ASTROCYTE-MEDIATED SHORT-TERM SYNAPTIC DEPRESSION

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ASTROCYTE-MEDIATED SHORT-TERM SYNAPTIC PLASTICITY

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Abstract
Short-term synaptic plasticity, the activity-dependent regulation of synaptic efficacy that occurs in the timeframe of milliseconds to seconds, is a fundamental property of the synapse, mostly attributed to changes in release probability. These changes are commonly ascribed to intrinsic mechanisms in the presynaptic terminal and to different transmitters acting on the presynaptic terminal. Astrocytes are the most abundant cell type in the brain. It has become increasingly clear that they can have a more active role in regulating neuronal signalling than their first established role of providing neuronal support. Astrocytes send out processes, which enwrap the synapses, in an ideal position to respond to synaptic transmission and in turn modulate synaptic function, such as short-term plasticity. However, not much is known about how astrocytes affect short-term synaptic plasticity.

The overall objective of this thesis was to examine the possible involvement of astrocyte-synapse signalling in short-term synaptic plasticity in the hippocampus. We used the acute rat hippocampal slice preparation and recorded the transmission at the glutamatergic CA3-CA1 synapses using extracellular and whole-cell patch-clamp recordings.

Hippocampal CA3-CA1 synapses as a population exhibit facilitation or augmentation milliseconds and seconds after a brief synaptic burst. However, we found that in the intermediate timeframe, between a couple of hundred milliseconds to seconds, these synapses exhibit a postburst depression (PBD). This PBD was found to be expressed as a reduction of release probability. The PBD displayed a cooperativity threshold as it was necessary to activate a critical number of synapses in order to elicit the depression. We found that the PBD develops over the first three postnatal weeks and that it is blocked when astrocyte metabolism is compromised. The PBD was blocked when a calcium chelator was delivered into the astrocytic network through a patch pipette, showing a requirement for astrocytic signalling.

Activation leading to PBD homosynaptically, also gave rise to a decrease in release probability in neighbouring inactive synapses, a transient heterosynaptic depression (tHeSD). The tHeSD developed over the same period as the PBD and was blocked by a blocker of astrocyte metabolism. In addition, the tHeSD was blocked by application of gap junction blockers. The tHeSD relied on $GABA_B$ and mGlu II/III receptors, but not on NMDA, adenosine A1 or mGlu I receptors.

Analysis of paired-pulse plasticity and relative vesicle pool size suggest that the tHeSD is expressed as a depression of resting vesicular release probability, causing a large increase of the paired-pulse ratio. In addition, the PBD was suggested to be a combination of vesicle depletion and augmentation, causing no change and a large decrease in paired-pulse ratio, respectively.

Hippocampal pyramidal neurons typically fire action potentials in short bursts in the behaving animal, at frequencies suitable for eliciting the PBD and the tHeSD. This suggests that astrocytes are critically involved in mediating a negative feedback on synaptic transmission after a burst of synaptic activity.

LIST OF PUBLICATIONS

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

I. My Andersson and Eric Hanse. Astrocytes impose post burst depression of release probability at hippocampal glutamate synapses. *Manuscript*.

II. My Andersson, Fredrik Blomstrand and Eric Hanse. Astrocytes mediate transient heterosynaptic depression at rat hippocampal synapses. *Journal of Physiology*. (2007) 585;843-852

III. My Andersson and Eric Hanse. Astrocyte-mediated short-term synaptic depression in the rat hippocampal CA1 area: two modes of decreasing release probability. *Manuscript*.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vår personlighet och våra tankar och känslor utgörs av aktivitet i nätverk av nervceller i hjärnan. Aktiviteten är elektrisk och förmedlas mellan nervcellerna över tunna spalter som kallas synapser genom att s.k. neurotransmittorer frisätts från den aktiva cellen. De flesta synapser i hjärnan använder glutamat som huvudtransmittor.

Effektiviteten hos synapsen kan ändras både över korta tidspann (millisekunder till minuter) och långa tidspann (timmar till år) beroende av hur aktiviteten ser ut, tidigare aktivitet och av aktiviteten hos närliggande synapser. Denna s.k. synaptiska plasticitet gör att synapser både kan bearbeta och lagra information. Förutom nervceller, består vår hjärna av en stor mängd s.k. astrocyter. Astrocyterna är, trots sitt namn, mer lika buskar än stjärnor och de använder sina små tunna utskott till att trycka sig mot synapserna medan de skickar iväg ett enda stort utskott till kontakt med hjärnans blodkärl. De har främst setts som hjärnans hushållningsceller vars främsta uppgift är att rensa synapsklyftan från neurotransmittor, hålla nervcellerna med transmittorbyggstenar och reglera blodflöde/syretillgång.

Syftet med min avhandling var att undersöka om astrocyter aktivt deltar i synaptisk korttids-plasticitet. Genom att mäta synaptiska strömmar i hippocampus-skivor från råtta, har jag visat att synapser i hippocampus uppvisar en tidigare okänd depression dvs en minskning av det synaptiska svaret efter kortvarig högfrekvent aktivitet (burst), som vi kallar en post-burst depression (PBD). Denna synaptiska depression beror på en minskad sannolikhet för frisättning av transmittor och påverkar inte bara de aktiva synapserna utan även närliggande inaktiva grannar, ett fenomen som vi kallat transient heterosynaptisk depression (tHeSD).

Genom att behandla astrocyterna med en drog som hämmar deras energiproduktion blockerades både PBD och tHeSD. Vidare kunde jag visa att PBD var beroende av kalcium-signalering i astrocyterna. Nervcellerna i hippocampus signallerar ofta i korta burst av aktivitet vilket gör vårt stimuleringsprotokoll efterliknar den naturligt förekommande aktiviteten i hippocampus.

Slutsatsen är att astrocyterna har en viktig funktion i regleringen av synaptisk kort-tids plasticitet genom att fungera som en negativ återkoppling efter hög-frekvent kortvarig aktivitet i aktiva synapser och genom att minska sannolikheten för frisättning av neurotransmittor i närliggande inaktiva synapser. Mina resultat bidrar till den begynnande insikten att astrocyter direkt kan påverka hur hjärnan bearbetar och lagrar information. Denna kunskap gör att man framöver i högre utsträckning bör överväga astrocyterna när man letar efter orsaker till olika sjukdomar i hjärnan.

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ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
AMPA	α-amino-hydroxy-5-methylisoxazole-4-propionic acid
ATP	Adenosine triphosphate
APCD	(2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CA	Cornu ammonis
cAMP	3'-5'-cyclic adenosine monophosphate
CGP 52432	3-[[(3,4-Dichlorophenyl)methyl]amino]propyl] diethoxymethyl)phosphinic acid
CV	Coefficient of variation
DAG	Diacyl glycerol
D-AP5	D-(-2)-amino-5-phosphopentanoic acid
DG	Dentate gyrus
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
EAATs	Excitatory amino acid transporters
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
Et-1	Endothelin-1
FAc	Fluoroacetate
fEPSP	Field excitatory postsynaptic potential
GABA	Gamma-aminobutyric acid
GAT	GABA transporter
$GABA_BR$	$GABA_B$-receptor
GLAST	Glutamate/aspartate transporter (EAAT1)
GLT-1	Glutamate transporter-1 (EAAT2)
GTP	Guanosine triphosphate
GFAP	Glial fibrillary acidic protein
5-HT	5-hydroxytryptamine, serotonin
IP_3	Inositol 1,4,5-triphosphate
IPSC	Inhibitory postsynaptic current
L-AP4	(2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate
LPP	Lateral perforant path
LTP	Long term potentiation

LTD	Long term depression
m	Quantal content
mf	Mossy fibres
mGluR	Metabotropic glutamate receptor
MPP	Medial perforant path
Munc-13	Mammalian homologue of *Caenorhabditis elegans* unc-13
MK-801	(5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cycl ohepten-5,10-imine maleate
n	Number of functional release sites
NMDA	N-methyl D-aspartate
p	Release probability
PBD	Postburst depression
PLC	Phospholipase
PIP_2	Phosphatidylinositol 4,5-biphosphat
PPF	paired-pulse facilitation
P_{ves}	Release probability of a single vesicle
PPR	Paired-pulse ratio
PSD	Postsynaptic density
PTP	Post tetanic potentiation
Ptx	Picrotoxin
q	Quantal size
RIM	Rab interacting molecule
SIC	Slow inward current
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SVLM	Small vesicle-like microvesicle
tHeSD	Transient heterosynaptic depression
TNF-α	Tumor necrosis factor-alpha
VGCCs	Voltage-gated calcium channels

INTRODUCTION

Information in the brain is passed on from one neuron to another via synaptic connections. This synaptic activity is the foundation for our person, our thoughts, and emotions. The information transfer in the brain is regulated at every synapse. Processes inherent to the neuron, as well as from the surrounding cells, take part in forming both long and short lasting changes in synaptic efficacy. Astrocytes, a subtype of glia cells, enwrap the synapses providing neurons with metabolic support *and*, as I will try to show in this thesis, take an active part in regulating synaptic transmission.

The tripartite synapse

The word synapse derives from the Greek word synapsis, meaning clasp, and was chosen by Sir Charles Sherrington in the late 1800s after advice from a friend working with classical languages (Tansey, 1997). In our body, we have two major types of synapses, the electrical and the chemical synapse. Electrical synapses are found in many cell types, not only neurons, and are especially important in heart, smooth muscle cells and astrocytes. The connection in these synapses is formed by gap junctions and transmission can be either unidirectional or bidirectional. In the central nervous system, chemical synapses are the most abundant ones. They are a unit composed of a presynaptic terminal and a postsynaptic density with astrocytic processes ensheathing the synapse, forming the tripartite synapse (Araque *et al.*, 1999). From the presynaptic terminal, transmitter filled vesicles can be released in response to a presynaptic action potential. This released transmitter diffuse over the synaptic cleft, and binds to its receptors on the postsynaptic membrane, causing opening of ion channels and thereby altering the membrane potential. This effect can be either excitatory or inhibitory, increasing or decreasing the probability for eliciting an action potential (respectively). The transmitters glutamate and GABA (γ-aminobutyric acid) are the most common excitatory and inhibitory transmitters, respectively, in the central nervous system (Ottersen & Storm-Mathisen, 1984). Synaptic effectiveness is not fixed but constantly regulated by previous activity in the synapse (synaptic plasticity), the activity of adjacent synapses, hormones, neuromodulatory transmitters (such as serotonin, dopamine, noradrenalin and acetylcholine) and gliotransmitters.

Presynaptic vesicular release

The presynaptic terminal visualised using electron microscopy shows hundreds of vesicles near and close to the active zone. A few of the vesicles are in direct contact with the cell membrane, thought to make up the so called readily releasable pool, while the majority of vesicles are not associated with the membrane, called the reserve pool. The synaptic vesicle travels through several stages in its lifecycle (Sudhof, 2004). It is born by budding of membrane from the endosome. To be able to fill the vesicles with transmitter, the vesicles express an ATP-dependent proton pump that creates a proton gradient, which then transmitter transporters use to fill the vesicle with transmitter. The vesicle can then be recruited to the cellular membrane, dock, and become primed for release. Following fusion of the vesicle, collapsing and releasing its contents, the vesicle is retrieved by chlatrin-meditated endocytosis (Granseth *et al.*, 2007) and finally fused with the endosome again. When the action potential depolarises the membrane, voltage-gated calcium channels (VGCCs) open and calcium can follow its steep electrochemical gradient into the terminal ($[Ca]_e$ 1.2 mM to $[Ca]_i$ 0.1 μM). Release of vesicles is triggered by the high calcium concentration (20-50 μM) in microdomains, within 100 nm from the VGCCs and the dominating forms of VGCCs, N and P/Q type, in the central nervous system have been shown to interact closely with the release machinery (Catterall, 1999; Khanna *et al.*, 2006). When the channels close, calcium returns to near baseline values due to diffusion, buffering and pumps (into internal stores and out to the extracellular space) leaving only a small residual calcium concentration (Lisman *et al.*, 2007). Due to the non-linear relationship between calcium concentration and probability of fusion (the power of four to five), small changes in the calcium concentration give rise to large changes of the vesicular release probability (see short-term plasticity). For the vesicle to be released it has to dock at the active zone and be primed for release (Neher & Sakaba, 2008). The small GPT-binding protein Rab3A, a vesicle-bound protein, is thought to be important in the docking and priming through the interaction with RIM (Rab interacting molecule) and its binding partner Munc-13 (Sudhof, 2004). For the vesicle to fuse, a release-machinery, the SNAP receptor (SNARE) complex, must form. This is composed of the synaptic vesicle protein VAMP/synaptobrevin and the cellular membrane associated proteins syntaxin and SNAP-25 (Sutton *et al.*, 1998). The calcium sensor for release has been proposed to be synaptotagmin 1, a synaptic vesicle protein, which binds calcium and interacts with syntaxin in a calcium-dependent manner (Lisman *et al.*, 2007). The docked fusion-complex is stabilised by the protein complexin, interacting with the SNARE proteins, until synaptotagmin

1 competes for complexin's binding site and the vesicle can collapse into the membrane (Rizo *et al.*, 2006; Sudhof & Rothman, 2009).

