

Regulation of serine in excitatory synapses in the mammalian brain

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. It contains some material (results, figures and discussion) that has been published in a journal article mentioned below. To the best of the author's knowledge, it does not contain material written by another person, except where due reference is made in the text.

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Abstract

D-serine (DS) and L-serine (LS) are important signalling molecules in the brain. DS acts as an endogenous co-agonist of N-methyl-D-aspartate receptors (NMDARs), which play a crucial role in synaptic function and plasticity. LS serves as a precursor for DS synthesis. Although neurons are the primary DS source, derived from astrocyte-produced LS, the mechanisms governing its clearance/regulation remain enigmatic. This thesis explores the topic of serine regulation, focussing on the role of transporters responsible for DS and LS uptake. Given that the astrocytes intricately envelop the excitatory synapses in the brain, it is hypothesised that the astrocytes are likely candidates to mediate the uptake of DS, after its action on the postsynaptic NMDARs.

To understand the uptake phenomenon, *in situ* patch-clamp recordings from acutely isolated mouse brain slices, focussing on the hippocampal region were conducted. Chapter 3 presents direct *in situ* patch clamp recordings of DS uptake into astrocytes, revealing transport-associated currents upon 10 mM DS application. Pharmacological inhibition experiments, utilising O-benzyl-L-serine and trans-4-hydroxy-L-proline, known substrate inhibitors of the sodium-dependent alanine serine cysteine transporter ASCT1 (*Slc1a4*), unveiled ASCT1's involvement in DS uptake into astrocytes adjacent to synapses in the *stratum radiatum* layer of the CA1 hippocampal region. Similar results were observed in other brain regions, including the primary somatosensory cortex, and cerebellum. These findings emphasise the central role of ASCT1 in mediating DS transport in astrocytes, thereby influencing NMDAR function and synaptic plasticity.

Chapter 4 extends the investigation to LS transport at the CA3-CA1 hippocampal excitatory synapses, revealing ASCT1's involvement in this essential astrocytic function. Transport-associated currents induced by the application of 10 mM LS on *stratum radiatum* astrocytes sensitive to the administration of O-benzyl-L-serine and trans-4-hydroxy-L-proline were observed. These results support the role of ASCT1 in mediating LS transport in the astrocytes. Since neurons require a constant supply of LS from the astrocytes, for DS synthesis, this result implies that astrocytic ASCT1 could mediate the LS shuttle transport. In CA1 pyramidal neurons, the transport of LS was highly sensitive to inhibition by α -(methylamino) isobutyric acid (MeAIB), implicating the involvement of system A transporters in this process.

Having established the functional presence of ASCT1 in the astrocytes surrounding excitatory synapses, Chapter 5 aimed to delve into the physiological role of ASCT1 in governing the

synaptic concentrations of DS and LS. Surprisingly, the addition of exogenous 100 μ M DS or LS did not alter NMDAR-mediated excitatory postsynaptic current (EPSC) responses in the hippocampal CA3-CA1 synapses, indicating glycine binding site saturation under periods of low synaptic activity or low frequency of synaptic stimulation. Further pharmacological inhibition of ASCT1 using 10 mM benzyl serine or trans-4-hydroxy-L-proline did not significantly alter the NMDAR-mediated EPSC responses under low synaptic activity. These results not only highlight the complexities of agonist and co-agonist activation at excitatory synapses but also emphasise the need for further exploration into the underlying mechanisms by which ASCT1 regulates synaptic DS levels.

In essence, this thesis characterises the functional presence of the astrocytic ASCT1, an elusive DS transporter pivotal in orchestrating synaptic DS dynamics. The implications of these findings resonate deeply within the realm of fundamental neuroscience and in the development of new drug targets for neurological disorders. For example, inhibiting ASCT1 may be a potential therapeutic strategy for reducing synaptic DS levels in diseases associated with DS hyperactivity, such as schizophrenia. Conversely, enhancing ASCT1 activity could be beneficial for increasing synaptic DS levels in conditions associated with DS deficiency, such as Alzheimer's disease.

List of Abbreviations

3PGDH	3-Phosphoglycerate dehydrogenase
aCSF	Artificial Cerebrospinal fluid
ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
AMPA	α -Amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid
AMPA	α -Amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid Receptors
ANOVA	Analysis of variance
ANU	Australian National University
APF	Australian Phenomics Facility
APV / AP5	((2R)-Amino-5-Phosphono Valeric acid / (2R)-Amino-5-Phosphono pentanoate)
asc1	Sodium Independent Alanine Serine Cysteine transporter
ASCT	Sodium-dependent Alanine Serine Cysteine Transporter
ASCT1	Alanine Serine Cysteine Transporter Isoform 1
ASCT2	Alanine Serine Cysteine Transporter Isoform 2
ATP	Adenosine monophosphate
BBB	Blood-brain barrier
BCH	2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid
Bic	Bicuculline Methiodide
CA	<i>Cornu Ammonis</i>
cAMP	Cyclic adenosine monophosphate
CAMPK	Calmodulin-dependent protein kinase
CNS	Central nervous system
CREB	cyclic-AMP response element binding protein
dH ₂ O	Distilled water
DAAO	D-amino acid oxidase
DAG	Diacylglycerol
DG	Dentate Gyrus
DIC	Differential interference contrast
DS	<i>Dextro</i> -Serine
EAAT	Excitatory Amino Acid Transporters
EC ₅₀	Half maximal effective concentration
EGTA	Ethylene Glycol-bis(β -aminoethyl ether)-N,N,N',N'-Tetra Acetic acid
EM	Electron microscopy
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
GABA	γ -Aminobutyric acid
GBS	Glycine binding site
GDH	Glutamate dehydrogenase
GFAP	Glial fibrillary acidic protein
GGs	Glutamate-Glutamine cycle
GLUT	Glucose transporter
GLT	Glutamate transporter
GPCR	G-protein coupled receptor
GS	Glutamine synthetase
HEPES	4-(2-Hydroxy Ethyl)-1-Piperazine Ethane Sulfonic acid
HEK	Human embryonic kidney cells
HP	Helical hairpin
IC ₅₀	Half maximal inhibitory concentration
IP ₃	Inositol 1,4,5-triphosphate
IP ₃ R2	Inositol 1,4,5-triphosphate receptor type 2

JCSMR	John Curtin School of Medical Research
KO	Knockout
LAT1	Large neutral Amino acid Transporter isoform 1
LBD	Ligand binding domain
LFS	Low-Frequency Stimulation
LME	Linear Mixed Effect Model
LS	<i>Laevo</i> -Serine
Ltd.	Limited
LTD	Long Term Depression
LTP	Long Term Potentiation
MCPG	(+)- α -Methyl-4-carboxyphenylglycine
MeAiB	Methyl Amino Iso-Butyric acid
mGluR	Metabotropic glutamate receptor
Mk801	Dizocilpine
MNTB	The medial nucleus of the trapezoid body
mRNA	Messenger Ribonucleic acid
NBQX	2,3-dihydroxy-6-Nitro-7-sulfamoyl-Benzo[f]Quinoxaline
NMDA	N-Methyl-D-Aspartic acid
NMDARs	N-Methyl-D-Aspartic acid receptors
NTT4	Neurotransmitter transporter
OMIM	Online Mendelian Inheritance in Man
P	Postnatal day
PAG	Phosphate-activated glutaminase
PAT	Proton-coupled amino acid transporter
PGDH/PHGDH	Phosphoglycerate dehydrogenase
PKC	Protein kinase C
PLP	Phospholipase-C
PPR	Paired Pulse Ratio
PSA	Phosphoserine aminotransferase
PSD	Post-synaptic density
PSPH	Phosphoserine phosphatase
PV	Parvalbumin
SC	Schaffer collateral
SHMT	Serine hydroxymethyl transferase
SLC 1	Solute Carrier family 1
SMLC	S-methyl-L-cysteine
SNAT1	System N Amino acid Transporter isoform 1
SNAT2	System N Amino acid Transporter isoform 2
SNP	Single nucleotide polymorphism
SOM	Somatostatin
SPATCCM	Spastic tetraplegia, thin corpus callosum, and progressive microcephaly
SR	Serine Racemase
str	<i>Stratum radiatum</i>
TASK	TWIK-related acid-sensitive potassium channels
TCA	Tricarboxylic acid cycle
TEA	Tetra ethyl ammonium chloride
TMD/TM	Trans-membrane domain
TREK	2-pore domain potassium channels
TTX	Tetrodotoxin (Citrate free)
T-Pro	Trans-4-Hydroxy-L-proline
VGLUT	Vesicular glutamate transporters
WHO	World Health Organisation

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Chapter 1 Introduction

The astounding complexity of life on Earth, which is thought to be unique in this universe, is due to the richness of carbon, enabling the formation of countless biologically active molecules, notably amino acids. A typical human brain comprises about a hundred billion neurons and several hundred trillion synapses or connections formed through highly organised neural networks, which process information. Traditionally in the central nervous system, memory, thoughts, and behaviour were assumed to be a manifestation of synchronised neural activity. However, evidence has been accumulating over the recent years indicating that crosstalk between the neurons and the astrocytes, a specific type of star-shaped glial cell, is crucial for proper neurotransmission (Araque et al., 1999). From the earliest stages of existence to the final moments of life, the brain exhibits remarkable plasticity, ceaselessly evolving. A fundamental question emerges within the realm of modern neuroscience: how does the brain orchestrate its intricate chemical signalling precisely? Central to this investigation is serine, an amino acid molecule renowned for its physiological functions and its influence on the plasticity of excitatory networks. This thesis embarks on an exploration of the intricate regulatory mechanisms governing Dextro-Serine, a pivotal molecule that governs excitatory signalling within the brain. However, there remains an element of complexity in the way crosstalk occurs between neurons and surrounding glial cells, which adds a whole new layer of intrigue to the precise control of D-serine-mediated excitatory signalling. While our understanding remains incomplete regarding the fundamental clearance mechanisms responsible for regulating D-serine levels in the brain, this thesis delves into a cutting-edge inquiry: identifying the elusive D-serine transporter in the brain—a pursuit of utmost significance for both fundamental and clinical research.

In the first chapter, heading 1.1 covers a broad introduction to the fundamental concepts dealt with within this thesis. Headings 1.2 and 1.3 delve into an overview of how excitatory networks function in the brain, with an emphasis on the N-methyl-D-aspartate type of excitatory receptors. In headings 1.4 and 1.5, information on how astrocytes play a role in several brain functions is summarised. In subsequent heading 1.6, a thorough examination of the Dextro isomeric form of the amino acid, D-serine takes centre stage. In this section, a review of information concerning D-serine's biosynthesis within the brain, its pivotal role in neurotransmission, particularly in facilitating excitatory glutamatergic signals and a profound exploration of the cell types responsible for regulating its concentration at central excitatory synapses are presented. Headings 1.7 presents a meticulous introduction to the vital concepts regarding serine transporters, laying the groundwork for further scientific inquiry. This chapter

gives a more profound comprehension of D-serine's contribution to excitatory synaptic transmission in the brain.

1.1 General Introduction

1.1.1 The compartmentalisation of the information processing

The brain serves as the central and vital organ of the body, which aids in processing information about our surroundings and facilitating survival. The sensory organs serve as a gateway or interface for the brain to communicate with the surroundings. Information from the external world, in the form of energy forges simultaneously and explodes into an enormous collage of signals in our sensory organs. These signals converge forming a vast array of electrical and chemical information, which is then processed by brain cells called neurons.

All sensory information except smell gets processed through a relay station called the thalamus and gets abstracted into specialized cortical and subcortical brain areas (Sherman, 2016). Broadly the cortex comprises four lobes: the frontal lobe, involved in decision-making, and higher-order information processing; the parietal lobe, responsible for object identification, and spatial memory; the temporal lobe, dedicated to auditory processing, speech, and music; and the occipital lobes, specialised in visual processing (Figure 1.1A).

These specialised areas of the brain have evolved to be organised into different layers, each populated by various types of cells, including excitatory neurons, inhibitory neurons, and glial cells. Each cell type portrays a different morphological structure while serving diverse physiological functions (Markram et al., 2015; Rojo et al., 2016). For example, the mammalian cortex is organised into six distinct layers, where specific thalamic or sensory inputs are received in layers 1 and 4, while layers 2 and 3 contain excitatory and inhibitory neurons. The primary cortical output layers, layers 5 and 6, consist of pyramid-shaped excitatory neurons, forming interconnected feed-forward and feed-backward circuits that regulate circuit homeostasis (Markram et al., 1997; Markram et al., 2015).

In addition to the cortex, the hippocampus, a seahorse-shaped archaean cortical area in the brain, plays a crucial role in processing input signals from parietal, temporal, and occipital cortices via the entorhinal cortex (Figure 1.1B). This diverse information gets processed in the three-layered hippocampus area, contributing to our feelings, emotions, and memory. In this thesis, the hippocampal area is primarily used as a model system (see 1.6.8). Overall, it is

evident that the intricate networks of our brains have evolved uniquely, to piece together complex puzzling information and solve complex mysteries.

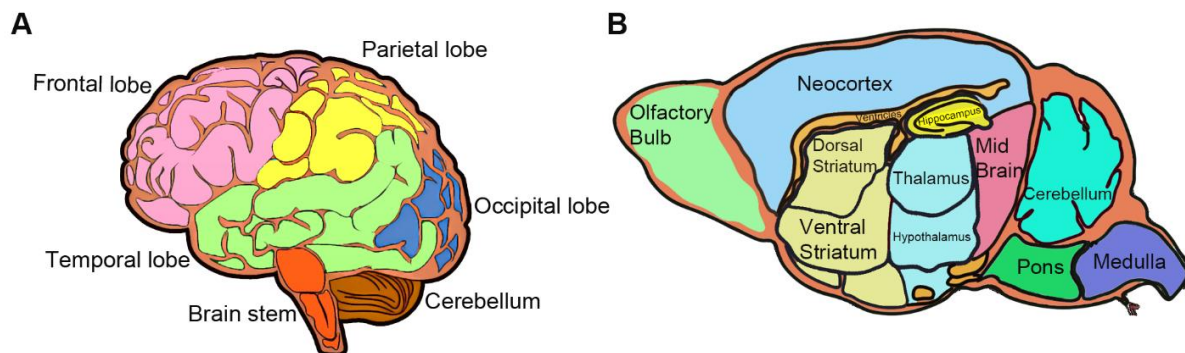


Figure 1.1: Picture of human and mouse brain with different regions of the brain

A panel depicts the human brain and B panel depicts the mouse brain, highlighting different important structures, each processing unique sensory and motor information. In panel B, the hippocampus area of the mouse brain (in yellow) will be of primary focus for this thesis.

1.1.2 Information processing unit of the brain

Pyramidal neurons, as the most prevalent type of excitatory neurons in the brain, have captivated the attention of brain scientists for over a century. They were correlated with all the information processing in the brain. Exploring them has widened our understanding of how the brain processes sensory information from the world around us. Despite structural diversity among pyramidal cells across different brain areas, they share a common pyramid-shaped cell body or soma, typically measuring approximately 10 to 20 μm in diameter. Pyramidal neurons usually possess thin cellular projections extending from the top (apex) and bottom (base) of the soma, known as apical dendrites and basal dendritic projections, respectively (Figure 1.2). From the base of the soma, long threadlike axons arise, sending signals to the subsequent neurons. These dendrites and axons serve as communication cables, transmitting information from one neuron to another. Remarkably, these projections have been observed to span across various brain areas, extending along approximately 200 - 800 μm and even across hemispheres. For instance, CA1 and CA3 pyramidal neurons in the hippocampus have somas measuring 10-15 μm and exhibit dense apical and distal dendrites, facilitating intricate neural communication and information transfer within this crucial brain region (Benavides-Piccione et al., 2019).

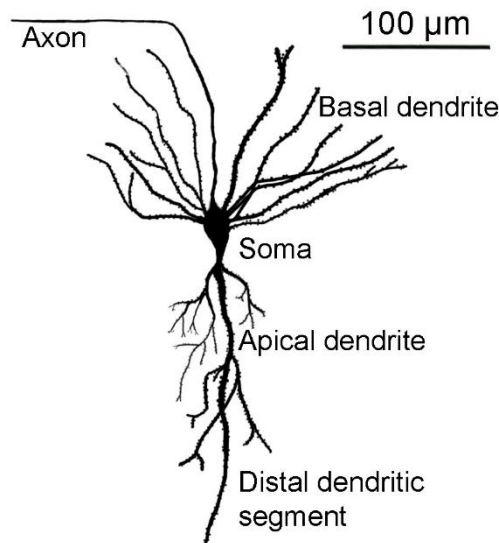


Figure 1.2: Typical structure of a pyramidal neuron found in the hippocampal CA1 region

A drawing of a CA1 pyramidal neuron highlighting the main segments – Soma or cell body which forms the core, basal dendrites and axon which profusely arise from the base of the soma and apical dendrites which arise from the apex of the soma. Dendrites contain numerous tiny actin-rich protrusions called spines which aid in information processing, whereas the axon contains axon terminals but lacks spine-like structures. The soma also lacks such spine-like structures.

1.1.3 Neurotransmission in the brain

Neurons in the brain communicate with each other in the form of electrical and chemical events, namely action potential and chemical neurotransmission. The process of neurotransmission involves three main components (Figure 1.3). Firstly, the pre-synaptic component, represented by a neuron or its terminal, is responsible for sending the input signals by releasing chemical neurotransmitters. The intermediate component is the synapse or the meeting point between the two neurons, where chemical neurotransmitters are released, and ions, nutrients, and signalling molecules are bathed, facilitating communication across neurons. Remarkably, there are more such synaptic connections in our brains than the total number of stars in the night sky. The last component is the post-synaptic neuron, which receives the incoming signals via various receptors and proteins expressed on their cellular membranes. When a chemical neurotransmitter is capable of activating the post-synaptic receptor, it is referred to as an agonist. If the molecule is synthesised by the host cells or artificial, it is deemed endogenous or exogenous. Depending on the chemical released by the pre-synaptic neuron, the synapse is classified as either excitatory or inhibitory in nature. For instance, neurons releasing the major excitatory neurotransmitter, glutamate, and expressing receptors capable of transducing glutamate-mediated information are called glutamatergic neurons. The synapses associated with these neurons are called glutamatergic synapses. On the other hand, neurons releasing the major inhibitory molecule gamma-aminobutyric acid

(GABA) are called GABAergic neurons, and the associated synapses are called GABAergic synapses (Purves, 2017). In summary, the process of signal transduction is called neurotransmission and it serves as the main mode of communication between neurons. Through electrical and chemical signals, neurons relay and process information, establishing the basis for the intricate functioning of the mammalian brains.

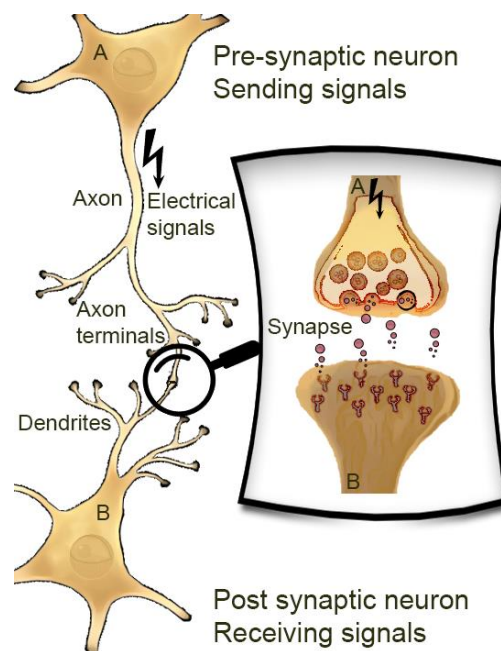


Figure 1.3: A synapse is the meeting point of two neurons

Electrical information in the form of action potential (lightning bolt) travels from neuron A, along the axon of neuron A to the dendrites of neuron B. The junction between neuron A and neuron B is called a synapse (zoomed image) and is a gap where the electrical action potentials are converted into chemical signals. This orchestra of chemical signalling occurs via the release of neurotransmitter molecules contained within tiny vesicles, from the pre-synaptic terminals of neuron A, and acts on the neurotransmitter receptors expressed on the receiving neuron B.

1.1.4 Synapses that shape our sensory experience

When all aspects of synapses in the brain work in synchrony, we are immersed in a symphony of multisensory experiences or sensations. This coordinated dance of 20 million billion bits of information that is processed every second by the brain, culminates in our conscious construct of the 3-dimensional world, coupled with deeply personal experiences. At birth, a child's brain is a flurry of superfluous networks, with almost every part communicating with every other part of the developing brain. During the first two years of childhood, the child's brain embarks on a lifelong process of fine-tuning the neural circuits. This process, popularly referred to as synaptic pruning, is a complex mechanism through which synapses or neural connections in the brain get selectively eliminated. While synapse formation is crucial for establishing new memories and storing more information in the brain, pruning synaptic connections is equally

important for maintaining global homeostasis (Huttenlocher and Dabholkar, 1997). This shedding of unnecessary synapses adheres to the well-known principle of “Use it or lose it”. This process is commonly referred to as “neurons that fire together, wire together” (Purves and Lichtman, 1980). In other words, necessary connections are strengthened, while unnecessary or rarely used connections are eliminated. This refinement of synaptic connections aids in the survival of the species and the mastery of the environment we inhabit.

The process of synaptic pruning is widely influenced by the stimulus from the environment and therefore thought to underlie the cellular process of learning and memory in the brain (Bliss and Collingridge, 1993; Moser et al., 1998). For example, the simple circuits and associations that are pruned during early childhood are replaced by much more complex circuits that are suited for survival during adulthood (Purves and Lichtman, 1980; Lichtman and Colman, 2000; Kano and Hashimoto, 2009). Furthermore, synaptic pruning has been associated with the concept of neural Darwinism. In a similar manner to how the slowest deer in a herd is more likely to fall prey to a lion, synaptic pruning allows the brain to shed its weakest connections, making it faster and more energy efficient. Just as the herd becomes swifter and more adept at evading predators, the brain becomes more efficient in its neural communication by eliminating weaker connections.

The adult human brain, despite comprising only about 2% of the total body weight, consumes approximately 25% of the total calories consumed. In contrast, during childhood, the brain's energy consumption is reported to be even higher, ranging from 44% to 87% of the total energy utilized by the resting body. This observation is in line with the 'expensive tissue hypothesis,' which emphasises the critical role of energy conservation in adult brain functioning (Aiello and Wheeler, 1995). Additionally, this hypothesis sheds light on the metabolic differences of synapses in the brain during different stages of life. Consequently, the cellular correlates of memory formation and synapse strengthening are likely to vary during different life stages.

To sum up, the intricate process of synaptic pruning contributes to the pristine functioning of the brain, enabling us to adapt to our environment, learn from our experiences, and navigate the complexities of the world around us. It exemplifies the remarkable plasticity and adaptability of the mammalian brain, shaping our cognitive abilities and influencing our survival as a species. Overall, extensive research done to understand synaptic pruning has contributed to the emergence of an exciting field in neuroscience known as neuroplasticity or synaptic plasticity. The interplay between synaptic plasticity and energy efficiency is fundamental to the brain's functioning and is an intriguing area of present investigation in the field of neuroscience.

1.2 Excitatory synapses in the brain

In this thesis, the primary focus lies in investigating the excitatory synapses in the brain. Reflecting on the role of the excitatory synapses in the information processing of the brain, it is imperative to consider the intricacies of excitatory neurotransmitters and the receptors that are involved in the process of excitatory neurotransmission.

1.2.1 Glutamate release from the pre-synaptic neuron

In mammalian excitatory synapses, the presynaptic neurons release the excitatory neurotransmitter, glutamate. Initially, the glutamate molecules are packaged into synaptic vesicles via vesicular glutamate transporters (VGLUTs). There are three subtypes of VGLUTs, namely VGLUT1, VGLUT2, and VGLUT3 (encoded by *Slc17a7*, *Slc17a6* and *Slc17a8* respectively). VGLUT1 is mainly expressed in the cortex, hippocampus, and cerebellum, and is essential for the plasticity of synapses. VGLUT2 is expressed in subcortical regions, such as the thalamus, basal ganglia, and brainstem and is essential for motor control and sensory processing (Danbolt, 2001; Billups, 2005; Takagi et al., 2022). Unlike the other two subtypes, VGLUT3 is expressed in inhibitory interneurons, mediating the release of GABA (Herzog et al., 2004). VGLUTs actively transport glutamate into vesicles, against its concentration gradient, utilising the energy obtained from the electrochemical gradient generated by vacuolar proton pumps (V-ATPase). This process is followed by the fusion of glutamate-packed synaptic vesicles with the presynaptic membrane. Upon the arrival of pre-synaptic input, the pre-synaptic membrane depolarises, causing an influx of Ca^{2+} ions through voltage-gated calcium channels. This event acts as a trigger to expel glutamate into the synaptic cleft, facilitated via a Ca^{2+} -dependent process of exocytosis. This VGLUT-mediated packaging of glutamate can be inhibited by lowering the concentration of calcium in the extracellular medium (Yeates and Frank, 2021).

After the exocytosis, the empty synaptic vesicles are refilled with glutamate for subsequent release. This recycling process involves clathrin-mediated endocytosis, which forms new vesicles needed for the high-turnover release of glutamate underlying efficient excitatory neurotransmission (Li and Kavalali, 2017).

Glutamatergic synapses in the brain can be categorised into two distinct types based on their pre-synaptic terminals, which differentiate them as either drivers or modulators of neuronal networks. The drivers are characterised by thick axons ending with large boutons or terminals and usually target proximal parts of the dendrites to form synapses. These boutons have a

high probability of glutamate release (P_r) when stimulated, leading to the coordination of large but depressive post-synaptic responses. These driver synapses are typically found in the cortex, thalamus, and brain stem. On the other hand, the modulators are distinguished by their thin axons, ending with small boutons and usually target distal parts of the dendrites to establish synapses. These boutons have low P_r compared to driver synapses, resulting in the coordination of smaller but facilitative postsynaptic responses. The modulator synapses are commonly found in the hippocampus (Sherman and Guillery, 2011; Sherman, 2016).

1.2.2 Action of glutamate on the post-synaptic receptors

When an action potential arrives at the presynaptic terminal of a neuron, it triggers the release of glutamate, which diffuses across the synapse and binds to the glutamate receptors on the postsynaptic membrane. Glutamate acts upon two main classes of post-synaptic receptors: ligand-gated ionotropic glutamate receptors (iGluRs) and G-protein-coupled metabotropic glutamate receptors (mGluRs). The iGluRs are ionotropic in nature, meaning their activation is coupled with the opening of ion channels directly, which are a part of the receptor structure. iGluRs can be further classified into three subtypes based on their specific responses to glutamate: N-methyl D Aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate postsynaptic receptors (Paoletti et al., 2013). On the other hand, the mGluRs are G-protein-coupled metabotropic in nature, meaning their activation is coupled with intracellular signalling processes mediated via secondary messenger molecules. Regardless of the type of post-synaptic receptor that glutamate activates, the post-synaptic membrane experiences a change in voltage, primarily due to an influx of sodium. This change in voltage results in the generation of a fast excitatory response referred to as excitatory post-synaptic potential (EPSP). The EPSP increases the odds of the post-synaptic neuron firing an action potential, allowing the transit of information to subsequent neurons or brain structures. Overall, the interplay between the glutamate release, activation of the post-synaptic glutamatergic receptors, and the subsequent generation of EPSPs is fundamental to excitatory neurotransmission in the brain.

1.2.3 AMPA and Kainate receptors

The AMPARs are tetrameric complexes usually composed of 4 subunits (GluA1, GluA2, GluA3 and GluA4; encoded by GRIA1 to GRIA4 genes respectively). The endogenous agonist, glutamate binds to AMPARs with low affinity (EC_{50} of 20-200 μ M), giving rise to the fast component of an excitatory postsynaptic current (EPSC) response. Such an EPSC response is routinely recorded by voltage clamping the cell, whilst electrically stimulating glutamate

release from the pre-synaptic cells (Banks and Smith, 1992). This fast AMPAR component of the EPSC response arises due to the influx of Na^+ and K^+ ions through their cationic pore. To prevent continuous activation by glutamate, AMPARs undergo an inbuilt process referred to as 'desensitization', which makes the receptor less responsive or non-responsive to glutamate, post its initial activation (Tang et al., 1989). This process involves several changes to the pore conformation and modulation by AMPAR auxiliary proteins, and typically lasts only a few milliseconds to seconds. Since this process is faster than deactivation or recovery from an open state, the AMPARs adapt rapidly to changes in synaptic glutamate concentrations.

Typical GluA2 subunit-containing AMPARs have lower Ca^{2+} permeability compared to AMPARs lacking GluA2 subunit, which have higher Ca^{2+} permeability. This implies that subunit composition determines the synaptic plasticity and excitotoxicity associated with AMPARs. Moreover, alternate splicing and the presence of a 'Q/R RNA editing site' in the GluA2 subunit, allow post-transcriptional editing, replacing the glutamine (Q) with an arginine (R) residue (Sommer et al., 1990; Quirk et al., 2004). This process influences the Ca^{2+} ion permeability, thereby giving rise to multiple different variants of AMPARs.

Kainate receptors are tetrameric complexes composed of 5 main subunits - GluR5 or GluK1, GluR6 or GluK2, GluR7 or GluK3, GluK4, and GluK5; encoded by *GRIK1* to *GRIK5* genes respectively (Bettler and Mülle, 1995). Physiologically, they are activated by kainic acid, a glutamate analogue. Pharmacologically, they are challenging to differentiate from AMPARs, as they allow Na^+ , K^+ and Ca^{2+} to a certain extent, through their central pore. Due to their greater Ca^{2+} permeability compared to AMPARs, their EPSC rise time is slightly slower. They are expressed in both pre- and post-synaptic membranes and have a subtle influence on plasticity, compared to AMPARs (Jin et al., 2006; Jin and Smith, 2011).

Analogues of quinoxaline such as 2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline (NBQX) have been used experimentally to non-specifically and competitively inhibit both AMPAR and kainate-mediated EPSCs (IC_{50} of 0.15 μM and 4.8 μM , respectively). These inhibitors are effective in reducing excitatory neurotransmission and therefore could potentially act as neuroprotective and anti-epileptic (Sheardown et al., 1990; Zeman and Lodge, 1992).

1.2.4 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are seven transmembrane G-protein coupled receptors (GPCR), categorized into three groups, based on their sequence homology and intracellular signalling mechanisms (Niswender and Conn, 2010). Group I (mGluR1 and mGluR5; encoded by *Grm1* and *Grm5* genes respectively) are coupled to G_q protein and activate phospholipase-C (PLC). This further activates Inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), which triggers the release of calcium from various intracellular stores and modulates various cellular processes associated with calcium. Due to their predominant expression in the post-synaptic neurons, their activation augments the activity of NMDAR and Ca^{2+} permeable AMPARs, thereby increasing neuronal excitability. On the other hand, group II (mGluR2 and mGluR3; *Grm2* and *Grm5* respectively) and group III (mGluR4, mGluR6, mGluR7, and mGluR8; *Grm4*, *Grm6-8* respectively) are coupled to G_i protein and inhibits adenylyl cyclase. This further prevents the conversion of cyclic adenosine monophosphate (cAMP) from Adenosine triphosphate (ATP) and regulates K^+ entry via potassium channels, thereby reducing neuronal excitability (Bailey et al., 2008). They are expressed across several cell types, including pre- and post-synaptic excitatory and inhibitory neurons and glial cells including astrocytes (Petrulia et al., 1996).

1.2.5 AMPARs and NMDARs are important transducers of synaptic transmission in glutamatergic synapses

In the postsynaptic glutamatergic neurons, a combination of glutamatergic receptors is expressed, to coordinate the neurotransmission. However, the two major receptors that are of primary importance to synchronised excitatory neurotransmission in the central nervous system are the AMPA receptors (AMPARs) and NMDA receptors (NMDARs). While AMPARs are activated by the presence of glutamate alone in the synaptic cleft, the NMDAR is unique and requires the presence of a co-agonist molecule, in addition to glutamate in the synaptic cleft, to be activated. In other words, the binding of glutamate alone to NMDARs is insufficient to trigger the opening of their ion channels. The NMDARs and AMPARs produce kinetically slow and fast excitation of the post-synaptic neuron, respectively (Bekkers and Stevens, 1989; Mothet et al., 2000).

To elaborate on the activation process, NMDAR activation alone is insufficient to depolarise the post-synaptic neuron. Concurrently, glutamate released into the synaptic cleft activates the AMPARs, which are found in proximity to the NMDARs (Figure 1.4). This leads to the depolarisation of the postsynaptic neuronal membrane, resulting in the removal of the

voltage-dependent Mg^{2+} ion block from the pore of the NMDAR structure. The Mg^{2+} ion removal then allows Na^+ and Ca^{2+} ions to influx into the post-synaptic neuron (Ascher and Nowak, 1988). Collectively, the Ca^{2+} ion permeability and the Mg^{2+} ion block properties of the NMDARs allow them to function as molecular coincidence detectors (Tang et al., 1999). Due to this unique feature, the NMDARs have been linked with several forms of plasticity of glutamatergic synapses such as Long-Term Potentiation (LTP) and Long-Term Depression (LTD). Moreover, NMDARs are also implicated in forming the cellular basis of memory, learning, neuronal development, and pain perception, making them essential for many neurological processes (Collingridge and Bliss, 1987; Bender et al., 2006). Consequently, in this thesis, the focus will be on the NMDA type of glutamatergic receptors.

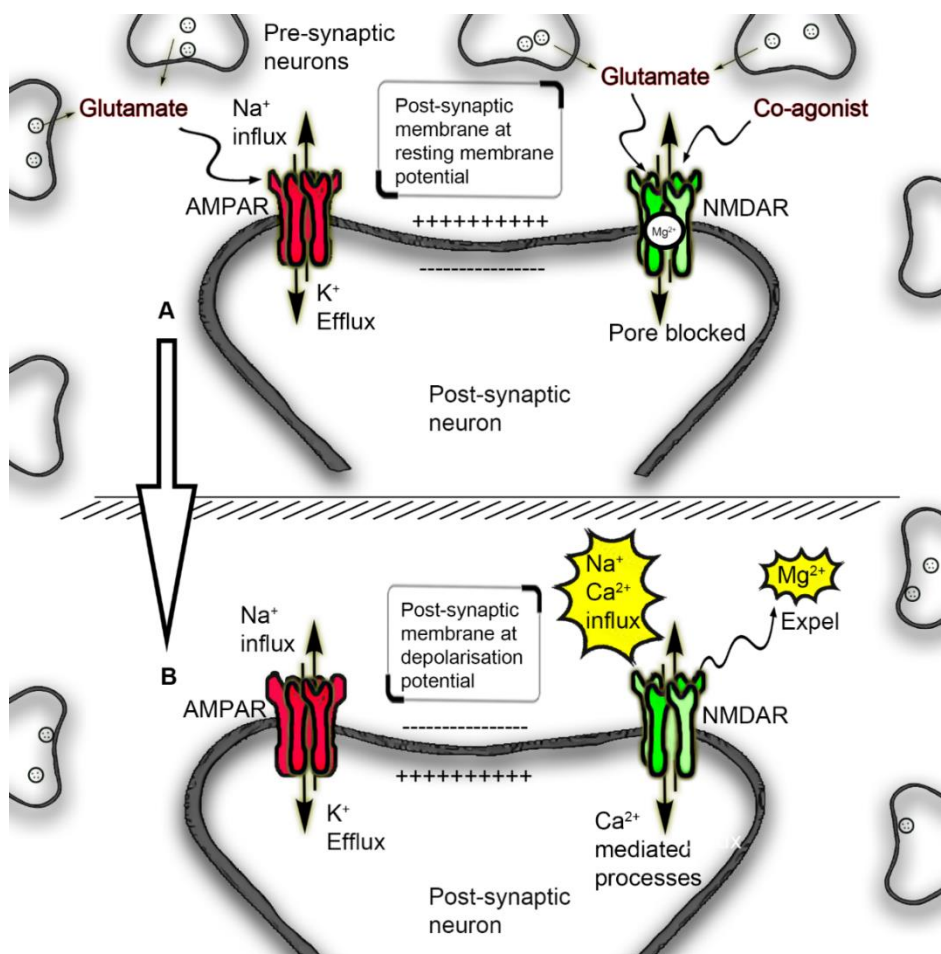


Figure 1.4: NMDAR activity is governed by AMPAR activation

The cartoon shows the co-expression of NMDARs and AMPARs at the post-synaptic membrane. A | The agonist glutamate that is released from pre-synaptic terminals activates the AMPAR, causing an influx of Na^+ and a depolarisation of the membrane. Whereas NMDAR activation requires the presence of glutamate in addition to a co-agonist molecule. When the neuron is at a resting state, Mg^{2+} ions block the pore of the NMDAR. B | The AMPAR-mediated depolarisation expels the Mg^{2+} ion from the NMDAR pore. This expulsion allows the Na^+ and Ca^{2+} ions to freely influx through the NMDAR. The Ca^{2+} entry further mediates several intracellular signalling processes and coordinates plasticity associated with the excitatory synapse.

1.3 NMDA glutamatergic receptors – Structure to function

NMDARs are composed of four subunits arranged in a tetrameric structure assembled around a central ion channel, with each subunit comprising several transmembrane domains. Each subunit has four parts: an amino-terminal domain (ATD), a ligand-binding domain (LBD, also called an agonist-binding domain or ABD), a transmembrane domain (TMD), and an intracellular or cytoplasmic C-terminal domain (Figure 1.5). The LBDs have a structure that looks like two lobes connected by a hinge, and when an agonist binds to them, the lobes close. This causes tension in the LBD-TMD linkers, which helps to open and close the TMD channel. The ATDs can affect how the channel works in a subtype-specific way by interacting with the LBDs. So, the LBDs play a key role in controlling how the channel responds to agonists (Chou et al., 2020). NMDARs contain multiple pharmacologically important sites, such as glycine binding site (GBS), phencyclidine, Zn^{2+} , polyamine, glycosylation, and polyamine sites. The GBS located in the GluN1 subunit is a pharmacologically important site that plays a crucial role in the functioning and regulation of the activity of the NMDAR.

The subunits can be divided into three main categories: GluN1 (previously known as NR1 subunit, a product of *Grin1* gene), GluN2 (NR2, *Grin2*), and GluN3 (NR3, *Grin3*). GluN1 is the most abundant and essential subunit, responsible for determining the receptor's affinity for its endogenous ligands, D-serine (will be addressed as DS) or glycine (apparent affinity of glycine $\sim 0.6 \mu\text{M}$). GluN2 subunits come in four different isoforms (2A-2D subunits) and contain the binding site for its endogenous ligand, glutamate (EC_{50} of $2.3 \mu\text{M}$, apparent affinity K_d of glutamate $\sim 4.8 \mu\text{M}$). This low K_d value, indicative of high binding affinity accounts for the longer duration of the EPSC response (Patneau and Mayer, 1990; Lester and Jahr, 1992; Buller et al., 1994). While DS or glycine binds to the GluN1 subunit, the glutamate binding to the GluN2 subunits also regulates the DS/glycine sensitivity (Kuryatov et al., 1994; Furukawa et al., 2005). GLUN3 (3A and 3B) subunits are less common and are thought to modulate the function of NMDARs (Sanz-Clemente et al., 2013; Tu and Kuo, 2015; Pérez-Otaño et al., 2016).

Two obligatory GluN1 subunits and two GluN2 or GluN3 subunits, either as di-heteromers or tri-heteromers can lead to the formation of typical NMDARs (Paoletti and Neyton, 2007). The different combinations determine their functional properties, such as their sensitivity to glutamate, DS, and glycine as well as their pharmacological and signalling characteristics (Sanz-Clemente et al., 2013). Unlike AMPARs or the kainate receptors, the activation of NMDAR happens only when its endogenous co-agonists DS or glycine bind to the GluN1 subunit (Paoletti et al., 2013) along with its fellow agonist glutamate, which binds to the GluN2 subunit. Being an ionotropic receptor, its transmembrane pore allows the flow of Na^+ , K^+ , and

Ca^{2+} ions which control various intracellular signalling in the neurons. Unlike AMPARs, the NMDARs do not undergo desensitisation but get deactivated between 50 ms and a few seconds. This deactivation phase of EPSC depends on the concentration of the ligands and other modulatory factors in the synaptic cleft. Some modulatory factors include Zn^{2+} released from pre-synaptic terminals (Frederickson et al., 2000), the phosphorylation state of the subunits, and interactions with scaffolding proteins, such as PSD-95, which can regulate the trafficking, localisation, and signalling of the GluN1 and GluN2 subunits.

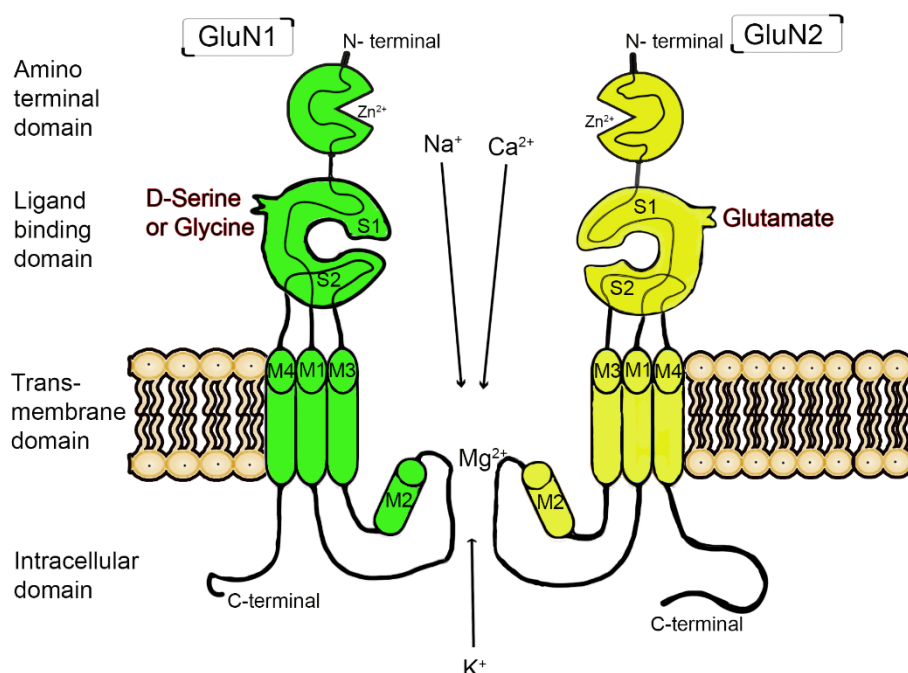


Figure 1.5: Schematic representative structure of N-methyl-D-aspartate type of glutamatergic receptors

At the molecular level, the NMDAR is composed of several domains, including the extracellular domain, the transmembrane domain, and the intracellular domain. The extracellular domain contains the binding site for the co-agonists, glutamate (in the GLUN2 subunit) and D-serine/glycine (in the GLUN1 subunit- referred to as the glycine binding site). The transmembrane domain (M1, M3, M4) located within the cell membrane contains the ion channel pore, blocked by Mg^{2+} ions at resting state. Ca^{2+} and Na^{+} ions flow into and K^{+} out of the cell when the membrane is depolarised, often caused by activation of adjacent AMPA receptors. The intracellular domain is involved in signalling cellular processes associated with Ca^{2+} such as plasticity and memory. The Figure was drawn using information from Benske et al. (2022).

The specific subunit composition of NMDAR can vary depending on the location in the brain and can define the different properties and physiological role that the receptor plays. NMDARs containing GluN2A subunits are more sensitive to activation by glutamate and to the inhibitory effects of Zn^{2+} ions, compared to NMDARs containing GluN2B subunit. NMDARs containing GluN2A and GluN2B are more sensitive to the inhibitory action of the Mg^{2+} block than NMDARs containing GluN2C and GluN2D subunits (Kuner and Schoepfer, 1996; Paoletti and Neyton,

2007). GluN2A-containing NMDARs are found adjacent to the synapses, whereas the GluN2B-containing NMDARs are found in the spaces away from the synapses (extra-synaptic space). Hence, they are referred to as synaptic and extra-synaptic NMDARs respectively. NMDARs in the hippocampus specialise in learning and memory-related processes and have a different subunit composition than NMDARs in the spinal cord, where they are involved in pain perception and motor control (Cull-Candy et al., 2001; Shimomura et al., 2015). The GluN3-containing NMDARs are more abundantly expressed during early development and are less Ca^{2+} permeable. Their role in shaping synaptic connections is not extensively studied compared to other types. Their expression in immature neurons indicates that they may not contribute to any excitatory neurotransmission in adulthood (Pérez-Otaño et al., 2016).

AP5 (K_d of 24 nM; 2-amino-5-phosphonopentanoic acid) or APV (DL-2-amino-5-phosphonovaleric acid) is commonly used as a selective competitive antagonist binding at the co-agonist binding site of the GLUN1 subunit, while MK-801 (K_d of 37.2 nM; dizocilpine) is used as a selective pore-blocking antagonist of NMDARs (Wong et al., 1986; Fletcher and Lodge, 1988; Murphy et al., 1988). Kynurenate, an endogenous antagonist, is a small molecule that can bind to the glycine site on the NMDAR and inhibit its activity (Birch et al., 1988).

In summary, NMDARs play a pivotal role in excitatory neurotransmission, by requiring the presence of an agonist, glutamate and either of the co-agonists, DS, or glycine, to fully open and allow cations to freely flow across the membrane. Their involvement in plasticity and various cellular processes emphasises their importance as molecular coincidence detectors.

1.3.1 Saturation of the glycine binding site

Saturation of the glycine binding site (GBS) refers to the extent to which the NMDAR is occupied by agonists such as glycine and DS. In this heading, the evidence for and against the saturation of the glycine binding site is summarised.

Early evidence suggests that the glycine binding site may not be fully saturated under physiological conditions. Johnson and Ascher (1987) first reported that even sub μM concentration of glycine (as low as 10 nM) can potentiate the response of cultured neurons to NMDA application. Another landmark study found that the application of low concentrations of glycine (1 μM) increases NMDAR-mediated responses in cultured neurons, suggesting that the glycine binding site was not fully saturated (Forsythe et al., 1988). Berger et al. (1998) reported the enhancement of miniature NMDAR currents associated with glycine application

only at concentrations 100 μ M or above in rat hypoglossal brain stem slices. In cortical brain slices, electrophoretic application of 200mM glycine enhanced NMDAR-mediated responses (Thomson et al., 1989). However, much of the early evidence of unsaturation must be carefully interpreted as the studies were conducted in a significant amount of contaminating glycine or in cultured conditions, wherein the metabolic breakdown from cells or bacteria could produce glycine.

Evidence indicates that the concentration of glycine or DS required to saturate the glycine binding site is much higher than the concentration of glycine or DS found in the brain under normal *in vivo* conditions (7-10 μ M glycine and 5-8 μ M DS, as determined by micro-dialysis of free moving rats (Hashimoto et al., 1995; Matsui et al., 1995), particularly in the prefrontal cortex and striatum. Further, due to highly efficient glycine uptake systems expressed on the astrocytes (a type of glial cell, 1.5) surrounding synapses, the synaptic glycine concentrations were suggested to be as low as <1 μ M. It is important to note (above study) that the *in vivo* concentration of DS is higher in the hippocampus and lowest in the cerebellum, suggesting variability between different brain areas. Moreover, the estimation of extracellular DS is subject to careful interpretation as the levels could primarily be dependent on the traffic between neuronal and astrocytic compartments viz. a dynamic concentration range rather than a static and stringently regulated concentration (Wolosker and Radzishevsky, 2013). Secondly, the extracellular concentration of DS is influenced by the expression of D-amino acid oxidase (DAAO; See section 1.6.5), the enzyme that breaks down DS, which is expressed highly in the cerebellum, but almost negligible in the hippocampus (Suzuki et al., 2017). Additionally, it is unclear whether the glycine binding site can remain partially or fully empty under conditions of high NMDAR activity, such as when the animals are actively exploring the environment.

High-affinity glycine transporters, GlyT1 (a product of the *Slc6a9* gene) and GlyT2 (*Slc6a5*) have been extensively studied in heterologous systems to regulate glycine levels (Supplisson and Bergman, 1997), especially in brain areas where glycine could act as a preferred co-agonist. It has been scrutinised whether this mechanism could reduce the physiological glycine levels below a few μ M, as early measurements estimated extracellular glycine concentration to be 1-10 μ M (Westergren et al., 1994), which is above saturation concentration. However, calculating the thermodynamic efficiency of the glycine transporters expressed on the astrocytes, Attwell et al. (1993) suggested that glycine concentrations could be restricted to less than ~200 nM, which is less than the saturation concentration of the binding site. Moreover, exogenous addition of 10-100 μ M DS or glycine or blockade of GlyT1 potentiates NMDAR response in prefrontal cortex and hippocampal slices (Bergeron et al.,

1998; Chen et al., 2003; Martina et al., 2003), suggesting that the glycine binding site might be unsaturated in certain brain area. However, this question is unresolved due to contradictory observations.

On the other hand, there is also evidence that suggests that the glycine binding site of NMDARs can be saturated under certain conditions. One study found that even high concentrations (1 μ M – 10 mM) of glycine are incapable of enhancing NMDAR-mediated responses in rat cortical slices (Fletcher and Lodge, 1988). Another study found that the application of high concentrations of glycine (~2mM) can inhibit NMDAR-mediated responses in CA1 hippocampal neurons (Zhang et al., 2014). In line with these findings, in the mossy fibre-granule cell synapses of cerebellar brain slices, Billups and Attwell (2003) reported saturation of glycine binding site and no enhancement of NMDAR responses to the application of 10-100 μ M DS or glycine. To sum up, there is strong evidence for saturation of the glycine binding site, which can lead to a decrease in NMDAR function and potentially contribute to the development of certain neurological disorders.

Despite efforts centred on the regulation of glycine about its action on the NMDARS, not much is known about the key regulators of DS in brain areas like the hippocampus, where DS acts as the preferred co-agonist (Papouin et al., 2017). Given the significant effect, DS has been shown to have on NMDAR activity, maintaining DS homeostasis is essential for proper NMDAR-mediated neurotransmission. Therefore, it is rudimentary to understand the transporters, that are involved in the regulation of synaptic DS concentrations, that are essential for NMDAR function. The saturation of GBS can be studied in acute brain slices by measuring NMDAR currents in response to glutamate release, stimulated via a stimulating electrode. Endogenous compounds like kynurenic acid can be used to competitively unblock the DS or glycine if they saturate the receptor (Kessler et al., 1989). Further, the relative role of potential DS transporters in governing the synaptic concentrations of DS can be determined.

Overall, the evidence for and against the saturation of the glycine binding site of NMDARs is mixed. The only logical interpretation of the literature on the saturation of the glycine binding site of NMDAR is that the regulation of the co-agonist and the binding site is complex and not fully understood. Further research is needed to fully understand the role of the glycine binding site in the regulation of NMDAR activity, function, and its potential role in neurological disorders. The degree of saturation of NMDARs at the GluN1 site is regulated by various mechanisms, including the release and uptake of co-agonists, the activity of enzymes involved in the synthesis and degradation of co-agonists, and the activity of transporters that mediate

the uptake of co-agonists into cells. Dysregulation of these mechanisms can lead to abnormal levels of saturation of NMDARs and impaired neurotransmission and neural plasticity in the brain.

1.4 Glial cells in the brain

“Any study of synaptic physiology will need to consider glial biology if scientists hope to achieve a comprehensive understanding of brain function”. -Mirko Santello and Andrea Volterra.

Glial cells compose approximately 90% of the total cells in the brain (Herculano-Houzel, 2014). The term “glia” derived from ancient Greek, was first identified by 19th-century scientists including Rudolph Virchow and Otto Deiters. They were initially thought to perform supplementary functions in the brain, while neurons were considered to be the primary information-processing cells in the brain (Chvátal and Verkhratsky, 2018). There are two major types of glial cells in the brain – 1. Macroglia which includes astrocytes, oligodendrocytes, progenitor NG2 cells and ependymal cells and 2. Microglia, a specialised immune cell in the CNS (Figure 1.6).

Astrocytes: They are the most prominent type of macroglia found in the CNS including the brain stem and spinal cord. Most astrocytes reside close to neurons, actively participating in synaptic transmission and helping regulate the balance of neurotransmitters in the synaptic cleft. Therefore, this thesis will exclusively focus on the astrocytes in the brain.

Vascular Astrocytes: These are a type of specialised astrocytes that reside close to blood vessels and contribute to energy demands by supplying nutrients to the neighbouring neurons. These astrocytes have specialized end plates that attach to the walls of blood vessels, forming part of the blood-brain barrier, where they stringently regulate the exchange of ions and nutrients between the blood and brain cells.

Oligodendrocytes: Oligodendrocytes are responsible for forming a protective insulating layer called myelin around axons in the CNS, which increases electrical conductivity and facilitates rapid signal transmission in neurons. Oligodendrocyte equivalents in the periphery make a cuff around the node of Ranvier, thereby wrapping around the neurons (Schwann cells).

Microglia: Microglia are the resident immune cells of the brain, which aid in detecting and engulfing foreign pathogens and cellular debris. They transform into phagocytic cells when activated and destroy the pathogens through the process of engulfing or phagocytosis.

Ependymal cells: They line the ventricles of the brain and are involved in the production of cerebrospinal fluid (CSF). They also form a barrier between the brain and the CSF, thereby contributing as gatekeepers of the brain's internal environment.

Overall, these diverse types of glial cells work together to maintain global homeostasis and support neuronal activities.

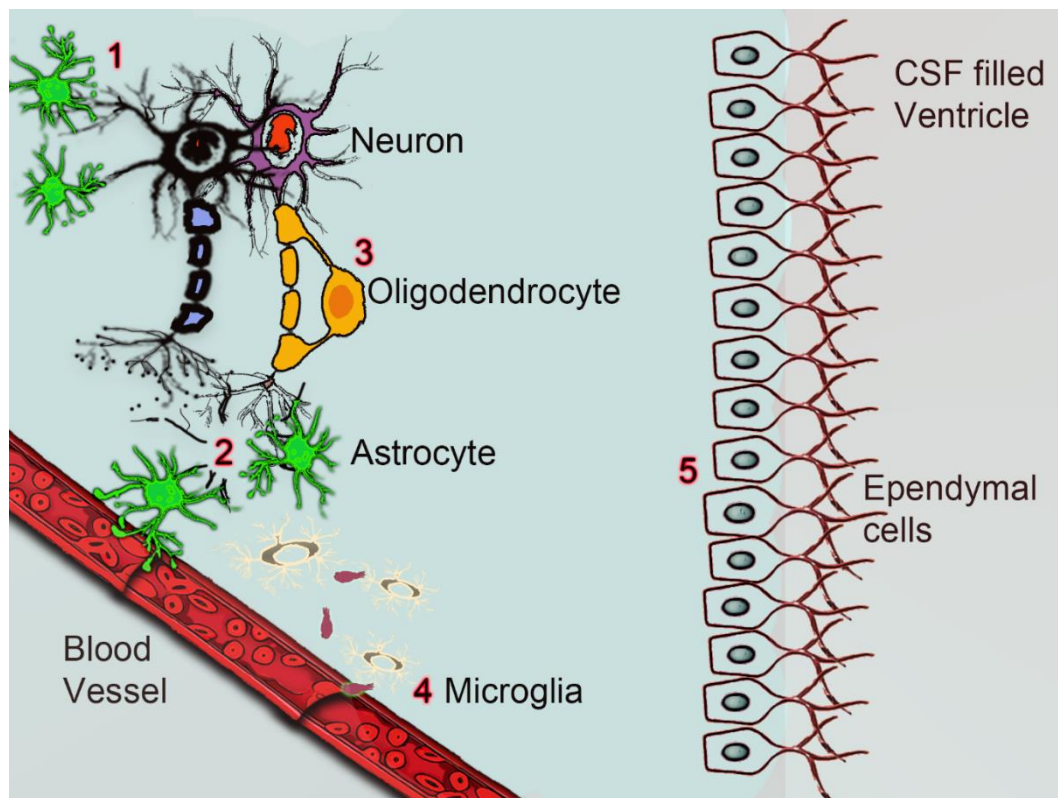


Figure 1.6: Different types of glial cells in the brain

1 – Astrocytes that reside close to the neurons and modulate synaptic activity. 2 – Vascular astrocytes that reside close to the blood vessels and help in supplying nutrition to the neurons 3- Oligodendrocytes help in forming a protective insulating layer around the axons, thereby increasing the electrical conductivity of neurons. 4- Microglia, the native immune cells of the brain. They engulf invading pathogens and transform into activated microglia, which destroy the pathogens via phagocytosis. 5 – Ependymal cells lining the ventricles of the brain, where they form a brain-CSF barrier

1.5 Astrocytes – Structure to functions in the brain.

Astrocytes are star-shaped cells bearing several tiny processes originating from the soma. They are distributed throughout the central nervous system in most known mammalian species and can make up around 12-50% of the brain's volume. For years, the role played by astrocytes in the synapse was thought to be passive, providing a structural scaffold to the neurons, leading them to be designated as neuroglia, which means nerve glue. In the literature, they have also been referred to as astroglia. The discovery that astrocytes intimately make contact with synapses in the brain, has challenged the traditional concept of a partite synapse, which only involved the pre- and post-synaptic neuronal components. Early observations of astrocytes expressing neurotransmitter receptors that can modulate neuronal activity have introduced the novel approach of a tripartite synapse, which incorporates the astrocytic component (Araque et al., 1999). In line with this, researchers such as Mirko Santello and Andrea Volterra, affiliated with the Department of Cell Biology and Morphology at the University of Lausanne, Switzerland, have notably articulated, "Memory formation is known to occur at the level of synaptic contact between neurons. It, therefore, comes as a surprise that another type of brain cell, the astrocyte, is also involved in establishing memory".

To understand the dynamic role played by the glial cells and the neurons in regulating proper neurotransmission, scientists have studied similar processes with a popularly referred model called "The tripartite synapse". This model is composed of three main processes (Araque et al., 1999); a. Neurotransmitters are typically released from pre-synaptic neuronal terminals b. Occasionally, neurotransmitters can "spill over" and bind to the surrounding glial cells c. Astrocytes release neurotransmitter modulatory molecules, often referred to as gliotransmitters, which can then modulate the release of neurotransmitters from pre-synaptic neurons. This model helps to portray the synapses as composed of dynamic players (involving both neurons and astrocytes) rather than a static neurotransmitter release model, which involves neurotransmitter release followed by a sequence of post-synaptic events. For instance, during ongoing glutamatergic neurotransmission, the glutamate that is released into the synapse can also bind to the glutamatergic receptors in the astrocytes. This causes a rise in astrocytic Ca^{2+} concentrations and a subsequent release of gliotransmitters (D'Ascenzo et al., 2007).

1.5.1 Astrocytes – Network of cells

Astrocytes display the unique property of being able to function as globally connected supercells rather than acting as individual astrocytes. They express connexins (Cx43 and Cx30) proteins in abundance, forming gap junctions between hundreds of neighbouring astrocytes (Xing et al., 2019). These gap junctions are crucial for inter-astrocytic communication, maintaining ion and neurotransmitter homeostasis in thousands of synapses. Despite being globally connected, they occupy a specific spatial domain, that enables them to control local neurotransmission by interacting with neighbouring neurons. This astrocytic network connectivity adds a whole new level of complexity to the physiological functions they coordinate (Figure 1.7A). One of the essential roles of astrocytes is regulating extracellular potassium levels, by rapidly clearing potassium released during normal synaptic activity. Although pre-synaptic neurons release potassium (Orkand et al., 1966), post-synaptic neurons are the primary contributors, releasing nearly 80% of the potassium during normal synaptic activity (Poolos et al., 1987). Uptake of K^+ is facilitated by inward-rectifier potassium channels (K_{ir} channels), $Na^+/K^+/2Cl^-$ cotransporters and the activity of the Na^+-K^+ ATPase pumps. Additionally, astrocytes engage in spatial buffering utilising their interconnected network via gap junctions (Bellot-Saez et al., 2017). These combined mechanisms contribute to the effective management of extracellular K^+ levels. In summary, astrocytes interact with neurons forming the tripartite synapses. Their global connectivity and specific spatial domains allow them to coordinate local neurotransmission, making them an indispensable component of a tripartite synapse.

1.5.2 Astrocytes in the Hippocampus

Astrocytes in different brain areas perform a wide variety of specialised functions. In most excitatory and inhibitory connections in the hippocampus, they enwrap the synaptic junction and form a typical tripartite synapse. Despite forming intimate connections with neurons, astrocytes are not electrical excitability. They are hyperpolarised cells with lower resting membrane potential (-80 mV) than the neurons (-60 to -70 mV), with low membrane resistance (Bolton et al., 2006). Typically, mature astrocytes have distinct stellate morphology and can be classified as passive glia (Zhou et al., 2006). The typical somatic volume of a CA1 astrocyte is approximately $500 \mu m^3$, whereas the total cell volume has been estimated to be ten times larger. Reconstructing the morphology of the processes indicated that a single astrocyte creates a territory volume of around $30000 \mu m^3$. The hippocampal astrocytes studied in this thesis typically have a soma diameter of 3-5 μm , while their tiny processes span up to 100 μm , as previously reported by Halassa et al. (2007). They described the hippocampus as an island

comprising thousands of synapses, estimated to be around 140,000 in number. The primary branches, about seven per astrocyte, typically target the cell bodies, axons and dendrites of surrounding neurons (Figure 1.7B). The secondary branches and the finer branchlets (thousands) intimately interact with synapses, axons, dendrites, neighbouring astrocytic branches, other glial cells like oligodendrocytes, and blood vessels via end plate (Bushong et al., 2002; Moye et al., 2019). In conclusion, astrocytes in the hippocampus play active roles in synaptic microenvironment. Their interactions with neighbouring neurons and other glial cells underscore their physiological importance to overall brain functioning.

