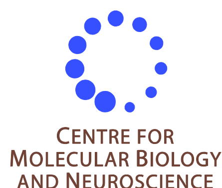
Elements of glutamate release, action and reuptake

THESIS

Simen Gylterud Owe



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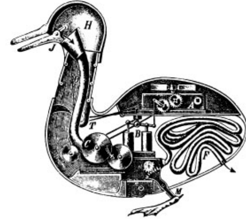
PREFACE

Cogito ergo sum – I think, therefore I am. So, is being the same as thinking? Can the true essence of thought, consciousness and feelings be unravelled? These are bold questions still, after several millennia of studies. And they are certainly not solved here, in this thesis. Rather, in the spirit of Descartes, the bits and pieces of the brain are approached mechanistically.



Drawing of the Duck of Vaucanson (1739)

Nowadays we do know some of the workings of the brain; as for example, the brain's enormous computing power seems to reside in the way electrical signals are processed; integrated or differentiated, enhanced or depressed, dependent on neuronal networks and properties of connections. Neurons conduct electrical signals, and signals pass from one neuron to the next by the use of chemical substances in specialized contacts, synapses. In synapses, the most abundant transmitter is the amino acid glutamate.

A machinery of proteins conducts the task of glutamate release, binding/effect (action) and reuptake, at speeds of up to hundreds of times every second. The bits of this machinery are not at all fully understood and differ between areas of the brain. It is the principal aim of this thesis to discuss the role of some of the proteins that are involved and considered essential. The findings can be summarized as follows:

First, signal transmission was shown to be enhanced in mossy fibre terminals of the hippocampus when stimulated at a specific range of frequencies, by a mechanism dependent on the proteins synapsin I+II and actin. This was shown by a loss of function and change in morphology in mice genetically altered to lack synapsin I+II. Also, the localization of synapsin III was described at high resolution.

Secondly, in the mice described above, signal receiving neurons were found to have increased sensitivity. This was described as a change in composition of receptor proteins (GluR1 and GluR2/3) which glutamate acts upon. We suggest that this change can contribute to epileptic seizures seen in the genetically altered mice.

Signals are terminated by the removal of glutamate from the extracellular space. Last, a protein (GLAST) that transports glutamate away from the synapse into surrounding cells (glia) was characterized. Specifically, the study determined the number and identity of ions involved in and driving its transporter cycle.

It is my hope that this attempt to describe brain mechanics is not understood as a simplification, but merely a contribution that adds complexity to this most mysterious of organs.

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ORIGINAL PAPERS

Paper I: Gylterud Owe S, Bogen IL, Walaas SI, Storm-Mathisen J, & Bergersen LH (2005). Ultrastructural quantification of glutamate receptors at excitatory synapses in hippocampus of synapsin I+II double knock-out mice. *Neuroscience* **136**, 769-777.

Paper II: Owe SG, Marcaggi P, & Attwell D (2006). The ionic stoichiometry of the GLAST glutamate transporter in salamander retinal glia. *J Physiol* **577**, 591-599.

Paper III: Owe SG, Jensen V, Evergren E, Ruiz A, Shupliakov O, Kullmann DM, Storm-Mathisen J, Walaas SI, Hvalby O, & Bergersen LH (2008). Synapsin- and Actin-Dependent Frequency Enhancement in Mouse Hippocampal Mossy Fiber Synapses. *Cereb Cortex* **[Epub ahead of print]**.

ABBREVIATIONS

aa: amino acid

AC: Associational/Commissural fiber

ATP: adenosine triphosphate

AZ: active zone

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate

CA: Cornu Ammonis

cAMP: cyclic adenosine monophosphate

CaMK: Ca2+/calmodulin-dependent protein kinase

Cdk: Cyclin dependent kinase

CNS: central nervous system

DKO: double knockout (Synapsin I and II)

EAAT: excitatory amino acid transporter

F-actin: filamentous actin

GLAST: glutamate aspartate transporter

GLT: glutamate transporter

GluR: glutamate receptor

KA: kainate receptor

LTP: long-term potentiation

MAPK: Mitogen-activated protein kinase

MF: mossy fiber

NR: N-methyl-D-aspartate receptor

NMDA: N-methyl-D-aspartate

PKA: protein kinase A

PSD: post synaptic density

STE: short-term enhancment

RP: reserve pool

RRP: readily releasable pool

ser: serine

Trk: family of tyrosine kinase receptors

tyr: tyrosine

WT: wildtype

SYNOPSIS

A neuron in a network is not simply a relay station through which signals pass. Rather, signal processing is performed at multiple sites along the pathway of a signal. This property adds unique features to the brain, such as the ability to adapt to changing conditions and to modulate output. One site of modulation is the synapse, the basic connection between neurons. In this thesis, localization and function of specific protein components of synapses are investigated.

BACKGROUND AND AIMS OF THE PRESENT STUDY

Proteins are responsible for cellular functions. The three compartments of the glutamatergic synapse are shown in Figure 1. For each compartment, one class of proteins has been studied in this thesis, corresponding to the roman numerals in Figure 1: the neurotransmitter glutamate is released from round synaptic vesicles, seen in the terminal (I). First, synapsin proteins, which govern synaptic vesicle organization, were studied in the terminal and their interaction with the cytoskeletal protein actin examined in relation to enhancement of synaptic responses. Glutamate acts upon receptors on a dendrite (II), which are situated along the characteristic postsynaptic density (PSD) facing the terminal and the synaptic cleft. In the second project, the AMPA and NMDA types of glutamate receptors in dendrites and their responses to altered input were investigated. Transporter proteins remove glutamate by uptake into an astrocyte (III), which surrounds both terminal and dendrite. In the third study, the stoichiometric properties of GLAST, a glial glutamate transporter protein, were determined.