The glutamatergic synapse

About 85 % of the synapses in the central nervous system are glutamatergic (Megias *et al.*, 2001). These synapses are morphologically different depending on their postsynaptic target. Synapses between glutamatergic neurons are mostly spine synapses, located on dendritic spines, whereas synapses between glutamatergic and GABAergic interneurons are often shaft synapses, located directly on the dendritic shaft. The ionotropic glutamatergic receptors have been classified based on their ability to bind the different agonists, NMDA, AMPA and kainate. The main ionotropic receptors in the postsynaptic density are the AMPA and the NMDA receptors. The AMPA receptors are low affinity glutamate receptors permeable to monovalent cations and mediate the main part of the excitatory post synaptic current (EPSP) (Davies & Collingridge, 1989). The NMDA receptor is a high affinity glutamate receptor (Cull-Candy *et al.*, 2001) that requires the binding of a co-agonist to its (strychnine-insensitive) glycine site e.g. D-Serine. The NMDA receptor is voltage-gated because magnesium blocks the channel pore at resting membrane potentials. In addition to sodium and potassium permeability, the NMDA receptor also allows passage of calcium, making it a key player in the induction of long lasting synaptic plasticites. Both AMPA and NMDA receptors can have different properties depending on subunit composition and these subunits are also targets for posttranslational modification (Kohr, 2006; Greger *et al.*, 2007). Glutamate also binds to kainate receptors and metabotropic glutamate receptors on either side of the synaptic cleft. There are both ionotropic and metabotropic kainate receptors, which presynaptically act to decrease release probability (Lerma, 2006). The metabotropic glutamate receptor family is divided in to three groups. Activation of presynaptically located mGluR group II (mGluR 2-3) and mGluR group III (4, 6-8) inhibit glutamate release through inhibition of VGCCs (Schoepp, 2001). Postsynaptically located mGluR I (mGluR 1 and 5), whose activation induces calcium release from internal stores via the production of inositol 1,4,5-triphosphate (IP_3), are also able to decrease presynaptic release of transmitter by inducing the release of endocannabinnoids, which acts as retrograde signal (Kano *et al.*, 2009).

The GABAergic synapses

Inhibitory synapses are mostly GABAergic and express ionotropic $GABA_A$ receptors. GABAergic synapses are found on the cell soma, axon initial segment and dendritic shafts

(Megias *et al.*, 2001; Spruston, 2008). The $GABA_A$ receptor belongs to the same ligand-gated ion channel family as the nicotine, the glycine and the $5\text{-}HT_3$ receptors (Jacob *et al.*, 2008). The $GABA_A$ receptor consists of five subunits and there are eighteen different subunits known. The majority of $GABA_A$ receptors are composed of two α, two β and one γ (or one δ) subunit (Jacob *et al.*, 2008). When GABA binds to its receptor, chloride and bicarbonate ions can flow through the channel (with a permeability ratio of about 4:1). Depending on the concentration of chloride in the cell, the $GABA_A$ receptor mediated current can either hyperpolarise (low chloride), or depolarise (high chloride), the cell. This is developmentally regulated so that early in development GABA acts depolarising while it has a hyperpolarising action in the adult (Cossart *et al.*, 2005). Presynaptic metabotropic $GABA_B$ receptors decrease the probability of vesicular release by inhibiting VGCCs and decrease vesicular recruitment by negatively regulating adenylate cyclase (Sakaba & Neher, 2003). Activation of postsynaptic $GABA_B$ receptors gives rise to a slow IPSC by activating an inwardly rectifying K^+ conductance. $GABA_B$ receptors regulating GABA release are called autoreceptors while those regulating the release of other neurotransmitters are called heteroreceptors (Kornau, 2006).

Quantal transmission

In 1954 J. Del Castillo and B. Katz deduced, from experiments on the frog neuromuscular junction, that neurotransmitters are released from presynaptic terminals in discrete quanta (vesicles) from independent release sites (Del Castillo & Katz, 1954). According to the quantal theory of release, the efficacy of the synaptic transmission between two cells depends on the number of functional release-sites (n), the probability that an action potential will result in the release of a vesicle from the release site (p) and the postsynaptic effect of one released vesicle of transmitter (quantal size, q) (Fig. 1). The quantal content, the amount of transmitter released, m, is the product of $n \times p$. An average synaptic response would be the product of $q \times n \times p$ (Del Castillo & Katz, 1954). The number of functional release sites, n, is small (<10) at the vast majority of central glutamate synapses. For example, in the glutamatergic connection between CA3 and CA1 pyramidal neurons in the hippocampus n is one early in development (Hsia *et al.*, 1998; Groc *et al.*, 2003) and increases to on average about five when the developmental period is completed (Hsia *et al.*, 1998).

Release probability, p, can vary from zero ("presynaptically silent") to almost one at different release sites in the brain (Branco & Staras, 2009). At synapses between CA3 and CA1

hippocampal pyramidal neurons, a seemingly homogenous population of synapses, there is a large heterogeneity in *p* (Hessler *et al.*, 1993; Dobrunz & Stevens, 1997; Hanse & Gustafsson, 2001a). This large variation in p between release sites is also reflected in a large variation of the two factors that determine *p*; the number of vesicles available for release (pool) and the release probability of each vesicle (P_{ves}) (Hanse & Gustafsson, 2001c).
The quantal size, *q*, is in theory determined by a number of factors, such as the vesicular content of transmitter, the width of the synaptic cleft, the location of release relative to the postsynaptic cluster of receptors, the number, or the density, of postsynaptic receptors (Lisman *et al.*, 2007). In practise, variation in quantal size, such as in association with synaptic plasticity is usually attributed to variations in the number of postsynaptic receptors.

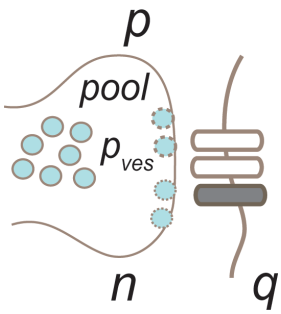

Figure 1. Quantal transmission
Presynaptic and postsynaptic quantal parameters.
Grey and blue circles represent the reserve pool while thin dotted circles and thick dotted circles represent docked and primed vesicles, respectively. Unfilled rods, AMPA receptors, filled rods, NMDA receptor.

Synaptic plasticity

Synaptic plasticity is the capacity of synapses to change their transmission efficacy in response to specific patterns of synaptic activity. The duration of such changes range from milliseconds to minutes, referred to as short-term plasticity, to minutes, hours, or longer, referred to as long-term plasticity. Homeostatic plasticity refers to the response to decreases or increases in firing rate with global up or down regulation of synaptic efficacy (Turrigiano, 2008). Plasticity of the plasticity itself (a change in the threshold for eliciting the plasticity) is called metaplasticity (Abraham & Bear, 1996).

Short-term plasticity

Short-term synaptic plasticity is in most cases a change in presynaptic release probability (*p*) (Zucker & Regehr, 2002). Activity-dependent increases of *p* are divided into facilitation, augmentation and post-tetanic potentiation (PTP). Facilitation can be induced by only a single action potential (referred to as paired-pulse facilitation, or PPF) and lasts tens to hundreds of

milliseconds. A short burst of high-frequency activation is necessary to induce augmentation, which typically decays with a time constant of about five seconds. Longer high-frequency trains of synaptic activation are required to induce PTP, which has a duration that depends on the number of stimuli in the activating train, and can last for minutes. Activity-dependent decreases of *p* are referred to as synaptic depression. Less is known about short-term synaptic depression, but both release-dependent (for example depletion of vesicles) and release-independent mechanisms are involved.

Long-term potentiation and long-term depression

An activity-dependent persistent enhancement of synaptic responses was first described in the intact rabbit dentate gyrus by Bliss and Lomo in 1973 (Bliss & Gardner-Medwin, 1973). A high frequency train (10-20 Hz for 10-15 s or 100 Hz, 1 s) resulted in an increase of the field excitatory potential (fEPSPs) that could last for days (Bliss & Gardner-Medwin, 1973) to a year (Abraham *et al.*, 2002), later named long-term potentiation (LTP). LTP was found to be input specific, only affecting the tetanised synapses (Andersen *et al.*, 1977) and its induction to exhibit a cooperativity requirement (a certain number of synapses needs to cooperate to induce LTP) (Gustafsson & Wigstrom, 1988). Postsynaptic depolarisation, activation of the NMDA receptors and an increase in postsynaptic calcium are necessary for the induction of LTP as voltage-clamping the postsynaptic cell, adding the NMDA receptor antagonist D-AP5 or injecting calcium chelating agents into the postsynaptic cell, blocked LTP (Collingridge *et al.*, 1983; Lynch *et al.*, 1983). The NMDA receptor was shown to mediate all of these requirements as Wigström and Gustafsson showed in 1986 that the NMDA receptor functions as a coincidence detector, sensing both presynaptically released glutamate and the postsynaptic membrane potential (Gustafsson & Wigstrom, 1986). In addition to the NMDA receptor-dependent LTP, there are also NMDA receptor-independent LTPs, e.g. at the mossy fibre – CA3 synapse (Malenka & Bear, 2004).

High frequency synaptic activity leading to LTP can also lead to a lasting depression of neighbouring non-tetanized synapses (Lynch *et al.*, 1977). This heterosynaptic depression is thus, in contrast to LTP, not input specific (see *Heterosynaptic depression*). It took until 1992 before homosynaptic long-term depression was described by Dudek and Bear (Dudek & Bear, 1992). Low frequency activation, 900 stimulations using a frequency of 1-3 Hz, was shown to result in a stable NMDA receptor-dependent depression of the fEPSP (Dudek & Bear, 1992). As for LTP, the induction of LTD depends on the induction frequency and the level of

postsynaptic depolarisation (Bear & Abraham, 1996). This latter criteria is also true for another form of LTD in the CA1 area whose induction does not require NMDA receptor activation, but instead relies on $mGluR_1$ activation and calcium-influx through T-type calcium channels (Oliet *et al.*, 1997). It is now clear that various forms of LTP and LTD exists, different forms in the same synapse, in different synapses and in the developing and aging brain (Malenka & Bear, 2004).

