Labile glutamate synaptic transmission onto CA1 hippocampal pyramidal neurons: regional, functional and developmental diversity

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Cover illustration: Hippocampal CA1 region

Microscope pictures of the hippocampal CA1 region in slices taken from a 2nd postnatal week and a one-month-old rat, respectively by Rong Ma.

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If you're not prepared to be (w)rong, you'll never come up with anything original.

Ken Robinson

ABSTRACT

A single neuron in the brain receives tens of thousands of glutamate synapses from other neurons, and these synapses can differ in their properties depending on their role in the neural circuit, during brain development as well as in the adult brain. In the hippocampus, a cortical region involved in mnemonic functions, CA1 pyramidal cells serve as the output stage for the return of information back to the cortical areas providing input to the hippocampus. On their proximal dendrites in the stratum radiatum (SR) these cells receive learned associations between multimodal sensory events while on their distal dendrites in the stratum-lacunosum-moleculare (SLM) they receive on-going sensory activity. I studied SLM glutamate synapses using an in vitro slice preparation from neonatal and adult rats. In the CA1 region, the neonatal state is a period of activity dependent organization of synaptic connectivity driven by spontaneous waves of activity. Thereafter, the hippocampus will develop into its adult state through sensory-motor interaction with the environment. For the SR synapses the neonatal period is characterized by labile synaptic transmission, i.e., that even sparse activity removes glutamate receptors of the AMPA-type from the postsynaptic membrane, creating AMPA-silent synapses. For a synapse to survive in this neonatal period, it must be active together with other synapses and participate in evoking postsynaptic activity to remain AMPA-stable. Thereafter, this developmental plasticity is replaced by an activity dependent adult plasticity strengthening the synapses associated with a morphological expansion of the SR region. The SLM synapses are much less studied, where previous work has only indicated some quantitative differences compared to SR synapses. However, the SLM region matures morphologically earlier than the SR region, suggesting a faster transition to adult plasticity. I found the neonatal SLM synapses to exhibit much less postsynaptic AMPA labile transmission compatible with this suggestion. The SLM synapses instead showed a presynaptic labile transmission that acted like a habituation-like process at the synaptic level, explained by activity dependent release site inactivation.

This lability decreases with age. In contrast, the postsynaptic lability did not disappear but rather enhanced with age and approached that of the neonatal SR synapses. Moreover, only a developmental plasticity was found in the adult SLM synapses and the SLM region did not expand morphologically. These results suggest that adult SLM synapses are adapted to allow the SLM region to serve as a flexible multimodal sensory map for novelty detection and proper backpropagation to neocortical areas.

Keywords: development, hippocampus, synaptic plasticity, short-term plasticity, long-term plasticity

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POPULÄRVETENSKAPLIG

Hjärnans aktivitet styrs i mångt och mycket av synapser mellan nervceller som använder sig av glutamat som transmittor, och hur dessa glutamatsynapser fungerar. Detta gäller även den region av hjärnan, hippocampus, som är starkt kopplad till hjärnans förmåga att skapa ett episodiskt minne, dvs var, vad och när något hände i det förflutna. Genom denna förmåga är hippocampus också viktig för att detektera vad som är nytt genom att jämföra en pågående händelse med en tidigare lagrad, och att se till att information om detta förmedlas till de hjärnbarksområden som förmedlat denna händelse till hippocampus. Pyramidceller i CA1 regionen av hippocampus som mottar såväl tidigare lagrad information från CA3 pyramidceller som pågående sensorisk information från övrig hjärnbark kan här spela en viktig roll.

I mitt avhandlingsarbete som utförts på in vitro-slicepreparat från råtta jämförs de glutamatsynapser som signalerar pågående information med de tidigare väl studerade CA3 - CA1 synapserna som förmedlar tidigare lagrad information. Dessa synapser har studerats både i en funktionellt omogen (2nd postnatala veckan) som mogen (~1 månad) hippocampus. Frågeställningen har varit om de glutamatsynapser som skall samverka med CA3 - CA1 synapserna för att producera ett funktionellt utflöde från hippocampus har egenskaper speciellt designade för denna funktion, t.ex. vad gäller aktivitetsberoende omformbarhet (plasticitet) och, om så, om dessa egenskaper finns från början eller utvecklas under hippocampus funktionella mognad. Tidigare studier har visat att CA3 - CA1 synapserna under hippocampus mognad förändrar sin plasticitet från att initialt medverka till att organisera upp ett nätverk för att sedan förstärka det. Mitt huvudfynd är att dessa andra synapser till CA1 cellerna i både omogen och mogen hippocampus skiljer sig från CA3 - CA1 synapserna i sina egenskaper, men förändrar sig under utvecklingen på så sätt att de i mogen hippocampus fungera som CA3 - CA1 synapser i omogen hippocampus. Denna förändring av dessa synapser kan medverka till att underlätta för en ständig uppdatering av

det pågående sensoriska inflödet för jämförelse med det inlärda, och att lärd information förmedlas till rätt hjärnbarksområden.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Ma, R., Xiao, M., Gustafsson, B.
Labile glutamate signaling onto CA1 pyramidal cells in the developing hippocampus depends mechanistically on input pathway.
Neuroscience 2016; 337: 27-36.

II. Ma, R., Hanse, E., Gustafsson, B.
Homosynaptic frequency-dependent depression by release site inactivation at neonatal hippocampal synapses in the stratum lacunosum-moleculare.

European Journal of Neuroscience 2021; 54(3):4838-4862

III. Ma, R., Hanse, E., Gustafsson, B.
Labile glutamate synaptic transmission in the adult CA1 stratum-lacunosum-moleculare region.

Accepted, pending revision, in European Journal of Neuroscience

CONTENT

ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CHA	N-cyclohexyladenosine
CGP	CGP 55845 hydrochloride
D-AP5	*D*-2-amino-5-phosphonopentanoic acid
DG	Dentate gyrus
eSWA	Early sharp wave activity
LEC	Lateral entorhinal cortex
MEC	Medial entorhinal cortex
PKA	Protein kinas A
fEPSP	Field excitatory postsynaptic potential
GABA	γ-aminobutyric acid
LTD	Long-term depression
LTP	Long-term potentiation
mGlu	Metabotropic glutamate
NMDA	N-methyl-D-aspartate
P	Postnatal day
PP	Paired pulse
PP_{50}	a 50-ms paired-pulse interval
PP_{1S}	a 1 second paired-pulse interval

P_{ves}	Release probability of a single vesicle
SC	Schaffer collateral
SLM	Stratum lacunosum-moleculare
SR	Stratum radiatum
TA	Temporoammonic

1 INTRODUCTION

While brain activity depends upon an immense number of various factors, the neurotransmitter glutamate stands out among these factors. The glutamate synapse constitutes the vast majority of the quadrillion (10^{15}) synapses connecting the nerve cells in our brain and is the main force behind driving neurons to nerve impulse activity. Most information forwarded from our sensory systems to the brain, most of the handling of that information in brain circuits including its storage, and the ensuing actions from the brain onto the brain stem/spinal cord, is mediated via the glutamate synapse. The glutamate synapse also has the potential to exist in manifold functional forms, created both by a variability in how glutamate is released and how glutamate interacts with receptors of varying properties. This variability could then implement specific forms of neural operations needed in the various brain circuits in which the glutamate synapse is embedded.

This thesis will deal with a specific subset of glutamate synapses in the hippocampus, a brain structure of decisive importance for learning and memory (Goode *et al.*, 2020). The hippocampus consists of several regions, each having a specific role in hippocampal function, as detailed below. In my thesis, I will study a group of glutamate synapses onto the pyramidal cells in the CA1 region, the output stage of the hippocampus. The apical dendritic tree of these CA1 pyramidal neurons can be subdivided into two regions, one region close to the cell body (SR; stratum radiatum) and one further away from the cell body (SLM; stratum lacunosum-moleculare). These two regions receive glutamate synapses carrying different kinds of information that together create the output activity of the hippocampus. The glutamate synapses in the SR region (SR synapses) are among the most well-studied synapses in the brain and are often seen as a prototype for a cortical glutamate synapse. In contrast, the SLM synapses onto the CA1 pyramidal neurons which form the subject of my thesis have been much less studied.

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While the hippocampus is commonly referred to as a single structure involved in learning and memory processes, there is a functional segmentation within the hippocampus along its dorso-ventral extension. Thus, the dorsal part is seen as the "cold" hippocampus, dealing with cognitive functions, while its ventral part is the "hot" hippocampus, involved in emotion and control of the hypothalamic-pituitary-adrenal gland axis (Fanselow & Dong, 2010). In my research, I have only focused on glutamate synaptic function in the dorsal hippocampus.

In the rat, the animal used for these studies, sensory information from the external world does not begin to reach the hippocampus until the beginning of the third postnatal week. Thereafter, hippocampus-controlled behavior develops gradually, and the hippocampus is functionally mature at the end of the 1st postnatal month (Albani *et al.*, 2014). During this month, the glutamate synapses will thus be used for various purposes. First, for a neonatal synapse to become organized in a crude topographic manner spontaneous waves of activity originating from these sensory organs play a crucial role. Thereafter, with the help of actual sensory information to create a functional hippocampus (Valeeva *et al.*, 2019). For the well-studied SR synapses, the 1st postnatal month is associated with considerable changes in both pre- and postsynaptic properties, as detailed below, in line with the different roles for these synapses in the neonatal vs adult brain. In the CA1 SLM region, morphological studies have shown an earlier maturation of this region than the SR region (Pokorný & Yamamoto, 1981; Jabès *et al.*, 2011), and much data, as detailed below, suggests that SLM and SR synapses play different functional roles in the adult rat. The question that will be explored in my thesis is to what extent this morphological earlier maturation of the SLM region and its different role in hippocampal function will be reflected in the electrophysiological properties of these SLM synapses, both as neonatal and as adult synapses.

Although the glutamate synapse is quantitatively the dominant synapse for most neurons, the activity of a brain circuit is also determined by

several other factors beyond the exact properties of glutamate synapses. For instance, the activity of inhibitory neurons releasing GABA, variable intrinsic excitability properties of neurons, and the activity of neurons releasing neuromodulators (e.g. noradrenaline, dopamine, serotonin), which affect pre- and postsynaptic properties of synapses as well as intrinsic excitability, are important in controlling how a brain circuit operates. My thesis project is thus very reductionistic, focusing primarily on the intrinsic properties of the glutamate synapses under the study, i.e., to what extent a variation in glutamate synapse function plays a vital role in shaping circuit function.