THE GLUTAMATE SIGNALLING SYSTEM

In an early study, it was shown that the amino acid glutamate produces motor effects when injected to cortical grey matter (Hayashi, 1954), followed by the demonstration of an excitatory effect on individual neurons (Curtis *et al.*, 1960). Several seminal studies have been published on glutamate as a neurotransmitter, including the evoked release of glutamate from excitatory nerve endings (Nadler *et al.*, 1976), development of antagonists (Haldeman *et al.*, 1972;Biscoe *et al.*, 1977), visualization of glutamate in nerve endings and synaptic vesicles (Storm-Mathisen *et al.*, 1983), and the first cloning of a glutamate receptor (Hollmann *et al.*, 1989).

Acknowledging the glutamate signalling system has led to remarkable progress in the understanding brain function. The overall aim here is to add details to some of the many aspects of glutamate signalling. More specific aims and choice of methods are described in the numbered (I-III) sections.

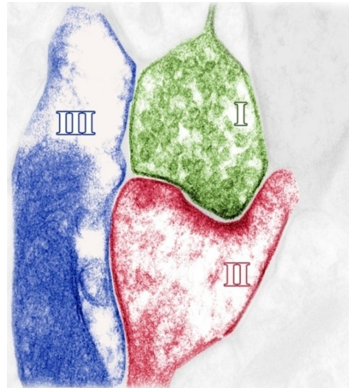

Figure 1. Artificially coloured electron micrograph of an axo-spinous glutamatergic synapse. The "tripartile" synapse consist of: the nerve terminal (I) which is recognized by its many synaptic vesicles, here approximately 35 nm in diameter. The terminal and dendritic spine (II, below) have protein rich, dense, areas close to the plasma membranes at the synaptic cleft, a gap of uniform distance between terminal and dendrite, here about 15 nm wide. In glutamatergic synapses the postsynaptic density (PSD) is particularly prominent. A glial cell (III) is seen left, ensheathing the synapse. The protrusion towards the synaptic cleft is characteristic of astroglial processes. The lower left, dense structure is a large mitochondrion. For clarity, the adjacent neuropil structures have been dimmed. The micrograph is from stratum radiatum of hippocampus CA1.

GLUTAMATE AND PLASTICITY

Adapting to and modulating signals are essential functions in the workings of the brain. In glutamatergic synapses this can be referred to as synaptic plasticity. A number of different mechanisms, that can be divided in short- and long-term synaptic plasticity, has recently been reviewed (Citri & Malenka, 2008).

As an example of short-term synaptic plasticity, the brain constantly receives an enormous number of input signals originating from our senses. Only a fraction of these will be responded to. Short-term enhancement (STE) at synapses offers one possible mechanism for signal filtering (Fisher *et al.*, 1997): in short, signals at given frequencies specific to the

type of synapse in question, are enhanced. This can be achieved through an increase in glutamate release by the nerve terminals, whereas other frequencies are not enhanced, or are even depressed by other mechanisms not described here. Hence, neurons can pick up signals coded at specific frequencies and ignore others, reducing the total number of signals. Also multiple other ways of filtering signals exist that modify the probability for a signal to be passed on, such as the interplay between excitatory and inhibitory synapses. However, synaptic inhibition will act on a different level by hyperpolarizing the signal receiving neuron, dependant on additional inhibitory neurons. STE acts on a timescale from milliseconds to tens of minutes, covering most of the signalling frequencies recorded in the brain. Complementary, the mechanisms of long term potentiation (LTP), act on timescales of up to a lifetime.

Removal of the hippocampus, a well studied part of the mesial temporal lobe, induces anterograde amnesia (Scoville & Milner, 1957). Based on this finding, it is evident that hippocampal neurons, through their array of neural pathways that can be modulated, are involved in the formation of memories. In paper I, a molecular mechanism is proposed for STE in a glutamatergic synapse of the hippocampal circuitry.

GLUTAMATE AND CNS PATHOLOGY

An adverse effect of glutamate is its toxicity at high extracellular concentrations in the brain. Excessive activation of glutamate receptors may trigger lethal cellular mechanisms through an increase in intracellular Ca^{2+} in the dendrites and perikarya of neurons. The excessive stimulation may have a subsequent effect on neighbouring synapses by glutamate spillover, leading to a vicious circle. Such a rise in extracellular glutamate concentration and its detrimental effect can be seen in ischemia, e.g. during a stroke. Paper III raises the question of what the extracellular glutamate concentration in the brain is, by looking at one major determinant, the transport stoichiometry of the glutamate transporter GLAST.

Other diseases, such as epilepsy, rather depend on an imbalance in the glutamatergic signalling system: an epileptic seizure is defined as “a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronized neuronal activity in the brain” (Fisher *et al.*, 2005). One causative theory is that glutamate, if not kept within very strict concentration limits in the extracellular space (paper III), will spontaneously activate dendrites. Faulty regulation in the glutamatergic system could potentially cause a sustained, autonomous firing in neurons. In paper II, one such malfunction is proposed, based on changes seen in glutamate receptor subunit distribution in epileptic mice.

