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THE INTERACTION BETWEEN ASTROCYTES AND EXCITATORY SYNAPSES IN HEALTH AND DISEASE

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Cover illustration: The painting titled “Witnessing the stars” is an attempt by me to capture the essence of this thesis. It depicts a person looking at the stars outside the window while the stars (astrocytes in green) and the neurons (in red) in the brain participate in the circuitry for perception of the night sky. The color codes of the astrocytes (green) and neurons (red) matches the violinist and the dancing girl as an attempt to depict the interplay of astrocytes and neurons together creating the neural symphony.
Ipsit

The interaction between astrocytes and excitatory synapses in health and disease
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The thesis will be defended in public at Eva och Georg Klein salen, Biomedicum, Floor 3, Solnavägen 9, Solna, Tuesday the 24th of November, 2020 at 10:00 am

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To Maa, Papa and Paiya

POPULAR SCIENCE SUMMARY OF THE THESIS

Everything, we do in our daily life from speaking Latin, to playing curling or diving with the sharks and also eating, walking, sleeping involves the activity of our brain cells. Our brain is composed of billions of neurons which form connections and communicate between themselves. These connections form specific circuits which are involved in all our behaviors. Along with neurons, the brain is also composed of another cell type called the glial cells. Studies have indicated that a type of glial cells called the **"astrocytes"** are also involved in modulating this neuronal communication and they have been found to be affected in disease conditions.

A large number of people are affected by mental disorders such as Alzheimer's disease (AD) and depression. Most of the available treatments designed for these diseases have met with limited success making it hard for the patients and their families. One of the reasons can be that these treatments have been designed with neurons as the central targets and by not considering astrocytic involvement in neuronal communication we might be missing the other half of the picture. Hence understanding the role of astrocytes in these diseases and developing treatments with astrocytes as pharmacological targets may be the answer. However, our understanding of the role of astrocytes in disease conditions is still very limited and it therefore becomes extremely important to investigate the communication between astrocytes and neurons in health and disease.

In this thesis, we investigated how the astrocyte-neuronal communication is affected in AD and depression. We found that indeed astrocytes are impaired both morphologically and functionally in these diseases which in turn affects the neuronal activity. In one of the studies (Paper **II**) we found that restoring the astrocytic function improves the impaired neuronal activity seen in depression. In summary, we found that astrocyte functions are impaired in diseased conditions and propose that pharmacological targeting of astrocytes aimed to restore their functions can help in improving the disease conditions.

ABSTRACT

Synaptic transmission forms the basis of neuronal activity and astrocytes play an integral part in this process. Glutamate is the major excitatory neurotransmitter in the brain and an important function of astrocyte during excitatory synaptic activity involves the uptake of glutamate through astrocyte glutamate transporters (EAATs) and hence shaping the excitatory neurotransmission. Recently astrocytes have also been shown to affect a sustained form of inhibition. In this thesis, we study these aspects of astrocyte functions and their role in affecting the excitatory synapse. In addition, using animal models of diseases we describe how the astrocyte synapse interaction is affected in diseased conditions.

In the **Paper I**, we confirmed previous studies showing that astrocytes respond by a long-lasting depolarization upon synaptic stimulation, mediated by an increase in extracellular potassium ions. We found that this long-lasting depolarization is enhanced when astrocytic glutamate transporters are blocked, whereas the neuronal EPSC is reduced under these conditions. Blocking the glutamate transporters reduces the AMPA receptor response whereas the NMDA receptor activation is increased, causing the enhancement seen in the astrocytic long-lasting depolarization. Since astrocyte glutamate transporters are impaired in many neurodegenerative diseases, this study gives us an idea about how the impairment of astrocytic glutamate transporters can influence synapse activity.

In the **Paper II** and **III** we used animal models of depression and AD respectively to understand the role of astrocytes in affecting synaptic transmission.

In **Paper II** we used the well characterized FSL rat model of depression and investigated how reactive astrocytes affect inhibition by producing and releasing GABA. We found that tonic inhibition of pyramidal neurons is increased in the FSL rat while the synaptic plasticity is impaired. We also found that this tonic inhibition was reduced by blocking the astrocytic GABA synthesis or by chelating intracellular Ca^{2+} in astrocytes in slices from the FSL rat, giving evidence for increased astrocytic involvement in tonic inhibition in an animal model of depression. Furthermore, blocking of astrocytic GABA synthesis restored the impaired synaptic plasticity seen in FSL rats.

In the **Paper III**, we explored the astrocyte mediated glutamate uptake in Alzheimer's disease model using a knock in AD mouse model $App^{NL\text{-}G\text{-}F}$. Astrocytes displayed a reactive morphology, with swollen cell bodies and increased number of processes. We found that though there was an increase in the protein expression levels of astrocytic glutamate transporters (EAATs), they were functionally impaired as reflected by the glutamate transporter current recordings.

LIST OF SCIENTIFIC PAPERS

I. **Ipsit Srivastava**, Erika Vazquez-Juarez, Maria Lindskog
"Reducing glutamate uptake in rat hippocampal slices enhances astrocytic membrane depolarization while down- regulating CA3-CA1 synaptic response."
Frontiers in Synaptic Neuroscience, 2020, 12:37; doi: 10.3389

II. **Ipsit Srivastava**, Erika Vazquez-Juarez, Lukas Henning, Marta Gómez-Galán and Maria Lindskog
"Blocking Astrocytic GABA Restores Synaptic Plasticity in Prefrontal Cortex of Rat Model of Depression."
Cells, 2020, 9, 1705; doi:10.3390

III. **Ipsit Srivastava**, Marta Pereira Iglesias, Tamer Ayberk Kaya, Luis Enrique Arroyo-García, Per Nilsson, André Fisahn, Maria Lindskog, Silvia Maioli and Raúl Loera-Valencia
"Increased levels but reduced function of astrocytic glutamate transporters in the hippocampus of the *App* $^{NL-G-F}$ knock-in mice"
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

Aß	Amyloid ß-peptide
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	Amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
CA	Cornu Ammonis
DL-TBOA	DL-threo-ß-Benzyloxyaspartic acid
EAAT	Excitatory amino acid transporter
EPSC	Excitatory post synaptic current
fEPSP	Field excitatory post synaptic potential
FSL	Flinders sensitive line
GABA	Gamma-Aminobutyric acid
GAT	GABA transporter
GFAP	Glial fibrillary acidic protein
GLT-1	Glutamate transporter-1
IPSC	Inhibitory post synaptic current
Kir	Inward-rectifier potassium channel
MAO-B	Monoamine oxidase B
MDD	Major depressive disorder
NMDA	N-methyl-D-aspartate receptor
STC	Synaptically activated, transporter-mediated current