1.1 BACKGROUND OF HIPPOCAMPUS

The hippocampus, located in the medial temporal lobe, is a structure chiefly known for its key role in learning and memory. It sits at the top of the associative hierarchy in the brain, targeted by all sensory modalities arising from high-level neocortical association areas as well

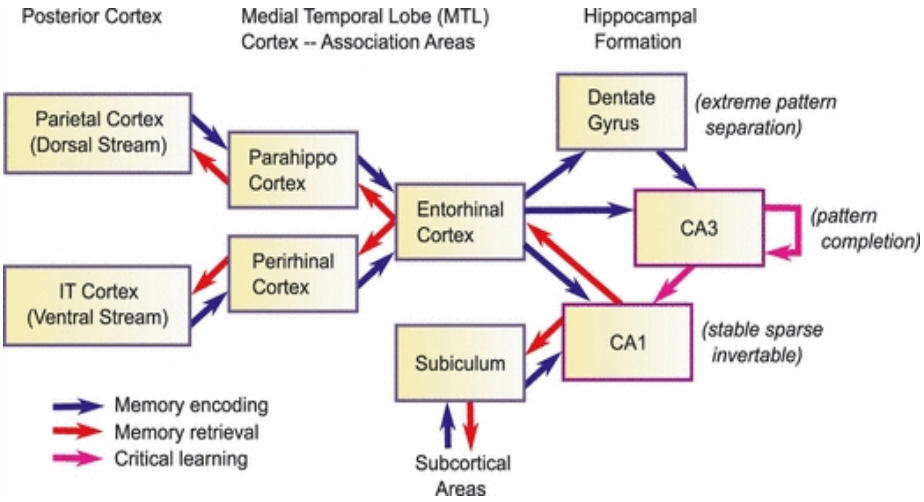

Figure 1. Memory formation in the hippocampus. (reprinted from (O'Reilly et al., 2014) with permission)

as from subcortical structures such as the thalamus, amygdala, septal nuclei etc., All these inputs thereby provide the hippocampus with both sensory information and with the context within which that occurs (O'Reilly *et al.*, 2014). As indicated in Fig. 1, (reprinted from (O'Reilly *et al.*, 2014), neocortical inputs do not reach the hippocampus directly but are channeled via other medial temporal lobe structures, including the parahippocampal and perirhinal cortices, and finally the entorhinal cortex. The output from the entorhinal cortex is channeled through hippocampus in two parallel paths, the trisynaptic circuit via the dentate gyrus - CA3 - CA1 region and the monosynaptic path directly to the CA1 region. The CA1 pyramidal neurons serve as the output stage of the hippocampus leading activity back to the entorhinal cortex and beyond.

The entorhinal cortex is subdivided into two major areas, the medial (MEC) and lateral (LEC) entorhinal cortex, respectively. The information channeled into these two areas differs, with MEC handling the position of objects while LEC handles the object itself (Rolls, 2018). In the dentate gyrus (DG) MEC and LEC axons reach the DG granule cells such that MEC axons make synapses onto the middle third of the dendritic tree of each granule cell, while LEC axons contact the distal third (Witter *et al.*, 1989). Each single granule cell may thus be activated by most sensory inputs, including vestibular, auditory, olfactory, visual, and somatosensory inputs that enter the DG via the entorhinal cortex. A key role of DG is to provide the sensory information with a spatial location, and then to encode and separate different spatial events from each other. The output from the DG in the form of mossy fiber synapses onto the CA3 pyramidal cells is thought to support a pattern separation function. The DG also exhibits an on-going neurogenesis (of granule cells) throughout the lifetime of the rat which is thought to play an important role in the spatial pattern separation and in time-based remote memory (Kesner, 2018).

The mossy fiber connectivity onto the CA3 pyramidal cells is rather circumscribed (Rolls, 2018), a single mossy fiber (granule cell)

innervates only ~14 of the ~300 000 CA3 pyramidal cells, and each CA3 pyramidal cell only receives synapses from ~50 mossy fibers (granule cells) out of the ~1 million granule cells present in the rat brain. Instead, each CA3 cell receives most of its synapses (~12000) from other CA3 pyramidal neurons, any CA3 pyramidal neuron sends out collaterals and forms synapses over a major portion (~70%) of the CA3 region, allowing the CA3 region to function almost as a single associative network (Le Duigou *et al.*, 2014; Rolls, 2018). As will be taken up later in the text, the CA3 region, through plasticity at these widespread recurrent connections, is capable of learning/storing complex multimodal sensory events that can be recalled later. Due to the auto-associative nature of this CA3 network, only part of this multimodal sensory event has to enter the network to recall the full event, a feature referred to as pattern completion. The output from the CA3 region will then be a prediction based on previous learning within the CA3 region based on the cue given by a subset of the multimodal sensory event (Barron *et al.*, 2020).

The information coming out of the CA3 region in the form of activity in a small, dedicated population of CA3 pyramidal neurons representing the most likely (predicted) multimodal sensory event is then sent to the CA1 pyramidal cells for further transmission back to the neocortical areas from which this sensory event originated. Within the CA1 region these CA3 axons, the so-called Schaffer collaterals, make synapses in the SR region of the CA1 pyramidal cell apical dendritic tree. Simultaneously with the activity reaching the CA1 pyramidal cells via this trisynaptic circuit, sensory activity also reaches the CA1 pyramidal cells in the SLM region via the monosynaptic circuit directly from the entorhinal cortex. In contrast to the DG, the MEC and LEC axons do not make synapses onto each CA1 pyramidal cell. Instead, they are distributed so that the MEC and LEC axons make synapses onto CA1 pyramidal cells closer/more distant to the CA3 region, respectively (Amaral, 1993).

1.2 BACKGROUND OF CA1 HIPPOCAMPAL REGION

Until about the end of the 2nd postnatal week, little sensory activity about the external world reaches the rat hippocampus. For instance, the eyelids are shut, the auditory canals are plugged, and the rat is fairly immobile. Despite this, sensory organs communicate with the cortex via spontaneous waves of activity, such as in retinal ganglion cells and cochlear hair cells (Valeeva *et al.*, 2019). This activity is important for the topographical organization of primary sensory neocortical areas and may extend its influence onto the hippocampus. In fact, in the sensorimotor system, spontaneously occurring myoclonic contractions generate sensory activity that not only reaches the somatosensory cortex but also reaches the hippocampus (Valeeva *et al.*, 2019; Cossart & Khazipov, 2022). As discussed by these authors, the predominant activity in the CA1 region at this age is an early sharp wave activity (eSWA), which originates from somatosensory activity and possibly from the other sense organs as well. Even though this activity, via the entorhinal cortex, is channeled through both the monosynaptic and trisynaptic pathways to the CA1 region, at this early age the eSWA activity is predominantly driven by the monosynaptic pathway in the SLM. As noted above, the monosynaptic circuitry from the entorhinal cortex to the CA1 SLM region also matures earlier than the trisynaptic circuitry. In fact, in SLM the adult extension of the dendritic tree is already achieved around postnatal day 10, whereas it takes until day 48 in the SR region. It seems likely that the eSWA activity in the early SLM CA1 region, driving action potential discharges in the CA1 pyramidal cells, establishes a topographic connectivity between the CA1 region and the neocortex.

During the 2nd postnatal week, pyramidal cells in the CA3 region start to expand their axonal distributions into the CA1 region with an extensive synaptogenesis. This expansion, combined with spontaneous waves of activity in the CA1 region originating from the CA3 region,

likely establishes an activity dependent organization between the CA3 and CA1 regions (Valeeva *et al.*, 2019).

After the 2nd postnatal week, as the rat starts to actively explore its environment, EEG recordings demonstrate a 5 - 12 Hz rhythmicity in the CA1 region, referred to as a theta rhythm. The theta oscillation is thought to indicate the on-line state of the hippocampus (Vanderwolf, 1969; Buzsáki, 2002) and is produced by alternating synaptic activation (at 5 - 12 Hz) of the SLM and SR regions, respectively. One phase is dominated by strong synaptic activity in the SLM region, with relatively limited SR activity. This phase is thought to be a period of information encoding through strengthening of the active SR synapses (Buzsáki, 2002; O'Reilly *et al.*, 2014). The other phase dominated by SR activity reflects the retrieval of information in that CA1 pyramidal cells whose SR synapses have been strengthened, may now fire action potentials and cause downstream activation that is subsequently channeled back to neocortical areas.

To examine the role of the various hippocampal regions in memory functions, researchers have used the rat's ability in spatial navigation as a proxy for episodic memory in humans, i.e., the memory for personal events regarding time, location etc. Tests of spatial navigation have revealed the existence of place cells in the CA1 region, where CA1 pyramidal cells, through learning, become active when the rat is in a certain location in a particular environment (O'Keefe & Dostrovsky, 1971). Considering this as a learning event, the role of the SLM vs SR regions in the establishment of such place cells has been of interest. Early experiments involving lesions in either the tri- or the mono-synaptic circuit suggested an important role for the monosynaptic circuit in the establishment of place cells (Brun *et al.*, 2002; Brun *et al.*, 2008). Later experiments using acute lesions which do not allow for compensatory changes, strongly indicated the predominant role of the CA3 region in the establishment of place cells (Davoudi & Foster, 2019). Lesioning of the entorhinal input to SLM also revealed that rats were able to acquire new memories for navigation but could not

consolidate such memories (Remondes & Schuman, 2004). Additionally, research exploring the interaction between SR and SLM synaptic responses has also indicated that SLM may play an instructive, rather than a direct role, in the memory formation in the CA1 region (Dudman *et al.*, 2007).

Based on the above findings, one can envisage functional distinctions between the SR and SLM regions. Thus, during encoding, the SR synapses undergo strengthening to better mediate the learned associations between sensory events stored in the CA3 region. On the other hand, the SLM synapses signaling on-going sensory input will identify the CA1 pyramidal cells that should have their simultaneously active SR synapses strengthened for backpropagation to the relevant neocortical areas. The fact that the CA3 output represents a prediction of a complete multimodal event, rather than the event itself, implies that on-going sensory activity reaching the CA1 pyramidal cells in the SLM region enables the CA1 region to operate as a comparator. That is, it can match previously learned sensory events with actual sensory information, thereby providing a mechanism for novelty and mismatch detection (Lisman & Grace, 2005; Kumaran & Maguire, 2007a; b; Duncan *et al.*, 2009; Duncan *et al.*, 2012). The question then arises whether the various roles of the SR and SLM regions are reflected in differences in glutamate synapse function.

1.3 THE GLUTAMATE SYNAPSE

The glutamate released from the presynaptic terminal can interact with three types of ionotropic glutamate receptors, named after their specific (synthesized) agonists AMPA, NMDA and kainate, as well as with several types of metabotropic glutamate receptors. Even though all these types of receptors are present in the SLM and SR regions, my thesis has primarily focused on the AMPA receptor-mediated transmission, which constitutes an obligatory component in the glutamatergic transmission. In contrast, although activation of the other glutamate receptors can also participate in synaptic activity, they often play a modulatory role. In

many of my experiments, these receptors are therefore pharmacologically blocked.

The AMPA receptor (AMPAR) is composed of four subunits, together forming an ion channel with sites for glutamate binding. These subunits, GluA1-4, usually form heteromers, GluA1/GluA2, or GluA3/GluA2 in the adult brain (Lu *et al.*, 2009). The GluA4-containing AMPARs are predominantly expressed in the early brain, mostly within the first postnatal week (Zhu *et al.*, 2000). For the SR synapses, the neonatal GluA4-containing AMPARs are replaced by GluA1/GluA2-containing AMPARs and within the third postnatal week also with GluA3/GluA2-containing AMPARs (Blair *et al.*, 2013). This latter replacement prolongs the AMPAR-mediated response duration at these synapses. As suggested by these authors, this prolongation supports the emergence of spatial navigation after the third postnatal week. For humans, the expression of this ability appears around 2 - 3 years of age (Huttenlocher, 2008). Regarding the SLM region, nothing is known about the subunit composition of the AMPARs, assuming it follows that of the SR region.