I. SYNAPSINS AS MODULATORS OF SYNAPTIC TRANSMISSION

Fundamental to glutamate signalling is its release from terminals by exocytosis of synaptic vesicles. A single such event has been termed a quantal release, based on vesicles presumably containing a set amount of glutamate. Consequently the release kinetics of glutamate is highly dependent on the number of vesicles released. Exocytosis of vesicles is supported by the synaptic *vesicle cycle; docking, priming, fusion and recycling*, describing the events from when a vesicle is bound to the plasma membrane to the retrieval and reuse of the vesicle, as reviewed in Murthy & De Camilli (2003) and Südhof et al. (2004). The synapsins have been proposed to be involved in several of these events.

VESICLE POOLS

A synaptic terminal contains clustered vesicles proximal to the synaptic cleft. This cluster can be subdivided into pools of vesicles defined by anatomical or physiological properties (Figure 2). Initially, if a synapse is subjected to repetitive nerve stimulation, the amplitude of synaptic signals declines from an initial high value. This is thought to reflect emptying of a readily releasable pool (RRP), proposed to correspond to vesicles that morphologically are docked to the plasma membrane (Schikorski & Stevens, 2001). The area of the plasma membrane where docking occurs is termed the active zone (AZ), situated along the synaptic cleft and in excitatory synapses opposed to the post synaptic density (PSD).

A second subset of vesicles is the reserve pool (RP), which under many conditions is not involved in exo- and endocytosis (Richards *et al.*, 2003). Based on the link between the readily releasable pool and the presynaptic membrane, the reserve pool consists of vesicles distant from the active zone. These criteria were used in this thesis to morphologically define distinct areas within the synapse (Figure 2B). Additionally, the periactive zone has been defined lateral to the active zone based on the presence of clathrin coated pits and endocytic machinery (Roos & Kelly, 1999;Shupliakov *et al.*, 2002). Importantly, the link between physiologically defined pools and morphology has been questioned (Figure 2A+B) (Rizzoli & Betz, 2004;Fdez & Hilfiker, 2006).

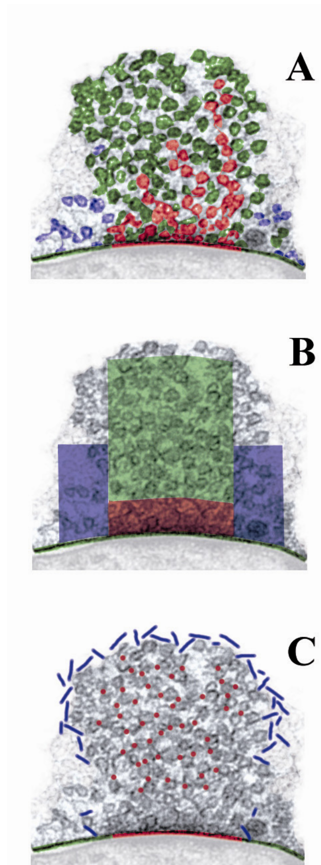

*Figure 2. Artificially colored and altered electron micrographs representing vesicular organization and localization of F-actin and synapsin. (*A*) Vesicles are artificially labeled to represent the physiologically distinct vesicle pools: the readily releasable pool (RRP, red), the reserve pool (RP, green), and the recycling pool comprised of both the RRP and endocytic vesicles (blue). The membrane is colored green in the endocytic zone and red in the active zone (AZ). (*B*) Regions used for morphological sampling of the vesicle pools: the first 100 nm from the synaptic cleft (red) contains mostly vesicles of the RRP docked at the AZ. The RP covers the next 400 nm in distance (green). Laterally, two areas of 150 x 250 nm (blue) cover endocytic zones. (*C*) The localization of synapsin (red dots) and F-actin (blue rods) are schematically indicated.*

Synapsins (Ia-b, IIa-b and IIIa-f isoforms) show a complex phosphorylation behaviour, synapsin Ia having at least eight phosphorylation sites (Evergren *et al.*, 2007). These sites may contribute to the four proposed actions of synapsin: binding to synaptic vesicles, binding to the actin cytoskeleton, polymerization of actin and synapsin dimerization. Also, synapsins show binding affinity to a wide array of vesicle related proteins that colocalize in the RP at rest, reviewed in Shupliakov (2008), with the possible addition of the piccolo protein (Leal-Ortiz *et al.*, 2008).

Taken together, the synapsins were first proposed to function as an anchor for synaptic vesicles to the actin cytoskeleton, especially in the RP, preventing release unless they are phosphorylated (Chi *et al.*, 2001). Later studies suggest additional roles: in recycling of vesicles from endocytic zones, in polymerization of actin, and in linkage to other synapsins by dimerization, or to other molecules. The latter two actions may explain why perturbation of the actin cytoskeleton does not lead to a loss of RP vesicles and that immunocytochemical staining of F-actin fails to show F-actin in the RP (Dunaevsky & Connor, 2000;Gustafsson *et al.*, 2002;Bloom *et al.*, 2003).

The kinases and phosphatases that set the phosphorylation state of synapsins are summarized in Table 1. Most of these enzymes are themselves controlled by the intracellular level of Ca^{2+}. Katz and Milledi (1968) proposed the residual calcium theory, stating that an increased Ca^{2+} level following an action potential can remain in the terminal causing the effect of subsequent action potentials to be enhanced. Synapsins are both regulated by Ca^{2+} dependent enzymes and involved in the regulation of vesicle dynamics. Hence, they offer a mechanism for coupling of Ca^{2+} concentration to STE.