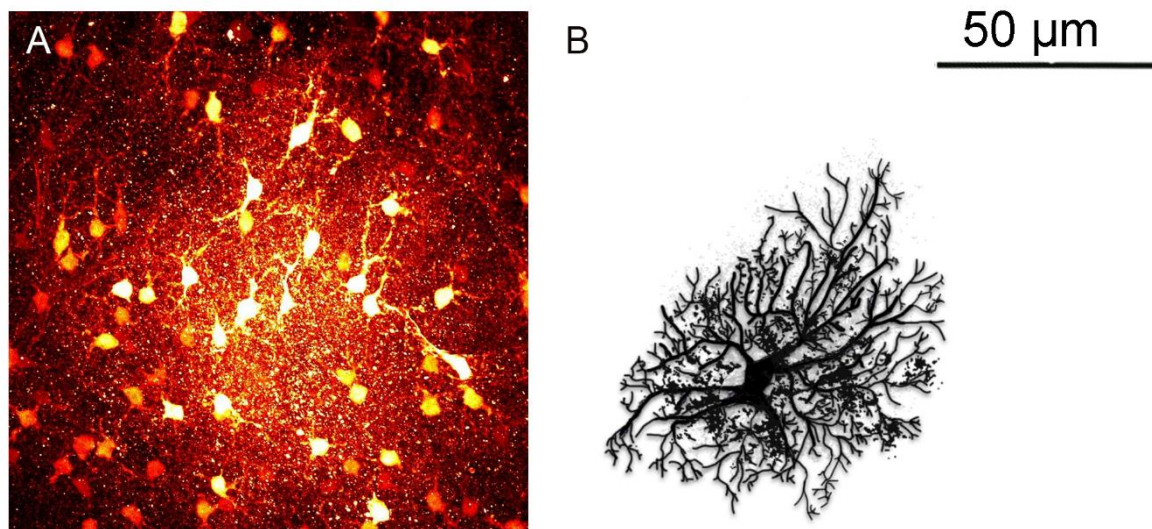


Figure 1.7: Morphology of an astrocyte

A | Confocal image of astrocytes in the *stratum radiatum* of the CA1 region in the mice hippocampus. Astrocytes were filled with biocytin and post hoc stained with Alexafluor-594. Stacked images were acquired using a Nikon A1 confocal microscope using the Nikon instruments elements software. Image stacks were acquired with a 20X objective lens with stacks of 2 μm resolution. B | A drawing showing the morphology of a single astrocyte. It shows the immense branching of the astrocytic network that arises from a single astrocytic soma.

Identifying the hippocampal astrocytes

Although astrocytes look like oligodendrocyte precursor cells (NG2) found in the hippocampus, these precursor cells have outward and variable rectifying potassium currents, with fast inward sodium currents (Xie et al., 2007; Bergles et al., 2010). This is in stark contrast to astrocytes, which express a wide range of voltage-dependent and voltage-independent ion channels, that help regulate extracellular Na^+ , K^+ , Ca^{2+} and Cl^- ions. The expression of voltage-dependent sodium and potassium channels is developmentally regulated across several brain areas including the hippocampus, cortex, and cerebellum (Kressin et al., 1995; Bordey and Sontheimer, 1997; Bordey and Sontheimer, 2000). After day P20 of brain development in mice, almost all mature astrocytes lack expression of voltage-gated Na^+ channels (Kressin et al.,

1995), suggesting that Na⁺ channels are characteristic of immature astrocytes and could be upregulated during early synaptic development to maintain ionic homeostasis. The presence of a wide variety of voltage-dependent and independent potassium channel subtypes, often characterized as leak channels, has been documented. Voltage-gated K⁺ channels (K_v channels), inwardly rectifying K⁺ channels (K_{ir} channels), Ca²⁺-dependent K⁺ channels (SK channels) and two-pore domain potassium channels (K₂P channels) identified as TREK1, TREK2, TASK1 and TWIK1 are essential components of the astrocytic membranes, contributing to the regulation of ion fluxes, K⁺ buffering capacity and maintenance of membrane potential (Du et al., 2016; Seifert et al., 2018; McNeill et al., 2021). Pharmacologically, tetrodotoxin (TTX) and tetraethyl ammonia (TEA) can be used to inhibit voltage-gated sodium and potassium channels, thereby aiding in better clamping of astrocytic membrane voltage (Nowak et al., 1987).

The classification of cells as astrocytes is further complicated due to cellular heterogeneity. However, certain proteins such as GFAP (glial fibrillary acidic protein) and astrocyte-specific excitatory amino acid transporters like EAAT1 (GLAST) and EAAT2 (GLT-1) are considered more definitive markers (Walz, 2000), offering a degree of clarity in distinguishing astrocytes from other cell types. Electrophysiological criteria have been instrumental in differentiating astrocytes from interneurons found in the hippocampus, revealing several distinct features. Firstly, when subjected to voltage/current changes, CA1 interneurons but not astrocytes, exhibit spiking responses. Further, a significant number of interneurons display ongoing firing activity, even in the absence of any voltage or external stimulation (Kawaguchi and Hama, 1987). Overall, distinguishing astrocytes within the hippocampus from neighbouring glial cells and interneurons is facilitated by their distinct electrophysiological characteristics, making their identification relatively straightforward.

1.5.3 Cortical astrocytes

Astrocytes are abundantly found across all layers of the cortex and differ in their gene expression pattern (Bayraktar et al., 2020). In layers 1-2 of the cortex, the astrocytic processes project towards the surface of the pia mater, forming specialised structures called astrocytic end feet, which collectively constitute the glia limitans. In this area, they form a protective barrier between the capillary or blood vessels and the nerve cells, popularly referred to as the blood-brain barrier (Tabata, 2015). These astrocytic end feet also make contact with the capillary endothelial cells, where they take up glucose via glucose transporters (GLUTs) expressed on their membranes. This process becomes essential in supplying energy to the neurons, especially during periods of high neuronal activity (Sokoloff, 1981; Kacem et al.,

1998; Braet et al., 2001). This illustrates that astrocytes are important for any activity-dependent process in the brain, as without their involvement, the neurons would be deprived of proper blood, oxygen, and glucose flow for their functioning.

1.5.4 Astrocytes in the Cerebellum

In the cerebellum, the astrocytes residing in the cell layer are called Bergmann glia. Worthy of thy name, these astrocytes play an exemplified and dynamic role by actively providing feedback to the synaptic circuitry. One of the key mechanisms by which they exert this influence is by the rich expression of AMPARs and kainate receptors on their surface (Burnashev et al., 1992; Müller et al., 1992), which are widely known to influence synaptic functions. Additionally, Lino et al. (2001) demonstrated the importance of the astrocytic AMPARs on excitatory post-synaptic currents at Purkinje cell synapses. They conducted experiments using an adenoviral delivery system of the GLUR2 subunit, which effectively abraded the calcium permeability property of the AMPARs, subsequently affecting the EPSCs. Remarkably, this manipulation resulted in the retraction of the synapse-sealing glial processes, and as a consequence, climbing fibre inputs exhibited persistent multiple innervations. This interplay between Bergmann glia and the cerebellar circuitry exemplifies the active and dynamic role that astrocytes play in shaping neuronal communication within the cerebellum.

1.5.5 Astrocytes in Culture

Much of our knowledge of astrocytes came from culturing them in a growth medium. Although culturing astrocytes has proven to be effective in understanding their functional roles in the brain, they may not fully represent their physiological roles in the complex and dynamic environment of a living brain. Astrocytes in culture behave as reactive astrocytes, where their structural morphology and protein expression change drastically (Cahoy et al., 2008; Buffo et al., 2010; Zamanian et al., 2012). Nevertheless, it is evident that even outside the living host, astrocytes are crucial for the survival of neurons. Culturing neurons in the absence of supporting astrocytes is not viable, indicating the vital role of astrocytes in meeting the energy demands of neuronal circuits and synapses. This aspect is further discussed in detail concerning the synthesis of DS in the brain. Overall, it is safe to conclude that without astrocytes, the energy demand of the local neuronal circuits and synapses will not be met.

1.5.6 Astrocytes and the Waves of Calcium

Astrocytic membranes possess a diverse array of ion channels, transporters and neurotransmitter receptors, that enable them to play active roles beyond being just passive

observers of neuronal activity. They sense the concentration of various ions and neurotransmitters and dynamically alter the functions of surrounding neurons and synapses. In familiar terms, the astrocytes sense synaptic activity and respond by increasing their intracellular calcium levels, a phenomenon well-documented in the literature (Haydon, 2001). Intracellular Ca^{2+} elevation mediated via IP_3 -dependent release of Ca^{2+} from internal stores, and its associated signalling in the astrocytes have been reported *in vivo* both in astrocytic soma and processes (Stobart et al., 2016).

The physiological consequences of the intracellular Ca^{2+} elevation in the astrocytes are universal and exhaustive (Shigetomi et al., 2013; Perez-Alvarez et al., 2014; Durkee et al., 2019). One unique feature of astrocytes is their ability to propagate these Ca^{2+} changes to the neighbouring astrocytes connected via gap junctions, mimicking waves of activity in the local astrocytic network (Dani et al., 1992; Nedergaard, 1994). Most of the astrocytic Ca^{2+} elevation associated with synaptic events reported to date have been associated with the activation of astrocytic G-protein coupled receptors (GPCRs). Depending on the specific G protein that is activated, astrocytic Ca^{2+} responses can vary. For instance, G_i -mediated signalling leads to a transient elevation followed by a sustained decrease in astrocytic Ca^{2+} levels (Durkee et al., 2019).

Impairment in astrocytic Ca^{2+} signalling has been shown to have deleterious effects on synaptic networks, significantly disrupting gliotransmission. Srinivasan et al. (2015) studied this phenomenon in mice deficient in Inositol 1,4,5-triphosphate type 2 receptors (IP_3R_2) and revealed impaired gliotransmitter release. Additionally, in the CA1 hippocampus, activation of the G_q pathway in the astrocyte's chemo-genetically (Adamsky et al., 2018) and through optogenetics (light activated channels; Mederos et al., 2019) has been demonstrated to elevate Ca^{2+} mediated LTP in the CA1 synapses. These findings highlight the active and dynamic consequences of astrocytic calcium changes in mediating synaptic communication and plasticity in the brain.

1.5.7 The astrocytes meet the metabolism and energy requirements of the brain.

Astrocytes play an intricate role in metabolic pathways and in maintaining energy demands in the brain. Early research pioneered in the early 1950s (Kety and Schmidt, 1948; Sokoloff, 1981) demonstrated that while most neurons are fully oxidative (breaking down glucose), glial cells such as astrocytes and oligodendrocytes have the capacity to utilise both glucose and glycogen to produce ATP. Glucose absorbed from the blood, not only acts as the primary source for ATP production in the brain but also as an essential precursor for glycogen synthesis in the astrocytes. As the astrocytes are closely entangled with the synapses, they have been shown to actively participate in glucose utilisation and lactate release to support neuronal energy demands. This process of lactate release is well documented in the literature and is referred to as the lactate shuttle (Brooks, 2018). Pellerin and Magistretti (1994) and Voutsinos-Porche et al. (2003) have shown that glutamate uptake in the astrocytes triggers aerobic glycolysis, a process contributing to the supply of energy substrates for neurons.

On the contrary, Parpura et al. (1994) introduced a novel mechanism through which astrocytes contribute to the release of glutamate into synapses. Through the utilisation of confocal microscopy, photo-stimulation techniques, as well as appropriate pharmacological agents such as bradykinins, Haydon and colleagues demonstrated, both in neuronal cultures and in astrocyte-neuron cocultures, that bradykinin induces a selective elevation in neuronal calcium levels only within the coculture environment. Moreover, they established that this calcium increase is contingent upon glutamate release from the astrocytes. This investigation provided pioneering evidence that astrocytes play a role in modulating neuronal calcium levels by means of glutamate release.

In addition to their metabolic functions, astrocytes are involved in regulating several neurotransmitter levels in the synapses, contributing to proper neurotransmission in the brain. Recent research has hypothesised their role in regulating DS levels at synapses (Wolosker et al., 2016), a topic further explored in this thesis. Moreover, astrocytes have been extensively studied for their involvement in modulating glutamate concentrations at synapses, a critical aspect of synaptic functioning.

1.5.8 Role of astrocytes in the glutamate-glutamine cycle

In line with the metabolic role astrocytes play, they also act as native housekeeping cells of the brain. One crucial housekeeping function of astrocytes is the active removal of excess glutamate from the synaptic cleft or extracellular space. This process prevents excitotoxicity, a condition where the leftover glutamate overstimulates the neurons and causes damage. Astrocytes achieve this by sequestration of synaptic glutamate via a plasma membrane sodium coupled-excitatory amino acid transporter isoform 2 (EAAT2 or GLT-1, a product of the *Slc1a2* gene; See 1.7.2) mediated uptake mechanism (Billups et al., 1998). Once inside the astrocytic soma, they quickly neutralise the glutamate by converting it into glutamine by the astrocyte-exclusive enzyme glutamine synthetase (GS). Glutamine is a relatively less toxic amino acid and has been hypothesised to be shuttled back into neighbouring pre-synaptic neurons to replenish the glutamine pools (Bak et al., 2006).

This synaptic activity-dependent glutamine efflux from the astrocytes occurs via the sodium coupled-neutral amino acid transporter isoform 3 (SNAT3, a product of the *Slc38a3* gene) and has been observed in the medial nucleus of the trapezoid body (MNTB) area of the brain stem (Todd et al., 2017). The precise mechanism of glutamine uptake in the pre-synaptic terminals is an ongoing area of research. Sodium-dependent neurotransmitter transporter (NTT4, a product of the *Slc6a17* gene) has been hypothesised to be a likely candidate involved in refilling the pre-synaptic glutamine pools (Nicoli et al., unpublished; Jia et al., 2023). The recycled glutamine from astrocytes can be converted back into glutamate inside the pre-synaptic terminals, via phosphate-activated glutaminase (PAG) within the mitochondria, as a crucial step in the glutaminolysis pathway (Curthoys and Watford, 1995). Additionally, the glutamate can be further converted into GABA inside the GABAergic neurons and the process of glutamine uptake in the GABAergic synapses is dependent on the sodium-coupled neutral amino acid transporter isoform 1 (SNAT1, a product of the *Slc38a1* gene). This metabolic cycle involving glutamine and glutamate or GABA has gained therapeutic interest in the context of many glutamate/GABA-related brain disorders, and metabolic research (Bröer and Brookes, 2001; Qureshi et al., 2020), holding promise for plausible therapeutic intervention. Overall, the 'Glutamate-Glutamine cycle' (GGS; Figure 1.8) highlights the importance of neighbouring astrocytes in serving the synaptic demands, making this process of interplay between neurons and astrocytes highly efficient and recycling-oriented.

Alternatively, the glutamate sequestered into the astrocytic soma can be converted into α -ketoglutarate by the enzyme glutamate dehydrogenase (GDH). This α -ketoglutarate can enter the tricarboxylic acid (TCA) cycle, generating more energy for the astrocytes. Astrocytic

α -ketoglutarate, as well as other TCA cycle intermediates like citrate, have been hypothesised to be shuttled into the neuronal compartment, instead of glutamine, to synthesise glutamate (Andersen et al., 2021). This shuttle mechanism is referred to as the α -ketoglutarate–glutamate cycle or citrate–glutamate cycle, respectively.

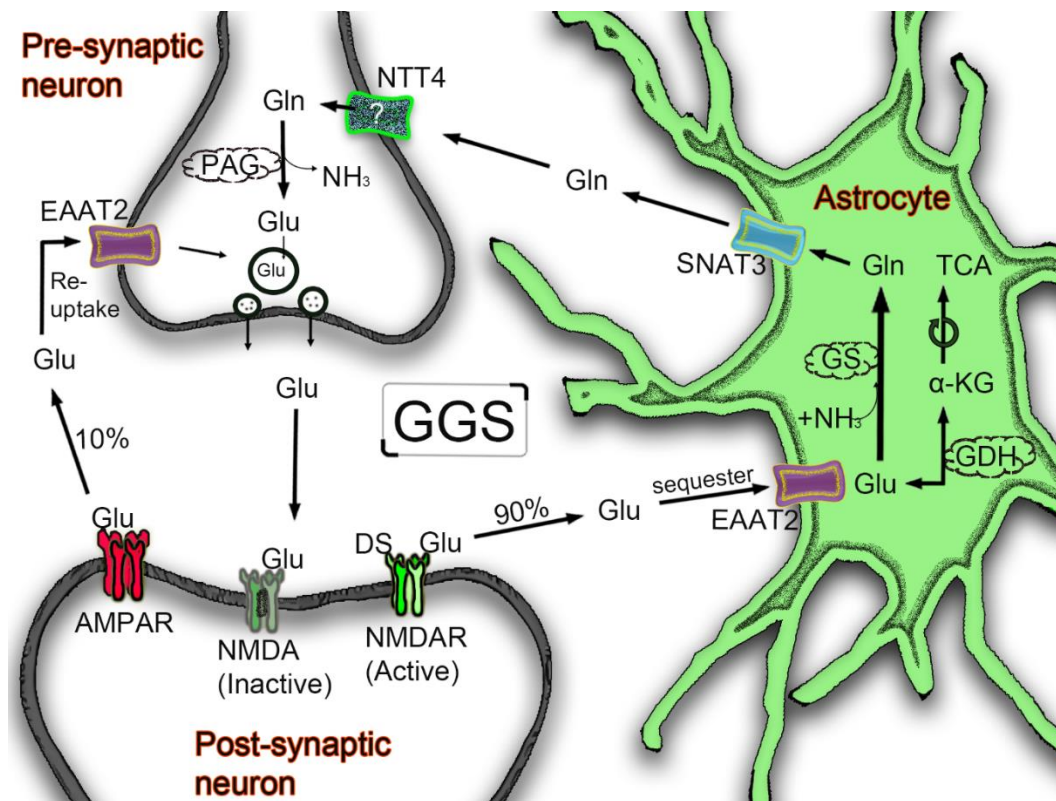


Figure 1.8: Role of astrocytes in the glutamate-glutamine cycle

Astrocytes play an integral role in clearing up glutamate (Glu) rapidly from the synapses after its action on the post-synaptic glutamate receptors. The glutamate sequestered by the astrocytic excitatory amino acid transporter (EAAT2) is converted into glutamine (Gln) by glutamine synthetase (GS). The non-toxic glutamine is then released back into the synapse via the system N transporter (SNAT3). Once the glutamine gets into the pre-synaptic neurons via an unknown transporter (could be neurotransmitter transporter NTT4), it can be recycled back into glutamate by phosphate-activated glutaminase (PAG). This process is referred to as the glutamate-glutamine cycle (GGC). Alternatively, the glutamate that is sequestered into the astrocytes can be converted into α -ketoglutarate by glutamate dehydrogenase (GDH). α -ketoglutarate can enter the tricarboxylic acid (TCA) cycle to undergo further metabolic reactions to produce energy.

1.6 Serine is a unique biologically active molecule

An essential component for various cellular functions such as protein synthesis, nucleic acid synthesis, energy production, metabolism, and intracellular signalling are amino acids. Serine is a truly unique and biologically active molecule, serving as a vital component in a plethora of cellular processes and biochemical pathways. As a non-essential amino acid, serine is indispensable for the biosynthesis of important molecules including phospholipids, and nucleotides, thereby contributing to protein synthesis, cell membrane formation and energy production (Genchi, 2017; Holeček, 2022). In the mammalian brain, serine takes a specialised role in the process of excitatory synaptic transmission. Dextro-serine (D-serine or DS; dextrorotatory) and Laevo-serine (L-serine or LS; laevorotatory) are two enantiomers of the same amino acid, serine, and are uncanny mirror images of each other. The primary chemical distinction between DS and LS lies in the spatial orientation of atoms around the central α -chiral carbon atom (Figure 1.9). Although DS and LS have similar physical and chemical properties, their physiological functions are completely different. Although D-amino acids are less common in nature, alongside D-aspartate (D'Aniello et al., 2011), DS is one of the known D-isomers found in the human body. It is mainly produced from LS through a racemisation reaction catalysed by the enzyme serine racemase (Wolosker et al., 1999a). In the mammalian forebrain, DS is relatively less abundant compared to LS, contributing approximately 25–30% of the total serine content (Hashimoto et al., 1992). Unlike LS, DS is not involved in protein synthesis, instead serves as a significant neuromodulator through its interaction with NMDARs. Together with glutamate, DS or glycine acts as co-agonist molecules, regulating the activity of excitatory synapses. Co-binding of these molecules to the postsynaptic NMDARs is crucial for their activation, leading to the designation of 'co-agonists'. The presence of DS as a neuromodulator, alongside another co-agonist like glycine, highlights the complexity of synaptic transmission and the fine-tuned regulation of NMDARs. Overall, this multifaceted biomolecule showcases its versatility and indispensability, underscoring its significance in many fundamental biological processes. Understanding the role of DS and its interactions with NMDARs is essential for gaining insights into various neurological processes and disorders related to excitatory neurotransmission.

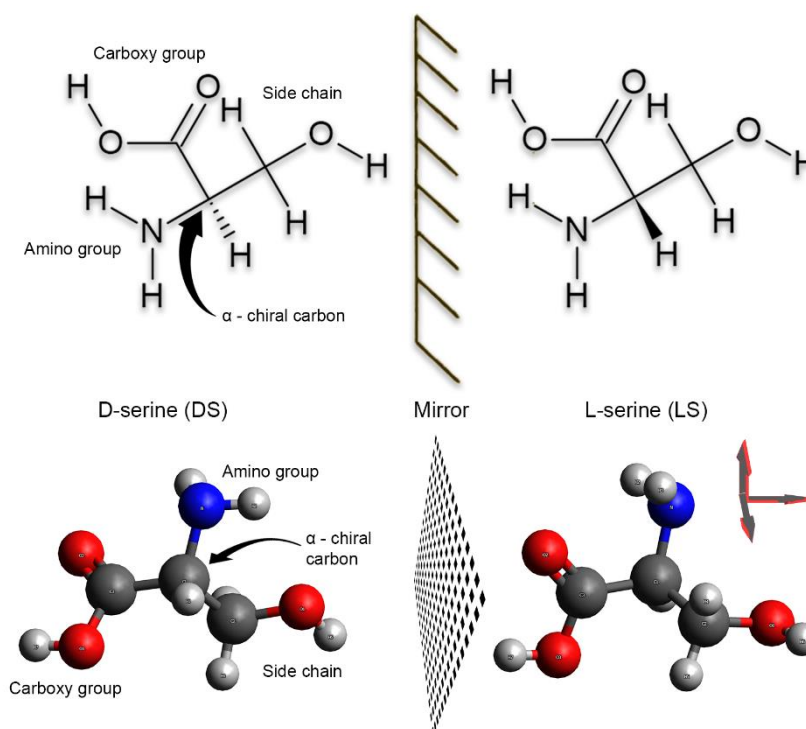


Figure 1.9: Structure of serine isomers

Both D (Dextro) and L (Laevo) are mirror images of each other. (2R)-2-amino-3-hydroxy propanoic acid. The dashed line and bold-wedged H bond indicate that the atom extends behind and protrudes out of the plane, respectively. The structures were created using ACD/Chemsketch 2020.1.2, Open Babel 2.4.1 and Avogadro 1.2.0.

1.6.1 L-serine synthesis in the brain

L-serine (LS) is widely researched compared to DS, due to its involvement in the synthesis of intracellular proteins, its role as a potent neuronal neurotrophic factor and its functional role serving as a precursor to producing several vital biomolecules including D-serine, glycine, phosphatidylserine, and sphingomyelin (Holeček, 2022). LS can be obtained from different sources, including the diet (crossing the BBB), the breakdown of proteins or sphingolipids, and directly from glycine. However, they are minor contributors to the LS supply compared to the *de-novo* synthesis, which is the primary pathway for LS production. In the brain, astrocytes synthesise LS *de-novo* from the precursor glucose, which undergoes glycolysis. Glucose is taken up by the astrocytes from the blood vessels through the glucose transporter isoform 4 (GLUT4) expressed on their plasma membrane (El Messari et al., 2002). The glycolytic intermediate 3-phosphoglycerate is then utilised to be converted to 3-phosphohydroxypyruvate by the enzyme phosphoglycerate dehydrogenase (3 PGDH; a product of *Phgdh* gene; Jaeken et al., 1996; Yamasaki et al., 2001; Jeon et al., 2009; Ehmsen et al., 2013). Subsequently, 3-phosphohydroxypyruvate is converted to 3-phosphoserine by the enzyme phosphoserine aminotransferase (PSAT; a product of *Psat1* gene). Finally, 3-phosphoserine is converted into

LS by the enzyme phosphoserine phosphatase (PSP; a product of *Psph* gene; van der Crabben et al., 2013).

The *de novo* biosynthesis is the main metabolic pathway unique to astrocytes to produce LS (Figure 1.10), as defects in the *Phgdh*, *Psat1*, and *Psph* genes can lead to severe serine deficiency disorders in infants and adults (Jaeken et al., 1996; Acuna-Hidalgo et al., 2014; El-Hattab, 2016; El-Hattab et al., 2016; Abdelfattah et al., 2020). Inactivation of the 3-PGDH in mice has been shown to have deleterious consequences, such as brain microcephaly and embryonic death, due to the overall reduction in DS and glycine synthesis in the body (Furuya et al., 2008). Research on LS is extensive because of its fundamental role in various cellular processes and its critical contributions to supplying DS, for maintaining excitatory neurotransmission.

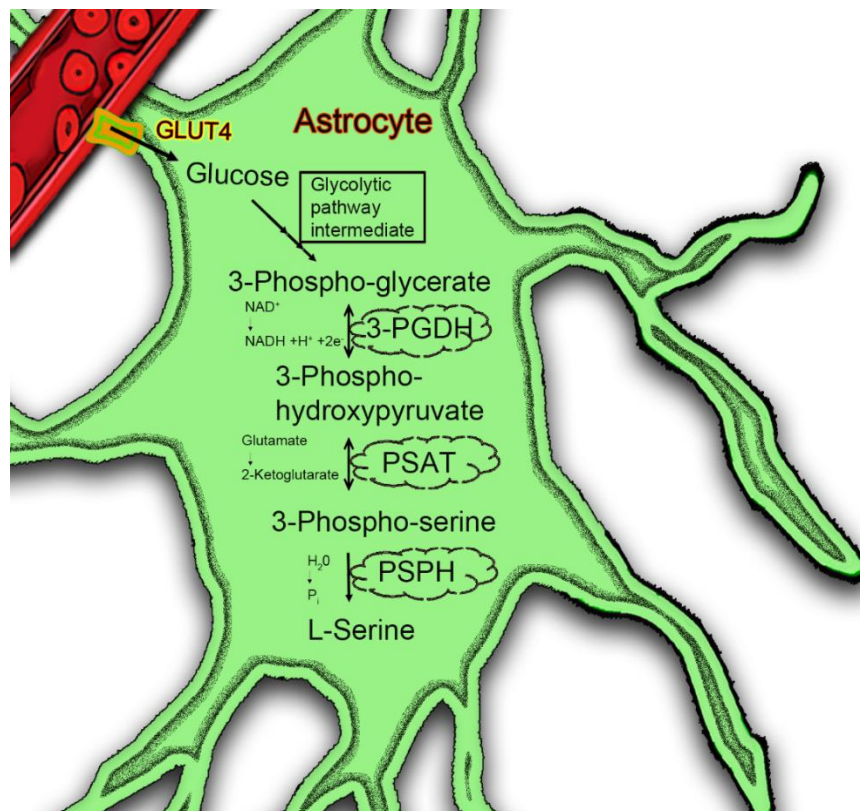


Figure 1.10: *De-novo* synthesis of L-serine in the astrocytes

De-novo synthesis of L-serine occurs exclusively in the astrocytes. Glucose is taken up by the astrocytes via glucose transporter isoform 4 (GLUT4) from the blood vessels that are attached to the astrocyte end feet. 3-Phosphoglycerate is an intermediate obtained from the glycolytic breakdown of glucose. The enzyme 3-phosphoglycerate dehydrogenase (PGDH) is a key enzyme that is exclusive to the astrocytes in the brain. Other enzymes include phospho-serine aminotransferase (PSAT) and phospho-serine phosphatase (PSPH).

1.6.2 Utilisation of L-serine produced in the astrocytes.

One major pathway involving the utilisation of LS is its contribution to the production of phosphatidylserine, a crucial type of phospholipid, which constitutes the lipid bilayer in cell membranes. This happens via a base exchange reaction, wherein the base ethanolamine in phosphatidylethanolamine (the second most abundant phospholipid in mammals) gets exchanged for a free serine (Vance, 2008). Although this reaction occurs in many cells capable of producing LS, the newly synthesised LS gets channelled into three major pathways in the brain. Firstly, LS is utilised in the production of sphingomyelin, a structural lipid that forms the myelin sheath surrounding neurons. This process occurs via a condensation reaction between LS and palmitoyl CoA, leading to the generation of sphinganine, which is further converted to ceramide, and finally transformed into sphingomyelin (Weiss and Stoffel, 1997).

Furthermore, LS is utilised to synthesise DS, via the enzyme SR (discussed below). DS produced via this process functions as a co-agonist at the NMDAR, modulating excitatory neurotransmission. Finally, LS is utilised in the synthesis of glycine, facilitated by the enzyme serine hydroxymethyltransferase (SHMT, encoded by the *Shmt* gene). Glycine functions as a neurotransmitter, exerting its effects on the extrasynaptic NMDARs or glycinergic receptors. A by-product, N⁵, N¹⁰-methylenetetrahydrofolate generated in this reaction, can be used in the synthesis of nucleic acids like purines and pyrimidines, as it functions as a one-carbon donor (Sodulescu et al., 2018).

In addition to these pathways, LS can also be converted to pyruvate, which enters mitochondria and serves as an energy currency in cells (Richard and Pamela, 2011). Overall, LS plays a vital role in various metabolic pathways in the brain, influencing the production of important molecules like phosphatidylserine, sphingomyelin, DS, glycine, and pyruvate.

1.6.3 Neuronal neurotrophic factor

Apart from the above-mentioned role of LS in the production of membrane lipids, nucleic acid and neurotransmitters, LS also serves as a vital neuronal neurotrophic factor for neuronal growth and survival. This is evident from studies that demonstrated that exogenously supplied LS, at concentrations ranging from 10-200 nM, but not DS, promote various aspects of neuronal growth including survival, dendritic arborisation, and synapse formation in cultured neurons (Savoca et al., 1995; Furuya and Watanabe, 2003). This type of communication between astrocyte-derived LS and pyramidal neurons was first observed in studies where cultured neurons in a glia-free and LS-free medium had about 25% fewer neurons after 7 days

(Mitoma et al., 1998a; Mitoma et al., 1998b). This suggests that LS is one of the major neurotrophic factors essential for neuronal growth and survival. Moreover, the importance of LS in the production of intracellular proteins, glycine, 5,10-methylenetetrahydrofolate, phosphatidyl-L-serine, sphingolipids and other metabolic intermediates needed for energy production in neurons, further emphasises its role in promoting neuronal survival and proper functioning. Depriving cells of these essential molecules would ultimately lead to cell death.

Additionally, the secretion of LS from the astrocytes has been suggested to be stimulated by extracellular glutamate (Yamagata et al., 2006). This suggests that astrocytic LS may play a protective role in neuronal survival by countering glutamate-mediated excitotoxicity. Understanding the baseline levels of LS required for neuronal survival and proper functioning is crucial. The precise physiological stimulus and mechanism of LS release from the astrocytes is unclear. Nevertheless, the intricate interplay between LS and its various metabolic pathways in neurons and astrocytes underscores its importance as a multifaceted neurotrophic factor in the CNS.

1.6.4 Serine racemase mediates D-serine synthesis in the Brain

The initial immunohistochemical localisation of brain DS revealed its presence primarily within the astrocytes of the rat cerebral cortex and hippocampus (Schell et al., 1995). Further, purification of the Serine Racemase (SR), an enzyme that catalyses the interconversion of DS and LS (also known as D-serine ammonia-lyase or D-serine dehydratase or L-serine ammonia-lyase or L-serine dehydratase) by Wolosker et al. (1999b), from cultured cortical astrocytes led to the assumption that the astrocytes could be involved in DS synthesis from the precursor LS. SR, expressed in critical brain regions like the hippocampus, cortex, and striatum, exists in both neurons and astrocytes (Wolosker et al., 1999a; Yoshikawa et al., 2007; Miya et al., 2008).

SR is a bifunctional enzyme, dependent on pyridoxal 5'-phosphate (PLP) and cofactors such as ATP and Mg^{2+} (De Miranda et al., 2002). SR is also thought to dimerise with PXLK-K56 protein or to be in a state of dimer-tetramer equilibrium (Cook et al., 2002; Neidle and Dunlop, 2002). The K_m value (Michaelis constant) or the amount of substrate it takes for the enzyme SR to reach half of its maximum velocity ($V_{max}/2$), calculated using the Michaelis–Menten equation, was 9.8 mM for LS (with V_{max} of 5 $\mu M/mg/h$) and 60 mM for DS (with V_{max} of 22 $\mu M/mg/h$). Notably, the inverse correlation between the K_m value and the affinity of SR for its substrates, emphasises the likelihood of its predominant role in the conversion of the L form to the D form under physiological conditions, as observed in the comprehensive work by

Wolosker et al. (1999a). The presence of 1 mM ATP has been observed to decrease the K_m value for LS from 13 mM to 1.8 mM (Neidle and Dunlop, 2002). Interestingly, the same enzyme SR can catalyse either a racemisation or an elimination/deamination reaction resulting in either the racemisation product DS or the elimination product pyruvate, depending on the specific reaction mechanism (Graham et al., 2019).

The serine isomer transformation occurs mainly in the chiral centre at the α -carbon of serine. SR binds with its co-factor PLP, forming a serine-PLP complex that is necessary for the catalytic process. The cofactors Mg^{2+} ions and ATP, plays an essential role in stabilising enzyme conformation and providing the necessary energy for the reaction to occur (Foltyn et al., 2005). The binding of Mg^{2+} ions to SR enhances its affinity for LS and PLP, promoting the formation of the serine-PLP complex. On the other hand, ATP plays a role in the conformational changes of the enzyme, influencing the movement of substrates within the active site, thereby enhancing the SR's catalytic efficiency. In the racemisation pathway, SR acts on LS, and the enzyme's active site residues like Lysine57, serine308, and serine82 (highlighted in red in Figure 1.11) facilitate the transfer of a proton to the α -carbon of serine (Goto et al., 2009). This proton abstraction and donation lead to the formation of an aldimine (enamine) intermediate, leading to the formation of a resonance-stabilised carbanion. The carbanion undergoes tautomerisation to produce DS. In the elimination pathway, SR catalyses the elimination of a molecule of water from the carbanion intermediate to form pyruvate and ammonia. This involves the deprotonation of the hydroxyl group on the α -carbon, followed by the formation of a double bond between the α - and β -carbons and the release of water. To sum up, the outcome of this enzymatic reaction can be in any direction and depends on the interplay between the SR's active site residues, the binding and concentration of cofactors like Mg^{2+} ions and ATP, and the availability of substrates DS and LS (Pollegioni and Sacchi, 2010; Pollegioni et al., 2018). Overall, SR plays a crucial role in the regulation of DS levels, impacting NMDAR-mediated neurotransmission and synaptic plasticity. The balance between racemisation and elimination pathways is crucial for maintaining appropriate concentrations of synaptic DS.

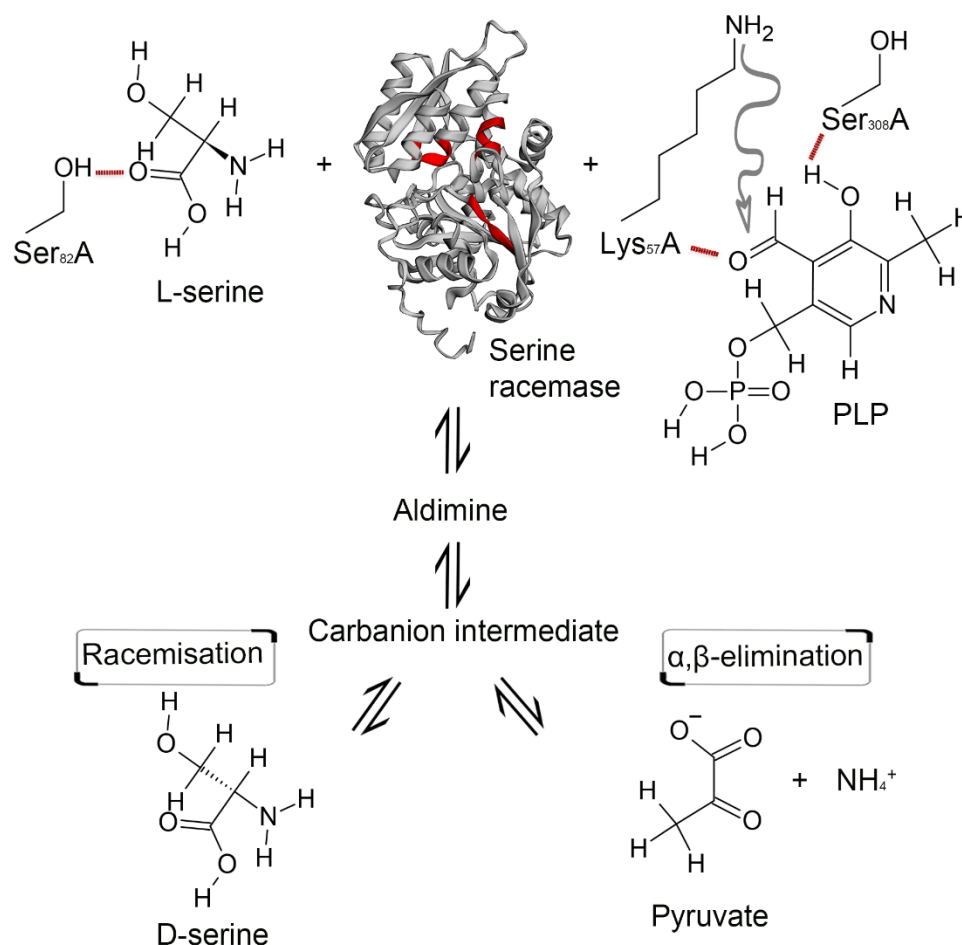


Figure 1.11: Synthesis of D-serine from L-serine

Reaction showing the serine racemisation and α,β -elimination catalysed by serine racemase enzyme. A molecule of LS or DS interacts with pyridoxal-5-phosphate co-factor (PLP) forming a serine-PLP complex, which forms an aldimine complex. Lysine57, serine308 and serine82 act as bases interacting with the LS and PLP. Their sequence positions are highlighted in red on the 3D alpha fold structure of serine racemase (Reynolds et al., 2018; Jumper et al., 2021; Varadi et al., 2021). Aldimine complex undergoes resonance stabilisation to form a carbanion intermediate, which can either undergo a racemisation or an α,β -elimination reaction. Racemisation yields DS/LS and the elimination yields, α -amino acrylate followed by pyruvate and ammonia.

Germline deletion of SR has been shown to have consequences on DS production and results in diminished long-term potentiation (LTP) in the hippocampal areas such as the CA1 region (Basu et al., 2009; Balu et al., 2016; Neame et al., 2019) and dentate gyrus (Balu et al., 2013). DS reduction also impacts synaptic plasticity in diverse brain regions, including the visual cortex, nucleus accumbens and lateral amygdala (Curcio et al., 2013; Meunier et al., 2016). Furthermore, targeted deletion of SR in neurons of mice showed reduced LTP, whereas deletion of astrocytic SR resulted in mice with normal LTP (Benneyworth et al., 2012; Perez et al., 2017). Similarly, the reduction of DS enzymatically also correlates to a decrease in the magnitude of LTP in the CA3-CA1 synapses (Papouin et al., 2012; Rosenberg et al., 2013; Le

Bail et al., 2015). Overall, evidence strongly indicates the central role of neuronal DS in synaptic plasticity and that SR is the key regulator of plasticity in those synapses.

A key element in unravelling the intricacies of DS regulation involves ascertaining the cellular origins and pathways for DS release and uptake. Recent scrutiny has been focused on identifying the primary cellular source of synaptic DS. Amidst the diverse abilities of distinct cell types to maintain a steady DS supply, it has been firmly established that a substantial portion of brain DS originates from its laevo enantiomer, LS. This transformative process is catalysed by SR, an enzyme integral to this isomerisation process (Wolosker et al., 1999a; Wolosker et al., 1999b; Ehmsen et al., 2013), taking place within both neurons (Kartvelishvili et al., 2006; Rosenberg et al., 2013) and astrocytes (Panatier et al., 2006; Henneberger et al., 2010). The constitutive knockout of SR has resulted in a substantial reduction of endogenous brain DS levels, by nearly 90% in comparison to control conditions (Basu et al., 2009; Horio et al., 2011). However, a constitutive knockout approach falls short of discerning the precise cell types responsible for DS production and release. To address this knowledge gap, the following paragraph 1.6.6 endeavours to illuminate the respective contributions of neurons and astrocytes to the concentrations of synaptic DS.

1.6.5 Role of D-amino acid oxidase in the brain

D-amino acid oxidase (DAAO) is a key enzyme that is involved in the metabolism of D-amino acids, including DS in the brain. DAAO is a flavoprotein belonging to the Flavin adenine dinucleotide (FAD)-dependent oxidoreductase family, which usually catalyses the conversion of D-amino acids into their corresponding aldehyde and ammonia. In the case of DS, DAAO converts it into pyruvate (an alpha-keto acid) and ammonia (Figure 1.12; Pollegioni et al., 2018). Strikingly, immunohistochemistry studies done in mice indicate that the regional localisation of DAAO in the brain is inversely correlated with the expression of DS. DAAO is predominantly expressed in the brain stem and cerebellum while being virtually absent in the forebrain, which includes the hippocampus, amygdala, and cortex (Schell et al., 1997). This regional localisation of DAAO suggests that it may play a role in regulating DS levels, by terminating its actions in the astrocytic compartments, particularly in the brain stem and cerebellum (Horiike et al., 1994; Hashimoto et al., 1995; Wolosker et al., 1999a). Similarly, low forebrain DAAO expression was observed in rats, cats, bovines and humans (Neims et al., 1966; Weimar and Neims, 1977).

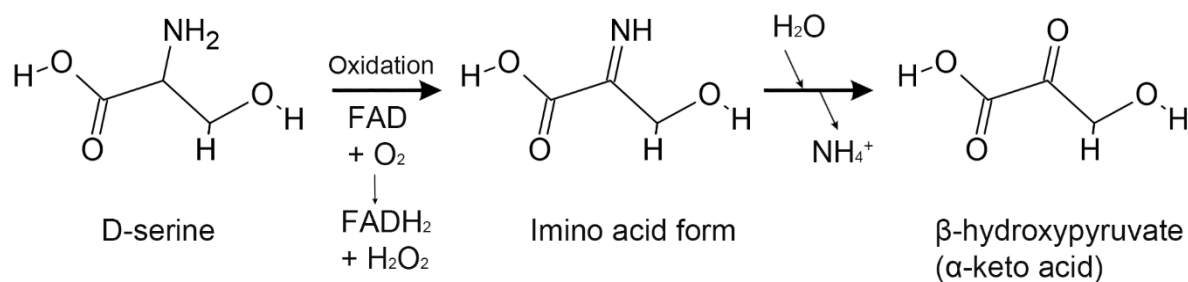


Figure 1.12: Degradation reaction of D-serine by D-amino acid oxidase

D-serine undergoes an oxidative breakdown in the presence of co-enzyme Flavin adenine dinucleotide (FAD). FADH₂ is the hydroxyquinone form of the coenzyme, which yields 2 ATP during oxidative phosphorylation. The intermediate imino form of D-serine undergoes further oxidation to produce β-hydroxy pyruvate.

Studies have shown that the highest level of DS expression in the hippocampus is observed at post-natal days P7-14 (Schell et al., 1997). This period corresponds to early postnatal development when synaptic connections are rapidly forming, and excitatory neurotransmission is maturing. However, after P15, DS levels start to drop significantly in the hippocampus. This decrease in DS expression is primarily attributed to the increased expression and activity of DAAO, which breaks down DS. Despite the drop in DS levels after P15, there is still a substantial amount of DS expression in the mouse hippocampus at P21-25 (Kim et al., 2019; Liu et al., 2019). This indicates that even after the predominant expression of DAAO, some DS continues to be present in the hippocampus during this period of development. On the contrary, the reduction in brain DS levels during ageing could be attributed to a decrease in SR expression, as a study by Potier et al. (2010) revealed that the DAAO levels remain constant throughout development. However, the potential role of DS transporters in the brain during ageing, which could contribute to the reduction of brain DS, remains unknown. The regulation of DS levels and the interplay between DS and DAAO during various stages of postnatal brain development are important factors in modulating excitatory neurotransmission and plasticity in the brain.

The addition of DAAO (0.1 - 0.2 U/ml) to brain slices, to metabolise endogenous DS, leads to a diminished NMDAR-mediated LTP in the hippocampus and supraoptic nucleus (Yang et al., 2003; Panatier et al., 2006). This suggests that despite the low expression of DAAO in the forebrain, it is likely to play a role in DS degradation in young (P16-18) and adult (1-3 months) animals. Almond et al. (2006) have also demonstrated, for the first time, that DAAO KO mice strain exhibits increased occupancy of the NMDAR glycine binding site due to elevated extracellular DS levels. Furthermore, they have shown that this enhanced DS presence leads to improved NMDAR function *in vivo*. Indeed, adult mice (2-4 months old) with single point

mutation of DAAO displayed significantly larger LTP in the hippocampal CA1 synapses. Moreover, the DAAO KO mice displayed a significantly shorter search time to find the hidden platform in a Morris water maze learning paradigm (Maekawa et al., 2005). This evidence suggests that DAAO is involved in several brain functions, including learning and memory. Overall, DAAO is an important enzyme in the brain that plays a role in the metabolism of d-amino acids like DS and therefore in the regulation of excitatory neurotransmission.

1.6.6 The co-agonist dilemma in the Synapses

This finding of DS co-localising with NMDARs challenged the understanding of excitatory neurotransmission and opened a myriad of questions. What might be the identity of the endogenous co-agonist present in the brain? Are there obvious distinctions between the co-agonists regulating synaptic and extra-synaptic NMDARs? Assuming DS functions as the preferred co-agonist, what are the primary sources responsible for its presence in the synaptic cleft? Do synaptic DS concentrations, pivotal for the activation of synaptic NMDARs, predominantly originate from astrocytic or neuronal contributions? Under this heading and in 1.6.7, succinct outlines of the important advancements that have significantly contributed to unravelling and clarifying several of these inquiries have been presented.

The question still lingers regarding whether DS or glycine could serve as the favoured co-agonist at the synaptic NMDARs. The role of DS as a primary co-agonist for synaptic NMDARs has been a subject of ongoing debate within the realm of synaptic physiology. Early research centred on glycine (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988) as the preferred co-agonist. However, mounting evidence has compellingly demonstrated that DS can bind to the GluN1 site with higher affinity and efficacy than glycine (with an EC_{50} of approximately 0.22 μ M for DS as opposed to 0.53 μ M for glycine, as observed in studies by Matsui et al., 1995; Yovanno et al., 2022). Targeted point mutations of the glycine binding site have been shown to significantly lower the affinity of DS. An 86-fold reduction in DS affinity was lethal, where the mice didn't feed and died within a few days of birth, highlighting the importance of the glycine binding site. A 5-fold reduction in glycine binding site affinity resulted in an impaired hippocampal LTP in the CA1 region, along with altered anxiety-associated behaviour and impaired spatial learning. Further addition of 30, 100, and 300 μ M DS significantly increased the NMDAR-mediated LTP in KO conditions compared to wild-type conditions (Kew et al., 2000).

A Study from Solomon Snyder's lab marks the initial indication that DS might function as an endogenous co-agonist, potentially superseding glycine, at the glycine site of the NMDAR.

Additionally, it serves as a foundational study highlighting the potential substantial involvement of astrocytes in the release of DS. Schell et al. (1995) utilised a custom-designed antibody with high specificity for DS that was conjugated to glutaraldehyde. Employing this polyclonal antibody, they unveiled the highest DS levels in specific brain regions including the grey matter of the hippocampus, anterior olfactory nucleus, and amygdala. Interestingly, the regional distribution of glycine, particularly within the telencephalon, did not align with the localisation of NMDARs. Notably, DS and NMDARs did not co-localise in the adult cerebellum; however, detailed scrutiny revealed selective DS presence in the cerebellum, specifically within Bergmann glia. Remarkably, this observation of DS colocalisation with the DAAO enzyme in the cerebellum adds further weight to the study's findings. This indicates that the preferred co-agonist at the NMDAR site could be glycine or DS depending on the area of the brain and might differ during various stages of brain development (Schell et al., 1997; Puyal et al., 2006). However, Mothet et al. (2000) performed whole-cell patch-clamp recordings from rat hippocampal pyramidal neurons and found that degradation of DS with the enzyme DAAO significantly diminished NMDAR-mediated neurotransmission. This strongly suggests that in the hippocampus, DS could be the preferred co-agonist that mediates NMDAR functioning.

A landmark study by Philip G. Haydon and colleagues utilised enzymes to specifically destroy either DS or glycine without affecting the basal glutamatergic neurotransmission. Eliminating DS inhibited LTP and NMDAR synaptic potentials at the CA1-CA3 hippocampal synapses while removing glycine had no effect (Papouin et al., 2012). It is important to highlight that the processes underlying long-term depression (LTD) involve both synaptic and extrasynaptic NMDARs, requiring the presence of glycine. By directly applying NMDA to slices and isolating the extra-synaptic component, the authors demonstrated that glycine, rather than DS, is the primary co-agonist of extra-synaptic NMDARs. Another crucial observation is that the synaptic NMDARs controlled solely by DS are responsible for neurotoxicity. This concept challenges the commonly held notion that increased activation of extra-synaptic NMDAR leads to neurotoxicity, whereas activation of synaptic NMDAR transmits signals that promote neuroprotection.

Henneberger et al. (2010) later went on to effectively prevent the Ca^{2+} -mediated exocytosis of DS from the astrocytes in rat hippocampal slices. They observed that NMDAR-associated LTP was diminished in the nearby synapses. Further, the exogenous addition of DS replenished LTP associated with those nearby synapses. This indicates that astrocyte-derived synaptic DS could be crucial for NMDAR functioning and that the synaptic DS concentration could determine the plasticity at the glutamatergic synapses. Of late, effective pharmacology

and electrophysiological techniques have allowed other researchers to deduce that the DS occupancy at the GluN1 site of the NMDARs could be responsible for plasticity associated with those synapses (Yang et al., 2003; Wolosker, 2006; Henneberger et al., 2010; Rosenberg et al., 2013). Besides, extrasynaptic GluN2B-containing NMDARs are more sensitive to activation by glycine than DS, whereas synaptic GluN2A-containing NMDARs are most sensitive to activation by DS (Papouin et al., 2012; Le Bail et al., 2015). Since GluN2A-containing NMDARs constitute about 70–75% of all NMDARs in the brain, DS rather than glycine, acts as a preferred endogenous co-agonist at most mammalian synapses (Mothet et al., 2000; Panatier et al., 2006). In summary, synaptic NMDARs in the hippocampus are primarily gated by the endogenous co-agonist DS and the DS potentially released from the astrocytes significantly modifies the NMDAR-mediated synaptic plasticity. Therefore, to understand the NMDAR-associated functions, it is important to understand how DS is regulated at the excitatory synapses.

Over the last two decades, a plethora of research findings have consistently converged towards demonstrating DS as the preferred co-agonist in major brain regions such as the cortex and hippocampus (Schell et al., 1995; Mothet et al., 2000). Robust evidence has highlighted the substantial impact of DS concentration within the synaptic cleft on synaptic transmission, underscoring its potency and affinity to NMDARs, both of which surpass (about three times higher) those of glycine, initially presumed to be the endogenous co-agonist (Matsui et al., 1995; Shleper et al., 2005; Balu and Coyle, 2015). Consequently, DS has been deemed to be essential for the process of memory formation and learning through a process called long-term potentiation (LTP; Mothet et al., 2000; Wolosker, 2007). Despite these advancements, DS remains less explored compared to its counterparts, glutamate and glycine, leading Coyle et al. (2020) to label it a versatile "shape-shifting agonist". Amidst our growing grasp of DS's role in modulating NMDAR activity and the plasticity associated with it, the conundrum surrounding the origin and fate of DS after its action on the NMDAR remains unresolved. This persistent enigma pertains to its cellular origin, a point of most contested dilemmas in the realm of synaptic physiology.

1.6.7 The Rise of serine shuttle hypothesis

The quest to unravel the precise mechanism by which DS could be regulated within the synaptic cleft, though subjected to rigorous investigation, remains a subject of ongoing research (Wolosker et al., 2016). Over the past two decades, insights have underscored the crosstalk between neurons and astrocytes, as a key determinant of proper excitatory neurotransmission (Araque et al., 1999; Barber and Raben, 2019). Cumulatively, the precise contributions of astrocytes and neurons in shaping DS-associated NMDAR neurotransmission remains a promising area of research in synaptic physiology.

Previously thought to be just supporting brain cells, studies are now gaining interest in the dynamic role played by astrocytes. A pioneering study showed that DS is prevalently distributed throughout the central nervous system and thus more likely to serve as an endogenous co-agonist for NMDARs (Hashimoto et al., 1992). Double immunostaining experiments aimed to pinpoint the cellular expression of DS and the enzyme SR have indicated that the expression is localised to the astrocytes (Balu and Coyle, 2014). Further, electrophysiology coupled with pharmacological studies done on astrocyte cultures, hypothalamic slices, and *in vivo* rodent models, have supported the notion that astrocytes could release DS through the process of exocytosis in a Ca^{2+} -dependent manner (Schell et al., 1995; Panatier et al., 2006; Henneberger et al., 2010). Additionally, Ribeiro et al. (2002) indicated that astrocytes can synthesise DS from other amino acids like glycine. Immunohistochemical studies have indicated that the enzyme 3-Phosphoglycerate dehydrogenase present exclusively in the astrocytes could catalyse the *de-novo* synthesis of LS from glucose (Yamasaki et al., 2001; Ehmsen et al., 2013). Collectively, these studies indicate that, the astrocytes could synthesise LS/DS and that they could release LS or DS into the synapses. Consequently, due to the popular concept of DS being derived from glial cells/astrocytes and being released in response to the activation of NMDARs, DS has earned the label to be a 'glio-transmitter' or 'glio-modulator' (Schell et al., 1995; Schell et al., 1997).

The localisation of SR, the key enzyme-mediator of DS synthesis in the brain, to the astrocytic compartments, led to the presumption that the astrocytes are the main producers of DS in the brain. However, the interpretation of these results is clouded, as some of the initial evidence came from astrocytes in cultures or from experiments involving the clamping of astrocytic calcium, which is deleterious. Cultured astrocytes differ in their gene expression profile from astrocytes *in vivo* and extrapolating these results to normal brain function is, therefore, non-physiological (Eddleston and Mucke, 1993; Cahoy et al., 2008; Liddel and Barres, 2017).

In parallel to investigations involving astrocytes, a series of studies focusing on the enzyme SR's immuno-localisation within distinct neuronal cultures have consistently demonstrated its robust expression in neurons (Miya et al., 2008). Kartvelishvily et al. (2006) conducted a meticulous purification of SR from cortical neurons, revealing the co-existence of DS and SR, using the double immunofluorescence staining, within the cortical neuronal culture and under *in vivo* conditions. Subsequently, knocking out SR conditionally in neurons yielded a more pronounced reduction in total brain DS levels, than knocking out SR from the astrocytes (Miya et al., 2008; Benneyworth et al., 2012). This conditional KO mouse model of SR reinforced the importance of neuronal localisation of SR. This was supported by *in-situ* hybridisation (Yoshikawa et al., 2007) and in the transgenic mice model where the SR encoding gene was replaced by a green fluorescent protein (Ehmsen et al., 2013). Recently, the localisation of SR to the neuronal compartment was confined to the apical dendrites and the post-synaptic densities in the CA1 region by Wong et al. (2020) through immunogold-electron microscopy. These findings firmly established that DS is synthesised from LS by the enzyme SR, which is mainly expressed in neurons (Miya et al., 2008), distinguishing it from the astrocyte-originated DS as described above. Studies with SR conditional KO mice have shown that neuronal DS is important for NMDAR-dependent LTP (Balu and Coyle, 2014; Wong et al., 2020). Meunier et al. (2016) reported that DS but not glycine mediates the induction and maintenance of NMDAR-mediated LTP in the visual cortex of P21-28 days old rats. Since neurons express the SR enzyme but lack the enzyme machinery to synthesise LS, the precursor for the biosynthesis of DS, it is hypothesised that the astrocytes provide LS, for the neuronal DS synthesis (Wolosker et al., 2016). Nonetheless, consensus remains elusive regarding the extent to which neurons and/or astrocytes contribute to synaptic DS concentrations. As such, the core objective of this project is to discern how glutamatergic synapses orchestrate DS levels, identify the participating cell types, and elucidate their influence on NMDAR activity and consequent brain plasticity. Given the polarity of DS and LS, their translocation between neurons and astrocytes necessitates membrane transporters. Nonetheless, the intricate mechanisms governing DS/LS release or uptake by these cell types have been insufficiently explored, particularly in intact tissue preparations preserving neuronal and neuron-astrocyte connections. **Thus, the overarching aim of this research project is to investigate the intricate regulatory mechanisms underlying serine dynamics within excitatory synapses, thereby advancing the comprehension of how these mechanisms finely modulate excitatory synaptic transmission within the synaptic landscapes of the mice brain.**

Indeed, the integration of evidence stemming from both *in vitro* and *in vivo* systems has illuminated the possibility of astrocytes serving as a source of LS for the orchestrated synthesis of DS within the neurons, a phenomenon termed the serine shuttle process (depicted in Figure 1.13; Ehmsen et al., 2013; Wolosker and Radzishevsky, 2013). Recent investigations have identified a route by which astrocytes can supply the essential LS precursor to neurons through a transporter-mediated mechanism (Kaplan et al., 2018). This mechanism entails the conversion of LS into DS, subsequently recognised as the serine shuttle pathway (Neame et al., 2019). The strategic release of DS by neurons via a transporter-mediated mechanism into the synaptic cleft introduces a pivotal regulatory mechanism, influencing NMDAR activity and thereby orchestrating excitatory synapse plasticity (Rosenberg et al., 2013).

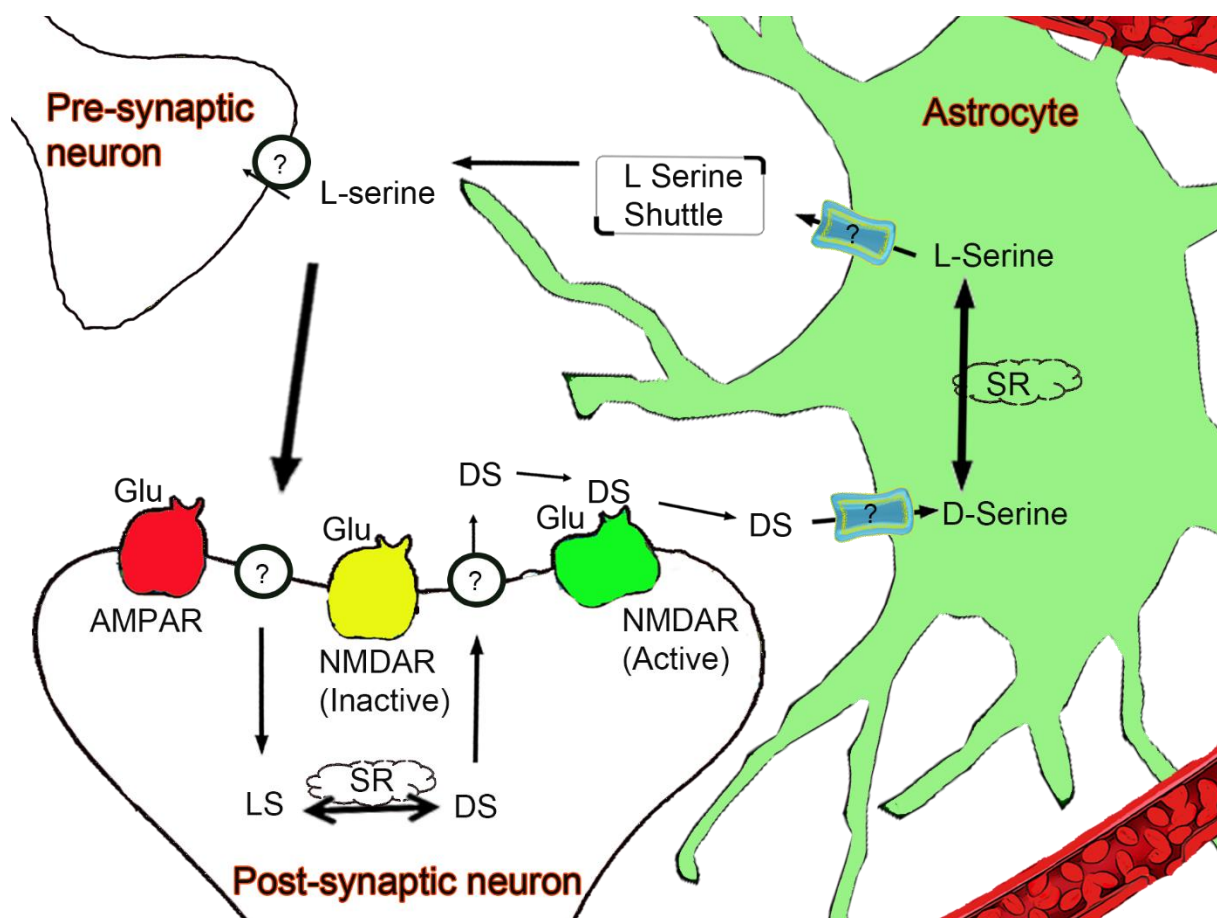


Figure 1.13: Illustration of Serine-shuttle hypothesis

Glutamate is released from the presynaptic neuron and binds to AMPARs on the postsynaptic neuron, leading to neuronal excitation. The source and sink of D-serine (DS) remains uncertain. DS is hypothesised to be cleared by astrocytes via plasma membrane transporters. Astrocytes convert DS to L-serine (LS) using the enzyme serine racemase (SR). L-serine is shuttled back to the presynaptic or postsynaptic neuron via serine transporters. In the postsynaptic neuron, LS may be converted to DS by SR. DS could be released by serine transporters or packaged into synaptic vesicles for release. DS released can bind to NMDARs on the postsynaptic neuron, leading to neuronal excitation or long-term potentiation.