In the adult rat brain, there is a notable distinction between the SR and SLM regions, with a higher proportion of SLM synapses being perforated and thus exhibiting two release sites (Nicholson *et al.*, 2006). For the SR synapses, the AMPAR number per synapse increases with the distance from the cell body, potentially serving as a compensation for the increasing distance from the action potential trigger zone. This compensation halts at the SR - SLM border, indicating that other mechanisms, such as extra-synaptic voltage dependent channels, contribute to boost the SLM synaptic activity (Nicholson *et al.*, 2006).

In terms of the presynaptic release of glutamate, there is a large heterogeneity in release properties among the SR synapses, both in the neonatal and adult rat (Dobrunz & Stevens, 1997; Hanse & Gustafsson, 2001a), Thus, the release probability, when activated by a single-pulse stimulation, can vary from 0.1 - 0.2 up to 0.9 among these synapses, with most of them operating within the lower range. While this variation

was at first related to a variation in the pool of ready releasable vesicles (Dobrunz & Stevens, 1997), subsequent studies attribute this variation to differences in P_{ves}, the release probability of an individual vesicle (Hanse & Gustafsson, 2001b). For the SR synapse population, the release probability shows a clear age dependence, being highest in the 1st postnatal week and thereafter decreasing during the 2nd postnatal week (Wasling *et al.*, 2004). In contrast, the earliest measure reported for the SLM synapses is from the 3rd postnatal week, indicating a substantially higher release probability than the SR synapses. However, notably, this difference has largely vanished in the adult rat (Speed & Dobrunz, 2009). Similar to the SR synapses, a large release heterogeneity in the SLM population was suggested in this study, although in an indirect manner.

The postsynaptic NMDA receptor as a coincidence detector

In many glutamate synapses, including those in the CA1 region, AMPA and NMDA receptors are co-localized in the postsynaptic density with the released glutamate binding to both these receptors. The NMDA receptor (NMDAR) channels are however largely blocked at resting levels of membrane potential by magnesium ions which may be relieved by membrane depolarization (Mayer *et al.*, 1984; Nowak *et al.*, 1984). The synaptic response to single-pulse stimulation, normally observed, is thus almost exclusively carried by currents through the AMPAR channels. In addition to the voltage dependence of NMDAR channel opening, the much higher affinity of these receptors to glutamate makes these synaptic responses, when present, markedly longer (in the hundreds of ms range) compared to the AMPAR-mediated responses. Moreover, when opened, the NMDAR channels also allow influx of calcium ions. These properties mean that the NMDAR can act as a coincidence detector for simultaneous presynaptic activity and strong postsynaptic depolarization. Upon detection of such activity, it allows for the influx of calcium ions in active synapses allowing for induction of synaptic plasticity (Gustafsson & Wigström, 1988). Like the

AMPARs, NMDARs are composed of four subunits that can be selected from more than a dozen various types of units, allowing for a wide variability of NMDAR properties (Dupuis *et al.*, 2023).

Short-term plasticity of the CA1 glutamate synapse

An important feature of synaptic transmission is its history dependence, meaning that the presynaptic release from a presynaptic action potential depends on the preceding action potentials. In this manner, the release becomes dynamic, increasing or decreasing with successive presynaptic activations, creating synapses that can convert a given presynaptic activity pattern into a separate pattern of postsynaptic activity. This presynaptic plasticity is generally in place within ranges of milliseconds to a few seconds, i.e., is short-term, and can either facilitate or depress the synaptic response. The most studied form of this plasticity is the paired-pulse (PP) plasticity in which the synaptic response to a single presynaptic action potential is compared to that evoked by a preceding presynaptic action potential, with the stimulus interval usually ranging from ~10 ms up to a few s. For each interval, the response to the 2^{nd} stimulus is divided by that evoked by the 1^{st} stimulus and used to create a PP ratio. A ratio above 1.0 signifies PP facilitation. Another measure of short-term plasticity can be obtained by using a brief train of presynaptic activations, usually 5 - 10 stimuli delivered at 20 or 50 Hz, to examine the development of either frequency facilitation or depression. These PP or train stimulations are then repeated at intervals considered long enough to avoid evoking any short-term plasticity, usually 30 - 60 s.

When examining PP plasticity in SR synapses, there is a clear age dependence in the PP ratio. While the neonatal population on average shows a PP ratio ~1.0, this ratio increases during the 2^{nd} postnatal week and thereafter becomes rather stable (Wasling *et al.*, 2004). The developmental increase in the PP ratio was found to be paralleled by a developmental reduction in release probability. This indicates that the SR synapses, as a population, transition from a neonatal state of high release probability to single-pulse activation to a lower such probability,

but to a higher sensitivity with respect to brief train (or burst) activation. PP plasticity for the SLM synapses has been examined, starting at the earliest in the 3rd postnatal week, a time when the SR synapses have already reached about adult values (Speed & Dobrunz, 2009). At this age, the SLM synapse population was also found to display a PP facilitation, but to a much smaller degree than that of the SR synapses, a difference that became much smaller with age. Thus, the SLM synapses would seem to retain a high release probability for a much longer developmental period than the SR synapses, indicating a different developmental trajectory for the SLM synapses. This late presynaptic development is noteworthy, especially when considering the early development of the SLM region.

Long-term plasticity of the adult CA1 glutamate synapse

While long-term plasticity was first described for entorhinal synapses onto dentate granule cells (Bliss & Lomo, 1973), from the 1980s onwards, the SR synapses became the preferred subject for studying long-term synaptic plasticity (> hours) in the brain. In such studies, the synapses are activated by a very low frequency (e.g., 0.05 Hz) of single-pulse stimulation to establish a stable baseline of the synaptic response. This stimulation is thereafter interrupted once or a few times, either by a brief high frequency burst of activity to induce long-term potentiation (LTP) or by prolonged (10 - 15 min) low frequency stimulation (1 Hz) to induce long-term depression (LTD). As shown in such studies, high frequency stimulation opens voltage dependent NMDAR channels, allowing for a large calcium influx that initiates processes within the synapse to strengthen it. On the other hand, the low frequency stimulation sufficiently activates the NMDARs to induce processes that reduce the synaptic response. In this manner, synapses that are simultaneously active and together can sufficiently depolarize the postsynaptic cell will be strengthened. On the other hand, synapses that are active at low frequency and out of phase with other synapses to the same postsynaptic cells become weakened. This form of conditions for LTP induction is often referred to as Hebbian induction conditions, named after the Canadian psychologist D. O. Hebb.

Controversy has long existed regarding what kind of processes are initiated to strengthen or weaken these synapses, and whether the synaptic modification is pre- or post-synaptically located, or both. However, for LTP in the adult SR synapses, evidence points to a strong involvement of Ca/calmodulin-kinase II as the calcium-target for initiating synapse strengthening and for the creation of new nanocolumns, i.e., a presynaptic release site with associated postsynaptic AMPARs (Choquet & Hosy, 2020; Yasuda *et al.*, 2022). For the SLM synapses, both NMDAR dependent LTP and LTD have been described in ≥ one-month-old rats (Colbert & Levy, 1993; Dvorak-Carbone & Schuman, 1999; Remondes & Schuman, 2002; 2003; Maurin *et al.*, 2014; Qi *et al.*, 2016).

1.4 THE AMPA LABILE GLUTAMATE SYNAPSE

A conceptual advance made in the middle of the 90s was that a glutamate synapse could be AMPA-silent, i.e., a single-pulse stimulation led to an NMDAR-mediated synaptic response (examined at a depolarized membrane potential) but failed to induce an AMPAR-mediated response (Isaac *et al.*, 1995; Liao *et al.*, 1995). Moreover, an AMPAR-mediated response emerged when the silent synapse was exposed to Hebbian induction conditions. Although the interpretation of the results was open to discussion, these results were generally perceived as indicating that glutamate synapses are born with only NMDA receptors, and that the AMPARs are subsequently inserted in an activity dependent manner. Furthermore, although these experiments were performed on slices from relatively young rats (2 - 3 weeks), and the number of AMPA silent synapses in the SR region quickly dwindles with age, un-silencing of AMPA silent synapses by insertion of AMPARs into the postsynaptic membrane has become a model for LTP expression (Kerchner & Nicoll, 2008).

Nevertheless, several results indicated that the glutamate synapse was not born without its AMPARs. For instance, when recording

spontaneous synaptic release events, there was no difference in the detection of AMPAR- and NMDAR-mediated events (Groc *et al.*, 2002). This raised the question of whether the absence of AMPAR-mediated responses during examination of stimulation-evoked responses was a result of stimulation-induced AMPA silencing. In fact, when exposing previously unstimulated (naïve) neonatal SR synapses to low frequency stimulation (0.05 - 0.1 Hz), believed not to affect the synaptic transmission, the synapses became AMPA silenced (Xiao *et al.*, 2004). When tested on slices from one-month-old rats, no such AMPA lability was observed. Later studies showed that this lability was also observed in neonatal entorhinal synapses onto granule cells in DG (Abrahamsson *et al.*, 2008), as well as for Schaffer collateral synapses onto SR interneurons (Riebe *et al.*, 2009). Interestingly, the AMPA lability in the synapses onto the interneurons was also observed in slices from adult rats, suggesting that AMPA lability might not only be restricted to the neonatal brain.

A notable feature of this AMPA lability was its frequency independence (at least in the 0.03 to 1 Hz range), indicating that its induction does not rely on the accumulation of an inducing agent. Moreover, most of the neonatal SR synapses seems susceptible to such silencing in that prolonged stimulation (2700 stimuli) can extend the 35 - 40 % depression obtained by around 100 stimuli, and the 60% depression by 900 stimuli, to an almost 80% depression of the naïve AMPAR-mediated response (Strandberg & Gustafsson, 2024).

It should be noted that neither NMDARs nor metabotropic glutamate receptors (mGluRs) are needed to induce this depression of the AMPAR-mediated response, even though they may affect its magnitude. An in vitro slice experiment where AMPA was applied to the bath solution was also found to result in a depression of the AMPAR-mediated response occluding the effect by low frequency stimulation (Wasling *et al.*, 2012). The working hypothesis arising from these findings was that the AMPA lability is associated with the presence of GluA4-containing AMPARs in the neonatal CA1 SR region, these

AMPARs unphosphorylated in a serine 842 site, and being removed from the postsynaptic site by glutamate binding alone (Abrahamsson *et al.*, 2008). The 842 site can be phosphorylated by protein kinas A (PKA), and the finding that forskolin, a PKA activator, can rescue the AMPAR-mediated synaptic response from this activity dependent depression provides further support of this hypothesis (Abrahamsson *et al.*, 2008).

AMPA un-silencing and developmental LTP

When induced by a relatively small number of stimuli (100), the depression of the AMPAR-mediated synaptic response reverses within 20 - 30 min by stimulus interruption alone (Abrahamsson *et al.*, 2007). However, an extended stimulation period can result in depression that does not recover within 30 - 60 min, i.e., is an LTD (Strandberg & Gustafsson, 2011). Another way to reverse the AMPAR-mediated response is through Hebbian activity, a stimulation form that was demonstrated earlier to un-silence AMPA-silent synapses (Liao *et al.*, 1995). However, in such experiments, Hebbian activity should only restore the synaptic response to the level before any stimulation, referred to as the naïve level. In addition, if Hebbian activity is given at this naïve level, no LTP should be observed. Both predictions were borne out (Abrahamsson *et al.*, 2008). Since this form of LTP only represents an un-silencing of the previously silenced synapses it has been referred to as developmental LTP and was found to be the only form of LTP for the CA1 SR synapses up to ~postnatal day 12 (Abrahamsson *et al.*, 2008). Thereafter, throughout the 1st postnatal month, Hebbian activity leads to greater amounts of LTP overshooting the naïve level, this signifies an LTP not based on a postsynaptic un-silencing.