1 INTRODUCTION

1.1 ASTROCYTES: STARS IN THE BRAIN-VERSE

Stars have been fundamental in shaping the universe as we see it today. Life on earth would not have been possible without a star (*Sun*) and they play a pivotal role in the functioning of the universe. As much as the stars are important for the outside universe, equally fascinating and important is the role of non-neuronal star like cells called astrocytes inside our brain. In the human brain, neurons communicate with each other through synapses which forms the basis of all brain functions, and a specific type of glial cells called astrocytes have been found to be extremely important in maintaining this synaptic transmission. Since the early nineteenth century many neuroscientists including Michael von Lenhossék, Fernando De Castro and Carl Ludwig Schleich expressed the idea of glial involvement in brain functions[1]. Santiago Ramón y Cajal developed a staining method for astrocytes and his sketches showed astrocytes in close contact with the neurons[2], laying the ground for the idea that astrocytes might play a role in synaptic transmission. We now know that astrocytes perform numerous functions in the brain ranging from maintaining the ionic homeostasis, blood brain barrier, energy metabolism, protection against insults and inflammation and hence can affect synaptic activity[3]. Recent studies have shown them to be actively involved in modulating synaptic transmission as well as behavior[4, 5]. In this thesis, we investigate the interaction of astrocytes with synapses and their role in affecting the synaptic transmission in health and disease.

2 LITERATURE REVIEW

2.1 DYNAMIC AND DIVERSE

Most of the initial attempts on astrocyte classification were based on morphological studies. Astrocytes express glial fibrillary acidic protein (GFAP) and immunohistochemical staining of this protein has been the main method to visualize astrocytes in different anatomical regions of the human brain. Astrocytes have been characterized depending on their location in the grey matter as protoplasmic astrocytes and in the white mater as fibrous astrocytes[6]. Astrocytes tile the entire nervous system and in mature astrocytes this tiling follows a non-overlapping pattern giving astrocyte its domain specific arrangement which defines the territory of a particular astrocyte[7].

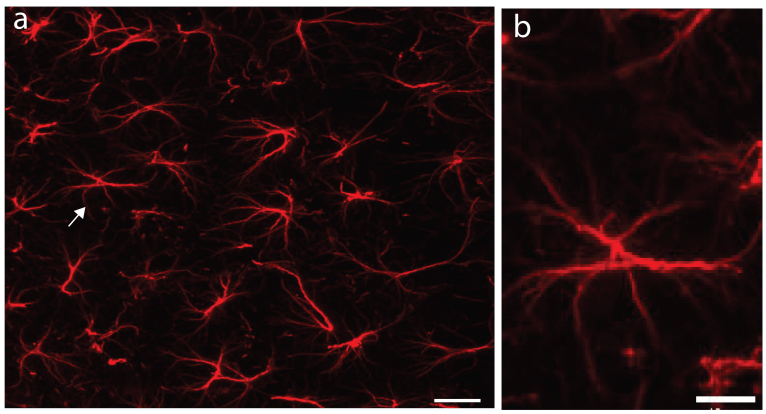

Figure 1: (a)Astrocyte seen by GFAP immunostaining (red) in the stratum radiatum of the mouse hippocampus (scale bar: 20μM) (b) enlarged view (scale bar: 10μM) (paper III)

Physiological studies have revealed that astrocytes show a high expression of potassium channels along with the calcium activated BEST-1 ion channels and hemichannels[8, 9]. The high expression of potassium channels set up the hyperpolarized resting membrane potential of astrocytes[10] and contributes to the low input resistance[11, 12]. Astrocytes are generally considered electrically passive due to the absence of action potential firing however they do depolarize in response to glutamate application[13, 14]. Though morphology and physiology gives us an idea about the basic characteristics of astrocytes, the multiple functions performed by them in the brain makes the astrocyte population functionally heterogeneous[15]. Astrocytes are dynamic and adapt constantly to the changing synaptic microenvironment around them. As the astrocytes play many roles to ensure proper functioning of synaptic activity, they show differences in aspects ranging from circuit specificity[4], gene expression profiles[16], ion channel and transporter expression, gap junction connectivity[17] as well as in their calcium activity[18] making functional classification complex.

2.2 THE POTASSIUM HOMEOSTASIS MANAGER

Potassium released as a result of neuronal activity needs to be taken up in order to maintain the ionic homeostasis. Astrocytes with their high potassium permeability and gap junction connectivity are ideally suited for this job. Studies in the amphibian astrocytes first showed slow depolarization following nerve stimulation. The explanation for this depolarization was that the K^+ ions accumulate in the extracellular space as a result of neuronal activity and are

taken up through the astrocytic potassium channels. The excess potassium is then redistributed through the glial syncytium restoring the ionic homeostasis and was proposed as the spatial potassium buffering hypothesis[19-21]. In the retinal Müller cells a specialized form of this potassium buffering has been suggested, called potassium siphoning, where K^+ ions enter the astrocyte at the region of higher concentration of K^+ and is expelled at another region in the same cell[22]. One of the major contributors in maintaining this potassium homeostasis have been the glial inward rectifier potassium channels (Kir). Among different glial inward rectifiers, Kir4.1 has been shown to be abundantly expressed in astrocytes and has been found to be located in the astrocytic processes surrounding the synapses and blood vessels, highlighting its importance in maintaining the synaptic micro environment[23, 24]. Several studies using knock out models or downregulating Kir4.1 channels have shown to affect the glia mediated potassium uptake and hence affect overall neuronal activity[25-27]. Apart from the inward rectifier potassium channels astrocytes also have two pore K^+ channels and voltage dependent potassium channels[28, 29].

2.3 THE THIRD PARTNER

Classically astrocytes were not considered to be involved in synaptic transmission and supposed to play supportive role for neurons. However, studies in the early 90s aimed to understand the role of astrocytes in synaptic transmission showed that astrocyte cultures had calcium activity in response to glutamate[30]. Studies using electron microscopy revealed that astrocytic processes ensheath the synapses[31]. This morphological proximity of the astrocytic process to the synapse gives the astrocyte access to closely monitor the synaptic activity. These studies suggested that there may be a bidirectional communication between the astrocytes and neurons which gave birth to the concept of the tripartite synapse in which astrocytes are active partners in synaptic activity[32, 33]. Recent studies have shed more light on the tripartite synapse as a morphological and functional unit. STED microscopy revealed the proposed morphology of the tripartite synapse comprising of a presynaptic axonal bouton, a post synaptic dendritic spine and an astrocyte process where astrocyte processes show close contacts with dendritic spines sometimes forming an `O´ ring structure[34]. Astrocytes as a part of the tripartite system have been shown to modulate basal synaptic transmission[35] and in basal ganglia have even been shown to participate in signaling specific to distinct neural circuits[4] establishing the idea of astrocyte-neuronal crosstalk proposed in the tripartite synapse model.