1.6.8 Hippocampus – Anatomy and a perfect model system

The hippocampus stands out as a highly plastic brain area, capable of processing incoming data in novel ways. It is primarily excitatory in nature and, unfortunately, also highly susceptible to uncontrolled neuronal firing, as seen in epilepsy. Therefore, decades of extensive research have been undertaken to comprehend how the hippocampal circuits keep the excitatory signals in control (Chatzikonstantinou, 2014). This knowledge is crucial for treating any mental ailment involving excitatory signal dysregulation.

Furthermore, the hippocampus has been extensively studied for its crucial role in the long-term storage of various types of information, particularly in the storage of declarative memory encompassing names, facts, events, and spatial memory. This process of memory formation occurs via a phenomenon called Long-Term Potentiation (LTP) of synaptic transmission, a process by which the strength of a synapse is increased following repeated activation. Despite decades of extensive research focussed on the hippocampal network functions, detailed molecular information essential to understanding the mechanisms underpinning LTP formation remains elusive (Lynch et al., 1990). It is evident now that the mechanisms underlying memory and learning are more complex involving multiple cellular and molecular interactions in dynamic ways, contrary to the previous notion of a unitary process for information storage in the brain.

This thesis focuses on elucidating the regulation of the amino acid Dextro-Serine (DS), a key molecule mediating excitatory signalling in the brain. Hence, this current research primarily employs the hippocampus as a model brain area. Traditionally, the hippocampus has been a fundamental model system to investigate mechanisms involved in glutamatergic synaptic transmission (Bekkers and Stevens, 1989; Stecher et al., 1997).

The hippocampus plays a significant role in memory consolidation and repetitive behaviours, making it a key structure within the limbic system. In the mouse hippocampus, information processing occurs between two structures: the hippocampus proper and the dentate gyrus (Figure 1.14). These two structures form two interlocked C's and hence were historically referred to as the seahorse in Greek. Typically regarded as the archaia cortex, the dentate gyrus, and the hippocampus proper are in fact phylogenetically evolved cortical structures; however, it is 3-layered rather than the 6-layered neocortex. It comprises the molecular layer (*stratum lacunosum-moleculare*), the middle cell layer (*stratum pyramidale*) and the polymorphic layer (includes *stratum lucidum*, *stratum oriens*, and *stratum radiatum*). In the dentate gyrus, the middle layer is populated by granule cells, whereas in the hippocampus

proper, the middle layer consists of pyramidal cells. The entorhinal cortex provides the major input to the dentate gyrus cells, through a pathway popularly referred to as the perforant path. Information is further transmitted from dentate gyrus granule cells to the CA3 (CA - *Cornu ammonis*) pyramidal cells via the mossy fibre tract. The principal neurons or the major excitatory pyramidal neurons of the CA3 region have their cell bodies stacked together, where they project onto the excitatory CA1 neurons via the Schaffer collaterals (Megias et al., 2001). Additionally, the CA1 neurons also receive some input from the CA2 neurons, recently categorised as distinct areas with distinct functions related to social memory (Tzakis and Holahan, 2019). Interneurons expressing the markers parvalbumin (PV) and somatostatin (SOM) participate in intrinsic rhythm generation and regulate excitatory-inhibitory balance in principal cells in distal CA1 and subiculum regions (Sik et al., 1995).

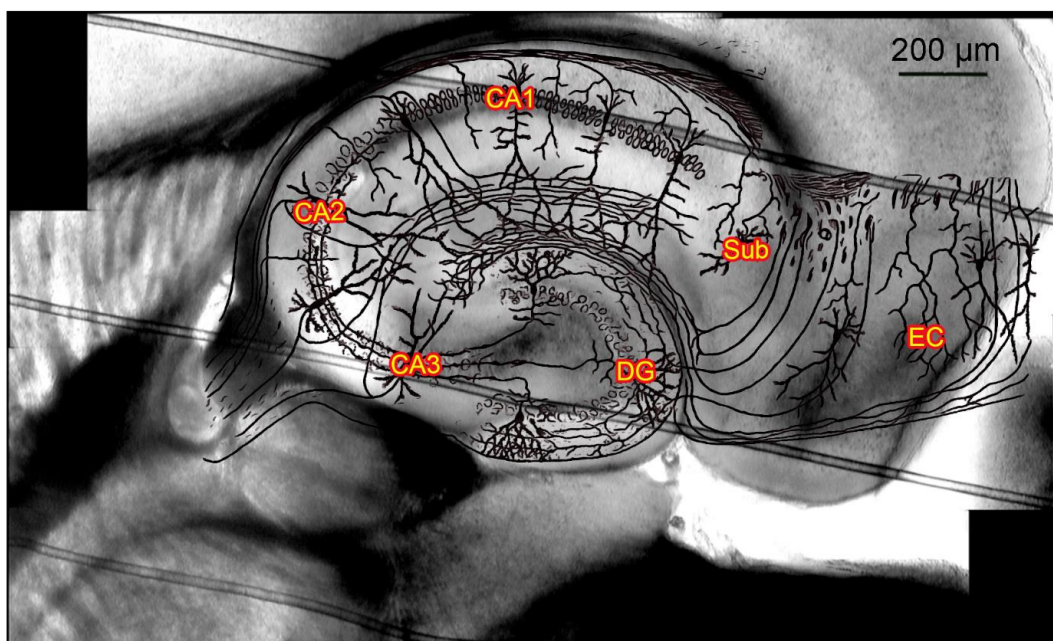


Figure 1.14: Hippocampal Architecture and synaptic connections

A differential interference contrast (DIC) image of a mouse hippocampal brain slice, highlighting major landmarks of the hippocampal architecture. The picture is overlaid with the original drawings of Santiago Ramon Y Cajal to accommodate the DIC image. Highlighted regions- DG-Dentate gyrus (C-shaped granule cell layer), CA3, CA2 and CA1 cell layers (contains pyramidal neurons), *Stratum radiatum* layer is below the CA1 cell layer and is rich in glutamatergic synapses and astrocytes. Excitatory input to the CA1 cell layer predominantly arises from the Schaffer collaterals pathway (CA3 to CA1 pyramidal neurons).

Finally, the CA1 area is characterised by the abundance of postsynaptic NMDARs, that drive synaptic plasticity (Monyer et al., 1994; Li and Tsien, 2009). In the hippocampus, NMDARs expressed on pyramidal neurons typically contain GluN1 and GluN2A subunits (Le Bail et al., 2015). These receptors play a pivotal role in the formation of LTP (Gao et al., 2010). GluN2C and GluN2D are expressed in the interneurons of the hippocampus (Martina et al., 2003).

Moreover, the CA1 area houses a high proportion of astrocytes (Bushong et al., 2002). Consequently, the mouse hippocampal CA3 to CA1 synapses serve as a practicable model for comprehending the intricate process of excitatory neurotransmission. By focusing on the hippocampus, this thesis aims to shed light on the regulation of DS and its impact on excitatory neurotransmission.

1.6.9 D-serine-dependent synaptic plasticity and memory

Learning and memory depend on 'activity dependent changes' in the strength of central glutamatergic synapses (i.e. Synaptic plasticity). Conventionally, glutamatergic synaptic plasticity is dependent on various cellular components – Presynaptic mechanisms (modulation of glutamate release by changes in vesicular release probability, amount of neurotransmitter packed in the individual vesicles, recycling of readily releasable pool of neurotransmitters etc.), post synaptic mechanisms (number and state of NMDAR and AMPAR, secondary messenger pathways such as cAMP, phosphatases, protein kinases etc.) and neuromodulator mediated pathways (adenosine, endocannabinoids, mGLUR).

Synaptic plasticity exhibits bidirectionality and is influenced by the patterns of incoming synaptic activity, such as long-term potentiation (LTP) and long-term depression (LTD). LTP involves a prolonged enhancement of synaptic strength, causing long-lasting changes in synaptic activity. The high-frequency stimulation protocol (HFS; 100 to 200 Hz; Tani et al., 2014) is usually employed to induce LTP in brain slices, involving the application of repetitive electrical stimuli to the presynaptic neuron. On the other hand, a Low-frequency stimulation (LFS; range of 0.1 Hz to 5 Hz) is more likely to induce LTD, representing a decrease in synaptic strength (Mayford et al., 1995; Billups et al., 2013).

Both LTP and LTD can coexist within the same synapse, despite distinct molecular mechanisms. The precise mechanism of origin, whether presynaptic or postsynaptic, varies depending on the specific brain region. In the CA1 area of the hippocampus, where synaptic plasticity is prominently postsynaptic, both LTP and LTD necessitate Ca^{2+} and Na^{+} influx through NMDARs. This can then trigger a variety of downstream signalling pathways linked to alterations in the properties and trafficking of synaptic AMPARs, that are important for learning and memory. Hence these receptors are often the target of cellular signalling pathway/s, which in turn are responsible for regulating synaptic strength during plasticity (Malenka and Bear, 2004; Kumar, 2011). Distinguishing between induction and maintenance mechanisms is crucial, where for conventional NMDA-dependent LTP in the hippocampus, NMDARs are important for the induction and the maintenance is by upregulation of the AMPARs in the

postsynaptic membrane (Sumi and Harada, 2020). Additionally, to these conventionally thought regulators of plasticity in the glutamatergic synapses, understanding molecular pathways involving various transporters expressed on neurons and the surrounding astrocytes, modulators of this LTP, act as key regulators in this process.

Christian Henneberger and Colleagues (Henneberger et al., 2010; Henneberger and Rusakov, 2012) employed a calcium clamping strategy, utilising calcium buffering agents, to prevent any increase in intracellular Ca^{2+} , ensuring the absence of LTP induction at surrounding hippocampal CA1 synapses. Their manipulation led to a 25% reduction in NMDAR-mediated current in the neighbouring hippocampal synapses, and, this reduction was reversed upon the addition of DS. While this calcium clamping process may have deleterious effects, its significance lies in revealing the crucial role of astrocytes and calcium signalling in mediating plasticity within the hippocampal synapses. Further, their findings indicated that the introduction of HOAsp, a blocker of serine racemase, into the astrocytes, effectively inhibited LTP, establishing a direct link between astrocyte DS synthesis and the local induction of LTP in hippocampal synapses. However, the serine racemase inhibitor might also impact pyruvate production, potentially influencing intra-astrocytic glutamate levels, suggesting the importance of metabolic balance within astrocytes.

DS administration has consistently demonstrated its positive impact on memory task performance. Duffy et al. (2008) observed that administering 600 mg/kg subcutaneously improves the hippocampus-dependent spatial reversal learning in the Morris water maze paradigm (learning a new response, such as moving towards a new target location, in a familiar setting, where the surroundings remain the same during both the initial learning of the spatial arrangement and during the learning of the reversal). Further, 10 μM DS significantly increased hippocampal SC-CA1 LTD with 1Hz stimulus protocol, whereas even 100 μM DS did not increase the theta burst-induced LTP in 4-6 months old mice. This study highlights that the enhancement of LTD with DS could broaden the range of synaptic activity in the hippocampus, and as long as both LTD and LTP are triggered in appropriate situations, the learning process can be enhanced. In addition, Andersen and Pouzet (2004) observed a reversal effect of DS on spatial memory deficits induced by perinatal treatment with phencyclidine in rats. This reinforces the potential of DS in enhancing memory-related functions and highlights its relevance in synaptic plasticity.

The intriguing finding of a cell-autonomous loss of LTP, rescued by exogenous DS, suggests a potential autocrine mode of DS action (Wong et al., 2020). This implies that DS levels in

postsynaptic neurons are regulated by SR, indirectly influencing NMDAR activity. Surprisingly, this loss of LTP occurred without a significant reduction in the amplitude of the NMDAR-EPSCs, arguing against any alterations to the baseline co-agonist occupancy. This phenomenon perhaps occurs through compensation by glycine, or through DS diffusing from neighbouring neurons. However, during HFS induction of LTP, DS may become limiting. This indicates the complex and context-dependent nature of DS modulation in synaptic function, especially during plasticity, where higher concentrations of co-agonists may be required. Finally, a recent study by Tapanes et al. (2022) demonstrated that modulation of DS levels through inhibition of SR can affect dendritic spine density and morphology, highlighting the importance of DS signalling in shaping neuronal connectivity and function.

Overall, there is a growing body of evidence highlighting the role of DS in NMDAR-mediated synaptic plasticity (Panatier et al., 2006; Henneberger et al., 2010; Bodner et al., 2020), which is essential for the proper function of the hippocampus and other areas of the brain. Moreover, the intricacies of hippocampal LTP involve a complex interplay of signalling components, including kinases, phosphatases, scaffolding proteins, AMPARs, cytoskeletal proteins, local dendritic protein synthesis, and other receptor proteins. In the broader perspective, these signalling and structural components are functionally interconnected, highlighting the complex nature of hippocampal synaptic plasticity.

1.7 Solute carrier family of transporters

Among the 20 amino acids found in nature, all essential amino acids need to be transported into the cells, as they need to be absorbed from various food sources or blood. Some non-essential amino acids are synthesised in certain cell types, whereas the cells, incapable of synthesising on their own, need a supply from other cell types. This leads to the compartmentalisation of amino acid transport within the brain, which mandates a need for membrane transport proteins that are capable of amino acid transport across different cells. Generally, amino acid transporters are transmembrane proteins that belong to the solute carrier family (SLC) and other putative SLCs. To date, over 400 members have been classified into 66 main families that are capable of transporting a variety of molecules that are charged or uncharged organic molecules, some inorganic ions (Cl^- , NO_3^-) and ammonia gas (Hediger et al., 2013). Their nomenclature follows the HUGO Gene Nomenclature Committee standards and has a general trend. For instance, SLC n X m family - SLC is the familial name, n is a number representing the family (1-66), X represents the subfamily and m is a number representing an individual isoform.

Different transport systems are responsible for bringing amino acids into mammalian cells, and these systems often have similar capabilities in terms of the types of amino acids they can transport. One specific system first discovered and studied by Christensen is called system ASC. It is found in all cells and is responsible for transporting small zwitterionic amino acids using Na⁺ ions, but it does not transport branched-chain amino acids (Christensen et al., 1965; Christensen et al., 1967; Christensen, 1990). To understand which plasma membrane transporters of this system could potentially mediate the transport of DS and LS in astrocytes and neurons, this heading highlights the properties of the relevant amino acid transporters.

In mammalian glutamatergic synapses, the Solute Carrier Protein 1 (*SLC1*) family of transporters, is relatively small but consists of a physiologically important bunch of plasma membrane transporters that govern the trafficking of anionic and zwitterionic amino acids in cells. This family is broadly classified into two groups which share substrate specificity and transport properties. The first group consists of five sodium-dependent excitatory amino acid transporters (EAATs), namely EAAT1 to 5 (encoded by *Slc1a3*, 2, 1, 6 and 7 genes respectively) and the second group consist of two sodium-dependent neutral amino acid transporters, namely Alanine, Serine and Cysteine Transporters ASCT1 and 2 (encoded by *Slc1a4* and *Slc1a5* genes). Notably, the EAATs are primarily associated with glutamate transport, while the ASCTs may play a role in DS homeostasis (Kanai, 1997; Kanai et al., 2013).

This investigation seeks to shed light on the intricate mechanisms governing serine regulation at synapses and contribute to a deeper understanding of synaptic physiology. Hence, the broad aim of this project is to investigate the transport mechanism/s of serine regulation in the excitatory synapses and to understand how these transport mechanisms modulate excitatory synaptic transmission in the brain. Two proposed models, the gliotransmitter and the serine shuttle, underscore the significance of understanding these transporters, particularly in the context of shuttling DS/LS between neurons and astrocytes. To identify amino acid transporters that regulate serine *in situ*, experiments have been designed utilising currently available substrates and inhibitors. Experiments have been designed to understand the biophysical properties of serine transporters such as ion dependence, substrate specificity etc. To achieve a comprehensive understanding, this project involves recording electrophysiological signals from these amino acid transporters expressed in excitatory neurons and astrocytes.

1.7.1 D-serine transport mechanism

According to an evolutionary analysis of the seven members of the SLC1 family, a very low percentage of identity of 21% was identified. While analysing them separately, the percentage of identity increased up to 29% for EAATs and up to 58% for ASCTs. Given their evolutionary proximity, these transporter subtypes share sequence similarities of approximately 40-44% (Arriza et al., 1993; Utsunomiya-Tate et al., 1996). Despite their differences in substrate specificity and sensitivity to different inhibitors, their 3D architecture is almost overlapping. The one-gate elevator transport mechanism is a common transport cycle of all SLC1A family of transporters. The schematic of this transport cycle shown below (Figure 1.15), involves several key components: the Scaffold domain; a structural domain that provides stability or support to the transporter protein and the transport domain; a domain that is actively involved in binding and transporting the amino acid substrate (Garaeva et al., 2018; Garaeva et al., 2019).

The transport domain is an integral component that acts as a gate. It consists of an extra- and intracellular gate (HP2 loop), controlling the entry and exit of amino acids and an HP1 loop (Yernool et al., 2004; Reyes et al., 2009). At the start of the transport cycle, when the transporter is in the outward-facing conformation, the extracellular HP2 gate is open (outward-open), allowing DS to bind to the transporter. During this phase, a concomitant neutral amino acid exits the transport domain (antiport). After DS binds, the HP2 gate closes (outward-occluded), allowing DS to interact with various residues within the transporter. This first stage is referred to as the "outward facing" state of the transporter and is thought to be the ready state, facilitating a fast uptake mechanism of the substrates from the synaptic cleft (Yernool et al., 2004; Reyes et al., 2009).

Subsequently, the transporter goes through a conformational change, acting as an elevator, and moves into the "inward facing" conformation (Focke et al., 2013). At the start, the intracellular gate remains closed (inwards-occluded). Once the conformational change is complete, the intracellular gate is opened (inwards-open), allowing DS to be released into the intracellular side. Then, the transporter undergoes a second round of conformational change, allowing the extracellular gate to open once again, and bind to a new molecule of DS in the extracellular space.

An anionic channel is thought to open while the protein undergoes such a conformational change. Molecules like O-benzyl-L-serine, DL-threo- β -hydroxyaspartate (TBOA) and trifluoromethyl benzoyl derivatives of TBOA (TFB-TBOA) can hold the transporter in such a conformational stage, preventing the transporter from undergoing the whole transport cycle

(Grewer and Grabsch, 2004). Nevertheless, the precise physiological role of the metabolically uncoupled anionic channel is not known. It could be a by-product of the evolution of life in salty waters, serving as a protective mechanism to expel chloride ions from the cells. The existence of an anionic channel is demonstrated to be ancient in evolutionary terms, as evidenced by its presence in bacterial orthologs, such as GltPh (Machtens et al., 2015). Moreover, it is unclear if such an anionic conducting state occurs during the transport process for different substrates, and if the antiport is symmetrical or asymmetrical, viz., exchange mechanisms involving the same or different amino acid substrates respectively (Wang et al., 2022; Gauthier-Coles et al., 2023).

Overall, the one-gate elevator transport mechanism alternates between inward-facing and outward-facing conformations, transporting amino acid substrates across the plasma membrane. This allows continuous homeostatic regulation of the concentration of amino acids inside and outside the cells. Hence, this family of antiporters is referred to as ‘homeostatic exchangers’.

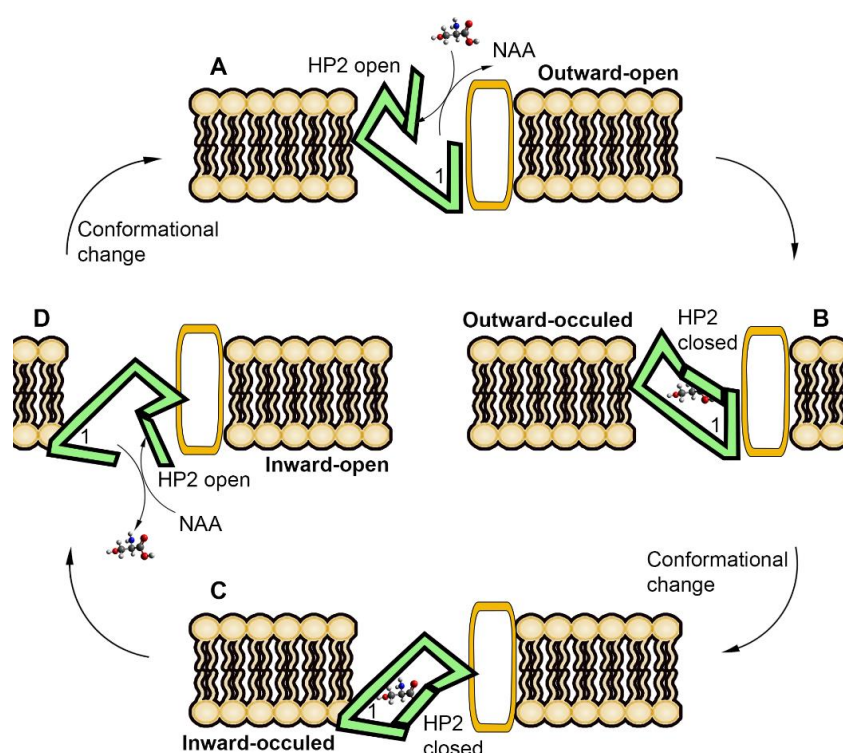


Figure 1.15: One-gate elevator mechanism of D-serine transport

A schematic representation of the transport cycle that is commonly seen in the SLC1 family of transporters. The scaffold domain is represented as a yellow rectangle, the extra- and intracellular gate (HP2 loop) is represented in green, 1 represents the HP1 loop, NAA represents a neutral amino acid substrate, and the example molecule is D-serine. This figure is made using information from structural elucidation (Garaeva et al., 2019; Stehantsev et al., 2021).

1.7.2 The excitatory amino acid transporters

The first group of transporters is widely studied and is known as EAATs (excitatory amino acid transporters). They predominantly transport negatively charged amino acids such as glutamate and aspartate. The glutamate concentration in the extracellular space has been estimated to be in nanomolar concentrations. Chiu and Jahr (2017) estimated an average of 25 nM at the nucleus accumbens and Cavelier and Attwell (2005) estimated about 35-90 nM in rat hippocampal slices. Particularly in the CA1 region of rat hippocampal slices, glutamate concentration has been observed to be in the 25 nM range (Herman and Jahr, 2007). Although during basal activity in the brain, the extracellular glutamate concentrations are non-toxic, during an action potential the concentration rises to sub-toxic levels and therefore needs to be cleared rapidly. For instance, in the synaptic clefts near the climbing fibre-Purkinje cells in the cerebellum, the glutamate concentrations have been estimated to be above 1mM during synaptic activity. At the same time, the extra-synaptic glutamate concentration was estimated to be elevated to almost 200 μ M (Dzubay and Jahr, 1999). It is therefore critical to reduce the glutamate concentrations in the synaptic cleft. This is achieved by EAATs, which maintain optimal glutamate concentrations required for proper excitatory synaptic neurotransmission in the brain. Their expression in the astrocytes surrounding such glutamatergic synapses makes it highly convenient to control synaptic glutamate concentration (Bergles and Jahr, 1998; Oliet et al., 2001). They are also avid regulators of glutamate concentrations below toxic levels (K_m of EAATs is approximately 18-28 μ M) to avoid any excitotoxicity associated with glutamate levels in the synapses. Due to this, EAATs have been studied extensively in the past with direct application to physiological conditions and pathological states associated with glutamate such as stroke (For review see Billups et al., 1998; Murphy-Royal et al., 2017).

There are five different EAATs in the mammalian central nervous system. EAAT 1 is expressed in the astrocytes (Lehre et al., 1995), EAAT2 is predominantly expressed in the astrocytes (about 90%) and to a lesser extent in pre-synaptic terminals (about 10%; Danbolt et al., 1992; Levy et al., 1993), EAAT 3 and EAAT 4 are expressed in the soma and dendrites of neurons (Dehnes et al., 1998; Holmseth et al., 2012). EAAT2 is the most important transporter of this family as it plays an integral role in clearing almost 90-95% of the extracellular glutamate (Otis and Kavanaugh, 2000). Knockout of EAAT2 has been observed to be lethal as the mice develop seizures even at three weeks of age (Tanaka et al., 1997). Astrocyte-specific KO of EAAT2 has also been observed to cause spontaneous seizures in mice (Petr et al., 2015).

The transport mechanism of glutamate via the EAATs involves a coupling process where the uptake of one glutamate molecule from the extracellular space or synaptic cleft is coupled with

the co-transport of 3 Na^+ and 1 H^+ ions, while simultaneously counter-transporting 1 K^+ ion (Figure 1.16). This process leads to a net accumulation of positive charge (+2) within the intracellular space, making the entire transport cycle electrogenic. In addition, during its transport cycle, EAATs allow an uncoupled movement of Cl^- under physiological conditions or other anions like SCN^- or NO_3^- or ClO_4^- non-physiologically, in either direction, depending on the membrane voltage (Billups et al., 1996). This anionic channel emerges within the transporter's structural framework, enabling a leaky movement of anions without any direct metabolic coupling.

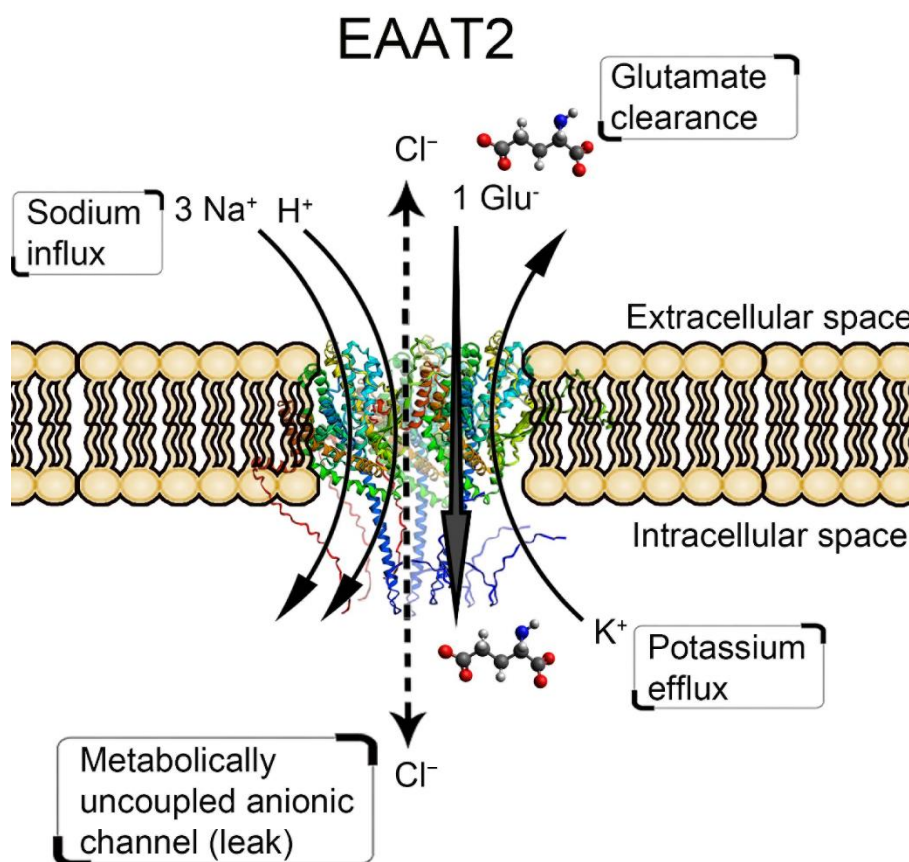


Figure 1.16: Functional structure of excitatory amino acid transporter isoform 2

Transport of a molecule of glutamate from the extracellular space or synaptic cleft via EAATs is coupled with co-transport of 3 Na^+ and 1 H^+ , with a counter-transport of 1 K^+ . This results in a net positive charge accumulation (+2) in the intracellular space. During its transport cycle, EAATs allow a metabolically uncoupled movement of anions (Cl^- physiologically or SCN^- / NO_3^- non-physiologically) in either direction through an anionic channel found within the transporter structure.

1.7.3 Alanine serine cysteine transporters

The second group of SLC1 transporters includes the two plasma membrane transporters that traffic neutral amino acid substrates. Alanine serine cysteine transporters (ASCT), also known as Neutral Amino Acid Transporter and as the Sodium-dependent neutral Amino acid Transporter SATT. ASCT 1 and ASCT2, although belonging to the same family lack such in-depth research compared to EAATs. ASCT1 is severely understudied, whereas ASCT2 has become a hot topic in cancer research in recent years because of its over-expression in cancer cells (Bröer et al., 2019; Luo et al., 2020; Zhang et al., 2020; Lopes et al., 2021). Hence an array of publications is available on the structure, functional, pharmacological, and pathological aspects of ASCT2 but not ASCT1. Structurally, ASCTs form homotrimeric structures similar to EAATs, but display distinct substrate specificities compared to their familial counterparts, due to variations in amino acid residues (Arriza et al., 1993; Scalise et al., 2021). For instance, the basic amino acid side chain of arginine in EAATs is replaced by a neutral amino acid side chain of cysteine in ASCT2 (Bendahan et al., 2000). Consequently, ASCTs prefer neutral amino acid substrates to anionic amino acids. Conversely, mutation of Arginine447 in EAAT3 has been observed to allow the transport of neutral amino acids via EAAT3 (Bendahan et al., 2000).

ASCTs are responsible for transporting a wide array of neutral and zwitter ionic amino acids including alanine, serine, cysteine, and threonine across cellular membranes. They share most of their substrates, however ASCT2 also recognises L-glutamine, while ASCT1 does not translocate it (Pinilla-Tenas et al., 2003; Foster et al., 2016). They facilitate the antiport movement of amino acids, a process also referred to as obligatory exchange or trans-stimulation. In essence, operating at physiological pH, ASCTs are primarily involved in the exchange of intracellular neutral or zwitterionic amino acids with their extracellular counterparts. It is less likely to facilitate a net uptake or release of amino acids. This process is tightly coupled with the reciprocal movement of Na⁺ ions and hence they function in a sodium-dependent manner. Their physiological role involves maintaining neutral amino acid balance and availability for various intracellular processes, qualifying them to be termed 'homeostatic exchangers'. ASCT2 is particularly prominent in facilitating the uptake of essential amino acids that are crucial for protein synthesis and in meeting the metabolic demands of growing/ proliferating cells, making ASCT2 an intriguing target for studying amino acid transport in the context of cancer and other diseases (Fuchs and Bode, 2005).

ASCT1 and ASCT2 exhibit distinct affinities for D- amino acids compared to their L- counterparts. The IC₅₀ values of these DS transporters span the range of 10⁻⁵ to 10⁻³ M. DS

demonstrates a higher IC_{50} value compared to LS for both ASCT1 and ASCT2 indicating that LS has a higher (10-fold) affinity for these transporters. The IC_{50} values for inhibition by DS of [3H] LS transport via ASCT1 and ASCT2 in HEK cells are approximately 4 and 5 mM respectively; The IC_{50} values for inhibition by LS of [3H] LS transport via ASCT1 and ASCT2 are approximately 300 and 700 μ M respectively (Foster et al., 2016). These affinity differences suggest that ASCT1 and ASCT2 have distinct preferences for the two serine isomers. While Foster et al. (2016) primarily focused on serine transport, their work also provides insights into L-glutamine and T-Pro affinities with ASCTs. They reported that L-glutamine inhibits [3H] LS uptake via ASCT1 and ASCT2 with IC_{50} values of approximately 5 mM and 600 μ M, respectively. Similarly, T-Pro inhibits [3H] LS uptake via ASCT1 and ASCT2 with IC_{50} values of approximately 200 μ M and 4 mM, respectively. These differential affinities suggest that L-glutamine and T-Pro are likely to significantly impede LS uptake under normal physiological conditions. The dissociation constants (K_i) for LS binding to these transporters range from 29 to 88 μ M, as established by Arriza et al. (1993) and Yamamoto et al. (2003) while the EC_{50} for DS transport is significantly higher at about 2 mM (Foster et al., 2016).

Furthermore, the concentrations of DS and LS used in the experiments were selected based on their markedly different affinities for ASCT1. For these experiments, a concentration of 10 mM was chosen to demonstrate the functional expression of the transporter on the astrocytic membrane. Given the approximately 10-fold difference in affinity, DS and LS are likely to produce different effects at this concentration. Theoretical dose-response curves using different substrate concentrations would be required to determine the precise affinity of each substrate for the transporter. Moreover, it is important to note the limitation posed by puff-applying the substrate near the cell body and the potential dilution effect due to substrate diffusion, which could hinder accurate measurement.

1.7.3.1 Gene, Chromosomal location, and Genetic variants of ASCT transporters:

The human ASCT1 and 2 genes (*SLC1A4* and *A5*) spans across 8 exons are located on chromosomes 2p13-p15 and 19q13.32 respectively (Hofmann et al., 1994). ASCT1 shares a sequence identity exceeding 56% with ASCT2. The cDNA sequence of ASCTs encodes a protein consisting of approximately 529 amino acids, sharing a sequence identity of 39–44% with the EAAT family of glutamate/aspartate transporters (Arriza et al., 1993).

The ASCT1 gene is prevalent in 341 organisms, while the ASCT2 sequence is present in 56 diverse organisms. Notably, both genes are ubiquitous in lot of vertebrates. Within the various ASCT1 isoforms listed in the NCBI database, the longest isoform encodes a protein comprising

532 amino acids. Additionally, three other transcripts are shorter, with lengths of 312, 214, and 312 amino acids, respectively. As for ASCT2, the primary isoform codes for a peptide consisting of 541 amino acids, with an estimated molecular mass of 57 kDa. A second isoform features a truncated peptide spanning 313 amino acids, while the third isoform lacks the initial exon, resulting in a peptide sequence of 339 amino acids (National Library of Medicine (US), 2004a, b).

Numerous variations in single-nucleotide polymorphisms (SNPs) have been identified within the human ASCT1 and ASCT2 genes, with certain variants linked to clinical conditions. Notably, mutations in ASCT1 such as rs201278558 and rs761533681, results in the substitution of the conserved glutamate 256 and arginine 457 residues with lysine and tryptophan, respectively. Interestingly, these mutations are linked to the medical condition SPATCCM (Online Mendelian Inheritance in Man OMIM - 616657; 600229), characterised by spastic tetraplegia, thin corpus callosum, and progressive post-natal microcephaly (Damseh et al., 2015; Abdelrahman et al., 2019). The majority of individuals affected struggle to attain autonomy in walking or speech, with certain cases also experiencing seizures (Heimer et al., 2015; Srour et al., 2015). Similarly, mutations in ASCT2 such as rs3027956 and rs11668878, are linked to breast cancer and chronic lymphocytic leukaemia, respectively (Savas et al., 2006; Sillé et al., 2012).

1.7.3.2 Expression of ASCT1

ASCT1 cDNA was first isolated by two independent research teams from the human motor cortex and hippocampus (Arriza et al., 1993; Shafqat et al., 1993). ASCT1 transporter is widely expressed in the plasma membrane of the mouse, with its highest expression observed in the brain followed by skeletal muscles, the pancreas and the adrenal gland (Arriza et al., 1993). ASCT1 is ubiquitously expressed in several regions in the brain with the highest expression in the cerebral cortex, thalamus, and amygdala, followed by the hypothalamus and the hippocampus. Single-cell RNA sequencing data suggests expression to be highest in melanocytes and dendritic cells followed by astrocytes & and plasma cells, granulosa cells and basal squamous epithelial cells (Atlas; Sjöstedt et al., 2020).

In the hippocampus, like the cortex, (Sakai et al., 2003) observed ASCT1 in small star-shaped cells and in ringed structures in the cell bodies. The presence of ASCT1 in the astrocytes was confirmed through co-staining with Glial fibrillar acidic protein (GFAP, glial cell marker for astrocytes and some oligodendrocytes) and double immunofluorescent staining with 3PGDH (3-Phosphoglycerate dehydrogenase, an astrocytic enzyme regulating the committed step in

LS synthesis). Further, Immuno-electron microscopy revealed the expression of ASCT1 in astrocytic processes that wrap around neuronal soma, dendrites, and synapses. Despite the detection of ASCT1 mRNA in the pyramidal cell layer, ASCT1 was barely noticeable in the soma and dendrites of pyramidal cells (marked by Microtubule-Associated Protein 2 or MAP-2). Scanning electron microscopic evaluation further revealed weaker and smaller labelling in the soma of pyramidal cells, near cisterns of the endoplasmic reticulum (ER), compared to the astrocytes. Further, the presence of ASCT1 near the ER could be interpreted as the trafficking of ASCT1 onto the membrane or ASCT1 could serve some function on the ER membrane, possibly to buffer intracellular amino acid between the cytoplasm and ER (Sakai et al., 2003). Additionally, they observed ASCT1 expression to gradually reduce when the brain develops, suggesting that ASCT1 is less likely to mediate the transport of DS/LS in pyramidal neurons of adult mice. Since ASCT1 mRNA is detectable in adult neurons, the potential role of ASCT1 in DS/LS transport in the adult brain cannot be completely ruled off (Cahoy et al., 2008).

In the cerebellum, ASCT1 was localised to the Bergmann glial cells in the cell layer and its processes (overlapped with 3PGDH enzyme expression). Additionally, ASCT1 expression was observed in cell bodies and dendrites of Purkinje cells, but absent in presynaptic terminals, post-synaptic spines and endothelial cells (Furuya et al., 2000; Sakai et al., 2003).

A similar ASCT1 expression pattern was observed in developing rat brains (Weiss et al., 2001), suggesting similarities in the physiological role played by ASCT1 in the mammalian brain. On the other hand, Jeon et al. (2009) reported a predominant expression of ASCT1 in normal mice brains and an enhanced astrocytic ASCT1 expression in KA-treated (kainic acid) hippocampus mice. This discrepancy could have arisen due to the ASCT1 antibodies used or the KA treatment, which is routinely used to induce excitation or epilepsy in mouse models (Lévesque and Avoli, 2013).

Another glial cell that has low expression of 3PGDH, but lacks ASCT1 expression are the oligodendrocytes (Yamasaki et al., 2001; Sakai et al., 2003). The small amount of LS synthesised inside the oligodendrocytes is likely used for protein synthesis, and hence unlikely to mediate the LS shuttle.

1.7.3.3 Expression of ASCT2

Expression of ASCT2 was first reported in astroglial cultures of newborn rat brains (Broer et al., 1999). Later, using confocal immune-fluorescence microscopy and anti-mrASCT2-N

antibody, mice ASCT2 protein was observed in capillary endothelial cells and the localisation was observed to be different from that of GFAP (Tetsuka et al., 2003). Supporting this, Utsunomiya-Tate et al. (1996) did not detect the presence of ASCT2 in the brain. It remains a matter of debate whether ASCT2 is developmentally regulated like its sister isoform ASCT1 (Sakai et al., 2003). Although Tetsuka et al. (2003) reported no ASCT2 immunoreactivity in adult mice brains, the localisation and expression of ASCT2 might change during various stages of development. Therefore, the expression of ASCT2 cannot be completely ruled out in the brain.

1.7.3.4 Functional Structure of ASCTs

At the project's inception, a comprehensive understanding of ASCT1's molecular structure in different conformations throughout its transport cycle remained elusive, although models had been established based on the sequence and structural similarities with its sister isoform ASCT2 (Figure 1.15; Zander et al., 2013; Garaeva et al., 2018). With the recent emergence of the cryo-electron microscopy (cryo-EM) structure of ASCT1, the intricacies and similarities are more evident (Stehantsev et al., 2021). ASCT1 is a transmembrane protein composed of twelve membrane-spanning helices (TMs), forming a complex 3D architecture (Figure 1.17). The core scaffold domain consists of TMs 1, 2, 4, and 5, while the three independent elevator or transport domains consist of TMs 3, 6, 7, and 8. Notably, two (a pair of) pseudo-symmetrical helical hairpins (HPs), HP1 and HP2, located between TM6 and TM8, surround the binding site, with only HP2 functioning as a gate during both inward and outward conformations. The structural components of ASCT1, including the scaffold domains, the 3-4 loop between TMs 3 and 4, along with the N- and C-terminal residues 1-37 and 482–532, contribute to its overall stability and functionality of the transporter (Stehantsev et al., 2021).

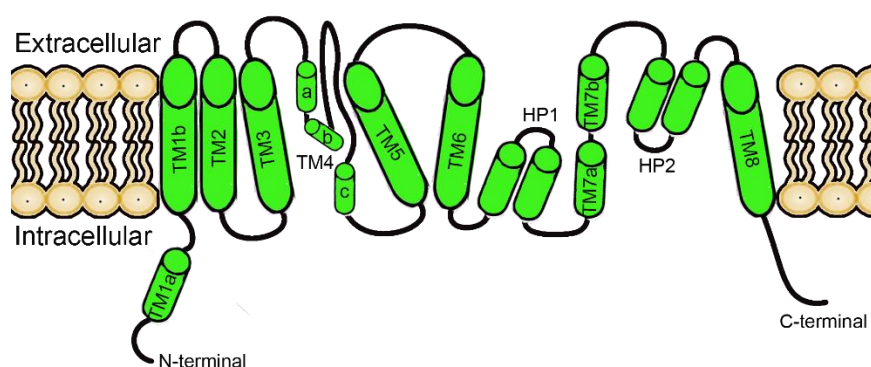


Figure 1.17: Secondary structure diagram of the ASC transporters

The ASC transporters are transmembrane proteins primarily composed of alpha helices, as depicted by the green helical coils (TMs represent transmembrane-spanning helices; HP represents helical hairpins). The Image was drawn (or) adapted from Stehantsev et al. (2021).

Functionally, the ASCTs are obligatory exchangers of amino acids across the membrane. The substrate binding site of ASCT1 closely resembles that of ASCT2, with the notable distinction of a single substitution, namely, C467 (in ASCT2) to T459 (in ASCT1). Both ASCT1 and ASCT2 contain a negatively charged substrate binding pocket, which hinders the binding of negatively charged glutamate and aspartate at neutral physiological pH. Additionally, the pocket's geometry could also hinder the entry of positively charged lysine, arginine, and histidine (Stehantsev et al., 2021). However, it's important to note that the cryo-EM resolution was limited to 4.2 Å, which is insufficient to allow precise identification and comparison of substrate binding states.

Determining the precise count of sodium binding sites within the transporter remains complicated, yet recent studies suggest the presence of three plausible sodium binding sites, localised within these residues; E465, D467; T376, V418; and F121, T124, T125, N378, D380 (Stehantsev et al., 2021). The stoichiometry of sodium binding to the outward/inward-facing configuration is predicted to involve 2 or 3 sodium ions, as predicted through homology modelling (Scalise et al., 2021). ASCT2 interacts more strongly with Na^+ with a K_m of 0.3 to 2 mM compared to EAATs (K_m of 80 mM for EAAT3; Broer et al., 2000; Grewer and Grabsch, 2004), suggesting that Na^+ binding sites of both transporters have different properties. Further, the high affinity for Na^+ indicates that the ASCT2 is more likely to be in a Na^+ bound state in the outward-facing configuration, which would allow the extracellular amino acid substrate to readily bind to the substrate binding domain (Figure 1.18). This intricate sodium binding mechanism underscores the complexity of the ASCT's sodium-dependent dynamics and its crucial role in the transport of DS, in an electrogenic manner. In addition, the ASCT transporters could also display a metabolically uncoupled anionic leak/ conductance

associated with its transport cycle (which follows the anion permeability sequence of $\text{SCN}^- > \text{NO}_3^- > \text{Cl}^-$) similar to its familial counterpart, the EAATs (40-45% similarity, Arriza et al., 1993). The metabolically uncoupled movement of anions (Cl^- physiologically or $\text{SCN}^-/\text{NO}_3^-$ non-physiologically) can occur in either direction through an anionic channel found within the transporter structure. Electrophysiological recording of sodium movement, coupled with substrate transport and potential uncoupled anionic conductance, can be recorded through the application of substrates in close proximity to the membrane. This approach allows for the visualisation of transport-associated currents, shedding light on the transport of substrates across the cell membrane.

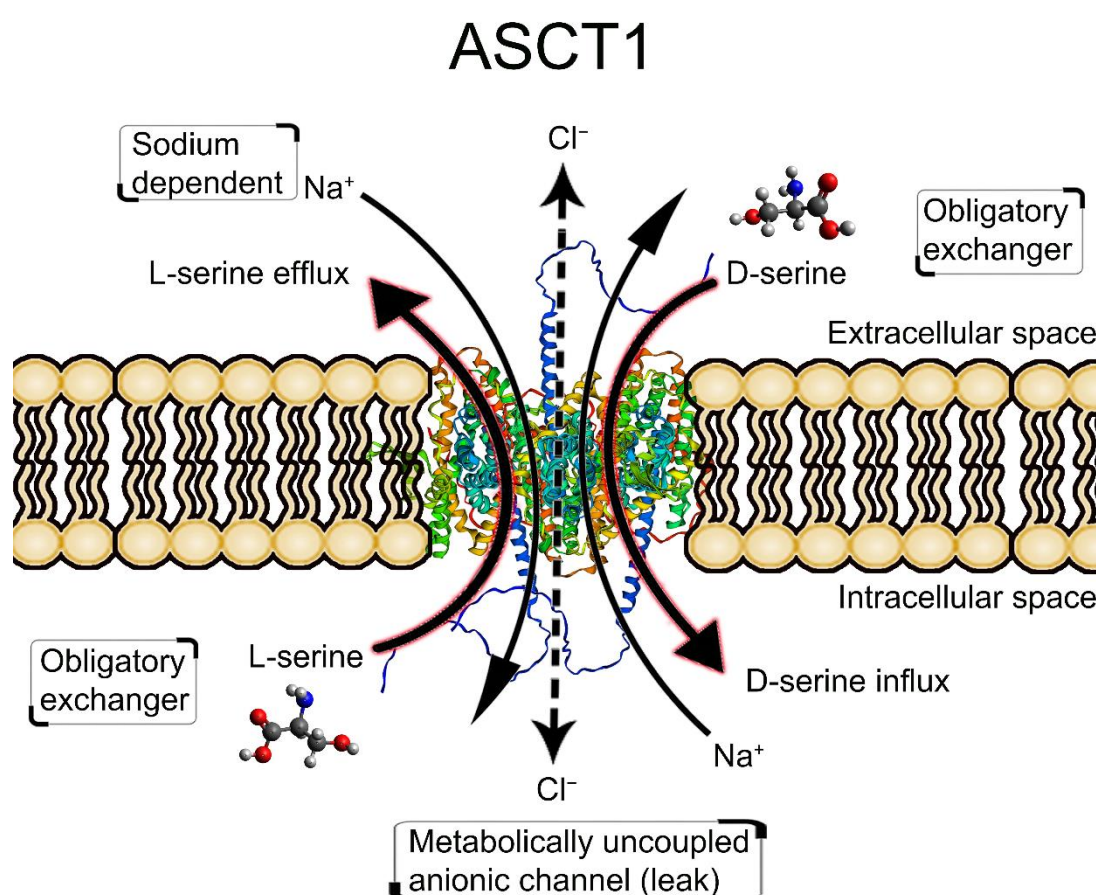


Figure 1.18: Functional structure of alanine serine cysteine transporter isoform 1 (ASCT1)

(A) Illustration of the functional structure of mouse ASCT1, emphasising its dependency on sodium ions (2 or 3 Na^+), its obligatory antiport mechanism (involving the exchange of one intracellular amino acid for one extracellular amino acid), and the presence of a metabolically uncoupled anionic channel. The 3D alpha fold structure was derived from an open-source database developed by DeepMind (Jumper et al., 2021; Varadi et al., 2021) and visualised using the open-source online platform EzMol (Reynolds et al., 2018).

1.7.3.5 Thermodynamics of ASCT1 Transporter

ASCT1 transporters, belonging to the SLC1 family, function as obligate exchangers, where the transport of one neutral amino acid in one direction is directly coupled to the transport of another neutral amino acid in the opposite direction. Understanding the thermodynamics of amino acid transport by ASCT1 is crucial for comprehending its role in the movement of D- and L-serine across excitatory synapses. The transport cycle of the ASCT1 family of transporters involves the co-transport of an extracellular amino acid with two or three Na^+ ions in one direction, followed by the exchange of another intracellular amino acid and the return of the Na^+ ions in the opposite direction (Boudker et al., 2007; Machtens et al., 2015). The efficiency of ASCT1 transport depends on two main factors: Electrochemical and Amino Acid gradient.

Electrochemical Gradients: The electrochemical gradient of Na^+ ions across the membrane is a key driver in the transport process (Boudker et al., 2007), but since ASCT1 exchanges two/three extracellular Na^+ ions for two/three intracellular Na^+ ions, the net electrochemical gradient of Na^+ may balance out. Therefore, while the Na^+ gradient, maintained by the Na^+/K^+ -ATPase pump, is crucial, it does not solely drive the amino acid transport process.

Amino Acid Gradients: The concentration gradients of D- and L-serine across the membrane are crucial for determining the direction and efficiency of their transport, assuming that the same number of Na^+ ions are transported in and out per transport cycle. The major driving force for ASCT1 transport is the amino acid concentration gradients across the membrane. The coupling of Na^+ and D- or L-serine transport ensures that the movement of each substrate is energetically favourable, while the outward flow during exchange resets the transporter efficiently to its outward configuration, minimising energy expenditure (Machtens et al. 2015). This cycle tends to equalise substrate concentrations on both sides of the membrane.

In summary, the inward flow of Na^+ ions drive the co-transport of D- and L-serine into the cell, while the outward flow with a different accompanying amino acid during exchange resets the transporter for another cycle. At excitatory synapses, such as the CA3-CA1 synapses of the hippocampus, understanding the thermodynamics of ASCT1 transport is crucial for regulating extracellular D- and L-serine levels, thereby influencing NMDAR activation and synaptic plasticity.

1.7.3.6 Physiological role of ASCT1

Despite the knowledge of the cellular localisation of the ASCT1 transporter, its physiological importance is yet to be reported. One presumed role of ASCT1 in the brain is to maintain the homeostasis of neutral amino acids, such as DS. The latest work from ASCT1 KO mice has demonstrated severe disruption to the levels of DS and LS compared to the wild-type conditions (Kaplan et al., 2018), suggesting the crucial involvement of ASCT1 in the efficient transport of DS and LS. Another possible role played by ASCT1 could be the maintenance of cysteine levels by reuptake of cysteine into the astrocytes to prevent the synergistic effect of cysteine–glutamate–induced toxicity (Puka-Sundvall et al., 1995).

1.7.4 Asc-1 transporter

System asc (alanine, serine, cysteine) contains two sodium-independent transporters – asc-1 and asc-2. The asc-1 transporter (encoded by the *Slc7a10* gene, alanine serine cysteine transporter 1, asc- type amino acid transporter-1, cationic amino acid transporter, y⁺ system) has been identified as one of the main regulators of DS in the central nervous system of mice (Rutter et al., 2007). The sodium-independent asc-1 has been extensively studied in oocyte expression systems and in astrocyte/neuronal cultures to mediate the transport of several small and neutral amino acids including DS and LS across the plasma membrane (Broer et al., 1999; Broer et al., 2000; Ribeiro et al., 2002; Foster et al., 2016). It has been suggested to be involved in NMDAR regulation in the glutamatergic by mediating the clearance of DS and glycine from the synaptic cleft (Xie et al., 2005) with higher affinity than ASCT1 (Affinity K_i ~8–12 μ M, K_m value ~10–50 μ M, Fukasawa et al., 2000; Nakauchi et al., 2000). Since the *in vivo* extracellular concentrations of DS in the prefrontal cortex and striatum of rats (~6.5 μ M, Hashimoto et al., 1995) match up to asc-1 transporter's affinity, it is likely to regulate synaptic DS concentrations, especially during periods of synaptic activity *in vivo* (Maucler et al., 2013). Moreover, asc-1 has been suggested to be involved in the release of DS in the synaptic cleft (Rosenberg et al., 2013). As asc-1 is an antiporter, it is likely to function as a mediator of DS release from neurons as well.

Asc-1 is widely distributed across the CNS, where its expression has been observed to be exclusive to pre-synaptic terminals and dendrites (soma to a lesser extent) of principal neurons in the rat cortex, hippocampus, and cerebellum (Matsuo et al., 2004). Although the relative expression of asc-1 is only moderate in the hippocampus and cortex when compared to other brain areas such as the hypothalamus, the medial septal area, globus pallidus, entopeduncular

nucleus, cingulate and retrosplenial cortices, it is likely to contribute to synaptic DS concentrations in the hilus of the dentate gyrus and CA3-CA1 region (Helboe et al., 2003).

While the expression of asc-1 was initially believed to be exclusive to neurons, a study by Ehmsen et al. (2016) reported its presence in a subset of astrocytes in the caudal brain stem and spinal cord. However, asc-1 expression was undetectable in regions such as the cortex, hippocampus, and cerebellum in mice. These findings suggest that asc-1 likely functions as a neuronal DS transporter.

Sakimura et al. (2016) employed targeted inhibition of asc-1 in both *in vitro* primary neuronal-astrocyte cultures and CHO cell line expressions. Their investigations established that the extracellular DS levels exhibited a substantial reduction in the asc-1 inhibited conditions in comparison to control conditions. This finding strongly suggests the plausible involvement of asc-1 in mediating the efflux of DS either from neurons or astrocytes. In a more recent study, Sason et al. (2017) delved into the functional implications of asc-1 through examination of both neuronal cell cultures and HEK cell lines, further supplemented by investigations involving asc-1 KO mice. Their results illuminated a prominent role for asc-1 in the tonic release or efflux of DS within neurons.

Furthermore, asc-1 is involved in the transport of glycine through hetero-exchange mechanisms (Mesuret et al., 2018), which adds complexity to understanding its specific physiological role in DS transport. Recording electrical signals resulting from substrate transport via asc-1 is challenging due to the transporter's electroneutrality and its pH-independent nature. Nevertheless, to investigate the function of asc-1 in DS transport, researchers have employed drugs such as the transportable inhibitor D-isoleucine (D-Ile), the non-transportable blocker Lu AE00527 (Lu), and S-methyl-L-cysteine (SMLC) to block the asc-1's activity (Sakimura et al., 2016; Sason et al., 2017; Mesuret et al., 2018).

A related transporter designated as asc-2 (encoded by *Slc7a12*, asc-type amino acid transporter-2) was identified due to its sequence similarity to asc-1 (Chairoungdua et al., 2001). However, asc-2 was observed to exhibit more selectivity for LS compared to DS (Yamamoto et al., 2003). This suggests that asc-2 is less likely to play a significant role in mediating DS transport. Furthermore, asc-2 is predominantly expressed in the kidneys, placenta, and skeletal muscles and is almost undetectable in the brain (Chairoungdua et al., 2001). It is worth noting that Humans don't have an orthologous asc-2 transporter in the genome, so it will not be addressed in this thesis.

1.7.5 System A family of transporters

One family of amino acid transporters, popularly referred to as system A transporters, has gained recent interest in the transport of DS and LS. System A transporter family contains three transporters; SNAT1 (encoded by *Slc38a1* gene, also previously referred as ATA1, GlnT, SA2, SAT1, NAT2), SNAT2 (encoded by *Slc38a5* gene, ATA2, SA1, SAT2) and SNAT4 (ATA3, NAT3). SNAT1 and 2 are expressed in the brain, which function primarily as high-affinity glutamine transporters (K_m ~230 μ M for SNAT1 and ~1.65 mM for SNAT2; Figure 1.19; Sugawara et al., 2000; Yao et al., 2000; Mackenzie et al., 2003; Mackenzie and Erickson, 2004). System A has been recognised for its unidirectional uptake characteristics, rendering it an improbable candidate for facilitating DS efflux from neurons or astrocytes (Mackenzie and Erickson, 2004). Typically, they transport Na^+ along with glutamine, but recently been shown to be capable of DS and LS transport, in an electrogenic manner (Broer et al., 1995; Bodner et al., 2020). SNAT1 has been observed to be more substrate-specific to glutamine and asparagine than serine, but capable of transporting serine and alanine. SNAT2 prefers serine, alanine and cysteine over glutamine, proline and glycine (Yao et al., 2000; Chaudhry et al., 2002).

In situ hybridisation data from Chaudhry et al. (2002) and immunocytochemical studies from several groups (Melone et al., 2006; Blot et al., 2009; Jenstad et al., 2009) have shown that both neurons and astrocytes across different brain areas express SNAT1 and 2, particularly in somatodendritic compartments. There is, however, a controversy regarding the localisation and function of SNAT1 and SNAT2. Erickson's group, using antibodies against SNAT1 and SNAT2, reported that SNAT1 is expressed in glutamatergic, GABAergic, monoaminergic, and cholinergic neurons, but not in astroglial cells (Mackenzie et al., 2003). In contrast, Conti's group found that both SNAT1 and SNAT2 are localised in a variety of cells, including glutamatergic, GABAergic, ependymal cells, and astroglial cells (Melone et al., 2004; Melone et al., 2006). SNAT1 expression has been observed in the glutamatergic pyramidal neurons and also in the GABA-releasing interneurons (Melone et al., 2004). Furthermore, *in situ* hybridisation and highly specific antibody studies suggest that SNAT1 mRNA and protein are primarily enriched in GABAergic cells (Chaudhry et al., 2002). SNAT1 expression has also been observed in cultured astrocytes, but not in hippocampal astrocytes of adult rats (Mackenzie et al., 2003; Menchini and Chaudhry, 2019). On the other hand, SNAT2 expression has been observed in the glutamatergic neurons and astrocytes across several brain areas (González-González et al., 2005). Patch-clamp recording from presynaptic terminals in the calyx of Held synapses has indicated that these transporters could be inserted quickly into the

plasma membrane upon synaptic activity (Billups et al., 2013). Nevertheless, it is unclear whether the insertion of SNAT1 or 2 on the cell membrane could be synaptic activity-dependent.

Compounds like MeAIB (α -(Methylamino) isobutyric acid) have been used as a classical substrate for system A due to its N-methyl substitution (Christensen et al., 1965). MeAIB competes with alanine or other substrates for binding sites, thereby reducing the uptake of alanine and other substrates via the transporter.

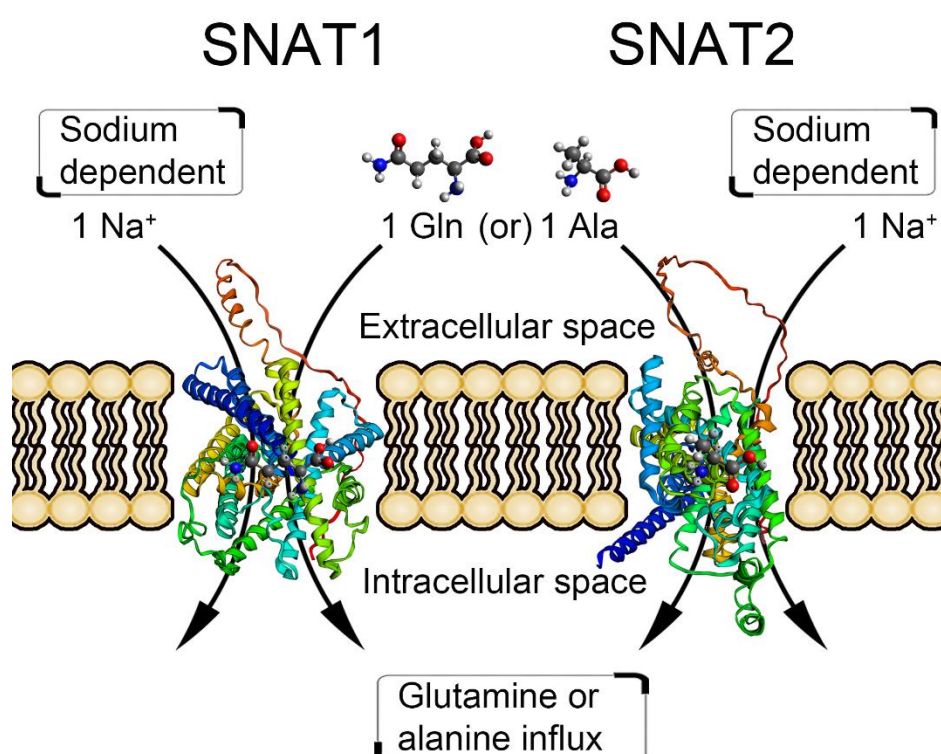


Figure 1.19: Functional structure of sodium-coupled neutral amino acid transporter isoforms 1 and 2 (SNAT1 and SNAT2)

Transport of a molecule of glutamine from the extracellular space or synaptic cleft via SNAT1 is coupled with the co-transport of 1 Na^+ . This results in the accumulation of a net positive charge (+1) in the intracellular space. The example molecule of transport via SNAT1 is glutamine and for SNAT2 is alanine.

