independent in juvenile WT mice

In young mice, the best known form of LTD is mediated by mGluRs[15]. This form of plasticity is largely known to rely on novel protein synthesis at the hippocampal-CA1 dendrites, since isolated dendrites still support LTD[24]. Briefly, mGluRs activate the expression of several proteins, such as Arc/Arg3.1[52], MAP1B[143], FMRP[144] which regulate AMPARs internalization and promote LTD. The removal of synaptic glutamate receptors, such as AMPARs, is a well-established mechanism for the expression of mGluR-LTD in the hippocampus[145].

Surprisingly, our study shows a form of mGluR-LTD independent on novel protein synthesis. Indeed, CHX does not show any effect on DHPG-inducing LTD, in any DHPG- concentration used.

The protein synthesis dependence of DHPG-induced LTD is known to be developmentally regulated[28]. In fact, previous studies on protein synthesis concluded that DHPG-LTD is independent of protein synthesis at P8-P15 and becomes dependent between P21-P35. Here, we show that at P21-P23 mGluR-LTD, in WT mice, is still protein synthesis independent. Whether this changes at older ages, remains unknown.

Moreover, it has been reported that the induction of stable LTD in the CA1 region of the hippocampus of awake adult rats is facilitated by exposure of mild stress[146] Our mice were housed in the animal facility, picked up in the morning and directly anaesthetized and sacrificed in our laboratory. The animals were neither acclimatized nor handled before the procedures. Thus, we suggest that the mice might have experienced mild stress before slicing. Preparation for transport by caretakers in the

basement, transport from the basement to the laboratory, changes in room temperature and the exposure to novel environmental cues could be a source of mild stress for rodents and the stress might promote LTD even when blocking protein synthesis. Interestingly, it has also been documented that the time window in which LTD could be reliably elicited was prolonged by inducing anesthesia immediately after the stress[146]. Although it has been reported that the effects of stress on LTD appear to be mediated primarily by NMDARs activation[147], we hypothesize that a similar effect might happen also in our LTD mediated by mGluRs.

Recent evidence showed that the circadian rhythm can alter synapse number or strength[148]. For instance, hippocampal LTP is facilitated during the dark cycle, when mice are active[139]. On the other hand, during sleep cellular mechanisms involved in decreasing synaptic transmission in hippocampus and cortex are active. Studies on changes in GluR1-containing AMPAR levels in mice during wakefulness and sleep show that a significant increase in total GluR1 level is detected in synaptoneurosomes of the waking group[149]. Interestingly, it was found that during sleep the ratio of nuclear to cytoplasmic Arc/Arg3.1 expression is higher, compared to wakefulness and sleep deprivation in supragranular cortical layers[150]. Arc/Arg3.1 localizaton to the nucleus promotes an increase in promyelocytic leukemia nuclear bodies, which decrease GluR1-containing AMPARs transcription[151]. Since our experiments were all performed during the mice's light-cycle (i.e. sleep phase), it is possible that a decrease of GluR1 transcription, due to an increased nuclear Arc/Arg3.1, promotes LTD even in absence of novel protein synthesis.

5.7 Normal mGluR-LTD in Juvenile KO mice

Previous reported suggested that Arc/Arg3.1 is essential for mGluR-mediated LTD in juvenile mice, since Arc/Arg3.1 KO showed impaired mGluR-LTD[52]. It had been shown that activation of mGluRs triggers dendritic Arc/Arg3.1 translation, which eventually promotes AMPARs internalization and LTD maintenance[152]. Surprisingly, in our experiments mGluR-LTD was preserved in juvenile KO mice, and was protein synthesis independent in both WT and KO mice. These findings are at odds with previous publications[24]. Arc/Arg3.1 KO mice used in these publications were identical to ours as well experimental procedures including slicing, recovery time, solutions, chamber perfusion and stimulation. Subjective time of day at brain preparation was not reported in these publications and may be different to ours. On a cellular level, it is known that mGluR activity stimulates translation through two major signaling pathways, ERK-MAPK and PI3K-mTOR pathways[153]. It is still unknown whether these pathways are influenced by light/dark cycle. A first preliminary examination might be to check whether these pathways are also activated by mGluRs following DHPG application in juvenile KO slices under our experimental conditions.LTP is facilitated during the dark cycle and more interestingly, LTP of slices prepared during the light-phase but recorded during the dark-phase has a profile remarkably similar to the dark group, i.e. LTP was preserved[139]. Additionally, molecular and electrophysiological evidence suggest that sleep is linked with LTD and memory consolidation[149]. The role of LTD during sleep makes sense since activity-dependent down regulation of synaptic strength, potentiated after wake, restores cellular homeostasis and favors memory consolidation[140]. LTD experiments in our hands were performed during the sleep phase of the mice. It might be possible that in KO mice different states of metaplasticity promote the persistent LTD following DHPG application. Furthermore, no differences in basal synaptic transmission were found