Evolutionary conserved structural domains A, C and E are distinguished. Domains A and C are featured in all synapsins, whereas the E domain is specific to synapsin a isoforms; Ia, IIa and IIIa (Kao *et al.*, 1999). Domain C is linked to most of synapsin's proposed functions: F-actin interaction (Bahler & Greengard, 1987;Petrucci & Morrow, 1987), synaptic vesicle binding through three amphiphatic regions (Cheetham *et al.*, 2001), ATP hydrolysis, synapsin dimerization (Benfenati *et al.*, 1993): it contains the Src phosphorylation site. Domain A is proposed to be regulatory through the PKA and CaMKI/IV binding site. Domain E is less extensively studied, but is involved in synaptic vesicle cycling (Fassio *et al.*, 2006) and retension of the RP (Pieribone *et al.*, 1995;Hilfiker *et al.*, 1998). Remaining domains are more variable between species and synapsin isoforms. Domain B, present in all synapsins, has additional phosphorylation sites for MAPK and possibly calcineurin, and is involved in

nerve terminal targeting. In contrast, domain D, found in synapsin I isoforms, inhibits targeting. Domain E, present in all synapsin "a" isoforms alleviates this inhibition (Gitler *et al.*, 2004). Domain D also plays a regulatory role, containing phosphorylation sites for CaMKII, cdk1, cdk5 and MAPK.

Enzyme	Site (aa)	Function	Activation	Synapsins	Reference
PKA CaMKI/IV	ser^{9}	Affinity ↓ SV Affinity ↓ F-actin Bundling ↓ F-actin	cAMP, Ca^{2+}/Cal-modulin	All	(Huttner & Greengard, 1979;Schiebler *et al.*, 1986;Bahler & Greengard, 1987;Czernik *et al.*, 1987)
MAPK	ser^{62} ser^{67} (ser^{549})	Affinity ↓ F-actin Bundling ↓ F-actin Polymerization ↓, F-actin	High freq. st. Neurotrophins Trk	I II? III?	(Jovanovic *et al.*, 1996;Matsubara *et al.*, 1996)
Src	tyr^{301}	Affinity ↑ F-Actin		All	(Onofri *et al.*, 1997;2007;Foster-Barber & Bishop, 1998)
cdk1	(ser^{549})	Affinity ↓ F-actin		I III?	(Hall *et al.*, 1990;Jovanovic *et al.*, 1996)
cdk5	(ser^{549}) (ser^{551})	Affinity ↓ F-actin		I III?	(Matsubara *et al.*, 1996)
CaMKII	(ser^{566}) (ser^{603})	Affinity ↓ SV Affinity ↓ F-actin Bundling ↓↓ F-actin	Low freq. st. Ca^{2+}/Cal-modulin	I III?	(Huttner *et al.*, 1981;Kennedy & Greengard, 1981;Schiebler *et al.*, 1986;Bahler & Greengard, 1987;Czernik *et al.*, 1987)
Calcineurin ?	ser^{62} ser^{67} (ser^{549})	Dephosphorylates Affinity ↑ F-Actin	Ca^{2+}/Cal-modulin dependent	I II? III?	(Jovanovic *et al.*, 1996)

Table 1: Kinases and a putative phosphatase (calcineurin) that act on synapsins. Amino acid (aa) residues marked by parentheses are not present in all synapsins. Question marks indicate that the site has not been described in the actual synapsin type, but that it could be present in one or more subtypes based on sequence similarity. Difference in methods and/or species has lead to controversy about the exact position of the cdk5 sites, by two aminoacids, not specified in the table. Polymerization and bundling of actin probably represent the same process, but studied differently.

Synapsins I and II are widespread throughout all areas of the brain, but mainly restricted to synaptic terminals (De Camilli *et al.*, 1983;Kao *et al.*, 2008). Synapsin III shares structural similarities to synapsin I and II, but has a different expression pattern: light-microscopic studies indicate, in addition to showing puncta staining with a high degree of regional variation, that synapsin III stains the soma and processes of immature neurons (Pieribone *et al.*, 2002). Within the terminal, panspecific synapsin antibodies strongly label an area

apparently corresponding to the RP (Figure 2B). During activity immunoreactivity is relocated to the endocytic zones, responsible for recycling of vesicles, and to some extent to the active zone (Bloom *et al.*, 2003).

The inherent structural differences among isoforms offer interesting explanations for the varied expression pattern described (Mandell *et al.*, 1992;Kielland *et al.*, 2006), and led us to focus on the precise localization of synapsins within the terminals, and the localization of synapsin III within mossy fibre terminals (MF), which have been reported to selectively contain this subtype (Pieribone *et al.*, 2002).

THE ROLE OF ACTIN IN THE PRESYNAPTIC TERMINAL

The actin cytoskeleton is highly dynamic; driven by ATP-hydrolysis, globular actin monomers can form filamentous actin (F-actin). Similarly, filaments can be depolymerised into monomers. A large number of proteins control these two events to define the outer shape of and organize the interior of a cell. At the synapse, F-actin is most notable in dendrites, being involved in cytoarchitectural changes associated with LTP, but has also important roles in the presynaptic machinery (Cingolani & Goda, 2008).

Actin has been proposed to have a central role in the organization of vesicle pools (Dillon & Goda, 2005). In electron microscopic studies (Bloom *et al.*, 2003;Sankaranarayanan *et al.*, 2003) of synapses using immunocytochemical staining for F-actin, actin is situated around the vesicle pools and to a smaller extent in the AZ and lateral endocytic zones (Figure 2A).

Several functions have been proposed for actin in the terminal: as a scaffold restricting vesicle movement, as a conduit for transfer of vesicles between pools and as a constituent of the active zone, either as a guide for arriving vesicles or as a physical barrier inhibiting exocytosis (Cingolani & Goda, 2008). In a recent review the actin scaffold observed around the RP has also been suggested as a binding site for the enzyme CaMKII (Fdez & Hilfiker, 2006).

