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DIFFERENCES IN GLUTAMATERGIC TRANSMISSION ONTO INTERNEURONS AND PYRAMIDAL CELLS IN THE RAT HIPPOCAMPUS

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Abstract
In the human brain there are about 100 billion excitatory glutamatergic neurons and 10 billion inhibitory GABAergic neurons. During development, these neurons are synaptically wired together into neural networks, functionally prepared to perform the full register of brain functions, and to learn from experiences. Much research has focused on the understanding of synapses onto excitatory neurons. Considerably less, however, is known about the properties of synapses onto the inhibitory neurons, information necessary to achieve a more complete picture of network function.

The aim of this thesis was to examine the effects of glutamate on inhibitory interneurons by comparing them to those on excitatory pyramidal cells in the same region of the hippocampus. Electrophysiological methods were used in the acute rat hippocampal slice preparation.

This thesis shows that glutamate synapses formed onto interneurons, in contrast to those formed onto pyramidal cells maintain a developmental phenotype into adulthood. First, interneurons in adult rats have AMPA silent synapses, which are created by activity dependent AMPA silencing. Second, the average number of synapses connecting a presynaptic excitatory cell and an interneuron remains only one in the adult rat. Furthermore, inhibitory interneurons rely more on tonically active extrasynaptic NMDA receptors for their excitability than do excitatory pyramidal cells.

The findings presented in this thesis suggest that some fundamental aspects of glutamate transmission onto interneurons do not change during development, as they do in synapses onto pyramidal cells. This differential development results in different phenotypes of glutamatergic transmission onto these two major groups of neurons. This difference is likely critical for the optimized functioning of the adult neural network.

Keywords: AMPA, development, glutamate transmission, hippocampus, interneuron, NMDA, synapse

POPULÄRVETENSKAPLIG SAMMANFATTNING

Människans hjärna innehåller drygt 100 miljarder nervceller. Nervcellens uppgift är att ta emot nervsignaler från andra celler, summera ihop dem och, om summan är tillräckligt stor, skicka signalen vidare till andra nervceller. Det finns två huvudtyper av nervceller. De som skickar signaler vilka ökar sannolikheten för att nästa cell ska skicka vidare signalen kallas retande nervceller och utgör ungefär 90 % av nervcellerna, medan de som minskar sannolikheten kallas hämmande och utgör ungefär 10 % av nervcellerna. Under hjärnans utveckling kopplas dessa celler ihop till stora nätverk där varje cell bildar specialiserade kopplingar, kallade synapser, med många tusen andra celler. När en elektrisk signal når till synapsen frisätts en signalsubstans, som binder till receptorer på nästa cell. Aktiveringen av dessa receptorer ger upphov till en ny elektrisk signal som ökar, eller minskar sannolikheten att mottagarcellen ska leda nervsignalen vidare. De vanligaste signalsubstanserna är glutamat som är retande och GABA som är hämmande. När en signal skickas in i nätverket kommer den att spridas enligt ett visst mönster, t ex ger doften av nybakade bullar upphov till ett specifikt mönster av aktivitet. Det här mönstret kan ändras om effektiviteten i överföringen i vissa synapser ändras, på så sätt kan doften istället bli ”doften av mormors nybakade bullar”. Förmågan till förändring i signalöverföring kallas för synaptisk plasticitet och anses vara avgörande för att vi ska kunna lära oss nya saker och att bilda minnen, dvs. för att skapa nya mönster av aktivitet i nätverket. Idag vet man rätt mycket om hur synapser på retande celler fungerar och hur deras styrka kan ändras beroende på vilka mönster av aktivitet de utsätts för. För att vi ska få en bättre förståelse för hur hela nätverket fungerar krävs en bättre förståelse även för hur synapser på hämmande celler fungerar.

Syftet med min avhandling är att studera hur retande, glutamaterga, synapser på hämmande, GABAerga, celler fungerar och att därmed bidra till att öka förståelsen för hur hela nätverket fungerar. För att göra det har jag mätt elektriska strömmar från synapser i skivor av råtthjärna från olika utvecklingsstadier. Den hjärnstruktur som jag har undersökt heter hippocampus och den är nödvändig för att vi ska kunna skapa nya fakta- och händelseminnen. I hippocampus är de retande cellerna väl karaktäriserade, vilket underlättar för mig då jag har använt dem som jämförelse när jag studerar de GABAerga cellerna.

I min avhandling visar jag att glutamatsynapser på retande och hämmande nervceller har liknande egenskaper i det nyfödda djuret. Under råttans utveckling ändras dock synapserna på de retande cellerna och därmed får glutamatsynapser på retande och hämmande celler i den vuxna råttan väldigt olika egenskaper. Den här skillnaden uppstår under den utvecklings-

period i råttans nervsystem som grovt motsvarar människans första 20 år. Under den perioden är det vanligt att neuropsykiatriska åkommor, såsom schizofreni, debuterar. Om vi bättre förstår den friska hjärnans utveckling under den här perioden så får vi bättre förutsättningar att också förstå vad som kan gå fel.

Jag visar också att glutamat har olika effekt på den inneboende retbarheten i de olika cellerna, dvs. på cellens benägenhet att skicka iväg en elektrisk signal som svar på en inkommande signal. De låga nivåer av glutamat som finns runt cellerna i hjärnan retar de GABAerga mer än de retar de glutamaterga cellerna. Den här skillnaden är sannolikt också viktig för att balansera retande och hämmande signaler i nätverket. Mina resultat tyder också på att memantine, den aktiva substansen i läkemedlet Ibixa© som används vid behandling av Alzheimers sjukdom, framför allt påverkar den här glutamateffekten på den inneboende retbarheten. Behandling med memantine minskar därför troligen aktiviteten i de GABAerga cellerna, vilket leder till en ökad retning i relation till hämning i hjärnan. Detta ökar möjligheten för glutamatsynapser att bli mer effektiva, något som är avgörande för bildandet av nya minnen.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Ilse Riebe, Bengt Gustafsson, and Eric Hanse. Silent synapses onto interneurons in the rat CA1 stratum radiatum. *European Journal of Neuroscience* **29**(9): 1870-1882.

II. Ilse Riebe and Eric Hanse. Development of synaptic connectivity onto interneurons in the CA1 region of the rat hippocampus. *Manuscript*

III. Ilse Riebe and Eric Hanse. Tonically active NMDA receptors – a signalling mechanism critical for interneuronal excitability in the CA1 stratum radiatum. *Manuscript*

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ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CA	cornu ammonis
αCaMKII	α-calcium-calmodulin-dependent kinase II
CV	coefficient of variation
D-AP5	D(-)-2-amino-5-phosphonopentanoic acid
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
fEPSP	field EPSP
GABA	γ-aminobutyric acid
GluA	AMPA receptor subunit
GluK	kainate receptor subunit
GluN	NMDA receptor subunit
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
LTD	long-term depression
LTP	long-term potentiation
mEPSC	miniature EPSC
mGluR	metabotropic glutamate receptor
mIPSC	miniature IPSC
n	number of release sites
NMDA	N-methyl-D-aspartate
P	postnatal day
p	release probability
PCP	phencyclidine
PKA	protein kinase A
PP	paired-pulse
PPR	paired-pulse ratio
PSD	postsynaptic density
PV	parvalbumin
q	quantal size

SEM	standard error of the mean
sEPSC	spontaneous EPSC
sIPSC	spontaneous IPSC
TARP	transmembrane AMPA receptor regulatory protein
TTX	tetrodotoxin
VGCC	votage gated calcium channel
VGlut	glutamate transporter

1 INTRODUCTION

In the human brain there are about 100 billion excitatory and 10 billion inhibitory neurons that are wired together during development to form functional networks. For these networks to function properly, it is essential that there is a correct balance between excitation and inhibition. There is a growing awareness that an incorrect balance may contribute to many brain disorders like epilepsy, mental retardation, schizophrenia and Alzheimer's disease. Some of these disorders, like schizophrenia, exhibit a prominent development profile, suggesting that developmental changes in neuronal properties may trigger imbalances between excitation and inhibition. One critical player controlling the excitation - inhibition balance, which has been paid relatively little attention, is the glutamatergic excitation of the inhibitory interneurons. My aim with this thesis work is to examine how glutamatergic excitation of inhibitory interneurons develops and whether this glutamatergic excitation is different from that onto excitatory pyramidal cells.

1.1 Hippocampus

The hippocampus is a brain structure that got its name from its morphological resemblance to a seahorse (*hippocampus* in Latin). The hippocampal formation, including the hippocampus proper, dentate gyrus, subiculum and the entorhinal cortex, is situated in the temporal lobe. The hippocampus can be functionally divided into three parts; the ventral, the intermediate and the dorsal hippocampus. These parts differ with regard to neuronal connectivity and gene expression. The ventral hippocampus is involved in processes regarding emotions whereas the dorsal hippocampus is essential for the formation of declarative memories (Moser and Moser, 1998, Fanselow and Dong, 2010). This thesis is based on studies performed exclusively on slices from dorsal hippocampus, and so are all studies referred to, unless otherwise is indicated.

1.1.1 The excitatory circuitry of the hippocampal formation

The excitatory circuitry of the hippocampal formation is well defined and often somewhat simplified into a trisynaptic circuit starting with the projection of axons from layer II cells in the entorhinal cortex to the granule cell layer of the dentate gyrus, forming the first synapse. The axons of these granule cells, the mossy fibres, in turn form the second synapse on

pyramidal cells in the CA3 region of the hippocampus. The third synapse is the connection between the CA3 and the CA1 pyramidal cells. CA1 pyramidal cells then project either directly, or via the subiculum, back to deep layers of the entorhinal cortex. This flow of neuronal signals can be “short-circuited” by the direct connection from layer III cells in the entorhinal cortex to CA1 pyramidal cells. One recent study showed a previously unrecognised disynaptic circuitry, where inputs from both layer II and layer III cells in the entorhinal cortex excite CA2 pyramidal cells, which in turn excite the CA1 cells (Chevaleyre and Siegelbaum, 2010). Chevaleyre and Siegelbaum (2010) also show that there is a strong feed-forward inhibition (see further below) from CA3 pyramidal cells to CA2 pyramidal cells. This feed-forward inhibition may turn the disynaptic circuitry off and thus help to keep the two circuits working independently. This thesis is focused on the CA1 area of the hippocampus, more specifically the stratum radiatum of the CA1. Stratum radiatum is the layer beneath the pyramidal cell layer (stratum pyramidale), where the CA3 axons connect to the CA1 pyramidal cells. Below the stratum radiatum is the stratum lacunosum-moleculare and the most superficial layer of the CA1 is called stratum oriens. These excitatory connections and layers of the hippocampal formation are illustrated in Fig. 1.