between WT and KO mice, suggesting that on a baseline level fast excitatory transmission is comparable between genotypes. Since LTD is supposed to occur during the night in order to restores synaptic network for proper memory storage, the persistence of mGluR-LTD in KO mice might explain the consequent lack of memory consolidation show in these mice.

5.8 Arc/Arg3.1 expression during development in adult plasticity

Proper development of cortical circuitry is influenced in part by ongoing sensory events, occurring in distinct postnatal time-windows[56].Arc/Arg3.1 mRNA is detected at P7 with upregulation peaking between P14 and P21[133],[134]. In this study we show that following LTD and LTP protocols early cKO mice exhibit an enhanced E-LTD and E-LTP and sustained L-LTD and L-LTP. Late cKO mice show an overall preserved synaptic plasticity.

LTD and LTP are conventionally separated into two-phases: the early-phase and the late-phase. The early-phase is known to be unaffected by transcriptional and translational inhibition[30] and rather relies on protein phosphorylation (PTMs), which is known to be a postsynaptic mechanism[154].

PTMs are mechanisms for regulating ionotropic glutamate receptors, including AMPARs and NMDARs[154]. One of the PTMs involved in regulating synaptic plasticity is phosphorylation[155]. It was reported that phosphorylation at 831 and 845 sites (S831 and S845, respectively) of the GluR1 subunit of AMPARs are modulated during LTD and LTP[156]. Previous studies found that GluA1 S831 phosphorylation by CaMKII[157] and PKC[158] increased single channel conductance, whereas S845 phosphorylation enhanced the channel open probability and the current peak of

GluA1-containing AMPARs[159]. It might be possible that the enhanced E-LTD and E-LTP detected in early cKO mice as well as the enhanced E-LTD and E-LTP in KO mice are the consequence of an alteration of the state of AMPARs phosphorylation sites occurred upon stimulation. In fact, phosphorylation of the GluR1 subunit on S831 is significantly increased in visual cortex of KO mice[50]. It would be interesting to examine the state of AMPAR-phosphorylation sites, in particular at S831 and at S845, in CA1 of our KO and early cKO slices before and within 20 min of following LTD and LTP protocols. Intriguing, our work demonstrates that the presence of Arc/Arg3.1 until the end of the third postnatal week lacks a change in E-LTD and E-LTP. In order to test whether the changes in the state of phosphorylation sites are a developmental issue, it might be interesting to test S831 and S845 before and after LTP and LTD protocols in the late cKO as well, and compare the results with the results of KO and early cKO mice. If the phosphorylation state of AMPARs is different at the baseline level in late cKO mice compared to KO mice, we might suggest that this difference is due to the role of Arc/Arg3.1 during development in regulating synaptic function. We show that late cKO mice exhibit seemingly normal LTD, LTP and basal synaptic transmission. However, the mechanisms of LTD and LTP maintenance like KO mice, late cKO mice show are protein synthesis-independent, as in KO mice. Taking together these results, it seems that the presence of Arc/Arg3.1 during early postnatal development, affects the size of E-LTP and E-LTD, which were abnormally elevated in the KO and early-cKO but not the late cKO. In contrast, protein synthesis dependence of the LTD and LTP was impaired in all Ko and cKO mice demonstrating that presence of Arc/Arg3.1 at the time of activity-dependent plasticity events is fundamental for engaging the protein synthesis machinery. In this case, the link between protein synthesis and Arc/Arg3.1 expression might not be a developmental issue.