Heterosynaptic depression

As noted above, the induction of LTP using high-frequency stimulation is often accompanied by a heterosynaptic depression in neighbouring non-tetanized synapses. A picture is also emerging that this heterosynaptic depression is not a uniform entity, but can be divided into long-term (hours), intermediate (minutes) and transient (seconds) heterosynaptic depression with different pharmacological profiles. Long-term heterosynaptic depression requires strong, repeated, high-frequency activation and the induction relies on NMDA, but not adenosine A1, receptor activation (Lynch *et al.*, 1977; Scanziani *et al.*, 1996). The induction of intermediate lasting heterosynaptic depressions, on the other hand, requires activation of $GABA_B$ and adenosine A1 receptors in addition to NMDA receptor activation (Grover & Teyler, 1993; Manzoni *et al.*, 1994; Zhang *et al.*, 2003; Pascual *et al.*, 2005; Serrano *et al.*, 2006). Finally, transient heterosynaptic depression (tHeSD) does not rely on NMDA receptor activation (Gustafsson *et al.*, 1989; Isaacson *et al.*, 1993).

Glia, astrocytes and the definition of an astrocyte

The first glia cell recognized was the Müller cell in the retina named after its discoverer, Heinrich Müller, in 1851. Seven years later, Rudolph Virchow refers to the tissue between neurons as neuroglia, (glia, Greek for glue), thus implicating the lesser importance of these cells, making up the connective tissue between neurons (Kettenmann & Ransom, 2005). Today we recognize three major cell types in the glial cell-group in the central nervous system; astrocytes, microglia (bone marrow derived macrophages of the central nervous system) and oligodendrocytes (providing the axons with their myelin sheet) (Fields & Stevens-Graham, 2002).

Astrocytes are the most abundant cell type in the central nervous system outnumbering neurons by a factor 3 in rats and 4 in humans (Nedergaard *et al.*, 2003). They can be further divided in to three major subgroups; radial astrocytes (e.g. the Bergmann glia of the

cerebellum), fibrous astrocytes (white matter astrocytes) and protoplasmic astrocytes (grey matter astrocytes), which are the most abundant. Moreover, the morphology and function of the astrocytes are defined by their anatomical region yielding further distinct subsets of astrocytes, for example the retinal Müller cells (Emsley & Macklis, 2006). Astrocytes have been characterised by their ability to express the intermediate filament protein GFAP (glial fibrillary acidic protein) and the small calcium binding protein S100β. This classification can cause problems as all astrocytes do not express these proteins (Emsley & Macklis, 2006). Astrocytes were originally named after their star-shaped appearance in silver staining techniques. This star-shaped appearance of the astrocytes is also seen in immunohistochemical GFAP staining. With intracellular dye-filling it became clear that staining of GFAP only revealed the major astrocytic processes. Instead, the astrocyte looked more like a bush when the thin distal processes were visualised, with very little process overlap between astrocytes (Bushong *et al.*, 2002). The thin processes of astrocytes contact the synapses and it has been shown, using electron microscopy, that about half of the synapses in the hippocampus are covered with astrocytic processes (Witcher *et al.*, 2007). Most astrocytes send out major processes, called end feet, contacting a blood vessel or ventricular wall, forming an integral part of the blood-brain barrier. By close contact with the arterioles, astrocytes can give rise to both dilatation and constriction of the vessels, thereby regulating blood flow after the metabolic demand (Koehler *et al.*, 2009). This ability to regulate blood flow fits well with the established functions of astrocytes as the house-keeping cells of the brain e.g. clearing the extracellular space of transmitter, providing neurons with substrate for glutamate and GABA, and redistributing potassium (Ransom *et al.*, 2003).

Glutamate and GABA uptake

Astrocytes are responsible for a major part of glutamate and GABA clearance from the synaptic cleft (Danbolt, 2001; Conti *et al.*, 2004). The excitatory amino acid transporters (EAATs) are transcribed from a family of five genes, EAAT 1-5 and astrocytes express EAAT1 (GLAST) and EAAT2 (GLT) while neurons in the hippocampus express EAAT3 (Danbolt, 2001). The GABA transporters GAT 1 to GAT 3 are expressed in both neurons and astrocytes and GAT 3 is responsible for most of the astrocytic GABA uptake (Conti *et al.*, 2004). Both EAATs and GATs mediate glutamate uptake using the electrochemical gradient of sodium and potassium. In the cerebellum, glutamate uptake has been shown to be directly coupled to the Na/K-ATPase, as a large macromolecular protein complex (Rose *et al.*, 2009). Glutamate uptake is electrogenic and can be recorded as an inward current in patch-clamp

whole-cell recordings (Takahashi *et al.*, 1996; Bergles & Jahr, 1997). In addition, both glutamate and GABA binding to its transporter gates an anion flux (Sonders & Amara, 1996). Mice lacking the astrocyte GLT-1 transporter exhibit lethal seizures and their brains become more damaged in response to injury (Tanaka *et al.*, 1997) while astrocyte targeted GABA uptake blockers decrease seizure susceptibility (Schousboe, 2000). Retraction of astrocyte processes or blockade of glutamate uptake has been shown to mediate activation of presynaptic mGluRs in the supraoptic nucleus of the hypothalamus (Oliet *et al.*, 2001). This suggests that astrocytic glutamate and GABA uptake have an important role in controlling ambient levels of these transmitters and in limiting intersynaptic crosstalk.

Spill-over

Spill-over, the activation of receptors by transmitter diffusion beyond the synaptic cleft, has been reported for both glutamate and GABA. (e.g. Isaacson *et al.*, 1993; Asztely *et al.*, 1997) and has been suggested to be a key mechanism in heterosynaptic depression involving these transmitters (Isaacson *et al.*, 1993; Mitchell & Silver, 2000). Retraction of astrocyte processes, expressing transporters, has been shown to facilitate spill-over (Oliet *et al.*, 2001; Piet *et al.*, 2004) making astrocytes important contributors, both for preventing and mediating, intersynaptic crosstalk.

The "passive" astrocyte

In the 1960, Kuffler and colleagues showed that glial cells in the CNS of aquatic salamander, *Necturus*, exhibited passive membrane properties, with a linear current-voltage relationship consisting of a potassium leak conductance (Kuffler et al, 1966). Blocking gap junctions do not alter the passive properties and excised outside-out patches exhibit the same current-voltage relationship as whole cell currents (Blomstrand *et al.*, 2004; Schools *et al.*, 2006). This shows that the passive properties of the astrocytes do not depend on their extensive coupling, but on their large potassium conductance, mainly composed of K_{2P}-channels and K_{IR}-channels (Seifert et al, 2009; Zhou et al, 2009).

Potassium redistribution

Astrocytes control the extracellular concentration of potassium by uptake and intracellular redistribution (Simard & Nedergaard, 2004). When the extracellular concentration of potassium increases, for example as a consequence of intense synaptic and neuronal activity, there will be an inward potassium current in the astrocytes. This fast net-uptake of potassium

is balanced by influx of chloride through chloride channels and potassium is also transported together with chloride through the N, K, Cl-transporter (Su *et al.*, 2002). When potassium increases locally, influx of potassium in one region of the astrocyte is spatially buffered both in the individual astrocyte and in the astrocytic network, via the current flow through gap junctions (Wallraff *et al.*, 2006).

Gap junction coupling

The majority of mature astrocytes are coupled to each other, forming large astrocyte networks (Giaume, 2001, Blomstrand, 2004). This coupling is mediated by docking of two hexamer transmembrane spanning protein rings, one from each cell that together lines the gap junction pore. There are two protein families that make gap junctions in astrocytes; the connexins and the pannexins (Scemes *et al.*, 2009). The gap junctions make the astrocytes electrically coupled and are permeable to ions and molecules smaller than 1 kDa. Communication through gap junctions is regulated by e.g. second messengers and intracellular pH (Scemes *et al.*, 2009). Endothelin-1, an agonist of the astrocyte-expressed endothelin receptor A (Cahoy *et al.*, 2008) closes gap junctions via de-phosphorylation of connexin-43, the major astrocyte gap junction forming connexin (Blomstrand *et al.*, 2004).

Activating astrocytes

Astrocytes have been shown to respond to neuronal activity with increases in intracellular calcium (see *Calcium signalling*) both in brain slices and *in vivo* (Pasti *et al.*, 1997; Winship *et al.*, 2007). They express a variety of G-protein coupled receptors for neurotransmitters, such as acetylcholine and endogenous cannabinnoids (Araque *et al.*, 2002; Navarrete & Araque, 2008), as well as for hormones and cytokines (De Keyser *et al.*, 2008). Although the relative expression of various receptors may differ between different types of astrocytes, the main pathways described for activating astrocytes are through glutamate, GABA and ATP receptors, whose activation can lead to calcium increases in the astrocytes (Perea *et al.*, 2009b).

Calcium signalling

In 1988, MacVicar and Tse showed that although the astrocytes are not electrically excitable, they can respond to noradrenaline and cAMP with intracellular changes in calcium (MacVicar & Tse, 1988). This was followed by Cornell-Bell et al in 1990, who showed that increases in calcium, in response to glutamate application could spread to adjacent astrocytes, creating

waves of intracellular calcium rises (Cornell-Bell *et al.*, 1990). Calcium waves and oscillations do not depend on influx of calcium trough voltage-gated calcium channels (Verkhratsky *et al.*, 1998) although astrocytes *in vitro* have been shown to express both L-type and T-type calcium channels which are up-regulated in reactive astrocytes (Westenbroek *et al.*, 1998). The main way of triggering astrocytic calcium increases is through activation of G-protein coupled receptors in the astrocyte membrane, e.g. mGluR5, which are coupled to phospholipase C (PLC), cleaving phosphatidylinositol 4,5-biphosphate (PIP_2). This generates DAG and IP_3. IP_3 binds to IP_3 receptors (IP_3R) on the endoplasmatic reticulum (ER), which opens and allows passage of calcium to the cytosol (Verkhratsky *et al.*, 1998).
One could roughly divide astrocytic calcium increases into three qualitatively different types. These are the calcium waves, propagating to adjacent cells (Cornell-Bell *et al.*, 1990), the calcium oscillations, a global increase in calcium in the individual astrocyte (Zur Nieden & Deitmer, 2006) and finally the calcium transients, local calcium increases in astrocyte microdomains (Grosche *et al.*, 1999).

Calcium waves have yet to be shown under physiological conditions *in vivo*. For example, transgenic mice designed to mimic Alzheimer's disease (human amyloid precursor protein in combination with mutated presenilin), but not wild-type mice, exhibit spontaneous astrocytic calcium waves (Kuchibhotla *et al.*, 2009). Calcium oscillations and calcium transients in astrocyte soma or processes, on the other hand, have been shown *in vivo* in response to neuronal activity (Tian *et al.*, 2006; Winship *et al.*, 2007).

Gliotransmitter release

Several substances have been shown to be released in response to calcium increases in the astrocyte e.g. glutamate, ATP, D-serine and TNFα (tumor necrosis factor-alpha) (Theodosis *et al.*, 2008). Several possible release pathways have been described for these gliotransmitters.

Vesicular release

In 2000, Araque and co-workers showed that astrocytes are competent of SNARE-mediated vesicular release (Araque *et al.*, 2000). Since this article was published, investigations in several different preparations have shown gliotransmitter release via SNARE-mediated exocytosis (Santello & Volterra, 2009). Astrocytes express proteins associated with vesicular release (SNAREs etc) and contain small synaptic vesicle-like microvesicles (SVLM) (Bergersen & Gundersen, 2009).

Astrocyte vesicular release is voltage independent and instead relies on calcium increases induced by activation of metabotropic receptors. Although this discrepancy exists between neuronal and astrocytic release, astrocytes can release SVLM within tens of millisecond in response to microdomain increases in calcium (Marchaland *et al.*, 2008). Both full fusion and restricted fusion, so called kiss-and-run, have been described. Marchaland *et al* also showed that endoplasmatic reticulum and the SVLM are spatially co-localised giving a precise spatio-temporal relationship between release from internal stores and vesicular release (Marchaland *et al.*, 2008). Slower forms of vesicular release have also been described e.g. the lysosomal release of ATP (Jaiswal *et al.*, 2007; Zhang *et al.*, 2007).