Early experiments indicated that Hebbian activity not only unsilenced the synapses but also rendered them AMPA-stable (Xiao *et al.*, 2004; Abrahamsson *et al.*, 2008). Later experiments have, however, shown that this stabilization is only temporary. Prolonged low frequency stimulation (>1 - 2 hours) was found to reduce the AMPAR-mediated

synaptic response to levels similar to synapses that were not exposed to Hebbian activity (Strandberg & Gustafsson, 2024).

During this neonatal period, there is an intense synaptogenesis and an activity dependent selection of these synapses. A developmental form of LTP based on an activity dependent selection of synapses such that only those synapses consistently active alongside the other synapses will retain their AMPARs, and thus avoid elimination. This may be an effective and dynamic manner to establish an activity dependent circuit organization (Groc *et al.*, 2002).

For the SLM synapses, no data exists for either neonatal AMPA lability or neonatal LTP. Considering the early morphological maturation of the SLM region, one could predict, based on the reasoning above, that AMPA lability and developmental LTP would be much less expressed in the SLM compared to the SR region.

2 AIM

The overall aim of my thesis project was to examine to what extent the glutamate synapses in two functionally distinct regions in the CA1 region of the hippocampus differ in their properties. Specifically, the thesis will examine if labile glutamate transmission, a form of neonatal synaptic plasticity earlier observed in the SR regions, is a feature of both regions and exhibits similar developmentally regulation.

Specific aims

i) To explore whether the postsynaptically labile AMPA signalling differs neonatally between glutamate synapses in the early- and late-maturing region.

ii) To explore differences in presynaptic signalling between the neonatal glutamate synapses in these two regions.

iii) To explore how the postsynaptically labile AMPA signalling has altered during the functional maturation of the hippocampus.

iv) To explore how the presynaptic signalling has altered in these two different regions during the functional maturation of the hippocampus.

3 METHODOLOGICAL CONSIDERATIONS

The experimental details used in my thesis are described in the articles.

3.1 AGE CONSIDERATIONS

My experiments were performed on the hippocampus using Wistar rats, either 8 to 12-day-old (either sex) or one to two-month-old (male). These age groups were chosen because they represent two different stages of hippocampal function. As noted in the Introduction, in the 2nd postnatal week little external sensory input reaches the hippocampus because of, e.g. closed eyelids and plugged auditory canals. Hippocampal activity is instead dominated by spontaneous activity from the sensory organs as well as from within the hippocampus itself and is associated with the early organization of the hippocampal circuits. In one to two-month-old rats, the hippocampus is functionally mature (Blair *et al.*, 2013; Albani *et al.*, 2014). The synapses from these older rats will therefore be referred to as adults in this thesis, even though the one-month-old rats are no more than adolescents (see below).

The rat is considered as a 'postnatal brain developer', so that at birth the rat brain corresponds to that of a half-term human fetus (Hagberg *et al.*, 2002) At the end of the 2nd postnatal week, the rat cerebral cortex is comparable to that of the newborn human baby (Romijn *et al.*, 1991), this implies that the neonatal (2nd postnatal week) rats in this thesis correspond to a human fetus in the last trimester. A one-month-old rat has reached puberty, while the complete development of the CNS takes an additional 2 - 3 months. The one-month-old rats in this study correspond to that of humans in early adulthood. Hence, rats experience greater CNS immaturity at birth compared to humans but have an accelerated postnatal development, a major part of which is compressed within the 1st postnatal month.

3.2 HIPPOCAMPAL SLICE PREPARATION

My thesis work was performed in acute in vitro slices of the CA1 region of the dorsal hippocampus, a brain region involved in episodic memories

and spatial navigation. This slice preparation is commonly used for the kind of electrophysiological study of synaptic transmission that I have performed because of its well-organized anatomical structure with a single pyramidal cell body layer, apical dendrites projecting at right angles from the cell body layer, and the synapses to be examined are well separated in the proximal vs distal part, respectively, of the apical dendrite. This organization thus provides easy access for stimulation and recording of synaptic activity. The slice preparation also offers possibilities for altering the extracellular environment and for application of various drugs e.g. to isolate the form of synaptic transmission to be studied. The disadvantage is of course that the hippocampal slice is severed from its normal environment and often kept at non-physiological extracellular environments and temperature (in my case ~30°C).

The bulk of my experiments were performed in the distal layer of the CA1 region (SLM region), mostly containing axons (and synapses) from layer III of the entorhinal cortex, the so called temporo-ammonic (TA) pathway. These synapses have historically been much less studied than the Schaffer collateral synapses from the CA3 region, possibly because of their distal location from the cell body layer. Another possible reason may be that while stimulation in the SR region is thought to only activate the Schaffer collateral synapses, other axons than those of the TA pathway may be present in the SLM, such as axons from nucleus reuniens (a midline thalamic nucleus). However, only a minor fraction of this thalamic pathway projects to the dorsal hippocampus which is the part of the hippocampus I have studied, most of it going to the ventral hippocampus (Vertes *et al.*, 2007). Nevertheless, in my thesis work, I have referred to the activated synapses as SLM synapses, rather than TA synapses, but it is likely that most of the glutamatergic transmission I have studied arise from TA synapses.

3.3 FIELD EPSP RECORDINGS

In my thesis work, I examined synaptic transmission using extracellular field recordings which represent the synaptic actions from a large population of synapses (Fig. 2). There are several advantages to this type of recording. It is a non-invasive technique versus the neurons themselves, thus avoiding wash-out effects disturbing the processes you want to study. Being a population response, the variability in successive synaptic responses is low. By simultaneously recording the extracellular action potential of the activated axons (presynaptic volley), one can ascertain the stability in the number of activated presynaptic axons. The disadvantage is that an analysis of synaptic transmission on the quantal level, i.e., whether the processes studied are due to changes in vesicle pool, in the release probability of individual vesicles (p_{ves}), or in release site number, is impossible and must be inferred in indirect manners.

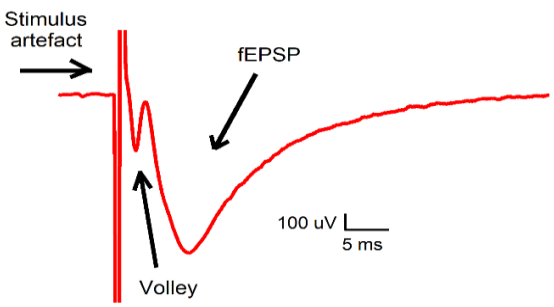

Figure 2. Example of a field EPSP from single-pulse stimuli (n = 10 sweeps)

To ascertain that the synaptic response only represents the AMPAR-mediated component of the transmission both $GABA_A$ and $GABA_B$ receptors were routinely blocked by picrotoxin (100 μM) and CGP (1 μM). The spontaneous activity that might arise due to such a blockade of inhibitory transmission was prevented by raising the extracellular concentrations of divalent cations (calcium and magnesium) to higher-than-normal levels. By concurrently increasing both calcium and magnesium, the release-enhancing effect of the higher calcium is at least partly counteracted by the higher magnesium concentration. Moreover,

these higher concentrations of divalent cations increased action potential thresholds and thereby also helped to avoid the contamination of population spike activity on evoked fEPSPs. When brief high-frequency trains (Fig. 3) were used as test stimuli, NMDA and mGlu receptor antagonists were always present in the perfusion solution to ascertain that the synaptic response during the train did not contain components related to the activation of these receptors, as well as of components arising because of synaptic plasticity induced by the activation of these receptors.

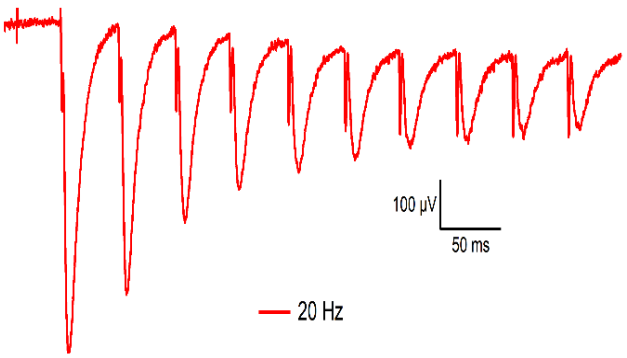

Figure 3. Example of field EPSPs during a high-frequency train stimulation (n =10 sweeps).

As the stimulation strength was adjusted to a level that the fEPSPs produced were below the threshold for exhibiting signs of population spike activity, the fEPSP magnitude was estimated from its mean amplitude during a 2-ms window around the negative peak (see Figure 4). This amplitude measurement was preferred since it gave less noisy measurements than estimation of the fEPSP magnitude from its initial slope (see Fig. 4). In the LTP induction experiments (**Paper III**, Fig. 5) where the fEPSP was expected to become enhanced, I also measured the initial slope of the fEPSP and had the same result as with the amplitude measurements (Figure 4). When estimating the magnitude of the successive fEPSPs during a brief train activation (Fig. 3), amplitude measurements were also made. In some of these cases (n = 10), I also

made initial slope measurements, these measures giving the same ratio between the last fEPSPs and the 1st fEPSPs in the train ($fEPSP_{8-10}/fEPSP_1$) as that obtained using the amplitude measurements (unpublished data).

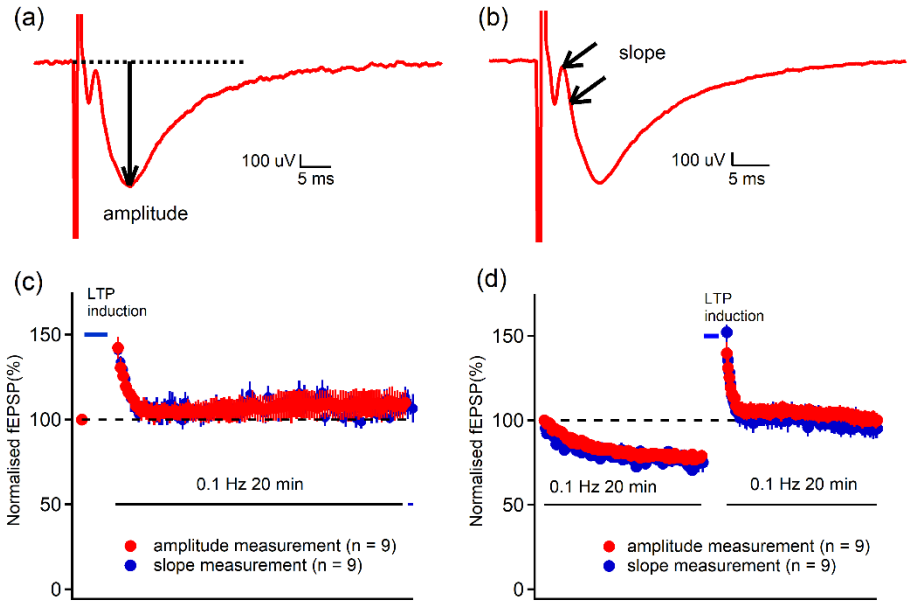

*Figure 4. LTP experiments using both amplitude and slope measurements (adapted from **Paper III**, Fig. 5).*

3.4 STIMULATION CONDITIONS

A very important aspect of my thesis project was to examine synaptic transmission from a naïve level, i.e., from a level at which none of the glutamate synapses were AMPA-silent. After positioning the stimulating and recording electrodes, the slice was allowed to rest in the recording chamber for 30 - 60 min after its transferal from the incubation chamber (~26°C) to the recording solution (~30°C) before any stimulation. The duration of this resting period relied on initial experiments in which I applied single-pulse stimulation every 5 min to test how long it took for a slice to adapt to the recording situation, i.e.,

for the stimulation to result in a stable fEPSP. This stabile fEPSP was taken to reflect the naïve level for the synapses. To test the stability of the naïve level, in some experiments (n = 7) I kept a separate input and recorded a single-pulse-induced fEPSP at the beginning of the experiment. I thereafter left this input non-stimulated until the end of the experiment. At that time, I evoked another single-pulse-induced fEPSP for comparison with that evoked at the beginning of the experiment, this fEPSP being 101 ± 1 % of that of the initial fEPSP.