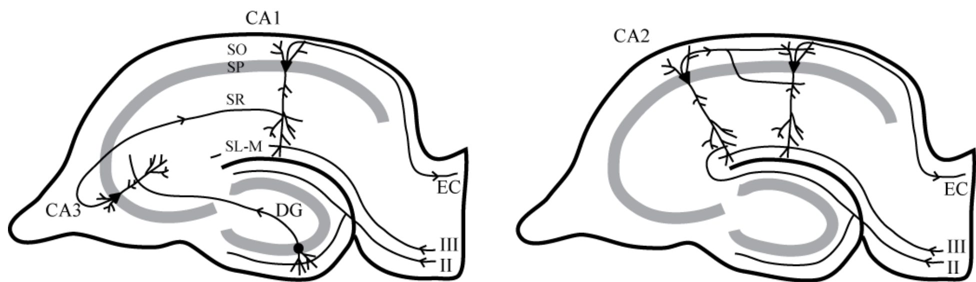

Figure 1. The hippocampal circuitry

The left panel shows the classical trisynaptic circuitry. The recently proposed disynaptic circuitry is shown to the right. EC entorhinal cortex, DG dentate gyrus, SO stratum oriens, SP stratum pyramidale, SR stratum radiatum, SL-M stratum lacunosum-moleculare

1.1.2 Inhibitory interneurons in the CA1

The inhibitory, GABAergic cells of the CA1 subfield in the hippocampus is a highly diverse cell population. When characterizing cells using a combination of their axonal projections, firing patterns and expression of neurochemical markers at least 21 classes of interneurons have been identified (Ascoli et al., 2008, Klausberger and Somogyi, 2008).

GABAergic interneurons are the conductors of neuronal activity. They control neuronal plasticity, restrict pathological excitation and are instrumental for the occurrence of synchronous oscillatory activity in the pyramidal cells, both for the low frequency (theta) oscillations and for higher frequency (gamma) oscillations (Bartos et al., 2007, Buzsaki, 2002, Buzsaki et al., 2007). A large amount of research has focused on the mapping of when during the oscillatory cycles different classes of interneurons fire action potentials (Klausberger, 2009). The synchronization of activity in a population of neurons may help the target population to extract that particular signal from the background noise from other, asynchronous, populations (Akam and Kullmann, 2010). Synchronization of neuronal activity in the theta range (~6-12 Hz) appears in the hippocampus during exploratory behaviour (Buzsaki, 2002), whereas gamma oscillations (~20-100 Hz) seem to arise during many different conditions, like selective attention or working memory maintenance (Fries et al., 2007). The large frequency range of gamma oscillations may be important for routing the flow of information; in the hippocampus fast gamma oscillations are synchronized with fast gamma oscillations in the medial entorhinal cortex, whereas slow oscillations in the CA1 region are correlated with slow oscillations in the CA3 region (Colgin et al., 2009). Oscillations thus seem critically important for the processing and routing of information in the brain, and these oscillations are governed by interneurons.

1.1.3 The CA1 pyramidal cell - interneuron micro circuitry

There are four different basic local circuit elements in the CA1 region; feedforward inhibition, feedback inhibition, recurrent excitation and mutual inhibition (Fig. 2, Andersen et al., 2007). In feedforward inhibition the afferent excitatory axon makes synaptic contacts with inhibitory interneurons as well as with pyramidal cells, this leads to a slightly delayed inhibition from the feedforward interneuron. In feedback inhibition the inhibitory interneuron is excited by the cells that it in turn inhibits. This provides a period of inhibition to the pyramidal cell after

the firing of an action potential. Recurrent excitation is sparsely present in the CA1. There is evidence suggesting that it is normally strongly suppressed by adenosine activation of A_1 adenosine receptors (Klishin et al., 1995), and that the number of recurrent connections might be higher in an experimental model of epilepsy (Shao and Dudek, 2004). Mutual inhibition is also present in the CA1 area, where most interneurons innervate both pyramidal cells and other interneurons. There are also three classes of interneurons (interneuron-specific interneurons or disinhibitory cells) that exclusively form synapses on other interneurons (Klausberger and Somogyi, 2008). In addition to these microcircuits produced by chemical synapses, specific groups of interneurons are also connected via electrical synapses (gap junctions) (Kosaka and Hama, 1985).

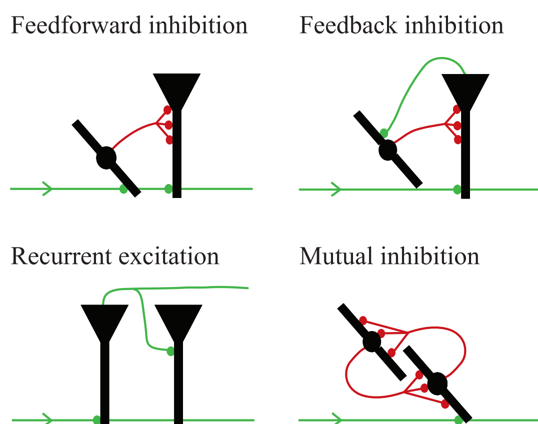

Figure 2. Local micro circuits. *Excitatory connections are shown in green, inhibitory in red.*

1.2 The glutamatergic synapse

The vast majority of projecting neurons, i.e. neurons that carry the signal (as opposed to the ones modifying it) are excitatory glutamatergic neurons. Glutamate is thus the major neuro-transmitter in the central nervous system and about 95 % of the synapses onto pyramidal cells in the CA1 area of the hippocampus are glutamatergic (Megias et al., 2001). The glutamatergic synapse consists of a presynaptic terminal, the synaptic cleft and a postsynaptic compartment (Fig. 3). In the presynaptic terminal the transmitter is stored in synaptic vesicles and may be released into the synaptic cleft as a result of an action potential. In the synaptic

cleft the distance between the presynaptic and the postsynaptic membrane is very short, about 20 nm (Savtchenko and Rusakov, 2007), which allows for an almost instant increase in transmitter concentration at the postsynaptic membrane following the release of a synaptic vesicle. At the postsynaptic membrane glutamate binds to, and opens, ionotropic receptors which will depolarize the cell membrane. This depolarization increases the probability of the generation of an action potential in the postsynaptic neuron. The postsynaptic compartment contains the postsynaptic density (PSD), a specialization containing a large number of proteins that interact with the membrane receptors (Kim and Sheng, 2004). In adult animals the postsynaptic compartment in pyramidal cells is usually situated on a membrane protrusion, called a spine, whereas the postsynaptic compartment in inhibitory interneurons of the hippocampus is usually situated directly onto the dendritic shaft (Craig et al., 1993).

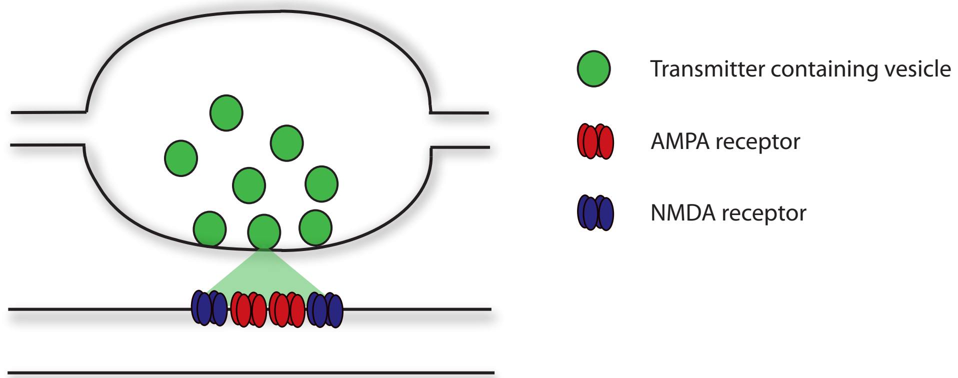

Figure 3. Schematic drawing of a glutamate synapse.

1.2.1 Glutamate release

A presynaptic terminal contains up to hundreds of small synaptic vesicles filled with glutamate, which is a small, negatively charged amino acid that can not pass over the blood-brain barrier, and thus is synthesized from glutamine locally in the neuron. Glutamine is mainly provided from astrocytes, which convert glutamate to glutamine and have the ability to produce large amounts of glutamine from glucose (Hertz and Zielke, 2004). In the presynaptic terminal glutamate is transported from the cytoplasm into the vesicles by vesicular glutamate transporters (VGlut1-3), which use the electrochemical gradient that is created over the

vesicular membrane by a proton pump (Takamori, 2006). Synaptic vesicles are distributed in different functional pools in the presynaptic terminal, with the majority of vesicles not being available for immediate release. A small portion of the vesicles, called primed vesicles, is however held in close proximity to the cell membrane of the active zone by the SNARE-complex and they are thus immediately available for release (Südhof, 2004). In pyramidal cells of the CA1 area in the hippocampus, the size of this vesicle pool was estimated, using electrophysiological methods, to be very small with an average size of 1 vesicle (Hanse and Gustafsson, 2001c). When an action potential reaches the presynaptic terminal and depolarizes the membrane, voltage-gated calcium channels (VGCCs) are opened and Ca^{2+} enters the terminal. This leads to a very local elevation in [Ca^{2+}] in the active zone and this may induce the release, exocytosis, of a primed synaptic vesicle (Pang and Südhof, 2010). After vesicle fusion the vesicular membrane, with important vesicular proteins such as synaptotagmins, is endocytosed and recycled, either directly or via the endosome (Chua et al., 2010).

The release probability (*p*) at synapses onto CA1 pyramidal cells is highly variable (Hanse and Gustafsson, 2001c, Dobrunz and Stevens, 1997, Hessler et al., 1993), for example varying between 0.04 and 0.94 in a group of 43 glutamatergic synapses formed onto CA1 pyramidal cells (Hanse and Gustafsson, 2001c). Sun and Dobrunz (2006) showed that synapses onto interneurons positive for the neuropeptide somatostatin have a very low *p*, whereas somatostatin negative interneurons have a *p* that is higher than that at synapses formed onto pyramidal cells.

The release probability is not fixed, but constantly changes depending on the recent synaptic activity and on the presence of modulatory neurotransmitters. Action potentials change *p* in response to a subsequent action potential if the time interval is short enough (< seconds) (Branco and Staras, 2009). In synapses with a low *p*, the subsequent action potential normally has an increased release probability (called facilitation and augmentation), whereas the opposite is true for synapses with a high *p* (called depression). These activity-dependent changes in *p* are the basis for short-term synaptic plasticity and enables synapses to transmit information in a frequency-dependent manner. Modulatory transmitters (see Modulation) can bind to the receptors on presynaptic terminal and either lower *p* (presynaptic inhibition) or heighten *p* (presynaptic facilitation). In this way modulatory transmitters have the capacity to gate information to different groups of synapses, which are equipped with different types of receptors for modulatory transmitters.

After exocytosis, glutamate diffuse very rapidly (ms) from the synaptic cleft (and the postsynaptic receptors), and is taken up by transporters primarily in the astrocytes, and to a lesser extent in the neurons. The glutamate molecules are subsequently recycled back into synaptic vesicles (Edwards, 2007). The peak concentration of glutamate at the postsynaptic receptors immediately after exocytosis (within 100 µs) has been estimated to be about 10 mM. This can be compared to the extracellular background concentration of glutamate, which is at least 10000 times lower (see Tonic glutamate receptor currents).