2.4 GLIOTRANSMISSION, LANGUAGE OF THE ASTROCYTES

Extensive research in the last decade has focused on understanding if there is an active bidirectional signaling between the astrocytes and the neurons. Astrocytes respond to neuronal activity through calcium activity and calcium waves were recorded in astrocytes in response to neuronal glutamate release[30]. In vivo calcium imaging of astrocytes has revealed the

complexity of astrocytic calcium activity as it shows a difference in specific microdomains in the astrocytic processes as well as in the soma[36]. Astrocytic calcium activity has also been shown to be involved in basal synaptic transmission[35].

To have an active signaling from the astrocytes to the neurons there should be an active machinery involved. Astrocytic calcium activity has been suggested to trigger an active release of signaling molecules from the astrocytes, called gliotransmission[37]. Calcium activity in astrocytes have been shown to release these active compounds called gliotransmitter through exocytosis[38]. Immunogold labelling had shown presence of vesicular glutamate transporters in astrocytes[39] however vesicular release is still debatable. Activation of endocannabinoid receptors on astrocytes lead to release of glutamate from the astrocytes which in turn affect the NMDA receptors on neurons[40] and have been shown to control synaptic strength[41]. Astrocytes have been shown to release D-Serine which has been shown to control NMDA receptor dependent long term plasticity[42]. Astrocytes have also been shown to express proBDNF[43] and release ATP[35]. Astrocytes have also been shown to synthesize as well as release GABA and affect LTP[44].

Another important challenge is to investigate if there is an astrocytic specificity for release of a particular gliotransmitter[45]. One study has shown that a single astrocyte can release glutamate and ATP in different temporal pattern which cause an initial glutamate mediated potentiation followed by ATP mediated depression[46]. However, to understand this temporal difference in gliotransmitter release from a single astrocyte, we need to study and interpret the complexities and regional compartmentalization of the astrocytic calcium activity[47, 48].

2.5 THE GLUTAMATE CARETAKERS

Glutamate is the main neurotransmitter in the CNS involved in excitatory neurotransmission and astrocytes play an important role to take up glutamate from the synaptic cleft to tune synaptic transmission and prevent excitotoxicity. Astrocytes take up glutamate through glutamate transporters called the excitatory amino acid transporters (EAATs). Among five different types of EAATs, EAAT-1 and EAAT-2 are expressed on astrocytes[49-51]. Glutamate uptake through the EAATs is electrogenic involving inward movement of one glutamate along with 3 Na^+ ions and 1Cl^- ion with 1K^+ moving outside and this net ionic movement allows to record EAAT current from these transporters[52]. In adult animals, the majority of glutamate is taken up by EAAT-2[53, 54] that is expressed mainly in astrocytic processes. The localization of EAAT-2 on astrocytic processes has been shown to be dynamic and the transporters form stable clusters which disperse upon glutamate treatment[55]. This EAAT-2 surface mobility is pronounced in regions of increased synaptic activity thus allowing for activity dependent modulation. Impairing EAAT-2 surface mobility affects the kinetics of neuronal EPSC[56]. Reduction in glutamate uptake has been shown to affect synaptic transmission by enhanced activation of the extra synaptic NMDA receptors and presynaptic mGlu receptor activation[57]. Blockage of glutamate transporters have also been shown to potentiate postsynaptic excitation[58] and prevent remodeling of dendritic spine head protrusions[59].

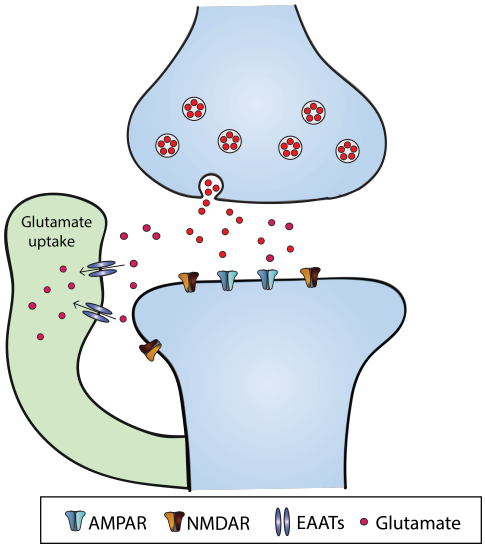

Figure 2: A tripartite synapse model with a presynaptic and a post synaptic neuron and an astrocyte process. Glutamate released by the presynaptic neuron is uptaken by the post synaptic glutamate receptors and the remaining glutamate is uptaken by the astrocyte glutamate transporters (EAATs).

2.6 THE INHIBITORY ASPECT

Inhibition shapes neural circuit activity. Along with interneurons, astrocytes have also been shown to play an important role in inhibitory synaptic transmission. Astrocytes take up GABA through the astrocyte GABA transporter GAT-3[60]. GAT-3 is expressed in the astrocytic process and have been shown to affect excitatory[61] synaptic transmission. In addition, astrocytes have also been shown to synthesize GABA. In a mouse model of Alzheimer's disease (AD), the mechanisms of astrocyte GABA synthesis involves the putrescine dependent pathway and the MAO-B enzyme[44]. This astrocytic GABA has been shown to be released by the specific anion channels called Best-1and contributes to tonic inhibition which is a form of persistent inhibition affecting the extra synaptic $GABA_A$ receptors and is distinct from the phasic inhibition[62]. GABA synthesis and its subsequent release have been shown to be feature of astrocytes in animal models of AD[44], stab wound injury[43] and depression[63] and have been shown to affect long term potentiation (LTP)[63]. Astrocytes have also been shown to express ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors[64]. Recently it has been shown that astrocytes respond differentially to specific interneuron subtypes affecting synaptic activity[65]. These studies give us an idea about the extremely intricate machinery through which astrocytes can affect inhibition and hence synaptic transmission.

2.7 FROM SYNAPSE TO BEHAVIOR

As astrocytes affect the synaptic activity, memory and are part of specific brain circuits it becomes extremely interesting to dissect the role of astrocytes in different behaviors. Recent application of optogentic tools to manipulate astrocytes have helped us in answering these questions. Optogentic manipulation of astrocytes in the hypothalamus and cortex have shown to affect feeding and sleep behavior respectively[66, 67]. Recent studies manipulating the G-protein coupled receptors have revealed multiple pathway through which astrocytes affect

different aspects of memory. Selective activation of astrocytic G_q proteins in mice have been shown to increase spontaneous synaptic activity and enhance learning and memory in fear conditioning[68] whereas G_s protein activation coupled to astrocytic A_{2A} receptors have shown to reduce long term memory[69]. Similarly, activation of G_i protein affected the remote memory with no effect on recent memory[70]. These studies point towards the extremely complex yet distinct mechanisms between astrocytes and neural circuits to regulate different aspects of the same behavior.