In my study of the synaptic plasticity of the SLM synapses, I exposed these synapses to a prolonged single-pulse stimulation at low frequencies (0.05 - 1 Hz) as well as to brief high frequency (20 Hz) trains to induce various forms of plasticity. The question arises to what extent such stimulation protocols are relevant with respect to the natural firing of the entorhinal cells whose axonal projections I activate with my stimulation electrode. In fact, in vivo recording of the action potential activity of entorhinal cortex cells projecting to the SLM during running for food indicated a wide frequency range peaking around 10 Hz, but also extending to 1 Hz and below (Frank *et al.*, 2001), compatible with the presently used stimulation frequencies.

3.5 SHORT-TERM PLASTICITY AS AN ANALYTICAL TOOL

A well-established tool to examine changes in the release properties of synapses is to examine changes in the short-term plasticity, such as the PP ratio (see Introduction, Short-term plasticity of the CA1 glutamate synapse). Specifically, manipulations of synaptic transmission that cause changes in this PP ratio are generally thought to reflect changes on the presynaptic rather than the postsynaptic side of the synapse. For example, when applying drugs, or altering extracellular concentrations of calcium and magnesium ions, to reduce the release probability, there is an almost linear relation between the decrease of the 1st fEPSP in the PP stimulation and the increase of the PP ratio (**Paper II**, Fig. 6b). In my thesis, I have used this relation to estimate changes in a single-pulse-

induced fEPSP based on changes in the PP ratio (**Paper II,** 3.8; see also Discussion, facilitation - unmasking.

This relationship between changes in PP ratio and presynaptic release has led authors to directly refer actions of neuromodulators not resulting in PP ratio changes as being postsynaptic (Otmakhova *et al.*, 2005). However, release probability can be altered in manners that may not result in changes of this ratio (Hanse & Gustafsson, 2001b; Burke *et al.*, 2018) indicating that this measure of release probability must be used with some caution. Moreover, in a population of synapses in which the release probability, and thus PP ratio, can vary among the synapses, any synaptic alteration that results in a selective effect on high, or low, release probability synapses will result in changes in the PP ratio.

3.6 NEUROMODULATORY EFFECTS

In addition to glutamate release from axons from the entorhinal cortex, the SLM layer is also targeted by axons from subcortical structures (Rosen *et al.*, 2015). These axons might be activated by the stimulation currents and thus release neuromodulators such as dopamine, noradrenaline etc. Application of such neuromodulators have been found to have effects on the glutamatergic transmission causing e.g. presynaptic inhibition of release via metabotropic receptor activation (Otmakhova & Lisman, 1999; 2000; Otmakhova *et al.*, 2005). To ascertain that the AMPA lability was not influenced by such heterosynaptic modulation, instead of using a homosynaptic PP stimulation (1 s interval). I activated two separate inputs on either side of the recording electrode with such a 1 s interval, once per minute (**Paper I,** Experimental procedures; Recordings and analysis). The absence of any such heterosynaptic depression compared to the depression of ~20 % using the homosynaptic PP stimulation speaks for the homosynaptic nature of this depression. I also compared the depression evoked by a prolonged 1 Hz stimulation using either a single input or two separate inputs (stimulated 0.5 Hz apart) and observed no significant difference in the depression (**Paper I,** Fig. 3c3). If stimulation and release of neuromodulators were important for this

depression, one might expect that a larger number of such axons likely activated using two rather than a single input might be expected to result in a greater depression. However, while these experiments indicate that the depression induced by such low frequency stimulation is homosynaptic, they do not exclude the possibility that different stimulation parameters, such as when a brief high frequency train is used, might not be more conducive to neuromodulator release (Delaney *et al.*, 2007). This possibility will be taken up in the Discussion section.

3.7 ETHICAL CONSIDERATIONS

The experiments in this thesis were performed in vitro, using acute hippocampal slices from Wistar rats, and were approved by the Gothenburg Ethics Committee for animal research. The animals were transferred from the animal center and were kept in the lab for 30 min in a quiet environment. They were thereafter decapitated by using a guillotine under isoflurane anesthesia, the brain was then removed and cut into slices.

To minimize the stress of the animals before being used in research, the rats were kept in spacious, clean, and relatively enriched environments, usually with a tunnel and shelters in the cage with free access to food and water to suit their natural behaviors. The lighting, temperature, and noise levels were maintained stable. Social housing was also considered for their well-being, the rats were kept together with their mothers until they were one month old. After that they were caged same-sex pairs or in small groups.

To minimize the number of rats used for the experiments, I planned the experiments meticulously to maximize the use of the slices. Generally, a few different experimental protocols were performed on the hippocampal slices from the same rat.

4 SUMMARY OF RESULTS

4.1 Single-pulse-induced axonal and synaptic activations

As mentioned in the Introduction, the SLM region matures morphologically earlier than the SR region, with the dendritic branches reaching adult values within the 2nd postnatal week in SLM as compared to after 4-8 weeks for the SR region (Pokorný & Yamamoto, 1981). This developmental difference agrees with my observation that the width of the SLM layer is unaltered from the 2nd postnatal week to the end of the 2nd month, while that of the SR layer keeps expanding until the end of the 1st month (**Paper III,** Fig, 7a, b, c). During this time, there is a shift from the SLM to the SR layer as being the dominant dendritic layer. From the 2nd postnatal week to the end of the 1st postnatal month, there is also a large decrease within the SR layer in the stimulation current (22 to 13 µA) needed to produce a naïve fEPSP of ~0,5 mV in peak amplitude with a proportional decrease in volley amplitude (0.17 - 0.09 mV), implicating an increase in the fEPSP/volley ratio (5.4 vs 8.0). In contrast, the currents and presynaptic volleys required to induce fEPSPs of this amplitude in the SLM layer grew larger with time (28 to 46 µA, and 0.22 to 0.51 mV, respectively), implicating a substantial decrease in the fEPSP/volley ratio (3.2 to 1.3) (**Paper III**, Fig. 7e). The synaptic machinery in these two regions thus moves in diametrically different directions during this time.

In further contrast to the SR synapses (Blair *et al.*, 2013), the fEPSP in the SLM region did not display any change in the fEPSP half-width in the latter half of the 1st postnatal month (**Paper III**, Fig. 7d). This shift for the SR synapses was explained by a change from GluA1 to GluA3 subunits in the AMPAR receptors, a subunit shifts thus not likely occurring at the SLM synapses.

4.2 Postsynaptic labile AMPA signaling in neonatal SLM synapses

If the SLM region has matured earlier than the SR region, would the SLM synapses then show less of the postsynaptic AMPA lability presumed to be important for an early activity-dependent organization of a neuronal circuit? I thus first exposed naïve 2nd week SLM synapses to low frequency stimulation (0.2 Hz) that at 2nd week SR synapses results in a fEPSP depression of 37% after ~100 stimulations (Abrahamsson *et al.*, 2007). For the naïve SLM synapses, this stimulation resulted in a fEPSP depression of 44% (**Paper I**, Fig. 2a) which thus exceeded that previously found for the SR synapses as well as significantly exceeded that for the SR synapses (~31%) examined in this thesis (**Paper I**, Fig. 2b).

The low-frequency-induced depression of the neonatal SR synapses depends on the number of stimuli but not on their frequency, at least not within a 0. 03 - 1 Hz range (Abrahamsson *et al.*, 2007; Strandberg *et al.*, 2009). Such frequency independence was also observed for SR synapses examined in this thesis (**Paper I**, Fig. 3b1). In contrast, I found that the low-frequency-induced depression of the neonatal SLM synapses varied with the frequency such that it incrementally increased in magnitude when the frequency was raised from 0.05 Hz to 1 Hz. In contrast, reducing the stimulation frequency to 0.033 Hz failed to further decrease the depression (**Paper I**, Fig. 3a1). These results suggest that the neonatal SLM synapses may also become postsynaptically AMPA silent but that an additional form of AMPA lability is added to these synapses at frequencies above 0.05 Hz.

For the neonatal SR synapses, the low-frequency-induced depression was facilitated by NMDAR activation, i.e., reduced by the NMDAR antagonist *D*-AP5, while being slightly facilitated by the broad-spectrum mGluR antagonist LY-341495. When tested on the neonatal SLM synapses, I found that *D*-AP5 reduced the depression in the 0.05 - 1 Hz range, the effect being most pronounced at the lower frequencies. Thus,

while almost half the 0.05 Hz-induced depression was removed by the NMDAR antagonist, three quarters of the 1 Hz-induced depression remained (**Paper I**, Fig. 5a). On the other hand, LY-341495 application gave no, or only a small non-significant, facilitation of the depression (**Paper I**, Fig. 5b). These results support a contribution of a postsynaptic AMPA lability in the neonatal SLM synapses like that of the neonatal SR synapses.

For the low-frequency-induced depression of the neonatal SR synapses, its longevity had been probed by stimulus interruption followed by application of single-pulse stimulations at various times after the end of the stimulation (Abrahamsson *et al.*, 2007; Strandberg & Gustafsson, 2011). Depending on the induction conditions, such studies had shown that the low-frequency-induced depression could result in an LTD component, i.e., a depression still present at 30 min after the stimulation interruption, but also to a component that reverses over a period of 20 - 30 min. When testing for recovery from the low-frequency-induced depression for the neonatal SR synapses I found that 85 % of the initial depression remained at 3 min post-stimulation, the depression thereafter slowly reversing within 30 min (**Paper I**, Fig. 6a). In contrast, for the neonatal SLM synapses only one quarter of the induced depression remained at 3 min post-stimulation. However, after an additional 5 min ~one-fifth of the depression remained indicating the presence of a small component that took an additional period of 30 min to reverse (**Paper I**, Fig. 6b). These results thus support the presence of a low-frequency-induced depression in the neonatal SLM synapses similar to but clearly smaller than that explained by a postsynaptic AMPA silencing in the neonatal SR synapses.

Bath application of AMPA has been found to induce a depression that occludes the low-frequency-induced depression of neonatal SR synapses, and that is occluded by a prior such stimulation (Wasling *et al.*, 2012). I found that while AMPA application resulted in a depression of the neonatal SR synapses to ~two thirds of the naïve level when stimulation was resumed 10 minutes after AMPA application (**Paper I**,

Fig. 7b), the corresponding depression of the neonatal SLM synapses was only ~one-third of that (**Paper I**, Fig. 7a). Moreover, while the ensuing 0.2 stimulation resulted in no further depression of the SR synapses, this stimulation led to a large additional depression of the SLM synapses.