6. Abstract

The expression of the immediate early gene Arc/Arg3.1 is rapidly upregulated following neuronal activity, synaptic plasticity and learning. The loss of Arc/Arg3.1 has been well-correlated with the impairment of long term depression (LTD) and long term potentiation (LTP), as well as with deficits in consolidation of long term memory in mice. In order to further investigate adult hippocampal plasticity in acute slices of Arc/Arg3.1 knock-out (KO) mice, a novel form of LTD is initially established in wild type mice. The novel LTD is dependent on NMDARs activation and supported by the synthesis of novel proteins and by the protein degradation through the lysosomal pathway. In KO mice, NMDAR-LTD was enhanced in the early phase but, surprisingly, it remains stable throughout the entire recording. Further investigations on the mechanisms underlying the LTD maintenance reveal that the LTD in KO mice is independent on both protein synthesis and lysosomal degradation. These data demonstrate that, under certain circumstances, the maintenance of the LTD is not directly linked with memory consolidation, since KO mice still show deficit in memory consolidation. In this regard, if we want to directly correlate the time needed for the memory to evolve from short term to long term memory, i.e. days and weeks, with the time-window investigated for LTD experiments, 2 hours, and the discrepancy is pretty remarkable. These results acknowledge the role of Arc/Arg3.1 in linking protein synthesis dependent-synaptic plasticity with memory consolidation.

Proper development of cortical circuitry is influenced in part by ongoing sensory events, occurring in distinct postnatal time-windows. Since Arc/Arg3.1 mRNA and protein are expressed in the hippocampus early after birth, we were interested in understanding whether this gene acts during neuronal development in shaping adult plasticity. Indeed, adult conditional mice in which Arc/Arg3.1 is ablated after 7 days

postnatally (early cKO) show a LTD comparable to KO mice, i.e. enhanced E-LTD and normal L-LTD. On the other hand, the ablation of Arc/Arg3.1 after the third postnatal week (late cKO) do not show any significant effect on LTD. The role of Arg 3.1 in linking long lasting synaptic plasticity to memory is supported by the results of NMDAR-LTD experiments in KO mice. However, it is still under debate whether this link matures during development or later in life. In order to consider a developmental role of Arc/Arg3.1 in settling the association between protein synthesis and maintenance of synaptic plasticity, LTD was induced in late cKO mice in the presence on the protein synthesis blocker cycloheximide (CHX). Here, in these mice LTD was independent on novel protein synthesis, like the LTD expressed in KO mice. Together these results validate Arc/Arg3.1 as a crucial bond during the stimulation time in order to link the novel protein synthesis with the consolidation of synaptic plasticity associated with memory.

Growing evidence suggests the role of local protein synthesis in supporting several forms of synaptic plasticity related with memory formation. The unique feature of Arc/Arg3.1 gene to be locally translated gives the opportunity to determine whether the dendritic Arc/Arg3.1 translation is directly involved in synaptic plasticity, i.e. NMDAR-LTD. To examine this possibility, LTD is induced in mice lacking dendritic Arc/Arg3.1 mRNA (Tg (3'UTR) Arc/Arg3.1). Interestingly, they show impaired LTD induction and lack of LTD maintenance, confirming the role of local Arc/Arg3.1 translation in NMDAR-LTD.

Next attempt is to directly evaluate hippocampal plasticity in slices derived from juvenile KO mice. In juvenile mice the best known form of LTD is mediated by mGluRs. Therefore, we induce a chemical mGluR-LTD by bath-application of DHPG, the well-known mGluRs agonist. This form of mGluR-LTD, known to rely on novel

protein synthesis, unexpectedly is not protein synthesis dependent in our wild type mice. Additionally, this form of plasticity does not show any impairment in KO mice, peculiar results since the scientific community agrees that Arc/Arg3.1 is essential in regulating mGluR-dependent LTD. Our data suggest that, under certain circumstances, mGluR-LTD might be sustained by signaling mechanisms unrelated to novel protein synthesis. However, these signaling mechanisms remain to be identify both for the LTD in wild type and in KO mice.

The other model of synaptic plasticity strongly linked with memory is long term potentiation (LTP). LTP is induced initially in KO mice by application of the high frequency stimulation (HFS) protocol, which consists of three train of HFS (100 Hz each train). In KO mice, HFS induces an enhanced E-LTP, as previously reported. However, in this set of experiments, the L-LTP was surprisingly preserved. It is well-accepted that HFS induces protein synthesis dependent L-LTP. Therefore, we next aim to assess this statement by inducing HFS-LTP in the presence of the protein synthesis blocker CHX in wild type and KO mice. Indeed, HFS-LTP is protein synthesis dependent in WT mice. Conversely, KO mice show a protein synthesis independent L-LTP. The developmental role of Arc/Arg3.1 in HFS-inducing LTP is assessed in late cKO mice as well. Yet, in late cKO mice HFS-LTP is unaffected by the CHX, confirming the independence on protein synthesis. Together these results point to the conclusion that Arc/Arg3.1 is essential in mature neurons in order to link protein synthesis with LTP maintenance. This assumption goes eventually in line with previously reported results on NMDAR-LTD and protein synthesis in KO and late cKO mice.