EXPERIMENTS FOR PAPER I

The roles of synapsins were examined by comparison between double knockout mice (DKO), lacking both synapsin I and II (Ferreira *et al.*, 1998) and wild type (WT) mice. First, it was determined whether the loss of vesicles in a distinct area of the synapse, the RP, is general, i.e. equal in different excitatory synapses. Such loss of RP vesicles upon removal of synapsins has been thoroughly described in model systems such as cell cultures and

lamprey or aplysia (Hilfiker *et al.*, 1999). A suspicion that this was not the case for all synapses in the rodent brain was based on examination of the hippocampus with the electron microscope, where the MF terminals appeared to have a more pronounced reduction in vesicle density than associational commissural terminals (AC). Other areas of the brain, such as the cerebellum were briefly screened as well, but less obvious effects were observed. The MF terminals and the AC terminals (Figure 3) were chosen for further studies because they are part of the main circuitry of the hippocampus and have been extensively characterized before. Also they differ markedly in size and function and hence offer analytical power in the case of differential findings.

In brief, the reduction of vesicles in the AC terminals was smaller than in the MF terminals. The next step was to test whether this difference could be attributed to a functional difference between the two types of synapses, thereby connecting synapsin to a specific function. Extracellular synaptic responses to orthodromic stimuli at different frequencies were measured. In the WT MF terminals, the response is markedly enhanced at a stimulating frequency of 2 Hz in WT mice, a form of short term plasticity not seen in the AC terminals, and severely reduced in synapsin I+II knockout mice. Hence, we argue that this function is specifically linked to synapsin, in opposition to the view that synapsins serve to maintain glutamate release during high frequency or prolonged stimulation (Pieribone *et al.*, 1995).

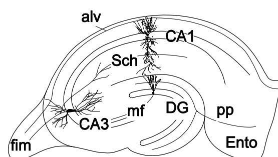

Figure 3. Schematic drawing of a cross section of hippocampus from paper I, figure 5A, showing the simplified, relevant excitatory network. Ento, entorhinal cortex; DG, dentate gyrus; pp, perforant path; fim, fimbria; alv, alveus; Sch, Schaffer collaterals (branches of AC collateral axons).

A NOVEL ROLE OF SYNAPSINS I AND II

The physiologic effect described was seen at a low firing rate, much lower than seen in previous studies on effects of synapsins on short term plasticity. At the frequency used for stimulation (2Hz), it is believed that the terminal can sustain the rate of exocytosis by recycling of vesicles and hence, does not need to recruit vesicles from the RP. Therefore, a possible role for synapsin in the recycling of vesicles to sustain synaptic response by actin polymerization was investigated. Indeed, a role for synapsin in recycling of synaptic vesicles

has previously been established in experiments using lamprey as a model system (Bloom *et al.*, 2003). Using similar methods, except in slices from WT and synapsin DKO mice, less actin was found to polymerize in endocytic zones of DKO mice, but only in MF, not in AC.

A system where synapsin is active in recycling through actin polymerization would be disrupted by drugs inhibiting the same polymerization. The actin stabilizing agent cytochalasin B was used to examine physiological as well as anatomical effects of the drug, which prevents both formation and breakdown of actin filaments (Cooper, 1987).

The main finding is the previously unknown role of synapsin in MF, that it is responsible for a substantial part of an output enhancement at a moderate firing frequency, and that this is not seen in another main type of excitatory synapse, the AC terminal. These studies support the finding of synapsin dependent actin polymerization, previously reported by Bloom et al. (2003) and emphasize that a common theory for synapsin function might not be found. Rather, several functions and probably also localizations should be sought for. For example, a more thorough study of the different synapsin subtypes could be valuable, as most work so far has been done on the a-subtypes.

Reduced function of the hippocampus, as can be assumed to result from the findings above, could lead to impaired learning. In Garcia et al. (2004) some, but not all subjects in a family with a mutated synapsin I gene are described as having learning difficulties. Also, a number of the family members experience epileptic seizures. Synapsin DKO mice are epileptic and a possible link between synapsin and epilepsy is proposed in paper II.

PRECISE LOCALIZATION OF SYNAPSIN III

Next, since function was different, could the levels of synapsin also be different between the synapse types? To answer this question synapsin was labelled immunohistochemically on electron microscopic sections, quantifying the level of synapsin in the different terminals and between compartments within the terminals. Synapsin was equally present, and could therefore serve another role in the AC (Jensen *et al.*, 2007) than in the MF. Interestingly, when a routine check was done of the synapsin antibody in synapsin DKO mice; an unexpectedly strong labelling was seen at the AZ. Since synapsin I and II are not present in the DKO, and the sections were processed with an antibody recognizing all three synapsins, this labelling probable represents synapsin III. It has been proposed that the function of synapsin III is different from synapsins I and II (Ferreira *et al.*, 2000), which is supported here by the finding of a difference in distribution at the ultrastructural level.

II. RECEPTOR ADAPTATION TO PRESYNAPTIC CHANGES

Theoretically, glutamate released in large amounts triggers AMPA and subsequently NMDA receptors in the dendrites, increasing the intracellular concentration of Ca^{2+}. As a result, AMPA and to a lesser extent NMDA receptors are translocated to the synaptic membrane, increasing the dendrites' ability to respond to a signal (Newpher & Ehlers, 2008). This is a form of LTP, as introduced in the Background section.