1.7.6 Large neutral amino acid transporters

Two major transporters belonging to system L, LAT1 and LAT2 (encoded by the *Slc7a5* and *Slc7a8* genes respectively) have been studied to be involved in the mediated transport of branched-chain amino acids like leucine, valine and Isoleucine (Kanai et al., 1998; Pineda et al., 1999). Although the expression of LAT 1 and LAT 2 has been observed in the rat blood-brain barrier, its cellular distribution remains unexplored (Boado et al., 1999; Segawa et al., 1999). Among the four LAT transporter families, only LAT2 has been reported to be capable

of serine transport (Lewis et al., 2007). Brown et al. (2014) reported that HEK293 cells transfected with LAT2 cDNA-mediated LS but not DS transport. Nevertheless, the specificity of LAT transporters to mediate DS or LS transport in the brain is yet to be established. This system could mediate DS release in exchange for an extracellular amino acid substrate such as alanine and leucine.

LAT1 (*SLC7A5*) consists of 12 transmembrane domains and the protein 4F2hc (*SLC3A2*) consists of one transmembrane domain. The two proteins form a heteromeric complex through a disulfide bridge (Figure 1.20). This complex stabilises the transporter on the plasma membrane and is non-functional without the 4F2hc heavy chain (Broer et al., 1995). The net charge transfer is thought to be neutral but could be electrogenic if the antiport is asymmetrical involving the exchange of extracellular neutral aromatic amino acids like tryptophan or leucine for an intracellular neutral aliphatic amino acid like glutamine. The transmembrane domain of 4F2hc is like the β subunit in eukaryotic sodium channels (Wagner et al., 2000) and hence could mediate a net influx of positive charge during its transport.

Compounds like 2-amino bicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) have been used to selectively inhibit amino acid transport via system L and have been demonstrated to be effective in reducing intracellular amino acids necessary for cancer cell survival (Kim et al., 2008).

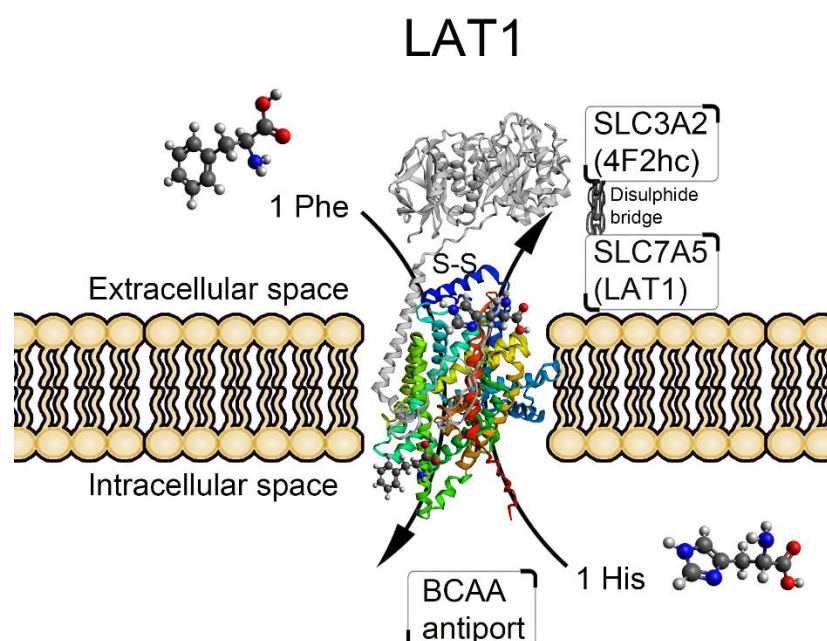


Figure 1.20: Functional structure of large neutral amino acid transporter isoform 1 (LAT1)

LAT1 (*SLC7A5*) the protein 4F2hc (*SLC3A2*) form a heteromeric complex through a disulfide bridge, stabilising the transporter on the plasma membrane. LAT1 is non-functional without the 4F2hc heavy

chain. Transport of a branched-chain amino acid (BCAA) from the extracellular space or synaptic cleft via LAT1 is coupled with the antiport of a branched-chain amino acid from the intracellular space. Example molecules shown in the figure are phenylalanine and histidine.

1.7.7 System N family of transporters

Alongside the other DS transporters, the transporters belonging to system N have also been identified as mediators of neutral amino acid transport (Bröer, 2014). Like system A, system N transporters are subject to intricate regulatory mechanisms. Within System N, there are two transporters, SNAT3 (encoded by the *Slc38a3* gene) and SNAT5 (encoded by the *Slc38a5* gene), which exhibit a distinct preference for glutamine as their substrate (Chaudhry et al., 2001; Nakanishi et al., 2001b; Nakanishi et al., 2001a; Hamdani et al., 2012). SNAT3 and SNAT5 facilitate Na⁺ coupled glutamine symport along with H⁺ exchange, rendering the overall transport process electroneutral. The direction of glutamine transport across the membrane is strictly contingent upon intracellular p^H and Na⁺ concentrations (Bröer et al., 2002). While SNAT5 has been observed to mediate LS transport, SNAT3 does not utilise DS as a substrate (Cubelos et al., 2005; Bodner et al., 2020). SNAT5 has also been demonstrated to exhibit electroneutral transport of LS in a p^H-sensitive manner in rat expression systems and human retinal epithelial cultures (Umapathy et al., 2008). Furthermore, the expression of SNAT3 and SNAT5 transporters has been localised in the astrocytes of rat brains (Boulland et al., 2003; Cubelos et al., 2005). Despite these insights, the actual transportation of DS and LS via the System N transporters within their native *in situ* environment remains a relatively unexplored area of study. As of the present time, a specific inhibitor targeting System N transporters remains unavailable, posing a significant challenge in exploring the role of this system, in modulating synaptic DS/LS concentrations.

1.7.8 PAT transporters

Finally, two transporters PAT1 (encoded by the *Slc36a1* gene, Proton/Amino acid Transporter1) and PAT2 (encoded by the *Slc36a2* gene, Proton/Amino acid Transporter2) transporter belonging to amino acid/auxin permease (AAP) family have been observed to be capable of DS, glycine, alanine, and proline transport in oocytes, in an electrogenic manner (Boll et al., 2002). PAT1 (referred to as LYAAT1) has been reported to be solely a lysosomal transporter (Sagné et al., 2001), although its surface expression remains to be fully explored (Wreden et al., 2003). PAT2 is widely expressed across the brain, but since PAT2 is localised in lysosomes, endoplasmic reticulum, and endosomes of neurons (Boll et al., 2002; Rubio-Aliaga et al., 2004), it is unlikely to mediate DS transport on the neuronal or astrocytic membranes. Nevertheless, it could aid in the packaging of DS into synaptic vesicles. Since the

PAT transporters are not expressed on the cell membranes, conventional electrophysiological techniques are not useful for detecting its signals.

Taken together, it is evident that multiple transporter systems may be implicated in the meticulous maintenance of DS/LS levels within both intracellular and extracellular environments. Despite extensive research into DS/LS transporters within the brain, a comprehensive understanding of the distinct contributions of various transporters, particularly the ASCTs in native physiological setup, has remained elusive. Consequently, in the pursuit of elucidating serine regulation, this thesis investigates the role of ASCT transporters.

1.8 D-serine in pathological states of the brain

The field of mental health has gained significant attention in recent times, driven by the pressing issue of increasing rates of brain disorders. Amidst a multi-million-dollar pharmaceutical industry focusing on mental health, approximately 300 million people worldwide tackle various forms of brain disorders (World Health Organization, 2020). In the quest to unravel and solve the complexities of brain disorders, neuroscientists have often looked for clues and turned their focus towards neurochemistry. This is hardly surprising as our brains are intricate bags full of chemicals, filled with neurotransmitter molecules such as glutamate, dopamine, serotonin, acetylcholine, etc., which influence our thoughts, emotions, and behaviour. Throughout history, philosophers and practitioners of traditional/Vedic medicine have been searching for a kind of “magic pill” capable of altering our neurochemistry, to enhance well-being and emotional resilience. However, this search for a magic pill has proven challenging for neuroscientists, especially because mimicking nature is no easy task. In essence, when there is an imbalance in the neurochemistry, such as in cases of depression, epilepsy, schizophrenia etc., the question arises: how can a magic pill change this neurochemistry in a manner that is most fruitful and that aligns with the body’s natural ways? Even if such a magic pill were to exist, it most often fails to show the desired effect/s for everybody suffering from such mental illness. Therefore, the concept of a magic pill appears somewhat immaterial, and it prompts us to look for more rational avenues to solve this mystery.

One promising approach to addressing/solving chemical imbalances in the brain, which has, time and again proven to be effective, involves delving into the fundamental neural circuitry at play and identifying the key players that govern and regulate these brain chemicals. This knowledge holds immense value, as it allows for the precise targeting of specific chemicals, circuits, and regulators to restore chemical balance and ultimately achieve homeostasis.

1.8.1 L-serine synthesis deficiency

Impairment of enzymes involved in the phosphorylated pathway of LS biosynthesis, notably phosphoglycerate dehydrogenase, phosphoserine aminotransferase, and phosphoserine phosphatase, can lead to a deficiency of LS widespread throughout the body. These LS biosynthesis defects span from severe conditions like Neu-Laxova syndrome, a deadly congenital disorder characterised by restricted growth, microcephaly, and multiple anomalies to infantile serine biosynthesis defects that lead to significant intellectual disability and stunted growth (Acuna-Hidalgo et al., 2014; El-Hattab, 2016; El-Hattab et al., 2016; Abdelfattah et al., 2020). Moreover, defects in DS, which is synthesised from LS primarily through the enzyme serine racemase, have been associated with neurological conditions such as schizophrenia and Alzheimer's dementia (Abdelfattah et al., 2020). Notably, defects in serine transport, arising due to mutation or deficiencies in sodium-dependent ASCT1, the predominant serine transporter in the brain, have been reported in schizophrenia (Weis et al., 2007; Kaplan et al., 2018). Furthermore, this transporter defect has been identified in children with severe neurological manifestations such as intellectual disability, microcephaly, and spasticity. These manifestations are similar to those reported in cases of defective serine biosynthesis (Damseh et al., 2015; Heimer et al., 2015; Srour et al., 2015).

Remarkably inhibitors targeting the ASCTs have been demonstrated to be beneficial for relieving symptoms associated with *in vivo* mice models of schizophrenia (Li et al., 2018). Consequently, gaining a comprehensive understanding of the transport mechanisms regulating serine levels in the brain holds significant promise not only for identifying specific targets but also for treating such debilitating neurological disorders.

1.8.2 D-serine-related disorders

DS has been linked to CNS disorders in humans, both when DS levels are decreased, resulting in altered neurotransmission, such as in schizophrenia and Alzheimer's disease, and when DS levels are increased, causing neuronal damage, such as in ischemia and Amyotrophic Lateral Sclerosis (ALS; Lee et al., 2017; Li et al., 2018).

1.8.2.1 Schizophrenia

One of the theories regarding the pathophysiology of schizophrenia involves the dysregulation of glutamatergic NMDAR-mediated neurotransmission, including abnormalities in DS. It has been established in early research that NMDAR antagonists can cause negative symptoms associated with schizophrenia (Javitt and Zukin, 1991), a disorder characterised by disruptions

in thought processes, emotions, and behaviour. Conversely, DS, the co-agonist of NMDARs, has also been known to potentiate the NMDAR activity and plasticity mediated by it (Van Horn et al., 2013). A double-blinded, placebo-controlled clinical trial conducted by Kantrowitz et al. (2015) provided a classical clinical implication of fundamental research. The patients on DS therapy showed improvement in their negative symptoms when compared with placebo groups. Although the therapeutic potency of DS remains to be fully explored, this clinical trial provides positive evidence for considering DS therapy in the case of schizophrenia.

DS has also been implicated in the development of schizophrenia. Several studies have shown that levels of DS are altered in the brains of individuals with schizophrenia, leading to changes in excitatory neurotransmission (Bennett et al., 2000; Fujii et al., 2006; Bendikov et al., 2007; Balu et al., 2013; Kantrowitz et al., 2015). Specifically, lower levels of DS have been associated with reduced NMDAR activity, which is thought to contribute to the symptoms of schizophrenia, including positive symptoms such as hallucinations and delusions, as well as negative symptoms like apathy and decreased motivation. This has been strongly correlated with an increased/decreased expression of SR (Steffek et al., 2006; Verrall et al., 2007; Verrall et al., 2010) or an increased expression of DAAO (Madeira et al., 2015) or a decreased expression of asc-1 (DS transporter mediating release) without changes in expression of GlyT1 (glycine transporter) of postmortem schizophrenia brains (Burnet et al., 2008). Therefore, supplementing or increasing DS levels was considered a therapeutic approach to alleviate the symptoms (Tsai et al., 1998; Yamamori et al., 2014). Unfortunately, on the flip side, elevated levels of DS have been implicated in oxidative stress and neuronal damage, further exacerbating the symptoms of the disorder (for review Guercio and Panizzutti, 2018). Although DS has been shown to have a protective effect in some experimental models of schizophrenia, more research is needed to fully understand its role in this condition. Therefore, regulating DS levels in the brain would be a best-potential target for the development of new treatments for schizophrenia.

Conventionally, studies have focussed on increasing glycine levels by using GlyT1 inhibitors (sarcosine based), to treat schizophrenia (Lane et al., 2005). Although there are no drugs (apart from DS itself or D-cycloserine) that can effectively regulate DS levels in the central nervous system, pre-clinical studies have strongly suggested the possibility of elevating DS levels through inhibition of asc-1 or ASCT1 transporter or DAAO, to enhance the hypofunctional NMDARs in schizophrenia (Hashimoto, 2010; Pei et al., 2021). An important question arises out of these pre-clinical observations. Which approach is more effective in improving the functioning of underperforming NMDARs—targeting glycine or DS levels? Are

the newer GlyT1 inhibitors distinct from conventional therapy by not utilising sarcosine nuclei, overcome the adverse effects associated with standard treatments? Alternatively, are there more favourable alternatives, such as selective glycine binding site agonists/antagonists/modulators or inhibitors of ASCT1/asc-1/DAAO that cross the blood-brain barrier? These crucial questions need to be thoroughly considered, before determining the usefulness of these types of compounds in treating neuropsychiatric disorders.

1.8.2.2 Alzheimer's disease

In Alzheimer's disease, excitatory neurotransmission is disrupted due to the death of neurons and loss of synapses. This consequently impairs the ability of the brain to process information and form new memories, which is an underlying aspect of cognitive decline observed in Alzheimer's patients. This decline during the early stages of the disease is thought to be due to a reduction in the expression of SR, leading to a decrease in DS levels. On the contrary, during the late stages, there is increased expression of SR, phosphoglycerate dehydrogenase (responsible for LS synthesis in astrocytes) and DS levels (Yan et al., 2020). This was evident from the severe inconsistencies while monitoring brain DS levels after a postmortem (Chouinard et al., 1993; Kumashiro et al., 1995; Nagata et al., 1995; Biemans et al., 2016). Additionally, SR has also been implicated in the formation of neurofibrillary tangles, another hallmark of Alzheimer's disease, through its role in the regulation of tau protein phosphorylation (Wu et al., 2004). KO of SR has been observed to alleviate the Amyloid β -induced neurotoxicity in mice, further suggesting that targeting SR and DS could be an effective strategy to counter the progression of Alzheimer's dementia (Inoue et al., 2008).

1.8.2.3 Cerebral ischemia

Cerebral ischemia is a condition where there is a lack of blood flow and oxygen supply to an area of the brain, often leading to tissue damage or death of neurons. Elevated levels of DS have been observed in simulated ischemic conditions of rat cortical slices (Katsuki et al., 2004), in cultured cells and *in vivo* ischaemic conditions (Abe et al., 2014). Elevated DS and NMDAR over-activation are thought to contribute to neuronal damage, suggesting that DS may play an important role in ischemia. Elevated DS and glycine have also been reported in CSF from piglets exposed to hypoxia–ischemia and in human newborns affected by perinatal asphyxia (Fuchs et al., 2012). Deletion of SR has been explored as a potential way to reduce brain DS levels in mice models of cerebral ischemia (Mustafa et al., 2010). Conceivably, this approach could have fewer side effects compared to the traditional blockade of NMDARs with

memantine and like drugs (Lipton, 2006) or a complete deletion of NMDAR, which is lethal (Mohn et al., 1999).

1.8.2.4 Amyotrophic lateral sclerosis

ALS (Amyotrophic lateral sclerosis), commonly known as Lou Gehrig's disease is a neurodegenerative disease, characterised by the progressive loss of motor neurons, resulting in muscle weakness and eventual paralysis. In the context of ALS, increased levels of DS have been associated with neuronal damage. The exact role of DS in the development of ALS remains a question, but it has been suggested to play a role in the progression of the disease (Kondori et al., 2018). Sasabe and Colleagues (Sasabe et al., 2007; Sasabe et al., 2012) reported a decrease in DS and its production enzyme SR in the spinal cords of both ALS mice and ALS individuals. This indicates that enhanced DS and the NMDAR over-activation associated with the increased DS could underlay the disease progression of ALS. Further research is needed to determine the exact mechanisms involved and how DS levels can be effectively regulated to potentially slow or halt the progression of ALS. Moving forth, understanding the role of glial cells in regulating DS is an important area of research, to develop therapeutic targets for ALS.

1.9 Aim and Hypotheses

Synaptic D-serine (DS) is a critical component of normal excitatory neurotransmission in the brain, but its precise origin and regulation remain poorly understood. The central question revolves around whether neurons and/or astrocytes are responsible for supplying synaptic DS. To uncover the source(s) of DS, it is essential to understand the transporters present on astrocytes and neurons that can move DS into and out of cells. Moreover, understanding the intricate interplay between neurons and astrocytes in regulating synaptic L-serine (LS) levels is of paramount importance, especially considering that the neurons rely on LS transported from the astrocytes to synthesise their own DS. Consequently, to elucidate the potential role of astrocytes in shuttling LS, it becomes imperative to gain insights into the specific transporters expressed on both astrocytic and neuronal membranes that facilitate the movement of LS into and out of these cells. Using CA1 hippocampal astrocytes and neurons as a model system, this study aims to shed light on the potential mechanisms behind DS regulation. Past *in vitro* research has suggested that Alanine Serine Cysteine Transporters (ASCT) may play a crucial role in DS regulation. However, no study to date has provided clear evidence of their functional expression in brain slices, a practical model for understanding ASCT transporter functions. **Therefore, the overarching aim of this project is to comprehensively investigate the regulatory mechanisms governing the amino acid serine within synapses of the central nervous system.**

Chapter 3

The primary aim of Chapter 3 is to elucidate the roles of neurons and astrocytes in modulating extracellular D-serine concentrations within the central nervous system.

Objectives

The overarching objectives of this chapter are as follows:

- ❖ To investigate the uptake of D-serine by plasma membrane transporters expressed in the astrocytes and neurons.
- ❖ To investigate whether Alanine Serine Cysteine transporters (ASCT) on astrocytic and neuronal membranes contribute to the uptake of D-serine in the CA3-CA1 glutamatergic synapses.
- ❖ To understand the contribution of other potential D-serine plasma membrane transporters in the uptake of D-serine.
 - System A (SNAT1 and SNAT2) transporters
 - L-type amino acid transporter (LAT1)
 - Sodium-independent alanine serine cysteine transporter (asc-1)

- ❖ To investigate the role of Alanine Serine Cysteine transporters (ASCT) on astrocytic membranes in the uptake of D-serine in the other brain areas such as the cerebellum and the somatosensory cortex.

Hypothesis

The hypothesis being tested is as follows:

- ❖ Astrocytes are likely candidates to mediate the uptake of D-serine from the extracellular space. The application of D-serine will evoke transport-associated currents in voltage-clamped CA1 *stratum radiatum* astrocytes in the hippocampus of mice brain.
- ❖ The astrocytes located in the CA1 *stratum radiatum* will exhibit functional expression of the ASCT1 transporter isoform, which will be substantiated by their responsiveness to ASCT inhibitors.
- ❖ The contribution of other D-serine transporters to D-serine uptake in the CA1 *stratum radiatum* astrocytes will be relatively minor. This expectation will be supported by their reduced sensitivity to specific transporter inhibitors.
- ❖ In voltage-clamped CA1 pyramidal neurons, it is hypothesised that the application of D-serine will induce transport-associated currents.

Chapter 4

The primary aim of Chapter 4 is to elucidate the roles of neurons and astrocytes in modulating extracellular L-serine concentrations within the central nervous system.

Objectives

- ❖ Explore the uptake of L-serine by plasma membrane transporters expressed in astrocytes and neurons.
- ❖ Investigate whether Alanine Serine Cysteine transporters (ASCT) on astrocytic and neuronal membranes contribute to the uptake of L-serine in the CA3-CA1 glutamatergic synapses.
- ❖ Investigate the contribution of other potential L-serine plasma membrane transporters in the uptake of L-serine.
 - System A (SNAT1 and SNAT2) transporters
 - L type amino acid transporter (LAT1)
 - Sodium-independent alanine serine cysteine transporter (asc-1)

Hypothesis

- ❖ Astrocytes are likely candidates to mediate the uptake of L-serine from the extracellular space, similar to their role in D-serine uptake.
- ❖ L-serine elicit transport-associated currents in voltage-clamped CA1 *Stratum radiatum* astrocytes, and these currents are specifically sensitive to inhibition by ASCT inhibitors but with some additional potential involvement of other transport systems.

- ❖ L-serine elicits transport-associated currents in voltage-clamped CA1 pyramidal neurons in the hippocampus of mice brain, indicating its role in neuronal uptake.

Chapter 5

The broad aim of Chapter 5 is to investigate the role of D-serine plasma membrane transporters in regulating extracellular DS/LS concentrations, with a focus on their role in modulating NMDAR-mediated excitatory neurotransmission in the mammalian brain.

Objectives

- ❖ To investigate whether D-serine or L-serine facilitates NMDAR-mediated synaptic transmission in the hippocampal CA3-CA1 synapses.
- ❖ To determine whether ASCT1 transporter is necessary to maintain D-serine mediated NMDAR synaptic transmission at glutamatergic synapses.
- ❖ To explore the relative contribution of the other D-serine transporters in maintaining D-serine-mediated NMDAR synaptic transmission at glutamatergic synapses.

Hypothesis

In line with the above-stated objectives, I hypothesised that,

- ❖ Exogenous L-serine enhances the ongoing NMDAR-mediated synaptic transmission in the hippocampal CA3-CA1 synapses, synapses by fuelling D-serine synthesis in neurons.
- ❖ Exogenous D-serine enhances the ongoing NMDAR-mediated synaptic transmission in the hippocampal CA3-CA1 synapses.
- ❖ Inhibition of the ASCT1 transporter decreases synaptic D-serine and therefore decreases the NMDAR-mediated response at the hippocampal CA3-CA1 synapses.
- ❖ Inhibition of SNAT1 and SNAT2 transporters reduces synaptic D-serine availability and therefore decreases the NMDAR-mediated response at the hippocampal CA3-CA1 synapses.
- ❖ Inhibition of asc-1 transporters decreases synaptic D-serine release and therefore decreases the NMDAR-mediated response at the hippocampal CA3-CA1 synapses.

Chapter 2 Materials and Methods

All chemicals used were of analytical or reagent grade and were sourced from Sigma and Chem-supply unless otherwise specified.

2.1 Animals

2.1.1 Ethics

All the animal experiments and protocols were performed in accordance with procedures approved by the Australian National University Animal Experimentation Ethics Committee (Approval Ethics protocol: A2020/38), National Health and Medical Research Council's (NHMRC) Australian Code for the Care and Use of Animals for Scientific Purposes and the Australian Capital Territory Animal Welfare Act 1992. Mice were euthanised by decapitation, as authorised by the Australian National University Animal Experimentation Ethics Committee.

2.1.2 Mice housing, age, and Strain

All mice utilised in this study were of the wild-type C57BL/6N strain (Charles River), encompassing both males and females and falling within the age range of post-natal day 21–25. However, two experiments included mice at P10 and P30 days old. They were bred and housed at the Australian Phenomics Facility (APF), Australian National University, at 24–26 °C and were exposed to a daily 12–12 hrs light-dark cycle. A maximum of five mice, all from the same weaned litter, were collectively housed in a shared cage. However, on the day of the experiment, a single mouse was selected, transported to the John Curtin School of Medical Research (JCSMR) PC2 facility, and used for experiments within 30 minutes post-weaning. Additionally, mice weighing less than 4g were excluded from the experiments.

2.2 Brain extraction procedure

To minimise the stress experienced by the mice during the experimental procedure, they were handled as little as possible before being humanely euthanised via decapitation using a guillotine. Following this, both brain hemispheres were carefully removed and placed into an ice-cold slicing solution that had been oxygenated with 100% O₂. The two hemispheres were then separated and cut at precise angles as described by Bischofberger et al. (2006), to preserve the axonal integrity of the hippocampus.

2.3 Brain slice preparation

Hippocampal brain slices were primarily used for experiments. Transverse slices, measuring 250 μm in thickness, were carefully cut from mouse brains under stereomicroscopic observation, using a VT1200S vibratome (Leica Biosystems, Nussloch, Germany) in an ice-cold slicing solution. These slices were then incubated for 15 minutes at 37°C before resting at room temperature, in artificial cerebrospinal fluid (aCSF) in a 250 ml borosilicate glass holding chamber. The brain slices were carefully placed on a sheet of nylon netting held between tightly fitting inert acrylic rings.

In the experiment involving the primary somatosensory cortex, transverse sections were obtained in a manner similar to the procedure employed for slicing the hippocampus. In experiments involving the extraction of the cerebellum, parasagittal slices with a thickness of 200 μm , as described previously by Edwards et al. (1989) and Llano et al. (1991) were collected. This choice of thinner cerebellar slices, compared to those used for the hippocampus or the cortex, was made to enhance visibility when directly observing the slices under the microscope. Subsequently, the slices were stored at room temperature until they were used (within 8 hours). When ready for recording, the slices were transferred to a recording chamber and perfused with aCSF.

2.4 Patch clamp rig

The patch clamp setup/rig was used to study the electrophysiological properties of individual cells and record the activity of ion channels, receptors, and transporters (Stuart et al., 1993). Prior to recording sessions, slices were moved from the room temperature holding chamber to a recording chamber. The submersion-type recording chamber was continuously perfused at a rate of 2 ml/min with oxygenated artificial cerebrospinal fluid (aCSF) or various bath solutions, including control and inhibitors. The chamber enabled visualisation of the slices using infrared differential interference contrast (DIC) on an Olympus BX50WI water immersion (Olympus Optical, Japan) connected to a light source-IR 1000EX camera (SciTech Private Ltd, USA).

All recordings were obtained in voltage clamp mode, which allows recording currents, using an amplifier (Acquisition device- Digidata 1440A, Axon CNS, Molecular devices, and a command device- Multiclamp 700B, Axon instruments) and its associated software (Clampex 10.7.0.3, Molecular devices 2016 and Multiclamp 2.2.1.0, Molecular devices 2017). An inbuilt 10 Hz low pass-filter was used, to acquire the current traces. The signals from the amplifiers

were tracked using a Pentium i7-based PC. The data were digitised at a frequency of half the sample interval, resulting in a sampling rate of 1000 Hz (1 ms), and a total of 60,000 samples per signal.

The regulation of puff pressure (2–20 psi) and duration of puff (5 sec) was controlled using Picospritzer 11 (from General Value Corporation). External solutions were applied to the bath using a circulating peristaltic pump (Gilson Miniplus 3) at a flow rate of 3 ml/min (gassed with 100% O₂ or carbogen).

2.5 Electrodes

A standard-walled borosilicate glass pipette (1.5 mm outer diameter*0.86 inner diameter) was used to achieve a high-resistance (GΩ) seal with the cellular membrane, thereby isolating a small patch of membrane for electrical recordings. Two types of glass electrodes were made using a two-stage vertical electrode puller-Narishige Model PC-10), with the pulling temperatures adjusted to achieve distinct tip resistances.

Recording electrode: Electrodes had tip resistances of 6–7 MΩ (for astrocytes) and 5-6 MΩ (for CA1 pyramidal neurons) when filled with an intracellular solution.

Puffer electrode: Puffer pipettes were also pulled from standard-walled borosilicate capillary glass and had tip resistances of approximately 5 MΩ when filled with aCSF.

The positioning of patch and puffer pipettes was controlled using electrode manipulators (Sutter Instruments & Co MP-285, USA).

2.6 Amino acid transport assays (Chapter 3 and 4)

The experimental setup for investigating serine transport dynamics is visually depicted in Figure 2.1, providing an overview of the key components.

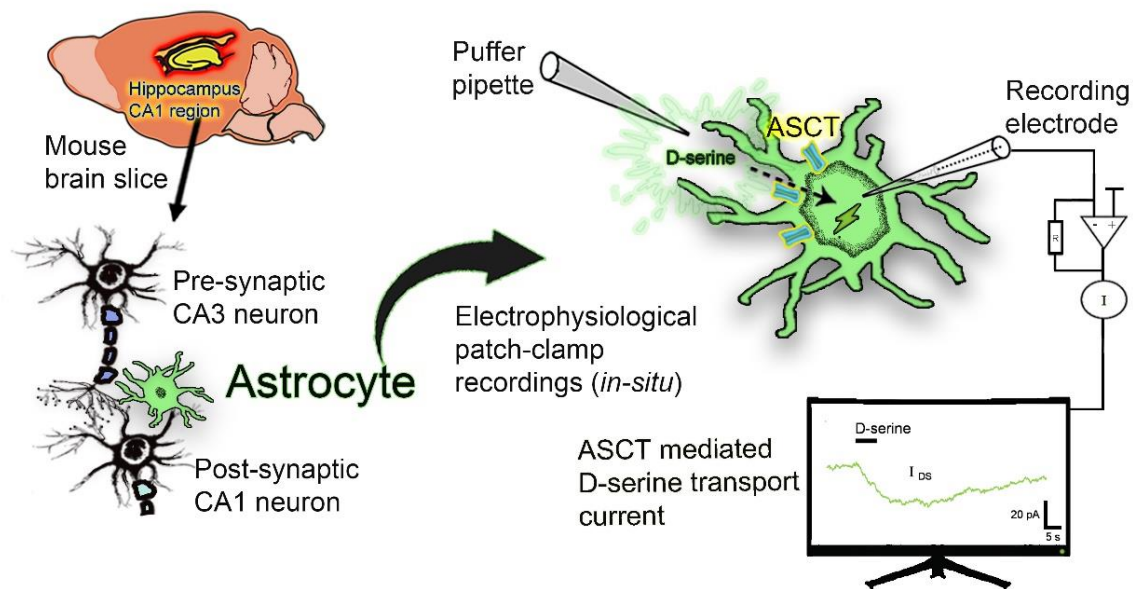


Figure 2.1: Pictorial representation of experimental setup employed in the study of serine transport dynamics.

In the top left corner, a mouse brain is depicted, with the hippocampus highlighted in yellow. A downward-pointing arrow leads to the lower left, directing attention to a representation of an excitatory CA3-CA1 synapse. This synapse comprises two pyramidal neurons with an ensheathing astrocyte (coloured green), emphasising the tripartite nature of the synaptic environment. The green arrow extending from the astrocyte directs focus to the top right of the image, where an enlarged view of an astrocyte is presented. This astrocyte is subjected to a voltage-clamp electrophysiological recording. To the left of the astrocyte is a puffer pipette, utilised for the controlled application of substrates such as D-serine or L-serine onto the voltage-clamped astrocyte. A recording pipette, situated to the right of the astrocyte, is used for recording any transport-associated electrical signals originating from the astrocytes. The patch pipette is connected to an amplifier setup positioned on the far right of the image. The representative amplifier is further linked to a computer screen, displayed on the bottom right, which exhibits the recorded DS transport-associated currents (trace shown in green).

2.6.1 Composition of slicing solution

High sucrose-based slicing solution contained (in mM): 290 Sucrose, 10 glucose, 2.5 KCl, 10 HEPES, 1.25 NaH₂PO₄, 4 MgCl₂, 0.1 CaCl₂. The pH was adjusted to a range of 7.3–7.4 using NaOH, and the solution was fine-tuned to an osmolarity of approximately 360–365 mOsmol. Additionally, it was bubbled with 100% Oxygen (O₂) throughout.

2.6.2 Composition of artificial Cerebrospinal Fluid (aCSF)

The extracellular aCSF solution contained (in mM) 145 NaCl, 10 glucose, 2.5 KCl, 10 HEPES, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂. The pH was adjusted to a range of 7.3–7.4 using NaOH, and the solution was fine-tuned to an osmolarity of approximately 315–325 mOsmol. Additionally, it was bubbled with 100% Oxygen (O₂) throughout.

2.6.3 Composition of drug cocktail, bath control and bath inhibitor solutions

All recordings were made in the presence of drug cocktail listed below in aCSF solution; to inhibit other ion channel and receptor-mediated currents containing: 40 μ M DL-2-amino-5-phosphonopentanoic acid (APV; a competitive NMDAR antagonist), 10 μ M dizocilpine maleate (MK801; an NMDAR open channel inhibitor), 20 μ M 3,4-dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoxaline-7-sulfonamide (NBQX; an AMPA and kainate receptor antagonist), 10 μ M bicuculline methiodide (a competitive GABA_A receptor antagonist), 1 μ M strychnine hydrochloride (a competitive glycine receptor antagonist), 1 μ M tetrodotoxin (TTX; a voltage-gated sodium channel inhibitor) and 10mM tetra-ethyl ammonium chloride (TEA; a potassium channel inhibitor).

The bath control solution is composed of the drug cocktail without any amino acid substrate or inhibitor. This control solution was bath applied i.e. to the recording chamber at a constant flow rate (3 ml/min was achieved using Minipuls 3 Osmotic pump, Gilson), for at least 7 minutes after the cells were voltage-clamped in whole-cell configuration and after obtaining the IV responses.

To prepare the bath inhibitor solutions, the necessary amounts of the transporter inhibitors were dissolved in the drug cocktail solution. These solutions were freshly prepared, thoroughly vortexed, and continuously oxygenated with 100% O₂ during the experiments. The inhibitors were applied by switching the bath control solution to one containing the respective compound. Considering a perfusion rate of 3 ml per minute, the length of the tubing, and the volume of the chamber (2 ml), a complete exchange of the external solution was accomplished in less than 7 minutes.

Only a single recording was obtained from a hippocampal brain slice, to avoid the residual effects of the drugs that were washed-in during the experiment. Although multiple recordings were obtained from the same animal, all the data sets included recordings from a minimum of two different animals.

All ingredients of aCSF, slicing and internal solutions were obtained from Sigma-Aldrich, except NBQX and D-APV were acquired from HelloBio.

2.6.4 Preparation of puffer solutions

The puffer solutions were used to apply amino acid substrates (such as DS, LS, L-cysteine, and L-asparagine) adjacent to the cells, for the activation of transporters on the cellular membranes. Puffer pipettes were filled with an adequate amount of puffer solution for this purpose. To make puffer solutions containing the appropriate transporter substrates, the necessary amounts of substrates were dissolved in the drug cocktail solution. These solutions were freshly prepared, vigorously mixed, and continuously oxygenated with 100% O₂ throughout the experiments.

2.6.5 Composition of Internal Solution

A thiocyanate-based [Sodium Thiocyanate (SCN⁻)] internal solution contained (in mM): 80 NaSCN, 10 EGTA, 2 MgCl₂, 10 HEPES, 10 L-alanine, 50 KCl, 1.4 ATP-Mg²⁺, and 0.6 GTP-Na⁺ was filled in the patch pipette, to record the transport associated currents (Modified from Foster et al., 2016). The pH was adjusted to approximately 7.2 with KOH and the osmolarity was adjusted to approximately 280 mOsmol. The internal solution was filtered using a GV 0.22 µm syringe filter unit (Merck Millipore Ltd., Ireland) before the experiments.

To test the effects of removing internal SCN⁻ anion, SCN⁻ ions were substituted with Cl⁻ ions. (NaCl 80 instead of NaSCN 80). The transport currents obtained from the SCN-based internal solution are expected to be higher because SCN⁻ generates more charge compared to Cl⁻ during its translocation, as predicted by the typical permeability sequence seen with anionic conductance (Wright and Diamond, 1977; Billups et al., 1996). This means that SCN⁻ can move more readily through the anionic channels found within the transporters, leading to a higher current if/when those channels are open.

2.6.6 Current Voltage response

Whole-cell voltage-clamp electrophysiological recordings were conducted on CA1 pyramidal neurons and *stratum radiatum* (str) astrocytes. Identification criteria for astrocytes followed Henneberger and Rusakov (2012). CA1 pyramidal neurons were identified visually in the hippocampal CA1 cell layer. CA1 pyramidal neurons were voltage-clamped at a holding potential of -70 mV, while astrocytes were held at -80 mV. Series and input resistances were continually monitored during the experiments to detect any electrode blockages. Cells were

excluded from analysis if series and input resistances increased by more than 30% compared to their initial values. Additionally, cells were rejected if the holding current exceeded a 20% increase from its initial value within the first 10 minutes after transitioning to the whole-cell configuration.

Cell type identification involved holding CA1 pyramidal neurons and *stratum radiatum* astrocytes within the ranges of -80 to +60 mV and -90 to +50 mV, respectively, with voltage steps of 10 mV. Current responses corresponding to these voltages were recorded from cells patched at their cell bodies, allowing differentiation between neurons, astrocytes, and other cell types such as interneurons and glial cells. I-V plots, representing the relationship between holding voltage on the X-axis and recorded current (near the last second of the recording) on the Y-axis, were generated for both neurons and astrocytes. From these plots, values for the resting membrane potential (the potential when the current is zero) and the membrane resistance (the inverse of the slope of the plot near the resting potential) were determined.

2.6.7 Post-Hoc Biocytin Filling and Staining Protocol:

Following electrophysiological recordings, post-hoc biocytin filling was performed to visualise the astrocyte morphology. The patch pipette contained a biocytin solution (2-5% in the internal solution). After achieving a stable whole-cell configuration, astrocytes were filled with biocytin during a recording session. Subsequently, cells were allowed to recover for about 15 to 30 minutes to facilitate biocytin diffusion. The patch pipette was quickly withdrawn from the brain slices to minimise biocytin spread. The brain slices were then fixed in paraformaldehyde (4% PFA in phosphate-buffered saline; filtered) for approximately 30-60 minutes at room temperature. After fixation, the slices were washed with standard phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, adjusted to pH 7.2 to 7.4) and then incubated in a streptavidin-Alexa Fluor 594 conjugate solution (1:500 in PBS) for 1-2 hours. Following thorough rinsing with PBS, the slices were mounted on glass slides using an anti-fading mounting medium. Confocal microscopy (using Nikon A1 controlled using NIS-Elements software) was employed to visualise and capture images of astrocytes filled with biocytin, as indicated by the presence of Alexa Fluor 594 fluorescence, allowing for subsequent morphological analysis.

2.6.8 Amino acid puff assay

To demonstrate the existence of serine plasma membrane transporters on the astrocytes and CA1 pyramidal neurons, amino acid puff assay was conducted (Figure 2.2). Following the assessment of I-V responses, the bath control solution was applied to the brain slices. Substrates were puff-applied to cells from a puffer-patch pipette positioned (10-20 μm) from the cell soma, unless stated. The only distinction between the external bath control solution and the puffer solution was the substrate. The duration of the substrate puffs was 5 seconds and the puff associated currents were recorded over a total duration of 40 seconds with an interval of 90 seconds between two puffs. The puff protocol was repeated on the same cell to obtain 5–8 stable recordings. The first 2–3 test puffs were excluded. Further, to test the effect of a particular transport inhibitor on the measured transporter currents, inhibitor solutions were bath applied separately.

All amino acid puff assay data were analysed using Clampfit V10.7 (Axon Instruments). The duration of the puff is depicted as a black bar in all current traces, and each recording session lasted for 40 seconds. The mean amplitude of current traces was measured at the last second of puff (4–5 s) for neurons and at 14–15 second intervals (14–15 s from the start of the protocol, to compensate for the slow current observed) for astrocytes. The individual data points in the scatter plots represent the average current values of 5 stable current traces from the same experiment (include data from different slices and mice). The transport-associated current values in the results, in-text and in the bar graphs are presented as mean $\pm$ SEM of all experimental repeats.

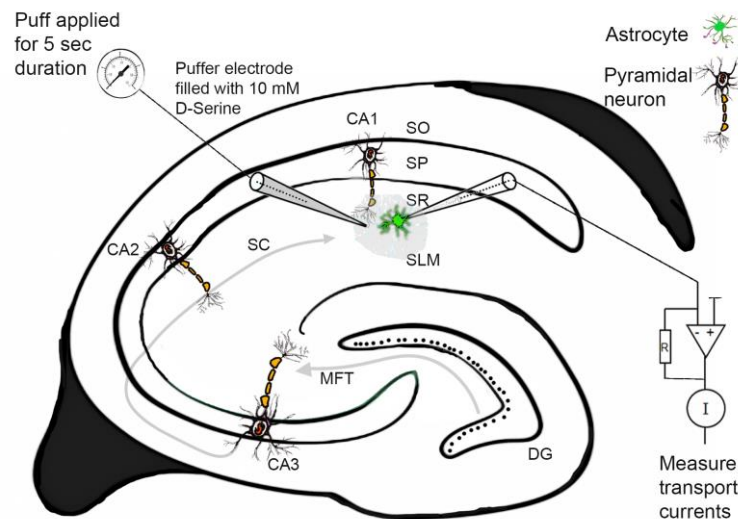


Figure 2.2: Pictorial representation of amino acid puff assay

Picture showing the exact position of the puffer electrode in the Schaffer collateral tract (SC- path) and the recording patch electrode used to record astrocytic (green) membrane currents.

2.6.9 Substrate current vs voltage relationship

DS or LS current vs voltage relationship: I–V plots. To test if DS/LS currents correspond to metabolically uncoupled anion currents, a ramp protocol was performed. Astrocytes were voltage-clamped at -80 mV with a Cl^- based internal solution. The membrane voltage was incrementally raised from -80 mV to +40 mV, at a rate of 100 mV per sec. The resulting ramp current at different holding voltages were derived by subtracting the data obtained before and after DS/LS application. The Cl^- ion reversal potential was theoretically calculated to be around -3 mV, using Nernst equation $E_{\text{Cl}} = \frac{RT}{ZF} \cdot \ln\left(\frac{[\text{Cl}^-]_{\text{in}}}{[\text{Cl}^-]_{\text{out}}}\right)$.

To simplify the analysis of the voltage ramp experiment, the acquired 1000 data points were averaged into discrete 5 mV intervals to facilitate a clearer interpretation. To assess the voltage dependence of ramp currents, a statistical analysis using LM (Fixed-effects model) was conducted. The voltage dependence was considered significant if the associated p-value reached conventional significance levels.

2.6.10 Statistical test

All data values are represented as mean $\pm$ SEM, with * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ convention. To determine any statistical significance among different experimental conditions, a linear mixed model (LME) was used in R Studio unless specified otherwise (R code in appendix). The cell was treated as the random effect in the model.

2.7 Synaptic transmission experiments (Chapter 5)

2.7.1 Composition of slicing solution

The slicing was performed in a bicarbonate-based ice-cold slicing solution containing (in mM): 75 sucrose, 25 glucose, 87 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 7 MgCl₂ and 0.5 CaCl₂. The solutions were continuously bubbled with carbogen gas (95% O₂ + 5% CO₂).

2.7.2 Composition of artificial Cerebrospinal fluid (aCSF)

A bicarbonate-based artificial cerebrospinal fluid (aCSF) contained (in mM) 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂ and 2 CaCl₂. The solutions were continuously bubbled with carbogen gas (95% O₂ + 5% CO₂) throughout.

2.7.3 Composition of Bath Control and bath inhibitor solutions

The bath control solution is composed of 20 μ M NBQX, an AMPA and kainate receptor antagonist. To prepare the bath inhibitor solutions, the necessary amounts of the transporter inhibitors were added to the bath control solution. In all experiments, AMPARs were blocked to prevent any plasticity-related changes to the post-synaptic NMDAR responses. All NMDAR responses were recorded at 34°C to mimic the physiological conditions, using an automatic temperature controller TC-324B (Warner instruments Corporation).

40 μ M APV, a selective antagonist of NMDAR that competes with glutamate for the ligand binding site, was added to confirm the NMDAR component of the EPSC response.

30 or 60 μ M Kynurenate or Kynurinic acid, a non-specific competitive antagonist having low affinity and fast unbinding rate at the glycine binding site of the NMDAR was used to desaturate the binding site.

2.7.4 Composition of internal solution

Electrodes filled with a CsMeSO₄-based internal solution (containing in mM; 85 CsMeSO₄, 40 HEPES, 10 TEA-Cl, 0.008 CaCl₂, 10 EGTA, 2 Mg-ATP, 0.5 Na-GTP, 2 QX-314 and pH adjusted to 7.2 with 0.1 CsOH) was inserted over Ag/AgCl recording electrode. QX314 was obtained from Tocris Bioscience. The internal solution used for the recordings consisted of cesium methanesulfonate to selectively block potassium channels, enabling the recording of large EPSCs from the soma of CA1 pyramidal neurons.

2.7.5 Electrophysiological recording

Whole-cell voltage-clamp recordings were employed to record NMDAR-mediated EPSCs in CA3 to CA1 glutamatergic synapses of the hippocampus (Figure 2.3A, B). To evoke EPSC responses, a presynaptic stimulation was applied using a stimulating electrode to the *Schaffer collateral* pathway, the primary excitatory pathway connecting CA3 to CA1 neurons. The corresponding EPSC responses were measured using the recording electrode or patch pipette from the soma of voltage clamped CA1 pyramidal neurons (Figure 2.3C).

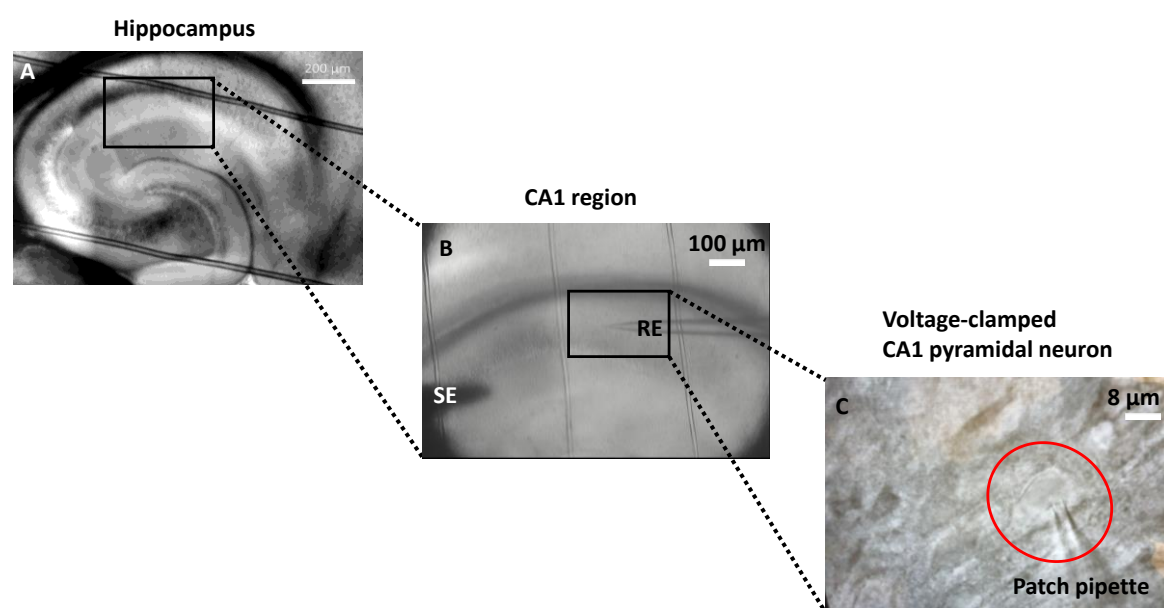


Figure 2.3: DIC image of patch clamp setup in the hippocampal brain slices

A & B | Mouse hippocampal brain slice focussed under 4x objective, showing the CA1 region, where the stimulating electrode (SE) was placed in the *stratum radiatum* and the recording electrode (RE) was positioned in the stratum pyramidale, where the cell bodies of CA1 pyramidal neurons reside C | Mouse hippocampal brain slice focussed under 60x objective showing an example CA1 pyramidal neuron that was recorded using a patch pipette.

2.7.6 Stimulating electrode and Optimisation of stimulation protocol

To record NMDAR-mediated post-synaptic currents at the CA3-CA1 synapses, a concentric bipolar tungsten electrode was used to stimulate glutamate release from the CA3 terminals. The stimulating electrode was carefully placed (using MP-285, Sutter Instruments) in Schaffer collateral (SC) in a parallel orientation with the axonal projection (Figure 2.4). An isolated current stimulator (custom-made in JCSMR,1996) was used to deliver current pulses. The stimulus strength was adjusted (between 20 to 200 μA pulses of 200 μs duration) to ensure a reliable starting postsynaptic NMDAR response, ranging between 10 to 100 pA. This low-frequency stimulation (LFS) condition, consisting of sets of paired pulses (40 ms apart) was presented every 20 seconds (frequency of 0.05 Hz). The ideal stimulation strength was

set up according to the individual cell, as the responses would vary depending on the placement of the stimulating electrode, amount of pre-synaptic terminals, amount of pre/post synaptic dendrites preserved after the slicing protocol, angle of slicing during that day etc.

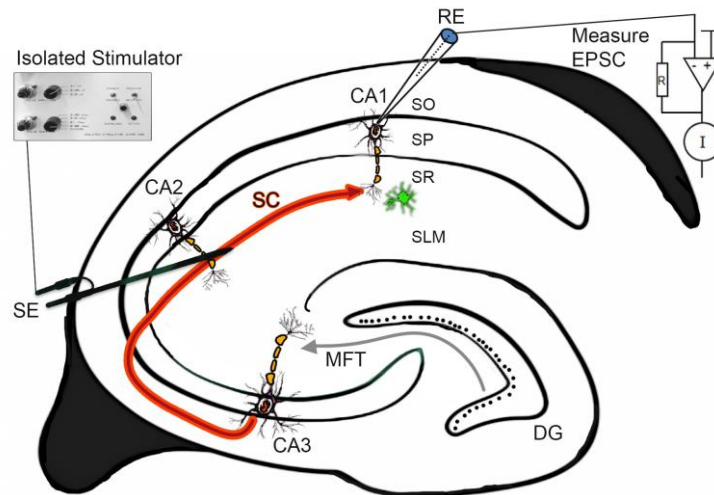


Figure 2.4: Pictorial representation of the stimulation setup

Picture showing the exact position of the stimulating electrode (SE) in the Schaffer collateral tract (SC- path highlighted in red) and the recording electrode (RE) used to record EPSCs from the CA1 pyramidal neuron.

2.7.7 Optimisation of the recording, Whole cell and R_s compensation

Mg^{2+} free solution ($0.2\mu M Mg^{2+}$) was avoided as the low Mg^{2+} concentration significantly affected the health of the cells (runaway NMDAR-associated excitotoxicity). NMDAR current responses were measured at negative resting potentials ($-70mV$), despite the voltage-dependent Mg^{2+} block (Kovalchuk et al., 2000). This is contrary to the conventional measurements of NMDAR current responses at positive potential ($+20 mV$), to prevent any changes to post-synaptic transporter activity at the positive potentials (Nowak et al., 1984). Inhibitory GABAergic activity was not blocked, as parvalbumin and somatostatin expressing GABAergic interneurons keep the local CA1 excitation under control (Udakis et al., 2020).

Whole-cell and Series Resistance (R_s) compensation was performed on the multiclamp commanding software. 60-70% of the initial R_s (less than $30 M\Omega$) was compensated throughout the recording. During the recording, if the R_s value changed by 30 % of the initial R_s value or if the baseline current changed by 30 % of the initial holding current, cells were excluded for recording and analysis.

2.7.8 Data analysis

All NMDAR-mediated EPSC amplitudes were analysed through Clampfit software 10.7.0.3 (Molecular Devices, 2016) and MATLAB R2022a 9.12.0.1927505 (custom MATLAB codes were written by Brian Billups). Stored Pclamp files (.abf) were loaded onto MATLAB using an extension (open-source code written by Forrest Collman, 2022 on GitHub). Boxcar filtering with a 1ms width was used to obtain a moving average.

A 10mV negative voltage step (ΔV) was included before delivering the stimulation pulses during each sweep, to monitor the cell's passive membrane properties (Figure 2.5). A holding or baseline current (I_{b1}) was measured in each recording sweep before the voltage step or test pulse. This step-down voltage results in a step-current response, displaying its characteristic peak capacitive current (I_p) and a steady state current (I_s), measured in relation to the I_{b1} , during each sweep. Series resistance (R_s) and input resistance (R_{in}) values were calculated using the formula, $R_s = \Delta V / I_p$ and $R_{in} = \Delta V / I_s$ respectively.

A baseline current (I_{b2}) was measured in each recording sweep before the start of the stimulus. The peak EPSCs were used as an indicator to measure NMDAR activation originating from the dendrites of the voltage-clamped CA1 pyramidal neurons. Peak 1 (P1) and peak 2 (P2) current responses were measured in relation to I_{b2} . Paired-pulse ratio (PPR) was calculated as the ration of the peak 2 to peak 1 amplitude. Any change to PPR (>30%) throughout the recording was excluded from analysis, as it would indicate that the post-synaptic responses were modified by changes in the pre-synaptic release probability of neurotransmitters. Cells with a holding current of -200pA or more at the start and a holding current of -500pA or more throughout the recording were excluded from recording and analysis.

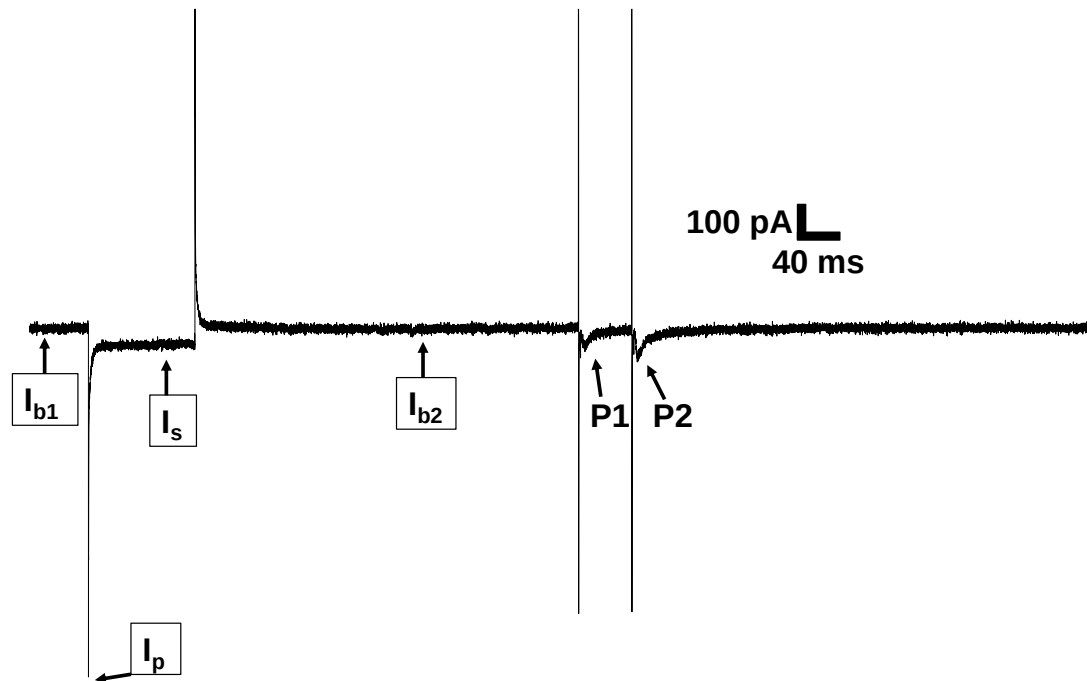


Figure 2.5: Example trace showing method for recording baseline, series and input resistance and NMDAR-mediated EPSCs.

The trace represents an example post-synaptic NMDAR-mediated EPSC response recorded from a voltage-clamped CA1 pyramidal neuron. Low-frequency stimulation protocol - initial 10 mV voltage step followed by the paired current pulse stimulus (interval 40 ms) delivered every 20 seconds (0.05 Hz) using a stimulating electrode. Response - Shows the step current that was measured at the beginning of each sweep in relation to the baseline I_{b1} current. It highlights a capacitive peak current I_p and steady-state current I_s , which was monitored as indicative of series R_s and input resistances R_{in} during the experiment. This was followed by the two NMDAR-mediated EPSC amplitude response (P1 and P2) that was measured at their peak in relation to the baseline I_{b2} currents.

To determine any statistical significance among different experimental conditions, a linear mixed model was used in R Studio (R code in appendix). The cell was used as the random effect in the model. Data are presented as means $\pm$ SEM, with * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ convention. NMDAR-mediated EPSCs (peak P1 amplitude) in the figures were normalised to the average EPSC value of 5 minutes before the drugs hit the bath solution. This was done to remove the discrepancy resulting from different starting response magnitudes. Comparisons were made between the average of the control - last 5 mins with the average of the test - last 5 mins (after 10 mins of the test drugs have been in the bath solution). The data were then plotted over the time course of the recording. PPR and baseline comparisons were also made at the same time point. Figures were made with Microsoft – Excel, PowerPoint (Office 365), MATLAB and Adobe Photoshop 2022.

Chapter 3 Transport of D-serine in the mammalian brain

3.1 Aim

A central question revolves around the regulatory mechanism governing synaptic DS, after its action on the NMDARs, addressing whether the neurons, astrocytes, or both primarily control this process. To elucidate the regulation of DS, it is imperative to comprehensively understand the repertoire of transporters expressed on astrocytic and neuronal membranes, which are capable of facilitating the uptake and release of DS. The involvement of perisynaptic astrocytes in neurotransmitter regulation has been a recurring theme in the literature (Rosenberg et al., 2013). Additionally, previous studies employing radiolabelling techniques have provided evidence of DS uptake into both astrocytes and C6 glioma cells (Hayashi et al., 1997; Ribeiro et al., 2002). Based on this existing body of research, it is hypothesised that astrocytes are strong candidates for mediating the uptake of DS from the extracellular space. Utilising CA1 hippocampal astrocytes and neurons as a model system, this chapter aims to provide novel insights into the intricate mechanisms that underlie DS regulation. Prior *in vitro* investigations have suggested a potential role for ASCTs in DS regulation (Foster et al., 2016). Nevertheless, to date, functional evidence of ASCT transporter expression within brain slices, a more physiologically relevant model, remains unexplored¹. I hypothesise that astrocytes express ASCT1 transporters, which mediate the uptake of DS from the synaptic space.

¹ Some of the findings, figures, and discussions presented in this chapter have been published in;
Krishnan KS, Billups B. ASC Transporters Mediate D-Serine Transport into Astrocytes Adjacent to Synapses in the Mouse Brain. *Biomolecules*. 2023 May 11;13(5):819. doi: 10.3390/biom13050819.

3.2 Results

3.2.1 Identification of *Stratum radiatum* astrocytes and CA1 pyramidal neurons

To study D-serine (DS) transport in astrocytes, whole-cell patch clamp recordings were made from astrocytes in the *stratum radiatum* (*str*) layer of the CA1 hippocampal region in mouse brain slices. Astrocytes were visually identified using a Differential Interference Contrast (DIC) microscope, primarily relying on their small size (3-5 μm) and stellate morphology (Figure 3.1A, B). Conversely, in the subsequent experiments on CA1 pyramidal neurons, these were identified based on their location within the CA1 cell layer and their distinct triangle-shaped soma (Figure 3.2A, B).

The *str* astrocytes and CA1 pyramidal neurons were whole-cell voltage-clamped at -80 and -70 mV respectively. Astrocytes exhibited passive current responses when the membrane potential was changed. In contrast, the CA1 pyramidal neurons exhibited a non-linear current response and demonstrated characteristic fast voltage-gated sodium currents (inward flux of positive charge across the membrane) upon stepping up the membrane voltage to the voltage-gated Na^+ channel activation voltage (-40 mV) and a delayed outward potassium current (an efflux of positive charge across the membrane). Hence, the current-voltage (IV) relationships were used to confirm the cell identity (Figure 3.1C, Figure 3.2C). The input resistance, determined by the ratio of voltage to current responses in accordance with the classical Ohm's law ($V = IR$), was $43.0 \pm 3.0 \text{ M}\Omega$, $n=20$ (lower range) for *str* astrocytes and $303.4 \pm 30.0 \text{ M}\Omega$, $n=20$ (higher range) for CA1 pyramidal neurons respectively.