Hemichannels

Several groups report that unopposed gap junction channels can release transmitters, such as glutamate, during conditions of low extracellular calcium concentration (Stridh *et al.*, 2008; Scemes *et al.*, 2009). These so called hemichannels are composed of the pore-forming transmembrane peptides connexin and pannexins. Their opening can be regulated by several stimuli including voltage, intracellular calcium, mechanical stretch (pannexins), extracellular calcium and pH (connexins) (Scemes *et al.*, 2009).

Volume-sensitive outwardly rectifying anion channels

Volume-sensitive outwardly rectifying anion channels are large transmembrane spanning channels that open in response to the mechanical stretch of the cellular membrane occurring when the intracellular volume increases (Kimelberg *et al.*, 2006). These channels have been shown to release glutamate and ATP in response to hypotonic conditions (Kimelberg *et al.*, 2006).

Reversed transport

The glutamate transporter is able to reverse since glutamate on either side of the membrane can activate the transporter (Szatkowski *et al.*, 1990; Wadiche & Kavanaugh, 1998). Reverse transport of glutamate has been suggested to mediate glutamate excitotoxicity and cell death in cases of energy deprivation (Hazell, 2007).

Astrocytes and synaptic transmission/plasticity

Early studies showed that astrocyte-derived glutamate depresses excitatory synaptic transmission in hippocampal cultures (Araque et al., 1998a; Araque et al., 1998b) and increases the frequency of inhibory spontanous currents in CA1 pyramidal cells (Kang *et al.*, 1998). Another powerful way in which astrocytes regulate synaptic transmission is through release of the inhibitory transmitter adenosine (Halassa *et al.*, 2009a). Knock-down of SNARE-proteins in astrocytes inhibits the tonic adenosine inhibition of release probability in slices (Pascual *et al.*, 2005) and astrocyte-derived adenosine plays a part in sleep-wake homeostasis (Halassa *et al.*, 2009b).

LTP and LTD

Activation of the NMDA receptor requires the binding of agonists to the glycine sites, in addition to the binding of glutamate. The endogenous ligand for this glycine site is likely D-serine which has many times greater affinity for this site than does glycine (Oliet & Mothet, 2009). Extracellular D-serine is derived from astrocytes and retraction of astrocyte processes in the supraoptic nucleus of the lactating rat impairs LTP, indicating that astrocyte-derived D-serine is necessary for NMDA receptor function, such as the induction of LTP (Panatier *et al.*, 2006). Astrocytes have also been implicated in cerebellar LTD, which is impaired in a $GFAP^{-/-}$ transgenic mouse (Shibuki *et al.*, 1996).

Astrocytes and heterosynaptic depressions

Astrocytes have been shown to both prevent and mediate heterosynaptic depression. Piet and colleagues showed that retraction of astrocyte processes in the hypothalamus allowed diffusion of glutamate to reach GABAergic terminals, resulting in inhibited release of tranmitter (Piet *et al.*, 2004). Astrocyte-derived adenosine has been shown to mediate heterosynaptic depression in the hippocampus (Zhang *et al.*, 2003; Pascual *et al.*, 2005) and the release of adenosine is inhibited by introducing the calcium chelator BAPTA into the astrocytic network (Serrano *et al.*, 2006). As indicated above (see *Heterosynaptic depression*) heterosynaptic depressions are not a homogenous group and it is not known whether briefer stimulation, a few impulses at high frequency (Gustafsson *et al.*, 1989; Isaacson *et al.*, 1993), is sufficient to activate the astrocytes, or if the astrocytes contribute to transient heterosynaptic depression.

Homeostatic plasticity

Astrocytes have been implicated in homeostatic scaling of synaptic transmission (Stellwagen & Malenka, 2006). Blockade of action potentials for two days causes a global increase in action potential independent, spontaneous EPSC amplitude in both neuronal-astrocyte co-cultures and in cultured hippocampal slices. This homeostatic scaling was shown to depend on astrocyte-derived TNFα, which leads to the postsynaptic insertion of AMPA receptors (Stellwagen & Malenka, 2006).

Slow inward currents

One form of astrocyte-to-neuron signalling that has received considerable attention is the astrocyte-mediated slow inward currents (SICs) through extrasynaptic neuronal NMDA receptors (Araque *et al.*, 1998; Angulo *et al.*, 2004; Fellin *et al.*, 2004). However, SICs now rather appear to be of pathophysiological interest. Kang and co-workers (Kang et al, 2004) showed SIC-like events when blocking potassium channels in the hippocampal slice. They referred to these SIC-like events as paroxysomal depolarising shifts, which are typical for epileptiform activity. Fiacco et al (Fiacco *et al.*, 2007) used a knock-in mouse expressing an exogenous type of metabotropic receptor under the GFAP-promoter, which is able to activate the endogenous IP_3 cascade. Activation of these metabotropic receptors did not evoke any SICs under normal conditions. In fact, the authors did not reveal any marked changes on synaptic transmission by activation of these receptors. However, SICs were observed during hypertonic conditions. Observations like these have raised doubts whether astrocytes participate in synaptic transmission and plasticity at all during physiological conditions (Fiacco *et al.*, 2009).

Development

The cells that constitute the hippocampal CA1 and CA3 area proliferate and migrate from the subventricular zone. These cells are formed in succession with neurons first, followed by the astrocytes and finally the oligodendrocytes (Sauvageot & Stiles, 2002). Neurogenesis is completed just before birth in the rat and mouse and neurons migrate to form the pyramidal cell layer during the first postnatal week (Altman & Bayer, 1990), a time point corresponding to about half way through human gestation (Hagberg *et al.*, 2002). A number of key events related to the development of astrocytes in the rat hippocampus are schematically depicted in Figure 2. Astrocytogenesis follows neurogenesis and the majority of astrocytes are formed during the first two postnatal weeks (Gressens *et al.*, 1992; Nixdorf-Bergweiler *et al.*, 1994; Setkowicz *et al.*, 1999). The formation of gap junctions coupled astrocytic networks in the

CA1 region parallels the main astrocytogenesis during the first two postnatal weeks (Schools *et al.*, 2006). Synaptogenesis starts during the first postnatal week, and the number of synapses is rapidly increasing during the first postnatal month (Steward & Falk, 1991). Astrocytes have been shown to secrete molecules capable of inducing synaptic formation (thrombospondin), inducing the expression of AMPA receptors in these synapses (unknown molecule) and enhancing presynaptic function (cholesterol) (Barres, 2008). Already during the first postnatal days it is possible to demonstrate gap junction coupled astrocytes in the stratum radiatum of the CA1 region, and the number of coupled astrocytes in these networks increase during the following two weeks (Konietzko & Muller, 1994; Schools *et al.*, 2006). The astrocytes gradually take on a more adult morphological appearance with fine spongiform processes appearing and astrocyte domains forming during the third postnatal week (Nixdorf-Bergweiler *et al.*, 1994; Bushong *et al.*, 2004). At the beginning of the first postnatal week astrocytes exhibit two electrophysiological phenotypes; one group of outward rectifying glia expressing inward sodium currents with an outward transient A-current and a delayed Kdr current (Zhou *et al.*, 2006) and one group of variable rectifying glia expressing both inward and outward rectifying potassium currents, but no sodium currents (Zhou *et al.*, 2006). At the end of the second postnatal week the majority of astrocytes exhibit passive membrane properties (passive glia in Fig. 2) (Zhou *et al.*, 2006). In parallel, during this developmental period, the rate of astrocytic glutamate uptake becomes faster (Diamond, 2005). Thus, during the period of synaptogenesis, astrocytes have a prominent role in nurturing synapses. It is unclear when, during development, the synapse-regulating role of astrocytes emerges.

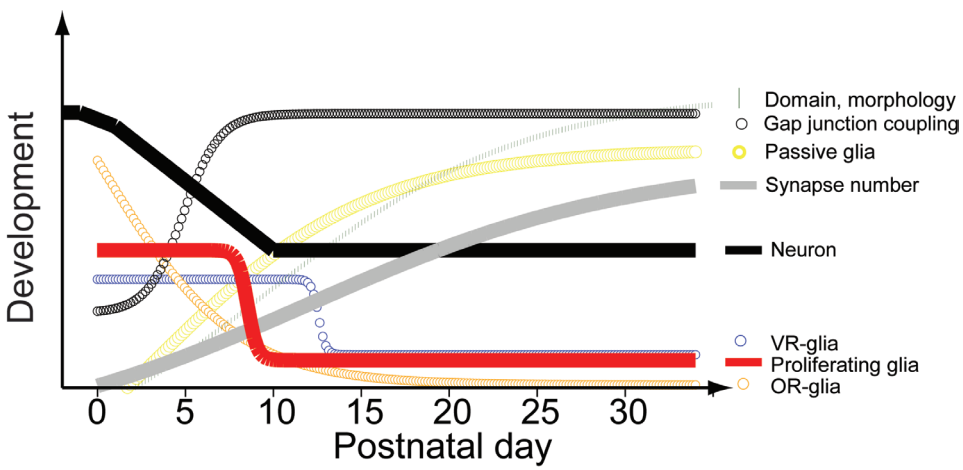

Fig. 2. The development of hippocampal astrocytes.

Figure depicting functional development of astrocytes with the arbitrary development on the Y-axis and postnatal day on the X-axis. Values are adapted from the following publications: Domain, morphology: The development of spongiform processes and domains in astrocytes (Bushong et al., 2004). Gap junction coupling: The number of astrocytes one (random) astrocyte couples to (Schools et al., 2006). Passive glia: The proportion of glia exhibiting passive membrane properties (Zhou et al., 2006). Synapse number: The number of synapses formed (Steward & Falk, 1991). Neuron: The number of pyramidal cells (Altman & Bayer, 1990). VR-glia: The proportion of glia exhibiting variable rectifying membrane properties (Zhou et al., 2006). Proliferating glia: Number of S100β expressing glia (Setkowicz et al., 1999). OR-glia: The proportion of glia exhibiting outward rectifying membrane properties (Zhou et al., 2006).

The hippocampus

The hippocampus is an integral area for declarative learning and memory, which in turn is vital for the planning and prediction of future events (Schacter & Addis, 2009). The CA1 region is the common output system from the hippocampal formation to the entorhinal cortex via the subiculum (Amaral & Witter, 1989). The hippocampal slices most commonly used for studying synaptic physiology are made from the dorsal hippocampus. This is an important point since the dorsal and ventral hippocampus differ from each other, both in terms of short (Izaki *et al.*, 2003) and long-term synaptic plasticity (Maggio & Segal, 2009). The Dorsal hippocampus is involved in cognitive and spatial aspects of memory whereas the ventral part of the hippocampus handles memories related to emotions and motivation (Bannerman *et al.*, 2004).

The hippocampus: endogenous activity

Hippocampal pyramidal neurons typically fire action potentials in short high frequency burst (40-100 Hz) (Buzsaki, 2002). This is especially evident during exploratory behaviour and during rapid eye movement sleep. These bursts appear at a frequency of 4-10 Hz, the theta frequency, thought to be critical for coding information in the hippocampus (Lisman & Buzsaki, 2008). To what extent astrocytes participate in this theta burst activity has not been examined, but it has been shown that synaptic activity at frequencies higher than 20 Hz activates calcium transients in astrocytes (Zur Nieden & Deitmer, 2006).

OBJECTIVE

The efficacy of synaptic transmission is constantly changing due to short-term synaptic plasticity. The astrocyte has emerged as a prominent signalling partner with the synapse, but little is known about to what extent astrocytes contribute to short-term plasticity. The overall aim of this thesis was to study the possible involvement of astrocyte-synapse signalling in short-term synaptic plasticity.