2.8 THE REACTIVE ONES

Astrocytes in many neurodegenerative diseases undergo changes in their morphology and domain specific arrangement, as well as in their protein expression profiles, including an increased GFAP expression[71, 72]. These astrocytes, termed reactive, show atrophy and changes in their volume and processes affecting the ensheathing of the synaptic terminals[73]. Apart from the morphological changes, recent experiments have highlighted the functional implications of these reactive astrocytes on the overall synaptic activity suggesting that astrocytes undergo a gain or loss of function under this condition[71, 74].

Glutamate uptake is one of the most important functions of astrocytes and functional changes have been shown in glial glutamate transporter, EAAT-2 in reactive astrocytes during ischemia[75]. This astrocyte mediated glutamate transport is also affected in cases of epilepsy[76] and amyotrophic lateral sclerosis[77] Astrocyte glutamate transporter genes are dysregulated in MDD[78] and glutamate transport have been shown to be impaired in mouse model of AD[79] These change in functions are not limited to affect synaptic transmission by glutamate uptake but also astrocyte mediated tonic inhibition. Reactive astrocytes show an increase in astrocyte specific tonic GABA current mediated hippocampal memory impairment in AD mouse model[44] as well as in cases of animal model of depression, astrocyte mediated increase in GABA release has been found to affect the LTP in prefrontal cortex[63]. Increased GABA release through astrocytes have also been seen in stab wound injury model[43]. Thus, reactive astrocytes affect the excitatory as well as the inhibitory aspects of synaptic activity.

Though the phenomena of reactive astrocytes have been popularly painted as being detrimental for the neurons, this does not represent a complete picture. Recent studies show that astrocyte react differently to different insults as LPS injection and ischemia in mice led to the appearance of two distinct populations of reactive astrocytes called A1 and A2 through the activation of different molecular pathways with the A1 reactive astrocytes being neurotoxic whereas the A2 astrocytes showing neuroprotective properties[80, 81]. This underscores the fact that astrocyte reactivity is not a single phenomenon, in addition to reactivity induced by inflammatory signals or trauma, reactivity can also be a finely tuned process which is governed by specific changes in the synaptic microenvironment. Astrocytic reactivity can thus be assumed as a response of these cells sensing major deflections from control conditions, leading to changes in their functional properties through triggering of different molecular pathways which can be

beneficial or detrimental for the adjacent synapse. As astrocyte activation involves microglia[80], studies aimed to understand reactive astrocytes in light of different types of microglial activation might make the picture clearer.

2.9 THE NOVEL TARGETS

Most of the treatments for neurological diseases have been designed to target neurons and have met with limited success. Recent studies emphasize the importance of astrocytic dysfunction in these diseases and suggest that attempts to restore them may help to improve disease conditions. In animal model of depression, astrocyte specific Kir4.1 channels responsible for maintaining potassium homeostasis has been shown to be upregulated and involved in neuronal bursting[82]. Inhibition of these Kir4.1 channels in astrocytic cultures have shown to increase BDNF expression which may be linked to the development of epilepsy[83] and have been suggested as a therapeutic target[84]. In AD mice models, reactive astrocytes secrete GABA which impairs memory in hippocampus and inhibiting the astrocytic GABA synthesis using selegiline restores memory[44]. Similarly, in animal model of depression, inhibition of astrocytic GABA improves LTP in the prefrontal cortex[63]. Glutamate uptake through astrocytic glutamate transporters have been shown to be affected in animal models of depression[85]. In case of Alzheimer's disease, glutamate uptake by astrocytes is impaired and upregulation of glial glutamate transporter EAAT-2 using ceftriaxone and other drugs improves cognition[86-88]. Thus, astrocytic channels and transporters involved in specific aspects of astrocytic function provide us with many novel pharmacological targets which can be useful while designing the treatment for these diseases[89].

3 RESEARCH AIMS

Astrocytes undergo functional changes in diseases which affect the astrocyte synapse crosstalk. The overall theme of this thesis is to study the interaction of astrocytes and excitatory synapses under control conditions and how it is affected in diseases.

The specific aims:

- To study the astrocyte-neuron interaction in conditions of reduced glutamate uptake as astrocyte mediated glutamate uptake is impaired in mental diseases (**Paper I**).

- To study the role of astrocytes in inhibition and how the inhibition by astrocytes affect synapse activity in an animal model of depression (**Paper II**).

- To study the astrocyte glutamate transporters in neurodegenerative disease using a knock in AD mouse model (**Paper III**).

4 MATERIALS AND METHODS

This section includes brief explanations of the main techniques used in the thesis. Kindly refer to the respective papers for a more detailed specification of the methods.

4.1 ANIMAL MODELS

In order to study the changes in astrocyte synapse interaction in case of depression and AD, we have used animal models of these diseases. Animal models though with their limitations and not being identical replicates of the human disease conditions, have been very helpful to understand specific aspects of different diseases. Here we have studied astrocyte neuronal interaction using two different animal models.

4.1.1 Flinders sensitive line (FSL)

In the **Paper II** to understand how astrocyte synapse interaction is affected in depression, we used an animal model of depression called the Flinders sensitive line (FSL). FSL is a selectively bred rat model of depression and it mimics behavioral alterations seen in depressed humans such as changes in sleeping behavior, social behavior and psychomotor retardation. It is a well validated rat model of depression and has been used to understand the underlying mechanisms of depression[90-92].

4.1.2 App^{NL-G-F}

In the **Paper III** we wanted to understand the role of astrocytes in Alzheimer's disease (AD). Most of the animal models of AD show an overexpression of APP which may have overexpression artifacts. For this study, we used a knock in mice model called the *App*$^{NL-G-F}$ where the Swedish, Beyreuther/Iberian and Arctic mutation has been introduced in the APP gene. The mice display robust Aβ pathology and behavioral deficits as seen in human AD patients[93-95].

4.2 SLICE ELECTROPHYSIOLOGY

Electrophysiological recordings is an important method used in this thesis in order to understand the functional interaction of synapses and astrocytes. It provides a valuable tool to record the electrical activities of the cells. As we were interested in studying the interaction between neurons and astrocytes we need to keep the neural circuitry intact. Hence, we performed slice electrophysiology in acute brain slices obtained from the region of interest in the brain. We performed field recordings and whole cell patch clamp electrophysiology depending on our questions.