To differentiate and confirm the identity of these astrocytes, a solution containing 5% biocytin hydrochloride was included in the patch pipette. Subsequently, post hoc staining was performed using streptavidin-Alexa fluor-594, allowing the visualisation of astrocytic morphology and confirming their cell type (Figure 3.1 D). This staining technique along with IV relationship (Figure 3.1C) ensured the accurate identification of astrocytes.

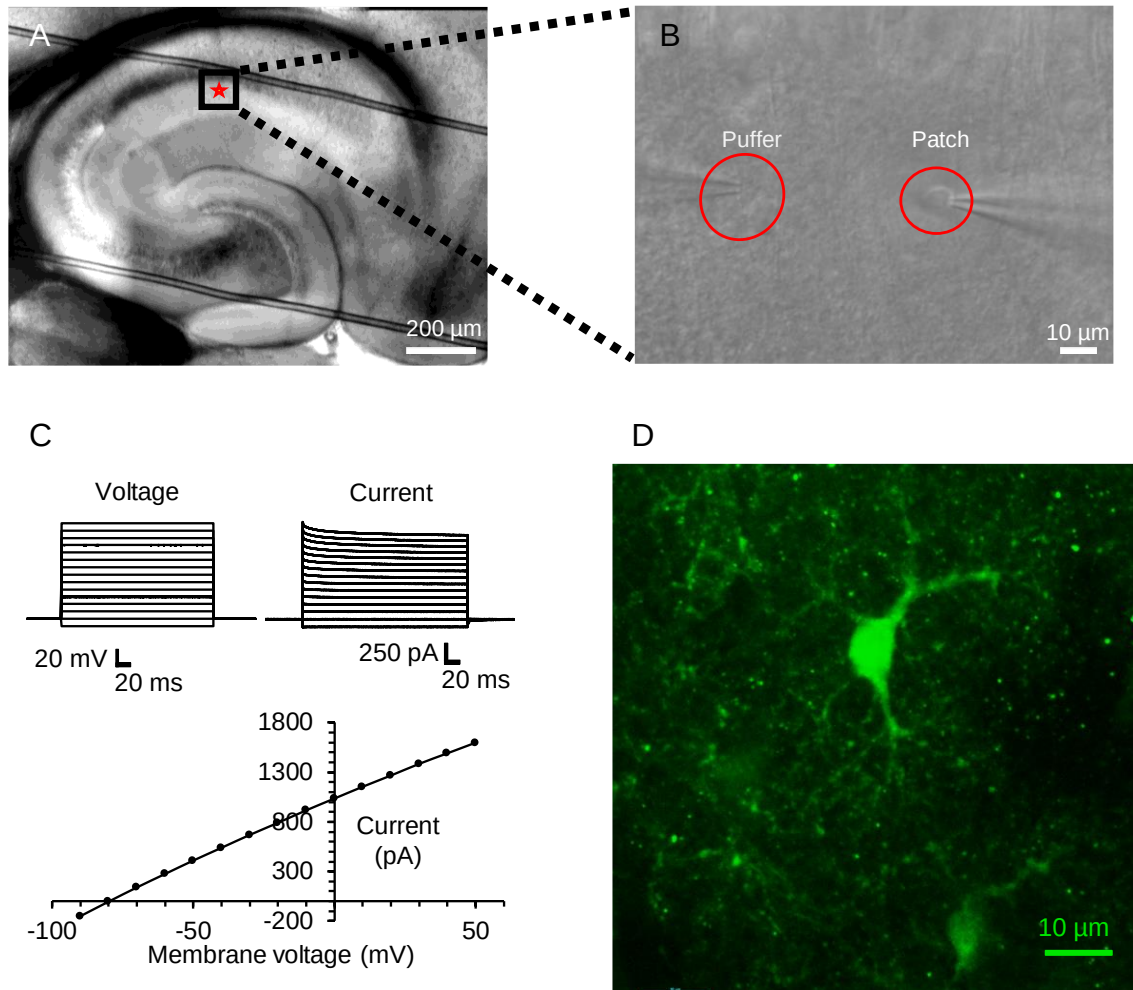


Figure 3.1: Visual and electrical properties used to positively identify CA1 astrocytes in whole-cell patch clamp recording.

(A) The Differential Interference Contrast (DIC) image depicts a mouse hippocampal brain slice, with a red star highlighting the recording location in the CA1 region's *stratum radiatum* layer. (B) A higher-magnification (x60 objective) DIC image of an astrocyte in the CA1 *stratum radiatum* layer. The astrocyte is subjected to whole-cell voltage-clamp at -80 mV via a glass patch pipette on the right. On the left, D-serine is applied via pressure ejection from a glass puffer pipette positioned about 40 μm away from the astrocytic soma. (C) The top panel displays a representative membrane current recorded from an astrocyte in response to 10 mV increments in membrane potential (-90 to +50 mV, on the left), indicating passive membrane properties. The bottom panel shows that the current-voltage relationship is approximately linear, indicating the absence of voltage-activated currents. The astrocyte has a negative resting potential of -80 mV and a low membrane resistance of 12.5 M Ω . (D) Post-hoc staining of a biocytin-filled astrocyte is visualised through confocal microscopy (Nikon A1) and displayed as a projected stack (captured using Elements software). This staining confirms the characteristic stellate morphology of the astrocyte.

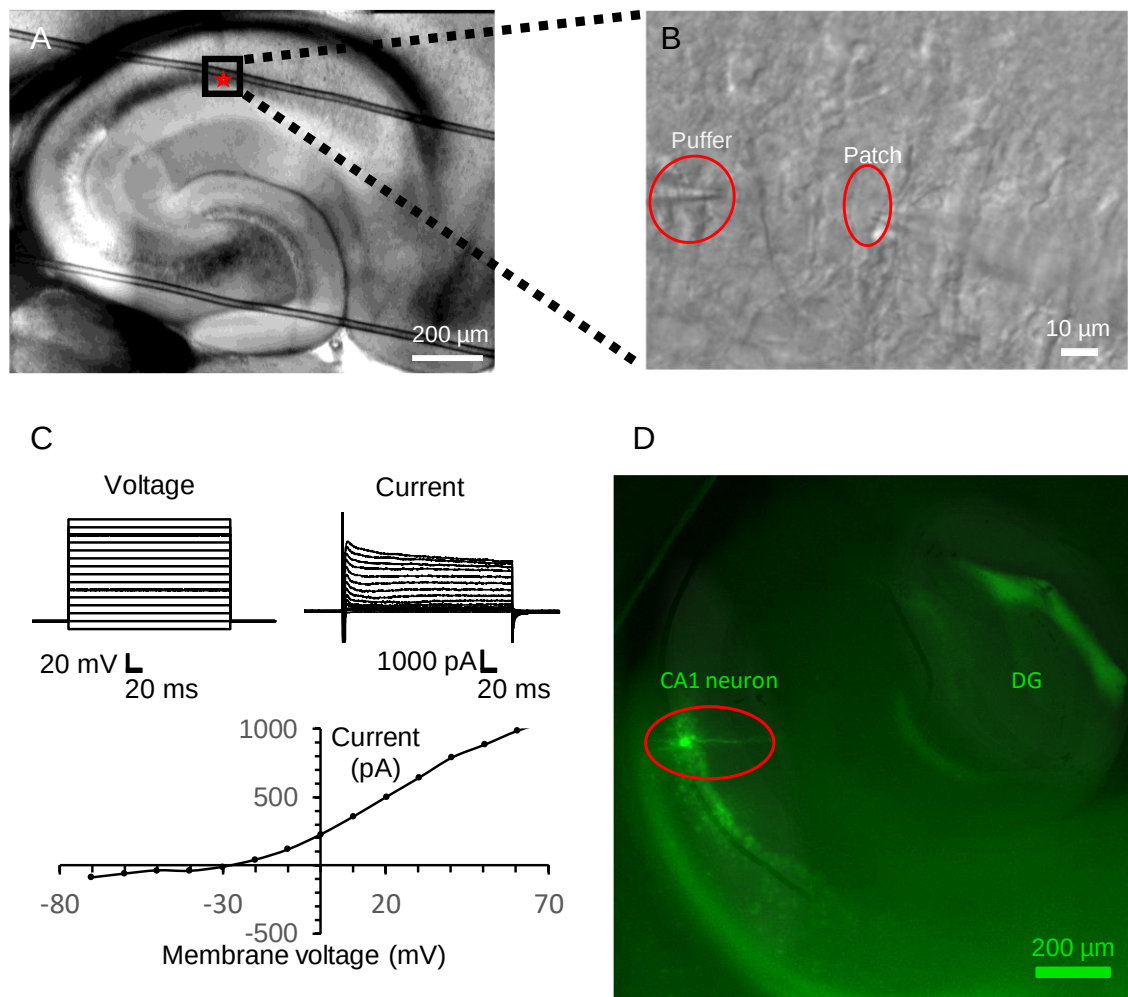


Figure 3.2: Visual and electrical properties used to identify CA1 pyramidal neurons in whole-cell patch clamp recording

(A) The Differential Interference Contrast (DIC) image depicts a mouse hippocampal brain slice, with a red star highlighting the recording location in the CA1 region's pyramidal cell layer. (B) A higher-magnification (x60 objective) DIC image of a pyramidal neuron in the CA1 cell layer. The pyramidal neuron is subjected to whole-cell voltage-clamp at -70 mV via a glass patch pipette on the right. On the left, D-serine is applied via pressure ejection from a glass puffer pipette positioned about 40 μm away from the neuronal soma. (C) The top panel displays a representative membrane current recorded from a CA1 pyramidal neuron in response to 10 mV increments in membrane potential (-80 to +60 mV, on the left), indicating active (voltage-dependent) properties. The bottom panel shows that the current-voltage relationship is non-linear, indicating the voltage-activated currents. The CA1 pyramidal neuron has a membrane resistance of 201.4 M Ω . (D) Post-hoc staining of a biocytin-filled neuron is visualised through confocal microscopy confirming the location of the neuron in the pyramidal cell layer. DG represents dentate gyrus.

3.2.2 D-serine uptake into the *stratum radiatum* astrocytes in the hippocampus

The first aim is to explore the transport mechanisms responsible for the uptake of synaptic DS by astrocytes surrounding excitatory synapses. Perisynaptic astrocytes have often been implicated in the regulation of neurotransmitters (Rosenberg et al., 2013), and prior radiolabelling investigations have indicated the uptake of DS into astrocytes and C6 glioma cells (Hayashi et al., 1997; Rosenberg et al., 2013). Hence, the potential mechanisms involved in the transport of synaptic DS into astrocytes in close proximity to the excitatory synapses in the *str* layer of CA1 was investigated.

3.2.2.1 Astrocytic D-serine-induced membrane currents I_{DS}

The initial objective was to determine the functional expression of DS transporters on the astrocytic plasma membranes. To achieve this, DS was used as a substrate to activate the DS transporters in *str* astrocytes. To provide more definitive evidence regarding the presence of functional transporters on the astrocytic membrane, direct electrophysiological recordings were conducted from these astrocytes. 10 mM DS was puff-applied via a glass pipette onto voltage-clamped *str* astrocytes (Figure 3.1B) and the resulting membrane currents were recorded. To specifically isolate the transporter currents induced by DS, all recordings were conducted in the presence of a cocktail of compounds that block ion channels and receptors (see methods section for details). A DS-induced membrane current (I_{DS}) was observed with an average amplitude of -18.8 ± 0.7 pA ($n = 68$; Figure 3.3A).

The application of a lower concentration of DS (1mM) onto voltage-clamped astrocytes did not elicit a significant ($p < 0.05$; compared to the control data set 3.2.2.2 with ACSF puff) membrane current response, with the observed I_{DS} amplitude averaging at -0.15 ± 0.8 pA ($n = 7$; Figure 3.3A). This finding is in line with the expected low affinity of ASCT1 for DS (Foster et al., 2016). It should be noted that puff-application of substrates in this manner results in a dilution of puff-pipette solution of approximately 1:10 (Todd et al., 2017). Hence, this technique does not allow an accurate measurement of the concentration of substrate reaching the transporter, or an assessment of the affinity of the transporter for the substrate.

3.2.2.2 Control experiment for puff application of substrates onto astrocytes

To ensure that the current observed during puff application was not a result of mechanical stimulation, a control experiment was performed. In this control, a puffer solution that did not contain DS was applied onto the astrocytes. The result was a negligible current of -0.15 ± 0.2

pA ($n = 7$; $p = 0.85$; one-sample t-test comparing the recorded current with a theoretical value of 0; Figure 3.3A), indicating that the astrocytic membrane current was not an artefact caused by the puff application itself. This confirms that the observed I_{DS} current is specifically linked to the presence of DS.

3.2.2.3 Current-voltage relationship of I_{DS}

To further understand the nature of I_{DS} , the current-voltage relationship was examined by comparing the current response to a ramp in membrane voltage both before and during the application of DS. I_{DS} remained relatively unaffected by changes in membrane voltage and did not exhibit a reversal of current at the reversal potential of any of the ions present in the recording solutions (Figure 3.3B, C). This finding strongly suggests that I_{DS} is minimally dependent on changes in membrane voltage, emphasising that I_{DS} is likely mediated by a membrane transporter current rather than being activated by an ion channel or ionotropic receptor.

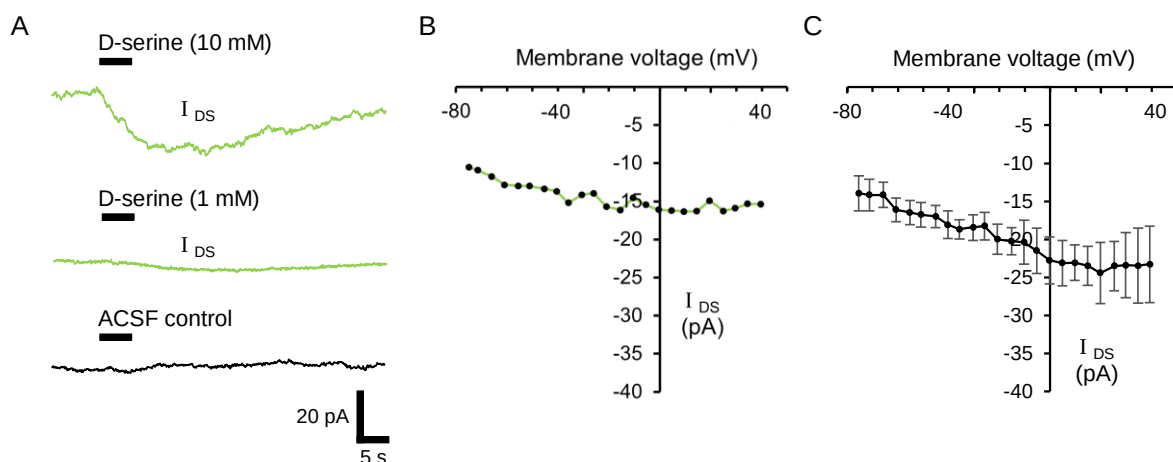


Figure 3.3: D-serine-mediated plasma membrane currents (I_{DS}) in CA1 stratum radiatum astrocytes are mediated by a transporter.

(A) A representative trace depicting an astrocytic membrane current induced by a 5-second puff application of 10 mM D-serine (I_{DS} ; top trace in green) and 1 mM D-serine (I_{DS} ; middle trace in green). Representative trace depicting an astrocytic membrane current in the control condition (external ACSF solution without D-serine; bottom trace in black). These traces are the average of three responses recorded at 1-minute intervals. (B) The current-voltage (IV) relationship of I_{DS} is illustrated using a voltage ramp protocol applied during the D-serine puff. Notably, this graph shows that there is no reversal of current at positive potentials, further supporting the involvement of a transporter in mediating these currents. (C) Average IV relationship of I_{DS} , with the error bars indicating the SEM values ($n = 6$).

3.2.2.4 Lack of D-serine uptake in CA1 pyramidal neurons, stratum radiatum interneurons and NG2 cells

An examination of DS uptake into CA1 pyramidal neurons, *str* interneurons and NG2 positive oligodendrocyte precursor cells was conducted to determine the functional expression of DS transporters on their plasma membranes. While identification of CA1 pyramidal neurons was relatively straightforward (Figure 3.2C), some NG2 positive glia and inhibitory interneurons were occasionally recorded, due to the visual resemblance in some soma shape and size with astrocytes. However, the putative NG2 positive glia could be distinguished by their high membrane resistance, outwardly rectifying current-voltage relationship and lack of action potential firing (Figure 3.4A) and the interneurons could consistently be distinguished by their depolarisation-induced inward Na^+ currents, as measured in their IV relationship (Figure 3.4B).

Further, utilising 10 mM DS as a substrate to activate DS transporters, the observations revealed a negligible current of -1.1 ± 0.4 pA ($n=19$) in CA1 pyramidal neurons, of -0.46 ± 0.6 pA ($n = 13$) in putative NG2 glial cells and of -0.36 ± 0.2 pA ($n = 10$) in *str* interneurons ($p > 0.05$; compared to the control data set with ACSF puff; Figure 3.4C). This indicates that DS does not evoke any current response through DS transporters in CA1 pyramidal neurons, putative NG2 precursor cells and *str* interneurons. These observations suggest a lack of functional electrogenic DS transporters on the neuronal and NG2 cell plasma membranes, contrasting with the observed uptake in perisynaptic astrocytes. These results contribute to our understanding of the cellular route of DS uptake within the hippocampal CA1 region.

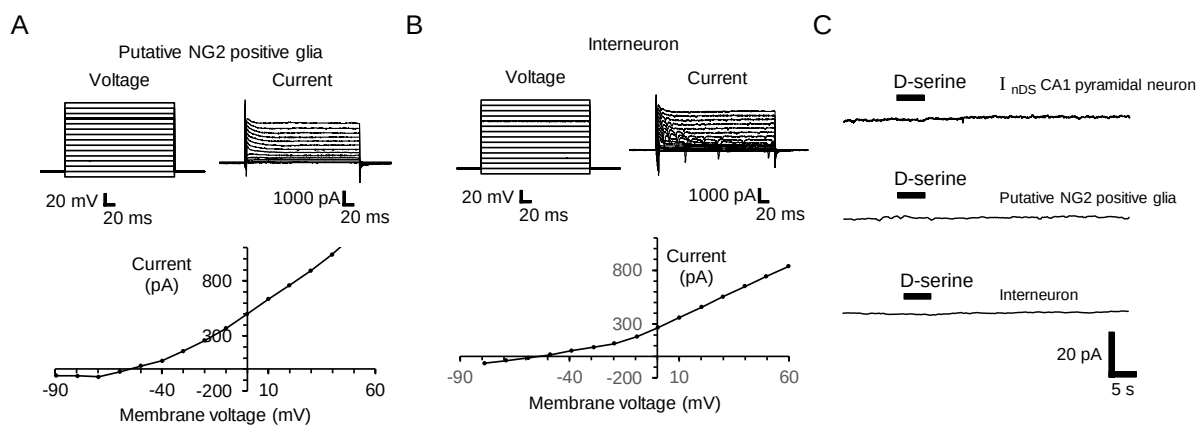


Figure 3.4: Lack of D-serine uptake in CA1 pyramidal neurons, stratum radiatum interneurons and putative NG2 positive glial cells.

(A and B) Top panel: Representative membrane current (on the right) recorded from a putative NG2 positive glia and *stratum radiatum* interneurons respectively, in response to 10 mV increments in membrane potential (-90 to +50 mV for glia and -80 to +60 mV for interneuron, on the left), indicating active (voltage-dependent) properties. Bottom panel: Non-linear current-voltage relationship indicating voltage-activated currents. (C) Utilising 10 mM D-serine as a substrate to activate D-serine transporters,

CA1 pyramidal neurons, *str* NG2 positive glial cells and interneurons exhibited no significant D-serine-induced membrane currents. All traces are averages of three responses recorded at 1-minute intervals.

3.2.3 Astrocytic transport of DS is mediated by ASCT transporters

After confirming the presence of a DS transport current in the *str* astrocytes, the objective was to identify which membrane transporter was responsible for this uptake. In the central nervous system, there are several DS transporters, including ASCT1 (*Slc1a4*), ASCT2 (*Slc1a5*), SNAT1 (*Slc38a1*), SNAT2 (*Slc38a2*), asc-1 (*Slc7a10*), and LAT1 (*Slc7a5*). To determine which transporter mediates I_{DS} , O-benzyl-L-serine was added to the external solution. This compound is a specific substrate inhibitor known to inhibit both isoforms of alanine serine cysteine transporters, ASCT1 and ASCT2 (Grewer and Grabsch, 2004). 10 mM O-benzyl-L-serine was bath applied prior to and during the DS puffs onto the *str* astrocytes. The addition of 10 mM O-benzyl-L-serine significantly reduced I_{DS} by 84.4% (from $I_{DS} = -22.4 \pm 1.3$ pA in control to -3.5 ± 0.4 pA in the presence of 10 mM benzyl serine; $n = 5$; $p < 0.001$; Figure 3.5A, B). This result indicates that I_{DS} is primarily mediated by ASCT transporters.

Moreover, experiments were conducted involving the application of 10 mM trans-4-hydroxy-proline (T-Pro) to bath solutions both before and during the administration of DS puffs onto the astrocytes in the *str* layer. T-Pro is a competitive substrate known for its specificity towards the ASCT1 isoform (Pinilla-Tenas et al., 2003; Foster et al., 2016). The application of T-Pro significantly reduced the recorded I_{DS} from astrocytes (from $I_{DS} = -17.8 \pm 3.0$ pA in control to I_{DS} in the presence of 10 mM T-Pro = -3.8 ± 1.4 pA; $n = 7$; $p < 0.001$; resulting in a reduction of 78.4%, Figure 3.5C, D). These results provide strong evidence that I_{DS} is predominantly mediated by ASCT transporters, implying a potential role for ASCT1 in the removal of DS from the synaptic cleft.

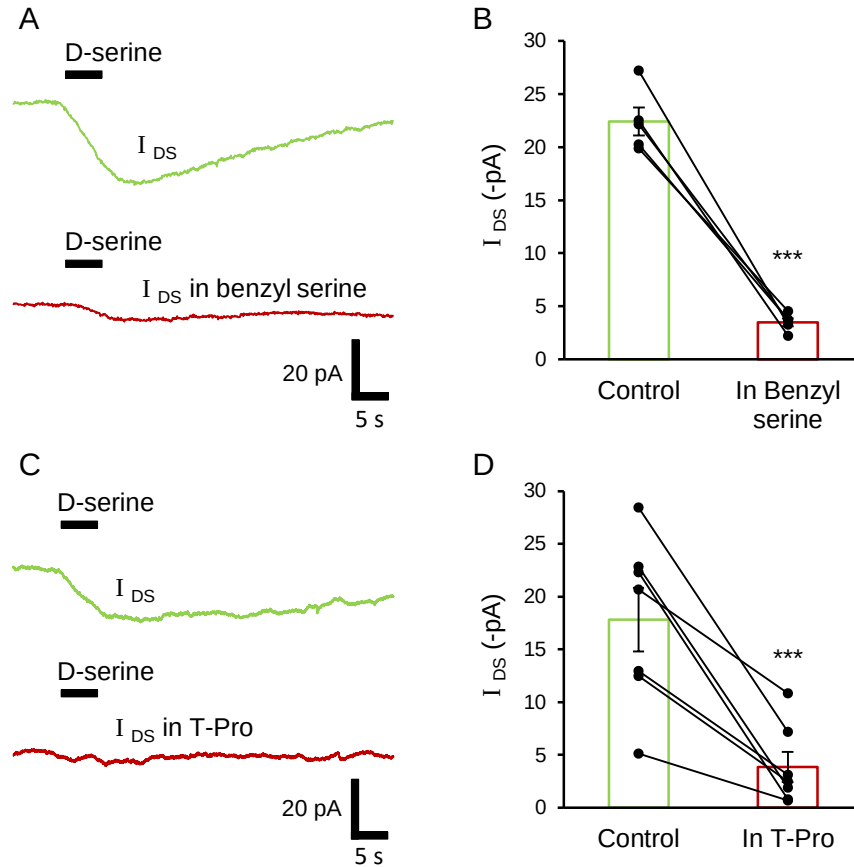


Figure 3.5: Astrocytic D-serine current (I_{DS}) is mediated by ASCT transporters: Inhibition by benzyl serine or trans-4-hydroxy-proline

The raw data trace presented in panels (A) and (C) demonstrate that the bath application of 10 mM benzyl serine or 10 mM trans-4-hydroxy-proline (T-Pro) which inhibits ASCTs, significantly decreased the D-serine induced membrane current I_{DS} . The green traces represent the current before the drug application, and the dark red traces represent the current after the drug application. (B) The averaged data demonstrate that bath application of benzyl-serine, an inhibitor of ASCT transporters, reduced I_{DS} by 84.4%. (D) Averaged data show that bath application of 10 mM T-Pro, an ASCT1-specific substrate, reduced I_{DS} to a similar extent as benzyl serine (78.4%), indicating the involvement of ASCT1 in mediating I_{DS} . *** represents a significant difference at $p \leq 0.001$.

Furthermore, experiments were conducted involving the application of 10 mM L-glutamine (L-gln; a non-specific ASCT substrate; Broer et al. 1999; Foster et al. 2016) to bath solutions both before and during the administration of DS puffs onto the astrocytes in the *str* layer. The application of L-gln significantly reduced the recorded I_{DS} from astrocytes (from $I_{DS} = -17.2 \pm 2.0$ pA in control to I_{DS} in the presence of 10 mM L-gln = -5.0 ± 1.1 pA; $n = 10$; $p < 0.001$; resulting in a reduction of 71.6%, Figure 3.6A, B). In addition, experiments were conducted involving the application of a combination of 10 mM T-Pro and 10 mM L-gln. The application of a combination of L-gln and T-Pro significantly reduced the recorded I_{DS} from astrocytes (from $I_{DS} = -18.8 \pm 3.0$ pA in control to I_{DS} in the presence of 10 mM T-Pro and L-gln = -2.5 ± 1.2 pA; $n = 6$; $p < 0.001$; resulting in a reduction of 86.5%, Figure 3.6C, D). These results

provide further supporting evidence that I_{DS} is predominantly mediated by ASCT transporters, underscoring their essential role in clearing DS from the synaptic cleft.

To investigate whether there was any additive effect of T-Pro and L-gln in the inhibition of I_{DS} , statistical comparisons were performed between the groups - T-Pro vs L-gln, T-Pro vs T-Pro + L-gln and L-gln vs T-Pro + L-gln. This did not reveal statistically significant differences in the measured responses ($p > 0.05$ for all comparisons), indicating a lack of any additive effect of T-Pro and L-gln and suggesting that they are both inhibiting the same transporter.

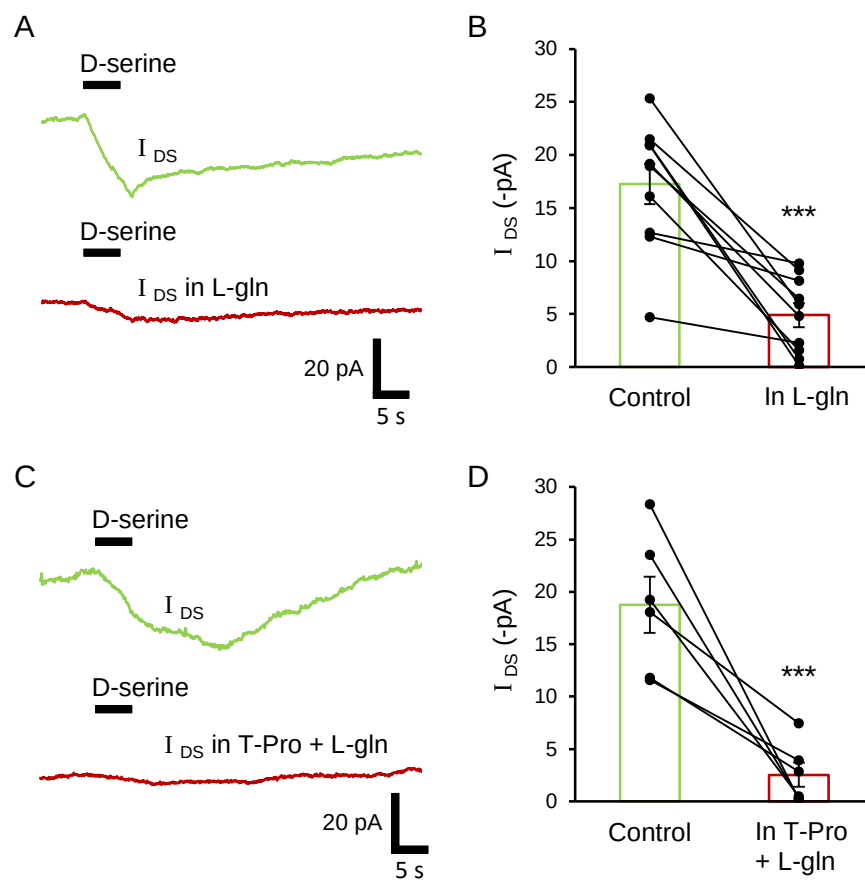


Figure 3.6: Astrocytic D-serine current (I_{DS}) indicating ASCT-mediated transport: Inhibition by L-glutamine or a combination of T-Pro and L-glutamine

The raw data trace presented in panels (A) and (C) demonstrate that the bath application of 10 mM L-glutamine (L-gln) or a combination of 10 mM L-glutamine and 10 mM T-Pro which inhibits ASCTs, significantly decreased the D-serine induced membrane current I_{DS} . The green traces represent the current before the drug application, and the dark red traces represent the current after the drug application. (B) The averaged data demonstrate that bath application of L-glutamine, an inhibitor of ASCT transporters, reduced I_{DS} by 71.6%. (D) Averaged data show that bath application of a combination of 10 mM T-Pro and 10 mM L-glutamine, reduced I_{DS} to a similar extent as benzyl serine (86.5%), supporting the involvement of ASCTs in mediating I_{DS} . *** represents a significant difference at $p \leq 0.001$.

Finally, the objective was to investigate whether DS uptake currents in *str* astrocytes altered with age of animal, as DS levels have previously been suggested to be developmentally regulated (Billard, 2015; Jagannath et al., 2017). Previously, I_{DS} was recorded in P21-25-day-old mice. In this experiment, older P30 and younger P6 mice were used. Puff application of 10 mM DS onto the *str* astrocytes evoked an I_{DS} of -17.1 ± 2.0 pA ($n = 4$; P30 day-old mice) and of -15.7 ± 7.0 pA ($n = 3$; P6 day-old mice). The analysis of baseline I_{DS} measurements at P6, P21-25, and P30 did not reveal any significant age-dependent differences ($p = 0.59$ between P21-25 and P30; $p = 0.82$ between P21-25 and P6). This suggests that the baseline current does not change with age.

Further, the addition of 10 mM O-benzyl-L-serine to the external bath solution significantly reduced I_{DS} in P30 mice (to -2.4 ± 1.0 pA; $n = 4$; reduction of 86.1%; $p < 0.001$; compared to control condition without benzyl serine; Figure 3.7A, B). Similarly, the addition of 10 mM O-benzyl-L-serine significantly reduced I_{DS} observed in P6 day-old mice (to -5.1 ± 4.0 pA; $n = 3$; reduction of 67.5%; $p < 0.01$; compared to control condition without benzyl serine; Figure 3.7C, D). These results suggest that the DS uptake is still mediated by the same process over development in the range P6 to 30.

Additionally, the analysis of percentage reduction in I_{DS} with benzyl serine across different age groups showed no significant differences ($p = 0.42$ between P21-25 and P30; $p = 0.74$ between P21-25 and P6). This shows that the effectiveness of benzyl serine in reducing I_{DS} is not significantly dependent on age. Together, these findings demonstrate that the reduction in I_{DS} with benzyl serine is effective across different developmental stages, supporting that the ASCTs mediate I_{DS} consistently.

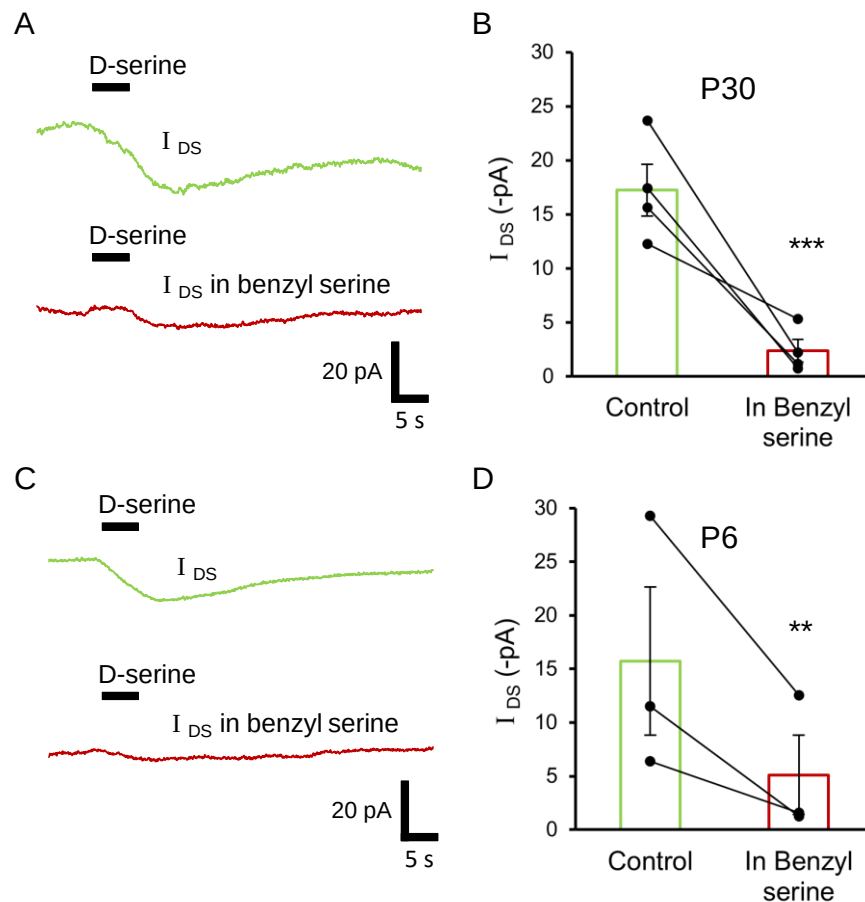


Figure 3.7: Astrocytic D-serine current (I_{DS}) is mediated by ASCT transporters in P30 and P6 mice

The raw data trace presented in panels (A) and (C) demonstrates that the bath application of 10 mM benzyl serine which inhibits ASCTs, significantly decreased the D-serine-induced membrane current I_{DS} in P30 and P6 mice respectively. The green traces represent the current before the benzyl serine application, and the dark red traces represent the current after the benzyl serine application. (B) and (D) The averaged data demonstrate that bath application of benzyl-serine, reduced I_{DS} by 86.1% in P30 mice and by 67.5% in P6 mice. ** and *** represents a significant difference at $p \leq 0.01$ $p \leq 0.001$ respectively.

3.2.4 System A transporters do not mediate the uptake of D-serine in *stratum radiatum* astrocytes

Apart from ASCT transporters, the system A transporters SNAT1 or 2 (*Slc38a1* or *Slc38a2*) could mediate an electrogenic DS uptake if they are expressed on the astrocytic membranes. To explore the contribution of potential alternative DS transporters responsible for I_{DS} , the pharmacological agent specifically, α -(methylamino) isobutyric acid (MeAIB) was added into the external solution, prior to and during the DS puffs onto the *str* astrocytes. This compound is known to inhibit System A transport activity (Christensen et al., 1965). More recent studies have demonstrated the impact of MeAIB on SNAT1 and SNAT2 transporters (Grewal et al., 2009; Takahashi et al., 2017; Yamada et al., 2019). However, I_{DS} remained insensitive to 10

mM MeAIB (I_{DS} control = -14.3 ± 1.2 pA; I_{DS} in presence of 10 mM MeAIB = -16.6 ± 1.2 pA; $n = 4$; $p = 0.60$; Figure 3.8A, B) or 30 mM MeAIB (I_{DS} control = -21.1 ± 3.6 pA; I_{DS} in presence of 30 mM MeAIB = -18.9 ± 2.6 pA; $n = 4$; $p = 0.88$; Figure 3.8C, D). This suggests that SNAT1 or 2 do not play a role in mediating astrocytic I_{DS} .

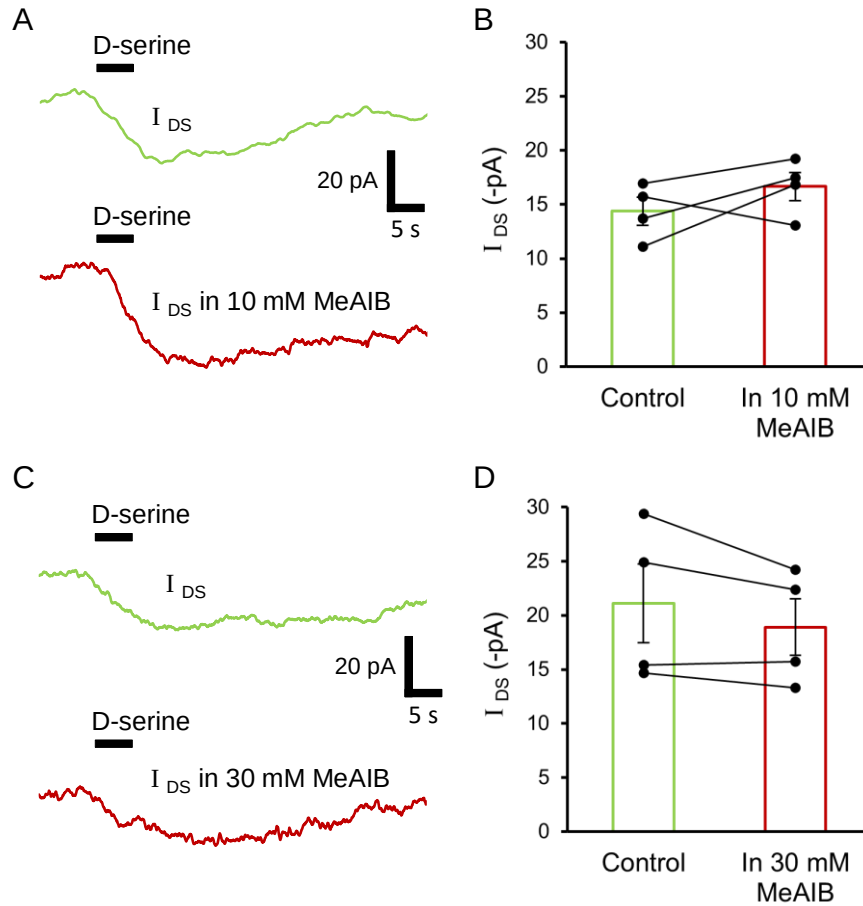


Figure 3.8: Astrocytic D-serine current (I_{DS}) is not mediated by SNAT1/2 transporters

The raw data trace presented in panels (A) and (C) demonstrate that the bath application of both 10 mM and 30 mM α -(methylamino) isobutyric acid (MeAIB), which specifically inhibits SNAT1 or SNAT2, did not have any significant effect on the D-serine induced membrane current I_{DS} . The green traces represent the current before the MeAIB application, and the dark red traces represent the current after the MeAIB application. (B) and (D) Averaged data show that bath application of 10 mM or 30 mM MeAIB respectively, a SNAT1/2 transporter substrate, did not reduce I_{DS} .

3.2.5 Asc-1 transporter does not mediate the electrogenic uptake of D-serine in *Stratum radiatum* astrocytes

To investigate whether the sodium-independent alanine-serine-cysteine transporter asc-1 transporter (*Slc7a10*) plays a role in generating I_{DS} , experiments were conducted in which S-methyl-L-cysteine (SMLC) was added to the external bath solution. This compound blocks any asc-1 transporter-mediated current (Ishiwata et al., 2013) and was applied prior to and during puff-applied 10 mM DS onto the *str* astrocytes. The observations showed that I_{DS} was not altered in the presence of either 5 mM SMLC (I_{DS} control = -15.8 ± 1.6 pA; I_{DS} in the presence of 5 mM SMLC = -17.8 ± 1.6 ; $n = 5$; $p = 0.86$; Figure 3.9A, B) or 10 mM SMLC (I_{DS} control = -20.7 ± 1.9 ; I_{DS} in the presence of 10 mM SMLC = -21.7 ± 2.5 ; $n = 5$; $p = 0.24$; Figure 3.9C, D). These results strongly indicate that the asc-1 transporter does not contribute to I_{DS} recorded from astrocytes.

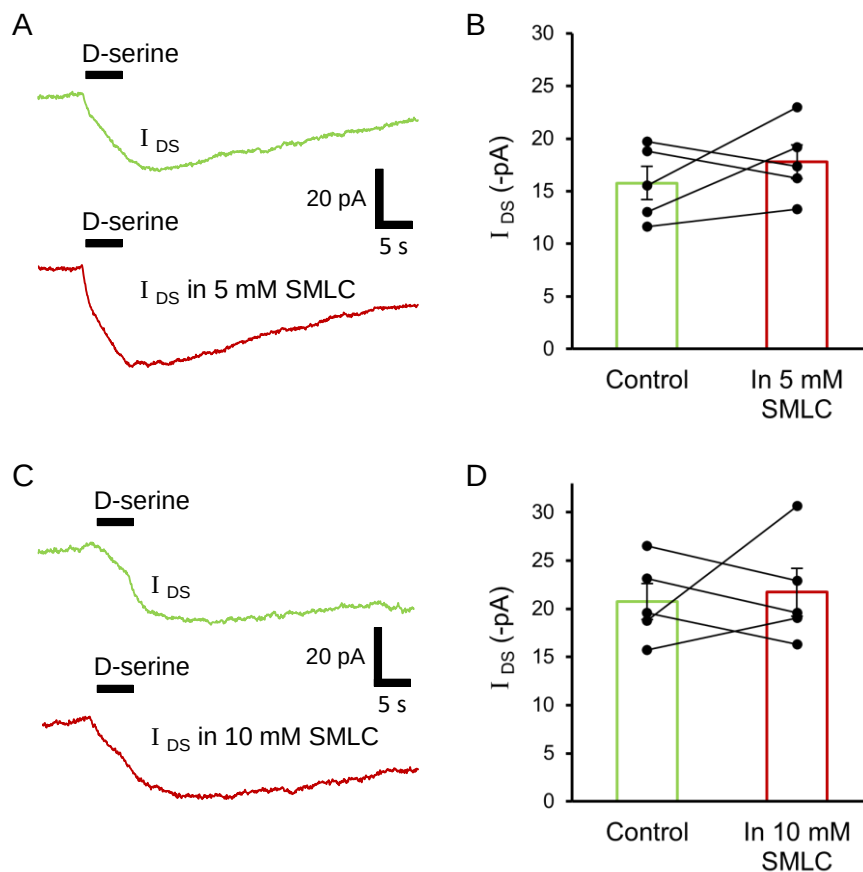


Figure 3.9: Astrocytic D-serine current (I_{DS}) is not mediated by asc-1 transporters.

The raw data trace presented in panels (A) and (C) demonstrate that the bath application of 5 mM and 10 mM S-methyl-L-cysteine (SMLC), which specifically inhibits the asc-1 transporter, did not have any significant effect on the D-serine-induced membrane current I_{DS} . The green traces represent the current before the SMLC application, and the dark red traces represent the current after the SMLC application. (B) and (D) Averaged data show that bath application of 5 mM or 10 mM SMLC respectively, did not reduce I_{DS} .

3.2.6 LAT transporters do not mediate the uptake of D-serine in *Stratum radiatum* astrocytes

Finally, to investigate whether the LAT1 transporter (*Slc7a5*) plays a role in generating I_{DS} , experiments were conducted in which 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) was added to the external bath solution. This compound blocks any LAT transporter-mediated current (Kanai et al., 1998) and was applied prior to and during puff-applied 10 mM DS onto the str astrocytes. The observations showed that I_{DS} was not altered in the presence of 5 mM BCH (I_{DS} control = -19.0 ± 1.7 ; I_{DS} in the presence of 5 mM BCH = -17.3 ± 0.7 ; $n = 8$; $p = 0.38$; Figure 3.10A, B), indicating that the LAT transporters do not contribute to I_{DS} recorded from astrocytes.

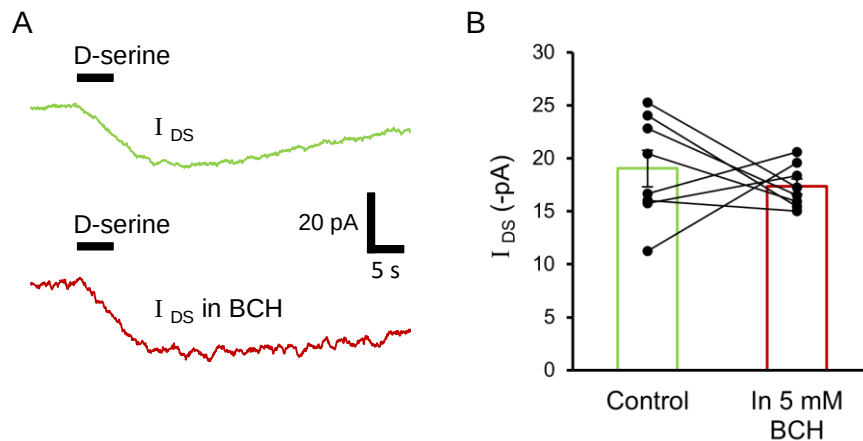


Figure 3.10: Astrocytic D-serine current (I_{DS}) is not mediated by LAT transporters

The raw data trace presented in panel (A) demonstrates that the bath application of 5 mM 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), which inhibits LAT transporters, did not have any significant effect on the D-serine-induced membrane current I_{DS} . The green traces represent the current before the BCH application, and the dark red traces represent the current after the BCH application. (B) Averaged data show that bath application of 5 mM BCH, did not reduce I_{DS} .

3.2.7 I_{DS} is not mediated by an uncoupled anion channel current

Having established that the uptake of DS is predominantly mediated by ASCT1 transporters and not by the SNAT1/2, asc-1 and LAT transporters, the nature of the DS-induced current was further examined. ASCT transporters can generate trans-membrane currents through the coupled transport of charged substrates such as Na^+ and via the opening of an integral anion channel, resulting in uncoupled anion currents (Zerangue and Kavanaugh, 1996; Scalise et al., 2014; Mazza et al., 2021).

In the previous experiments, I_{DS} was recorded with an intracellular recording solution containing sodium and alanine to provide substrates for ASCT-mediated amino acid exchange, and thiocyanate ions (SCN^-) to provide a permeant ion for any potential uncoupled anion channel. To discern the relative contributions of coupled charge movement and uncoupled anion channel activation in generating I_{DS} , experiments were conducted where intracellular SCN^- were replaced with Cl^- ions. Cl^- ions exhibit significantly reduced permeability through ASCT's integral anion channel compared to SCN^- , with an expected SCN^- current 100 to 1000 times more than that of Cl^- ions at -80 mV membrane potential (Zerangue and Kavanaugh, 1996; Broer et al., 2000). However, this replacement of SCN^- with Cl^- did not result in any significant alteration in I_{DS} (I_{DS} with intracellular SCN^- = -18.8 ± 0.7 pA; $n = 68$, and with intracellular Cl^- = -15.7 ± 0.9 pA; $n = 9$; $p = 0.07$; Figure 3.11A).

To confirm if I_{DS} still corresponds to activation of ASCT1 when the intracellular SCN^- were replaced with Cl^- ions, trans-4-hydroxy-proline (T-Pro), an ASCT1 inhibitor was added to the external solution. 10 mM T-Pro was bath applied prior to and during the DS puffs onto the *str* astrocytes. This addition significantly reduced I_{DS} by 84.0% (from $I_{DS} = -15.8 \pm 2.0$ pA in control to -2.5 ± 1.0 pA in the presence of 10 mM benzyl serine; $n = 4$; $p < 0.001$; Figure 3.11B, C). This result in conjunction with results from Figure 3.5C, D further supports that I_{DS} is indeed mediated by ASCT1 transporters.

These results, in conjunction with the absence of substantial voltage dependence (as shown in Figure 3.3B, C), suggests that I_{DS} is not primarily mediated by the gating of an uncoupled anion channel. Instead, it suggests that I_{DS} is generated predominantly through the coupled electrogenic transport of DS into astrocytes.

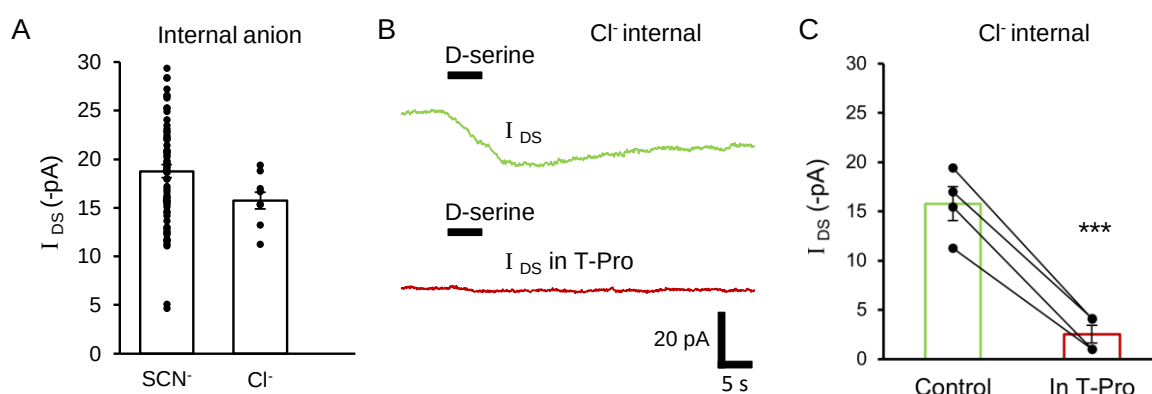


Figure 3.11: D-serine transport currents do not rely on uncoupled anion movement via ASCT1s integrated ion channels

(A) Astrocytic D-serine current (I_{DS}) remains unaltered when intracellular SCN^- is substituted with Cl^- . This suggests that the transport current is intricately linked to DS uptake and is not caused by the activation of an uncoupled anion channel within the transporter's structure. The raw data trace presented in panel (B) demonstrates that the bath application of trans-4-hydroxy-proline (T-Pro), an ASCT1 inhibitor, significantly reduced the D-serine-induced membrane current I_{DS} recorded from astrocytes voltage-clamped with a Cl^- based intracellular solution. The green traces represent the current before the T-Pro application, and the dark red traces represent the current after the T-Pro application. (C) Averaged data show that bath application of 10 mM T-Pro, reduced I_{DS} by 84.0%, supporting the involvement of ASCT1 activation in the astrocytes, measured by a coupled transporter current. *** represents a significant difference at $p \leq 0.001$.

3.2.8 D-serine transport in other brain areas

To explore whether glial cells in various brain regions also employ ASCT transporters for DS transport, astrocytes in the somatosensory cortex's layer 2 and Bergmann glia in the cerebellum were investigated (Figure 3.12Aa). Bergmann glial cell somata were identified based on their location within the Purkinje cell layer and their relatively small diameter, typically ranging from 5 to 10 micrometres (Figure 3.12Ab, c). Similar to the observations in hippocampal CA1 astrocytes, the application of 10 mM DS via puffing onto glial cells in the cortex and cerebellum induced transporter currents. These currents were found to be sensitive to O-benzyl-L-serine. In cortical astrocytes, an I_{DS} of approximately -20.1 ± 0.8 pA was recorded, which decreased significantly to -6.7 ± 1.0 pA in the presence of 10 mM O-benzyl-L-serine ($n = 4$; $p = 0.001$; Figure 3.12B, C). Likewise, cerebellar Bergmann glia exhibited an I_{DS} of approximately -17.0 ± 2.6 pA, which was notably reduced to -1.3 ± 1.3 pA when 10 mM O-benzyl-L-serine was introduced ($n = 4$; $p < 0.001$; Figure 3.12D, E). These findings illustrate that ASCT-mediated DS transport into glial cells is a widespread phenomenon occurring in various brain regions.

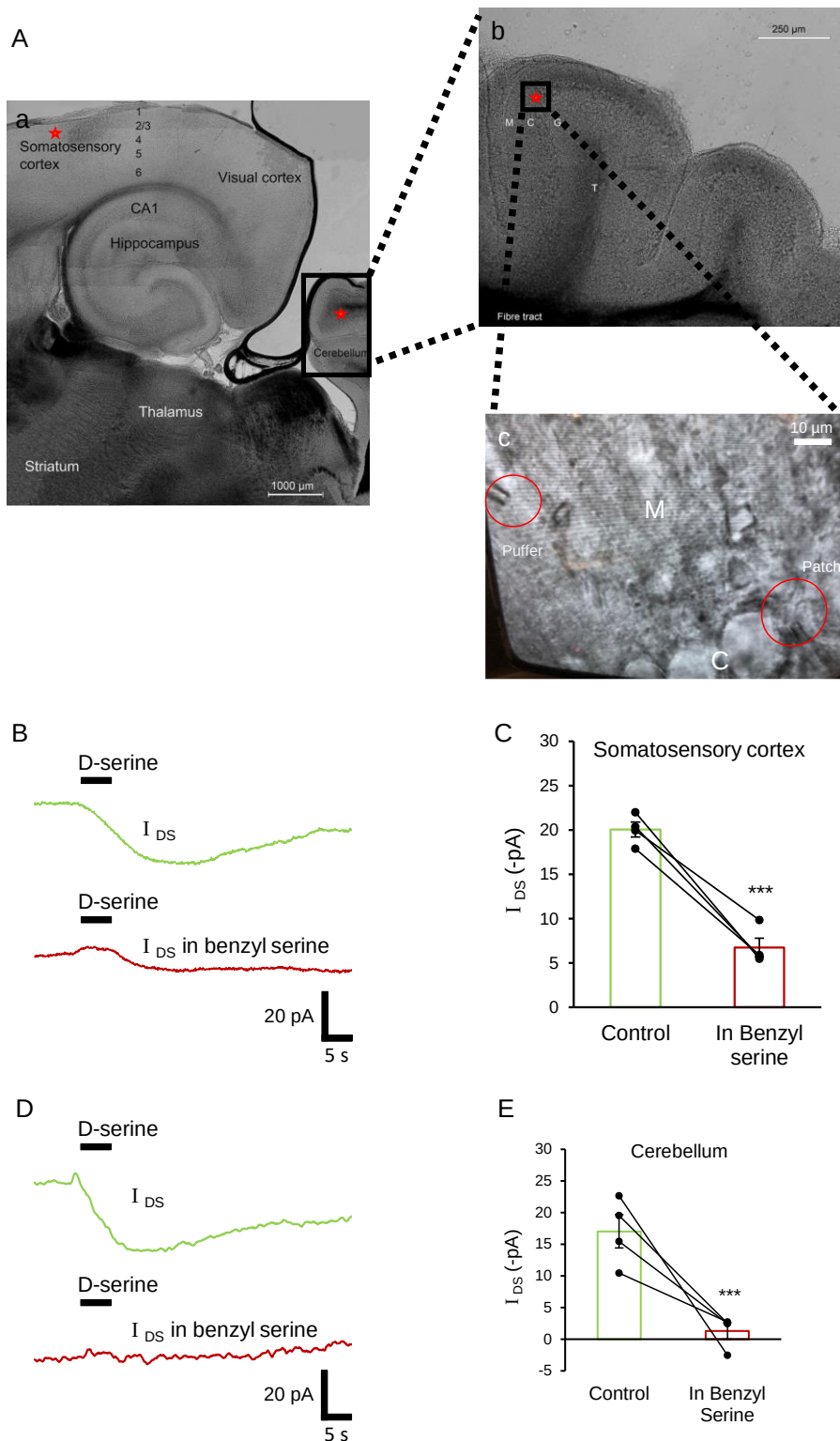


Figure 3.12: Glial D-serine transport is mediated by ASCT transporters in the somatosensory cortex and cerebellum

(A) In panel (a) sagittal section of a mouse brain focussed under a simple microscope is shown, with stars indicating the regions of recording, and numbers denoting the cortical layers. Panel (b) focuses on the cerebellum lobe, with labels M, C, G and T representing the molecular, Purkinje cell, granular layers and tract. A star highlights the location of Bergmann glial cells in the Purkinje cell layer. Panel (c) shows a higher-magnification (x60 objective) DIC image of Bergmann glia in the Purkinje cell layer (label C).

The glial cell is subjected to a whole-cell voltage-clamp at -80 mV via a glass patch pipette highlighted on the right. On the left, D-serine is applied via pressure ejection from a glass puffer pipette positioned about 70 μm away from the soma, in the molecular layer (label M). The raw data trace presented in panels (B) and (D) demonstrates that the bath application of 10 mM benzyl serine, significantly decreased the D-serine-induced membrane current I_{DS} in astrocytes of layer 2 somatosensory cortex and in Bergmann glia of cerebellum respectively. The green traces represent the current before the benzyl serine application, and the dark red traces represent the current after the benzyl serine application. (C and E) The averaged data demonstrate that bath application of benzyl-serine, reduced I_{DS} by 66.2% in the cortex and by 92.5% in the cerebellum, demonstrating ASCT-mediated DS transport in cortical and cerebellar glia. *** Indicates a significant difference at $p \leq 0.001$.

3.3 Discussion

D-serine (DS), an endogenous co-agonist at the synaptic NMDARs, plays a fundamental role in modulating excitatory synaptic neurotransmission and plasticity (Mothet et al., 2000; Panatier et al., 2006; Henneberger et al., 2010). However, the mechanisms governing its homeostatic regulation and clearance have remained a subject of extensive research. Perisynaptic astrocytes have frequently been implicated in the precise control of neurotransmitter levels within the synaptic cleft (Rosenberg et al., 2013). Previous studies employing radiolabelling techniques have provided compelling evidence of DS uptake into astrocytes as well as C6 glioma cells (Hayashi et al., 1997; Ribeiro et al., 2002). These investigations collectively underscore the role of astrocytes in modulating neurotransmitter dynamics, with a specific focus on the intriguing case of DS transport. In line with this, the overarching aim of this chapter was to gain insights into the transport mechanisms governing DS and consequently, to enhance our understanding of its intricate regulation within the mammalian brain. The working hypothesis posited that astrocytes represent likely candidates for facilitating the uptake of DS from the extracellular space. The results of this chapter shed light on the mechanism of DS uptake in astrocytes, a process that is expected to modulate excitatory neurotransmission and NMDAR activity. Through electrophysiological characterisation, compelling evidence has been provided revealing that DS uptake occurs within astrocytes positioned in close proximity to excitatory synapses, utilising acutely isolated brain tissue of mice. Previous investigations into DS transport have predominantly focused on cultured synaptosomes, cultured astrocytes, and transfected cell lines (Rosenberg et al., 2013; Foster et al., 2016; Kaplan et al., 2018). However, synaptosomes inherently comprise both neuronal and glial membranes, and cell culture conditions can influence the expression of membrane transporters (Cahoy et al., 2008). While the results presented here align broadly with those of prior research, the unique approach of conducting direct electrophysiological recordings from precisely identified individual astrocytes in their natural environment allows to comprehensively characterise DS transport within cells, preserving their native conditions and physiological context.

3.3.1 Uptake of D-serine is mediated by astrocytic ASCT1 transporter

Within the context of this investigation, the overarching objective was to elucidate the mechanisms underlying DS uptake in astrocytes and to understand the pivotal role of astrocytic sodium-dependent alanine serine cysteine transporters (ASCT; a product of *Slc1a4/5* genes) in this process. It is posited that ASCT transporters, expressed in astrocytic membranes, would be key contributors to the observed transport of DS. To address this, conventional electrophysiological voltage-clamp techniques were employed. Specifically, a substrate puffing approach was employed, enabling precise temporal control of DS application near the astrocytes of interest. This approach facilitated the measurement of small currents associated with the transport process. In line with this hypothesis, electrogenic DS transport was identified in hippocampal astrocytes located in the CA1 *stratum radiatum*, a region crucial for regulating excitatory neurotransmission at CA3-CA1 *Schaffer collateral* synapses (Perea et al., 2009; Papouin et al., 2017). This investigation reveals that this transport mechanism is highly sensitive to O-benzyl-L-serine, pointing to the involvement of ASCT transporters (Figure 3.5A, B and Figure 3.7). Notably, similar outcomes were observed in astrocytes from the somatosensory cortex and Bergmann glia in the cerebellum, indicating the ubiquity of this DS uptake process across diverse brain regions (Figure 3.12).

In addition, the recorded currents from voltage-clamped astrocytes exclusively originated from the astrocytes themselves and not from neighbouring neurons, given the absence of electrical coupling between adjacent neurons and astrocytes. Previous dual recordings of astrocytes and neurons demonstrated no astrocytic current responses when neighbouring neurons were firing action potentials (Pannasch et al., 2012).

The application of 1mM DS did not elicit a significant membrane current response, (Figure 3.3A). This finding is in line with the results reported by Foster et al. (2016), suggesting an affinity of DS in the low millimolar (mM) range. However, it is crucial to acknowledge the limitations associated with the experimental setup. One major consideration is the dilution factor resulting from the puffing technique, which hampers the accurate measurement of the actual DS concentration reaching the astrocytic soma. This dilution effect might have influenced the ability to detect a significant membrane current response at the 1 mM concentration. Assuming a dilution factor of 1:10 (Todd et al., 2017), the lack of response to 1 mM DS puffs (where 100 μ M reaches the cell) suggests an affinity for DS of at least several hundred μ M. The precise affinity of DS and its physiological implications warrant further investigation, considering the complexities introduced by the dilution factor in the current experimental approach.