Specific aims

I. Do astrocytes contribute to homosynaptic short-term plasticity?

II. Do astrocytes contribute to heterosynaptic short-term plasticity?

III. How is synaptic transmission affected by astrocyte signalling during short-term plasticity?

METHODOLOGICAL CONSIDERATIONS

The hippocampal slice preparation

The hippocampus is one of the most well studied regions of the brain. It has well-defined connections and a mostly laminar structure making it suitable for the *in vitro* slice preparation (Fig. 3) (Andersen *et al.*, 2007). The entorhinal cortex is the main gateway to the hippocampus. Axons from the medial and lateral entorhinal cortex form the medial (MPP) and lateral perforant path (LPP), respectively, which make excitatory synapses with the granule cells of the dentate gyrus (DG). The axons of the granule cells, the mossy fibres (mf), make large excitatory synapses with the proximal part of the CA3 pyramidal neurons. The axons of the CA3 pyramidal neurons, the Schaffer collaterals, make en passant excitatory synapses on the apical (in the stratum radiatum) and on the basal (in the stratum oriens) dendrites of the CA1 pyramidal neurons. The axons of the CA1 pyramidal neurons run in the stratum alveus and project out of the hippocampus to the subiculum, whose pyramidal neurons further project back to the entorhinal cortex.

The glutamatergic synapses in the CA1 region are very well studies and have become model synapses for small cortical glutamatergic synapses. These synapses are made onto dendrites of CA1 pyramidal neurons that are arranged more or less in parallel, perpendicular from the pyramidal cell body layer. This organisation is very favourable for extracellular recordings of synaptic responses (field potentials). The slice preparation provides precise control over experimental conditions (compared to *in vivo*) and an intact cell circuitry (compared to cell cultures). However, as the modulatory inputs are severed there are virtually no modulatory transmitters present. Not much work has been focused on the viability of glia cells in the slice preparation and there are reasons to suspect that glial functions could change during the lifetime of the slice (Fiala et al, 2003). It is difficult to predict what functional consequences culture systems have on astrocytes, or which developmental stage they are in after several days *in vitro*. *In vivo*, on the other hand, drug applications and recording access to hippocampus are extremely technically challenging making the reduced acute slice preparation more suited for the aim of this thesis.

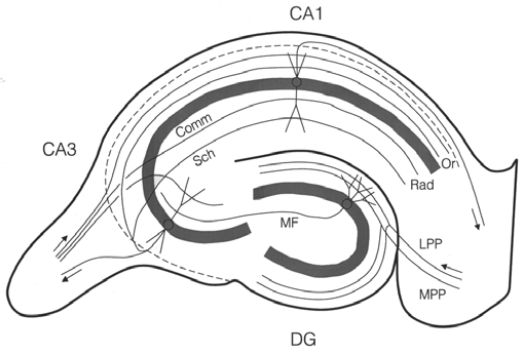

Fig. 3. ***Hippocampal slice*** *Comm, commisural fibres. Sch, Schaffer collaterals. Or, Stratum Oriens. Rad, Stratum Radiatum. LPP, lateral perforant path. MPP, medial parforant path. MF, mossy fibre. DG, dentate gyrus.*

Ethical permissions

My studies were performed in accordance with the regulations of the Swedish Animal Welfare law and approved by the local ethical committee for animal research of Gothenburg.

Preparation of hippocampal slices

Experiments were performed on dorsal hippocampal slices from 5- to 52-day-old Wistar rats. The rats were anesthetized with the inhalation anaesthetic isoflurane (Abbott) prior to decapitation. Isoflurane has a multitude of effects that might contribute to its anaesthetising potency including hyperpolarisation by activating two-pore domain K-leak channels, (Patel *et al.*, 1999), enhancing glutamate uptake (Zuo, 2001), affecting a wide range of voltage-gated channels (Campagna *et al.*, 2003) and inhibition of SNARE-mediated release (Herring *et al.*, 2009). Counterintuitively, a recent study suggests that the anaesthetising effect of isoflurane is related to cortical disinhibition (Ferron *et al.*, 2009). The hemispheres were dissected out and placed in an ice-cold (0-3°C) bubbled solution (95 % O_2 and 5 % CO_2) containing (in mM): 140 cholineCl, 2.5 KCl, 0.5 $CaCl_2$, 7 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1.3 ascorbic acid and 7 dextrose. Transverse hippocampal slices were cut (300-400 µm thick) with a vibratome. The slices were transferred to an incubation chamber that contained artificial cerebrospinal fluid (ACSF), bubbled with 95 % O_2 and 5 % CO_2, to recover from the preparation for at least one hour.

The recipe of the ACSF is made to mimic the salt composition of cerebrospinal fluid *in vivo*, although we have, depending on the experimental focus, changed the concentration of some of the salts. One example is the concentration of 4 mM calcium and 4 mM magnesium that I have used throughout my experiments, which are supraphysiological concentrations. The reason for this high concentration of divalent ions is to reduce neuronal excitability, which is

artificially increased in most of my experiments by blocking $GABA_A$-ergic inhibition by picrotoxin.

Extracellular field recordings

In my experiments, electrical stimulation of Schaffer collaterals/commissural axons and recordings of extracellular responses were carried out in the stratum radiatum of the CA1 region. The evoked response consists of a shock artefact, followed by the presynaptic volley (the reflexion of the spreading action potential through the axons) (Andersen *et al.*, 1978), and the field excitatory postsynaptic potential (fEPSP) (Fig. 4). The magnitude of the fEPSP was measured as the slope of the first 0.8 ms of the initial slope to avoid interference from spike activity. When recording fEPSPs one of the advantages is that the magnitude of the presynaptic volley can be monitored, together with the fEPSP, as a measure of the number of activated axons during the experiment. Another advantage, compared to the whole cell configuration, is that the cells that contribute to the response have their intracellular composition intact. A disadvantage, on the other hand, is that we do not know exactly which cells that contributes to our field response.

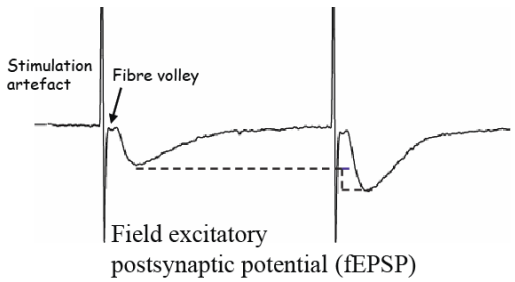

Fig. 4. The field response to a paired-pulse stimulus.

Whole-cell recordings

In whole-cell recordings, a high resistance seal (G Ω) is formed between the glass pipette and the cell membrane (Neher & Sakmann, 1976). Negative pressure is applied and a small hole in the membrane renders the cell interior in direct contact with the intracellular solution in the recording pipette. In whole-cell mode two different configurations are possible; voltage-clamp or current-clamp, monitoring whole-cell current or voltage, respectively.

Pyramidal cells were identified in the CA1 area by infrared differential contrast microscopy. The patch pipette contained a cesium-based solution and 20 mM BAPTA. The cesium helps in voltage-clamping the cell as it blocks potassium channels (Hille, 2001). BAPTA (1,2-**b**is(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) is a fast calcium chelator and 20 mM is expected to effectively buffer calcium in the pyramidal dendritic tree (Rozov *et al.*, 2001).

Astrocytes were visually identified in the stratum radiatum by their small soma (<10 µm) and their passive electrophysiological properties in the whole cell configuration (see Fig. 3, Paper I). The internal solution used was a potassium solution containing 50 mM BAPTA and the fluorescent dye, Lucifer Yellow. The diffusion of the dye is used to estimate the diffusion of the calcium chelator in the astrocytic network and is of comparable molecular size to BAPTA (Lucifer Yellow MW: 457 to BAPTA MW: 476). Both of these are well below the 1 kDa size limit for gap junction pores.

Drugs

Carbenoxolone is commonly used as a gap junction blocker, but it also has other effects, such as the inhibition of VGCCs (Rouach *et al.*, 2003; Vessey *et al.*, 2004). Therefore we also used an agonist of the endothelin A receptor, predominately expressed in astrocytes, endothelin-1, to block gap junctions (Cahoy *et al.*, 2008). The closure of astrocyte gap junctions is reversed after one hour of endothelin-1 application (Blomstrand & Giaume, 2006). The experiments were therefore performed within this time.

The metabolic inhibitor fluoroacetate (FAc) is selectively taken up by astrocytes due to their specific expression of acetate transporters (Fonnum *et al.*, 1997). Inside the astrocyte, FAc is converted to fluorocitrate which inhibits the aconitase in the Krebs cycle. We used FAc in paper II and observed a delayed depression on the baseline fEPSP, appearing after 45 minutes which was associated with an increase in the paired-pulse ratio (Fig. 3B, Paper II). The effect of FAc on the evoked synaptic depression was tested before this time point, between 20 and 40 minutes after the application of FAc.

Estimation of quantal parameters

In my studies, it has been pertinent to determine which of the quantal parameters *n*, *p* or *q* that is altered when synaptic efficacy is changed. I have used four different types of analysis

(paired-pulse ratio, coefficient of variance analysis, vesicle pool estimation and comparison of synaptic AMPA and NMDA EPSCs) to address this question.

Paired pulse ratio (PPR)

If two synaptic responses are elicited in close succession, the second response can be either facilitated or depressed depending on the initial release probability of the synapse population (Hanse & Gustafsson, 2001b). Changes in the PPR is frequently used to detect changes in release probability (Branco & Staras, 2009). A caveat of this is that changes in release probability caused by changes in the vesicle pool size results in no, or very small, changes in the PPR (Hanse & Gustafsson, 2001b; Abrahamsson *et al.*, 2005). Moreover, if postsynaptic changes occur in a subpopulation of low- or high release probability synapses a change in PPR might falsely be ascribed to a change in release probability.

Estimation of vesicle pool size

When synapses are activated by a train of high-frequency stimulation the vesicles available for release at the onset of the stimulation are rapidly depleted and new vesicles are quickly recruited in a calcium-dependent manner (Hanse & Gustafsson, 2001a; Neher & Sakaba, 2008). These considerations can be used as a basis for estimating the relative number of vesicles available for release at the onset of stimulation (Schneggenburger *et al.*, 1999; Abrahamsson *et al.*, 2005). The analysis is based on a plot of the cumulative EPSC amplitude. In such a plot, every value is the sum of the preceding values, resulting in a false decrease in variability. Nevertheless, by subtracting the contribution (back-extrapolating in the cumulative plot) of the newly recruited vesicles the vesicle pool size at the onset of the stimulation can be estimated (Fig. 3, Paper III). A problem with this analysis is that it (falsely) assumes that recruitment starts full throttle from the first impulse in the train. A peculiar consequence of this assumption is that if release probability during the recruitment phase is larger than during the initial depletion phase (as is the case for strongly facilitating synapses) the analysis will suggest a negative pool.

Coefficient of variance analysis

To determine if a change in synaptic efficacy is caused by a change in quantal content (m), or in quantal size (q), coefficient of variance analysis can be used (Korn & Faber, 1991). Since the evoked EPSC is the summation of multiple quanta released with a certain probability, then the standard deviation of the EPSC amplitudes normalised to the mean amplitude (the

coefficient of variation, CV) should vary reciprocally with quantal content, *m*. The inversed square, $1/CV^2$, is directly proportional to *m*. Thus, a change in $1/CV^2$ that is proportional to the change in synaptic efficacy is interpreted as a change in *m* (either *n* and/or *p*), whereas the absence of change in $1/CV^2$ is interpreted as a change in *q*.

Comparison of synaptic AMPA and NMDA EPSCs

Another test for discriminating between a change in quantal content, and a change in quantal size, is to compare the change in synaptic efficacy reported by the synaptic AMPA and NMDA receptors, respectively. An equal change is indicative of a change in *m*, whereas a selective change in, for example, the AMPA EPSC, is strong evidence for a change in the number of available postsynaptic AMPA receptors.

Statistics

Values are given as +/- standard error of the mean (SEM). Statistical significance for paired and independent samples was evaluated using Student´s *t* test.