The second form of LTP investigated is induced by theta burst stimulation (TBS). KO mice confirmed enhanced E-LTP but, yet, the L-LTP was maintained. Further

analysis on TBS-LTP recordings of KO mice reveals a correlation between the amplitude of the evoke response during baseline with the maintenance or decay of L-LTP. Furthermore, we confirm that TBS-LTP is protein synthesis independent. Late cKO mice exhibit normal TBS-induced plasticity, confirming the previously reported result of HFS-LTP in this mouse line. Taking together, these results suggest that Arc/Arg3.1 deficient mice, under certain circumstances, are able to undergo and maintained activity-dependent synaptic plasticity. However, this capability does not act in linking synaptic plasticity and memory consolidation. It remains still matter of debate which mechanisms support the abnormal long lasting plasticity in KO mice and, eventually, whether this abnormal plasticity might be rescue.

7. Zusammenfassung

Das unmittelbar-früh exprimierte Gen (immediate early gene, IEG) Arc/Arg3.1 wird nach neuronaler Aktivität, synaptischer Plastizität und Lernvorgängen exprimiert. Der Verlust von Arc/Arg3.1 korreliert mit einer Störung von synaptischer Langzeit-depression (long term depression, LTD) und Langzeitpotenzierung (long term potentiation, LTP), sowie fundmentalen Problemen bei der Konsolidierung des Langzeitgedächtnisses. Für ein tieferes Verständnis der Plastizität in akuten hippokampalen Hirnschnitten adulter konstitutiver Arc/Arg3.1 knockout Mäuse (KO) wurde eine neue Form von LTD in wildtyp Mäusen (WT) etabliert. Diese Form von LTD ist abhängig von NMDA-Rezeptoren (NMDAR) und geht einher mit *de novo* Proteinsynthese und lysosomalem Proteinabbau. In KO-Mäusen ist dieses NMDAR-LTD in der Anfangsphase verstärkt und bleibt während der gesamten Aufzeichnungsphase stabil. Eine weitere Untersuchung des zugrunde liegenden Prozesses ergab, dass das LTD in KO-Mäusen sowohl von Proteinsynthese als auch von Proteinabbau unabhängig ist. Diese Daten zeigen, dass die Stabilisierung von LTD und die Gedächtniskonsolidierung nicht direkt miteinander verknüpft sein müssen, da KO-Mäuse trotz LTD kein stabiles Langzeitgedächtnis ausbilden können. Hierbei ist zu bedenken, dass die Ausbildung des Langzeitgedächtnisses über Tage und Wochen erfolgt, während der Zeithorizont bei LTD-Experimenten mit ca. 2 Stunden wesentlich kürzer ist. Arc/Arg3.1 könnte in diesem Zusammenhang die Lücke zwischen Proteinsynthese-abhängiger synaptischer Plastizität und Gedächtniskonsolidierung überbrücken. Die Entwicklung kortikaler Netzwerke wird z. T. durch andauernde sensorische Erfahrung während eines begrenzten postnatalen Entwicklungsfensters beeinflusst. Da Arc/Arg3.1 mRNA und Protein im Hippokampus bereits kurz nach der Geburt exprimiert werden, wollten wir untersuchen, inwiefern dieses Gen bereits bei der neuronalen Entwicklung Einfluss auf die Ausbildung