It would appear from the results above, that the neonatal SLM synapses exhibit the form of activity dependent AMPA lability that has previously been described for the neonatal SR synapses and has been explained by a postsynaptic AMPA silencing (Xiao *et al.*, 2004). However, compared to the neonatal SR synapses, this depression process seems substantially less expressed in the neonatal SLM synapses.

4.3 Presynaptic labile AMPA signaling in neonatal SLM synapses

Inspection of the development of synaptic depression, when using stimulation trains at frequencies within the 0.05 to 1 Hz range revealed a large initial drop in the synaptic response from the 1st to the 2nd fEPSP in the train. This initial drop was larger the higher the frequency. Moreover, the higher frequency was associated with a larger final depression **(Paper I,** Fig. 3a). This result would suggest that each stimulation is followed by a synaptic depression lasting up to ~15 - 20 s that summates with each successive stimulation. To look for evidence for such a depression, I used a paired-pulse (PP) protocol with PP intervals varying from 20 s to 50 ms and with each PP stimulation delivered 1 min apart. For the SLM synapses, such a depression was indeed found, having a maximum at a 1s PP interval, and no longer observed at a 20s PP interval (**Paper II**, Fig. 1a). In contrast, the same protocol at SR synapses resulted in a PP facilitation that was largely complete within 1 s (**Paper II**, Fig. 1b). Studies of perforant path synapses onto granule cells in the dentate gyrus have shown the presence of a depression resembling that described above for the SLM synapses and explained as presynaptic depression (White *et al.*, 1979; Abrahamsson *et al.*, 2005). In agreement with results obtained from

these other synapses, I found that the NMDAR-mediated fEPSP also displayed a PP depression with about the same time course as that of the AMPAR-mediated fEPSP (**Paper II**, Fig. 9a). I will therefore refer to this depression as presynaptic depression in the following sections.

Since activation of a separate set of afferents did not induce depression in a test input (**Paper I**, Experimental Procedures; Recording and analysis), the depression appeared to be intrinsic to the synapse itself. Nonetheless I tested various receptor antagonists to exclude possible local heterosynaptic effects, or heterosynaptic effect arising during a prolonged low-frequency stimulation. Since these experiments were performed in the presence of GABAA, GABAB, and NMDA receptor antagonists, such receptor activations were directly excluded. As shown by the lack of effect of the broad spectrum mGlu receptor antagonist LY341495 (**Paper I**, Fig. 5b), mGluR activation seems not involved. Neither were kainate, adenosine A1 and endocannabinoid receptors found to be involved (**Paper II**, 3.12).

To examine in what manner the SLM synapses become presynaptically labile to stimulation in the low frequency range (0.2 and 0.1 Hz), I first looked at the effect not only on the synaptic response to a single-pulse stimulation but also to a brief train (10 impulses at 20 Hz). This test revealed that the depression preferentially acted on the 1st fEPSP in the train while the later responses were virtually unaffected (**Paper II**, Fig. 2a). The PP_{50} ratio (estimated from the first two fEPSPs in the 20 Hz train) was found to be increased, a result compatible with a decrease in P_{ves} and often explained by a reduced calcium influx. However, I found that altering P_{ves} by reducing calcium influx through changes in the extracellular calcium: magnesium (Ca: Mg) ratio, or by applying the adenosine A1 receptor antagonist CHA, altered the train response in a distinctly different manner from that of the presynaptic depression (**Paper II,** Fig. 3). Specifically, the later part of the synaptic response was not unaffected but instead facilitated by these procedures. This result suggests that a lowered presynaptic calcium influx does not explain the presynaptic depression.

The presynaptic depression at the perforant path synapses onto dentate granule cells was tentatively ascribed to vesicle depletion (Abrahamsson *et al.*, 2005). I tested for such an explanation by examining PP plasticity, as well as the low frequency-induced depression, at several different levels of release probability by using different Ca: Mg ratios in the perfusion solution. While this variation in Ca: Mg ratio altered the early PP plasticity, it did not alter depression at PP intervals of 1s or more (**Paper II**, Fig. 4a). Moreover, this variation failed to result in any depression at PP intervals of 1s or more for the SR synapses (**Paper II**, Fig. 4c). Such release independence of the presynaptic depression for the SLM synapses, coupled with a lack of such depression for the SR synapses even at high release levels, suggests that vesicle depletion cannot be the underlying mechanism.

Inspired by anecdotal data from work on the perforant path synapse onto dentate granule cells (White *et al.*, 1979) showing that a large early PP facilitation was associated with the absence of presynaptic depression, I plotted the values for early PP plasticity (PP_{50}) against those for the late PP plasticity (PP_{1s}) obtained in each experiment. This plot indicated a strong correlation between these PP values (**Paper II**, Fig. 5c). As a variation in PP_{50} does not result in a variation in presynaptic depression (**Paper II**, Fig. 4a), the above correlation would therefore rather suggest that the amount of presynaptic depression alters the early PP plasticity. If so, the presynaptic depression is not a late depression but should have a quick onset and be fully developed earlier than 50 ms after the stimulation of the synapse.

Building on this notion of a quick onset of the presynaptic depression and its accumulation with successive synaptic activations, I subsequently showed (**Paper II**, Fig. 10) how an activity dependent but release independent depression process could explain how the presynaptic depression affected the synaptic response to a brief train stimulation. That is, it explained both the change in PP_{50}, indicative of a P_{ves} change, as well as the lack of effect on the synaptic response in the

later part of the train. A release site inactivation was suggested as a candidate for such a depression process.

4.4 Short- and long-term plasticity of neonatal SLM synapses

While the neonatal SR synapses already from the end of the 1st postnatal week demonstrate an early PP facilitation that continues to grow over the next two weeks (Wasling *et al.*, 2004), I found the early PP plasticity of the SLM synapses to be depressing throughout the 2nd postnatal week (**Paper II**, Fig. 5a). Likewise, while the SR synapses, on average, were neither depressed nor facilitated when activated by a brief high frequency train, the SLM synapses were strongly depressing (**Paper II**, Fig. 2). However, based on a fast onset of the presynaptic depression process, my data suggest that much, but not all, of this difference between the SR and SLM synapses can be accounted for by the presence of this presynaptic depression in the neonatal SLM synapses while it is absent in the neonatal SR synapses (**Paper II**, Figs. 5e and Fig. 7c).

When a PP or a brief train stimulation was used to induce presynaptic depression, the additional stimulations added a facilitation based on an increased P_{ves} that masked the presynaptic depression as measured 1 s after the conditioning stimulation. Thus, I could calculate that while a prolonged 1 Hz stimulation seemingly induced a presynaptic depression of ~30%, the actual magnitude of the presynaptic depression process was appreciably larger, ~45% (**Paper II**, 3.10).

To examine the ability of the neonatal SLM synapses to undergo LTD after a prolonged low frequency stimulation, I adopted the procedure to estimate the magnitude of LTD as the amount of fEPSP depression remaining after a 30 min stimulus interruption following the stimulation (Strandberg & Gustafsson, 2011). I found that, as for the neonatal SR synapses, both 0.2 and 1 Hz stimulation resulted in LTD. This LTD was however only about half of that observed for the SR synapses (**Paper I**, Fig. 8). When I examined 3rd postnatal week SLM synapses, i.e., during

a time when LTD is often studied in SR synapses, no LTD was observed for these synapses (**Paper** III, Fig. 6).

In the 2nd postnatal week, no LTP is observed at the SR synapses unless the LTP induction was preceded by a very low frequency stimulation resulting in postsynaptic AMPA silencing (Abrahamsson *et al.*, 2008). This LTP thus represents an unsilencing of the recently silenced AMPA receptors and was referred to as a developmental LTP. When applying such a tetanization protocol to 2nd postnatal week SLM synapses, no such developmental LTP was observed (**Paper III**, Fig. 5e).

4.5 Postsynaptic labile AMPA signaling in adult SLM synapses

For the SR synapses, the labile AMPA signaling seems mostly to be a neonatal phenomenon, with low-frequency stimulation resulting in little (Abrahamsson *et al.*, 2007) or no (Wasling *et al.*, 2012) fEPSP depression when given in slices from one-month-old rats. When I applied low-frequency stimulation to SLM synapses in slices from one-month-old rats, a substantial fEPSP depression was observed (**Paper III**, Fig. 1a). As for the neonatal SLM synapses, this depression was frequency dependent, being greater using 0.2 than 0.05 Hz stimulation. This result indicates the presence in the mature SLM synapses of both forms of low-frequency-induced depression observed in the neonatal SLM synapses.

The depression induced by the 0.05 Hz stimulation of the adult SLM synapses reversed quite slowly with ~one-third of the depression remaining 5 min after stimulus interruption. This depression was significantly greater than that observed for the neonatal SLM synapses. Since for the adult SR synapses the low-frequency-induced depression had disappeared in parallel with the disappearance of bath-applied AMPA-induced depression (Wasling *et al.*, 2012), I tested such bath application to the adult SLM synapses. The depression that occurred in these adult SLM synapses was almost 5 times greater than that I had observed in the neonatal SLM synapses (**Paper III**, Fig. 3a). This

depression in the adult SLM synapses was also partly occluded when the AMPA application was preceded by stimulation inducing low-frequency-induced depression (**Paper III**, Fig. 3b).

For the neonatal SLM synapses, brief train stimulation at a low repetition rate (1/min) resulted in an accumulating depression that I attributed to postsynaptic AMPA silencing (**Paper II**, Fig. 8b). Such depression was also observed for the adult SLM synapses (**Paper III**, Figs. 8c) and had a magnitude ~twice that observed for the neonatal synapses. Likewise, when I used a conventional LTD induction protocol (900 stimuli at 1 Hz) during NMDAR blockade, the resulting prolonged depression of the adult SLM synapses was even greater than that observed in the neonatal SR synapses (**Paper III**, Fig. 2b). These results suggest that, in contrast to the SR synapses, the postsynaptic lability of AMPA signaling not only remains for the SLM synapses but is also enhanced during the functional maturation of the hippocampus.

4.6 Presynaptic labile signaling in adult SLM synapses

The larger depression induced by 0.2 than by 0.05 Hz stimulation showed that presynaptic depression remained in the adult SLM synapses. As for the neonatal synapses, this depression preferentially affected the 1st fEPSP in a brief train stimulation leaving the later fEPSPs unaffected (**Paper III**, Fig. 9b). However, compared with the neonatal SLM synapses, the presynaptic depression was smaller in the adult SLM synapses, being ~half the magnitude of that observed in the neonatal synapses (**Paper III**, Fig. 9a). Like the neonatal synapses, the presynaptic depression in the adult synapses became larger when applying a PP than a single conditioning stimulus, i.e., it accumulates with repetitive activation.

4.7 Developmental changes in short- and long-term plasticity

From the PP_{50} depression observed in the neonatal SLM synapses, this short-term plasticity was shifted to a PP_{50} facilitation in the adult synapses (**Paper III**, Fig. 9b). As for the neonatal synapses, there was a strong correlation between the PP_{50} and the PP_{1s} ratios in the adult synapses (**Paper III**, Fig. 9c), but with a smaller effect of the presynaptic depression on the PP_{50} values in line with the smaller presynaptic depression in the adult synapses. While the smaller presynaptic depression in the adult synapses partly contributed to the shift from depression to facilitation, a major part of it was found to be unrelated to this factor. I also examined neonatal and adult SR synapses with respect to their PP_{50} values and found a quantitatively similar developmental shift for these synapses as for the SLM synapses when the effect of the presynaptic depression on the PP_{50} values was removed.