SUMMARY OF RESULTS

Post-burst depression (PBD) of release probability

A synaptic burst consisting of 3 impulses at 50 Hz gave rise to a depression of the fEPSP of about 40% 500 ms after the burst. This depression is expressed as a reduction in release probability as the depression was associated with an increase in the PPR, was reported to the same extent by AMPA EPSCs and NMDA EPSCs and was associated with a comparable decrease in the $1/CV^2$–value (see Fig. 1, Paper I).

The PBD exhibits a distinct induction and cooperativity threshold

A stimulation pause was necessary for the expression of the PBD as continuous train stimulation resulted in a much smaller depression of the fEPSP (about 16%). Three impulses at frequencies above 20 Hz, 200-500 ms before testing produces maximal PBD. The PBD exhibited a cooperativity threshold, as a stimulation strength resulting in small EPSCs with frequent failures was not sufficient to elicit the PBD. By increasing the stimulation strength, and thereby recruiting more synapses, the PBD was induced (Fig. 1, Paper II). Further increase of the simulation strength did not increase the magnitude of the PBD suggesting that the PBD exhibits a distinct cooperativity threshold such that a certain number of synapses must be activated for the depression to be induced. However, this number is probably low, as we could not detect any threshold using field recordings. Stimulation intensities producing fEPSPs up to ten times smaller than those associated with signs of spike-activity were still sufficient to elicit PBD. The question then arises where the cooperativity is needed. While the postsynapse would be the first alternative, this seems unlikely as the PBD was still expressed when voltage-clamping the postsynaptic membrane potential (-80 mV, +40 mV) together with the calcium chelator BAPTA in the patch pipette. Two other possibilities, which could account for the cooperativity effect, are spill-over of neurotransmitter from many synaptic terminals (Arnth-Jensen *et al.*, 2002) and activity-induced release of gliotransmitters from astrocytes.

Astrocytes are necessary for the PBD

To test the involvement of astrocytes in the PBD we added the astrocytic metabolic inhibitor fluoroacetate, FAc, to the perfusion fluid. Fluoroacetate has been shown to be taken up only by astrocytes via an acetate transporter. Inside the astrocyte the FAc inhibit the enzyme

Acconitase of the Krebs cycle (Fonnum *et al.*, 1997). FAc completely blocked the PBD, indicating that astrocytes with intact metabolism are necessary for the generation of the PBD.

To investigate if the PBD was dependent on an astrocytic calcium increase we patched astrocytes with an intracellular solution containing 50 mM BAPTA. Due to the astrocytes extensive intercellular coupling, BAPTA can diffuse through the astrocyte network chelating calcium in the whole syncytium. To be able to estimate the extent of diffusion of BAPTA, we also included the fluorescent dye Lucifer Yellow. Lucifer Yellow is of a comparable size to BAPTA (Lucifer Yellow MW: 457 to BAPTA MW: 476) making us confident that when Lucifer Yellow is seen in an astrocyte then BAPTA is present there as well. Two similar but slightly different types of experiments were performed as seen in paper I, both blocking the PBD (Fig. 2, 3, Paper I).

Transient heterosynaptic depression (tHeSD)

If astrocytes can mediate synaptic depression to active synapses following a three-impulse burst, is this depression able to spread to neighbouring synapses? By using two independent synaptic inputs we gave the three impulse burst to one input and tested with paired-pulse stimulation in the other input 500 ms later (Fig. 1, Paper II). This group of synapses exhibited a decrease of the fEPSP of about 25%, a transient heterosynaptic depression, tHeSD. This depression was concomitant with an increase in PPR with 40% indicating that the tHeSD is expressed as a reduction in release probability (Fig. 2, Paper II).

The PBD and tHeSD develops over the first three postnatal weeks

In slices from six to eleven-day-old rats, neither the PBD nor the tHeSD could be elicited (Fig. 2, Paper I and Fig. 2, Paper II). This developmental profile of these plasticities is consistent with a critical involvement of the astrocytes.

tHeSD depends on astrocyte signalling and astrocyte gap junction coupling

To evaluate the involvement of astrocytes in the tHeSD we used fluoroacetate (Fig. 3, Paper II) which blocked the tHeSD showing that functionally intact astrocytes are necessary for this depression. As the heterosynaptic depression affects neighbouring synapses, we tested two gap junction blockers, carbenoxolone and endothelin-1, to determine if gap junction communication is required for the tHeSD. The tHeSD was indeed blocked by both of these substances (Fig. 3, Paper II).

The tHeSD depends on $GABA_B$ and mGlu receptors, but not on NMDA or adenosine A1 receptors

Previous reports have shown that activation of $GABA_B$ receptors, but not of NMDA receptors, is necessary for the generation of transient heterosynaptic depression (Gustafsson *et al.*, 1989; Isaacson *et al.*, 1993). Our results confirmed those findings. The $GABA_B$ antagonist CGP 52432 (3-[[(3,4-Dichlorophenyl)methyl]amino]propyl] diethoxymethyl)phosphinic acid, 4 μM), blocked the tHeSD without any effects on the baseline fEPSPs (Table 1, Paper II), or the PPR (Fig. 4, Paper II). The tHeSD was normal in the presence of the NMDAR antagonist D-AP5 (Fig. 4, Paper II). Adenosine have been shown to be a key player in both intermediate lasting forms and shorter lasting forms of heterosynaptic depression (Manzoni *et al.*, 1994; Zhang *et al.*, 2003; Pascual *et al.*, 2005; Serrano *et al.*, 2006). However, the adenosine 1 receptor antagonist DPCPX (8-Cyclopentyl-1,3-dipropylxanthine, 0.2 μM), although potently blocking the tonic adenosine inhibition, did not affect the tHeSD (Fig. 4, Paper II). Instead mGluRs seemed to play an essential role as the general metabotropic receptor antagonist, LY 341495, blocked the depression (Fig. 5, Paper II). The tHeSD was occluded by the mGluR group II agonist APCD ((2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylate, 50 μM) and partially occluded by a high concentration of the mGluR III agonist L-AP4 (L-2-amino-phosphonobutyric acid 1.2 mM, Fig.5, Paper II) that activates mGluR $_{4, 6, 7}$ and $_8$ (Conn & Pin, 1997). Activating only mGluR $_{4, 6}$ and $_8$ with a low concentration of L-AP4 (50 μM), (Conn & Pin, 1997) did not occlude the tHeSD. These results point more specifically to the involvement of $mGluR_7$ in the tHeSD.

The tHeSD partially occludes the PBD

The PBD and tHeSD are associated with different changes in paired-pulse ratio. In the case of the PBD, a relatively large depression of about 40% is associated with only a 15% increase in PPR. This contrasts to the tHeSD where the average 25% decrease is associated with a 40% increase in the PPR. Figure 1C, Paper III, shows that neither the second fEPSP nor the third is depressed by the tHeSD suggesting that this decrease in *p* is selective for synaptic activation occurring after a period of rest. The second fEPSP in the case of the PBD, on the other hand, is significantly depressed (Fig. 1D, Paper III).

Applying a three impulse burst simultaneously to the two independent synaptic inputs did not increase the homosynaptic depression, suggesting that the PBD already elicits the

"heterosynaptic depression" homosynaptically (Fig. 2, Paper III). When a heterosynaptic input was applied 500 ms before the homosynaptic, the homosynaptic increased the depression by about 10%, but reduced the increase in PPR by about 20%. These experiments suggest that the PBD can be separated into two components; one in common with the inactive synapses and one that is specific to the recently active synapses.

The PBD is associated with a reduction in the number of primed vesicles

To further examine the components that contribute to the PBD we used trains of ten action potentials as both conditioning and test stimulation. This gave us the opportunity to use the overall response during the train and estimate the relative number of vesicles, primed and ready for release when the train starts, see *Methodological considerations* (Schneggenburger *et al.*, 1999; Sakaba *et al.*, 2002; Abrahamsson *et al.*, 2005). The relative recruitment rate during high frequency stimulation was estimated as the slope of the linear part of the cumulative EPSC-stimulus number relationship. If this linear regression is extrapolated to the first stimulus, an estimate of the pre-primed pool at the onset of the stimulation can be made. This estimate of the pool showed a reduction of the pool by about 60% after a three-impulse conditioning and with about 90% after a ten-impulse conditioning. This analysis assumes that the activity-dependent recruitment starts with the first stimulus in the train and therefore most likely provides an overestimate of the depression (see *Methodological considerations*), but it nevertheless indicates that the PBD is associated with a reduction in the pre-primed pool of vesicles that increases with increasing number of stimuli in the conditioning train. A possible explanation for the similar amounts of total PBD after three and ten impulses is that although ten impulses further reduces the pool of vesicles it is more effective in eliciting augmentation, which is an increase in P_{ves} (Stevens & Wesseling, 1999). This augmentation may balance the reduction in the pre-primed pool, thereby maintaining the *p* (Garcia-Perez & Wesseling, 2008). If augmentation is enhanced after the ten impulses then PPR should be concomitantly decreased. Indeed, the PPR was smaller after a ten impulse conditioning than after a three impulse conditioning (Fig. 4, Paper III), indicating that the PBD is composed of a variable combination of astrocyte-mediated depression of "resting P_{ves}", augmentation and depletion of primed vesicles.

An alternative approach to estimate the reduction in the pre-primed pool during the PBD is to use these three different components differential effect on the paired-pulse ratio. The "resting P_{ves}" depression is associated with an increase in PPR (Paper II), augmentation with a

decrease in PPR (Granseth & Lindstrom, 2004) while changes in pool are associated with small or no changes at all in PPR (Abrahamsson *et al.*, 2005). In the interaction experiments (Fig. 2, Paper III), "resting P_{ves}" contributed with 28% to the PBD, leaving 10% for the homosynaptic components, augmentation and depletion. Resting P_{ves} and augmentation are expected to go with reciprocal changes in PPR (Fig. 4, Paper III). The pure homosynaptic component was associated with a decrease in the PPR of 23%, while the "resting P_{ves}" depression gave an increase in the PPR with 48% and the total PBD gave an increase of 25% (Fig.1, Paper III). If the 23% decrease in PPR is explained by augmentation, reciprocal to the "resting P_{ves}" depression, then augmentation counterbalance 48% of this depression (23/48=48). Thus, augmentation contributes with 13% of the total PBD PPR after a three impulse conditioning. With augmentation known we can estimate the contribution of depletion to 23%, by using total PBD subtracted with the "resting P_{ves}" depression and add augmentation (38% - 28% + 13% = 23%). This analysis indicates that the depletion-based depression increases from 23% to 54% when increasing the number of stimuli from three to ten in the conditioning train. With astrocytes compromised either by FAc or by BAPTA infusion, the same analysis as above indicates a much lower degree of depletion with only 12% for the three impulse burst. These results indicate that astrocytes contribute with a "resting P_{ves}" depression and, in addition, negatively affect recruitment to the pre-primed pool.

DISCUSSION

This thesis describes a novel form of homosynaptic short-term depression of release probability in response to a brief synaptic burst, a post-burst depression. This PBD relies on astrocyte calcium signalling and also affects neighbouring inactive synapses as a transient heterosynaptic depression. Neither the PBD nor the tHeSD are present during the first postnatal week. The tHeSD is expressed as an astrocyte-mediated reduction in "resting P_{ves}" while the PBD is composed of three plasticities; the reduction in "resting P_{ves}" concomitant with depletion of vesicles and augmentation. Inhibition of astrocyte signalling suggests that astrocytes also mediate a delay in the recovery from depletion after a burst.