adulter Plastizität nehmen kann. Konditionale Arc/Arg3.1 KO-Mäuse, in denen die Expression 7 Tage nach der Geburt ausgeschaltet wurde (early cKO), zeigten tatsächlich ein zu KO-Mäusen vergleichbares LTD mit einer verstärkten frühen (E-LTD) und normalen späten Phase (L-LTD). Eine weitere konditionale Mauslinie, in der Arc/Arg3.1 erst nach der dritten postnatalen Woche ausgeschaltet wird (late cKO), zeigte dagegen keine signifikante Veränderung der LTD. Die Rolle von Arc/Arg3.1 bei der Überbrückung von Langzeitplastizität und Gedächtnisausbildung wird somit durch die Resultate aus NMDAR-LTD Experimenten in KO Mäusen bestätigt. Unklar bleibt bisher, ob diese Verbindung schon während der Entwicklung oder erst später im Leben von Bedeutung ist. Um dies besser zu verstehen, wurde LTD in late cKO Tieren in Anwesenheit des Proteinsyntheseinhibitors Cycloheximid (CHX) untersucht. Hierbei zeigte sich, dass LTD unabhängig von *de novo* Proteinsythese exprimiert wurde, vergleichbar mit den Ergebnissen aus KO Mäusen. Dies bestätigt die Rolle von Arc/Arg3.1 als Bindeglied zwischen proteinsynthese-abhängier synaptischer Plastizität und Gedächtnisbildung in adulten Tieren.

Eine Vielzahl von Studien legt nahe, dass lokale Proteinsynthese eine wichtige Rolle für verschiedene Formen von synaptischer Plastizität und Gedächtnisbildung spielt. In diesem Zusammenhang sollte untersucht werden, ob lokale, dendritische Arc/Arg3.1 Translation eine Rolle bei NMDAR-LTD spielt. Hierfür wurde LTD in einem Mausmodel ohne dendritische mRNA (Tg (3'UTR) Arc/Arg3.1) induziert. Interessanterweise zeigten sich hier eine gestörte Induktion und Expression von LTD, wodurch die Rolle von Arc/Arg3.1 bei lokaler Translation während NMDAR-LTD unterstrichen wird.

Als nächstes sollte hippokampale Plastizität in Hirnschnitten junger Mäuse untersucht werden. Eine vielfach untersuchte Form von LTD wird hier über metabotrope

Glutamatrezeptoren (mGluRs) vermittelt, die durch Badapplikation von DHPG, einem mGluR-Agonisten, induziert werden kann. Diese Form von mGluR-LTD, die normalerweise proteinsynthese-abhängig ist, erwies sich unerwarteter Weise als nicht proteinsyntheseabhängig in unseren WT-Mäusen. Darüber hinaus zeigte sich auch keine Veränderung in unseren KO-Tieren. Beides unerwartete Ergebnisse, da eine Beteiligung von Arc/Arg3.1 an mGluR-LTD durch viele Studien belegt ist. Unsere Daten lassen den Schluss zu, dass mGluR-LTD unter bestimmten Umständen unabhängig von Proteinsynthese etabliert werden kann. Welche Signalwege einem solchen Prozess zu Grunde liegen, verbleibt sowohl für KO, als auch für WT Mäuse ungeklärt.

LTP ist eine andere Form synaptischer Plastizität, die als wesentliche Grundlage für Gedächtnisprozesse erachtet wird. Hier wurde LTD in KO Mäusen durch Hochfrequenzstimulation (high frequency stimulation, HFS) induziert, bei der jeweils drei Pulse HFS mit einer Frequenz von 100 Hz appliziert werden. In KO-Mäusen führt diese Stimulation, wie zuvor gezeigt, zu einer erhöhten E-LTP. Überraschender Weise blieb L-LTP in unserer Serie von Experimenten stabilisiert. Induktion über HFS führt nach allgemeiner Auffassung zu einer proteinsynthese-abhängigen L-LTP. Dies wollten wir durch Induktion von HFS-LTP in Anwesenheit des Proteinsynthese-blockers CHX in WT und KO-Mäusen überprüfen. Es stellte sich heraus, dass HFS-LTP in WT-Mäusen tatsächlich proteinsynthese-abhängig war. In KO-Mäusen dagegen war L-LTP proteinsynthese-unabhängig. Zusätzlich sollte die entwicklungs-biologische Rolle von Arc/Arg3.1 bei HFS-LTP in late cKO Mäusen untersucht werden. In diesem Modell erwies sich HFS-LTP als CHX-resistent und damit als proteinsynthese-unabhängig. Zusammengenommen deuten diese Ergebnisse darauf hin, dass Arc/Arg3.1 in reifen Neuronen ein Bindeglied zwischen Proteinsynthese-

abhängigkeit und LTP-Stabilisierung darstellt. Diese Interpretation der Ergebnisse stimmt mit den hier zuvor dargestellten Beobachtungen an NMDAR-LTD und der Proteinsyntheseabhängigkeit in KO und late cKO Tieren überein.