4.2.1 Field recordings

Field excitatory post synaptic potentials (fEPSPs) give us an idea about the activity of the population of neurons. Using 400 µM brain slices field recordings were performed in the hippocampus (**Paper I**) by stimulating the Schaffer collateral using a bipolar electrode and response was recorded using an Ag/AgCl electrode. Similarly, fEPSPs were recorded in the PFC by stimulating the layer two/three of the prelimbic cortex and recording from layer five (**Paper II**).

4.2.2 Whole cell path clamp electrophysiology

Whole cell patch clamp is one of the main methods which has been used to understand the astrocytic as well as neuronal responses in this thesis. Ervin Neher and Bert Sakmann[96, 97] developed this technique which allows us to record electrical activity of a single cell with an Ag/AgCl electrode and control and manipulate its electrical activity. It has helped neuroscientists enormously in understanding the synaptic and neuronal physiology, the function of ion channels, and the regulation of neural circuit activity[98]. This technique has also been extended to understand the physiology of the astrocytes. The fact that astrocytes can be patched allows for manipulation of astrocytic activity through clamping the astrocyte at specific potentials as well as by introducing specific drugs through the patch-pipette. This has helped us to understand more about the astrocytic contribution to different aspects of synaptic activity such as Ca^{2+} dependent D-serine release from astrocyte affecting LTP[42]. Due to the low membrane resistance of the astrocytes patch-clamp recordings of astrocytes can be used to record the field excitatory potential (a-fEPSP)[99]. Hence extending the patch clamp electrophysiology technique to dissect out the role of astrocytes in modulation of synaptic activity has enhanced our knowledge about astrocyte-synapse interaction. One of the main part of the thesis focuses on understanding the functional changes in astrocyte mediate glutamate uptake. As the glutamate transport is electrogenic it can be recorded through a patched astrocyte[100] and this method has been used in the **Paper I** and **Paper III** of the thesis. Similarly, we patched astrocytes to understand their membrane response in conditions of reduced glutamate uptake by blocking glutamate transporters (**Paper I).** In neurons, whole cell patch clamp has been used to record both the excitatory and inhibitory post synaptic currents.

4.3 IMMUNOHISTOCHEMISTRY

Morphological studies of astrocytes have played a major role in shaping our understanding of astrocytes as it revealed that astrocytes show morphological changes in disease conditions. The astrocyte proximity to neurons seen by Cajal with his own specific staining technique made him speculate about astrocyte synapse interaction[2] and hence morphological analysis gives us the first indications about the state of astrocytes in control and disease conditions. We performed morphological analysis as a part of the studies in **Paper II** and **III**. Using antibodies to stain astrocyte intermediate filaments called Glial fibrillary acidic protein (GFAP) and astrocyte cell body using s100ß, morphological differences were found between wild type

controls and the diseases models. Analysis of the stained astrocytes gave us an idea about morphological characteristics such as the soma size and process thickness.

5 RESULTS

5.1 PAPER I: REDUCING GLUTAMATE UPTAKE IN RAT HIPPOCAMPAL SLICES ENHANCES ASTROCYTIC MEMBRANE DEPOLARIZATION WHILE DOWN-REGULATING CA3-CA1 SYNAPTIC RESPONSE

A functional synaptic-astrocytic interaction would involve bidirectional communication between astrocytes and neurons. One of the ways to understand this astrocyte-synapse interaction is to study these responses during synaptic activity and how they are affected when an astrocytic function is impaired. Astrocytes have been shown to depolarize during synaptic stimulation. In this study, we aim to understand the underlying mechanisms involved in the astrocytic membrane response during excitatory synaptic transmission in control and under conditions of reduced glutamate uptake.

We performed whole cell patch clamp recordings on astrocytes in the stratum radiatum of the rat hippocampus while stimulating the Schaffer collaterals (SC) to mimic synaptic activity. Astrocyte responded to SC stimulation by a long-lasting depolarization. This astrocytic long-lasting depolarization was mediated by an increase in extracellular levels of potassium as it was reduced when astrocytic inward rectifier potassium channels were blocked.

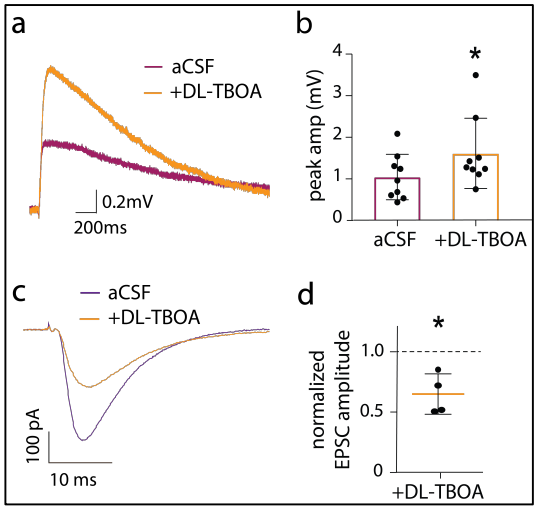

Figure 3 (a, b) Astrocyte response to SC stimulation. Astrocytes respond by a long-lasting depolarization on SC stimulation. This astrocytic depolarization is enhanced upon DL-TBOA application. (c, d) In the neurons, DL-TBOA application decreases the peak amplitude of the evoked neuronal EPSC.

To study the response of astrocytes and neurons in conditions of reduced glutamate uptake, the glutamate uptake was reduced using a pharmacological glutamate transporter blocker DL-TBOA. DL-TBOA application reduced evoked neuronal EPSCs on SC stimulation, however we found an increase in the astrocytic long lasting depolarization. Since astrocytic long lasting depolarization is dependent on the increase in potassium levels, the overall reduction in neuronal activity could not explain this response. To understand this response, we further dissected the AMPA vs NMDA receptor mediated neuronal response and found out that under control conditions the long -lasting astrocytic depolarization is governed mostly by AMPA receptors. However, in conditions of reduced glutamate uptake the activity of NMDA receptors is enhanced, generating an increase in extracellular potassium levels resulting in the enhancement of the astrocytic long-lasting membrane depolarization.