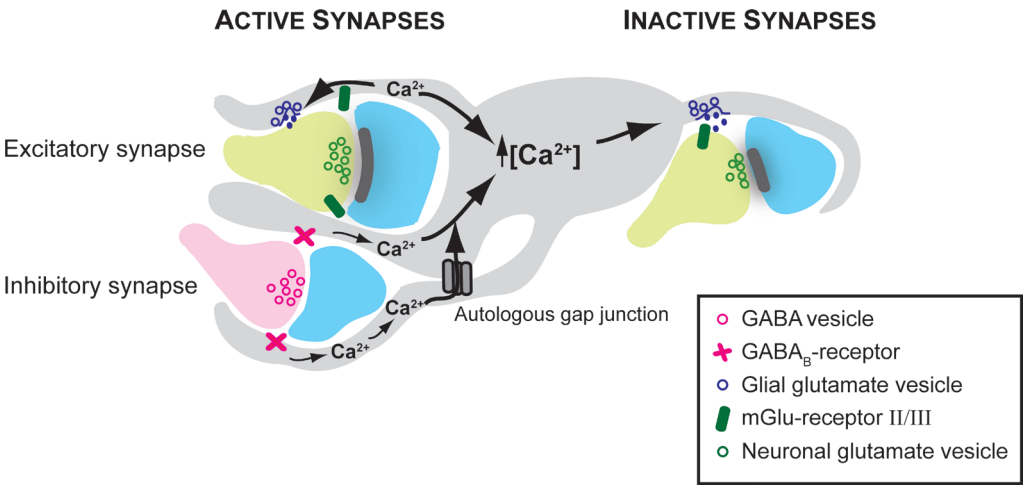

Fig. 5. Working hypothesis for astrocyte-mediated synaptic depression

I. Working hypothesis

From the results obtained in this thesis we have formulated a working hypothesis (Fig. 5). Glutamate released from the presynaptic terminals activates GABAergic interneurons and astrocytes directly. Astrocytes can also be activated by GABA released from interneuronal terminals. Activation of astrocytic mGluRs and $GABA_B$-receptors triggers a calcium increase

in the astrocyte, which in the case of the tHeSD spreads trough autologous gap junctions. The calcium increase induces gliotransmitter release, glutamate, onto the recently active presynaptic terminals decreasing release probability homosynaptically (PBD) as well as onto adjacent inactive synapses decreasing release probability heterosynaptically (tHeSD).

Homo and heterosynaptic depression - are astrocytes needed?

Synaptically released glutamate and GABA have been proposed to escape the synaptic cleft activating postsynaptic receptors extrasynaptically and heterosynaptically as well as activating presynaptic metabotropic receptors on its own terminal (auto-inhibition) and on neighbouring synapses (heterosynaptic inhibition) (Isaacson *et al.*, 1993; Kullmann *et al.*, 1996; Arnth-Jensen *et al.*, 2002; Losonczy *et al.*, 2003; Billups *et al.*, 2005). Astrocytes have the ability to hinder such intra- and intersynaptic crosstalk mediated via spill-over (Oliet *et al.*, 2001; Piet *et al.*, 2004).

Spill-over is highly dependent on the rate of transmitter uptake (Asztely *et al.*, 1997; Arnth-Jensen *et al.*, 2002). Since glutamate uptake progressively becomes more efficient during the first three postnatal weeks (Fiala et al, 1998, Diamond, 2005) spill-over is expected to decrease with development. Since the PBD, as well as the tHeSD, have the opposite developmental profile (Fig. 4, Paper I and Fig. 2, Paper II) spill-over was not a likely mechanism. As astrocyte uptake becomes faster, the astrocytes change morphology and electrophysiological phenotype towards their adult appearance (Fig. 6) (Konietzko & Muller, 1994; Bushong *et al.*, 2004; Schools *et al.*, 2006; Andersson *et al.*, 2007) and possibly also altering their function from nurturing newly established synapses to releasing gliotransmitters involved in synaptic physiology. Both the PBD and tHeSD exhibit a clear developmental profile, being absent the first postnatal week and becoming fully expressed after the third postnatal week, in agreement with an active participation of astrocytes.

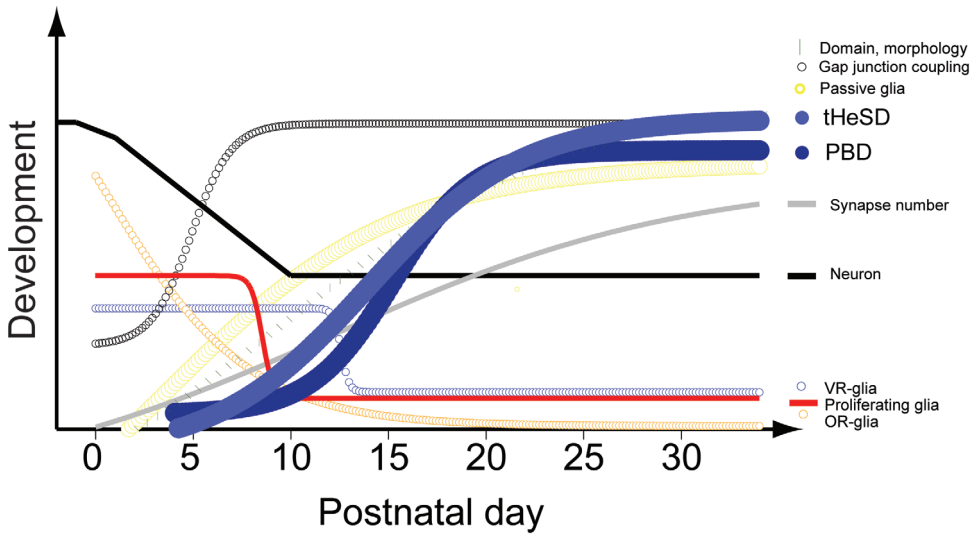

Fig. 6. Developmental profile of astrocyte-mediated synaptic depression.

However, the developmental profile merely suggests the involvement of astrocytes. To more directly investigate the possible role of astrocytes we used the astrocyte metabolic inhibitor FAc, which totally blocked both the PBD and the tHeSD. FAc is selectively taken up by glia because the expression of the acetate transporter is restricted to glia (Fonnum *et al.*, 1997). In addition, the tHeSD was blocked by application of endothelin-1, the agonist for the endothelin A receptor. This receptor has been shown with single cell PCR (polymerase chain reaction) to be profoundly expressed on astrocytes with a high degree of specificity (Cahoy *et al.*, 2008). When activated it blocks astrocyte gap junctions through de-phosphorylation of connexin-43, the major astrocytic gap junction forming protein (Blomstrand *et al.*, 2004). Delivery of BAPTA to the astrocytic network potently blocked the PBD, but this has not yet been tested for the tHeSD. Together these results show that astrocyte activation is necessary for mediating the PBD and the tHeSD. So how are the astrocytes activated?

Activating astrocytes

Membrane depolarisation

Synaptic activity could lead to the depolarisation of the astrocytic membrane by glutamate uptake as well as by the accumulation of extracellular potassium (Meeks & Mennerick, 2007). This depolarisation could be a possible candidate for activating the astrocytes and

depolarisation has been shown to be able to increase the conductance through gap junction channels (Enkvist & McCarthy, 1994). We have tried to voltage-clamp the astrocyte membrane at –80 mV and 0 mV, and simultaneously elicit the PBD, without being able to interfere with the PBD. As the input resistance of the astrocytes, ~3 MΩ, is very low, compared to the 100-300 MΩ for pyramidal cells, most of the potential drops over the pipette resistance (usually 10-20 MΩ) and dual patch clamp recordings from astrocytes have shown that the voltage error typically amounts to 80% (Zhou *et al.*, 2009). This makes any attempt to voltage-clamp the astrocyte membrane potential hard to evaluate. Still our attempts to voltage-clamp the astrocytes did not provide any preliminary support for membrane depolarisation as the activation mechanism.

Activation of metabotropic receptors

Astrocytes express a vast array of receptors and respond to activation by increases in calcium (Perea *et al.*, 2009b, a). As agonists and antagonists applied to the extracellular solution interact with receptors on both neurons and glia, it is hard to pin down the location of the different receptors in the circuit. The tHeSD relies on $GABA_BR$ and mGluR which are expressed on glutamatergic terminals, GABAergic terminals (Shigemoto *et al.*, 1997) and on astrocytes (Winder *et al.*, 1996). We cannot tell the location of the critical $GABA_BRs$, but it is likely that astrocytic $GABA_BRs$ contribute since interneurons have been shown to activate astrocytes via $GABA_BRs$ (Kang *et al.*, 1998; Liu *et al.*, 2004).

The application of the group II mGluR agonist APCD occluded both the tHeSD (Fig. 5, Paper II) and the PBD (preliminary results) which could have several different explanations. In rat cortical astrocytes, an mGluR II agonist caused an increase in calcium (Moldrich *et al.*, 2002). Zur Neiden and Deitmer (Zur Nieden & Deitmer, 2006) elicited astrocytic calcium waves with a general mGluR agonist. These calcium waves were blocked by group I and group II antagonists, but not by a group III antagonist. Accordingly, one possibility is that the presence of APCD occludes the activation of astrocytic calcium elevations. To test whether metabotropic receptors on astrocytes are indeed required for the PBD and the tHeSD one could apply the non-hydrolysable guanosine nucleotide GDP-βS through the recording pipette. This would lock the G protein in an inactive state (Sakaba & Neher, 2003) and prevent the activation of the astrocytes.

Calcium signalling

My preliminary results from calcium imaging of astrocytes loaded with Oregon green BAPTA-AM suggest that three impulses at 50 Hz are enough to induce calcium transients in stratum radiatum astrocytes. BAPTA delivered through a patch pipette to the astrocytic network effectively blocked the PBD (Fig. 3, Paper I), indicating that an astrocytic calcium rise is a necessary step in the generation of the PBD. We also expect that astrocytic BAPTA blocks the tHeSD, but that remains to be tested. BAPTA is a fast calcium chelator, able to buffer both fast microdomain calcium transients, and bulk increases in calcium. EGTA, a slow calcium chelator, could be used to discriminate the role of astrocyte calcium in these plasticities. If EGTA is equally effective as BAPTA in blocking the PBD, then the bulk increase in calcium is sufficient. If not, this would be an indication that an increase in microdomain calcium is necessary. Another way to test if bulk calcium is sufficient to elicit the PBD would be to uncage calcium or IP_3 in the astrocyte while simultaneously recording the synaptic responses.

The PBD exhibited a distinct cooperativity threshold (Fig.1, Paper I), an uncommon feature among short-term plasticises. As we did not find any evidence for the involvement of the postsynaptic neuron in the PBD, this threshold is likely to be a threshold for astrocyte activation. To investigate whether there is a correlation between the calcium increases and/or spread of calcium in the astrocyte and the generation of the PBD, astrocytic calcium could be imaged together with whole-cell recordings from a pyramidal cell. One would need to apply the calcium indicator intracellularly into the astrocytic network and also apply another fluorescent calcium-sensitive dye into the pyramidal cell to be able to identify locations where active spines are in very close contact with astrocyte processes. Increasing stimulation strength should reveal if there is a correlation of the calcium increase and/or spread of calcium in the astrocyte with the expression of PBD.

Gap junctions

The tHeSD was sensitive to the gap junction blocker carbenoxolone and endothelin-1 (Fig. 3, Paper II). As mentioned in the *Methodological considerations*, carbenoxolone is known to have other, unspecific, effects in addition to blocking gap junctions (Rouach *et al.*, 2003; Vessey *et al.*, 2004). The ET A receptor, which is only expressed on astrocytes(Cahoy *et al.*, 2008), is stimulated by Et-1, leading to closure of gap junctions (Blomstrand *et al.*, 2004). It also induces calcium oscillations in astrocytes (Verkhratsky *et al.*, 1998). It is thus possible

that application of Et-1 occludes the tHeSD by increasing astrocytic calcium. However, application of Et-1 did not alter the baseline fEPSP magnitude, indicating that the blocking effect of Et-1 on the tHeSD is more likely to be attributed to Et-1's ability to close gap junctions.