1.2.2 Glutamate receptors

Glutamate receptors are either ionotropic or metabotropic, i.e. G-protein coupled, receptors. They are expressed both pre- and postsynaptically. There are three different kinds of ionotropic glutamate receptors; AMPA, NMDA and kainate receptors, named after their respective main exogenous agonists. In the canonical glutamate synapse, AMPA and NMDA receptors are co-localized in the PSD.

1.2.2.1 The AMPA receptor

The AMPA receptor is a low affinity receptor mediating fast excitatory transmission. It contains a channel normally permeable to Na^+ and K^+ in a ratio such that the reversal potential is 0 mV. The receptor complex is a tetramer, i.e. it is built up from four subunits. There are four genes coding for these subunits and the gene products are named GluA1-A4 (Collingridge et al., 2009). One subunit consists of the extracellular N-terminal domain and the two ligand binding domains, three transmembrane domains and one segment that dips into the membrane from the intracellular compartment and, last, the C-terminal with PDZ binding motifs (Bredt and Nicoll, 2003). The AMPA mRNAs are subject to posttranscriptional modifications, such as alternative splicing and mRNA editing. Alternative splicing can produce either a flip or a flop version of the protein, rendering it different desensitization properties (Sommer et al., 1990). For GluA2 and GluA4 subunits the cytoplasmic tail is also alternatively spliced, such that they have a long and a short version with different motifs for binding to intracellular scaffolding proteins, GluA1 subunits have the long version and GluA3 have the short one (Bredt and Nicoll, 2003). GluA2 mRNA is posttranscriptionally edited in a way that makes the channel pore impermeable to Ca^{2+}. This is achieved through the substitution of a glutamine (Q) codon for an arginine (R) codon (Q/R-editing) (Seeburg et al.,

2001). The expression of subunits is developmentally regulated. In the hippocampus GluA4 is most abundant during the very first postnatal week, whereas the expression of GluA1-3 increases over the first two to three postnatal weeks (Zhu et al., 2000). The expression of GluA subunits in hippocampal interneurons is somewhat different from that in pyramidal cells. One interesting difference is that GluA4 subunits are highly expressed in parvalbumin (PV) expressing interneurons from adult rats (Catania et al., 1998). Posttranscriptional modifications are also developmentally regulated. The mRNA expression of the flop version of GluA subunits increases throughout the brain, reaching stable level during the second postnatal week, whereas the flip levels are constant (Monyer et al., 1991).

In the endoplasmic reticulum (ER) the AMPA receptors are assembled as dimers of dimers (Greger et al., 2007), and subsequently trafficked to the cell membrane. The trafficking of AMPA receptors out of the ER is dependent on correct interactions with auxiliary subunits and PDZ proteins at the C-terminus of the GluA subunits (Groc and Choquet, 2006). The auxiliary subunits described so far are the TARPs, the cornichons and CKAMP44. They have a large impact on deactivation and desensitization properties of the receptor. TARPs and cornichons both decrease deactivation and desensitization, whereas CKAMP44 decreases deactivation and increases desensitization (Guzman and Jonas, 2010).

1.2.2.2 The NMDA receptor

The NMDA receptor also participates in the fast excitatory synaptic transmission, its main importance is however in the induction of plastic changes in synaptic efficacy (see Plasticity and modulation). In contrast to the low affinity AMPA receptor, the NMDA receptor is a high affinity receptor, and importantly, it is also highly permeable to Ca^{2+}. There are seven sub-units that can take part in the formation of the NMDA receptor tetramer (Cull-Candy et al., 2001). They are named GluN1, GluN2A-D and GluN3A-B (Collingridge et al., 2009). Of these subunits only the GluN2 subunits carry the glutamate binding site, the GluN1 and GluN3 subunits instead have a binding site for the co-agonist glycine, or D-serine. The NMDA receptor is, like the AMPA receptor, assembled as a dimer of dimers. One NMDA receptor always contains the GluN1 subunit, usually in two copies, the remaining subunits can either be two GluN2 or one GluN2 and one GluN3 (Stephenson et al., 2008). In expression systems, pure glycine receptors, only containing GluN1 and GluN3, have been observed, but hitherto there is no evidence supporting the expression of these receptor complexes in neurons

(Cavara and Hollmann, 2008). NMDA receptors containing the GluN2A or 2B subunits have a high channel conductance (about 50 pS), but exhibit a strong inward rectification, due to a voltage dependent Mg^{2+}-block. This voltage dependency is much weaker in receptors containing the GluN2C or 2D subunits, which also have a considerably lower channel conductance (about 35 pS) (Cull-Candy et al., 2001). Whereas the expression of GluN2B (in rats) is constant, the expression of GluN2A starts before birth and is up-regulated during development, leading to a developmental change in relative expression (Sheng et al., 1994, Wenzel et al., 1997, Monyer et al., 1994). In pyramidal cells GluN2A containing receptors are preferentially located in the postsynaptic membrane, whereas Glu2B receptors can be either synaptically or extrasynaptically positioned (Groc et al., 2009). GluN2C is only expressed in interneurons in the hippocampus (Monyer et al., 1994), but is highly abundant in other brain areas, such as the cerebellum (Farrant et al., 1994). GluN2D is expressed in neonatal hippocampus, but the expression in pyramidal cells is decreasing with development and in two-week old rats GluN2D mRNA was not detected (Kirson et al., 1999). GluN2D was however still present in a subpopulation of interneurons (Monyer et al., 1994, Standaert et al., 1996). There are some indications, using the weakly GluN2D selective antagonist PPDA, that GluN2D containing receptors might be present in the extrasynaptic membrane of CA1 pyramidal cells from P21 rats, and that they can be activated by glutamate spill-over during high-frequency train stimulation (Lozovaya et al., 2004). NMDA receptors containing the GluN3 subunits have a low channel conductance, low Mg^{2+} sensitivity, and a low relative Ca^{2+} permeability (Cavara and Hollmann, 2008). The GluN3A subunit is widely expressed in the neonatal CNS and declines to its low adult levels around P21 (Ciabarra et al., 1995, Sucher et al., 1995, Wong et al., 2002). GluN3B has been thought to have a different expression pattern with high levels expressed exclusively in motoneurons of the spinal cord (Nishi et al., 2001, Chatterton et al., 2002). One quite recent study, however, suggested that it is also highly abundant in the adult rat brain (Wee et al., 2008).

The NMDA receptor heterogeneity is further increased by alternative splicing. All subunits, except for the GluN2A are subject to mRNA splicing (Cull-Candy et al., 2001). The GluN1 subunit, for example, is spliced in three different locations, one in the extracellular N-terminal domain and two in the intracellular C-terminal domain, giving rise to eight different splice variants (Zukin and Bennett, 1995). These splice variants differ for example in pharmacological properties (for the extracellular location) and in their trafficking (for the intracellular locations) (Chen and Roche, 2007).

1.2.2.3 The Kainate receptor

Kainate receptors are, like the other ionotropic glutamate receptors, tetramers. There are five identified kainate receptor subunits, named GluK1-5 (Collingridge et al., 2009). Kainate receptors can be either ionotropic or metabotropic and are expressed both pre- and post-synaptically (Lerma, 2006). In CA1 pyramidal cells, however, kainate receptors are not expressed postsynaptically, but rather presynaptically in the terminals of the CA3 cells, where they can modulate release probability (Jane et al., 2009). Early during postnatal development, for example, release probability is suppressed at a subset of these synapses via the tonic activation of a presynaptic high affinity, G-protein coupled, kainate receptor (Lauri et al., 2006). Interneurons in the CA1 stratum radiatum and lacunosum moleculare, on the other hand, express postsynaptic, GluK1 containing, kainate receptors that contribute to the evoked EPSC (Wondolowski and Frerking, 2009).

1.2.2.4 The metabotropic glutamate receptor

There are eight different metabotropic glutamate receptors (mGluR1-8), divided into three groups (Group I-III) depending on their pharmacology, signal transduction mechanisms and sequence homology (Ferraguti and Shigemoto, 2006). These receptors can be located pre-synaptically or postsynaptically, usually extrasynaptically, and are important in the regulation of release probability (via effects on VGCCs) and synaptic plasticity. The expression of mGluRs in the hippocampus is spatially regulated, for example mGluR7 seems to be preferentially expressed in terminals targeting interneurons (Shigemoto et al., 1996). Interestingly, this diverse expression of mGluRs has been suggested to be one important factor in the modulation of oscillatory activity in neuronal networks (LeBeau et al., 2005).

1.3 The GABAergic synapse

GABA is the most common inhibitory neurotransmitter, and about 5% of the total number of synapses formed onto CA1 pyramidal cells are GABAergic (Megias et al., 2001). The localization of these synapses on the pyramidal cell is very important in deciding the effect of them. Thus, GABAergic synapses in the distal dendrites will act locally to counteract the EPSPs, i.e. they will control the input to the cell. GABAergic synapses localized to the soma

or the initial segment will, on the other hand, control the spike probability, and thus the output from the cell (Miles et al., 1996).

There are two main groups of GABA receptors, $GABA_A$ and $GABA_B$ receptors. The $GABA_B$ receptor is responsible for slow synaptic inhibition via a K^+-channel coupled to a G-protein, whereas the $GABA_A$ receptor is ionotropic and responsible for the fast synaptic inhibition. The $GABA_A$ receptor contains an anion channel mainly permeable to Cl^- ions (Farrant and Nusser, 2005). The reversal potential of $GABA_A$ receptors is thus dependent on the intracellular $[Cl^-]$, which is developmentally regulated via the expression of the chloride extruder KCC2 (Rivera et al., 1999). The $GABA_A$ receptor is a pentamer built up from a large number of subunits, including α1-6, β1-3, γ1-3 and δ (Farrant and Nusser, 2005).

1.4 Intrinsic excitability and extrasynaptic receptors

The neuronal cell membrane contains a large variety of ion channels that regulate the cell's excitability. These channels include the two-pore domain channels (K^+ leak channels), the voltage gated ion channels and Ca^{2+} gated channels. The density of these channels and their open probabilities will decide the membrane permeability for different ions, and thus the membrane potential. Different neurons have their characteristic firing pattern, which is directly determined by their specific expression pattern of ion channels. In addition to these channels also ligand-gated ion channels are present in the membrane outside synapses. Like all membrane-bound receptors they are diffusing laterally in the membrane merely having a higher probability of being in the postsynaptic membrane (Gerrow and Triller, 2010). If the extracellular ligand concentration is high enough, receptors in the extrasynaptic membrane can be tonically active, and thereby contribute to the intrinsic excitability of the cell. Since uptake of glutamate and GABA is largely controlled by the astrocytes, these cells have a key role in the control of extracellular ligand concentrations (Schousboe, 2003).