Among the two ASCT transporter isoforms, ASCT1 (*Slc1a4*) and ASCT2 (*Slc1a5*), my data predominantly suggest the activation of ASCT1 based on the inhibition by trans-4-hydroxy-proline (Figure 3.5C, D and Figure 3.11B, C). This aligns with existing gene and protein expression studies, which indicate the presence of ASCT2 in cultured astrocytes (Broer et al., 1999; Yamamoto et al., 2003; Dolińska et al., 2004), but minimal or absent expression in intact brain astrocytes (Broer et al., 1999; Gliddon et al., 2009). In contrast, ASCT1 is found on astrocytic somata and processes throughout the brain (Weiss et al., 2001; Sakai et al., 2003; Jeon et al., 2009; Kaplan et al., 2018), making it the prime candidate for mediating astrocytic DS uptake.

The observation that L-glutamine and a combination of L-glutamine and trans-4-hydroxy-proline inhibit the DS-induced membrane current warrants careful interpretation (Figure 3.6). A previous study by Foster et al. (2018) characterised L-glutamine as being preferentially transported via ASCT2 isoform. Their finding indicated that ASCT1, insensitive to glutamine, and ASCT2, sensitive to glutamine, differ in their ability to recognise glutamine as a substrate. Consequently, L-glutamine can act as an inhibitor of DS uptake through ASCT2. Although Foster et al. (2018) established L-glutamine's effectiveness as an inhibitor, their study did not delve into the detailed transport mechanisms of L-glutamine via both isoforms. While the inhibition is evident, a more comprehensive understanding of the transport mechanisms would provide valuable insights into the nuanced interactions between L-glutamine and ASCT1/ASCT2, contributing to a deeper understanding of the observed inhibitory effects.

Although L-glutamine is not a good substrate for ASCT1, it does interact with the transporter and as a result, inhibits its transport activity. This is evident from the data from Pinilla-Tenas et al. (2003), where L-glutamine was shown to inhibit ASCT1-mediated alanine transport but not to be transported itself. Hence, the observation that L-glutamine inhibits the uptake of DS via ASCT1 is in line with expectations. It suggests that the application of glutamine in the current experiments may not be adequate to conclusively distinguish between ASCT1 and ASCT2 isoforms. To strengthen these observations, the use of specific small molecule inhibitors capable of selectively targeting ASCT isoforms could provide more definitive evidence. Given that the structure of ASCT1 and ASCT2 has been recently elucidated (Garaeva et al., 2018; Yu et al., 2019; Stehantsev et al., 2021), the development of specific small molecule inhibitors targeting ASCT isoforms would provide valuable support for the present observations. Nevertheless, considering the gene and protein expression data, it

strongly suggests the participation of the ASCT1 isoform in the observed DS uptake by astrocytes.

3.3.2 ASCT1 dominance: Minimal contribution of alternative transporters to D-serine uptake in astrocytes

The involvement of other potential DS transporters remains a topic that requires further investigation. While ASCT1 has been implicated as a significant player in DS transport in astrocytes, several other transporters may also play a role in this uptake process. The observation that benzyl serine does not completely block the observed DS-induced current (Figure 3.5A, B and Figure 3.7) could be attributed to its inability to competitively outperform all DS binding. Nevertheless, the possibility of another astrocytic transporter contributing a minor portion of the DS transport current cannot entirely be excluded. Understanding the contributions of these candidate transporters is essential for gaining a comprehensive understanding of DS regulation in the brain.

One potential contender might be the system A transporters SNAT1 and SNAT2 (a product of *Slc38a1/2* genes), which can transport DS, albeit less efficiently than other amino acids (Hatanaka et al., 2000; Reimer et al., 2000; Bodner et al., 2020). Experiments involving synaptosomes and neuronal cultures have clearly demonstrated DS uptake via SNAT1/2, sensitive to the classical system A inhibitor MeAIB (Bodner et al. 2020). While SNAT1/2 are believed to be primarily expressed in neurons (Melone et al. 2004; Mackenzie et al. 2004) and are thus associated with neuronal DS transport, there are reports of their expression in astrocytes as well (González-González et al. 2005). However, the insensitivity of astrocytic DS current to MeAIB rules out SNAT1/2 as a mediator of astrocytic DS uptake in the experiments (Figure 3.8).

In addition, the possibility of DS uptake in an electroneutral manner, completely undetectable via electrical changes cannot be ruled out. A potential astrocytic DS transporter that fits with the electroneutral uptake is asc-1 (*Slc7a10*), known to transport DS in neurons and contribute significantly to neuronal DS release (Rosenberg et al. 2013; Sason et al. 2017). Although the expression of asc-1 in astrocytes is minimal (Helboe et al., 2003; Matsuo et al., 2004), its presence has been detected (Lovatt et al., 2007; Cahoy et al., 2008; Doyle et al., 2008). According to the UCSC Genome Browser, the *Slc7a10* gene expression level is about 40% in glia and 55% in the choroid plexus, relative to housekeeping genes, indicating that the mRNA is predominantly expressed in glial cells in the mouse brain. Despite this, the pharmacological inhibition of asc-1 does not diminish the DS transporter currents observed in astrocytes (Figure

3.9). While the electroneutral uptake of DS via asc-1 remains a theoretical possibility, it appears unlikely, as studies employing radiolabelling techniques have failed to detect asc-1-mediated DS transport in astrocyte cultures (Rosenberg et al., 2013; Sakimura et al., 2016).

Another potential electroneutral DS transporter is LAT1 (system L; a product of *Slc7a5* gene), which is often co-expressed with ASCT2 and is known to play a role in cell growth and survival (Fuchs and Bode, 2005). While LAT1 primarily exchanges large neutral amino acids in a sodium-independent and pH-independent manner, it has the potential to transport small neutral amino acids as well (Bröer et al., 2016). However, the insensitivity of the observed DS transport currents to the inhibitor 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH; Figure 3.10), suggests that LAT1 may not play a detectable role in the observed uptake of DS into the astrocytes. Nevertheless, it is important to acknowledge the possibility of a LAT1 contribution that is not detectable by the experimental techniques. Collectively, the findings strongly support the conclusion that ASCT1, rather than ASCT2, SNAT1/2, asc-1 and LAT1 serves as the primary mediator of astrocytic DS uptake in its native environment.

3.3.3 D-serine generates coupled transport currents in astrocytes

The electrogenicity of the entire transport process refers to the net build-up of positive charges associated with ASCT1. This particular aspect has not received adequate attention for a long time, both in the context of EAATs (Excitatory Amino Acid Transporters) and ASCTs. The oversight is partly attributed to conflicting findings obtained with the bacterial transporter GltPh, which demonstrated a relatively low level of electrogenicity (Boudker et al., 2007). ASCT transporters (*Slc1a4* and *Slc1a5*) belong to the same transporter family as the high-affinity EAATs, including EAAT1 to EAAT5, encoded by the genes *Slc1a3*, *Slc1a2*, *Slc1a1*, *Slc1a6*, and *Slc1a7*, respectively (Kanai et al., 2013).

When substrates are transported by EAATs, it results in a membrane current that is closely linked to substrate translocation. Additionally, it induces the opening of an intrinsic anion channel, leading to an uncoupled anion channel current (Fairman et al., 1995; Billups et al., 1996). This anion channel exhibits significantly higher permeability to SCN^- compared to Cl^- , in accordance with thermodynamic studies on anion selectivity in channels (Wright and Diamond, 1977). However, the extent of coupled charge movement to uncoupled anion current varies widely among family members. For instance, EAAT1-3 have a relatively small proportion of current carried by the anion channel, whereas EAAT4-5 predominantly rely on the anion channel for current flow (Fahlke et al., 2016). Investigations of ASCT1 expressed in *Xenopus*

oocytes have also indicated the existence of an uncoupled anion conductance when the transporter is active (Zerangue and Kavanaugh, 1996). Nevertheless, the recordings demonstrate inconsistencies with this model, as the replacement of internal SCN^- with Cl^- and the absence of current reversal at positive membrane potentials did not yield the expected outcomes. If Cl^- was permeating such a channel, the addition of SCN^- would be expected to enhance the current, but this was not observed (Figure 3.11A). Also, the current-voltage relationship of an uncoupled current would show a strong voltage dependence, which is not observed in my experiments (Figure 3.3 B, C). These results indicate that ASCT1-mediated transport currents do not represent uncoupling of transport, from an anion current.

This observation suggests that the current associated with DS uptake is closely linked to the transport of DS itself and is not induced by the activation of an uncoupled anion channel within the ASCT1's structure. In line with this observation, previous studies have suggested that serine inhibits rather than activates the anion conductance in ASCT1 (Wang et al., 2022). Furthermore, electrogenic coupled amino acid transport has been previously reported for ASCT2 (Scalise et al., 2014; Mazza et al., 2021), and my data from ASCT1 align with this type of mechanism. This electrogenic transport mechanism observed with ASCT1 could ensure efficient uptake of DS by astrocytes, as the positive charge accumulation can help facilitate the movement of DS against its concentration gradient.

3.3.4 Physiological relevance of ASCT transporters in D-serine regulation

The extracellular concentration of DS significantly influences the activation of NMDA type of glutamate receptors and, consequently, shapes synaptic plasticity (Mothet et al., 2000; Panatier et al., 2006; Henneberger et al., 2010; Fossat et al., 2012). For an in-depth examination of this phenomenon, Chapter 5 delves into the role of DS transporters in modulating NMDAR-mediated neurotransmission. This DS concentration depends not only on the rate of DS release but also on its reuptake into cells. The astrocytic uptake pathway that has been identified involving ASCT1, is expected to exert a regulatory influence on the synaptic actions of glutamate. However, the constitutive knockout of ASCT1, as previously documented by Kaplan et al. (2018), has unveiled rather unexpected effects. This genetic alteration not only disrupts the DS transport but also significantly affects its precursor, L-serine (LS). Consequently, this leads to a global reduction in both DS and LS levels, accompanied by a compensatory increase in glycine production, which can serve as an alternative NMDAR co-agonist (Kaplan et al. 2018). This complicates the assessment of the specific effects of DS changes.

To gain a more nuanced understanding of the role of astrocytic ASCT1 in DS transport, future investigations should consider employing conditional knockout strategies tailored to ASCT1. This approach could provide deeper insights into the significance of astrocytic ASCT1 in DS transport while minimising potential impacts on alternative transporters or metabolic pathways. Given the impact of ASCT1 on both DS and LS concentrations, it becomes crucial to investigate the influence of ASCT on regulating LS levels. Furthermore, an intriguing avenue for prospective research lies in elucidating the transport mechanisms governing LS within astrocytes, a topic that Chapter 4 will comprehensively explore.

The fate of DS following its transport into astrocytes remains uncertain. It might be released from astrocytes in a stimulus-dependent manner, functioning as a gliotransmitter to modulate neuronal activity (Mothet et al., 2005; Martineau et al., 2008; Henneberger et al., 2010). Alternatively, DS can be metabolised within astrocytes through one of two potential metabolic pathways. One pathway involves D-amino acid oxidase (DAAO), which catalyses the oxidative deamination of DS to β -hydroxy pyruvate (Pollegioni and Sacchi, 2010). DAAO is functionally expressed in astrocytes and Bergmann glia in the cerebellum and other hindbrain regions but is seemingly absent from the forebrain (Horiike et al., 1987; Horiike et al., 1994; Moreno et al., 1999). Given the strong DAAO expression and activity in Bergmann glia, it is likely responsible for the oxidation of the DS observed to be transported into these cells (Figure 3.12B, C). The alternative DS metabolic pathway operates through serine racemase (SR; Wolosker et al. 1999), which, aside from converting LS into DS, can also catalyse an α , β -elimination pathway for both D- and L- forms to pyruvate (De Miranda et al., 2002; Foltyn et al., 2005). Unlike DAAO, SR is most prominently expressed in the forebrain, with significant levels observed in astrocytes (Wolosker et al., 1999a; Xia et al., 2004; Yoshikawa et al., 2007; Cahoy et al., 2008; Miya et al., 2008; Ding et al., 2011; Ehmsen et al., 2013). This enzyme is therefore likely to mediate the metabolism of the DS sequestered into astrocytes in the hippocampus and somatosensory cortex (Figure 3.5 and Figure 3.12D, E). While SR can convert the D- to L-form, this is unlikely to occur in astrocytes at physiological DS concentrations, with the α , β -elimination reaction being dominant under normal conditions (Foltyn et al. 2005). The production of astrocytic LS occurs through the alternative 3-phosphoglycerate dehydrogenase pathway (Yamasaki et al., 2001; Neame et al., 2019), which supplies the majority of LS for the "serine shuttle" transfer to neurons (Figure 3.13).

While my experiments do not directly investigate the release of DS through a transporter-mediated mechanism, it raises the intriguing possibility of whether ASCTs could potentially serve as mediators for the release of DS within astrocytes. ASCT1 operates as an

obligate amino acid exchanger (Zerangue and Kavanaugh, 1996), meaning that when DS enters the cell, it triggers the simultaneous antiport of another amino acid. Previous research has indicated that activating ASCT1 with transhydroxy-4-proline can lead to the release of LS from astrocytes (Kaplan et al. 2018). This intriguing observation suggests that the transport of DS into astrocytes through ASCT1 may stimulate the concurrent release of LS. Given that the release and subsequent uptake of DS by neurons could potentially deplete the neuronal supply or pool of DS, promoting the exchange of DS for LS via astrocytic ASCT1 could facilitate the replenishment of DS within neurons (Figure 3.13; discussed in detail in chapter 5).

However, it is important to note that there is limited evidence to suggest that under normal physiological conditions, astrocytic ASCT1 might release DS in exchange for another amino acid (Rosenberg et al. 2013; Kaplan et al. 2018). This absence of DS release could be attributed to its metabolic processes, which could sustain a low cytoplasmic DS concentration (Ehmsen et al., 2013; Balu and Coyle, 2014). Alternatively, ASCT1's ability to release DS may be constrained if the exchange of amino acids follows an asymmetrical pattern, allowing for different selectivity in import and export. Such asymmetric phenomenon has been observed in various antiporters, including ASCTs (Pingitore et al., 2013; Gauthier-Coles et al., 2023). Nevertheless, my findings pave the way for future investigations into the precise molecular and cellular mechanisms involved in the release of DS via ASCT1, as well as the therapeutic implications for disorders where DS regulation is perturbed.

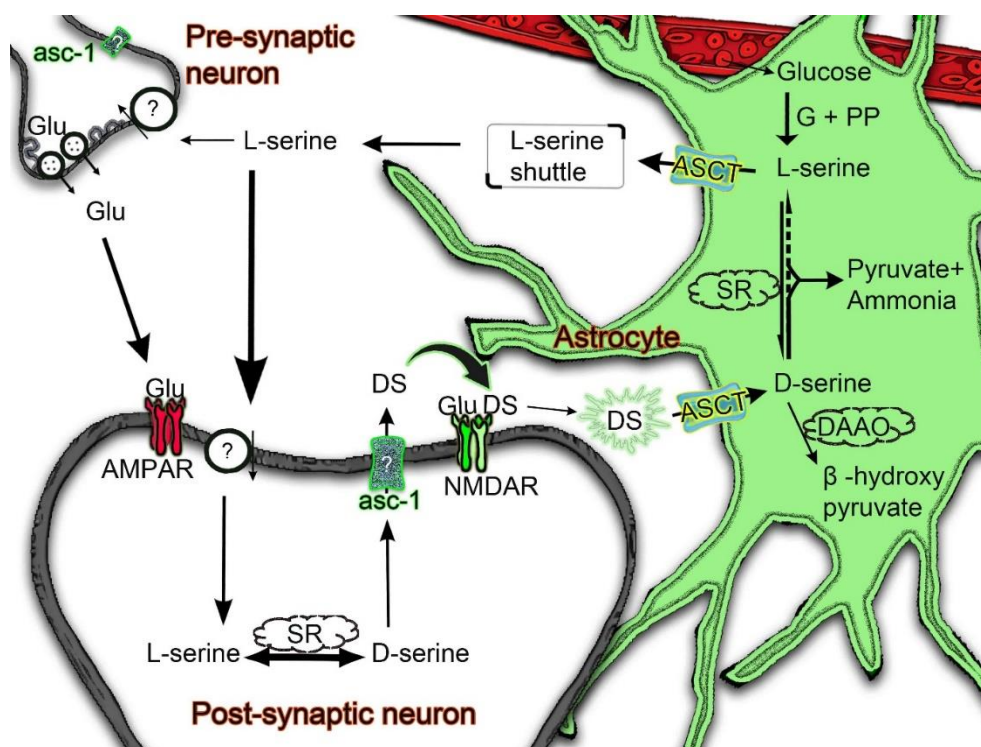


Figure 3.13: The physiological role of Alanine Serine Cysteine Transporters (ASCTs) in regulating the levels of L-serine and D-serine in the brain.

L-serine production (Top right): L-Serine is produced exclusively in astrocytes from glucose through glycolysis (G) and phosphorylated pathways (PP). Astrocytic L-serine transport: ASCTs expressed on astrocytic membranes could facilitate the transport of L-serine from astrocytes into the neuronal compartment, either presynaptic or postsynaptic. The specific neuronal L-serine transporter is yet to be characterised (represented as ?). Synthesis of D-serine (DS; Inside post-synaptic neuron): Neuronal L-serine is converted into DS by the enzyme serine racemase (SR) and released through asc-1 transporters. Enhancement of neuronal communication (highlighted green arrow): Released DS acts on NMDA receptors (NMDARs) in conjunction with glutamate (Glu) to enhance neuronal communication. Clearance of DS from the synaptic cleft (green cloud): Following its action at NMDARs, DS is cleared from the synaptic cleft. This process involves the characterised astrocytic ASCT1 transporters that transport DS back into astrocytes. Metabolism of DS: Inside astrocytes, DS can undergo two metabolic pathways: Metabolism by D-amino acid oxidase (DAAO) into β -hydroxy pyruvate, with significant expression in the cerebellum. Alternatively, astrocytic DS can be metabolized by α , β -elimination to form pyruvate and ammonia, catalysed by SR. Under pathological conditions: DS concentrations may increase in astrocytes, potentially through production from L-serine. ASCTs also may release DS, leading to the over-activation of NMDARs.

3.3.5 Concluding remarks

In summary, the data from this chapter provide direct electrophysiological recordings of transporters within identified cells in their natural environment, demonstrating the uptake of D-serine (DS) into glial cells in various brain regions, including the hippocampus, cortex, and cerebellum. My study for the first time shows that sodium-dependent alanine serine cysteine transporter ASCT is responsible for the uptake of DS into astrocytes adjacent to synapses in the *stratum radiatum* layer of CA1 hippocampal region and that the ASCT1 isoform plays the most dominant role in this process. It also highlights the central role of ASCT1 in mediating DS transport in astrocytes in the cortex and Bergmann glia of the cerebellum and indicates their role in influencing extracellular DS levels and participating in the metabolic breakdown of DS. These findings support the existing body of knowledge about the importance of astrocytes in regulating synaptic function and plasticity, which has implications for our understanding of brain functions.

Overall, my research findings are consistent with and extend previous studies that have investigated the role of astrocytes and ASCT transporters in the regulation of DS levels in the brain. For example, previous studies have shown that astrocytes are the primary source of L-serine, which is then transported into neurons and converted into DS by serine racemase. The DS is then released into the synaptic cleft where it acts on NMDARs to enhance neuronal communication. My study adds to this body of research by identifying ASCT1 transporters as the primary mechanism for the uptake of DS into astrocytes adjacent to synapses.

My findings implicate that under normal physiological conditions, ASCT1 may modulate the synaptic effects of glutamate, while in pathological conditions, it may contribute to the development of various neurological disorders, including traumatic brain injury, Alzheimer's disease, neuropathic pain, and schizophrenia, which are associated with alterations in DS levels. This research is important because it provides new insights into the mechanisms of specific transporters underlying the regulation of DS levels in the brain, which is crucial for proper NMDAR function and the development of new drug targets for neurological disorders.

Chapter 4 L-serine transport in the mammalian brain

4.1 Aim

The broad aim of this chapter is to explore the transport mechanisms responsible for the uptake of synaptic L-serine (LS) by astrocytes and the neighbouring excitatory neurons. Previous investigations employing radiolabelling techniques have provided valuable insights, demonstrating the uptake of LS into both astrocytes and C6 glioma cells (Hayashi et al., 1997; Ribeiro et al., 2002), indicating the active participation of astrocytes in LS regulation. The majority of research in the field has concentrated on understanding LS uptake in cultured synaptosomes, astrocytes, and transfected cell lines (Hayashi et al., 1997; Verleysdonk and Hamprecht, 2000; Yamamoto et al., 2001; Yamamoto et al., 2003; Rosenberg et al., 2013; Foster et al., 2016; Kaplan et al., 2018). However, the characterisation of LS transport within intact brain tissue, where the natural expression patterns of transporters are preserved, remains limited. This scarcity of studies exploring LS transport in the context of intact brain tissue has created a gap in our understanding of the true physiological mechanisms underlying LS regulation in the brain. This chapter endeavours to bridge this gap by investigating the transport of LS into astrocytes in close proximity to the excitatory synapses in the *str* layer and the neighbouring pyramidal neurons of CA1 in its native environment. I hypothesise that astrocytes and neurons transport LS, mediated by ASCT but with some additional potential involvement of other transport systems.

4.2 Results

4.2.1 The nature of L-serine mediated plasma membrane currents I_{LS} recorded from *stratum radiatum* astrocytes

The main objective was to determine the functional expression of LS transporters on the astrocytic plasma membranes. To achieve this, LS was used as a substrate to activate the LS transporters in *str* astrocytes. Direct electrophysiological recordings were conducted from these astrocytes to provide more definitive evidence regarding functional LS transporters on the astrocytic membrane.

In investigating whether astrocytes take up LS, 10 mM LS was puff-applied via a glass pipette onto voltage-clamped *str* astrocytes and recorded the resulting membrane currents. To specifically isolate the membrane currents induced by LS, all recordings were conducted in the presence of a cocktail of compounds that block ion channels and receptors, ensuring precise observation of the LS-induced effect. This approach resulted in an LS-induced

membrane current (I_{LS}) with an average amplitude of -43.0 ± 3.5 pA ($n = 37$; Figure 4.1A; blue trace). I_{LS} was significantly higher ($p < 0.001$) than puff control of ASCF, which only evoked a negligible current of -0.25 ± 0.2 pA ($n = 6$; Chapter 3 Figure 3.3A). This confirms that the astrocytic membrane current observed was not an artefact caused by the puff application itself, establishing that the observed current is specifically linked to the presence of LS.

In prior experiment, a 10 mM LS concentration was used as a substrate, due to the known micromolar affinity of serine for the transporters (Foster et al., 2016; Bodner et al., 2020). In this specific experiment, a lower concentration of LS (1 mM) was puff applied near the voltage-clamped astrocytes. This resulted in a significantly ($p < 0.001$) lower magnitude of I_{LS} , with an average amplitude of -7.5 ± 2.1 pA ($n = 5$; Figure 4.1A) compared to the responses elicited by the 10 mM LS application. This result indicates the low affinity of the LS transporters. The discrepancy in concentration reaching the astrocyte is likely due to the limitation of puff application, where the substrate concentration reaching the cell can be approximately one-tenth of the applied concentration (Todd et al., 2017), hinting at the involvement of dilution factor from the puffing which does not allow an accurate measure of the concentration reaching the astrocyte nor the affinity of the transporter.

The uptake of LS via transporters such as ASCT1 and System A can generate trans-membrane currents by transporting charged substances like Na^+ (Hatanaka et al., 2000; Albers et al., 2001; Scalise et al., 2014; Mazza et al., 2021). This process can also open an integral channel, leading to uncoupled currents (Zhang and Grever, 2007; Wang et al., 2022). To further understand the nature of I_{LS} , the current-voltage relationship was examined by comparing the current response to a ramp in membrane voltage both prior to and during the application of LS. I_{LS} remained relatively unaffected by changes in membrane voltage and did not exhibit a reversal of current at the reversal potential of any of the ions present in the recording solutions (Figure 4.1B, C). This finding strongly suggests that I_{LS} is independent of changes in membrane voltage, emphasising that I_{LS} is likely mediated by a coupled membrane transporter current rather than being mediated by an ion channel or ionotropic receptor.

I_{LS} was recorded with an intracellular recording solution containing sodium and alanine to provide substrates for ASCT-mediated amino acid exchange, and thiocyanate ions (SCN^-) to provide a permeant ion for any potential uncoupled anion channel. To discern the relative contributions of coupled charge movement and uncoupled anion channel activation in generating I_{LS} , in this specific experiment intracellular SCN^- was replaced with Cl^- ions, which exhibit significantly reduced permeability through the integral anion channel compared to

SCN^- (Zerangue and Kavanaugh, 1996). This manipulation would be predicted to reduce the observed current if it were mediated by an uncoupled anion channel. However, the replacement of SCN^- with Cl^- resulted in no significant alteration in I_{LS} (I_{LS} with intracellular $\text{SCN}^- = -43.0 \pm 3.5$ pA; $n = 37$, and with intracellular $\text{Cl}^- = -30.2 \pm 2.8$ pA; $n = 8$; $p = 0.07$; Figure 4.1D). This result, in conjunction with the absence of substantial voltage dependence (Figure 4.1B, C), suggests that I_{LS} is not primarily mediated by the gating of an uncoupled anion channel. Instead, it indicates that I_{LS} is generated predominantly through the coupled transport of LS into the astrocytes.

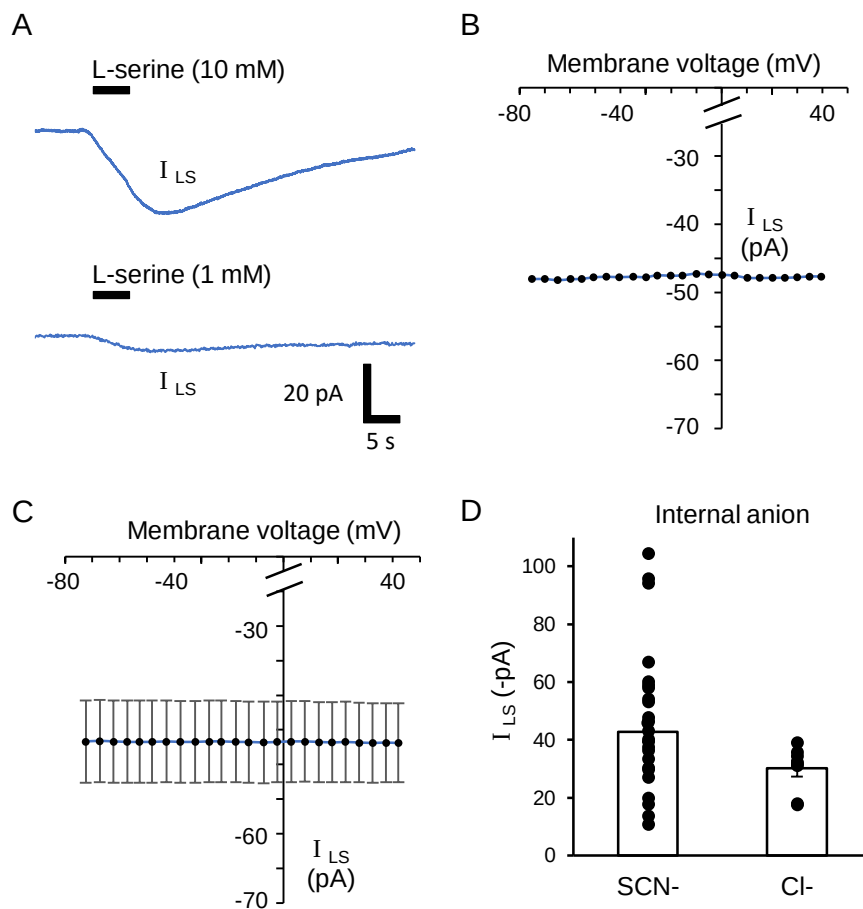


Figure 4.1: L-serine-mediated plasma membrane currents (I_{LS}) in CA1 stratum radiatum astrocytes are not mediated by uncoupled anion movement via an integrated ion channel.

Panel (A) show the raw data trace illustrating astrocytic 10 mM and 1 mM L-serine currents (I_{LS} ; in blue). (B) The current-voltage (IV) relationship of I_{LS} is illustrated through a subtraction approach, where a voltage ramp protocol applied during the L-serine puff is subtracted from a control ramp. (C) Average IV relationship of I_{LS} , with the error bars indicating the SEM values ($n = 8$). These graphs show that there is no reversal of current at positive potentials, further supporting the involvement of a transporter in mediating these currents. (D) Astrocytic L-serine current (I_{LS}) remained unchanged upon replacing intracellular SCN^- with Cl^- , indicating that the transport current is coupled to L-serine uptake and not due to activation of an uncoupled anion channel within the transporter structure.

4.2.2 ASCT transporters primarily mediate the astrocytic uptake of L-serine

After confirming the presence of an LS transport current in the *str* astrocytes, the next objective was to identify which membrane transporter was responsible for this uptake. Similar to the transporters facilitating the movement of DS across the membrane in the central nervous system, there are several LS transporters, including ASCT1 (*Slc1a4*), ASCT2 (*Slc1a5*), SNAT1 (*Slc38a1*), SNAT2 (*Slc38a2*), SNAT5 (*Slc38a5*), asc-1 (*Slc7a10*) and LAT1 (*Slc7a5*). To determine which transporter mediates I_{LS} , O-benzyl-L-serine was added to the external solution. This compound is a specific substrate inhibitor known to inhibit both isoforms of alanine serine cysteine transporters, ASCT1 and ASCT2 (Grewer and Grabsch, 2004). 10 mM O-benzyl-L-serine was bath applied before and during the LS puffs onto the *str* astrocytes. The addition of 10 mM O-benzyl-L-serine significantly reduced I_{LS} by 85.6% (from $I_{LS} = -33.6 \pm 2.1$ pA in control to -4.8 ± 0.8 pA in the presence of 10 mM benzyl serine; $n = 4$; $p < 0.001$; Figure 4.2A, B). This result indicates that I_{LS} is primarily mediated by ASCT transporters.

Moreover, experiments were conducted involving the application of 10 mM trans-4-hydroxy-proline (T-pro) to bath solutions both before and during the administration of LS puffs onto the astrocytes in the *str* layer. T-pro is a competitive substrate known for its specificity towards the ASCT1 isoform (Pinilla-Tenas et al., 2003; Foster et al., 2016). The application of T-pro significantly reduced the recorded I_{LS} from astrocytes (from $I_{LS} = -28.1 \pm 4.1$ pA in control to I_{LS} in the presence of 10 mM T-pro = -8.5 ± 1.3 pA; $n = 6$; $p < 0.001$; resulting in a reduction of 70.0%, Figure 4.2C, D).

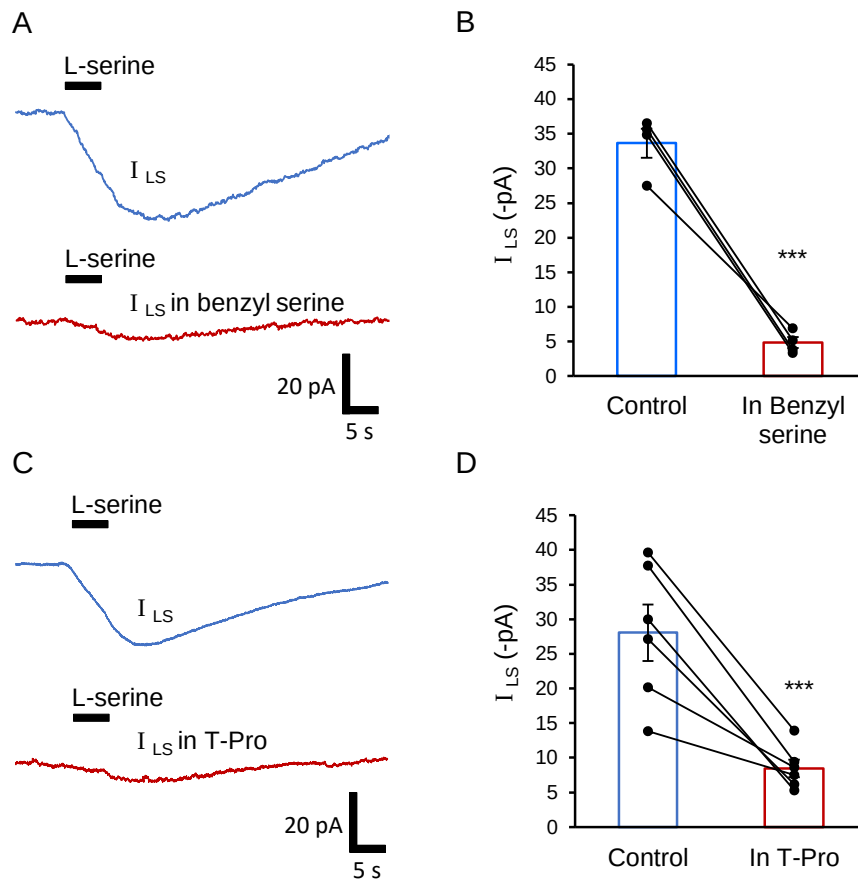


Figure 4.2: Astrocytic L-serine current (I_{LS}) is mediated by ASCT transporters

The raw data trace presented in panels (A) and (C) demonstrate that the bath application of 10 mM benzyl serine or 10 mM trans-4-hydroxy-proline (T-pro) which inhibits ASCTs, significantly decreased the L-serine induced membrane current I_{LS} . The blue traces show the current before the drug application, and the dark red traces show the current after the drug application. (B) The averaged data demonstrate that bath application of benzyl-serine, an inhibitor of ASCT transporters, reduced I_{LS} by 85.6%. (D) Averaged data show that bath application of 10 mM T-pro, an ASCT1-specific substrate, reduced I_{LS} to a similar extent as benzyl serine (70.0%), indicating the involvement of ASCT1 in mediating I_{LS} . *** represents a significant difference at $p \leq 0.001$.

Furthermore, experiments were conducted involving the application of 10 mM L-glutamine (L-gln; a non specific ASCT inhibitor; Broer et al., 1999; Foster et al., 2016) to bath solutions both before and during the administration of LS puffs onto the astrocytes in the *str* layer. The application of L-gln moderately reduced the recorded I_{LS} from astrocytes (from $I_{LS} = -66.2 \pm 20.7$ pA in control to I_{LS} in the presence of 10 mM L-gln = -39.0 ± 13.2 pA; $n = 3$; $p < 0.001$; resulting in a reduction of 41.5%, Figure 4.3A, B). In addition, experiments were conducted involving the application of a combination of 10 mM T-pro and 10 mM L-gln. The application of a combination of T-pro and L-gln significantly reduced the recorded I_{LS} from astrocytes (from $I_{LS} = -62.2 \pm 10.6$ pA in control to I_{LS} in the presence of 10 mM T-pro and L-gln = -18.5 ± 7.5 pA; $n = 7$; $p < 0.001$; resulting in a reduction of 70.4%, Figure 4.3C, D). These results provide

strong evidence that I_{LS} is predominantly mediated by ASCT transporters, implying a potential role for ASCT1 in the uptake of LS from the extracellular space.

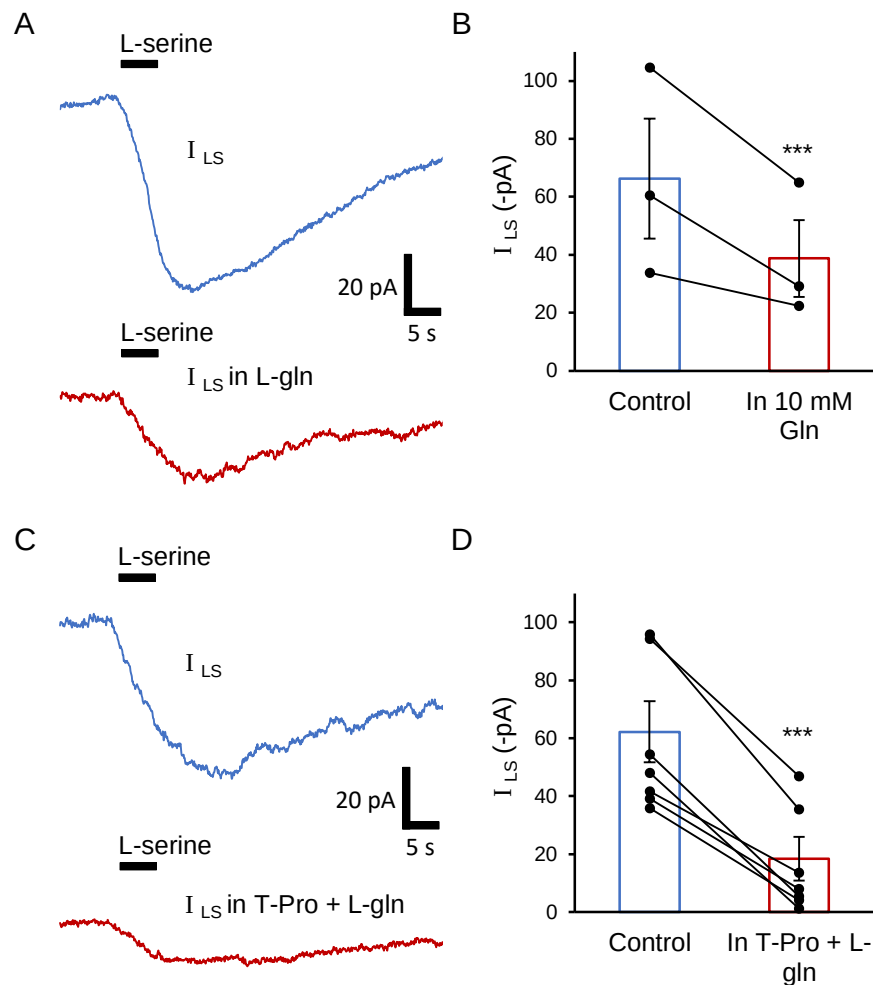


Figure 4.3: Astrocytic L-serine current (I_{LS}) is mediated by ASCT1 transporters

The raw data trace presented in panels (A) and (C) demonstrate that the bath application of 10 mM L-glutamine (L-gln) or a combination of 10 mM T-pro and 10 mM L-gln which inhibits ASCTs, significantly decreased the L-serine induced membrane current I_{LS} . The blue traces show the current before the drug application, and the dark red traces show the current after the drug application. (B) The averaged data demonstrate that bath application of L-gln, an inhibitor of ASCT transporters, reduced I_{LS} by 41.5%. (D) Averaged data show that bath application of a combination of 10 mM T-pro and 10 mM L-gln, reduced I_{LS} to a similar extent as benzyl serine and T-pro on its own (70.4%), supporting the involvement of ASCT1 in mediating I_{LS} . *** represents a significant difference at $p \leq 0.001$.

4.2.3 System A transporters mediate the uptake of L-serine in *stratum radiatum* astrocytes to a minor extent

Apart from ASCT transporters, the system A transporters SNAT1 or 2 (*Slc38a1* or *Slc38a2*) could mediate an electrogenic LS uptake if expressed on the astrocytic membranes. To explore this possibility, α -(methylamino) isobutyric acid (MeAIB) was added into the external solution, prior to and during the LS puffs onto the *str* astrocytes, which is known to inhibit SNAT1/2 transporters (Christensen et al., 1965). I_{LS} was partially sensitive to 10 mM MeAIB (I_{LS} control = -33.3 ± 6.0 pA; I_{LS} in the presence of 10 mM MeAIB = -20.7 ± 4.0 pA; $n = 8$; $p = 0.0001$; resulting in a marginal reduction of 37.8%, Figure 4.4A, B). This suggests that SNAT1 or 2 play a minor role in mediating astrocytic I_{LS} .

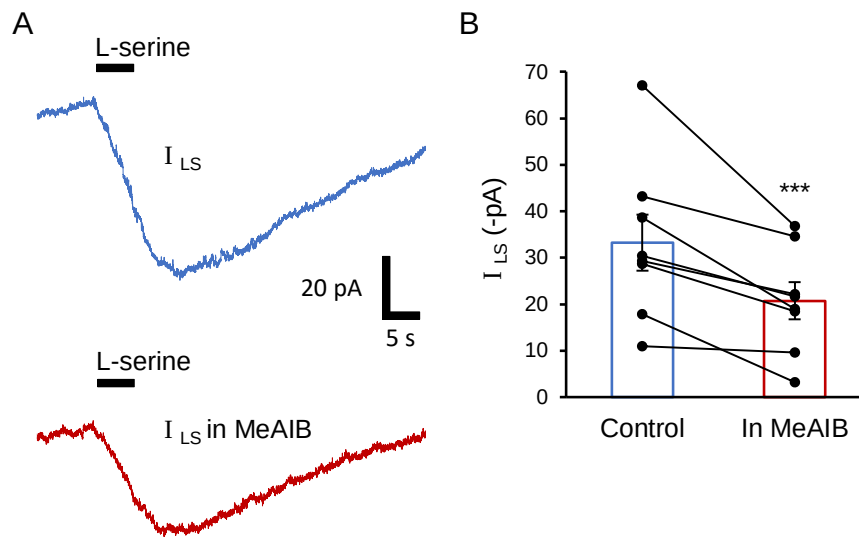


Figure 4.4: Astrocytic L-serine current (I_{LS}) is mediated by SNAT1/2 transporters to a minor extent.

The raw data trace presented in panel (A) demonstrates that the bath application of 10 mM α -(methylamino) isobutyric acid (MeAIB), which inhibits SNAT1 and SNAT2, had a marginally effect on the L-serine-induced membrane current I_{LS} . The blue traces show the current before the MeAIB application, and the dark red traces show the current after the MeAIB application. (B) Averaged data show that bath application of 10 mM MeAIB respectively, a SNAT1/2 transporter substrate, reduced I_{LS} by 37.8%. *** represents a significant difference at $p \leq 0.001$.

4.2.4 LAT transporters do not mediate the L-Serine transport current in *stratum radiatum* astrocytes

To investigate whether the LAT1 transporter (*Slc7a5*) plays a role in generating I_{LS} , experiments were conducted in which 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) was added to the external bath solution. This compound blocks any LAT transporter-mediated current (Kanai et al., 1998) and was applied prior to and during puff-applied 10 mM LS onto the str astrocytes. The observations showed that I_{LS} was not altered in the presence of 5 mM BCH (I_{LS} control = -46.0 ± 6.0 ; I_{LS} in the presence of 5 mM BCH = -44.5 ± 3.2 ; $n = 4$; $p = 0.81$; Figure 4.5A, B), indicating that the LAT transporters do not contribute to I_{LS} recorded from astrocytes.

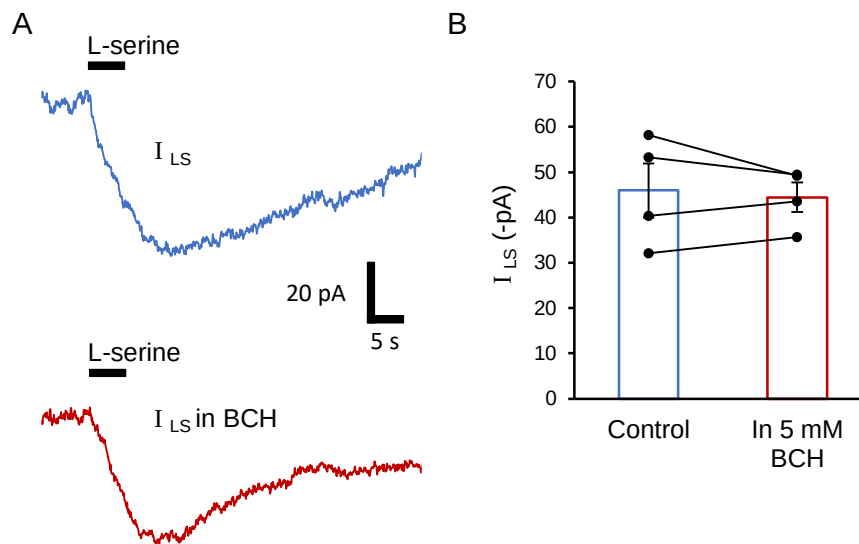


Figure 4.5: Astrocytic L serine current (I_{LS}) is not mediated by LAT transporters.

The raw data trace presented in panel (A) demonstrates that the bath application of 5 mM 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), which inhibits LAT transporters, did not have any significant effect on the L-serine-induced membrane current I_{LS} . The blue traces show the current before the BCH application, and the dark red traces show the current after the BCH application. (B) Averaged data show that bath application of 5 mM BCH, did not reduce I_{LS} .

4.2.5 Uptake of other ASCT substrates into the *stratum radiatum* astrocytes in the hippocampus

Having established the transport of serine (both D and L-isomers) is mediated by ASCT1 transporters into astrocytes, the next objective was to investigate whether ASCT1 transporters expressed on astrocytic membranes are capable of mediating the uptake of other substrates recognised by ASCTs. For this, L-cysteine and L-asparagine were used as alternative substrates for ASCTs to further confirm that the membrane currents arise from ASCT1 transporter activation. Electrophysiological patch clamp recordings were performed in which 10 mM L-cysteine and L-asparagine were puff applied onto voltage-clamped *stratum radiatum* astrocytes of the CA1 region in the hippocampus. These applications induced astrocytic membrane currents, namely, I_{L-cys} (-225.0 ± 55.8 pA; $n = 5$, Figure 4.6A), and I_{L-asn} (-213.0 ± 52.2 pA; $n = 4$; Figure 4.6C). This observation of a membrane current is consistent with the substrate profile of ASCT1 transport (Arriza et al., 1993).

Subsequently, 10 mM O-benzyl-L-serine was added to the external bath solution, and this addition significantly inhibited ($p < 0.001$) these currents. Specifically, for I_{L-cys} , the current in the presence of benzyl serine was -21.7 ± 10.6 pA ($n = 5$; Figure 4.6B), showing a 90.3% reduction. For I_{L-asn} , the current in the presence of benzyl serine was -15.0 ± 8.7 pA ($n = 4$; Figure 4.6D), indicating a 93.0% reduction. These findings strongly suggest that I_{L-cys} , and I_{L-asn} are indeed mediated by ASCT transporters.

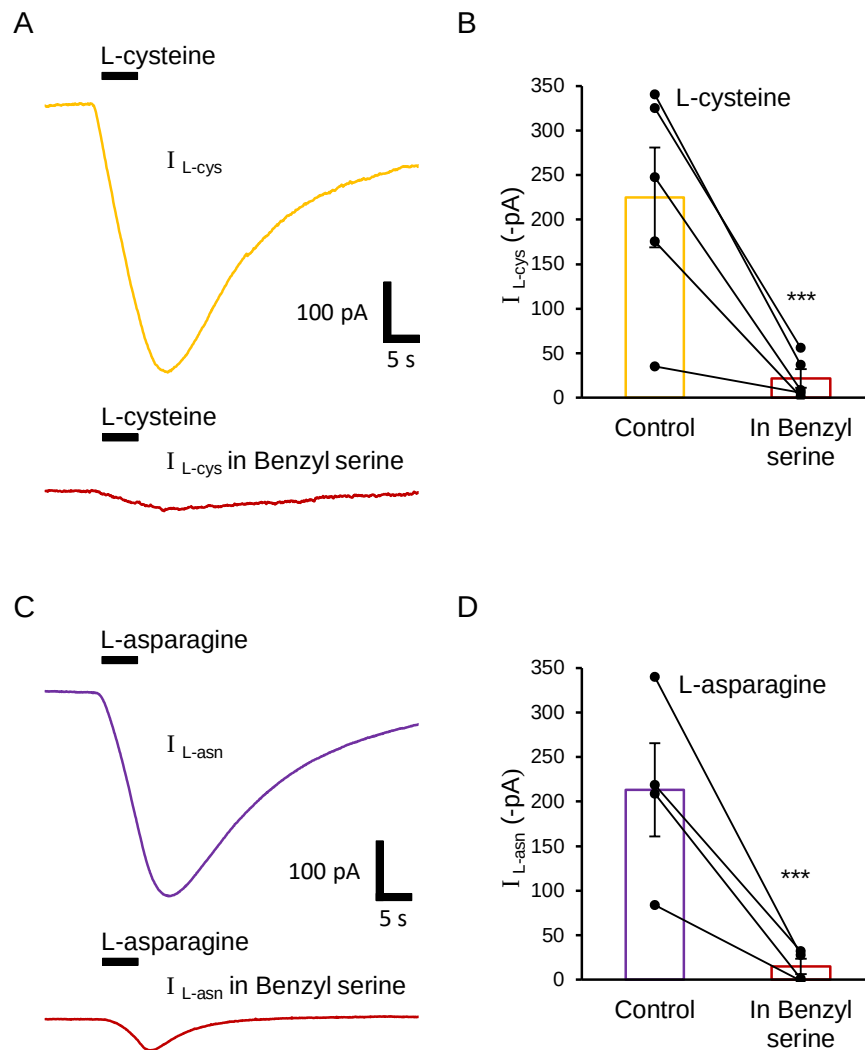


Figure 4.6: Astrocytic L-cysteine and L-asparagine currents (I_{L-cys} and I_{L-asn}) are mediated by ASCT transporters

Panel (A) show the raw data trace illustrating astrocytic L-cysteine currents (I_{L-cys}) before (in orange) and after (in dark red) the bath application of 10 mM O-benzyl-L-serine, which is a specific inhibitor of ASCT transporters. panel (C) show the raw data trace illustrating astrocytic L-asparagine currents (I_{L-asn}) before (in purple) and after (in dark red) the bath application of 10 mM O-benzyl-L-serine, which is a specific inhibitor of ASCT transporters. panels (B) and (D) display averaged data, indicating that the bath application of O-benzyl-L-serine, the specific ASCT transporter inhibitor, had a similar inhibitory effect on the I_{L-cys} (B) with a 90.3% reduction (orange represents before and dark red represents after benzyl serine application) and I_{L-asn} (D) with a 93.0% reduction (purple represents before and dark red represents after benzyl serine application) in astrocytes located in the *stratum radiatum* layer of CA1 hippocampal region. These results strongly suggest that ASCT transporters are crucial for mediating the uptake of these amino acids in astrocytes. *** Indicates a significant difference at $p \leq 0.001$.

4.2.6 Uptake of L-serine on CA1 pyramidal neurons in the hippocampus

Having established the presence of LS transporters on astrocytic membranes, the final objective was to investigate whether neighbouring pyramidal neurons participate in LS uptake, the succeeding step in revealing the physiological occurrence of the serine shuttle. In the context of CA1 pyramidal neurons, the uptake of LS is likely mediated by several transporters, with primary candidates being the widely expressed system A transporters, SNAT1 and SNAT2 (Melone et al., 2004; González-González et al., 2005; Bodner et al., 2020). While the involvement of ASCT1 cannot be completely ruled out despite its developmental downregulation (Sakai et al., 2003), it remains a potential candidate in this process. To investigate this, a controlled puff application of 10 mM LS was performed near the soma of CA1 pyramidal neurons. All recordings were conducted in the presence of an inhibitor cocktail, effectively blocking ion channels and receptors.

An LS-induced neuronal membrane current (I_{nLS}) was observed with an average amplitude of -46.5 ± 12.0 pA ($n = 10$; Figure 4.1A). To determine the specific transporter responsible for I_{nLS} , α -(methylamino) isobutyric acid (MeAIB) or O-benzyl-L-serine, known inhibitors of SNAT1/2 transporters (Christensen et al., 1965) and ASCTs (Grewer and Grabsch, 2004) respectively, were added to the external solution, prior to and during LS puff application onto the CA1 pyramidal neurons. The hypothesis posited system A transporters as primary mediators of LS influx. The addition of 10 mM MeAIB resulted in a substantial 83.0% reduction in I_{nLS} (from $I_{nLS} = -52.1 \pm 18.3$ pA in control to -9.0 ± 3.6 pA in the presence of 10 mM MeAIB; $n = 6$; $p < 0.05$; Figure 4.7A, B). However, the addition of O-benzyl-L-serine did not lead to a statistically significant reduction in I_{nLS} (from $I_{nLS} = -35.7 \pm 12.8$ pA in control to -10.4 ± 2.9 pA in the presence of 10 mM benzyl serine; $n = 4$; reduced by 70.7%; $p = 0.06$; Figure 4.7C, D). These findings indicate that I_{nLS} is predominantly mediated by System A transporters in CA1 pyramidal neurons, shedding light on the likely candidates involved in neuronal LS uptake.

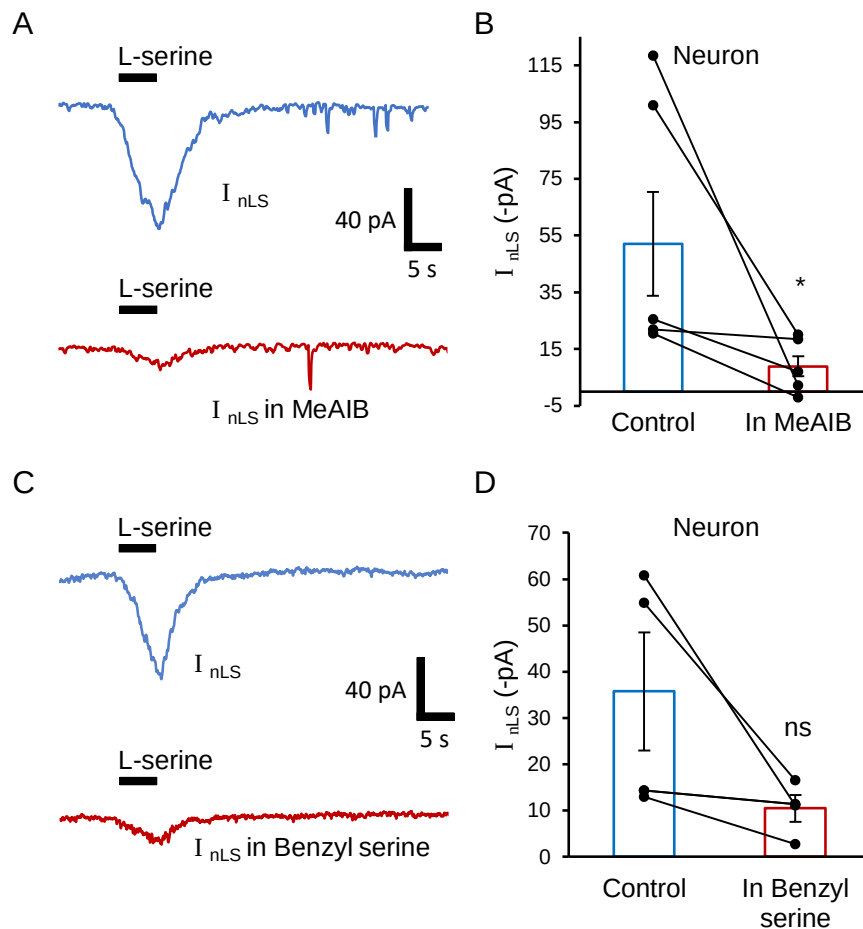


Figure 4.7: Neuronal L-serine currents (I_{nLS}) are mediated by System A transporters.

The raw data traces presented in panels (A) and (C) reveal that the bath application of 10 mM α -(methylamino) isobutyric acid (MeAIB) or 10 mM benzyl serine, which inhibit system A and ASCTs, respectively. MeAIB, but not benzyl serine led to a statistically significant reduction in the L-serine-induced neuronal membrane current (I_{nLS}). The blue traces show the current before the drug application, and the dark red traces show the current after the drug application. (B) The averaged data demonstrate that bath application of MeAIB, an inhibitor of System A transporters, reduced I_{nLS} by 83.0%. (D) Averaged data show that bath application of 10 mM benzyl serine, did not significantly reduce I_{nLS} , supporting the involvement of system A and potentially ASCT in mediating I_{nLS} . * Represents a significant difference at $p \leq 0.05$ and ns represents no statistical significance.

4.3 Discussion

In the context of NMDAR activation by synaptically released glutamate, the occupancy of the co-agonist binding site (glycine binding site) by either D-serine (DS) or glycine is essential (Mothet et al., 2000; Papouin et al., 2012). The endogenous co-agonist, DS is primarily synthesised by the enzyme serine racemase, which is predominantly present in adult neurons, with lesser expression in glial cells (Balu and Coyle, 2014). As neurons lack the enzymatic machinery for their own DS synthesis, they rely on the precursor L-serine (LS) for the racemisation reaction of serine. Astrocytes, major producers of LS derived from glycolysis and phosphorylated pathways (Yamasaki et al., 2001; Ehmsen et al., 2013), are hypothesised candidates for supplying LS to neighbouring neurons (Wolosker, 2011).

Identifying the specific plasma membrane transporter responsible for LS shuttle transport between astrocytes and neurons has remained elusive. Multiple carrier systems, including the sodium-dependent system ASC and system A, as well as the sodium-independent system L and system asc, mediate the transport of various neutral amino acids, including LS, DS, L-alanine, L-cysteine, and L-asparagine (Christensen et al., 1967; Arriza et al., 1993). To address this phenomenon in the astrocytes and neurons, a pragmatic approach was used by focusing on the measurement of LS uptake within these cells. This method provided an accessible and measurable parameter, offering valuable insights into the process of LS transport.

Therefore, the primary aim of this chapter was to explore the transport mechanisms governing LS and, by extension, enhance our understanding of its regulation within the mammalian brain. The main hypothesis posited astrocytes as potential candidates for mediating the transport of LS from the extracellular space. While a battery of literature focused largely on LS uptake in cultured synaptosomes, astrocytes, and transfected cell lines (Hayashi et al., 1997; Verleysdonk and Hamprecht, 2000; Yamamoto et al., 2003; Rosenberg et al., 2013; Foster et al., 2016; Kaplan et al., 2018), it is important to note that synaptosomes inherently contain both neuronal and glial membranes, and cell culture conditions can influence the expression of membrane transporters (Cahoy et al., 2008). This chapter's findings demonstrate the LS transport process within astrocytes and neurons, revealed through electrophysiological characterisation utilising acutely isolated hippocampal slices from mice.

4.3.1 L-serine uptake in astrocytes is mediated by ASCT1 transporter

Within the context of this investigation, the main objective was to elucidate the mechanisms underlying LS transport in astrocytes and to understand the pivotal role of astrocytic sodium-dependent alanine serine cysteine transporters (ASCT; a product of *Slc1a4/5* genes) in this process. It is posited that ASCT1 transporter, expressed in astrocytic membranes, would be a key contributor to the observed transport of LS. To address this, a substrate puffing approach was employed, which allowed the application of LS concentration (10 mM) directly near the astrocytes of interest while recording associated transport currents. In line with this hypothesis, electrogenic LS transport (I_{LS}) was identified in hippocampal astrocytes located in the CA1 *stratum radiatum*. Further investigation revealed that this transport mechanism was highly sensitive to O-benzyl-L-serine, pointing to the involvement of ASCT transporters (Figure 4.2A, B). This finding demonstrates that the primary mechanism facilitating the transport of LS in astrocytes predominantly relies on the ASCT transporter, accounting for approximately 80% of the uptake process.

In addition, using a lower 1 mM concentration of LS evoked only one-fifth of the astrocytic membrane currents compared to a 10 mM concentration (Figure 4.1A), indicating current amplitude is directly related to the substrate concentration reaching the cell and, consequently, the efficiency of the uptake process.

The gathered data strongly indicate that ASCT1 (*Slc1a4*) is the primary transporter responsible for LS uptake in astrocytes, highlighted by its inhibition in the presence of trans-4-hydroxy proline (Figure 4.1C, D). This pattern of inhibition mirrors the observed reduction in DS uptake in astrocytes reported in Chapter 3 (Figure 3.5). This expected link indicates that the transporters involved are the same, reinforcing the notion that there is not a dedicated LS transporter distinct from those transporting DS. This aligns with prior studies showing ASCT2's presence in cultured astrocytes but absence in intact brain astrocytes (Yamamoto et al., 2003; Dolińska et al., 2004; Gliddon et al., 2009), unlike ASCT1, which is widely distributed in astrocytic somata and processes throughout the brain (Weiss et al. 2001; Sakai et al. 2003; Kaplan et al. 2018). The critical role of ASCT1 is further highlighted by knockout studies, revealing severe consequences. The absence of ASCT1 not only disrupts the transport of DS but also significantly impacts its precursor, LS (Kaplan et al., 2018), underscoring the role of astrocytic ASCT1 in the regulation of DS and LS.

Furthermore, the observation that L-glutamine moderately inhibits LS uptake in astrocytes (Figure 4.3A, B) provides further support for the previously reported interaction of L-glutamine

with the ASCT1 transporter, leading to the inhibition of its transport activity (Pinilla-Tenas et al., 2003). This interaction becomes apparent when L-glutamine and trans-4-hydroxy proline are combined inhibiting LS-induced membrane currents (Figure 4.3C, D) to the same extent as observed with trans-4-hydroxy proline alone or O-benzyl-L-serine alone (Figure 4.2B, D). This finding underscores the regulatory interplay between LS and different inhibitors with ASCT1, shedding light on the intricate mechanisms governing LS transport dynamics within astrocytes.