The profound frequency depression during brief high frequency activation found in the neonatal SLM synapses was considerably less pronounced in the adult SLM synapses (Paper **III**, Fig. 12). Again, the reduction in presynaptic depression was found to contribute to this smaller frequency depression but only to a minor part of it. The adult SR synapses showed on average a strong facilitation during such train activation, i.e., the $fEPSP_{8\text{-}10}/fEPSP_1$ ratio was well over 1(Paper **III**, Fig. 12). As for the early PP plasticity, the percentual change in this ratio when comparing the neonatal and adult synapses was quite similar for the SLM and SR synapses when the effect of the presynaptic depression was removed.

Thus, apart from the influence of the presynaptic depression on early short-term synaptic plasticity, my results suggest that short-term plasticity is affected in much the same manner for the SLM and SR synapses during hippocampal maturation.

A notable contrast between the neonatal and the adult SLM synapses was the virtual absence in the adult synapses of a presynaptic depression at 1s after a PP_{50} stimulation (**Paper III**, Fig. 10a, b). Based on the change in PP_{50} ratio that was induced 1 s following the conditioning PP stimulation I could estimate that the facilitation given by this PP stimulation would be almost three times that observed for the neonatal SLM synapses, and thus could fully mask the presynaptic depression.

In contrast to the neonatal SLM synapses, no lasting depression could be induced in the adult SLM synapses despite the development of a strong depression during the on-going stimulation (**Paper III**, Fig. 6b, c). Thus, even though the postsynaptic AMPA silencing is larger at the adult than at the neonatal synapses, it does not translate into an LTD. I also observed that the depression induced during the stimulation (**Paper III**, Fig. 6a), as well as the recovery from the depression (**Paper III**, Fig. 6c), was quite unaffected by the presence/absence of NMDAR activation.

For the SR synapses, the neonatal developmental LTP which was explained by an unsilencing of recently AMPA-silenced synapses (Abrahamsson *et al.*, 2008), is to an increasing extent replaced by an LTP overshooting the naïve fEPSP level during the remaining part of the 1st postnatal month (Abrahamsson *et al.*, 2008). I found that adult SLM synapses, on average, did not display any significant LTP (**Paper III**, Fig. 5a) unless pre-stimulated to produce low-frequency-induced depression (**Paper III**, Fig. 5b). In fact, the LTP of the adult SLM synapses seemed quite like that of the neonatal SR synapses.

In conformity with the neonatal SR synapses, the LTP-inducing stimulation to the adult SLM synapses also resulted in a potentiation component above the naïve level. This component was not stable but decayed with the subsequent low frequency stimulation. (**Paper III**, Fig. 5).

There was a recurring theme in the experiments on the adult SLM synapses that the fEPSP increased above the naïve level following recovery from depression induced by low frequency stimulation (**Paper**

III, Figs. 2, 6) or by AMPA application (**Paper III**, Fig. 4). This potentiation was associated with a proportional increase in the presynaptic depression, indicative of the addition of a new set of synapses from the activated axons. Like the potentiation following LTP-inducing stimulation from the naïve level, this potentiation was not stable but decayed with low frequency stimulation.

5 DISCUSSION

My study on the glutamate synapses in the SLM layer of the hippocampal CA1 region has generated two main new insights into labile glutamate transmission. First, the postsynaptic form of the lability may even be enhanced with age for some synapses, seemingly playing a larger role in the adult hippocampus than during early development. Second, the presynaptic form of the lability may be explained by activity-dependent release site inactivation, a depression mechanism not previously suggested for a cortical synapse.

5.1 POSTSYNAPTIC LABILE TRANSMISSION

My discovery of postsynaptic lability of the SLM synapses in the adult hippocampus should be considered in the context of the role of such lability proposed earlier (Hanse *et al.*, 2009). That is, by having such lability, a synapse must continuously participate in Hebbian activity to maintain its AMPA signaling. Any synaptic activity outside this context may lead to AMPA silencing and potential elimination. Such lability could be crucial for building up and refining the synaptic circuitry during development, i.e., in creating topographic maps of the sensory environment. AMPA lability in the adult brain was previously observed for Schaffer collateral synapses onto interneurons (Riebe *et al.*, 2009), suggesting that such lability in the adult brain might be relevant for synapses in circuits constantly requiring some form of activity dependent reorganization. I will argue below (see Functional considerations) that the postsynaptic lability in the SLM region may also serve a similar role.

My initial rationale for examining SLM synapses for postsynaptic lability was driven by the early maturation of the SLM region compared to that of the SR region. This difference allowed me to examine the possible causal connection between postsynaptic AMPA lability and developmental circuit organization. My finding of a much smaller degree of postsynaptic lability in SLM than in SR in the 2nd postnatal week can thus be considered supportive of this idea. Implicit in this

reasoning is a higher degree of postsynaptic lability in SLM during the 1st postnatal week, a proposition not presently tested. However, based on the findings presented here, both the growth of the SLM region and the postsynaptic AMPA lability in this period should be addressed.

What I also found was an essential absence of adult LTP in the SLM region of the functionally mature hippocampus, i.e., an LTP exceeding the naïve level. Whether the LTP observed in previous studies (Colbert & Levy, 1993; Dvorak-Carbone & Schuman, 1999; Remondes & Schuman, 2002; 2003; Maurin *et al.*, 2014; Qi *et al.*, 2016) reflects an adult or a developmental LTP is not known since these studies did not consider the naïve level as a baseline level. Viewed conservatively, my results suggest that a developmental LTP is an important part of SLM LTP. However, further studies will be needed to exclude an adult LTP in SLM. Nonetheless, it is tempting to correlate the lack of expansion of the SLM area with age, compared to that in SR, with the absence of adult LTP in SLM.

A notable finding was that the postsynaptic lability in the adult brain is not only a preservation and extension of that expressed in the neonatal brain. Thus, while NMDAR activation facilitated the lability in both the SR and SLM neonatal synapses, no such facilitation was observed in the adult brain. Similarly, while NMDAR activation stabilized the activity-induced depression in the neonatal brain, no such NMDAR dependent LTD was observed from the 3rd postnatal week and onwards. As no depression was observed following a prolonged stimulus interruption, the results indicate that NMDAR activation could not stabilize the depression. NMDAR dependent LTD has previously been described for adult SLM synapses (Dvorak-Carbone & Schuman, 1999). However, since no stimulus interruption test was performed in these experiments, its stability cannot be assessed. In the neonatal synapses, the NMDAR-mediated facilitation of the postsynaptic lability was tentatively attributed to the action of NMDAR-mediated protein phosphatase 2B activation on GluA4, or possibly $GluA2_{long}$, containing AMPARs, making these receptors susceptible to ligand - induced synaptic removal

(Strandberg & Gustafsson, 2011). As also discussed in that article, the NMDAR-mediated stabilization of the AMPA silent synapses should be related to an action on protein phosphatase 2A implementing a stable AMPAR removal.

While the presence of GluA4-containing AMPARs on the adult CA1 pyramidal cells has been negated (Lu *et al.*, 2009), only the SR region was in fact examined in these experiments. A recent immunohistochemical study has instead suggested the presence of such AMPARs in the SLM layer of the adult hippocampus (Seth, H, Bjorefeldt A, Hanse E, personal communication), constituting a possible substrate for the AMPA lability. If so, these AMPARs must be differentially controlled by the above-mentioned phosphatases than in the neonatal hippocampus, but this feature remains to be studied.

Hebbian stabilization of AMPA signalling

Early studies on postsynaptic AMPA lability reported that Hebbian activity not only unsilenced the silenced synapses but also made them AMPA-stable, i.e., resistant towards a low frequency silencing (Xiao *et al.*, 2004; Abrahamsson *et al.*, 2008). However, a later study (Strandberg & Gustafsson, 2024) showed that this apparent stabilization was only temporary, and that prolonged (2 - 4 hours) low frequency (0.2 Hz) stimulation reduced the fEPSP to similar levels as those observed when applied to naïve inputs. Thus, throughout the 2nd postnatal week the synapses are likely exposed to an activity dependent selection pressure where only the synapses persistently participating in Hebbian activity will survive. The sensory map onto a region containing such synapses will thus not be static but flexible, constantly reorganising itself to new input patterns. Whether the adult SLM synapses also exhibit such a temporary stabilization is not clear since I did not test such prolonged low frequency stimulation on the tetanized adult SLM synapses. However, the fact that prolonged low frequency stimulation can depress the fEPSP in the naïve adult SLM as much as it does in naïve 2nd postnatal week SR synapses suggests that not much AMPA-stable transmission is present in the adult SLM. However, further studies using

more prolonged post-tetanus stimulation will be needed to establish whether the adult SLM synapses, similar to the 2nd postnatal week SR synapses, are exposed to such activity dependent selection pressure.

Adult synapse production/elimination or homeostatic plasticity?

While the adult SLM glutamate synapse behaves essentially like the 2nd postnatal week SR synapse, these synapses clearly differ in the origin of the synapses subjected to an activity dependent selection pressure. In the 2nd postnatal week, the synapses to be selected arise from a strong on-going synaptogenesis producing synapses that will either survive or be eliminated, as discussed above. Such synaptogenesis is not expected in the adult SLM layer. Based on a seemingly absent NMDAR-mediated LTD, silencing of the adult SLM synapses may not result in the elimination of the synapses but rather determine which synapses will be active/silent at a certain moment. This would allow an activity dependent flexibility in the sensory map in SLM.

Hypothetically, there could be an on-going synaptogenesis as well as elimination in the adult SLM. In experiments using bath application of AMPA, as well as after prolonged low frequency activation, I observed an unexpected potentiation above the naïve level most easily explained by the generation of new synapses. This effect has previously not been reported from similar experiments in the 2nd postnatal week in the SR or SLM regions. Moreover, the large decrease in the fEPSP/volley relation in the SLM layer occurring during the 1st postnatal month indicates an on-going refinement of the synaptic connectivity possibly explained by synapse elimination. The observed absence of an NMDAR-mediated stabilization of the silenced state could then be accounted for by a concurrent synaptogenesis masking the depression.

On the other hand, the potentiation above the naïve level may not directly be seen as a means for synaptogenesis but as a reflection of a homeostatic response to the induced AMPA silencing. Thus, a partial blockade of AMPARs by the AMPA/kainate receptor blocker GYKI in adult stratum oriens - CA1 synapses results in the insertion of a whole

nanocolumn, i.e., both a presynaptic nanomodule and its postsynaptic counterpart, in a pre-existing synapse (Chipman *et al.*, 2022; Muttathukunnel *et al.*, 2022). This effect was seen as a homeostatic response, the operation of which could potentially explain this form of potentiation above the naïve level of the SLM synapses.

5.2 PRESYNAPTIC LABILE TRANSMISSION

I found that the SLM synapses, in common with the entorhinal projections to the dentate gyrus, displayed a frequency dependent depression in the 0.05 - 1 Hz range. This depression in the dentate gyrus was described as a habituation-like depression, a form of sensory adaptation in which the response to repeated stimulation becomes reduced and thus acting as a filter favoring novel information (Teyler & Alger, 1976). The presence of such presynaptic plasticity for the SLM synapses appears in line with their role in forwarding a representation of sensory activity into the CA1 region. In paper II, this depression was referred to as late depression since it appeared as a prolonged PP depression that had a maximum at a 1 s PP interval and thereafter decayed within ~20s. However, my further analysis of this depression provided evidence that this depression is initiated soon after the presynaptic action potential and is important in shaping the very strong depression during a brief high-frequency train observed for the SLM synapses compared to the SR synapses. A similar robust depression during such stimulation is also observed in the dentate gyrus (Hanse & Gustafsson, 1992).