1.4.1 Tonic GABA receptor currents

The existence of tonic inhibition via $GABA_A$ receptors in the extrasynaptic membrane is well established (Belelli et al., 2009). Three of the $GABA_A$ receptor subunits, α4, α6 and δ, are preferentially located in the extrasynaptic receptor pool. Receptors containing these receptor subunits have characteristics that make them optimized for tonic activity, i.e. they have a high

GABA affinity and exhibit little desensitization. Importantly, they are also differently modulated, compared to the synaptic receptors. For example, they are insensitive to benzodiazepine modulation but positively modulated by neurosteroids already at quite low concentrations. Abnormalities in tonic $GABA_A$ receptor signalling have been implicated in several forms of epilepsy (Fritschy, 2008).

1.4.2 Tonic glutamate receptor currents

While tonic GABA currents are rather well characterized, tonic current arising from glutamate receptors is not a well established phenomenon. The first report of tonic NMDA receptor mediated currents came 1989 when Sah et al (1989) showed that, in the absence of Mg^{2+}, ambient levels of glutamate can indeed activate NMDA receptors. They further showed that this activation increases the excitability of pyramidal cells. After wash-in of 1.3 mM of Mg^{2+} the tonic activation, however, decreased dramatically. More recently LeMeur et al (2007) repeated these findings in pyramidal cells from P14-29 rats. Their study is exclusively performed in pyramidal cells voltage-clamped at +40 mV, where the Mg^{2+}-block is relieved. Interestingly, they show that the tonic current is independent of synaptically released glutamate, but rather dependent on glutamate of astrocytic origin, and, using PPDA, they imply that there might be a contribution from receptors containing the GluN2D subunit. This finding is in contrast to the report of Monyer et al (1994), stating that GluN2D mRNA is not present in pyramidal cells in the adult rat. Cavelier et al (2005) failed to reproduce the findings of Sah et al, and suggested that Sah's results might have been an artefact arising from low oxygenation in their rather thick slices (400 µm). Using exogenous glutamate and NMDA and endogenous NMDA receptors as reporters, Herman and Jahr (2007) concluded that endogenous glutamate levels are too low, around 25 nM, to tonically activate NMDA receptors.

Under conditions of elevated ligand concentration, i.e. after bath application of NMDA or in models of excitotoxicity, activation of extrasynaptic NMDA receptors is thought to be responsible for cell death (Moskowitz et al., 2010, Hardingham and Bading, 2003, Leveille et al., 2008). Interestingly, interneurons are less sensitive to excitotoxic insults (Avignone et al., 2005), suggesting differences in density, or composition, of extrasynaptic NMDA receptors.

1.5 Plasticity and modulation

The route neuronal signals take through the neuronal network can be changed by different processes, called plasticity and modulation. Plastic modifications of synapses or intrinsic excitability are activity dependent and help to create new signal paths in the network and to "broaden", or "narrowing", existing paths. A modulatory modification of the network, on the other hand, serves the purpose of helping the neuronal signals to choose between different paths in the network, on a moment-to-moment basis. Modulatory inputs typically originate in brain stem areas that are involved in circadian regulation of alertness and wakefulness.

1.5.1 Synaptic plasticity

Synaptic plasticity is the use dependent regulation of synaptic strength. The duration of these plastic changes can vary from ms-min (short-term plasticity) up to hours-months (long-term plasticity). Plastic changes can go in two directions; either the synaptic connection is strengthened, i.e. potentiated; or it is weakened, i.e. depressed. Long-term synaptic plasticity is essential for the development of neuronal circuits and for learning and memory. Over the last decade, or so, it has also been recognized that the threshold for the induction of synaptic plasticity can change in an activity-dependent manner, referred to as metaplasticity (Abraham, 2008). In addition to these forms of plasticity, more or less restricted to individual synapses, there are also global (cell-wide) changes in synaptic efficacy in response to increased or decreased overall activity of the neuron, referred to as homeostatic plasticity (Turrigiano, 2008). Interestingly, recent data indicate that homeostatic plasticity at glutamatergic synapses onto principal cells and onto interneurons go in opposite directions. Thus, if the neural network is exposed to elevated activity during a prolonged period of time transmission at glutamate synapses onto principal cells will scale down, whereas transmission at glutamate synapses onto interneurons will scale up, leading to an overall downscaling of neural activity. At principal cell synapses Arc/Arg3.1, whose expression increases by increased activity, has been proposed to mediate synaptic downscaling by promoting endocytosis of synaptic AMPA receptors (Shepherd et al., 2006, Chowdhury et al., 2006). At inhibitory neuron synapses, on the other hand, Narp, a pentraxin whose expression increases by increased activity, contributes to scaling up of synaptic efficacy by promoting synaptic clustering of AMPA receptors (Chang et al., 2010).

1.5.1.1 Long-term potentiation

In 1949 Donald Hebb proposed that "cells that fire together wire together", meaning that the connection between a pair of cells that are active at the same time will be strengthened (Hebb, 1949). More than 20 years later the first evidence in a chain leading to the confirmation of Hebb's postulate was presented. Thus, in 1973, Bliss and Lømo (1973) showed that a brief tetanization in the dentate gyrus, resulted in a potentiation of the field excitatory potential (fEPSP). This phenomenon was later named long-term potentiation (LTP) and shown to be input specific (only affecting the stimulated synapses) and dependent on a postsynaptic elevation in [Ca^{2+}] and a postsynaptic depolarization (Andersen et al., 1977, Lynch et al., 1983). In 1986, Wigström and Gustafsson suggested that the NMDA receptor was acting as the coincidence detector, sensing the presynaptically released glutamate, as well as the postsynaptic depolarization arising from the simultaneous activation of many synapses (Gustafsson and Wigström, 1986). The existence of the Hebb synapse was thus finally nailed. It is now evident that there exist several different forms of LTP, with different induction and expression mechanisms (Malenka and Bear, 2004). Different forms of LTP can even coexist in the same cell, and properties may change with development.

Synapses onto interneurons were long thought to be much less plastic than those onto principal cells (McBain et al., 1999), but in 2001 a mGluR dependent, NMDA receptor independent, Hebbian LTP was shown in interneurons from stratum oriens of the CA1 area (Perez et al., 2001). The same LTP was not found in interneurons in the stratum radiatum. In stratum radiatum about 50% of the interneurons was later shown to exhibit an NMDA receptor dependent Hebbian LTP (Lamsa et al., 2005). For interneurons in the stratum oriens also an "anti-Hebbian" LTP was shown (Lamsa et al., 2007). This LTP is dependent on postsynaptic hyperpolarization, rather than the classical depolarization, and on Ca^{2+} entering GluA2-lacking AMPA receptors. Thus, glutamate synapses onto interneurons appear to have specialized forms of long-term synaptic potentiation.

1.5.1.2 Long-term depression

"What goes up must come down" (Morris and Willshaw, 1989, Pitman and Waddell, 2009) - it is generally believed that each form of LTP has its counterpart, a long-term depression (LTD). LTD was first described in the early 1990s by Dudek and Bear (1992) and this NMDA

receptor dependent form of LTD was shown to be complementary to NMDA receptor dependent LTP in the hippocampal CA1 region (Mulkey and Malenka, 1992). Subsequently, many different forms of LTD have been found at many different synapses in the brain (Massey and Bashir, 2007). LTD has also been described at glutamatergic synapses onto hippocampal interneurons in the CA1 region (Gibson et al., 2008, McMahon and Kauer, 1997, Nissen et al., 2010). This LTD is NMDA receptor independent and appears expressed as a reduction in presynaptic release probability at synapses exhibiting calcium permeable AMPA receptors, thus apt to counteract the NMDA receptor independent LTP at these synapses (Perez et al., 2001, Lamsa et al., 2007). Since a different form of NMDA receptor-dependent, Hebbian LTP has been described at interneuronal glutamate synapses expressing calcium impermeable AMPA receptors (Lamsa et al., 2005), one would expect that also this LTP should have a LTD counterpart. This LTD has however so far not been described.

1.5.2 Intrinsic plasticity

Long-term plastic changes in intrinsic excitability, both increasing and decreasing excitability, have been shown for principal cells in different brain regions (Daoudal and Debanne, 2003). These changes include E-S plasticity (EPSP-Spike plasticity), i.e. changes in the probability of an EPSP evoking action potential, which directly affects the input-output relationship of the cell. The first evidence of a potentiation of intrinsic excitability in interneurons is an mGluR-mediated down regulation of a K^+ current in putative PV+ basket cells of the CA1 area (Campanac et al., 2010). This potentiation was not present in PV-interneurons.

1.5.3 Modulation

Both synaptic transmission and intrinsic excitability are extensively modulated. Modulation of synaptic transmission is mainly achieved through depression of release probability. One example of this kind of modulation in the CA1 region is the adenosine dependent tonic suppression of release in the recurrent excitation of CA1 pyramidal cells (Klishin et al., 1995). Also release from GABAergic terminals is modulated. For example, activation of the CB1 cannabinoid receptor, expressed by CCK+ basket cells of the hippocampus, reduces release probability (Katona et al., 1999). One example of modulation of pyramidal cell intrinsic excitability is the kainate-induced inhibition of the after-hyperpolarization, leading to an increased firing frequency (Melyan et al., 2002). The classic modulatory transmitters are

serotonin (or 5-HT), dopamine, histamine, acetylcholine, and noradrenalin, but several other substances, such as hormones, gliotransmitters, cytokines and endocannabinoids, also act as modulatory neurotransmitters.

1.6 Development

Figure 4 provides a schematic overview of the development of neurons and synapses in the rat hippocampus. Pyramidal cells of the rat hippocampus proliferate between the embryonic days 17-19 (E17-19) in the subventricular zone, and then migrate to their final destination (Bayer, 1980). GABAergic interneurons are born earlier in the medial and lateral ganglionic eminences and first migrate tangentially along the migratory zone before they turn radially into their cortical destination layer. Various interneuronal classes are born in different parts of the ganglionic eminence at different embryonal time points between E13-18, with CA interneurons proliferating before interneurons of the dentate gyrus (Danglot et al., 2006). Before the number of neurons is stabilized the general idea is that about half of the cells undergo apoptosis (Cowan et al., 1984).

The neurons of the hippocampus start forming synaptic connections already before birth, first GABAergic connections, followed by glutamatergic ones. This sequence is the same for pyramidal cells (Ben-Ari, 2001) and interneurons (Hennou et al., 2002), with interneurons preceding the pyramidal cells. Because of the low expression of the Cl^- extruder KCC2, the GABA synapses are depolarizing at this early developmental stage. In the pyramidal cells mature spines begin to appear during the second postnatal week and in the adult animal a majority of the synapses are located on spines (Fiala et al., 1998). In interneurons the majority of synapses are shaft synapses (Seress and Ribak, 1985). During the first postnatal month the rate of synaptogenesis is higher than that of synapse elimination, leading to a net increase in number of synapses. Towards the end of this period synaptogenesis may decline faster than the elimination.