4.3.2 System A transporters partially mediate uptake of L-serine in astrocytes

The observed incomplete inhibition by ASCT inhibitors implies the involvement of multiple transport systems in uptake, each possibly exhibiting varied affinities for LS. The other potential candidates include the sodium-dependent system A transporters (*Slc38a1* and *Slc38a2*; SNAT1 and 2 respectively), sodium-independent system L (*Slc7a5* or LAT1) and system asc (*Slc7a10*; asc-1) transporters. Previous data demonstrating the insensitivity of DS-evoked membrane currents to SMLC, an asc-1 inhibitor (Chapter 3; Figure 3.9), suggests that amino acid uptake via asc-1 is likely electroneutral, making the detection of asc-1 mediated LS uptake challenging with voltage clamp techniques. Similarly, the involvement of the sodium-independent System L in LS uptake appears to be predominantly electroneutral and minimal. This is evidenced by the lack of inhibition observed with BCH, a substrate specific to System L (Figure 4.5A, B). However, there are no specific studies reporting the radiolabelling of LS transport via the System L transporter. Therefore, the role of sodium-independent transporters in LS uptake remains elusive, narrowing down the potential LS transporter candidates, which could be contributing to the current, to likely include system A.

The observed partial inhibition (37.7%; Figure 4.4A, B) of I_{LS} in astrocytes by MeAIB, a substrate-specific to system A transporters, indicates that system A transporters are also involved in LS uptake, albeit to a lesser extent than ASCT. While DS uptake was observed to be insensitive to MeAIB (Figure 3.8), the uptake of LS in astrocytes was partially inhibited by MeAIB (Figure 4.4). This observation aligns with previous studies indicating that SNAT2's efficiency for LS surpasses its efficiency for DS uptake by 20-fold, whereas SNAT1's efficiency for LS uptake is three times higher than its efficiency for DS uptake (Bodner et al. 2020). These transporters are estimated to contribute approximately 30-50% to both DS and LS transport in synaptosomes (Bodner et al. 2020). Although Hatanaka et al. (2000) reported minimal DS and

LS transport via these transporters, the observed transport was not significant enough to have a meaningful impact on the physiological processes in human retinal pigment epithelial cells.

The distinct expression pattern of these transporters further underscores their role in LS uptake. The expression of SNAT2 in specific neuronal subtypes and regions of the brain suggests that it might not play a role in the astrocytic uptake of LS (Yao et al., 2000; González-González et al., 2005). While studies reported a lack of immunofluorescence in astrocytes, it was clarified that the localisation of SNAT2 is more restricted to the perivascular end-feet of astrocytes (González-González et al., 2005; Jenstad et al., 2009). Conversely, SNAT1 is expressed in both neurons and astrocytes, with only some astrocytic populations, alongside ependymocytes, and endothelial cells showing moderate expression levels (Chaudhry et al., 2002; Solbu et al., 2010).

Beyond the expression pattern, the highly regulatory mechanisms governing these transporter's surface expression add depth to our understanding of astrocytic LS transport. The amino acid response elements present within the promoter regions are activated during amino acid deprivation, leading to an increased membrane expression of SNAT2 transporter (Gazzola et al., 2001; Ling et al., 2001; Palii et al., 2006). This regulatory variability likely contributes to some disparities in LS-mediated membrane currents. It is hypothesised that astrocytes with higher LS currents might possess elevated SNAT2 expression, while those with lower currents might exhibit lower expression levels. To support this hypothesis, a post-hoc staining procedure or single-cell quantitative PCR after recording from each cell could be employed, verifying the correlation between LS currents and SNAT2 expression levels. This investigation would further strengthen our understanding of the intricate regulatory interplay underlying astrocytic LS transport via system A transporters.

Overall, the protein expression profile and variability observed in LS uptake suggest that astrocytic LS uptake could be influenced by SNAT2, though to a minor extent in most cells and a moderate extent in some cells. While System A transporters contribute to LS uptake in astrocytes to a minor extent, the predominant role in facilitating LS uptake seems to be played by the ASCT1 transporter. However, pharmacological inhibitors might lack high specificity. A more precise understanding of the roles and characteristics of System A (SNAT1 and SNAT2) and System ASC (ASCT1 and ASCT2) transporters could be obtained using genetically modified animal models where specific transporters (SNAT1, SNAT2, ASCT1, or ASCT2) are knocked out. These models would allow for a more targeted investigation into the physiological roles of these transporters in LS uptake.

4.3.3 The uptake of different ASCT1 substrates in astrocytes and the electrogenicity associated with the transport

One key question arising from the results of Chapters 3 and 4 pertains to the preference of ASCT1 transporters for DS over other L-enantiomers in physiological conditions. It is evident from my observations that ASCT1 transporters can handle the transport of both DS and certain L-enantiomers (LS, L-cysteine and L-asparagine) under physiological conditions. However, the degree of preference will depend on their respective extracellular concentrations and affinities. This phenomenon is in line with the transport kinetics of ASCT transporters, where competitive interactions among substrates are influenced by their relative extra and intracellular concentrations (Kaplan et al. 2018). This observation underscores the dynamic nature of serine transport and the possible interplay among different ASCT1 substrates in the extracellular environment surrounding the astrocytes.

In the context of amino acid transport via the ASCT1, the passage of substrates appears to occur independently of the activation of an intrinsically dormant chloride channel (Wang et al 2022). The behaviour of anion conductance in ASCT1 appears to vary depending on the specific amino acid being transported. Interestingly, while 10 mM DS and LS do not induce uncoupled charge movements (Figure 3.9A, Figure 4.5 C), certain other substrates might trigger this activation phenomenon. Evidently, serine has been demonstrated to function as a suppressor, rather than an activator of the anion conductance in ASCT1 (Wang et al. 2022). This is further supported as the magnitude of transmembrane currents evoked by L-asparagine and L-cysteine are approximately 20 times higher compared to DS and LS-evoked currents (Figure 4.7). Thus, the logical interpretation is that the electrogenicity associated with L-cysteine and L-asparagine could arise from an uncoupled anion movement in addition to a coupled charge movement. This activation is hypothesised to occur during an intermediate stage of the amino acid transport cycle. Overall, the variations in the magnitude of transmembrane currents observed across different substrates suggest that these differences could arise from the differential activation of the associated anionic channels in astrocytes. This hypothesis offers insights into the differential interactions of substrates within the ASCT1 and by extension its regulation of the integral anionic channel in cellular processes.

The currents generated by L-cysteine and L-asparagine were significantly greater than those observed for DS or LS. This is in contrast to experiments on ASCT1 expressed in oocytes where similar magnitude of currents was observed with these substrates (Arriza et al. 1993). This could be explained by differences in expression systems, or it is plausible that other transporters specific to L-cysteine or L-asparagine could contribute to these electrogenic

effects in a physiological context. Additionally, higher transport rates of L-cysteine and L-asparagine could occur if intracellular concentrations are low, promoting their influx. Thus, understanding these mechanisms in various expression systems is crucial, as it highlights the balance of transporter activity and its potential physiological relevance.

Moreover, ASCT1's behaviour in recombinant systems might differ significantly from its expression in the brain. While recombinant systems can show variability in the ratio of transport to anion conductance depending on the substrate, native brain tissue likely involves additional regulatory mechanisms that modulate these processes. These differences may be due to various post-translational modifications and unique intracellular interactions in mammalian cells that are not present in recombinant systems. For instance, ASCT1 in the brain might engage in distinct cell type-specific regulatory interactions and modifications, influencing its electrogenicity and stoichiometry. To better understand these phenomena, further proteomic investigations are necessary to elucidate the specific roles of ASCT1 in different cellular contexts.

4.3.4 The role of System N transporters in the regulation of L-serine needs investigation

The investigation into the potential involvement of astrocytic membrane transporters in LS uptake and release should extend to exploring the roles of System N: SNAT3 and SNAT5 transporters (encoded by the *Slc38a1* and *Slc38a5* genes, respectively). SNAT3 exhibit specific substrate preferences, favouring amino acids like glutamine, asparagine, and histidine over serine (Chaudhry et al., 1999). SNAT5, in particular, has been implicated in mediating LS transport and its expression has been reported in hippocampal astrocytes (Cubelos et al., 2005; Hamdani et al., 2012; Bröer, 2014). Given that transport via SNAT5 is sodium-dependent and associated with pH changes, employing techniques such as sodium and pH imaging becomes crucial to detect intracellular sodium and pH changes associated with these transport processes (Chaudhry et al., 1999).

Prior research from the Billups Lab has demonstrated the role of the SNAT3 transporter expressed in astrocytes, particularly its involvement in regulating glutamine transport within the brain (Todd et al., 2017). Following a similar investigative approach, sodium and pH imaging techniques can be effectively employed to explore the functions of SNAT5 transporter in the transport of LS within astrocytes.

The observed LS uptake in astrocytes (Figure 4.2) may be attributed to system N transporters, such as SNAT3 and SNAT5, which are known to facilitate electroneutral amino acid transport (Hamdani et al., 2012). This suggests that the mechanism of LS uptake in astrocytes could involve these electroneutral transporters, complementing the role of ASCTs in regulating amino acid levels in the brain.

Moreover, the remaining currents observed upon inhibition of ASCT1 (Figure 4.6) could be attributed to the activity of SNAT3 and SNAT5. Both SNAT3 and SNAT5 are electroneutral transporters but can generate uncoupled currents in astroglial cells upon stimulation with L-asparagine (Chaudhry et al., 2001). These system N transporters, particularly SNAT3 are known to transport L-asparagine and their activity is modulated by the membrane potential (Chaudhry et al., 1999; Hamdani et al., 2012). Depending on the membrane potential, they may contribute to the transport of L-asparagine and/or the generation of uncoupled currents. Thus, the remaining currents seen in Figure 4.6 upon inhibition of ASCT1 may indicate functional system N transport.

4.3.5 Role of System A in the uptake of L-serine in CA1 pyramidal neurons

The final objective was to investigate the mechanisms underlying LS uptake in pyramidal neurons and to understand the pivotal role of neuronal LS plasma membrane transporters in this process. To address this, 10 mM LS was applied directly near the voltage-clamped CA1 pyramidal neurons of interest while recording associated transport currents. Electrogenic LS transport (I_{nLS}) was identified in hippocampal pyramidal neurons located in the CA1 cell layer. Further investigation revealed that this transport mechanism was highly sensitive to MeAIB (Figure 4.7A, B), suggesting an important role of system A transporters. In addition, this transport mechanism was insensitive to O-benzyl-L-serine (Figure 4.7C, D), indicating a less prominent role for ASCT transporters in CA1 pyramidal neurons compared to astrocytes.

Although O-benzyl-L-serine reduced LS uptake in CA1 pyramidal neurons, the reduction was not statistically significant. This does not rule out the potential contribution of ASCT1 in the uptake of LS. The observed reduction of 70.7% indicates a potential effect that warrants further investigation. More data would be necessary to definitively determine ASCT1's involvement. Even with the current sample size of 4 cells, the results provide valuable insights.

The significant reduction observed with MeAIB (83%) in 6 cells strengthens the hypothesis that system A transporters play a major role in LS uptake. Although the statistical significance was

not reached for O-benzyl-L-serine, the trend suggests that ASCT1 could still be involved, albeit to a lesser extent.

Previously reported gene expression data helped identify the presence of specific transporters in CA1 pyramidal neurons that mediate the uptake of LS. The presence of ASCT1 immunoreactivities in early embryonic stages suggested their existence in immature neurons. However, as neurons matured, the expression of ASCT1 was downregulated, becoming diminished in mature neurons. Although ASCT1 mRNA persisted in adult neurons, its immunoreactivities were not detected on their cell membranes (Sakai et al. 2003). Interestingly, ASCT1 was found to be expressed in the dendrites and axons of adult human hippocampal neurons (Weis et al., 2007), implicating an underlying species difference. In contrast, ASCT2 was primarily observed in cultured neurons and not in mature neurons, making its role in LS uptake within neurons less likely. On the other hand, SNAT1 and SNAT2 were detected in neurons, present in both somatic and dendritic compartments (Reimer et al., 2000; Varoqui et al., 2000; Yao et al., 2000; Armano et al., 2002; Chaudhry et al., 2002). This intricate expression pattern implicates the involvement of System A predominantly in LS uptake within pyramidal neurons. The potential role of ASCT1 transporter in early development is unclear. Hence, further investigating these transporter dynamics in pyramidal neuronal at different developmental stages would offer deeper insights into the regulatory mechanisms governing LS transport.

In addition to being expressed in neurons and astrocytes, SNAT1 and SNAT2 are also found in interneurons. Therefore, for future research, it would be valuable to explore LS uptake in interneurons. The extent to which SNAT1 and SNAT2 transport LS could vary based on the type of synapse involved. In the hippocampus CA1 region, GABAergic interneurons exhibit high expression of SNAT1 (Chaudhry et al., 2002; Mackenzie et al., 2003; Melone et al., 2004) while lacking SNAT2 expression (González-González et al., 2005). This specific transporter profile raises interesting questions regarding LS uptake mechanisms in these interneurons. It is hypothesised that SNAT1 in GABAergic interneurons might serve as the primary mediator of LS uptake. Despite lower levels of SR in comparison to glutamatergic neurons, interneurons show strong staining for DS (Miya et al., 2008; Balu et al., 2014). Consequently, the LS sequestered into these cell types via SNAT1 could potentially contribute to the observed elevated levels of DS within these hippocampal interneurons.

4.4 Concluding remarks

In this chapter, the electrophysiological investigation of astrocytic and neuronal membrane transporters involved in LS uptake has provided valuable insights into the complex mechanisms underlying LS regulation within the mammalian brain. From the data gathered, it is evident that astrocytes can mediate an electrogenic transport of LS. Using pharmacological manipulation of the LS-mediated transporter currents, ASCT1 emerges as a key player in the astrocytic uptake of LS, implicating its role in the shuttle of LS between astrocytes and neurons. Its bidirectional capability positions it as a likely candidate for the exchange of extracellular DS for intracellular LS, a process crucial for promoting the replenishment of DS in neurons. Astrocytes are also equipped with the enzyme 3-phosphoglycerate dehydrogenase and exhibit an intrinsic capacity to synthesise LS. Therefore, this abundant astrocytic LS can serve as a readily available supply, facilitating its efficient transfer to neighbouring neurons.

Additionally, CA1 pyramidal neurons were also capable of mediating an electrogenic uptake of LS. From the pharmacological inhibition profile observed, along with previously reported gene expression data, SNAT1 and SNAT2 emerge as likely candidates mediating LS uptake in pyramidal neurons, emphasising the intricate astrocyte-neuron signalling essential for intercellular communication within the mammalian brain.

Dysfunctional LS biosynthesis pathways have been previously observed in Alzheimer's disease, underscoring the pivotal role of astrocytic LS in synaptic plasticity and cognitive function. Although dietary LS supplementation has proven to be a promising avenue, my current finding implies that targeting ASCT1 or system A transporter-mediated LS uptake could help prevent synaptic and behavioural impairments in AD. The findings from this chapter provide a platform for future research exploring the therapeutic potential of regulating LS, in preserving cognitive health.

Chapter 5 Role of ASCT1 transporter in mediating NMDA receptor-mediated excitatory neurotransmission.

5.1 Aim

The regulatory dynamics between glutamate and its co-agonists, D-serine (DS) and glycine, within the synaptic cleft determine NMDAR activation. While glycine, a presumed co-agonist in extra synaptic NMDAR, exhibits controlled concentrations through efficient transporters, DS's synaptic levels remain less characterised. Having established the functional presence of ASCT1 (*Slc1a4*) in the astrocytes, the next chapter delves into the landscape of CA3-CA1 hippocampal excitatory synapses, aiming to elucidate the regulatory mechanisms governing synaptic DS concentration, with a specific focus on the ASCT1 and other transporters. In particular, the main objective is to study synaptic serine regulation via monitoring the NMDAR currents in CA1 pyramidal cells. By pharmacologically manipulating ASCT1-mediated DS/LS transport and recording NMDAR-mediated excitatory postsynaptic currents (EPSCs) in mouse hippocampal slices, the aim is to discern ASCT1's impact on synaptic DS levels and, consequently, NMDAR-mediated neurotransmission. I hypothesise that inhibition of the ASCT1 transporter decreases synaptic DS and therefore decreases the NMDAR-mediated response at the hippocampal CA3-CA1 synapses.

5.2 Results

5.2.1 NMDAR currents in hippocampal SC-CA1 synapses

NMDAR-mediated EPSCs were recorded at CA3-CA1 glutamatergic synapses using whole-cell voltage-clamp recording with a holding potential of -70 mV. A cesium methanesulfonate-based internal solution was used to block potassium channels and this allowed for the recording of large EPSCs from the soma of CA1 pyramidal neurons. A presynaptic stimulation (see methods) was delivered to the *Schaffer collateral* pathway, the main excitatory pathway from CA3 to CA1, to evoke EPSC responses. A consistent pattern of two stimuli yielded two peak EPSC responses. The amplitude of first EPSC response is reported while the second EPSC was utilised to calculate Paired Pulse Ratio (PPR), with any changes exceeding 30% throughout the recording period being excluded. In addition, cells were excluded, if input resistance (R_i) and series resistance (R_s) values and baseline current changes exceeding 30% throughout the recording period in all subsequent experiments.

To make sure that the recorded currents are due to an isolated or uncontaminated NMDAR-mediated synaptic current, the following control experiments were performed. EPSCs were recorded before and after the application of NBQX to isolate the AMPAR component of the response. Compared to AMPAR currents, which govern the initial rise of the EPSC response, NMDAR currents rise more gradually and contribute to the extended deactivation phase. Bath application of 20 μ M NBQX abolished the fast AMPA component of the EPSC response recorded from the soma of the CA1 pyramidal neurons. In every experiment, a significant ($p < 0.001$; $n = 5$; Figure 5.1A) measurable inward synaptic current (slower component) remained in the presence of NBQX (-38.0 ± 7.3 pA) compared to the current before NBQX application (-121.6 ± 35.6 pA). This demonstrates that NBQX effectively inhibits the AMPA component, allowing the slower component to be attributed to NMDAR currents recorded from the CA1 neuronal cell bodies.

To further confirm this, a GluN2 site competitive antagonist of NMDAR, 40 μ M D/L-APV/AP5 was bath applied along with 20 μ M NBQX, and the EPSC responses were recorded from the CA1 pyramidal neurons. APV significantly ($p < 0.001$; $n=4$; Figure 5.1B) reduced the slow component of the EPSC response (-2.4 ± 0.4 pA) compared to control condition in the presence of NBQX alone (-25.3 ± 6.0 pA). This observation indicates that synaptically active NMDAR currents can be recorded from the postsynaptic neuron voltage-clamped at -70 mV, despite the presumed block by external Mg^{2+} ions. At this membrane voltage NMDAR currents are predominantly influenced by Na^+ and Ca^{2+} influx.

To assess the stability of NMDAR-mediated EPSCs during LFS stimulation, a control experiment was conducted. The initial average amplitude of EPSCs over the first 5 minutes was 34.7 ± 10.0 pA, with no statistically significant difference ($p = 0.43$; $n=5$; Figure 5.1 C, D) observed compared to the final amplitude of 31.0 ± 6.8 pA at 15 – 20 minutes. This finding suggests that the currents did not exhibit rundown during the experimental protocol.

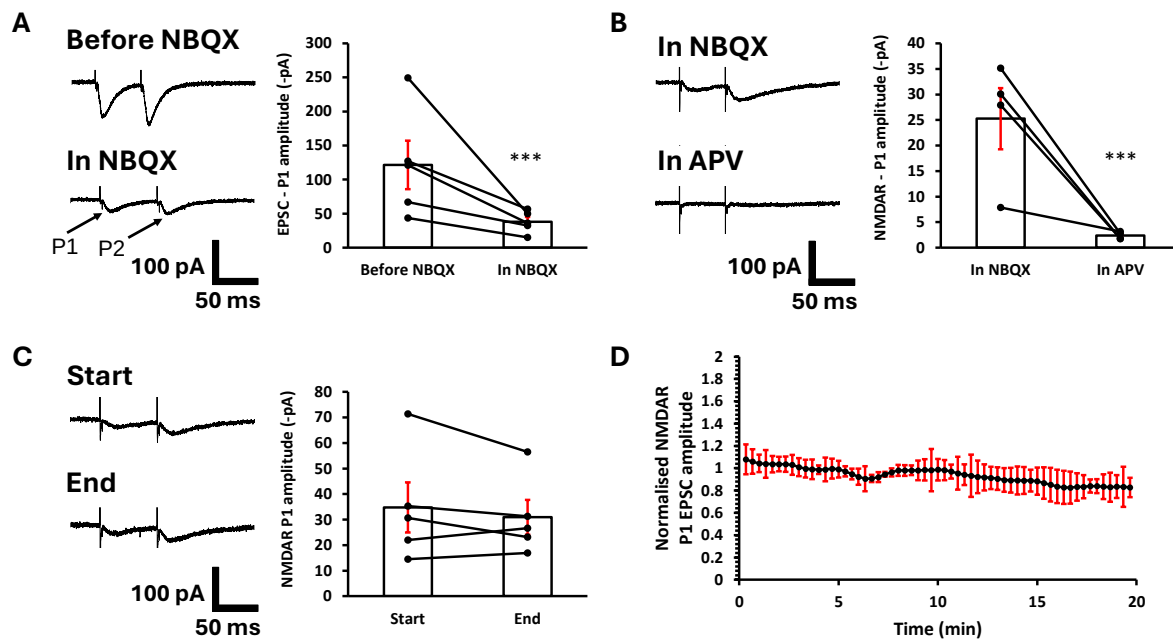


Figure 5.1: Characterisation of NMDAR-mediated excitatory postsynaptic currents

(A) Example recording of EPSC from a CA1 pyramidal neuron during presynaptic stimulation in control conditions (before application of NBQX) and post-NBQX application. Averaged data demonstrating a significant remaining inward synaptic current (slower component) in the presence of NBQX, confirming the contribution of NMDAR currents. Arrows point towards the peaks (P1 and P2) of NMDAR-mediated EPSC response. (B) Example recording of NMDAR-mediated EPSC in the presence of 20 μ M NBQX and both NBQX and 40 μ M APV/AP5 respectively. Averaged data showing a significant reduction in the slow component with APV/AP5 application, supporting the involvement of NMDAR currents. (C, D) Example recording of EPSC at the start and the end of the experiment. Averaged data showing no statistically significant difference in EPSC amplitude between the initial 5 minutes and the final (15 – 20 minutes) recording period. *** represents a significant difference at $p \leq 0.001$.

5.2.2 Exogenous L-serine application does not enhance NMDAR currents

During conditions of normal synaptic transmission, neurons require a supply of LS from the neighbouring astrocytes to synthesise DS. To explore whether exogenous application of LS enhances the DS synthesis and consequently enhances NMDAR response, NMDAR-mediated EPSCs were recorded before and after bath applying 100 μ M LS.

The NMDAR responses did not significantly change ($p = 0.11$) during the bath application of LS (-26.8 ± 2.8 pA; $n = 6$; 10 – 15 minutes) when compared to the control conditions (-32.7 ± 5.6 pA; $n = 6$; 5 minutes prior LS application; Figure 5.2). This suggests that during LFS conditions, the endogenous/extracellular concentrations of DS might saturate the NMDARs and that the exogenous addition of LS does not alter the recorded EPSC response. This result also suggests that the endogenously occurring LS shuttle from the astrocytes might be sufficient to maintain basal levels of neurotransmission in the CA3-CA1 synapses.

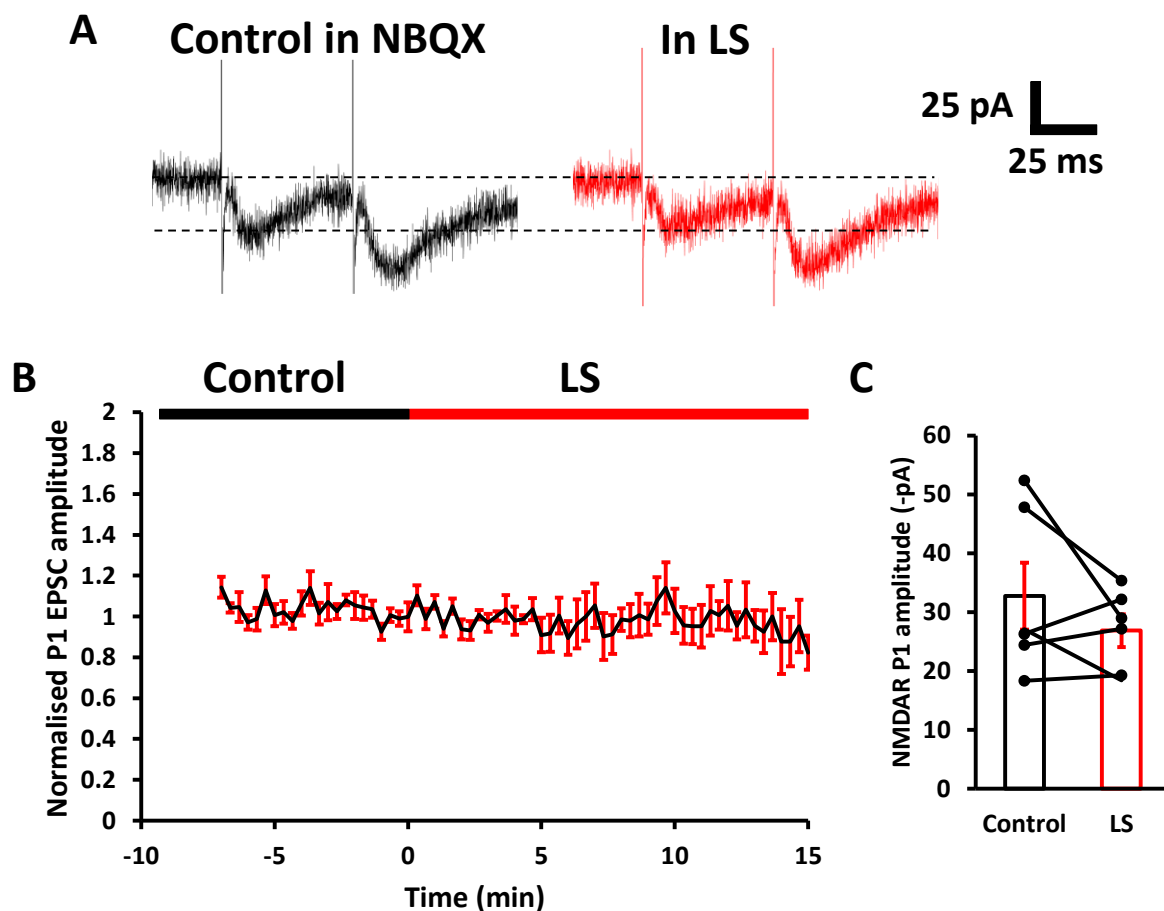


Figure 5.2: Exogenous LS does not enhance P1 amplitudes associated with NMDAR-mediated EPSC

(A) Example NMDAR-mediated EPSC recording from a CA1 pyramidal neuron during a single sweep in control conditions (in presence of 20 μM NBQX, black) and during bath application of 100 μM LS (red). (B) Averaged data showing that the application of LS (100 μM) had no effect to the normalised P1 EPSC amplitude, compared to the control condition. Normalisation was done with respect to 5 min before LS application. (C) Bar graph showing no difference in average P1 amplitudes between control condition (black; 5 minutes prior LS application) and LS condition (red; 10 – 15 minutes).

5.2.3 Exogenous D-serine application does not enhance NMDAR currents

The next objective was to directly investigate the ability of exogenous DS to modulate the NMDAR activation. If the glycine binding site (GBS) of the NMDAR is saturated under *in-situ* physiological conditions, the external application of DS will not increase the NMDAR response. On the contrary, if the *in-situ* concentrations of DS are below the saturation concentration of the GBS of the NMDAR, the exogenous application of DS will enhance the NMDAR-mediated EPSC response. To investigate this, a saturating concentration (100 μM) DS was bath applied and the post-synaptic NMDAR responses were monitored. However, the exogenous application of DS did not alter ($p = 0.31$) the observed NMDAR-mediated EPSC responses (-40.9 ± 8.9 pA, $n = 4$) compared to control conditions (-47.8 ± 8.8 pA, $n = 4$, Figure 5.3). This

suggests that the GBS of the NMDAR located in the CA3-CA1 synapses is likely saturated under *in-situ* physiological conditions.

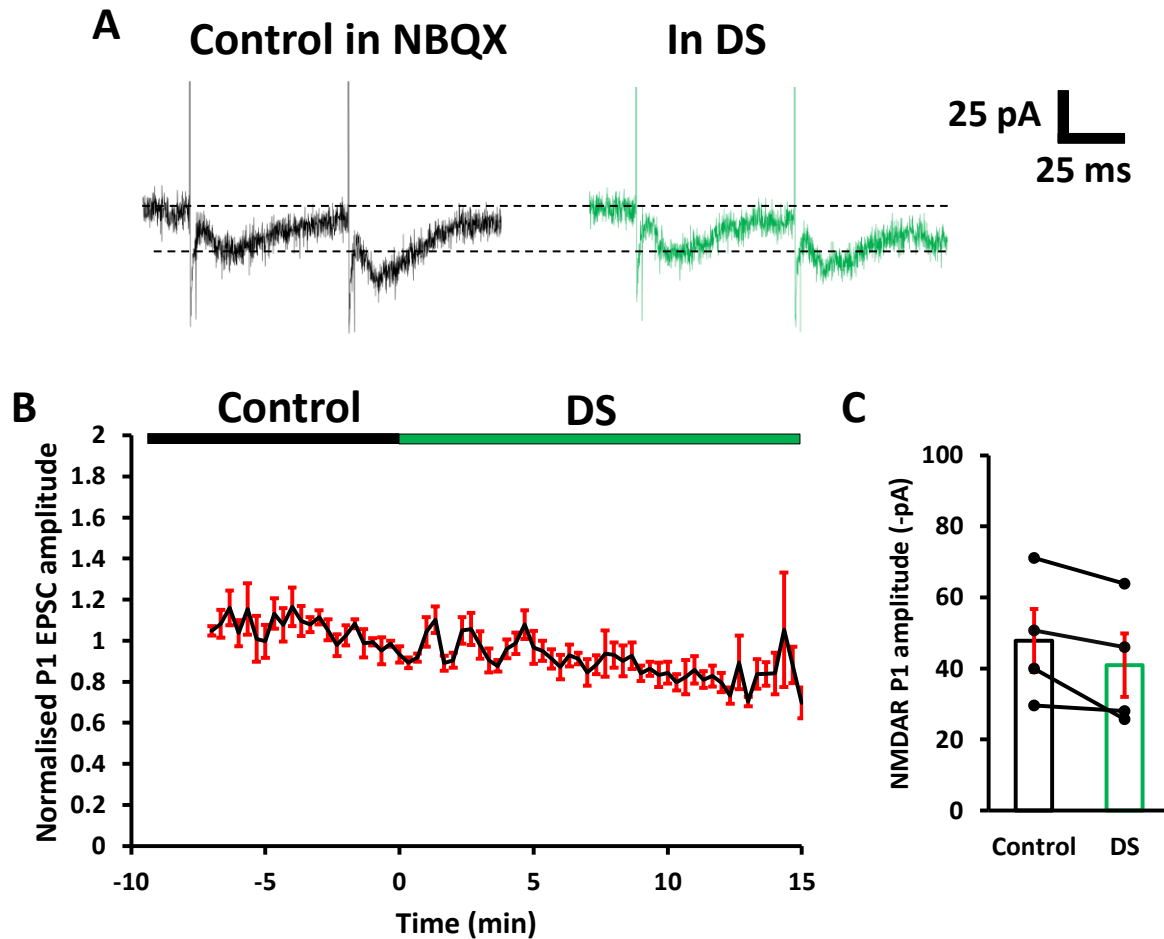


Figure 5.3: Exogenous DS does not enhance P1 amplitudes associated with NMDAR-mediated EPSC

(A) Example NMDAR-mediated EPSC recording from a CA1 pyramidal neuron during a single sweep in control conditions (in presence of 20 μ M NBQX, black) and during bath application of 100 μ M DS (green). (B) Averaged data showing that the application of DS (100 μ M) had no effect to the normalised P1 EPSC amplitude, compared to the control condition. Normalisation was done with respect to 5 min before DS application. (C) Bar graph showing no difference in average P1 amplitudes between control condition (black; 5 minutes prior DS application) and DS condition (green; 10 – 15 minutes).

5.2.4 Fine-tuning of NMDAR-mediated EPSC response

Since experiments so far indicated that the GBS of NMDAR could be saturated under *in-situ* conditions. The addition of exogenous DS nor LS did not enhance the EPSC responses (Figure 5.2, Figure 5.3). Therefore, desaturation or partial inhibition of the GBS was attempted to modulate the GBS-mediated NMDAR response.

To achieve this, a low affinity and fast unbinding inhibitor of the GBS of the GluN1 subunit was used. To assess what level of extracellular DS is activating the NMDARs, a low concentration of 30 μM or 60 μM kynurenate, capable of inhibiting the GluN1 site was used (Birch et al., 1988; Henderson et al., 1990). It was hypothesised that kynurenate would significantly inhibit the NMDAR-mediated EPSC response. For 30 μM kynurenate, there was no significant inhibition ($p = 0.29$, $n = 4$) of NMDAR mediated EPSCs (-32.9 ± 3.5 pA; $n = 4$) compared to control conditions (-38.2 ± 5.0 pA; $n = 4$; Figure 5.4). Bath application of 60 μM kynurenate significantly inhibited ($p < 0.001$, $n = 7$) NMDAR mediated EPSCs (-33.2 ± 4.5 pA; $n = 7$) compared to control conditions (-54.2 ± 4.0 pA; $n = 7$; Figure 5.5). Although 30 μM kynurenate was not capable of significantly inhibiting the NMDAR-mediated EPSCs, it could have resulted in partial desaturation of some GBS. Results on using 60 μM kynurenate show that the concentration (60 μM) is capable of significant inhibition (reduced by 39.0%) of NMDAR-mediated EPSCs. This result also suggests that the GBS of the GluN1 subunit can be functional or subject to further manipulations.

The mean baseline current, P2/P1 ratio, input resistance (R_i) and series resistance (R_s) values did not change significantly ($p > 0.05$; $n = 4$) over the course of the recording between NBQX (-120.2 ± 27.2 pA, 1.45 ± 0.27 , 235.57 ± 45.48 M Ω , 22.84 ± 2.49 M Ω) and NBQX + 30 μM kynurenate (-147.2 ± 27.4 pA, 1.40 ± 0.21 , 237.12 ± 44.59 M Ω , 26.66 ± 3.76 M Ω). Similarly, there were no significant differences ($p > 0.05$; $n = 7$) between NBQX (-165.8 ± 41.2 pA, 1.15 ± 0.19 , 197.89 ± 37.72 M Ω , 18.36 ± 2.49 M Ω) and NBQX + 60 μM kynurenate (-203.3 ± 45.4 pA, 1.03 ± 0.17 , 167.79 ± 20.44 M Ω , 22.59 ± 3.73 M Ω) in baseline current, P2/P1 ratio, R_i , and R_s respectively. This stability in PPR suggests that this observation is consistent with a mechanism of kynurenate binding to the post-synaptic receptor and not affecting pre-synaptic release probability of neurotransmitters. These observations support the idea that any changes observed in NMDAR-mediated EPSCs are not likely attributed to alterations in these other parameters.

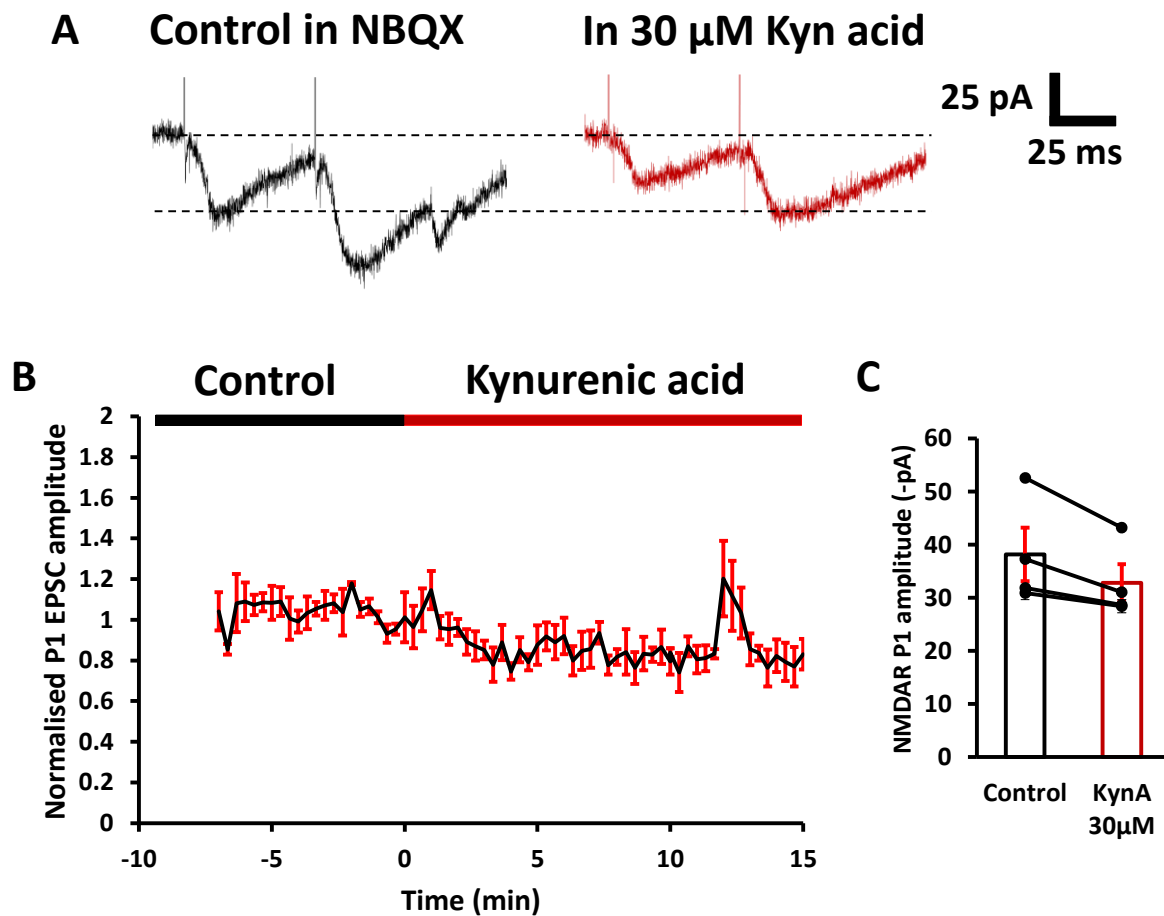


Figure 5.4: 30 μ M Kynurenate does not significantly desaturate the glycine-binding site of GLUN1 subunit of NMDARs

(A) Example NMDAR-mediated EPSC recording from a CA1 pyramidal neuron during a single sweep in control conditions (in presence of 20 μ M NBQX, black) and during bath application of 30 μ M kynurenate/ kynurenic acid (brown). (B) Averaged data showing that the application of kynurenate (30 μ M) had no effect to the normalised P1 EPSC amplitude, compared to the control condition. Normalisation was done with respect to 5 min before kynurenate application. (C) Bar graph showing no difference in average P1 amplitudes between control condition (black; 5 minutes prior kynurenate application) and kynurenate condition (brown; 10 – 15 minutes).

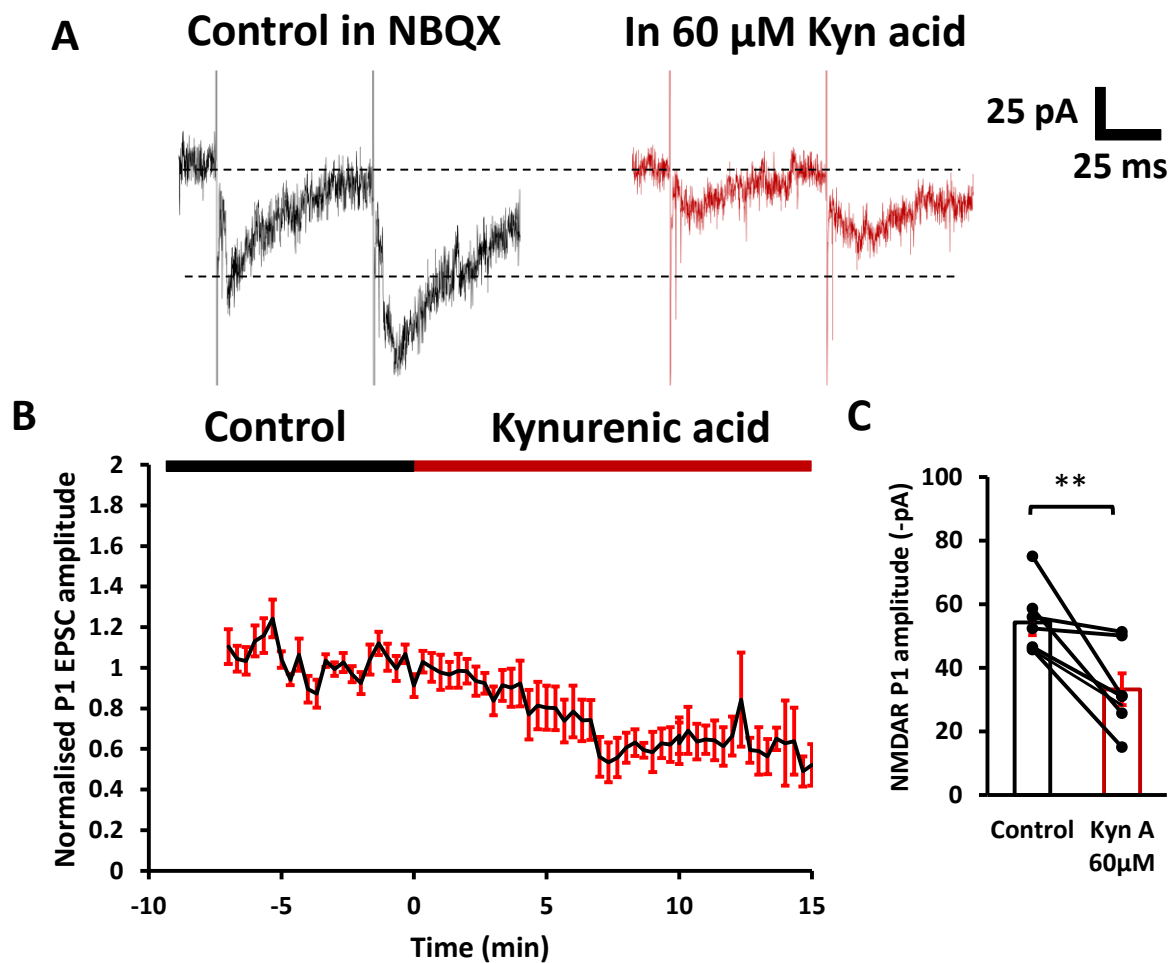


Figure 5.5: 60 μ M Kynurenic acid significantly desaturates the glycine- binding site of GLUN1 subunit of NMDARs

(A) Example NMDAR-mediated EPSC recording from a CA1 pyramidal neuron during a single sweep in control conditions (in presence of 20 μ M NBQX, black) and during bath application of 60 μ M kynurenic acid/ kynurenic acid (brown). (B) Averaged data showing that the application of kynurenic acid (60 μ M) significantly decreased the normalised P1 EPSC amplitude, compared to the control condition. Normalisation was done with respect to 5 min before kynurenic acid application. (C) Bar graph showing a significant difference in average P1 amplitudes between control condition (black; 5 minutes prior kynurenic acid application) and kynurenic acid condition (brown; 10 – 15 minutes). *** represents a significant difference at $p \leq 0.001$.

The next objective was to investigate whether LS can enhance the NMDAR-mediated EPSCs after partial desaturation of the GBS. For this, EPSC responses from CA1 pyramidal neurons were recorded, before and after bath application of 100 μ M LS, but in the presence of 20 μ M NBQX and 30 μ M kynureate during recording. Contrary to the hypothesis, exogenous application of LS did not significantly ($p = 0.90$; $n = 3$) enhance the EPSC responses recorded (-47.8 ± 10.8 pA; $n = 3$), compared to the control conditions (-46.5 ± 9.2 pA; $n = 3$; Figure 5.6).

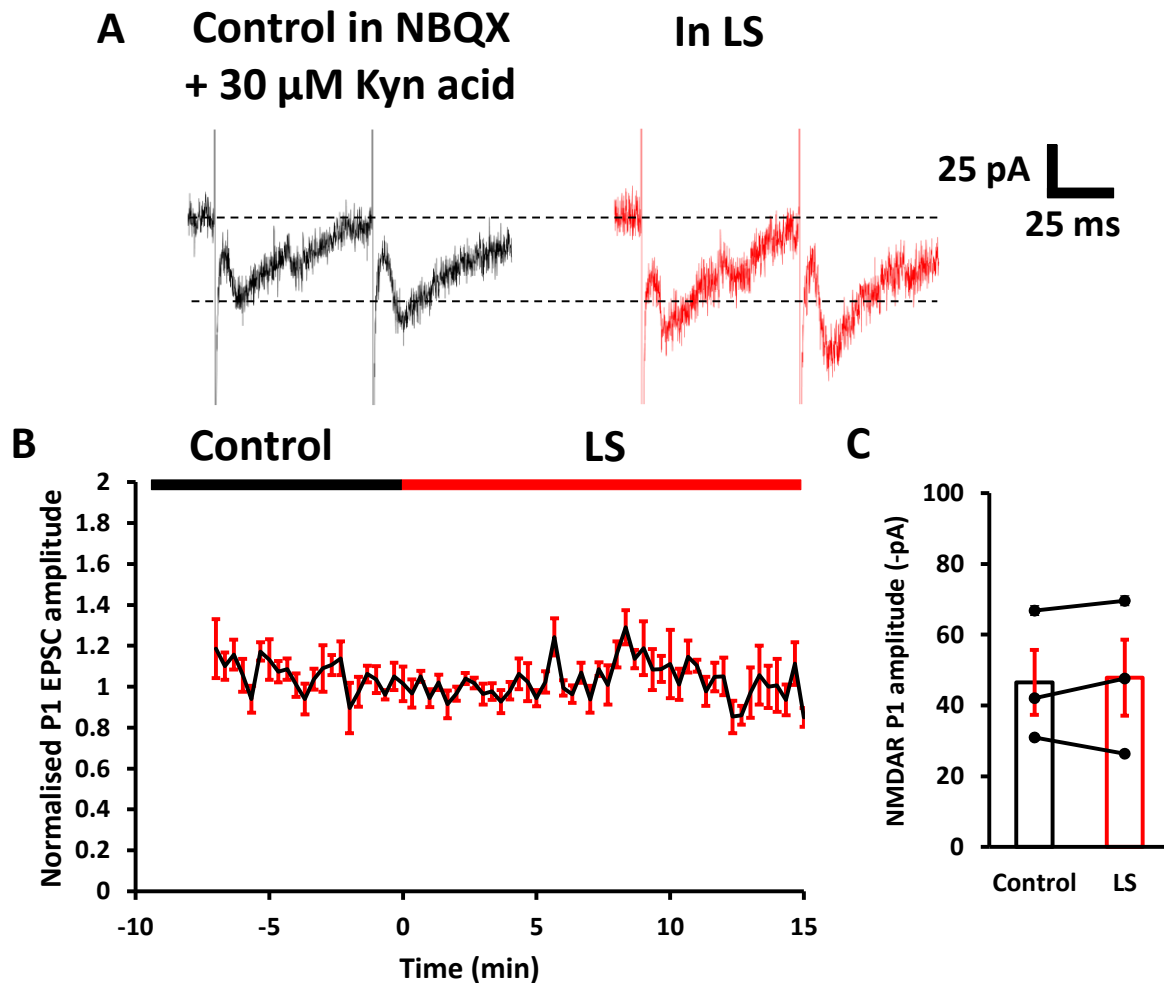


Figure 5.6: Exogenous LS does not enhance NMDAR-mediated EPSCs in presence of 30 μ M kynureate

(A) Example NMDAR-mediated EPSC recording from a CA1 pyramidal neuron during a single sweep in control conditions (in presence of 20 μ M NBQX and 30 μ M kynureate/ kynurenic acid, black) and during bath application of 100 μ M LS (red). (B) Averaged data showing that the application of LS (100 μ M) had no effect to the normalised P1 EPSC amplitude, compared to the control condition. Normalisation was done with respect to 5 min before LS application. (C) Bar graph showing no difference in average P1 amplitudes between control condition (black; 5 minutes prior LS application) and LS condition (red; 10 – 15 minutes).

Similarly, to investigate whether application of DS can enhance the NMDAR-mediated EPSCs after partial desaturation of the GBS, EPSC responses were recorded from CA1 pyramidal neurons, before and after bath application of 100 μ M DS, but in the presence of 20 μ M NBQX and 30 μ M or 60 μ M kynurenate during recording.

Exogenous application of DS in 30 μ M kynurenate (-56.1 ± 14.0 pA; $n = 5$) did not significantly ($p = 0.24$, $n = 5$) enhance the EPSC responses recorded, compared to the control conditions of 30 μ M kynurenate (-49.1 ± 8.2 pA; $n = 5$; Figure 5.7). Similarly, exogenous application of DS in 60 μ M kynurenate (-26.3 ± 6.4 pA; $n = 5$) did not significantly ($p = 0.34$, $n = 5$) enhance the EPSC responses recorded, compared to the control conditions of 60 μ M kynurenate (-33.0 ± 8.0 pA, $n = 5$, Figure 5.8). These observations align with the earlier observation (Figure 5.3), that the exogenous DS does not enhance NMDAR-mediated EPSCs in the CA3-CA1 synapses, despite the desaturation effect of 60 μ M kynurenate.

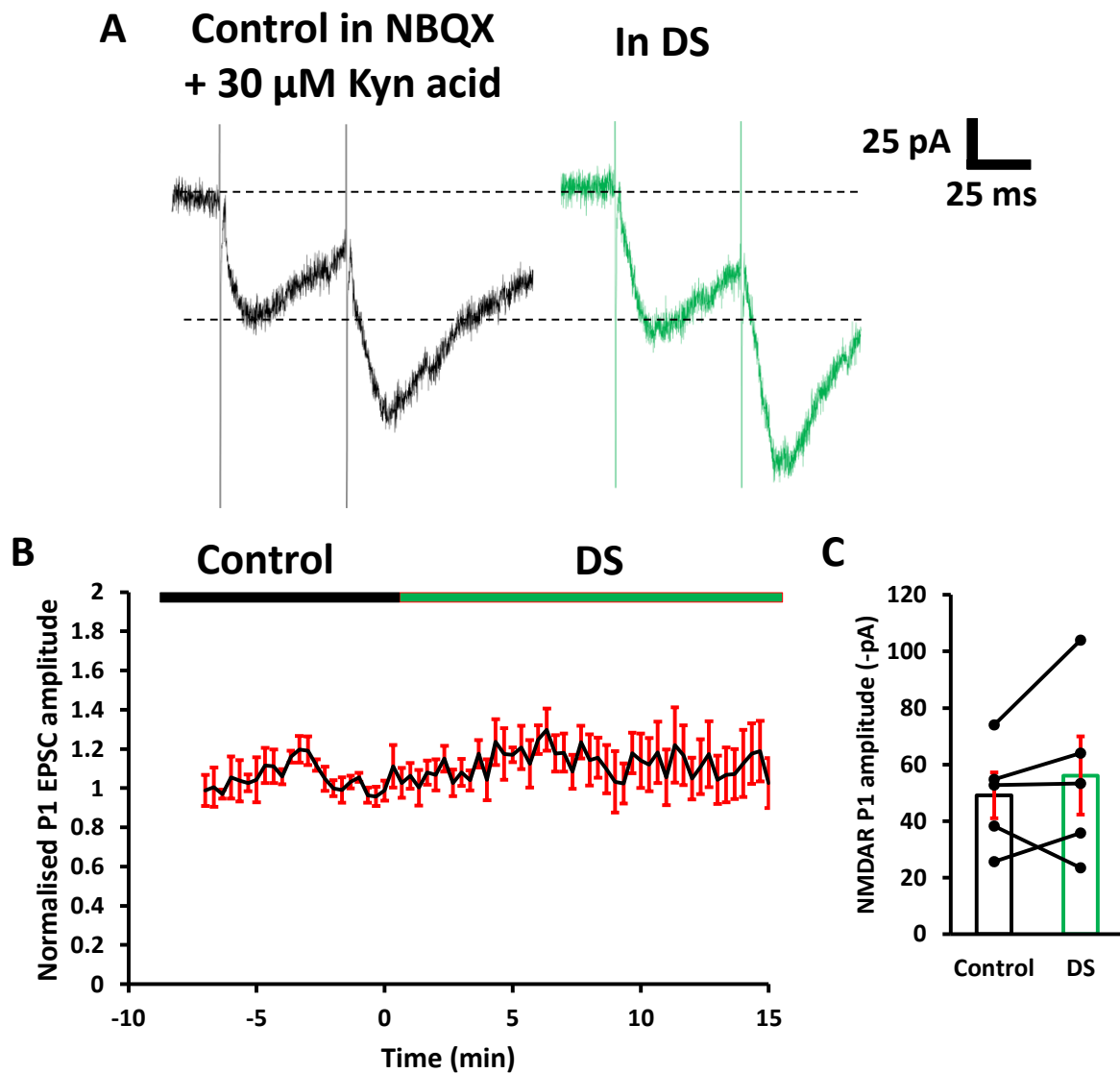


Figure 5.7: Exogenous DS does not enhance NMDAR-mediated EPSCs in presence of 30 μ M kynurenic acid

(A) Example NMDAR-mediated EPSC recording from a CA1 pyramidal neuron during a single sweep in control conditions (in presence of 20 μ M NBQX and 30 μ M kynurenic acid, black) and during bath application of 100 μ M DS (green). (B) Averaged data showing that the application of DS (100 μ M) had no effect to the normalised P1 EPSC amplitude, compared to the control condition. Normalisation was done with respect to 5 min before DS application. (C) Bar graph showing no difference in average P1 amplitudes between control condition (black; 5 minutes prior DS application) and DS condition (green; 10 – 15 minutes).

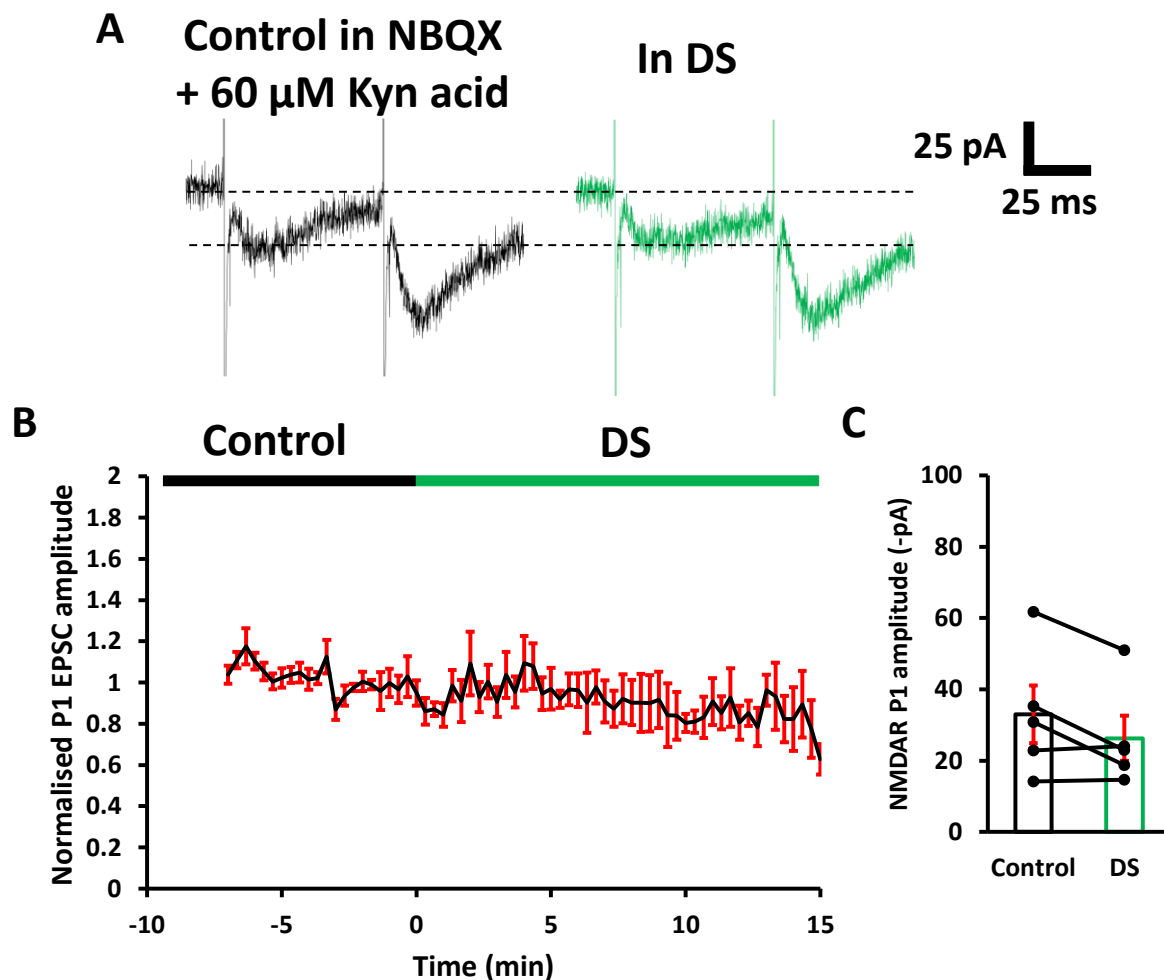


Figure 5.8: Exogenous DS does not enhance NMDAR-mediated EPSCs after desaturation of glycine binding site - using 60 μ M kynureinate

(A) Example NMDAR-mediated EPSC recording from a CA1 pyramidal neuron during a single sweep in control conditions (in presence of 20 μ M NBQX and 60 μ M kynureinate, black) and during bath application of 100 μ M DS (green). (B) Averaged data showing that the application of DS (100 μ M) had no effect to the normalised P1 EPSC amplitude, compared to the control condition. Normalisation was done with respect to 5 min before DS application. (C) Bar graph showing no difference in average P1 amplitudes between control condition (black; 5 minutes prior DS application) and DS condition (green; 10 – 15 minutes).

5.2.5 Inhibition of ASCT1 does not alter NMDAR-mediated EPSCs

The next objective was to investigate the role of ASCT1 (*Slc1a4*) in maintaining NMDAR-mediated neurotransmission in the hippocampal SC-CA1 synapses. As the LS shuttle plays an important role in the maintenance of constant DS levels in the synapses, the objective was to investigate whether inhibiting the LS shuttle affects the synaptic concentrations of DS. If ASCT1 plays an important role in regulating the synaptic DS concentrations, inhibition of ASCT1 will decrease the NMDAR-mediated EPSC response. To test this, NMDAR-mediated EPSCs were recorded before and after bath application of 10 mM benzyl serine or Trans-4-hydroxy-L-Proline (T-Pro), known inhibitors of ASCT1 transporter (Pinilla-Tenas et al., 2003; Grewer and Grabsch, 2004; Foster et al., 2016).

However, the application of 10 mM benzyl serine did not significantly ($p = 0.82$; $n = 6$) affect the NMDAR component of the EPSC response (-50.6 ± 21.9 pA; $n = 6$) compared to the control conditions (-53.7 ± 19.7 pA; $n = 6$; Figure 5.9). Similarly, the application of T-Pro also did not significantly ($p = 0.91$; $n = 6$) affect the NMDAR component of the EPSC response (-38.3 ± 11.1 pA, $n = 6$) compared to the control conditions (-36.8 ± 6.9 pA; $n = 6$; Figure 5.10). Contrary to the hypothesis, this observation indicates that ASCT1 does not play a significant role in governing the synaptic DS concentrations during the recording conditions.