In paper I, differences between two major types of synapses in the hippocampus of synapsin I + II double knockout mice (DKO) was established: in the MF terminals, synapsins are involved in the organisation of vesicles and short term enhancement when stimulated at moderate frequencies, whereas the AC terminals had a less pronounced morphological phenotype and did not exhibit the mechanism of synaptic enhancement at the same firing rate. However, faster depression in the AC terminals of DKO mice compared to WT mice has been observed after stimuli at a high frequency (Jensen *et al.*, 2007). Hence, MF terminals from synapsin DKO mice release less glutamate at moderate frequencies as compared to WT mice, while AC terminals from synapsin DKO mice release less glutamate during high frequency activity than WT mice.

The differences between MF and AC terminals described above are exploited in paper II to see if two types of altered glutamate release between synapsin DKO and WT mice could be associated with postsynaptic alterations in receptor density, as in LTP.

THE AMPA AND NMDA TYPES OF GLUTAMATE RECEPTORS

Two main categories of glutamate receptors have been described: ionotropic and metabotropic, of which the first class will be dealt with here. Ionotropic glutamate receptors contain an external part binding glutamate and a transmembrane channel that is opened for Na^{+} and Ca^{2+} upon ligand binding (Ozawa *et al.*, 1998). This influx can cause a depolarization of the dendrite and neuron. The absolute and relative permeabilities of the two ions differ between types of ionotropic receptors. By contrast, metabotropic receptors induce a second messenger cascade (Pinheiro & Mulle, 2008).

Ionotropic receptors are of three subtypes, AMPA, kainate and NMDA receptors. AMPA, NMDA and probably also kainate receptors consist of oligomers; AMPA receptors being formed by compositions of the proteins GluR1-4, NMDA receptors formed by NR1 and NR2A-D and kainite receptors formed by GluR5-7 and KA1-2. In short, AMPA receptors induce a rapid depolarization followed by a fast deactivation, whereas NMDA receptors

exhibit a voltage dependent Mg^{2+} block and slow gating kinetics. Generally, AMPA receptors are less Ca^{2+} permeable than NMDA receptors, but there are differences among AMPA receptors, the edited AMPA GluR2 subunit being less Ca^{2+} permeable than the GluR1 subunit (Hollmann & Heinemann, 1994) . The kainate receptors are highly enriched in MF terminals and are, in contrast to other ionotrophic receptors, both pre- and postsynaptic (Darstein *et al.*, 2003), they are Ca^{2+} permeable and rapidly desensitized (Ozawa *et al.*, 1998).

Previously, a difference in distribution between AMPA and NMDA receptors in the AC as compared to the MF terminals has been found, the MF terminals having the highest density of AMPA receptors, whereas the AC terminals have the most NMDA receptors (Takumi *et al.*, 1999). Correspondingly, LTP is NMDA receptor dependent in AC and NMDA receptor independent in MF, probably relying on presynaptic action of kainate receptors (Citri & Malenka, 2008).

EXPERIMENTS FOR PAPER II

Based on the findings described in paper I, manifested by the reduced MF STE, we asked the question of whether reduced glutamate release would affect density or subunit composition of AMPA and NMDA glutamate receptors, as previously described in response to NMDA receptor activation in LTP (Citri & Malenka, 2008). Again, the experimental setup from paper I was used; two of the main synapses of the basic circuitry of hippocampus with vastly different properties, MF and AC, in the paper referred to as Schaffer collateral/commissural synapses, scc, were examined in WT and synapsin DKO.

Receptor levels were first crudely assessed using western blotting on whole, homogenized hippocampus. This also served as a control of antibody specificity. Four glutamate receptor antibodies were chosen: against GluR1, GluR2/3, NR1 and NR2A/B, on the basis of the previously described involvement in STE and LTP. No differences were found, but any complementary regulation between different classes of neurons could mask such effects.

A higher level of resolution was obtained using the same antibodies on sections prepared for electron microscopy. The immunogold postembedding technique used has recently been discussed in a methodological paper (Bergersen *et al.*, 2008). Two of the antibodies, NR1 and NR2A/B (detecting both subunits), were mixed to increase sensitivity. Besides, the total amount of analyses was reduced (5 WT+5 DKO = 10 x MF+AC = 20 x 3 antibodies = 60 x 20 electron micrographs = 1200 quantifications), and we did not expect to find changes between them as NMDA receptors usually consist of NR1 in complex with varying proportions of NR2A and NR2B subunits (Ozawa *et al.*, 1998). Similarly, a dispecific GluR2/3

antibody was used. Hence we could not discriminate between GluR2 and GluR3, which probably are both expressed in hippocampus (Keinanen *et al.*, 1990).

In conclusion, an increase in labelling of AMPA receptor GluR1 subunit was observed in the MF terminals and a tendency for increased NMDA NR1+2A/B staining in AC terminals. This is proposed to induce more Ca^{2+} influx during stimuli and hence make cells more excitable.

POSTSYNAPTIC RESPONSE TO A PRESYNAPTIC PHENOMENON

We propose a mechanism whereby impaired synaptic enhancement at moderate stimulating frequencies leads to an increase in permeability to Ca^{2+}. However, the adaptive mechanisms are unclear. In the following section pathways known to alter glutamate receptor subunit composition or may have effect on dendrites are discussed. A central question is how the dendrite can sense that it is stimulated, but not with the desired intensity. In theory, this signal can come from any of the neighbouring synaptic compartments, as well as by central inputs (Figure 4).

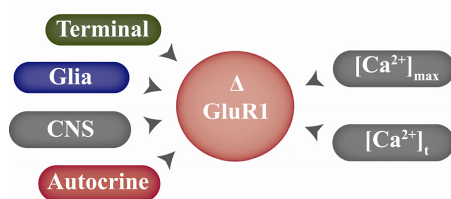

Figure 4. Sources of stimulants that potentially can trigger increase in GluR1 are summarized left. Right side represents the internal factor calcium: maximal (max) and residual (t=time) calcium concentration, controlling trafficking of glutamate receptors.