Autologus gap junctions comprise up to 75% of astrocytic gap junctions (Nagy & Rash, 2003). Diffusion of calcium, or IP_3, through these gap junctions could be an important mechanism for recruiting astrocytic microdomains in close vicinity to other synapses contacted by the same astrocyte. Such a scenario could explain the effect on inactive synapses during the tHeSD. However, it does likely not explain the cooperativity threshold exhibited by the PBD, because preliminary results show that Et-1 did not affect the magnitude of the PBD.

Release from glia

As discussed in the *Introduction* there are many potential routes by which gliotransmitters can be released from astrocytes. My present results do not allow me to discriminate among these possibilities. This is of course an important issue, and a pertinent first question to address is whether SNARE-mediated exocytosis is involved. One approach to address this question would be to infuse the catalytic subunit of tetanus, or botulinus, toxin, which specifically cleaves SNARE proteins, into the astrocytic network via the patch pipette and test whether the PBD and the tHeSD persist. If the astrocyte-mediated depressions are inhibited by this treatment, it would be strong evidence in favour of the requirement of astrocytic SNARE-mediated release. However, it should be noted that there are several forms of SNARE-mediated release, from small vesicle-like microvesicles, lysosomes and dense core vesicles. As endothelin-1 did not affect the PBD, it is unlikely that gliotransmitter release onto the presynaptic terminal passes through hemichannels.

Astrocyte-mediated decrease of release probability

Depression of "resting P_{ves}"

Our results where we see a selective depression of the first fEPSP in a pair, or triplet, of fEPSPs for the tHeSD are not consistent with a reduction in the number of release-ready vesicles or by a general reduction in P_{ves}, both of which would not be expected to be restricted to the first fEPSP (Hanse & Gustafsson, 2001b). We therefore propose that the tHeSD is based on a selective reduction of the P_{ves} at synaptic terminals not exposed to "residual calcium", which we call "resting P_{ves}". As a corollary, we propose that P_{ves} at synaptic

terminals exposed to "residual calcium" are resistant to this astrocyte-mediated depression. A depression of *p* selectively at resting presynaptic terminals is very similar to a phenotype exhibited by the $Rab3^{A\text{-}D\text{-}/\text{-}}$ knock out mice (Schluter *et al.*, 2006). Hippocampal autaptic synapses from these mice exhibited a reduction in P_r of about 30% when they were activated with a low frequency, but very little during high frequency stimulation (Schluter *et al.*, 2006).

Rab3A, B, C, D are a family of small GTPases associated with presynaptic vesicles. Rab $3A^{-/-}$ knockout mice are viable (Geppert *et al.*, 1994) while mice exhibiting the quadruple knockout $Rab3^{A,B,C,D\text{-}/\text{-}}$ die shortly after birth because of respiratory failure (Schluter *et al.*, 2004).

Hippocampal synapses from these mice exhibit a reduction in *p* during low frequency activation that seems to affect a subset of synapses at which Rab3 increases the calcium sensitivity of docked, fusion-competent vesicles (Schluter *et al.*, 2006). These synapses would then be "super-primed" for release, exhibiting a higher *p* than the general synapse population. "Super-primed" synapses correspond well to the population of high-*p* synapses among the CA3-CA1 synapses (Hessler *et al.*, 1993; Rosenmund *et al.*, 1993; Dobrunz & Stevens, 1997; Hanse & Gustafsson, 2001c). Thus, an astrocyte-mediated reduction in resting P_{ves} could be explained by an astrocyte-mediated reversal of Rab3-dependent "super-priming" at high-*p* synapses. This "super-priming", in turn, could correspond to the positional priming postulated by Neher and Sakaba (Neher & Sakaba, 2008). They suggested that a vesicle is first made release-competent by molecular priming, equipping it with all the necessary components for release, and that the vesicle then can undergo positional priming when it becomes located near the calcium source, the VGCCs. Opening of these channels then have a high probability of releasing a vesicle, thus a high *p*. During a high frequency train, the calcium concentration builds up, likely allowing also vesicles that are merely molecularly primed to be released. Since these vesicles are not positionally primed they will, according to our reasoning above, not be affected by the depressing astrocyte-derived signal. Whether high-*p* synapses are in fact selectively affected by the tHeSD can be tested using the use-dependent, irreversible NMDA receptor antagonist MK-801. Thus, only the time constant representing the high- *p* synapses should be affected when comparing the depression of the NMDA EPSC in the presence MK-801 with and without a preceding heterosynaptic conditioning burst.

The PBD consists of "resting P_{ves}"-depression, depletion and augmentation

The interaction between the tHeSD and the PBD (Fig. 2, Paper III) indicated that a part of the PBD is expressed as a reduction in "resting P_{ves}", expected to increase the PPR. The remaining part of the PBD is then specific for recently active synapses. The analysis of the cumulative response during a ten impulse test train (Fig. 3, Paper III) indicated that a part of this homosynaptic depression is a depletion of vesicles and that increasing the conditioning stimuli from three to ten impulses reveals the contribution of augmentation, reported as a decrease in the PPR (Fig. 4, Paper III) (Granseth & Lindstrom, 2004).

Astrocyte-mediated delay in recovery after depletion

Analysing the depletion component of the PBD using the PPR-analysis indicated that this depletion was reduced when the astrocytes were compromised by FAc or BAPTA. These treatments did, however, not affect the pre-primed pool of vesicles, or the depletion, per se, during the conditioning train. A larger pool after 500 ms could then be explained by an astrocyte-imposed delay in re-priming after depletion. This line of reasoning has previously been forwarded for results from the Calyx of Held where stimulus interruption was needed to reveal the effects of activating metabotropic glutamate autoreceptors, slowing down the recovery after a burst (Billups *et al.*, 2005). Further experiments are needed to elucidate the role of mGluRs in the PBD.

Recovery of primed vesicles have been divided into a fast calcium-dependent recovery and a slow calcium independent recovery (Neher & Sakaba, 2008). Based on our findings that the recruitment rate during a high-frequency train is unaffected by a three impulse burst (Fig. 3, Paper III) it is less likely that the putative gliotransmitter directly inhibits the calcium-dependent recruitment. Instead astrocytes could either slow down the calcium-independent priming or accelerate the decay of the calcium-dependent one when the stimulation stops. Calcium-dependent recruitment is thought to rely on calmodulin interaction with Munc-13 and is also dependent on the levels of cAMP (Sakaba & Neher, 2001a, b; Junge *et al.*, 2004). This is presumably mediated via the interaction of Rim (which also binds Munc-13) with the cAMP-GEFII complex and can be affected by $GABA_B$-receptor activation, which reduces cAMP levels (Sakaba & Neher, 2003). In this scenario, an astrocyte-mediated acceleration of fast recruitment-decay could be mediated by a reduction in cAMP levels. The activator of adenylate cyclase, forskolin, could be used to test if increased cAMP levels could counteract the delay in recovery. Experiments using a calmodulin blocker would be of interest both for

investigating effects on the astrocyte-mediated delay in recovery and for investigating the role of "super-priming"/positional priming.

Calmodulin can be involved in positional priming by inducing GTP binding to Rab3A, which leads to the formation of the active GTP-bound form of the Rab3A-calcium/calmodulin complex (Park *et al.*, 2002). A calmodulin blocker could then be able to interfere with the generation of "super-primed" vesicles, thus occluding the tHeSD.

Yet another possible way of delaying recruitment of vesicles is through the inhibition of endocytosis (Hosoi *et al.*, 2009). Retrieval of a fused vesicle via endocytosis is calcium-dependent and was suggested to be rate-limiting for the recruitment of new vesicles (Hosoi *et al.*, 2009).

II. Twitching of astrocyte processes as a mechanism for homosynaptic depression

The working model described above suggests a sequential synapse-glia-synapse activation. An alternative model explaining how the astrocyte may allow the PBD to occur could be that the brief train activates the astrocyte and causes the finest, veil-like astrocyte processes to retract, thereby enabling glutamate to activate presynaptic glutamate autoreceptors (Oliet *et al.*, 2001). A pause would then be necessary to reveal the impact of the synaptically released glutamate on presynaptic autoreceptors, similar to the previously mentioned results from the Calyx of Held (Billups *et al.*, 2005). The activation of the astrocyte should give gliotransmitter release at neighbouring synapses, mediating the tHeSD, while it should induce a twitch at the activated synapses, mediating the PBD. This scenario presupposes the ability of the astrocyte process to change its morphology within a hundred milliseconds. Although very fast, it is not unrealistic considering the fact that spines have been shown to twitch on time scales faster than 500 ms (Roelandse *et al.*, 2003). A first step to elucidate the role of a possible twitch of astrocyte processes could be to interfere with actin dynamics.

Heterosynaptic depression

Paper II shows the critical participation of astrocytes in a previously described transient heterosynaptic depression (Gustafsson *et al.*, 1989; Isaacson *et al.*, 1993). The tHeSD did neither rely on adenosine, nor on NMDA receptor activation, but instead required $GABA_B$ and mGluR activation. The $mGluR_7R$ was implied in the tHeSD by the partial occlusion of tHeSD seen at a high concentration of L-AP4. These receptors are found predominately on the

presynaptic terminals of the glutamatergic synapses (Shigemoto *et al.*, 1997). My results indicate how these autoreceptors might be activated by physiologically relevant activity.

As mentioned in the *Introduction*, long and intermediate lasting forms of heterosynaptic depressions have been shown to rely on astrocytes. My findings close the circle by showing astrocyte involvement also in the transient heterosynaptic depression. Astrocytes are in an ideal position to mediate such intersynaptic communication and to coordinate the activity of the population synapses located within their domain. It is also becoming clear that the astrocytes, depending on the nature of the triggering synaptic signal, can mediate different forms of heterosynaptic depressions, with different duration and different gliotransmitters involved.

Functional considerations

Hippocampal pyramidal neurons in the exploring animal typically fire action potentials in short bursts (Lisman & Buzsaki, 2008), in the frequency range eliciting both homo and heterosynaptic depression, the PBD and tHeSD, respectively. This suggests that astrocytes are critically involved in mediating a negative feedback after a burst of synaptic activity, functioning as a high pass filter, reducing the spatial and temporal extension of neural activity.

Astrocytes in physiology and pathology

Neuropathologies and brain trauma are typically associated with reactive astrocytes, which are defined by their high expression of the intermediary filament, GFAP (Wilhelmsson *et al.*, 2006). The slice preparation itself is of course a major trauma and we do not know how astrocytes change during the lifetime of the slice. Since the acute hippocampus slice preparation is maintained only for a day, GFAP up-regulation might not be possible to observe in these slices. In a paper examining different aspects of tissue recovery after slice preparation, an initial depletion of astrocyte glycogen was recovered during the first three hours (Fiala *et al.*, 2003). My preliminary results suggest that the ability to elicit the PBD is decreased seven hours after the preparation. The reduction of the PBD was observed even though synaptic transmission (EPSP/volley ratio) and paired-pulse plasticity appeared normal. Is this an early sign of reactivity and do reactive astrocytes maintain the ability to mediate the PBD? This question could be addressed by promoting, and/or inhibiting, the development of reactive astrocytosis in the slice.

CONCLUSIONS

I. The present study shows that astrocytes do contribute to homosynaptic short-term plasticity. They impose a previously unrecognized postburst depression at hippocampal glutamate synapses.

II. Astrocytes mediate short-term heterosynaptic depression at hippocampal glutamate synapses, which requires functional gap junctions in the astrocyte network.

III. Astrocyte-mediated short-term synaptic depression is expressed as a reduction in release probability. More precisely, as a decrease in vesicular release probability at inactive synaspes, but not at active synapses, and as delay in the recovery of primed vesicles following depletion at active synapses.

The results from my thesis support the growing body of evidence suggesting that astrocytes contribute actively to synaptic signalling in the brain. This knowledge should increase the awareness of astrocytes as potential targets when looking for mechanisms underlying various brain pathologies.

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