The CNS development of the rat and the human differs considerably (Hagberg et al., 2002), with the developmental stage of the neonatal rat (P0) corresponding roughly to the half-term human foetus. At two weeks age, the rat brain maturity resembles that of the newborn human, and about P30 the rat reaches puberty.

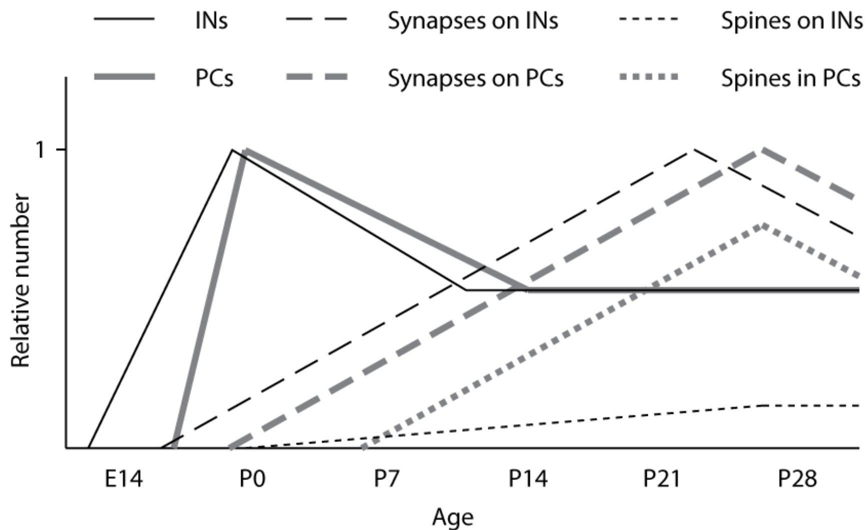

__Figure 4.__ Development of neurons, synapses and spines in the hippocampal CA1 region presented schematically.

1.6.1 AMPA silent synapses and AMPA silencing

One of the most prominent hallmarks of developing glutamate synapses are the so called AMPA silent synapses. These synapses lack functional AMPA receptors, while displaying functional NMDA receptors. AMPA silent synapses have been shown to exist on principal cells in many parts of the developing CNS, including the CA1 area of the hippocampus (Bardoni et al., 1998, Durand et al., 1996, Isaac et al., 1997, Isaac et al., 1995, Li and Zhuo, 1998, Liao et al., 1995, Rumpel et al., 1998, Wu et al., 1996). The number of silent synapses decreases during development, reaching a very low level in the adult animal (Abrahamsson et al., 2008, Hsia et al., 1998, Xiao et al., 2004).

Originally glutamate synapses were thought to be born AMPA silent, acquiring AMPA receptors only later as a result of LTP (Durand et al., 1996). This idea was challenged by the finding that very young CA1 pyramidal neurons exhibited about the same frequency of spontaneous AMPA and NMDA EPSCs (Groc et al., 2002). Subsequently it was shown that simply synaptic activation renders immature AMPA signalling synapses AMPA silent, a form of plasticity named AMPA silencing (Abrahamsson et al., 2005, Xiao et al., 2004), and that absence of synaptic activity allows these synapses to regain their AMPA signalling

(Abrahamsson et al., 2008, Strandberg et al., 2009). Thus, the transmission at the newborn glutamate synapse is unreliable, switching between a silent and a signalling state (Groc et al., 2006). Prolonged synaptic activation, of the kind traditionally used to induce LTD, makes the silent state of these synapses more stable (Strandberg, 2010). This study thus suggests that AMPA silencing and LTD at these synapses are the same synaptic change, i.e. a total loss of AMPA receptors, the only difference being the relative stability.

AMPA silent synapses have not been shown on inhibitory interneurons. On the contrary, Hennou et al (2002) stated that their results on AMPA and NMDA mEPSCs in embryonal and neonatal rats suggested the absence of silent synapses in interneurons. As also noted by these authors, this method is blunt and further studies using minimal stimulation are needed.

2 AIM

The properties of glutamatergic transmission onto principal cells, and how they change with development, have been extensively studied in different parts of the central nervous system, especially so for the pyramidal cells in the CA1 region of the hippocampus. Considerably less is known about glutamatergic transmission onto GABAergic interneurons, a piece of information necessary to better understand the functional properties of neuronal circuits at different developmental stages. The overall aim of this thesis work is therefore to examine glutamate effects on interneurons by comparing them to those on pyramidal cells.

2.1 Specific aims

I. Are AMPA silent synapses present onto interneurons? If so, is their existence developmentally restricted, as in the pyramidal cells?

II. Do pyramidal cell - interneuron cell pairs acquire multiple release site connections with development as do pyramidal - pyramidal cell pairs?

III. Is tonic NMDA receptor-mediated signalling expressed in interneurons to a larger, or to a smaller extent than in pyramidal cells?

3 METHODOLOGICAL CONSIDERATIONS

3.1 The hippocampal slice preparation

The studies presented in this thesis are performed on the hippocampal slice preparation. There are several reasons to use this preparation when studying synaptic functions. First, the hippocampal formation is essential for the formation of new long-term memories and hence it is highly relevant to study synaptic plasticity in the hippocampal circuitry. Second, the hippocampal circuit is relatively laminar in its structure (Andersen et al., 1971), and thus it is left fairly intact by the slicing procedure. It should be noted that the majority of modulatory inputs are cut during the slicing, making the slice a “sleeping” preparation. Last, the CA3-CA1 glutamate synapse is probably the most studied synapse in the brain. Since the objective of this thesis is to compare glutamate transmission onto interneurons to that onto principal cells it is thus natural to choose the CA1 region for these studies, since the principal cells here are already well characterized.

3.1.1 Preparation and storage of slices

The slices used in the studies presented here were prepared from rats aged 7 to 60 days. For the younger rats, up to 4-week old, both females and males were used, for the older rats, however, only male rats were used. The reason for this was to avoid the variability in spine density occurring over the estrous cycle in fertile female rats (Woolley and McEwen, 1992). After the rats were anaesthetized with isoflurane (1 ml in a box containing 5 l air) and decapitated, the hemispheres were placed in a cold solution containing (in mM): 140 cholineCl, 2.5 KCl, 0.5 $CaCl_2$, 7 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 0.5 ascorbic acid, and 7 D-glucose. Subsequently, transverse slices, 300 or 400 µm thick, were cut using a vibratome (Slicer HR 2, Sigmann Elektronik, Hueffenhardt, Germany). The idea is that the low temperature (approximately 2-3 °C) should decrease the metabolic demand in the tissue and hence increases cell survival, and that the substitution of Na^+ in the extracellular solution to the membrane impermeable choline ion should further reduce the cellular activity in the tissue. After slicing, the hippocampal part was dissected out and stored in a solution containing (in mM): 124 NaCl, 3 KCl, 2 $CaCl_2$, 4 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 0.5 ascorbic acid, 3 myo-inositol, 4 D,L-lactic acid, and 10 D-glucose. This solution contains

antioxidants to minimize oxidative stress. Initially the temperature of the storage solution was 32 °C but after the initial one hour of recovery it was lowered to 24 °C, where it was kept for the remainder of the storage. The initial elevation in temperature enhances the slice quality, and especially so in the slices from adult rats (Bischofberger et al., 2006).

All extracellular solutions were bubbled with 95% O_2 and 5% CO_2 in order to supply the cells with sufficient amounts of O_2 and to keep the pH around 7.3. Since the solubility of gasses in the extracellular solution is highly dependent on temperature, such that more is dissolved at lower temperatures, the content of $NaHCO_3$ is slightly higher in the choline solution, compared to the solution used during recordings. This since the choline solution is kept very cold during the slicing procedure.

3.2 Recordings

After at least one hour of recovery, slices were moved to a recording chamber (with a volume of ~ 1 ml) and continuously perfused at a rate of ~ 4.4 ml/min, with an extracellular solution containing (in mM): 124 NaCl, 3 KCl, 2-4 $CaCl_2$, 0.5-4 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 10 D-glucose.

3.2.1 Intracellular recordings

In intracellular recordings the recording electrode is in contact with the cell interior. This opens the possibility to record single synaptic events as well as study intrinsic properties of individual neurons.

3.2.1.1 Patch-clamp recordings

The patch-clamp technique was developed in the 1970s by Neher and Sakmann (1976). There are four different modes or patch-clamp recordings. They are all based on the initial formation of a high resistance seal (> 1 GΩ) between the recording pipette and the cell membrane. The recording pipette is of glass and filled with a salt solution. The pipette opening has a diameter of ~ 1 µm. In this *cell-attached mode* it is possible to record the activity of single ion channels, with the cell interior preserved. A second possibility is to pull the pipette away from the cell, thus creating an *inside-out patch*, where it is possible to record single channels and

manipulate the cytosolic part of the channels. If the membrane patch under the pipette tip is ruptured the recording electrode gets in contact with the cell interior. In this mode, called the *whole-cell mode*, activity from channels in the whole cell is recorded. If the pipette is pulled away from the cell in this mode, an *out-side out* patch may be formed. In this configuration it is possible to record single channel activity and manipulate the extracellular parts of the channel proteins. Of these four modes only the whole-cell mode is used in this thesis. Whole-cell recordings can be made either in current-clamp (measures membrane potential) or in voltage-clamp (measures membrane current). One major drawback with whole-cell recordings is that essential components of the cell interior can be washed out from the cell. This wash-out phenomenon is believed to explain the fact that it is very hard to induce LTP after more than 10 minutes of whole-cell recording (Malinow and Tsien, 1990).

In the studies presented in this thesis either pyramidal cells from the stratum pyramidale or interneurons with their soma in the stratum radiatum were visually identified using infrared-differential interference contrast video microscopy mounted on a NIKON E600FN microscope (Nikon, Japan). The recording pipettes used for patch-clamp recordings were filled with a solution where K^+ was substituted with Cs^+. A majority of K^+-channels are not permeable to Cs^+ (Hille, 2001) and this substitution thus helped to reduce the background noise in the recording, and improved the voltage control of the cell. The addition of QX-314, a blocker of voltage sensitive Na^+-channels further improved the recording conditions. Further, the [Cl^-] was very low, creating a rather hyperpolarized reversal potential of $GABA_A$ receptors (approximately -80 mV). This made it possible to record AMPA EPSPs at a holding potential of -80 mV without contamination of GABA IPSPs (inhibitory postsynaptic potentials). At a membrane potential of 0 mV it was instead possible to record isolated GABA IPSPs, since this is the reversal potential of the AMPA receptor. When recording NMDA EPSPs at +40 mV the $GABA_A$ receptor antagonist picrotoxin was always present.

3.2.1.2 Perforated patch recordings

The perforated patch-clamp technique is used to eliminate the wash-out problems of whole-cell recordings. This is achieved by the use of antifungal substances, most commonly amphotericine B or gramicidine, which make small holes in the membrane patch under the pipette opening (Rae et al., 1991). These pores are permeable to small monovalent cations, such as K^+, Cs^+ and Na^+, and the amphotericine B pore is also permeable to monovalent anions, like

Cl^-. Larger molecules do not pass through these pores, thus leaving the intracellular milieu fairly intact.