A well described determinant for synaptic LTP is the dendritic intracellular Ca^{2+}level, either observed as potentiation triggered by high levels of intracellular Ca^{2+}, or depression triggered by prolonged moderate Ca^{2+}levels (Cummings *et al.*, 1996). The balance between the two is finely tuned by Ca^{2+} buffers (Harney *et al.*, 2006;Nishiyama *et al.*, 2000), and could possibly be disturbed by changes in glutamate release.

Numerous other local and central signal systems exist to finely tune synapses: terminal release of large dense core vesicles containing neuropeptides, or release of secondary neurotransmitters (Gundersen, 2008), release of gliotransmitters (Volterra & Meldolesi, 2005;Haydon & Carmignoto, 2006) and higher order stimulation from either a local network of neurons or other sites within the CNS that shift the threshold for potentiation (Abraham

& Bear, 1996) or scale dendrites to be more or less excitable (Turrigiano & Nelson, 2004). Also, retrograde signals from the dendrite can possibly act in an autocrine fashion (Shin *et al.*, 2008).

A PUTATIVE ROLE IN EPILEPSY

If one of the roles of the MF terminal synapse is to pick out signals at specific frequencies, CA3 pyramidal cells will transmit signals periodically. That is, whenever struck by signals within a given frequency range, short term synaptic enhancement will increase the probability that the signal is passed on. If indeed the sensitivity of the MF synaptic dendrites is altered, it could mean that the synapse more easily responds to any signal, since the ability to pick out specific frequencies is not in order. Hence the system could instead pass signals tonically, in the next round affecting the AC synapses, which are tuned to periodic input and have high numbers of NMDA receptors, making them very susceptible to damage or unwanted output, a notion supported by the finding that CA1 pyramidal cells are especially vulnerable in ischemia (Kirino *et al.*, 1992). A possible mechanism could be that the AC synapse is vulnerable, but protected by the high pass filtering of the MF synapse, and that this protection could be somewhat alleviated by the adaptation seen in DKO mice. In addition, we did see a tendency of increased NMDA labelling in AC terminals, which could make the AC synapses in synapsin DKO mice more susceptible to seizures than in WT mice. A link between altered AMPA receptors and epilepsy is supported by studies of mice in which the expression of GluR2 subtypes has been altered to increase Ca^{2+} permeability. These mice also display an epileptic phenotype (Brusa *et al.*, 1995;Feldmeyer *et al.*, 1999).

In studies of humans with mesial temporal lobe epilepsy, an up-regulation of GluR1 has been found in the CA3 region of surgically removed hippocampi (de Lanerolle *et al.*, 1998).The up-regulation was later localized to dendrites postsynaptic to MF terminals and proposed to increase seizure susceptibility in these patients (Eid *et al.*, 2002). These observations point towards the relevance of the synapsin I + II DKO for human epilepsy and a possible clinical benefit of understanding the mechanism discussed here.

III. EXTRACELLULAR GLUTAMATE CONCENTRATION IN THE BRAIN

The Background section introduced the effects and toxicity of increased glutamate concentration in the extracellular space, and the ability to end chemical signalling between synapses by glutamate transport into glia cells. Also, the concentration of extracellular glutamate "at rest", i.e. without stimuli, has a possibly important effect on the synapse. In particular, low levels of glutamate can trigger or alter glutamate receptors, eliciting responses that are sub-threshold for evoking an action potential, as discussed in Cavelier et al. (2005). A controversy exists over the exact concentration of glutamate in the extracellular space, because microdialysis measurements (Westergren *et al.*, 1995) give much higher values than what could be expected if the concentration mainly depends on properties of glutamate uptake.

Glial glutamate uptake is accomplished by specific excitatory amino acid transporters (EAATs). Transporter function is dependent on which and how many ions that are co- or countertransported together with glutamate. If the reaction is allowed to proceed to equilibrium, ultimately the concentration of glutamate in the extracellular space should depend on the properties of these transporters and on the prevailing concentrations of transported ions. The most abundant such transporter, GLT-1 (EAAT2) was characterized by Levy et al. (1998). The current work explores the properties of the transporter GLAST (EAAT1), predominant in areas of the brain such as the retina and cerebellum and throughout the brain early in development, but also abundant in adult cortex and hippocampus (Danbolt, 2001). More precisely, the stoichiometry is determined using electrophysiological recordings and comparing the results to theoretical predictions of transporter behaviour.

EXPERIMENTS FOR PAPER III

Retinal Müller cells from salamander (*Ambystoma tigrinum*) were chosen because of their favourable expression pattern of glutamate transporters, expressing mainly GLAST and only minor amounts of GLT-1 and EAAT5 (Eliasof *et al.*, 1998), and their amenability to investigation by the whole cell patch clamping technique. In our experimental setup, transmembrane current was measured in a voltage clamp configuration. Transporter activity was manipulated by altering ion concentrations mainly in extracellular solutions, and the results monitored by blocking transporter activity. Hence, absolute values were not measured, rather the relative GLAST-dependent current. Results supported previous

suggestions based on sequence similarity, that GLAST shares the same stoichiometry as GLT-1 and EAAT3 (Zerangue & Kavanaugh, 1996).