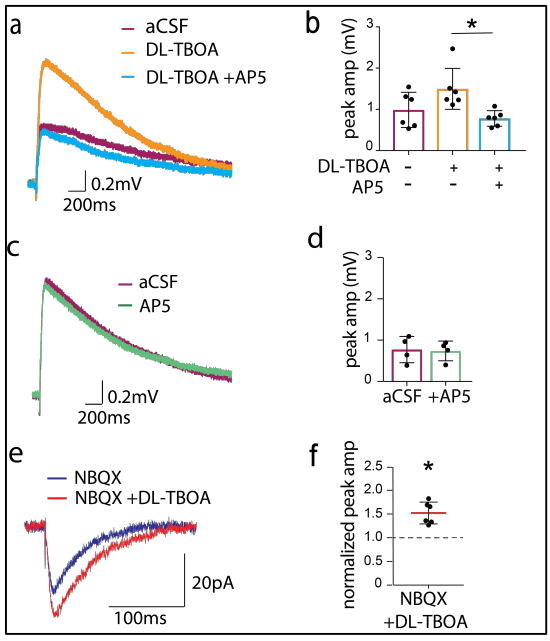

Figure 4: (a,b) Astrocyte show a long-lasting depolarization upon DL-TBOA application. This enhancement in astrocytic depolarization is restored by blocking NMDAR with AP5. (c,d) AP5 alone do not change the long lasting depolarization compared to control. (e,f) Evoked EPSC recorded in neurons in the AMPR blocker NBQX. This evoked EPSC is increased in presence of DL-TBOA.

In summary, our findings show that astrocytes respond to synaptic stimulation with a long-lasting depolarization, which is enhanced in case of reduced glutamate uptake, and this enhanced astrocytic depolarization is mediated by the NMDA receptor activation.

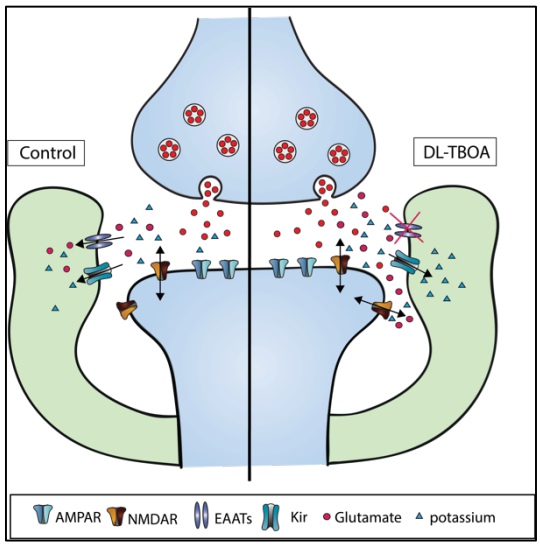

Figure 5: Tripartite synapse function under control and DL-TBOA application. Under control conditions the extra glutamate as well as the potassium released due to neuronal activity is taken up by the astrocyte glutamate transporters (EAATs) and potassium channels respectively. This potassium uptake by astrocytes leads to a long-lasting depolarization in the astrocyte (b) Upon DL-TBOA application, glutamate cannot be removed by the EAATs causing an over activation of NMDA receptors releasing more potassium and hence an enhancement of astrocyte long –lasting depolarization.

5.2 PAPER II: BLOCKING ASTROCYTIC GABA RESTORES SYNAPTIC PLASTICITY IN PREFRONTAL CORTEX OF RAT MODEL OF DEPRESSION

Recent studies have shown that astrocytes in an animal model of Alzheimer's disease actively release GABA and hence assert a tonic inhibition on neurons[44]. We started this study by asking the question if this aspect of astrocyte function is affected in other diseases, such as depression, since astrocytes have been shown to be affected in depression. Thus, we investigated the role of astrocytes in tonic inhibition and its effect on the overall synaptic activity in the medial

prefrontal cortex (mPFC) of a well validated rat model of depression the Flinders sensitive line (FSL). Firstly, using whole cell patch clamp recordings of neurons from the prelimbic area of mPFC in the FSL rats, we found an increase in the tonic current compared to control Sprague Dawley (SD) rats. Astrocytes have previously been reported to synthesize GABA through the MAO-B enzyme[44], and GABA is subsequently released through the Ca^{2+} activated Best-1 channels[62]. Using the MAO-B inhibitor Selegiline, we compared the tonic current between FSL and FSL brain slices incubated with Selegiline. The tonic current was found to be reduced in Selegiline treated slices from FSL rats. Similarly, calcium clamping the astrocytes in the FSL rat also reduced the tonic current. These finding showed that astrocytes in the PFC in FSL rat produce and release GABA contributing to the tonic inhibition.

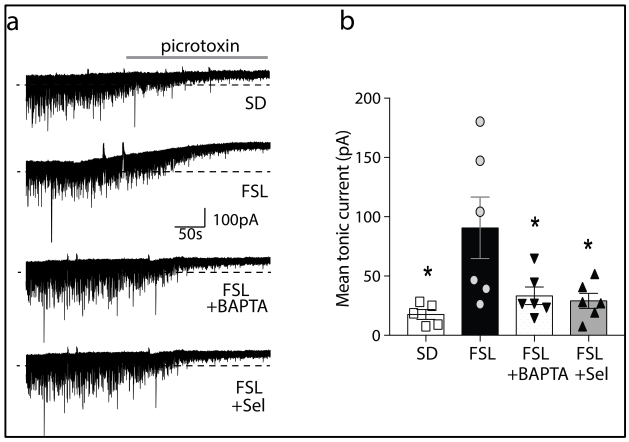

Figure 6: Tonic GABA current recordings in the presence of picrotoxin from pyramidal neurons in the layer V of the prelimbic cortex. Tonic current is significantly increased in FSL compared to SD controls. FSL show reduction in tonic current when FSL astrocytes are Ca^{2+} clamping using BAPTA. MAO-B inhibitor Selegiline also reduced the tonic current in FSL.

Our next approach was to see if this increased tonic inhibition affects synaptic plasticity. FSL animals showed impaired synaptic plasticity in the PFC compared to SD as seen in LTP recordings. In order to understand if the increased astrocyte mediated tonic inhibition contributed to this LTP impairment seen in FSL, we pretreated slices from the FSL rat with Selegiline and found that it restored the impaired LTP.

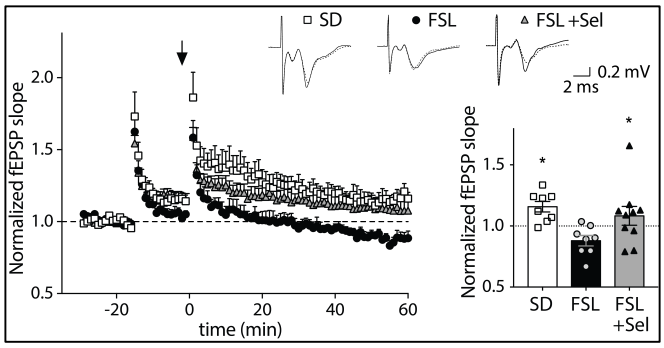

Figure 7: LTP recording from PFC. FSL show impaired LTP compared to SD. This impaired LTP in FSL is restored when astrocyte GABA producing enzyme MAO-B is blocked using selegiline

In summary, these findings show that astrocytes in rat model of depression have an increased level of GABA compared to healthy SD rats, which contributes to the increased tonic current and can impair synaptic plasticity. Moreover, blocking GABA synthesis in astrocytes restores the impaired synaptic plasticity.