The perforated patch-clamp recordings presented here were performed using gramicidine in a pipette solution containing CsCl and the pH buffer HEPES. The fluorescent dye Lucifer yellow was routinely added to the solution in order to improve the detection of spontaneous membrane rupture above that which is evident by monitoring the series resistance and the holding current. The higher than normal pipette resistance (6-10 MΩ, compared to 2-5 MΩ for whole-cell recordings) decrease the risk of membrane rupture.

3.2.2 Extracellular field recordings

In the extracellular recordings performed here the pipette was instead filled with a 1M NaCl solution, and placed in the extracellular compartment. Afferent axons, Schaffer collaterals/ commissural fibres, were activated by a biphasic constant current pulse, delivered through a tungsten electrode. The parallel organization of the apical dendrites of CA1 pyramidal cells allows for summation of the extracellular field potentials from individual cells. The straggling nature of interneuron dendrites, however, evens the interneuron potentials out. Thus, extra-cellular field potentials of the stratum radiatum are almost exclusively composed of EPSPs from pyramidal cell dendrites. This compound EPSP recorded in the stratum radiatum is preceded by the fibre volley, which is the extracellular field arising from the axonal action potentials and commonly used as a control for the stability of axon recruitment. Extracellular recordings were also made in the stratum pyramidale/oriens border, where the reversed EPSP and the population spike, i.e. the compound action potential from the pyramidal cells close to the recording pipette, were recorded.

3.3 Analysis of data

3.3.1 Quantal parameters

In the studies presented here, analysis of the quantal parameters, n (number of release sites), p (release probability) and q (quantal size) were used to determine where the observed changes in synaptic transmission occurred.

3.3.1.1 Coefficient of variation analysis

The coefficient of variation (CV) is the normalized variance (standard deviation divided by the mean) and is useful for the study of changes in quantal content ($m = n \times p$) (Korn and Faber, 1991), since m is proportional to $1/CV^2$. Thus, changes in n or p will affect the $1/CV^2$ measurement, while changes in q will not. This method was first used in the neuromuscular junction and relies on a few assumptions, such as a uniform p and a large n, which are not always fulfilled in recordings from CNS synapses (Korn and Faber, 1991). Nevertheless, the use of CV analysis on EPSC recordings from CA1 pyramidal cell synapses has been empirically validated (Manabe et al., 1993, Chen et al., 1998).

3.3.1.2 Paired-pulse recordings

When the same set of synapses is stimulated twice within a short time, the two compound post synaptic currents will not have the same size (see Glutamate release in the Introduction). The paired-pulse ratio (PPR, the size of the 2nd divided by the size of the 1st PSC) is commonly used as a measurement of p, where a high p results in a low PPR (Zucker and Regehr, 2002). Changes or the absence of changes, in PPR can also be used as a complement to the $1/CV^2$ analysis, to distinguish between changes in n and p. There are, however, some problems with the interpretation of PPR analysis. First, if the release probability changes as a result of changes in vesicle pool size, the resulting change in PPR is very small or undetectable (Hanse and Gustafsson, 2001a). Second, if a change in n or q is specific for a subpopulation of synapses with a high, or a low, release probability this will also change the PPR, and thus falsely be interpreted as a change in p.

3.3.2 The multiplicity method

The multiplicity method was introduced in 1998 by Hsia et al (1998), and it compares the amplitude of spontaneous action potential dependent EPSCs to the amplitude of action potential independent EPSCs (mEPSCs). This ratio is calculated from the frequency and average amplitude of spontaneous EPSCs (F_s and Q_s, respectively) and mEPSCs (F_m and Q_m, respectively) as follows:

$$\text{Multiplicity} = [(F_s \times Q_s) - F_m \times Q_m)] / [(F_s - F_m) \times Q_m]$$

If the release probability in the studied synapse population is known, this multiplicity measure can be converted to an average connectivity, i.e. the average number of release sites connecting a pair of cells. The fact that the multiplicity method gives an average estimate of the connectivity is actually one of its main limitations; if there is heterogeneity in connectivity depending on the presynaptic cell, this is not revealed using this method. Another limitation is that only synaptic events from proximal synapses are assumed to be detected in recordings of spontaneous synaptic currents (Soltesz et al., 1995, Williams and Mitchell, 2008). Thus, results obtained using the multiplicity method are preferentially relevant for these synapses. Further, stability in F_m and Q_m is assumed for this analysis. Hsia et al (1998) performed control experiments, confirming that these factors are indeed stable over time in whole-cell recordings. There is however one study showing that *p* can change rapidly and persistently in individual synapses, due to changes in the size of the active zone (Matz et al., 2010). However, that study was performed in cultured hippocampal neurons, and the relevance of their finding for our rather brief recordings in acute slices is hard to estimate.

3.4 Statistics

Data is expressed as mean ± SEM (standard error of the mean). Statistical significance for paired and independent samples was evaluated using *t*-test, except for PPR values, where geometric mean and SEM was used, in order to circumvent the problem of averaging ratios.

3.5 Drugs

Tetrodotoxin (TTX) was used to block voltage gated sodium channels, and thus action potentials. The expression of sodium channels differs between cell types. Interneurons in the mouse hippocampus are more dependent on the 1.1 subtype than are pyramidal cells (Mullen and Scheffer, 2009). TTX has, however, similar IC_{50} values for all sodium channels expressed in the hippocampus (Clare, 2010). There should thus be no difference in the effectiveness of TTX in blocking action potentials in interneurons compared to pyramidal cells.

Two different NMDA receptor blocking agents were used in **Paper III**, the commonly used NMDA receptor antagonist D-AP5 and the open channel blocker memantine. Memantine is

the only NMDA receptor antagonist that is approved for clinical use. It exhibits a voltage dependent block, such that it leaves the channel pore when the cell membrane is depolarized (Blanpied et al., 1997). It has also been suggested to have relatively larger effect on extrasynaptic receptors than on synaptic ones (Leveille et al., 2008, Milnerwood et al., 2010, Okamoto et al., 2009, Xia et al., 2010).

4 RESULTS AND DISCUSSION

4.1 Silent synapses and AMPA silencing in interneurons

In **Paper I** we showed that AMPA silent synapses are present in interneurons of the CA1 stratum radiatum. We showed this directly using minimal stimulation of Schaffer collaterals and recordings from single synapses in hippocampal slices from rats aged 7 to 12 days. These AMPA silent synapses show distinct NMDA receptor mediated EPSCs when the cell is clamped at a positive holding potential (+40 mV), however, when switching to a negative holding potential (-80 mV) no AMPA receptor mediated EPSCs are observed (**Paper I**, Figs. 2A and B). We also compared failure frequencies at negative and positive holding potentials (**Paper I**, Fig. 2C). These data indicated that, in interneurons, there are not only AMPA silent synapses, but also NMDA silent ones. In two cells we demonstrated this directly by recording single NMDA silent synapses (**Paper I**, Fig 2D). These NMDA silent synapses probably corresponding to the synapses containing GluA2 lacking AMPA receptors previously described in stratum oriens interneurons (Lamsa et al., 2007). This is supported by our observation that the NMDA silent synapses lacked AMPA receptor mediated EPSCs at positive holding potentials (**Paper I**, Fig. 2D), typical for GluA2 lacking receptors.

It has been suggested that all glutamatergic synapses onto principal cells are formed with functional, although labile, AMPA receptors (Groc et al., 2006). Upon activation, a subset of the synapses is AMPA silenced (see Introduction, Xiao et al., 2004, Abrahamsson et al., 2005, Abrahamsson et al., 2007, Abrahamsson et al., 2008). We show here that glutamatergic synapses onto interneurons can also be AMPA silenced. AMPA silencing in interneurons share critical features with that in principal cells; while the magnitude of AMPA receptor mediated EPSCs decays, the NMDA receptor mediated EPSC is stable (**Paper I**, Figs. 3 and 8); AMPA silencing is independent of the activation of NMDA receptors and mGluRs (**Paper I**, Fig. 4); the PPR is unaffected by AMPA silencing (**Paper I**, Figs. 4 and 7); AMPA silencing is associated with a decrease in quantal content (**Paper I**, Figs. 5 and 7). In two experiments we also observed a total disappearance of the AMPA receptor mediated EPSC with a spared NMDA receptor mediated EPSC (**Paper I**, Fig. 6). Our most important finding here was that this AMPA silencing, and by implication AMPA silent synapses, is present in interneurons beyond the developmental period. This is in profound contrast to what has

previously been shown for principal cells (Table I, **Paper I**, Figs. 7 and 8). Since AMPA silent synapses on principal cells are restricted to the developmental period of intense synaptogenesis and synapse elimination, a possible implication of our finding would be that interneurons exhibit intense synaptic turnover throughout life. This idea is supported by the findings of Lee et al (Lee et al., 2008, Lee et al., 2006) that interneurons express a higher level of structural dendritic plasticity than do principal cells.

AMPA silencing could be explained by several different pre- or postsynaptic effects. Available data, however, point towards a postsynaptic removal of functional AMPA receptors in a subset of synapses (Kerchner and Nicoll, 2008). Since AMPA silencing was not associated with a change in PPR, a simple change in release probability is not likely. Alternative presynaptic events that could explain the existence of AMPA silencing include creation of presynaptically silent, "mute", synapses or a change in release mode, e.g. to a "kiss-and-run" release. An increased number of mute synapses should give rise to a decrease, not only of the AMPA EPSCs, but also of the NMDA EPSCs, which is not the case for AMPA silencing (Paper I, Figs. 3 and 8, Xiao et al., 2004). Xiao et al also used a low affinity NMDA receptor antagonist (L-AP5, 100µM) to exclude the possibility of a reduction in glutamate concentration in the synaptic cleft following a change in release mode. Possible postsynaptic mechanisms for AMPA silencing could be a reduced AMPA receptor conductance or a reduction in the number of functional synaptic AMPA receptors. The fact that AMPA silencing is associated with a decrease in quantal content (i.e. $1/CV^2$, **Paper I**, Figs. 5 and 7, (Abrahamsson et al., 2005, Xiao et al., 2004)) rules out a change in receptor conductance (since that would give a change in q, and thus would not be detected by the variance analysis, see Methodological considerations). In fact, since a change in release probability has already been ruled out, the reduced quantal content can only be explained by a decreased number of detected synapses, a reduced n. This could be achieved in at least two ways, either by an increase in endocytosis of AMPA receptors following glutamate binding (Ehlers, 2000), or by a rupture of the anchoring of receptors in the PSD, which would allow for the receptors to laterally diffuse out of the postsynaptic membrane (Gerrow and Triller, 2010).