If glutamate concentration depended solely on glutamate transporters with a known stoichiometry of 3 Na^+, 1 H^+, 1 $glutamate^-$ being cotransported and -1 K^+ being countertransported, and if uptake was allowed to reach equilibrium, then the extracellular glutamate concentration would be ~ 2 nM (Zerangue & Kavanaugh, 1996;Levy *et al.*, 1998). However, there are several other factors that could possibly disturb this picture. These factors may be grouped depending on their site of origin (terminal, dendrite or glia).

SOURCES AND SINKS OF EXTRACELLULAR GLUTAMATE

In terminals, spontaneous fusion events release quanta of glutamate, causing a more or less continuous outward flow of glutamate from neurons, as shown by the build-up of extracellular glutamate with the blockage of glutamate transporters (Jabaudon *et al.*, 1999). Hence, glial transport might not reach the equilibrium values predicted. There are also other sources of extracellular glutamate, indicated in Figure 5.

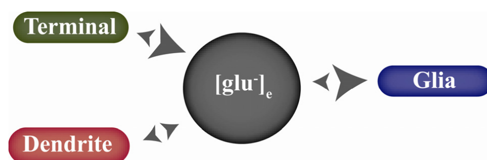

Figure 5. Origin of extracellular glutamate. Arrowheads indicate putative directions and proportions of glutamate flux. Glutamate diffusion out of the synaptic cleft is not shown.

Because glutamate also can be released when exocytosis is blocked (Jabaudon *et al.*, 1999), other sources must exist. In a review by Danbolt (2001) several mechanisms are proposed: transporter glutamate exchange for other amino acids, glutamate leak through vesicular glutamate transporters situated in the plasma membrane after exocytosis, and leakage through volume regulating channels associated with swelling.

The importance of glial uptake is attested by studies of glutamate toxicity. Presence of glia raises the glutamate dose needed to induce necrosis in slices and cultures (Garthwaite *et al.*, 1992;Rosenberg *et al.*, 1992). In addition to the ample glial glutamate transport, the kinetics of glutamate removal is influenced by the mere binding of glutamate to receptors and transporters, as reviewed by Tzingouni and Wadiche (2007).

EAATs are not restricted to glia, but are also found in dendrites and in terminals, though it has been argued that the latter transporters are less numerous than those in glia (Danbolt, 2001).

During the last decade, several studies have supported glial release of glutamate from vesicles through a Ca^{2+} dependent exocytotic pathway, triggered by metabotropic glutamate receptors or purinergic receptors (Volterra & Meldolesi, 2005;Bergersen & Gundersen, 2008). This offers a pathway for glial regulation of synaptic activity within an area consisting of several synapses. Glial glutamate release has been shown to be regulated by other compounds such as prostaglandins (Bezzi *et al.*, 1998), suggesting that vesicular glutamate release from glia can be part of a higher order of plasticity.

Against the importance of glial release, it can be argued that it is limited by the low number of vesicles seen in glia. On the other hand, the release could be more efficient if it was coordinated with the release of other gliotransmitters with synergistic effects (Volterra & Meldolesi, 2005). Glial glutamate release independent of exocytosis is evident (Kimelberg *et al.*, 1990;Sperlagh *et al.*, 2002;Ye *et al.*, 2003), but in general a substantial proportion of the release is blocked by bafilomycin and clostridium toxins, which inhibit exocytosis (Araque *et al.*, 2000;Pasti *et al.*, 2001).

A toxic effect of mercuric chloride is supposedly through an effect on GLAST, uncoupling transport of protons (Nagaraja & Brookes, 1996). Taking advantage of the method established to determine stoichiometry, this alleged effect was tested by changing the proton concentration when mercuric chloride was added to the extracellular bath. If indeed a proton uncoupling was induced by mercuric chloride, we would expect that the measured reversal potential (as described in paper III) was independent of protons. This was not the case; protons were still coupled to glutamate transport after application of mercuric chloride, suggesting that the toxic effect of mercuric chloride resides elsewhere.

To summarize, this thesis describes mechanisms for control of release, adaptation to altered release and reuptake of glutamate in the central nervous system, mechanisms that are previously unknown or have been incompletely described before.

Based on the results obtained and on the simplified understanding of hippocampus function described here, synapsin I + II DKO mice could have a significant impairment in their sorting of new impressions and learning. This hypothesis can be tested by functional studies on DKO mice, with the limitation that other structures can be equally impaired by the lack of synapsin, hence such studies cannot firmly establish whether the effect results from hippocampus only. It should also be noted that even though the findings here apply to an important part of hippocampus, they are still only pieces of a highly complex structure.

The seizure proneness in synapsin I+II DKO mice, and in human patients with mesial temporal lobe epilepsy, may partly depend on an upregulation of postsynaptic GluR1 glutamate receptor subunits. In future analyses it would be interesting to increase resolution further, by dividing the dendrite into different areas to see whether trafficking of receptors (Newpher & Ehlers, 2008) could play a role.

Stoichiometry of the glutamate transporter GLAST was identical to that of GLT-1 and EAAT3: 3 Na^+, 1 H^+, 1 glutamate$^-$ being cotransported and -1 K^+ being countertransported, hence a difference in extracellular glutamate concentration between areas expressing GLAST and areas expressing GLT-1 or EAAT3 was not supported.

There is a functional link between the synapsins (papers I and II) and the glutamate transporters (paper III) in so far as synapsins contribute to the release of glutamate, which is recaptured by glutamate transporters. A change in glutamate release could trigger a change in the glial expression of glutamate transporters in order to alter the reuptake kinetics, possibly in the direction of decreased uptake and thereby prolonged effect of each release quantum. This hypothesis remains to be tested.

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Simen Gylterud Owe
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