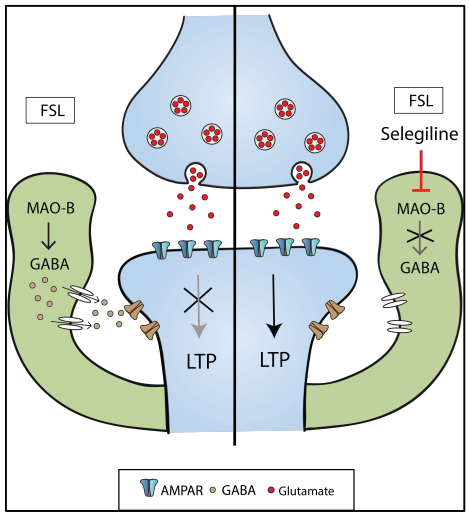

Figure 8: Role of astrocytic GABA in LTP in the FSL rat model of depression (a) The astrocyte release GABA which causes a tonic inhibition preventing the synapses to undergo LTP. (b) when the astrocytic GABA synthesis is blocked using selegiline, this leads to decrease in the tonic inhibition allowing LTP.

5.3 PAPER III: INCREASED LEVELS BUT REDUCED FUNCTION OF ASTROCYTIC GLUTAMATE TRANSPORTERS IN THE HIPPOCAMPUS OF THE *APP*$^{NL\text{-}G\text{-}F}$ MICE.

One of the most important aspects involved in maintaining excitatory neurotransmission is the proper uptake of glutamate by astrocytes and this glutamate uptake has been shown to be impaired in case of neurodegenerative diseases[101, 102]. Thus, we studied this aspect of astrocyte function using a knock-in AD mice model *App* $^{NL\text{-}G\text{-}F}$.[93]

To characterize the astrocyte morphologically in the hippocampus of the *App* $^{NL\text{-}G\text{-}F}$ mice, immunostaining was performed for GFAP and s100β which stained the astrocytic intermediate filaments and the cell body respectively. Astrocytes in the *App* $^{NL\text{-}G\text{-}F}$ mice showed morphological changes with increased number of processes and increased soma size, characterizing them as reactive astrocytes.

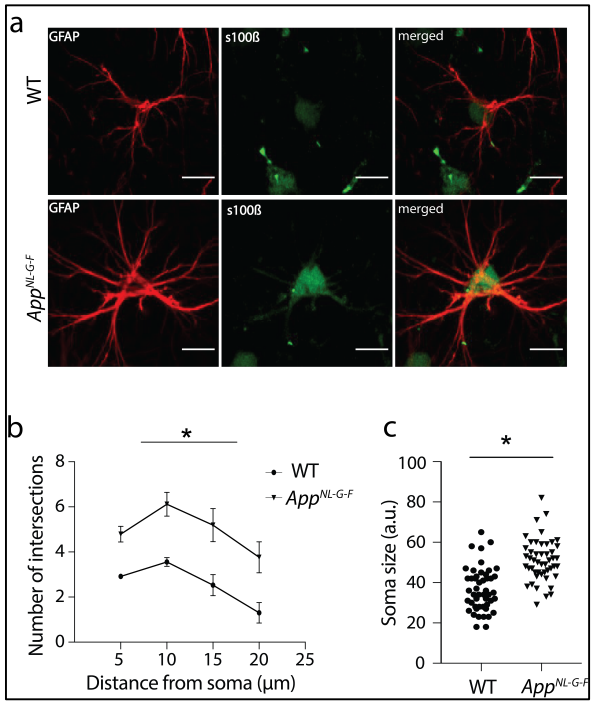

Figure 9: Immunostaing of astrocyte process and cell body with antibodies against GFAP(red) and s100β (green) respectively. Scholl analysis of GFAP positive processes at different distances from the astrocyte soma show *App*$^{NL\text{-}G\text{-}F}$ mice have significantly increased number of GFAP processes. Also astrocytes in *App*$^{NL\text{-}G\text{-}F}$ mice have bigger soma size compared to WT calculated as s100β positive area.

Glutamate is taken up by the astrocytic glutamate transporters EAATs[103]. Western blot analysis to estimate the expression levels of the astrocyte glutamate transporters showed an increase in the expression levels of astrocyte specific glutamate transporter EAAT-2 also referred as GLT-1 in the hippocampus of *App* $^{NL\text{-}G\text{-}F}$ mice. Considering that the astrocytes are reactive, an increase in EAAT-2 levels can be interpreted as an adaptive response in these animals. We wanted to understand if this increase in EAAT-2 protein levels is also accompanied by an increase in EAAT-2 function. The analysis of the synaptically activated transporter mediated current (STC) from astrocytic patch-clamp recordings will provide direct information on transporter function and glutamate clearance [53, 104]. We found that the glutamate transporter function is severely impaired in the *App*$^{NL\text{-}G\text{-}F}$ mice as reflected by the decreased amplitude and increase in decay time constant of STC in the *App*$^{NL\text{-}G\text{-}F}$ mice. Hence, though there is an increase in EAAT-2 protein levels, they are functionally impaired in the *App* $^{NL\text{-}G\text{-}F}$ mice.