My experimental data showed that an activity dependent release site inactivation could best explain the characteristics of this depression. To my knowledge, this is the first time a release site inactivation has been suggested to explain homosynaptic depression in a mammalian synapse. It should however be noted that this suggestion is not based on positive evidence for such inactivation, or a mechanistic understanding of it, but rather on my exclusion of other mechanisms such as vesicle depletion and a P_{ves} reduction. Nonetheless, release site inactivation has been demonstrated for a mammalian synapse but through a neuromodulator

action. Thus, in a multi-release site synapse in the central nucleus of the amygdala noradrenalin was found, via G-protein-mediated action on the SNARE-complex, to reduce the number of release sites participating in action potential-induced release (Delaney *et al.*, 2007). As will be discussed below (Is the presynaptic lability homosynaptic?), I believe my data are not consistent with an activity dependent release of a neuromodulator explaining the presynaptic lability. Nevertheless, this effect of noradrenaline shows that a mechanistic basis for release site inactivation seems to exist also for mammalian synapses.

While not suggested as a mechanism for activity dependent depression, an activity dependent increase of the vesicle pool was put forward as a possible, but not likely, explanation for an augmentation of release described for corticothalamic synapses in rat (Granseth & Lindström, 2004). An activity dependent, and release independent, reduction of an immediately releasable vesicle pool (Gustafsson *et al.*, 2019) could in fact tentatively explain the presynaptic depression in the SLM synapses. However, this mechanism fails to explain the good correlation observed between the depression measured at a long PP interval (1 s) and the synaptic depression during brief high frequency trains ($fEPSP_{8-10}/fEPSP_1$), since these latter synaptic responses would not be expected to be altered by such a mechanism.

My data also showed that in the absence of this presynaptic lability in the SLM synapses, most differences in PP plasticity between SLM and SR synapses would disappear. Moreover, the developmental change in short-term plasticity during the 1st postnatal month would become quite similar for both the SLM and SR synapses. These synapses thus seem to basically share the same release properties, excluding those related to the presynaptic lability process. This would also be supported by the similar effect on the release characteristics of the SLM and SR synapses following adenosine application and variation in the extracellular Ca: Mg ratio. The similarity might be taken as an indication that the presynaptic lability in the SLM synapses is not a result of a general

difference in release characteristics between the SLM and SR synapses, but due to a distinct process in the SLM synapses.

My data suggesting such a basic similarity between the SR and SLM synapses is however at odds with a previous study on the 3rd postnatal week SLM synapses (Speed & Dobrunz, 2009), indicating a substantially higher release probability in the SLM than in the SR synapses at that age. This conclusion was based on a large difference in the time course of MK-801 blockade of the NMDAR-mediated synaptic response, this time course was thought to reflect the release probability of the synapses. A possible problem with this often-used method is that synaptic activation may depress the NMDAR-mediated response for other reasons than MK-801-blockade, such as the removal of the NMDARs, possibly Ca-mediated (Gambrill *et al.*, 2011). When I tested for presynaptic depression of the NMDAR-mediated fEPSP by using PP stimulation (1 s PP interval at once per minute), I also observed the development of a very strong depression following each successive PP stimulation (**Paper II,** Fig 9b). While such a problem could, if mediated via calcium influx, be overcome by using a calcium chelator in the pipette solution (as used in the study of Speed and Dobrunz), it may be questioned whether the chelator can spread to the very distal SLM synapses as effectively as to the more proximal SR synapses. It cannot be excluded that the faster blockade of the SLM synapses in the Speed and Dobrunz experiments may reflect such a factor.

Facilitation – unmasking

A problematic issue in my analysis of the experimental data of the presynaptic lability was the temporal superposition of this depression with an activity dependent facilitation of the release. Such a homosynaptic facilitation following brief train activation is not uncommon and likely reflects the augmentation process, a calcium-induced increase in release probability based on an increased P_{ves} lasting around 10 -20 s (Kalkstein & Magleby, 2004; Garcia-Perez & Wesseling, 2008). To remove this facilitation in order to unmask the growth of the depression when I increased the number of presynaptic

activations, I had to make two assumptions. The first assumption was that the relation between the change in fEPSP amplitude and the resulting change in PP plasticity created by this facilitatory process is the same as that produced by adenosine application and changes in the extracellular Ca: Mg ratio. The second assumption was that the presynaptic lability itself was not associated with a change in PP plasticity, i.e., a variation in this plasticity exists among the SLM synapses, and the lability is neutral with respect to such a variation. By using these assumptions to unmask the facilitation, I collected data on the growth of the depression (at a 1 s interval) with increased number of presynaptic activations that well accounted for the depression during brief high-frequency train activation. In fact, the smaller magnitude of the presynaptic depression process that I observed in the one-month-old compared to the 2nd postnatal week rats was also reflected in smaller effect on these high-frequency trains. Even though both these assumptions may be considered reasonable, it should be noted that I do not have independent evidence for either of them. It should further be noted that an additional "result" emerging from this analysis was that the onset of this facilitation must be slow enough not to affect synaptic activity during a brief high-frequency train. While this "result" is in line with the proposition that the slower the decay the slower the onset (Fisher *et al.*, 1997), there is presently no experimental data supporting it.

Is the presynaptic lability homosynaptic?

In the Methodological Considerations section, I brought up the fact that the axons from subcortical structures releasing neuromodulators may run in the SLM layer and be activated by the stimulation current that supposed to only activate glutamate-releasing axons. Based on my own data as well as that of others, I concluded in that section that it was unlikely for such neuromodulator release to account for the depression following single-pulse stimulation at low frequency. Thus, the presynaptic lability should basically be an intrinsic property of the SLM synapses. For the multi-release site synapse in the central nucleus of amygdala previously referred to (Delaney *et al.*, 2007),

neuromodulatory effects were not observed during single-pulse stimulation. However, they were observed when a brief high frequency train was given, leading to depressed synaptic responses within the first second following stimulus onset. Such an effect might impact the depression presently observed both during the train stimulation and for some time afterwards. Many neuromodulators induce synaptic depression in the SLM region when applied pharmacologically to the bath solution (Otmakhova & Lisman, 1999; 2000). To directly reveal such a contribution, train activation should be applied also in the presence of receptor antagonists to such neuromodulators, experiments which remain to be performed. However, it should be noted that to the extent such train-evoked effects were present, they would only affect the depression during the train, i.e., the $fEPSP_{8\text{-}10}/fEPSP_1$ ratio, but not the depression found at 1 s after a single-pulse stimulation (PP_{1s}). Thus, it would not account for the correlation between the $fEPSP_{8\text{-}10}/fEPSP_1$ ratio and the PP_{1s} value observed for both the neonatal and adult SLM synapses (**Paper III,** Fig, 12b).

Release site inactivation – functional aspects

Modulation of presynaptic release can take various forms, resulting in different functional effects on the AMPA signaling. For example, the effect of a P_{ves} reduction induced by adenosine, observed presently, essentially impacted only the 1st synaptic response in a brief high frequency train, even causing a larger release when viewed over the whole train (**Paper II, Fig.** 3b). Such depression would thus mainly reduce low frequency presynaptic activity and might even enhance the synaptic response to a burst of presynaptic action potentials. Other neuromodulators may act by reducing the synaptic response independent of afferent input frequency, thus acting through gain modulation, selectively removing certain inputs, and enhancing others (Burke *et al.*, 2018). Thus, an absence of changes in PP plasticity by a neuromodulator, such as the depressing effect of noradrenalin and serotonin on the SLM synapses (Otmakhova *et al.*, 2005), does not exclude a presynaptic action of these modulators.

In this context, the presynaptic lability, based on release site inactivation, acts not only by reducing the number of synapses at low frequency activity but will also strongly reduce the number of synapses active during higher frequency bursts of presynaptic activity. On the other hand, the synapses that are not inactivated will display facilitation during such high frequency bursts, thereby depolarizing the CA1 cells they innervate more potently.

5.3 FUNCTIONAL CONSIDERATIONS

The operation of a cortical pyramidal cell is presumed to be based on that its dendritic tree does not work as a single compartment but is subdivided into at least two regions: a proximal and a distal dendritic region, each receiving different forms of information (Larkum, 2013). For a neocortical pyramidal cell, the proximal dendritic tree receives sensory feedforward information, while the distal dendrite receives feedback from other cortical regions, providing context and predictive information. To what extent the synapses in these regions differ does not seem to have been studied yet. However, in the auditory cortex, the topography was found to be more stable in the distal layer 1 region than in the more proximal layer 4 region, suggesting a possible difference in plasticity of these different synapses (Takesian *et al.*, 2018).

In the hippocampal CA1 region, the apical dendritic tree of the CA1 pyramidal cells displays a similar, albeit somewhat different, scenario. In this case, the distal part situated in the SLM region receives feed-forward sensory information, while the proximal part located in the SR region receives learned (predictive) information from the CA3 region. Following interaction between these two inputs, the output from the CA1, via the entorhinal cortex, returns to the cortical areas contributing to the layer 1 input to the neocortical pyramidal cells. In this context, the SLM region can be seen as having an instructive role, allowing for the induction of LTP in the SR region for those CA1 pyramidal cells that simultaneously receive sensory information via their SLM synapses. In that manner the output from the CA1 region when the CA3 region activates CA1 pyramidal cells via the potentiated SR synapses, can be

backpropagated towards the cortical areas from which the sensory information came, in a topographic manner.

As suggested by studies of the visual cortex, the critical period for the activity dependent topographic organization ends with the disappearance of labile AMPA transmission (Xu *et al.*, 2020). The SLM region, seen as a sensory cortical area, receives a more complex multimodal sensory activity compared to that of the primary cortical areas, and may constantly need updating of its sensory map. Thus, by retaining its postsynaptic AMPA lability, the SLM region may act like a primary cortical area even in its adult state.

In contrast to the postsynaptic lability, my data suggests that the presynaptic lability decreases with age and the presynaptic facilitation increases. Both these changes contribute to a stronger depolarizing action of the entorhinal inputs to the CA1 pyramidal cells. Considering the proposed role of the SLM synapses in creating sufficient depolarization to support the induction of LTP in the concurrently activated Schaffer collateral synapses to the same CA1 pyramidal cells (Takahashi & Magee, 2009; Grienberger & Magee, 2022), this developmental change in presynaptic plasticity appears functionally appropriate.

6 CONCLUDING REMARKS

My thesis is a tale of two glutamate synapses onto the same postsynaptic cell, the hippocampal CA1 pyramidal cell which is the output cell of the hippocampus. Located in the proximal and distal apical dendritic tree of the CA1 pyramidal cell, respectively, these two synapses interact to produce the hippocampal output. My thesis is focused on the labile signaling of these synapses, i.e., that even with very sparse activation, the synapses can become silenced. What my thesis demonstrates is that this lability can manifest in various forms, exhibiting both age-related differences within the same synapse and differences between the two synapses. This variation is found both when examined in the neonatal hippocampus as well as in the adult hippocampus. It appears that these two synapses are well-suited for their roles during both hippocampal development and in the adult state.

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