Eine zweite von uns untersuchte Form von LTP wird durch ein theta burst stimulation Protokoll (TBS) induziert. KO Mäuse zeigten ein verstärktes E-LTP und ein konsolidiertes L-LTP. Eine weiterführende Analyse in KO Tieren ergab einen Zusammenhang zwischen der Amplitude evozierter, basaler Feldpotentiale und der Stabilisierung von L-LTP. Darüber hinaus konnten wir bestätigen, dass TBS-LTP proteinsynthese-abhängig ist. Late cKO Mäuse zeigten eine unveränderte TBS-induzierte Plastizität, in Einklang mit zuvor gezeigten Eigenschaften von HFS-LTP in dieser Mauslinie. Zusammengefasst lassen diese Ergebnisse die Schlussfolgerung zu, dass in Arc/Arg3.1 KO Mäusen, unter bestimmten Umständen, aktivitäts-abhängige, synaptische Plastizität induziert und stabilisiert werden kann. Durch welche Mechanismen diese aberrant Formen synaptischer Plastizität in Arc/Arg3.1 KO Mäusen vermittelt werden, verbleibt kontrovers. Ebenso, ob und wie normale Plastizität in diesen Tieren wiederhergestellt werden kann.

8. List of abbreviation

aCSF	Artificial cerebro spinal fluid
AD	Alzheimer´s disease
Amp	Amplitude
AMPAR receptor	α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid
ANOVA RM	Analysis of Variance repeated measure
APV	DL-2-amino-5-phosphovaleric acid
Arc/Arg3.1	Activity-regulated cytoskeleton-associated protein Activity regulated gene 3.1 protein
AS	Angelman Syndrome
BDNF	Brain-derived neurotrophic factor
CA	Cornu ammonis
CamKII	Ca^{2+} /Calmodulin-dependent protein kinase II
CHX	Cycloheximide
cKO	Conditional knock-out
CNS	Central Nervous System
Ctrl	Control pathway
DDW	Distillated water
DG	Dentate gyrus
DHPG	(S)-3,5-Dihydroxyphenylglycine hydrate
Dyn	Dynamin
DMSO	Dimethylsulfoxid
EC	Entorhinal cortex
eEF2K	Eukariotic initiation factor 4E kinase
E-LTD/P	Early-Long term depression/potentiation

End	Endophilin
EPSP	Excitatoty post synaptic potential
fEPSP	Field Excitatory Post Synaptic Potential
FMRP	Fragile mental retardation protein
FV	Fiber volley
FXS	Fragile X syndrome
GluA1	Glutamate receptor 1 subunit
HFS	High frequency stimulation
Hz	Herz
IEG	Immediate early gene
KO	knock-out
Leu	Leupeptin
LFS	Low frequency stimulation
LFP	Local field potential
L-LTD/P	Late-Long term depression/potentiation
LTD	Long term depression
LTP	Long term potentiation
MCPG	(RS)-α-Methyl-4-carboxyphenylglycine
MF	Mossy fibers
MG132	carbobenzoxy-Leu-Leu-leucinal
mGluR	metabotropic glutamate receptor
N	number of mice
n	number of slices
Na	Natrium/Sodium
Nif	Nifedipine
NMDAR	N-methyl-D-aspartate receptor
NO	nitric oxide

NR2A/B	NMDAR subunit A/B
P8	Post-natal day8
Pop-spike	Population-spike
PP	Perforanth Path
	RO25-6981 (αR,βS)-**α**-(4-Hydroxyphenyl)-**β**-methyl-4-(phenylmethyl)-1-piperidinepropanol
SC	Schaffer collateral
SEM	Standard Error of the mean
SI	Stimulus Intensity
TBS	Theta burst stimulation
Tg	Transgenic mice with the 3´ UTR of Arc/Arg3.1 encoding region is replaced with the 3´UTR of Zif268
Ube3A	Ubiquitin-protein ligase E3A
UPS	Ubiquitin Proteasome System
VGCC	Voltage gated calcium channel
WT	Wild type

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12. CV

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Fazeli et al., 2013

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10. Eidesstattliche Versicherung *[als letztes Blatt in die Dissertation einzubinden]*

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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