4.2 LTP at AMPA silent interneuron synapses?

Shortly after the first indications of AMPA silent synapses on principal cells were presented, several studies showed that AMPA silent synapses can be unsilenced, i.e. converted to AMPA

signalling synapses after a Hebbian LTP induction (Durand et al., 1996, Isaac et al., 1995, Liao et al., 1995). This AMPA unsilencing is likely dependent on PKA activity (Yasuda et al., 2003) and LTP during the second postnatal week in the CA1 region appears to be exclusively explained by AMPA unsilencing (Abrahamsson et al., 2008). Thus, the existence of AMPA silencing seems necessary to allow for LTP in principal cells from young rats.

After the second postnatal week there is a developmental shift in LTP in principal cells, such that it gradually becomes dependent on αCaMKII (Yasuda et al., 2003). (I will, in accordance with Abrahamsson et al (2008) refer to this LTP as *mature LTP*.) Interestingly, this LTP switch coincides with the disappearance of AMPA silencing (Xiao et al., 2004) and, roughly, with the first evident signs of hippocampus dependent learning in the rat (Dumas, 2005). Mature LTP importantly differs from developmental LTP in that it actually strengthens the synapses, not merely de-depress them (Abrahamsson et al., 2008). Interneurons from the adult rat hippocampus do not express αCaMKII (Sik et al., 1998), and are consequently not expected to express mature LTP. Nevertheless, Lamsa et al (2005) showed that interneurons in the hippocampus from three- to four-week old rats do express Hebbian, NMDA receptor dependent LTP. This raises the question of whether this interneuronal LTP is also an *unsilencing LTP*. My preliminary results obtained in perforated-patch recordings from adult interneurons indeed indicate that LTP at these synapses converts AMPA silenced synapses to AMPA signalling synapses, a de-depression. A corollary of this reasoning is that AMPA silencing is in fact the predicted LTD that is complementary to Hebbian LTP at these synapses (see Long term depression in Introduction).

4.3 Development of multiple connectivity

The change from unsilencing LTP to mature LTP in pyramidal cells also parallels the development of glutamatergic multi-release site connections to these cells (Hsia et al., 1998). How this development of multi-release site connections comes about is not known. It is however tempting to speculate that it is a structural outcome of LTP expressed in these connections. This idea, in combination with our premise that the interneuronal LTP is actually an unsilencing LTP, lead us to the hypothesis that multi-release site glutamate connections might be largely absent in interneurons.

4.3.1 Multiplicity

In **Paper II** we have used the multiplicity method (see Methodological considerations) introduced by Hsia et al (1998) to examine how the connectivity of glutamate and GABA synapses develops in interneurons in the rat hippocampus. Previous studies (Hsia et al., 1998, Groc et al., 2003) have shown that, whereas GABAergic connections can form as multiple release site connections, glutamatergic connections form as single release site connections and can only later develop into multi-release sites connections. In **Paper II** (Fig. 1) we first confirmed the findings of Hsia et al regarding multiple glutamatergic connectivity onto adult CA1 pyramidal cells. When instead recording from CA1 stratum radiatum interneurons we found, however, that the multiplicity failed to develop above 1 (**Paper II**, Fig. 3), indicating that this glutamatergic connection maintains a single release site connection throughout development. We also studied GABAergic connections onto these interneurons, and found that they develop similarly to those onto principal cells, i.e. they start out as multiple release site connections in the neonatal rat and lack a developmental change in this respect (**Paper II**, Fig. 4). The findings of the previous two studies and the present one are summarized in a schematic figure (**Paper II**, Fig. 5).

Another way to articulate the meaning of these findings is that in adult animals the impact, or the weight, of all presynaptic cells on the firing probability of one specific interneuron will vary only with respect to release probability and quantal size. The impact of the cells contacting one pyramidal cell will, in addition, vary depending on the number of release sites connecting the two cells. For future experiments it would thus be very interesting to examine glutamatergic connectivity onto pyramidal cells in animals devoid of mature LTP, such as mice with a GluN1 deletion specific for CA1 pyramidal cells (Tsien et al., 1996), in order to map the importance of mature LTP for the development of multi-release site connections.

4.3.2 Structural basis for multiple connectivity

One major unanswered question in this thesis (**Paper II**) regards the structural basis for the glutamatergic multiple release site connections seen onto hippocampal CA1 pyramidal cells from adult rats. It is, however, possible to picture three different scenarios: i) a single release site synapse that allows multivesicular release, which will increase the transmitter concentration reaching the postsynaptic receptors, ii) a multi-release site synapse, where

released transmitter act on independent sets of postsynaptic receptors, iii) several distinct single release site synapses (**Paper II**, Fig. 5B). There are some indications that multivesicular release might occur at the mature CA3-CA1 synapse, at least under high release probability conditions (Christie and Jahr, 2006, Lisman and Raghavachari, 2006, Oertner et al., 2002). This does not seem to be the case for the same connection during the first two postnatal weeks, when release has proven to be univesicular (Hsia et al., 1998, Groc et al., 2002, Hanse and Gustafsson, 2001b, Raastad et al., 1992). This issue of uni- versus multivesicular release is, however, intricate due to the fact that the NMDA receptor has a higher affinity for glutamate than the AMPA receptor. This feature makes the NMDA receptor more apt to respond to intrasynaptic spillover of glutamate (Lisman and Raghavachari, 2006). The choice of postsynaptic reporter (AMPA or NMDA receptors) will thus possibly affect the results of this kind of studies. The remaining two scenarios (see **Paper II**, Fig. 5B, middle and lower panel) are difficult to distinguish with electrophysiological methods. There is, however, morphological evidence from the neocortex (Markram et al., 1997, Thomson and Lamy, 2007), and also from the CA1-CA3 connection (Sorra and Harris, 1993), showing the possibility of several morphologically distinct connections between two given cells. The structural basis for multiple connectivity remains, thus, an open question.

4.4 Extrasynaptic NMDA receptors

Although expected to counteract tonic GABAergic inhibition (Mann and Mody, 2010), glutamate's physiological effects on extrasynaptic NMDA receptors have been somewhat overlooked. On the other hand, extensive work has focused on the outcome of pathological activation of these receptors, and the current idea is that excess activation of extrasynaptic NMDA receptors activate intracellular signalling pathways eventually leading to cell death (Hardingham and Bading, 2003). This process is thought to be instrumental in the excite-toxicity in association with hypoxia (Moskowitz et al., 2010) and neurodegenerative diseases, such as Alzheimer's (Ittner et al., 2010) and Huntington's disease (Okamoto et al., 2009).

4.4.1 Tonically active extrasynaptic NMDA receptors

In **Paper III** we made use of the NMDA receptor open channel blocker memantine and the NMDA receptor antagonist D-AP5 to examine possible tonic excitation via NMDA receptors in stratum radiatum interneurons. We first show that 10 µM memantine, a concentration 10

times higher than what has been suggested to be therapeutically relevant, has no effect on isolated NMDA fEPSPs of the CA1 stratum radiatum (**Paper III**, Fig. 1). The same concentration nevertheless causes disinhibition of pyramidal cells (Dimpfel, 1995), as shown by reduction of disynaptic GABA receptor mediated IPSCs (**Paper III**, Fig. 2). It has been suggested the disinhibition caused by NMDA receptor antagonists could result from interneuronal synaptic NMDA receptors having a higher affinity for memantine than those of pyramidal cells (Lisman et al., 2008). We thus examined isolated NMDA receptor mediated EPSCs from pyramidal cells and interneurons, however, 10 µM memantine did not affect the EPSC magnitude in either cell type (**Paper III**, Fig. 4).

In Fig. 5 (**Paper III**) we showed that both pyramidal cells and interneurons express a tonic, D-AP5 sensitive current. This tonic NMDA receptor current appeared to run down during whole-cell conditions, which is consistent with previous reports for extrasynaptic NMDA receptors in pyramidal cells (Rosenmund and Westbrook, 1993) and synaptic NMDA receptors in relatively mature interneurons (Lamsa et al., 2005, Riebe et al., 2009). Nevertheless, at a holding potential of –40 mV this tonic NMDA receptor current was more prominent in interneurons than in pyramidal cells. We further showed that in interneurons there is a tonic memantine sensitive current with an I-V profile that fits well with the one for the AP5 sensitive current at negative holding potentials (**Paper III**, Fig. 6). At positive membrane potentials the blocking effect of memantine on the NMDA receptor is clearly reduced (Blanpied et al., 1997), explaining why the memantine sensitive current is smaller at positive membrane potentials. Thus, it seems as if extrasynaptic, tonically active NMDA receptors are more sensitive to memantine than the synaptic NMDA receptors are. Finally we recorded population spikes in the stratum pyramidale/oriens and observed a dramatic increase following application of memantine (**Paper III**, Fig. 7). This result is consistent with disinhibition of the pyramidal cells following reduced excitability in the interneurons.

4.4.2 Tonically active extrasynaptic NMDA receptors have a larger impact on the intrinsic excitability of interneurons than on pyramidal cells

We show here that the tonic NMDA current at +40 mV is of the same magnitude in both pyramidal cells and interneurons. At negative holding potentials (–40 mV), however, the current is larger in the interneurons (**Paper III**, Fig. 5). These data are not corrected for cell

size or total membrane conductance. The difference in relative current at –40 mV indicates a lower Mg^{2+} sensitivity of the NMDA receptors expressed in interneurons. NMDA receptors containing the GluN2D subunit display a lower Mg^{2+} sensitivity, and also a higher glutamate affinity (Traynelis et al., 2010). The GluN2D subunit has been suggested to be expressed in pyramidal cells of neonatal rats and to be down regulated during the first postnatal month (Kirson et al., 1999). Further, it has been suggested that the GluN2D subunit is expressed in, at least a subset of, interneurons in the adult rat (Monyer et al., 1994, Standaert et al., 1996) and hitherto there is, as far as we know, no evidence that they are synaptically located (Brickley et al., 2003). Since GluN2D containing NMDA receptors, in the presence of 1 mM extracellular Mg^{2+}, are more sensitive to memantine than receptors containing GluN2A or GluN2B (Kotermanski and Johnson, 2009), the presence of GluN2D in tonically active extrasynaptic NMDA receptors could explain the finding that these receptors are partially blocked by memantine in a concentration that has no effect on synaptic receptors (**Paper III**, Fig. 6).

4.4.3 Rundown of extrasynaptic NMDA receptors

Synaptic NMDA receptor mediated EPSCs in hippocampal interneurons from adult rats run down in whole-cell recordings (Lamsa et al., 2005, Riebe et al., 2009), which is in contrast to the stable conditions observed in both pyramidal cells (Xiao et al., 2004) and interneurons (Riebe et al., 2009) from young rats. As far as we know there are no observations of synaptic NMDA receptor rundown in pyramidal cells from adult rats. The data presented in **Paper III** (Figs. 3 and 5) of this thesis strongly indicate that tonically active extrasynaptic NMDA receptors also run down, at least in interneurons. This rundown will underestimate the tonic NMDA receptor mediated currents. The D-AP5 sensitive current recorded at +40 mV in our study, after more than 20 minutes of whole-cell recording, corresponds to a conductance of about 0.5 nS, which is approximately the size of the conductance that is washed out during 5 of the first 15 minutes of whole-cell recording (**Paper III**, Fig. 5). If an exponential decay is assumed (c.f. **Paper III**, Fig. 3), then this indicates that the tonic current in an intact interneuron would possibly be at least threefold larger than the one we have measured. The possibility that extrasynaptic NMDA receptors are very sensitive to rundown is further supported by the fact that the current response to bath-applied NMDA runs down in whole-cell recordings (Rosenmund and Westbrook, 1993). This rundown was however prevented by the use of an ATP-regenerating intracellular solution. For future experiments aimed at the

study of tonically active extrasynaptic NMDA receptors it would thus be interesting to use this kind of solution in order to try to prevent the rundown.