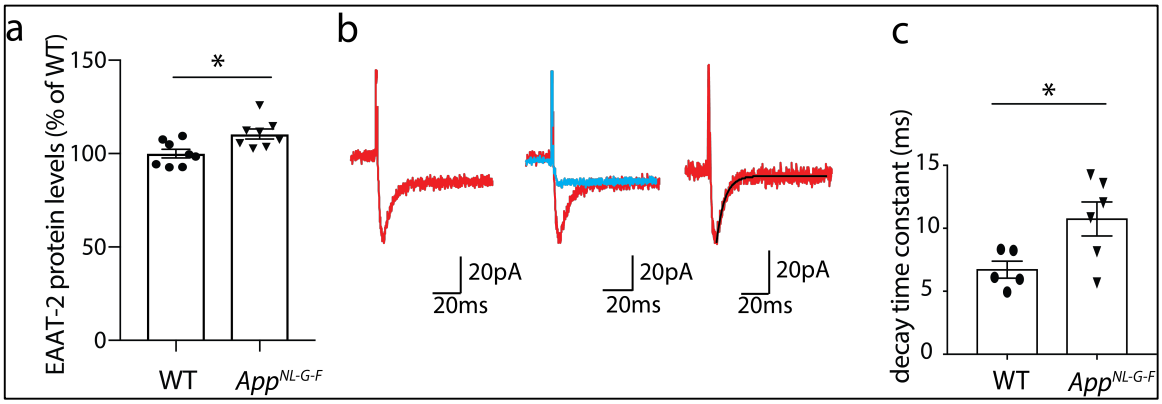

Figure 10 : Increased EAAT-2 protien levels in *App*$^{NL\text{-}G\text{-}F}$ mice. Astrocyte current response to SC stimulation (red) is reduced in the presence of glutamate receptor blocker TFB-TBOA (blue). Synaptically activated glutamate transporter current (STC) is obtained by subtracting the TFB-TBOA insensitive current (blue) from total astrocytic current (red). STC decay time constant obtained after best fitting to a single exponential function (black line) is increased in *App*$^{NL\text{-}G\text{-}F}$ mice compared to WT mice indicating impaired glutamate uptake. Bars show mean and SEM.

In summary, this study explores astrocytes in an animal model of AD, and shows that glutamate uptake is reduced in this model, which may contribute to the pathology of the disease. It also highlights the importance to consider astrocyte glutamate transporters as potential drug targets for AD treatment[88, 101].

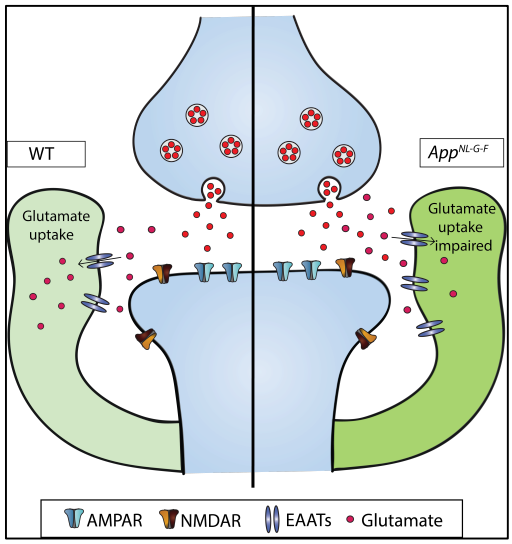

Figure11: The tripartite synapse in wild type and App^{NL-G-F} mice. (a) Under normal conditions glutamate released by the presynaptic terminals binds to the postsynaptic receptors and the remaining glutamate is taken up by the astrocyte glutamate transporters (EAATs). (b) In case of the AD mice, though we see that glutamate transporter protein expression is increased they are functionally impaired as the EAAT current recordings show that glutamate transport by astrocytes in App^{NL-G-F} mice is impaired compared to WT mice.

6 DISCUSSION

Astrocytes as a part of tripartite synapse perform numerous functions contributing to the physiology of the synapse[12, 32]. One of the important challenges is to understand how the specific astrocytic functions can affect synaptic transmission[12]. Another challenge is to understand how this astrocyte synapse interaction is affected in disease conditions. Recent studies have shown astrocytes contributing to both the excitatory and inhibitory aspects of synaptic transmission. In this thesis, we investigated the role of astrocytes in the excitatory aspects of synapse activity in health and disease.

In the **Paper I**, we show that astrocytes upon synaptic stimulation, respond by a long- lasting depolarization and this response is enhanced when the glutamate uptake by astrocytes is pharmacologically impaired. We also observed changes in neuronal glutamate receptor responses under these conditions, thus giving us an idea about how impairment in a specific astrocyte function is capable of modulating the astrocyte-synapse crosstalk. One of the questions raised by the results in **Paper I** is to understand the functional significance of this astrocytic depolarization and the mechanisms through which astrocytic depolarization can affect synaptic activity. Extending the application of optogentic tools to manipulate astrocyte membrane responses while simultaneously monitoring synapse activity may be helpful in answering such questions.

Astrocytic loss of function has been suggested to be involved in the underlying mechanism of depression and AD[85, 105, 106] and hence in **Paper II** and **III**, we studied aspects of astrocytic function in animal models of these disorders. In **Paper II**, we studied the astrocytic contribution to tonic inhibition in the FSL animal model of depression. FSL astrocytes in the prefrontal cortex were shown to impair synaptic plasticity by releasing GABA and hence contributing to the tonic inhibition. Pharmacological blocking of the astrocytic GABA release restored LTP in the FSL rat. In **Paper III**, we studied how astrocytes are affected in an AD mouse model App^{NL-G-F}. Astrocytes in the App^{NL-G-F} were found to display a reactive morphology and astrocyte mediated glutamate uptake was found to be impaired. Taken together the results from **Paper II** and **III** highlight the functional impairment of astrocytes in disease conditions and their effect on the synaptic activity. Studying astrocyte morphology gives an idea about their condition in disease state. In the FSL animals we found the astrocytes in the PFC to be atrophic whereas in the AD model we found them to be hypertrophic. It will be interesting to understand how the morphological changes translates to functional changes, and if the different morphology of astrocytes in different brain regions[18] leads to difference in their reaction to insult or injury. Also as astrocyte ensheathing influences the synapse physiology[3, 107], it will be interesting to understand if the different reactive profiles affect synapse activity differently. Overall these data suggest that restoring astrocyte functions can be helpful in improving the impaired synaptic activity and in turn disease conditions however in order to be able to target astrocytes we first need to understand different aspects of a dysfunctional astrocyte.

7 CONCLUSIONS

The field of astrocyte research has come a long way from considering astrocytes as supporting cells to understanding their role in modulating synaptic activity[4, 32, 35]. However, it is still not completely understood how the astrocyte synapse interaction is affected in cases of diseases that affects the brain[108, 109]. This thesis is an attempt to understand the mechanisms through which astrocytes can affect synaptic transmission and indicates that pharmacological manipulation of the astrocytes can help in recovery from disease conditions, presenting astrocytes as potential drug targets. It has been shown that astrocytes exhibit different reaction profiles[81] and understanding their functional impact on synapse activity will give us a better idea of the disease state. Recent studies have explored the role of astrocytes in behavior[68]. Extending these findings to study the behavioral changes observed in disease models[110] will help us to connect the dots starting from the impairment seen in astrocyte at the synapse level and its impact on the behavioral changes in these disease. Future work related to the identification of specific astrocytic targets causing the changes from synapse to behavior and an attempt to restore their function may be a step in developing the treatment.

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