4.4.4 Pathophysiological implications of tonic NMDA

The NMDA receptor open channel blocker memantine, used here to block tonically active NMDA receptors, is used clinically to treat patients with moderate to severe Alzheimer's disease (Chen and Lipton, 2006). One explanation for the positive effect of memantine on the symptoms of these patients is that it reduces excitotoxic cell death occurring as a result of excess activation of extrasynaptic NMDA receptors following increased glutamate concentrations (Hardingham and Bading, 2003, Leveille et al., 2008). Protection against excitotoxicity by memantine treatment has recently been shown to be beneficial also in models of Huntington's disease (Milnerwood et al., 2010, Okamoto et al., 2009). There is, however, some evidence that the doses used to prevent cell death in animal models are associated with negative side effects, i.e. severe motor impairment and reduced memory function (Creeley et al., 2006). It seems thus rather likely that doses used to treat patients with Alzheimer's disease are lower, since no severe adverse effects are observed (Chen and Lipton, 2006).

Another possible explanation for the therapeutic effect of memantine is given by the fact that low doses of NMDA receptor antagonists have been shown to disinhibit glutamatergic principal cells (Dimpfel, 1995, Greene, 2001, Grunze et al., 1996, Homayoun and Moghaddam, 2007, Li et al., 2002, Maccaferri and Dingledine, 2002) (Fig 2 and 7 Paper III), thus, probably facilitating LTP (Wigström and Gustafsson, 1983). The data presented here provides a plausible explanation for this disinhibition, since my data indicate that the inhibitory interneurons have a higher background excitation obtained via tonically active NMDA receptors. These data are obtained from healthy animals, and it would thus be very interesting to investigate if this relative difference in tonic NMDA receptor mediated excitation between interneurons and pyramidal cells persists in experimental models of Alzheimer's disease.

Disinhibition of pyramidal cells has also been suggested to causally underlie the symptoms of schizophrenia (Lisman et al., 2008). A commonly used model for schizophrenia is treatment with the NMDA receptor open channel blocker phencyclidine (PCP) (Mouri et al., 2007).

There is also evidence that memantine have behavioural effects similar to those of PCP (Erik Pålsson, personal communication and Wiley et al., 2003). These considerations, together with the fact that the gene coding for the GluN2D subunit has been suggested to be a susceptibility gene for schizophrenia (Makino et al., 2005), makes it highly attractive to investigate the possible involvement of tonically active NMDA receptors in the pathophysiology of schizophrenia.

5 GENERAL DISCUSSION

In this thesis I have taken a comparative approach to examine glutamate's effects on interneurons in the hippocampal CA1 area. Synaptic and extrasynaptic effects of glutamate have been compared with those on CA1 pyramidal cells. The main differences I have found are (Fig. 5); i) in interneurons AMPA silent synapses and AMPA silencing persist into adulthood, whereas they in pyramidal cells disappear during the first postnatal month, ii) in interneurons there is no developmental increase in glutamatergic synaptic connectivity, in contrast to the increase in pyramidal cells, iii) interneurons in adult rats are under a stronger influence of tonically active extrasynaptic NMDA receptors than are pyramidal cells. The data presented in this thesis show that glutamate transmission onto interneurons and pyramidal cells in the neonatal rat share common properties. It further shows that, whereas glutamate transmission onto pyramidal cells matures and changes some of its fundamental properties, the properties of glutamate transmission onto interneurons are largely preserved into adulthood. This would indicate that glutamate transmission onto interneurons is well adapted to act in the developing as well as in the mature network.

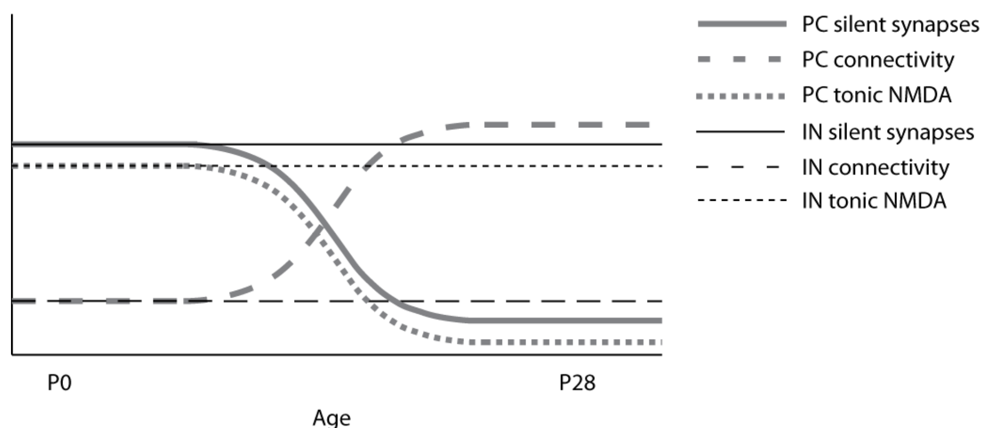

Figure 5. *Schematic illustration of the development of AMPA silent synapses, synaptic connectivity and tonic NMDA receptor currents in CA1 pyramidal cells and interneurons.*

5.1 Differences in glutamatergic transmission onto interneurons and pyramidal cells

Table I summarizes important differences between glutamatergic transmission onto interneurons and pyramidal cells in development and adulthood. The data in this table suggests three major phenotypes of the glutamate synapse. Below I will refer to them as “AMPA labile synapse”, “spine synapse” and “NMDA silent synapse” and they are schematically illustrated in Fig. 6.

	Young PC	Young IN	Adult PC	Adult IN	Refs
AMPA silent	+	+	-	+	**Paper I**; (Hsia et al., 1998); (Xiao et al., 2004)
NMDA silent	-	+	-	+	**Paper I**; (Lamsa et al., 2007); (Nyiri et al., 2003); (Laezza and Dingledine, 2004);
AMPA silencing	+	+	-	+	**Paper I**; (Xiao et al., 2004); (Abrahamsson et al., 2008)
Multi release site connections	-	-	+	-	**Paper II**; (Hsia et al., 1998)
Tonic NMDA	**0**	**0**	+	+	**Paper III**; (Le Meur et al., 2007); (Sah et al., 1989); (Herman and Jahr, 2007)
NMDA wash-out	-	-	**0**	+	**Paper I and III**, (Lamsa et al., 2005)
GluA2 lacking AMPARs	+	+	-	+	(Stubblefield and Benke, 2010); (Brill and Huguenard, 2008); (Shin et al., 2005); (Kumar et al., 2002); (Isaac et al., 2007)
GluA4 containing AMPARs	+	+	-	+	(Leranth et al., 1996); (Jensen et al., 2003); (Zhu et al., 2000)
Postsynaptic KARs	-	+	-	+	(Wondolowski and Frerking, 2009); (Cossart et al., 2002)
GluN2D	+	+	-	+	(Monyer et al., 1994); (Standaert et al., 1996)
Spines	-	-	+	-	(Fiala et al., 1998); (Seress and Ribak, 1985); (Craig et al., 1993);
αCaMKII	-	-	+	-	(Kelly et al., 1987); (Sik et al., 1998)
Arc	**0**	**0**	+	-	(Link et al., 1995); (Lyford et al., 1995)
Narp	+	+	-	+	(O'Brien et al., 1999)

Table I. Properties of glutamate synapses onto young and adult pyramidal cells and interneurons. *+ available data support the presence of, - available data indicates absence of, 0 to our knowledge no data available*

AMPA labile synapse

This type of glutamate synapse is found on pyramidal cells from developing rats and on interneurons throughout development. The “AMPA labile synapse” easily becomes AMPA silent by synaptic activation and regain AMPA signalling by absence of synaptic activation, or by NMDA receptor dependent LTP. This synapse is typically formed on the dendritic shaft, lacks αCaMKII and constitutes a single release site connection. (References in Table I)

Spine synapse

This glutamate synapse is typical for the pyramidal cells from adult rats. It has NMDA receptors and GluA2-containg AMPA receptors. It is located on dendritic spines and may be part of multi-release site connections from CA3 pyramidal cells. This synapse expresses classical NMDA receptor dependent LTP, which relies on αCaMKII. (References in Table I)

NMDA silent synapse

The third glutamate synapse phenotype is equipped with GluA2 lacking AMPA receptors and has few, if any, synaptically located NMDA receptors. It exists on interneurons and as has recently been shown on pyramidal cells from P5-7 rats. The induction of LTP at this synapse relies on Ca^{2+} influx through GluA2 lacking AMPA receptors (Lamsa et al., 2007) and perhaps also on activation of mGluRs (Perez et al., 2001). (Further references in Table I)

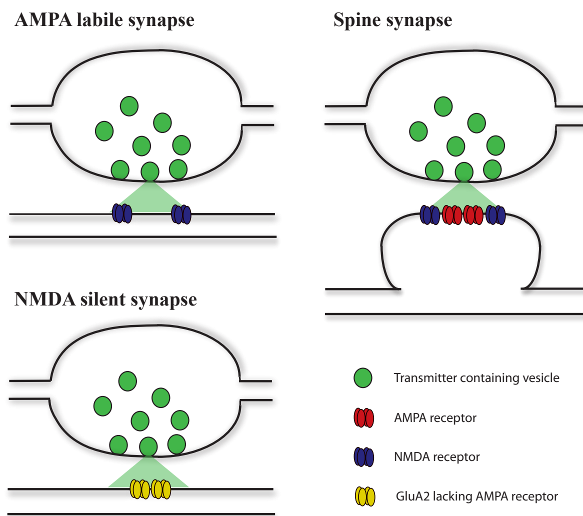

Figure 6. *Three different phenotypes of the glutamate synapse.*

6 CONCLUDING REMARKS

Based on the findings presented here, I suggest the following thesis: Glutamate signalling onto principal cells and interneurons differ in important aspects. These differences are brought about by a differential development from a common starting point.

The specific findings from my work in support of this thesis are the following:

I. AMPA silent synapses are present onto interneurons, and in contrast to AMPA silent synapses onto pyramidal cells, they are not restricted to the developmental period.

II. Pyramidal - interneuron cell pairs do not, in contrast to pyramidal - pyramidal cell pairs, acquire multiple release site connections during development.

III. Tonic NMDA receptor-mediated signalling has a lager impact on interneuron excitability than it has on pyramidal cell excitability.

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