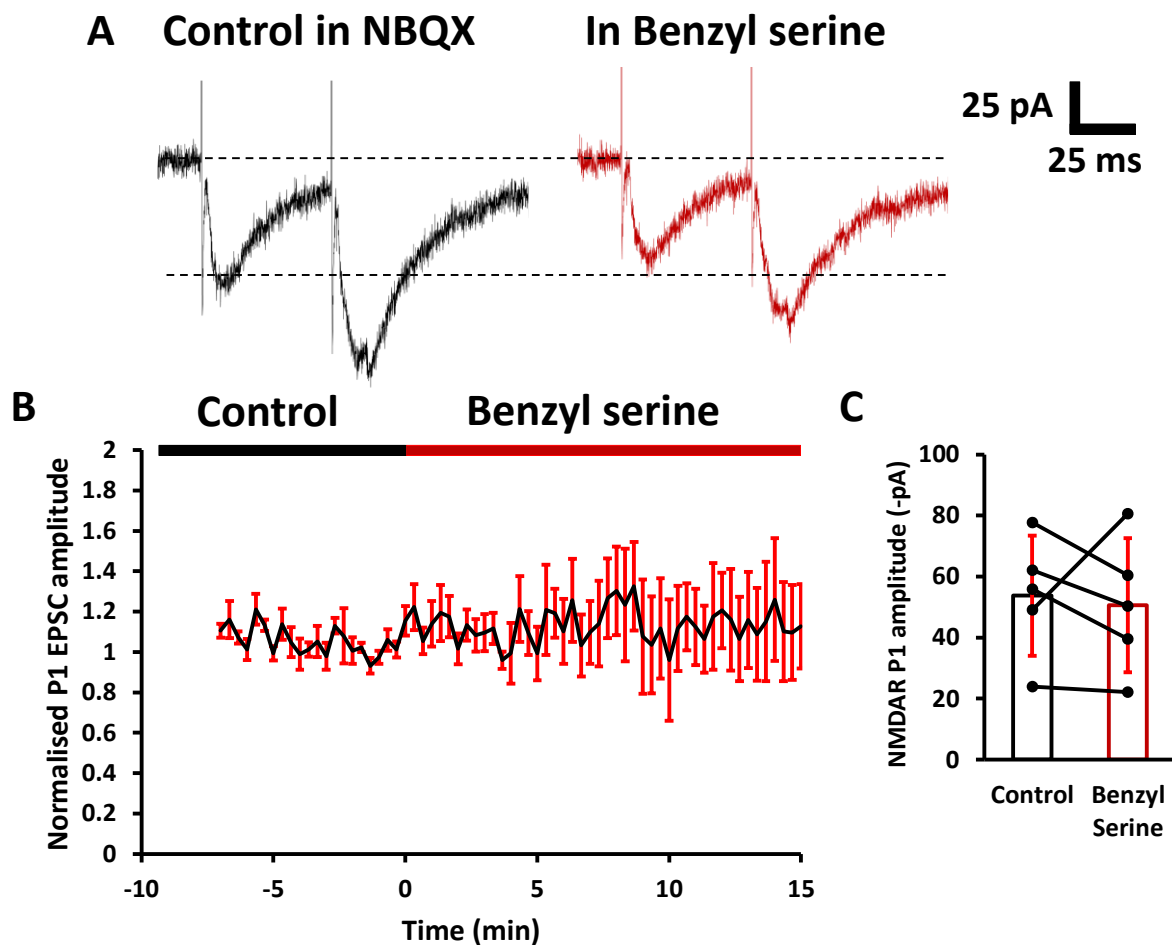


Figure 5.9: Inhibition of ASCT1 using O-benzyl-L-serine does not alter P1 amplitude associated with NMDAR-mediated EPSC

(A) Example NMDAR-mediated EPSC recording from a CA1 pyramidal neuron during a single sweep in control conditions (in presence of 20 μ M NBQX, black) and during bath application of 10 mM O-benzyl-L-serine/ benzyl serine (brown). (B) Averaged data showing that the application of benzyl serine (10 mM) had no effect to the normalised P1 EPSC amplitude, compared to the control condition. Normalisation was done with respect to 5 min before benzyl serine application. (C) Bar graph showing no difference in average P1 amplitudes between control condition (black; 5 minutes prior benzyl serine application) and benzyl serine condition (brown; 10 – 15 minutes).

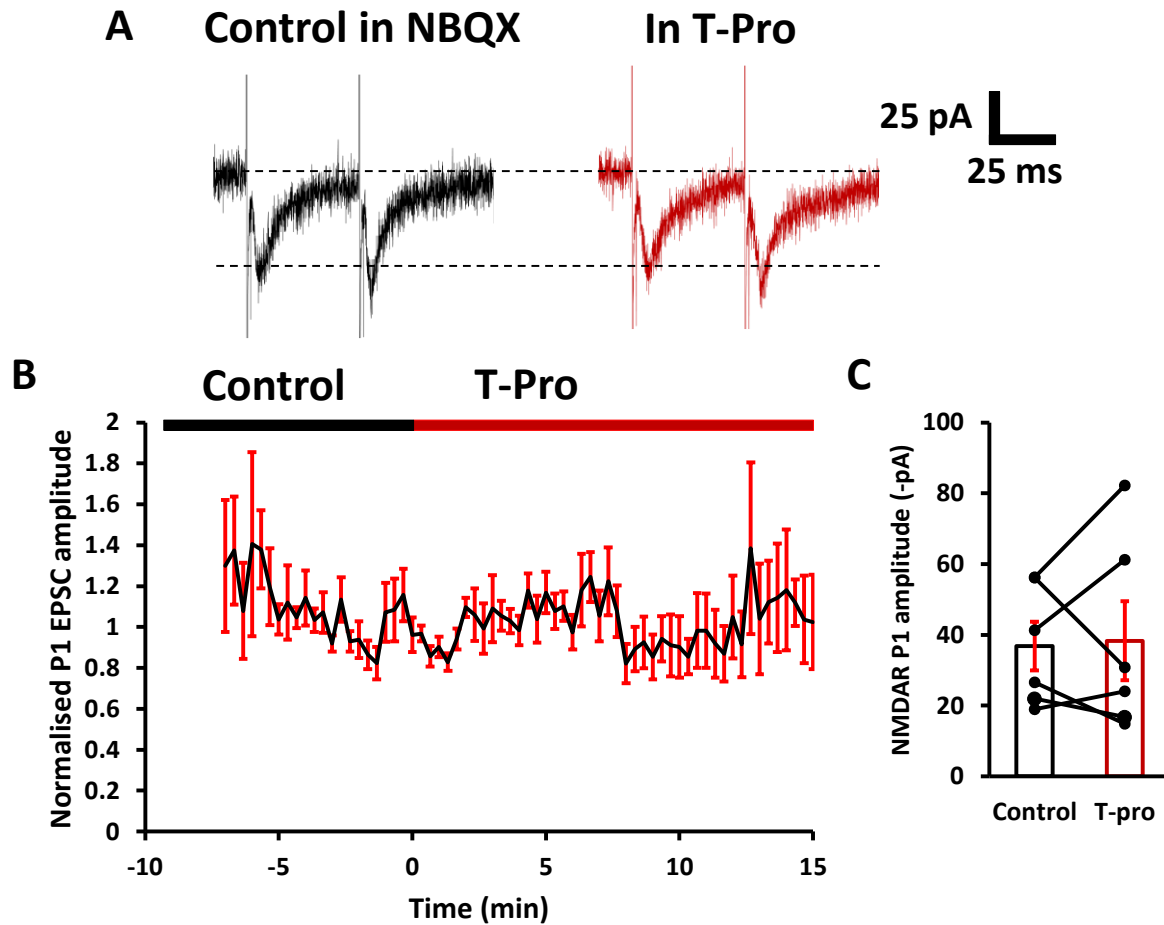


Figure 5.10: Inhibition of ASCT1 using Trans-4-hydroxy-L-proline does not alter P1 amplitude associated with NMDAR-mediated EPSC

(A) Example NMDAR-mediated EPSC recording from a CA1 pyramidal neuron during a single sweep in control conditions (in presence of 20 μ M NBQX, black) and during bath application of 10 mM Trans-4-hydroxy-L-proline/ T-Pro (brown). (B) Averaged data showing that the application of T-Pro (10 mM) had no effect to the normalised P1 EPSC amplitude, compared to the control condition. Normalisation was done with respect to 5 min before T-Pro application. (C) Bar graph showing no difference in average P1 amplitudes between control condition (black; 5 minutes prior T-Pro application) and T-Pro condition (brown; 10 – 15 minutes).

5.2.6 Inhibition of SNAT1 or 2 or asc-1 decreases NMDAR-mediated EPSCs

To investigate the regulation of NMDAR-mediated neurotransmission in hippocampal SC-CA1 synapses, the focus was on potential DS transporters that could influence DS concentrations within the synapses. As SNAT1 or 2 (*Slc38a1* or *Slc38a2*) and asc-1 (*Slc7a10*) are likely to play a role in generating DS from LS for its release into the synapses (Rosenberg et al., 2010; Bodner et al., 2020), these two candidate transporters were investigated further. If SNAT1/2 transport LS into postsynaptic cells, contributing to DS release, inhibiting these transporters might reduce DS availability and subsequently decrease NMDAR-mediated EPSCs. To test this, NMDAR-mediated EPSCs were recorded before and after bath application of 10 mM MeAIB (α -(MethylAmino) IsoButyric acid), known specific substrate of SNAT1 or 2 transporters (Armano et al., 2002). MeAIB was observed to significantly decrease ($p < 0.01$, $n = 6$) the NMDAR component of the EPSC response (-19.2 ± 3.5 pA, $n = 6$) compared to control conditions (-34.5 pA $\pm$ 4.4, $n = 6$, Figure 5.11). This observation indicates that SNAT1 or 2 could play a significant role in governing the synaptic DS concentrations.

Throughout the recording, baseline current, P2/P1 ratio, R_i , and R_s showed no significant changes ($p > 0.05$; $n = 6$) between NBQX (-87.7 ± 27.1 pA, 1.25 ± 0.20 , 324.19 ± 54.76 M Ω , 17.92 ± 2.31 M Ω) and NBQX + MeAIB (-98.5 ± 26.2 pA, 1.15 ± 0.19 , 327.07 ± 54.54 M Ω , 21.49 ± 2.64 M Ω), supporting the idea that the observed decrease in NMDAR-mediated EPSCs upon MeAIB application are not likely attributed to alterations in these other parameters.

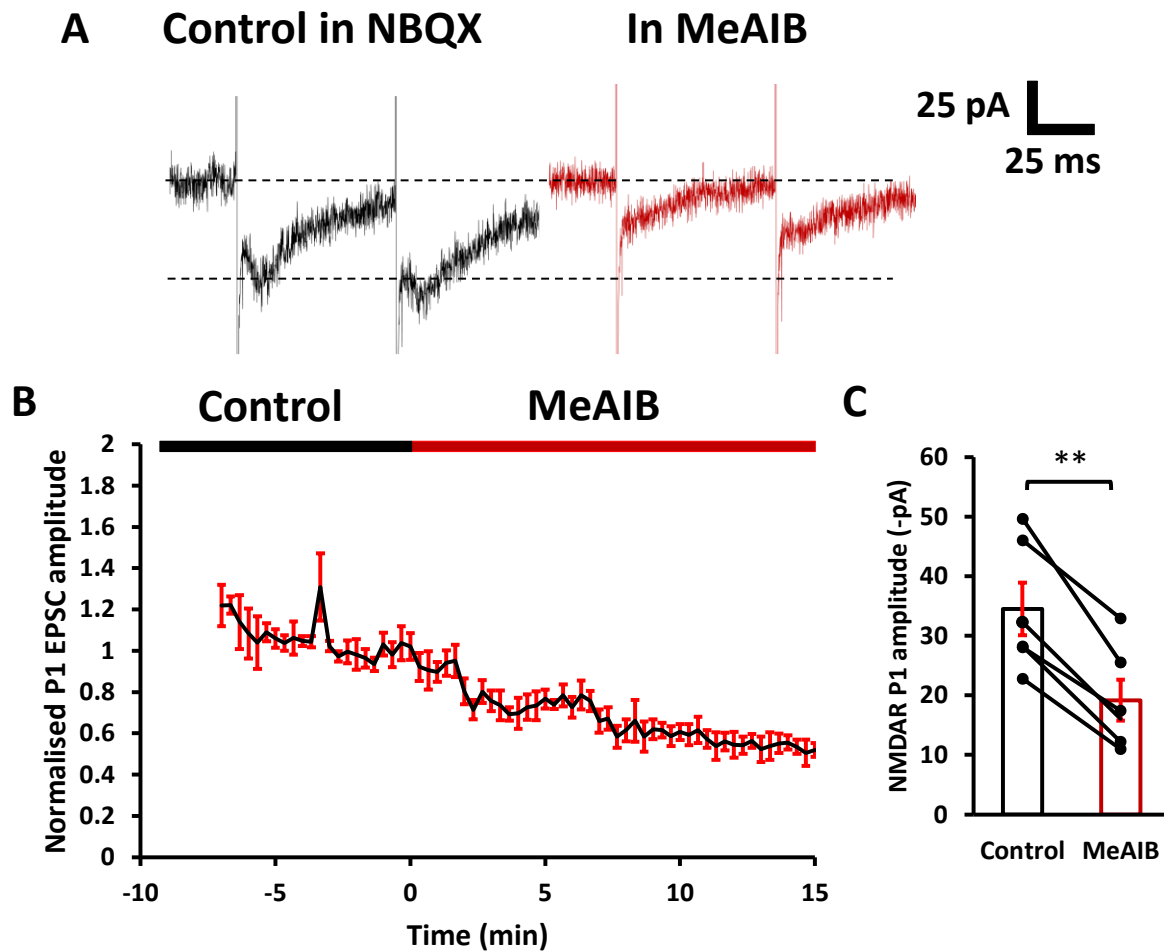


Figure 5.11: Inhibition of SNAT1 or 2 using α -(Methylamino) isobutyric acid decreases P1 amplitude associated with NMDAR-mediated EPSC

(A) Example NMDAR-mediated EPSC recording from a CA1 pyramidal neuron during a single sweep of LFS in control conditions (in presence of 20 μ M NBQX, black) and during bath application of 10 mM α -(Methylamino) isobutyric acid (MeAIB, brown). (B) Averaged data showing that the application of MeAIB (10 mM) significantly reduced the normalised P1 EPSC amplitude, compared to the control condition. Normalisation was done with respect to 5 min before MeAIB application. (C) Bar graph showing a significant difference in average P1 amplitudes between control condition (black; 5 minutes prior MeAIB application) and MeAIB condition (brown; 10 – 15 minutes). ** Represents a significant difference at $p \leq 0.01$.

Further, it was hypothesised that if asc-1 plays an important role in releasing DS into the synaptic cleft, inhibition of asc-1 will decrease the NMDAR-mediated EPSC response. To test this, NMDAR-mediated EPSCs were recorded before and after bath application of 10 mM S-Methyl-L-Cysteine (SMC), a known specific substrate of asc-1 (Sakimura et al., 2016). SMC was observed to significantly decrease ($p < 0.05$, $n = 5$) the NMDAR component of the EPSC response (-43.3 ± 6.7 pA, $n = 5$) compared to control conditions (-60.4 pA $\pm$ 12.1 pA, $n = 5$, Figure 5.12). This result suggests that the asc-1 transporter might also play a role in governing synaptic DS.

Throughout the recording, baseline current, P2/P1 ratio, R_i , and R_s showed no significant changes ($p > 0.05$; $n = 5$) between NBQX (-47.1 ± 17.0 pA, 0.91 ± 0.25 , 328.73 ± 54.38 M Ω , 14.16 ± 1.95 M Ω) and NBQX + SMLC (-77.0 ± 22.2 pA, 1.31 ± 0.41 , 297.64 ± 45.38 M Ω , 14.26 ± 1.90 M Ω), supporting the idea that the observed decrease in NMDAR-mediated EPSCs upon SMLC application are not likely attributed to alterations in these other parameters.

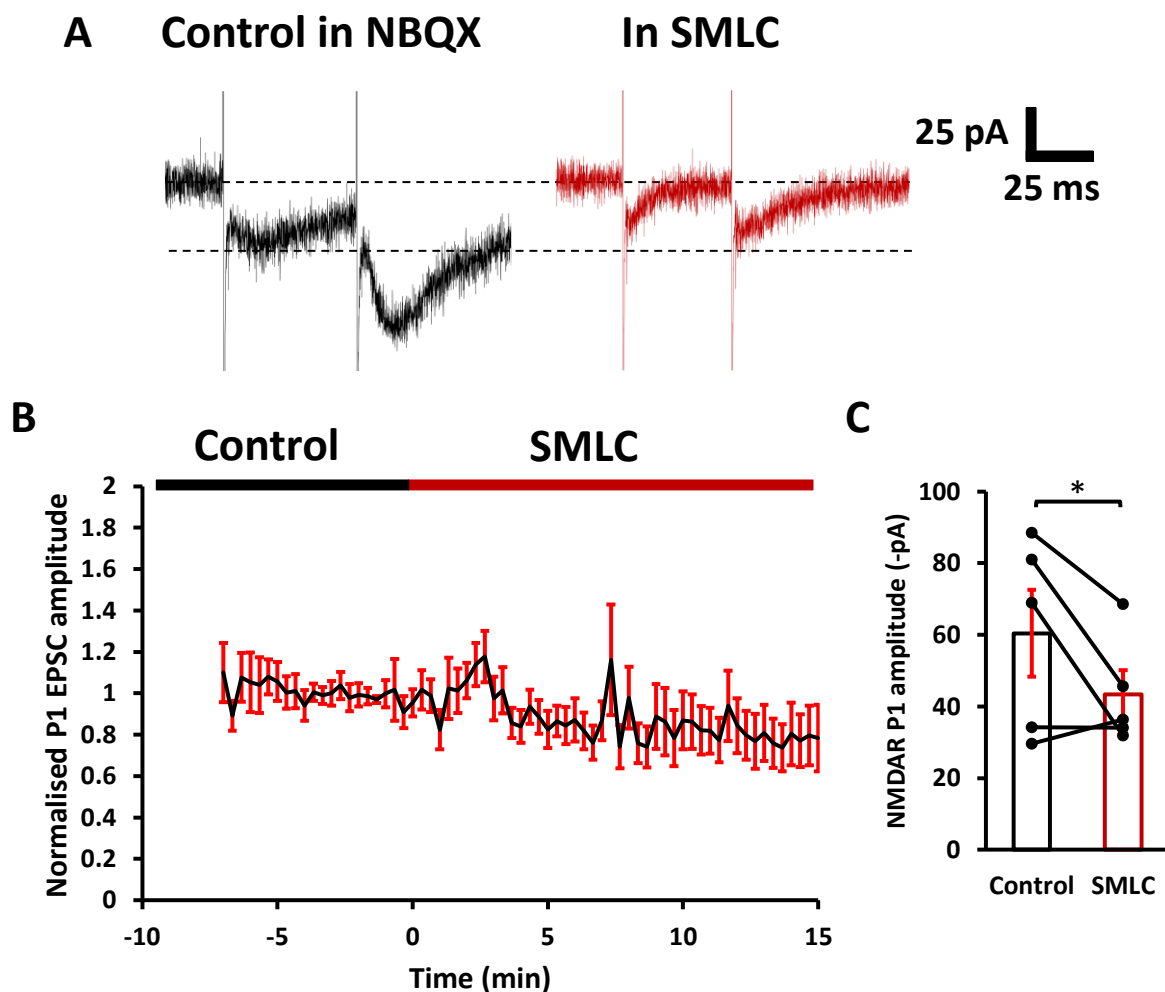


Figure 5.12: Inhibition of asc-1 using S-methyl-L-cysteine does not alter P1 amplitude associated with NMDAR-mediated EPSC

(A) Example NMDAR-mediated EPSC recording from a CA1 pyramidal neuron during a single sweep of LFS in control conditions (in presence of 20 μ M NBQX, black) and during bath application of 10 mM S-methyl-L-cysteine (SMLC, brown). (B) Averaged data showing that the application of SMLC (10 mM) significantly decreased the normalised P1 EPSC amplitude, compared to the control condition. Normalisation was done with respect to 5 min before SMLC application. (C) Bar graph showing significant difference in average P1 amplitudes between control condition (black; 5 minutes prior SMLC application) and SMLC condition (brown; 10 – 15 minutes). * Represents a significant difference at $p \leq 0.05$.

5.3 Discussion

NMDAR activation is governed by the presence of glutamate and DS (D-serine) or glycine in the synaptic cleft. It is widely known that glutamate is released into the synaptic cleft from the pre-synaptic terminals upon an incoming signal. Whereas the origin and fate of its fellow endogenous co-agonist, DS, has remained a matter of debate (Wolosker et al., 2016). Early research revolved around the assumption that glycine was the preferred co-agonist (Berger et al., 1998). Evidence suggests that in NMDARs (containing GluN2B), where glycine acts as the preferred co-agonist, the glycine concentrations are kept below the saturation concentration (below 1 μ M) at excitatory synapses (Roux and Supplisson, 2000). This is achieved by the high affinity and fast glycine transporters, GLYT1, expressed on the neighbouring astrocytes (Bergeron et al., 1998; Chen et al., 2003).

On the other hand, the concentration of DS in the excitatory synapses of the brain is not well characterized and may vary depending on various factors, including the brain region, age and physiological or pathological conditions. However, it is thought to be in the few μ M range in the synapses of the hippocampus and cortex (Matsui et al., 1995; Hashimoto and Oka, 1997). Synaptic concentrations of DS could be governed by broadly two modes. Firstly, by the DS uptake/release systems, namely ASCT1, asc-1 transporters to a major extent and other putative DS transporters like SNAT1 / 2, or LAT to a lesser or no extent (see chapter 3). Secondly, by the synthesis/degradation enzymes namely SR and DAAO. To date, there is no evidence of any transporter-mediated clearance mechanism for DS regulation in the central synapses. Some researchers have speculated that the extracellular concentration of DS is sufficient to saturate the glycine binding site (GBS) of the NMDARs (Billups and Attwell, 2003). It is therefore ambiguous whether DS has similar clearance mechanisms to keep the synaptic DS concentrations below the saturation concentrations and henceforth demands further research.

The main aim of this chapter is to determine whether the DS transporters contribute to synaptic DS concentrations and thereby the NMDAR-mediated neurotransmission. This was investigated by recording NMDAR-mediated excitatory postsynaptic currents (EPSCs) in the CA3-CA1 synapses of mice hippocampal slices, while manipulating DS transport pharmacologically.

5.3.1 LS does not enhance NMDAR activity

It has been a matter of contention whether the GBS could be saturated under physiological conditions in different brain areas. This debate holds significant implications as the saturation of the GBS is crucial for modulating DS concentrations. The impact of modulating DS concentrations becomes particularly important when considering the necessity of 'desaturating' the GBS, highlighting the critical need to unravel the saturation dynamics at the GBS for a comprehensive understanding of DS regulation.

To shed light on the occupancy of the GBS, 100 μM LS, the precursor for DS synthesis in the brain, was applied to the hippocampal CA3-CA1 synapses. The hypothesis posited that LS might enhance NMDAR-mediated EPSCs similar to DS. While the exact mechanism through which LS could boost synaptic DS levels remains speculative, both neurons and astrocytes possess the enzyme SR, allowing them to convert LS into active DS (discussed in 6.4). This enhanced DS presence could potentially enhance NMDAR activity and possibly induce LTP in these synapses. Contrary to the hypothesis, the addition of exogenous LS did not augment the EPSCs mediated by NMDAR activation (Figure 5.2). This finding suggests that the GBS of NMDARs could be saturated under *in-situ* conditions.

In Chapter 4 (Figure 4.7A, B), it has been established that the primary uptake of LS into the CA1 pyramidal neurons occurs via SNAT1/2 transporters. However, considering the reported K_m values for SNAT1/2, which range from 600 to 846 μM (Bodner et al. 2020), it appears that the concentration of 100 μM LS used in current experiment might be below the optimal range. Additionally, if LS is transported into CA1 pyramidal neurons via ASCT (Figure 4.7C, D), the reported EC_{50} values for the anion current in HEK cells are 60 and 146 μM (Foster et al. 2018). This suggests that the concentration used in current experiment aligns more closely with the reported affinities for ASCT. Further experiments could involve exploring the implications of using a higher concentration of LS (1 mM) to stimulate the uptake mechanisms in neurons.

Although the addition of LS might substantially increase DS concentrations, any such rise may not manifest as a discernible change in EPSC response. Therefore, it is essential to note that this experiment doesn't rule out the possibility that LS supplementation could fuel DS synthesis in neurons or astrocytes (as discussed in 6.4). To address this uncertainty, further measurements of synaptic DS concentrations through micro-dialysis, both before and after LS supplementation, would be necessary. For more precise measurements, employing the recently developed fluorescent DS probe (Vongsouthi et al., 2021) could offer a more refined investigation. This innovative probe, coupled with their fluorescent glycine probe (Zhang et al.,

2018), would allow for real-time monitoring of both DS and glycine concentrations, thereby ruling out any effects possibly caused by glycine. Incorporating such probes will provide more dynamic and detailed measurements of synaptic DS concentrations both before and after LS supplementation. These additional measurements could provide a clearer understanding of how LS influences DS synthesis and subsequent NMDAR activation.

5.3.2 The glycine binding site on the GluN1 subunit of NMDARs is saturated

The saturation of the GBS on the GluN1 subunit of NMDARs in CA3-CA1 glutamatergic synapses of the hippocampus was further investigated by adding 100 μ M DS to hippocampal brain slices and recording NMDAR-mediated EPSCs from CA1 pyramidal neurons. Contrary to the hypothesis, the addition of exogenous DS did not significantly alter the NMDAR-mediated EPSCs (Figure 5.3). This observation indicated that the GBS on the GluN1 subunit is saturated under low-frequency or basal levels of activity in the hippocampal CA3-CA1 synapses, implying that the endogenous concentrations of DS surpass the saturation level of the NMDARs.

The saturation of the GBS on NMDARs could potentially be influenced by the presence of either DS or glycine. Previous studies, pioneered by Mothet et al. (2003), have investigated the role of DS as a co-agonist at synaptic NMDARs. While DS acts as a primary modulator of synaptic NMDAR responses, glycine's contribution to site saturation cannot be discounted, especially in brain slice recordings where glycine spillover from other synapses can occur.

It is crucial to consider the competitive and non-competitive dynamics between glycine and DS at the GBS. For instance, the extracellular concentration of glycine in the brain is typically regulated by glycine transporters (GlyT1 and GlyT2; Supplisson and Bergman, 1997), which modulate synaptic glycine levels and, consequently, NMDAR activation. In conditions where glycine levels are high, it is plausible that glycine could occupy the binding site, thus diminishing the regulatory impact of DS on NMDAR activity. This scenario is particularly relevant during high-frequency synaptic stimulation (HFS), which is discussed in section 6.4, where increased synaptic activity may lead to elevated extracellular glycine concentrations.

Moreover, conducting further synaptic transmission experiments in the presence of glycine oxidase could help elucidate the specific contributions of glycine and DS to NMDAR activation. Glycine oxidase would selectively degrade extracellular glycine, allowing for a clearer assessment of DS's role in NMDAR modulation (Mothet et al. 2003). This approach could

provide more definitive evidence regarding which co-agonist predominates under physiological and experimental conditions.

The observed saturation at the GBS suggests the possibility of saturation by either glycine, DS, or a combination of both, given that they bind to the same site. While glycine may saturate the GBS, the addition of DS can enhance the NMDAR response. Two key factors could contribute to this potential enhancement. Firstly, the availability of GBS. Despite the saturation of some GBS by glycine, there may still be unoccupied binding sites. In this case, DS can effectively bind to these remaining sites, further activating the NMDAR. Secondly, the higher efficacy of DS compared to glycine. Even though DS binds to the same GBS as glycine, its higher efficacy could lead to a more pronounced NMDAR response.

The literature on glycine transporters indicates their high efficiency in clearing glycine (Betz et al., 2006), while the role of DS transporters in clearing DS remains unclear. Hence it is speculated that the possible reasons for this saturation could include less efficient DS clearance mechanisms compared to glycine transporters, leading to higher co-agonist concentrations at the GBS. Since the literature (apart from this current study) on the efficiency of DS uptake systems under physiological conditions is not established, further research is needed. Additionally, spontaneous, and evoked glutamate release could provide an excitatory drive to the neighbouring astrocytes. This could induce the release of DS close to synaptic NMDARs. This mechanism may explain why occupancy of the GBS is high at synaptic sites, whereas it is low at extra synaptic sites (Panatier et al., 2006). Recent evidence suggesting DS as a post-synaptically released autocrine molecule (Wong et al., 2020), raises the possibility of local control of DS concentration by the post-synaptic neuron. This hypothesis challenges our understanding of synaptic DS regulation, implying that the local DS concentrations and therefore the saturation of the GBS could be under the local control of the post-synaptic neuron.

Under low excitation states and controlled stimulation conditions, active DS release mechanisms from neighbouring post-synaptic neurons, astrocytes, or interneurons, as suggested by Mothet et al. (2005), cannot be completely ruled out, although they are less likely in the absence of high synaptic stimulation or increased excitation states such as epilepsy. The implementation of a low-frequency stimulation protocol without bicuculline and strychnine was intended to maintain excitation at a controlled level. Additionally, the use of high extracellular Mg^{2+} conditions instead of conventional low or zero Mg^{2+} was aimed at preventing

any increase in cell excitability (Hahin and Campbell, 1983) that might lead to elevated glycine or DS levels.

Furthermore, passive diffusion of DS across blood-brain barrier could contribute to high extracellular DS levels. While DS diffusion from the periphery may not be observed in brain slices, it is essential to recognise that *in-vivo* conditions may present a different scenario, where systemic circulation and passive transport mechanisms may enable the peripheral influx of DS into the brain, potentially influencing intra and extracellular concentrations at the GBS. Despite the limited diffusion of DS through the blood–brain barrier, Pernot et al. (2012) demonstrated that DS administration could significantly elevate intra and extracellular concentrations. Furthermore, DS obtained from food sources, or the gut microbiome could contribute to the extracellular DS levels. This influx is likely mediated by plasma LS elevation and local DS synthesis in the brain, thereby maintaining the DS/LS ratio (Savignac et al., 2013).

To sum up, a high synaptic concentration of DS could be due to local control of DS concentration or due to the low efficiency of the DS uptake system in the brain or due to passive diffusion of DS from the periphery. Despite these considerations, the absence of significant changes to NMDAR currents under low stimulation and controlled excitation conditions leads to the logical conclusion that the GBS remains saturated. It can therefore be hypothesised that the release of glutamate from pre-synaptic terminals primarily dictates the activation of post-synaptic NMDARs under basal levels of synaptic activity on its own, with no contribution of DS.

This phenomenon of GBS saturation likely serves a physiological advantage by enhancing faster excitatory neurotransmission and potentially influencing NMDAR function. With the GBS saturated, NMDARs do not have to rely on the simultaneous release of glutamate and DS to reach concentrations sufficient for activation. Additionally, the extracellular levels of DS, typically in the range of a few μM , are comparatively non-toxic when compared with its fellow co-agonist, glutamate (Piña-Crespo et al., 2014). DS's reduced toxicity could be attributed to its limited targets, unlike glutamate, which acts on various receptors like AMPAR, mGluRs, pre-synaptic glutamate transporters, and extra-synaptic NMDARs. If uncleared from central synapses, glutamate can significantly modify circuit dynamics. Saturating concentrations of DS might also facilitate GluN2B-dependent synaptic depression in the hippocampus, potentially enhancing performance in tasks related to reference memory and delayed-matching-to-place tasks (Steele and Morris, 1999).

While the precise physiological reason for this saturation discrepancy remains unclear, the current findings align with previous studies that reported that DS did not lead to an increase in the amplitude of synaptic and extra-synaptic NMDAR-mediated responses in mouse CA1 pyramidal neurons (Ballard et al., 2002; Scimemi et al., 2004; Le Meur et al., 2007). Similarly, previous studies have indicated that exogenously added DS does not potentiate the NMDAR-mediated response under basal conditions or during LTP in the hippocampal CA3-CA1 synapses of 4-6 months old mice (Duffy et al., 2008). This collective body of evidence suggests a consistent pattern across different investigations and reinforces the notion that the effects of DS on NMDAR-mediated responses may be influenced by specific experimental conditions or cellular contexts.

Contrary to my observation, previous evidence suggested that the GBS on NMDARs may not be fully saturated under certain conditions, that the GBS is only partially saturated at physiological glycine concentrations, and could potentially bind additional glycine molecules even when partially occupied (Bergeron et al., 1998). However, these early findings assumed glycine to be the preferred co-agonist and did not consider the endogenous concentrations of DS and its regulators under physiological conditions of synaptic activity. Martina et al. (2003) demonstrated that exogenous DS potentiated the NMDA component of synaptic responses evoked by *Schaffer collateral* stimulation in rat hippocampal slices. While Chen et al. (2003) reported a dose-dependent enhancement of NMDAR-EPSCs in individual pre-frontal cortical neurons in cortical rat brain slices, the effects of DS were not significant in group population data. Additionally, Neame et al. (2019) reported an enhancement of NMDAR- fEPSP upon application of 100 μ M DS in adult mice cortical slices, suggesting that the GBS might be unsaturated in certain contexts. Overall, although DS has been shown to potentiate the NMDAR-mediated EPSCs in certain regions like the spinal cord (Brugger et al., 1990), the current observations indicate that the NMDARs are saturated in CA3-CA1 hippocampal synapses. This saturation implies that the GBS are fully occupied by co-agonist molecules and cannot bind any additional co-agonists.

To deepen our understanding, investigating whether the GBS is saturated during spontaneous activity through miniature EPSCs (mEPSCs) recordings presents an intriguing avenue. In the context of *in-vivo* conditions, the GBS remain unsaturated (Wood et al., 1989; Singh et al., 1990; Chen et al., 2003). This observation challenges our existing understanding of GBS saturation levels, suggesting that under *in-vivo* circumstances, there might be intricate regulatory mechanisms at play, preventing the GBS from reaching their saturation points. Further, understanding whether the GBS remains unsaturated during heightened synaptic

demand, influenced by increased synaptic DS and glutamate utilisation or clearance, unveils a critical aspect of excitatory neurotransmission (discussed in 6.3).

5.3.3 Finetuning of NMDAR-mediated EPSCs requires better parameters.

The intricate interplay between DS and the GBS on NMDARs contributes significantly to our understanding of synaptic function. Despite the rich body of research, the question of GBS saturation remains elusive, especially within the complex milieu of hippocampal CA3-CA1 synapses. The current investigations delved into this saturation phenomenon, revealing that the GBS remains saturated in the hippocampal CA3-CA1 synapses *in situ*. Henceforth, desaturating the GBS is imperative to unravel the intricate dynamics of synaptic DS, LS, and the ASCT1 transporter. This essential step will provide better parameters to explore the contributions of these factors to synaptic DS levels.

Kynurenate, a low-affinity antagonist at the GBS (Birch et al., 1988), was employed in an attempt to desaturate the site. Its low affinity allows for ready displacement due to a fast off rate, and the lack of potency facilitates its use. Intriguingly, a concentration of 30 μM proved insufficient to significantly disrupt endogenous DS binding to GBS, evident from absence of reduction of the NMDAR-mediated EPSC responses from CA1 pyramidal neurons (Figure 5.4). The subsequent attempt to enhance the EPSC responses through the addition of exogenous 100 μM DS or LS in the presence of 30 μM kynurenate proved ineffective, contrary to the anticipated competitive displacement of kynurenate by DS, given its higher affinity (Figure 5.6, Figure 5.7).

Given the lower binding affinity of kynurenate with the GBS ($K_i = 15 \mu\text{M}$) than the affinity of DS or LS ($K_a = 0.2 \mu\text{M}$ and $0.45 \mu\text{M}$ respectively; with lower equilibrium association constant indicating stronger binding affinity), a higher concentration of kynurenate was used to try and desaturate the GBS (Kessler et al., 1989). Interestingly, doubling the concentration to 60 μM yielded a modest 38.81% reduction in NMDAR-mediated EPSC responses from CA1 pyramidal neurons (Figure 5.5). After exposure to 60 μM kynurenate in an attempt to desaturate the GBS, subsequent addition of exogenous 100 μM DS failed to enhance the EPSC responses recorded (Figure 5.8). This outcome is surprising because if the EPSC responses were solely dependent on the competitive binding between DS and kynurenate for the GBS, using DS, which has higher affinity and concentration should have dislodged the kynurenate from the GBS. The pharmacological logic dictates that using 100 μM DS should have been capable of dislodging 60 μM kynurenate. Together, this intriguing result warrants a deeper exploration into the molecular intricacies underlying kynurenate, DS, LS, and their

interactions with NMDARs, hinting at the potential involvement of allosteric modulation (such as Zinc) or additional binding partners (like glycine) that could influence GBS accessibility.

The intricate dynamics of the GBS demand a meticulous approach to unravelling its complexities. Using basic pharmacological principles, it should be possible to estimate the endogenous concentration of DS, given some assumptions, as outlined below. In this case, the inhibitor kynurenate competes with the substrate DS for binding to the GBS of NMDARs. The concentration of endogenous DS was estimated by using the Michaelis-Menten equation for competitive inhibition, $v = \frac{K_m(1+K_i[\text{Kynurenate}])}{[DS]V_{\max}}[DS]$. The equation was rearranged to calculate [DS]. IC_{50} of kynurenate for DS was derived from the experiment demonstrating the effective desaturation of the GBS by 39% at 60 μM [kynurenate] concentration (Figure 5.5). K_i represents the dissociation constant of the kynurenate-NMDAR complex. A K_i of 14 μM was utilised for calculation, a value reported within the range of 3-15 μM in previous literature (Henderson et al., 1990). In this case, K_m is the K_d value, representing the dissociation constant of the DS-NMDAR complex. The K_m value of DS for NMDAR was set at approximately 1 μM , in line with the previously reported values (EC_{50} of DS $\sim$ 0.46; Monihan et al., 1989; Thomson et al., 1989). A fraction fv_0 of velocity v_0 value of 0.6 was used, where $0 < fv_0 < 1$.

Upon solving this equation for [DS], the estimated endogenous concentration of DS within the CA3-CA1 synapses was determined to be approximately 6 μM . It is worth noting that the calculations assumed that the interaction of kynurenate and DS with the NMDAR is a simple reversible binding process and that the competition between the two ligands is limited by the availability of the GBS. It does not factor in the effects of various regulatory mechanisms and modulatory proteins. In addition, the calculation did not account for the recycling rate or turn-over rate of ASCT1 and other DS transporters (transporter kinetics), which can significantly affect the concentration of DS within the synapse. Therefore, it is likely that the calculated 6 μM DS concentration might overestimate the actual physiological values within the CA3-CA1 synapses. Nevertheless, this estimated value (6 μM) is in line with the previously reported DS levels in the brain (Hashimoto et al., 1992; Matsui et al., 1995).

The observed 39.0 % reduction in NMDAR-mediated EPSCs during the application of 60 μM kynurenate is consistent with the hypothesis of kynurenate inhibiting NMDAR activity (Figure 5.5). However, it is important to note that kynurenate can also act on the glutamate binding site, albeit less efficiently, with a K_i of 140 μM (Kessler et al., 1989). This dual action of kynurenate on GBS and glutamate binding site sheds light on the observed discrepancy in

present results (Figure 5.8), where the 60 μM kynurenate inhibition is not completely reversed by adding 100 μM DS. This dual action coupled with its inhibition of GBS-less AMPARs, indicates its complex non-specific modulation of NMDARs (Prescott et al., 2006). Other possible explanations for the reduction in EPSCs during kynurenate application, such as changes in the presynaptic release of glutamate or postsynaptic responsiveness to glutamate are less likely (5.2.4).

To delve deeper into the role of kynurenate in inhibiting NMDAR activity, experiments measuring NMDAR-mediated currents in the presence of varying kynurenate concentrations or employing other GBS site antagonists like (+)HA-966 and 7-chlorokynurenic acid could be invaluable (Kemp and Priestley, 1991; Foster et al., 1992). Furthermore, the limitations of using low concentrations of kynurenate as a desaturation tool, underscore the need to exploration of alternative desaturation strategies or more precise agents targeting the GBS. While it appears logical to use a higher concentration of kynurenate to significantly desaturate the GBS and investigate the effects of exogenous DS, it is worth noting a limitation with such an approach. The concentration of kynurenate must be restricted to below 100 μM , due to potential off-target effects on the GluN2 (glutamate binding site - at concentrations of 100 μM – 1 mM), even though its affinity for the glutamate binding site is 10 times higher than the affinity for GBS (Kessler et al., 1989). Elevated kynurenate concentrations could reduce the overall NMDAR-mediated EPSCs independently of their effects on the GBS. Alternatively, future experiments could consider using a lower concentration (10 μM) of exogenous DS in line with the physiological levels.

Overall, the estimated concentration of endogenous DS (6 μM) appears to surpass the NMDAR saturation point. This finding suggests that NMDARs in CA3-CA1 synapses during the developmental stage of P21-25 could already be saturated with endogenous DS, rendering additional supplementation ineffective. The forthcoming discussion (from 5.3.6) will delve deeper into the molecular nuances to theoretically decipher the intricacies of DS/NMDAR regulation and its potential impact on synaptic function employing targeted approaches.

5.3.4 ASCT1 transporters do not regulate synaptic D-serine levels during LFS conditions

Observations showed that neither DS nor LS supplementation can increase NMDAR-mediated EPSC responses in hippocampal CA3-CA1 synapses, further suggesting a saturation of the GBS. Results from Chapter 3 presented compelling *in-situ* evidence for a potential DS clearance mechanism mediated by astrocytic ASCT1. Given the observed GBS saturation, it

is rudimentary to precisely define the regulatory role of the ASCT1 transporter in synaptic DS levels. The hypothesis posits that the inhibition of astrocytic ASCT1 will modulate the synaptic DS levels and the NMDAR-mediated EPSC response. However, experiments involving wash-in of 10 mM benzyl serine or 10 mM trans-hydroxy proline failed to provoke discernible changes in the recorded EPSC responses from CA3-CA1 hippocampal synapses (Figure 5.9, Figure 5.10). This result suggests that inhibiting ASCT1 might not significantly impact DS concentrations when synaptic transmission at these synapses is in a quiescent state.

The lack of discernible changes in NMDAR-mediated EPSCs upon inhibiting ASCT1 aligns with expectations, indicating that the read-out (NMDAR-mediated EPSCs) is not sensitive in saturation conditions where the synapse is unresponsive to changes in DS concentration. While the direct effect of ASCT1 on DS concentration may not be apparent under saturating conditions, further investigation is required to explore the potential role of ASCT1 in non-saturating states and its broader implications for synaptic function (discussed in 6.3).

To delve deeper, a more robust desaturation of the GBS is warranted. The use of 60 μ M kynurenate, while effective in reducing NMDAR-mediated EPSCs, may not provide a sufficient baseline change to observe the impact of ASCT1 inhibition. Considering this, a stronger desaturation approach, such as employing a higher concentration of kynurenate or utilising an alternative GBS antagonist, could be employed. This would create a more discernible baseline, enabling a clearer assessment of ASCT1's influence on DS concentrations. Subsequently inhibiting ASCT1 using benzyl serine or trans-hydroxy proline presents an opportunity to decipher ASCT1's role in governing DS concentrations during basal synaptic activity.

While the actual findings indicate GBS saturation, the figure below illustrates a hypothetical scenario exploring the potential impact of ASCT1 blockade on NMDAR activation. The hypothesis posits that astrocytic ASCT1, despite not influencing NMDAR-mediated responses under saturating conditions, may still play a regulatory role in governing synaptic DS levels in non-saturating contexts. This potential effect is likely to be more pronounced during periods of higher activity. For instance, under non-saturating conditions, inhibition of ASCT1 is expected to significantly decrease DS clearance. This would elevate synaptic DS levels, potentially enhancing NMDAR-mediated EPSC responses (Figure 5.13A). Simultaneously, disrupting the LS shuttle, vital for maintaining neuronal DS supply, mediated by ASCT1 might result in decreased neuronal DS release and subsequently reduced NMDAR responses (Figure 5.13B).

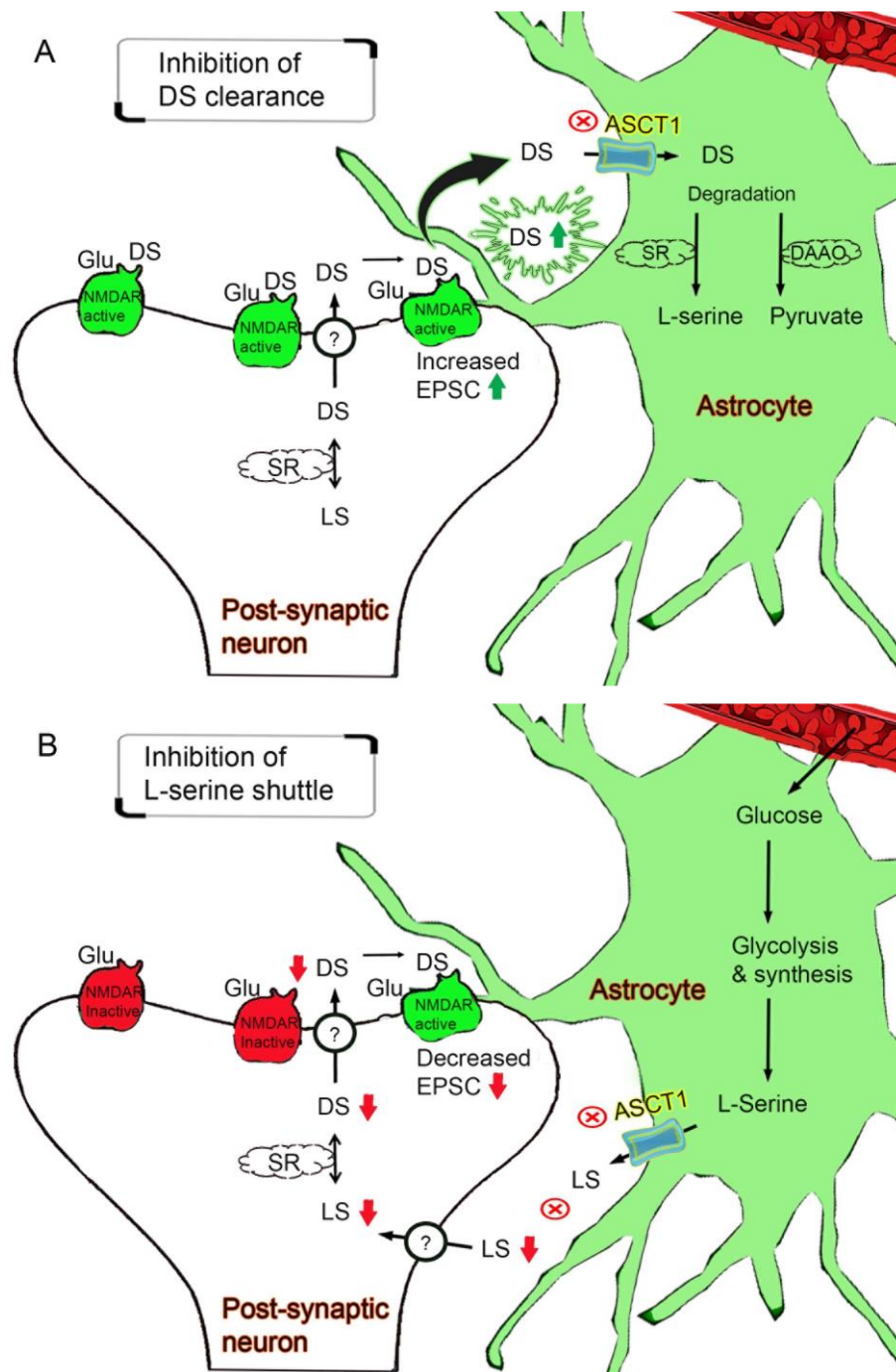


Figure 5.13: Hypothetical inhibition of ASCT1 transporter – Effects depend on its role in (A) D-serine clearance and (B) L-serine shuttle

(A) In normal conditions DS is released from post synaptic neuron and acts on the synaptic NMDARs. After its action, DS is cleared by astrocytic ASCT1 to reduce the activation of NMDARs. Inhibition of astrocytic ASCT1 elevates the synaptic DS concentration, which consequently increases the NMDAR mediated EPSC response. (B) Astrocytes synthesise LS from glucose and supplies it to neighbouring post synaptic neuron via a process called LS shuttle. Astrocytic ASCT1 has been hypothesised to play a role in this process. Inhibition of LS shuttle supply to the post synaptic neurons, will decrease the neuronal synthesis of DS and therefore the release of DS. Due to decreased availability of DS at the synaptic cleft, activation of NMDAR occurs to a less extent, which consequently decreases the overall NMDAR mediated EPSC response.

5.3.5 Role of SNAT1 or 2 and asc-1 in regulating synaptic DS concentration

The investigation into the key transporters that regulate synaptic DS has opened a compelling avenue of inquiry. Although ASCT1 has garnered attention for its role not only in DS clearance but also in the astrocyte-neuron LS shuttle (Kaplan et al. 2018), the involvement of other potential DS transporters remains an intriguing area to explore. Recent studies challenging the traditional perception of SNAT1 or 2 (*Slc38a1* and *Slc38a2*) as primarily glutamine transporters have presented a fresh perspective. These transporters have been shown to facilitate DS transport in primary neuronal cultures and synaptosomes, suggesting a complex role extending beyond mere glutamine transport (Bodner et al., 2020). Since *in-vivo* glutamine concentrations are below the saturation levels, SNAT1 or 2 could not only function as a glutamine transporter mediating glutamate-GABA synthesis (Qureshi et al., 2020) but also could aid in neuronal reuptake of DS or LS of the subsequent production of DS.

To understand the contribution of SNAT1 or 2 to synaptic DS concentrations, 10 mM MeAIB (α -(Methylamino) isobutyric acid, a specific SNAT1 or 2 inhibitor) was applied to the hippocampal brain slices while recording NMDAR-mediated EPSCs from CA1 pyramidal neurons. The results revealed a 44.4% decrease in EPSC responses compared to control conditions upon SNAT1 or 2 inhibition (Figure 5.11). This outcome implies that SNAT1 or 2 transporters may actively contribute to NMDAR-mediated EPSCs.

One possible interpretation of the current finding is that SNAT1 or 2 might be facilitating the LS shuttle by aiding LS uptake in the neuronal compartment. In this scenario, inhibiting SNAT1 or 2 would diminish the availability of LS in neurons, subsequently reducing synaptic DS levels and the associated NMDAR-mediated EPSCs (Figure 5.14A). However, this interpretation is complicated by MeAIB's potential impact on neuronal glutamine supply rather than DS concentration (Blot et al., 2009). In the context of the glutamate-glutamine cycle (GGC), where glutamine from astrocytes replenishes presynaptic glutamate pools, SNAT1 or 2, alongside other transporters like NTT4, plays a pivotal role (Leke and Schousboe, 2016; Jia et al., 2023). This alternative interpretation suggests that SNAT1 or 2 might be influencing the intricate dynamics of the GGC rather than directly modulating synaptic DS concentrations. Similar effects of MeAIB impacting hippocampal fEPSPs have been previously reported (Kam and Nicoll, 2007). However, the immediate impact of MeAIB observed on fEPSPs by Kam and Nicoll (2007) suggests that the effect may not result from an abrupt disruption of glutamine entry into neurons. The fact that the decrease was not sustained after extended incubation with MeAIB, unlike the prolonged potentiation seen with high exogenous glutamine, supports the idea of an effect of MeAIB that is not mediated via glutamine transport.

To discern whether the observed reduction in EPSCs upon MeAIB application is due to a DS mediated effect, adding exogenous DS after MeAIB inhibition could be used to reverse the effect. This would directly address the involvement of SNAT1 or SNAT2 in regulating DS levels. If the EPSC responses increase after the application of exogenous DS following MeAIB treatment, it strongly indicates that SNAT1 or SNAT2 plays a role in regulating DS concentration at the synaptic cleft. On the contrary, if there is no significant change in EPSC responses after the addition of exogenous DS, it implies that MeAIB's effects were not related to DS concentration.

Next, to investigate the contribution of asc-1 transporter to synaptic DS concentrations, 10 mM SMLC (S-Methyl-L-Cysteine, a specific asc-1 inhibitor) was applied to the hippocampal brain slices while recording NMDAR-mediated EPSCs from CA1 pyramidal neurons. The inhibition of asc-1 resulted in a slight reduction in EPSC amplitude (28.8%; Figure 5.12), suggesting a minor role in governing synaptic DS supply during periods of low synaptic activity. This result challenges the anticipated role of asc-1 in mediating DS release (Figure 5.14B). To further investigate the specific mechanism, a valuable approach would be to investigate if the observed reduction in EPSC amplitude can be reversed by adding exogenous DS after SMLC treatment. A reversal of the effect in the presence of additional DS would probe that the mechanism underlying asc-1 inhibition is indeed via a reduction in DS release, providing clearer insights into its role in regulating synaptic DS concentrations.

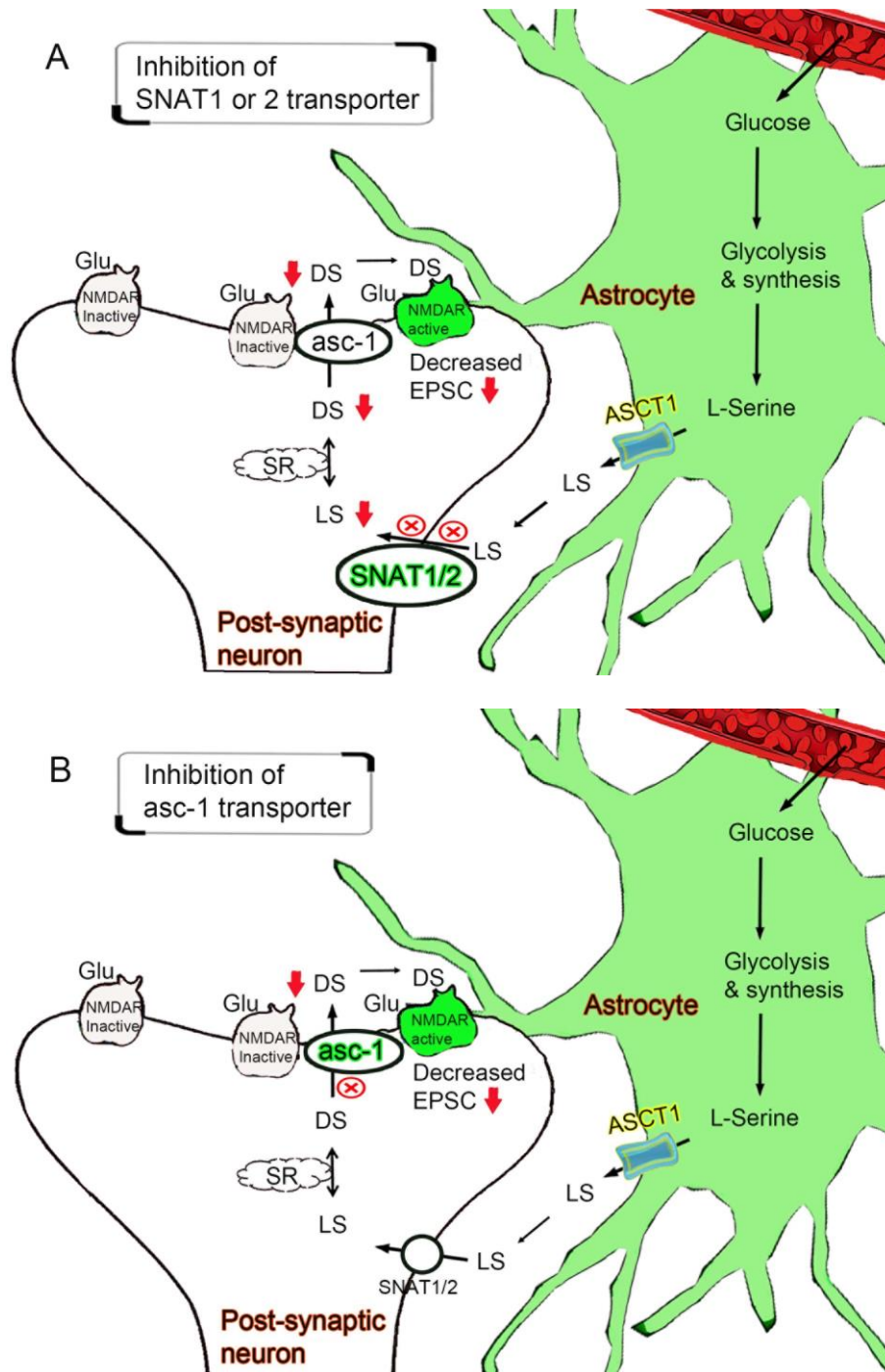


Figure 5.14: Hypothetical role of SNAT1 or 2 or asc-1 in governing synaptic DS concentration

(A) Shows the effect of inhibiting LS shuttle mediated by SNAT1 or 2 on the synaptic availability of DS. A decreased supply of LS to neurons leads to a decreased production and release of DS into the synaptic cleft, resulting in decreased NMDAR mediated EPSC response. (B) Shows the effect of inhibiting DS release mediated by asc-1 transporter. A reduction in the release of DS into the synaptic cleft results in a decreased activation of NMDAR and its associated EPSC response.

Previous studies investigating the effects of asc-1 inhibition have yielded some contradictory results, depending upon the exact method of inhibition used. Systemic administration of SMLC in rats (Onozato, 2016), or perfusion in the cortex (Ishiwata et al., 2013) or hippocampus (Thomsen, 2003) produced an increase in the levels of extracellular DS, along with increases of other asc-1 substrates such as LS, alanine and threonine. The mechanism for this is thought to be via hetero-exchange when high concentrations of SMLC are used, as SMLC is a weakly transported substrate of asc-1 (Maucler et al., 2013). Other studies using non-transported asc-1 inhibitors, Lu AE00527 (Sason et al., 2017) or ACPD (Sakimura et al., 2016), observed the opposite effect, demonstrating a reduction in external DS levels when asc-1 is inhibited in rat neocortical slices or hippocampus respectively. This highlights the importance of using a range of strategies to more thoroughly investigate the effects of asc-1 inhibition in further investigation of NMDAR activation. BMS-466442, a newly synthesised, highly-potent, non-competitive, asc-1 inhibitor (Brown et al., 2014) could be employed in these studies as it has been shown to be well tolerated in whole animals and inhibits asc-1 mediated DS transport *in vivo* (Tapanes et al., 2022). Further, inducing DS release mediated by asc-1 using D-Isoleucine (Rosenberg et al., 2010; Rosenberg et al., 2013), a transportable competitive substrate, might elucidate the intricacies of asc-1's role in DS modulation.

An additional avenue of future investigation would be the use of asc-1 knockout mice to provide more insights into this transporter's role in serine regulation. However, while these mice show reduced EPSC amplitudes in the hippocampus, they also have a severe neurological phenotype, exhibiting tremor, seizures, ataxia and early postnatal death (Xie et al., 2005). Although the symptoms reduced with an NMDAR antagonist, the exact reason for EPSC reduction is unclear. Given that glycine levels are regulated by glycine transporters, the KO of asc-1 in these mice likely affected DS regulation. Consequently, compensatory upregulation of other DS transporters in asc-1 KO mice, might lead to excessive synaptic DS and overactivation of NMDARs, highlighting the delicate balance in DS regulation within the synaptic environment. A more nuanced strategy of conditional knock-out mice would be required for further investigation in this area.

5.3.6 Concentration and subunit-dependency in the modulation of NMDAR currents by DS

In exploring the modulation of NMDAR currents by DS, it becomes essential to consider both the dynamic nature of NMDAR expression and function (Cull-Candy et al., 2001; Le Bail et al., 2015) and the levels of endogenous DS during development (Schell et al., 1997; Puyal et al., 2006). This consideration prompts an examination of the subunit dependency and concentration of DS, shedding light on the intricate dynamics of NMDARs.

One potential explanation for the variability in observing the effects of applying exogenous DS in current experiments (Figure 5.3, 5.7 and 5.8) may arise from the dual nature of DS modulation on NMDARs. While DS has been shown to enhance NMDAR EPSCs, its concurrent impact on NMDAR internalisation and its inhibitory effect at high concentrations may contribute to the observed complexities. Therefore, exploring the dynamics of receptor internalisation may offer insights into the observed variations in DS effects under different conditions. Not only are there differences in internalisation with NMDARs of different subunit compositions, but there are also differences in how the two co-agonists, glycine and DS, act. Therefore, it is crucial to consider the distinct roles of the different types of NMDARs in any future experiments, acknowledging that these differences may contribute to the observed complexities in NMDAR responses under varying co-agonist concentrations.

Physiological DS concentrations in the synaptic cleft typically range from 1-10 μ M (Hashimoto et al., 1995; Matsui et al., 1995; Hashimoto and Oka, 1997). These concentrations can effectively saturate the GBS of the GLUN1 subunit, ensuring full activation of NMDARs by glutamate (Matsui et al., 1995). It is crucial to recognise that lower concentrations of DS might yield more pronounced effects compared to higher concentrations. For instance, Zhang et al. (2008) demonstrated a bell-shaped concentration-dependent effect of exogenous DS on NMDAR-mediated fEPSPs in the hippocampal CA1 region of P13-18 Wistar rats. Specifically, 5 μ M DS, but not 100 μ M DS, significantly increased NMDAR-mediated fEPSPs post-induction of LTD. This suggests that the endogenous concentrations of DS also contribute to the bell-shaped concentration-dependent modulation of exogenous DS on LTD.

Recent studies, including molecular docking experiments and electrophysiological recordings from GluN1-2A expressing NMDAR in oocytes, have revealed that DS can function as an inhibitory molecule at high concentrations (1 mM), interacting with the GluN2A subunit and competing with glutamate (Yovanno et al., 2022). This concentration-dependent effect of DS on NMDAR function suggests that future experiments measuring EPSC responses should

consider using more physiologically relevant concentrations, such as 1-10 μ M DS, to avoid potential off-target effects.

A co-agonist concentration-dependent effect on NMDAR internalisation was first observed by Nong et al. (2003). They recorded stable NMDAR currents while applying 50 μ M NMDA + 1 μ M glycine for 45 mins, from isolated CA1 pyramidal neurons. However, a sharp decrease in NMDAR currents occurred when applying 1 mM NMDA + 100 μ M glycine, even after 2-3 mins. This rundown was attributed to NMDAR internalisation, a process triggered by exposure to high co-agonist concentrations.

Further evidence for this concentration-dependent effect was found when DS was manipulated instead of glycine. Burnet et al. (2011) knocked out DAAO in the cerebellum, where the expression of DAAO is abundant, as a means of increasing the overall DS concentrations (by 77%). This led to a reduction in GluN2A mRNA levels (by 22%) but did not affect the GluN1 or GluN2C mRNA levels. This decrease in mRNA levels suggests a reduction in transcription, which occurs in the nucleus, rather than a rapid effect on receptor internalisation at the cell membrane. This implies that the GluN2A containing NMDARs (synaptic) might not experience internalisation when the synapses experience high DS concentrations. However, this finding contrasts with the broader understanding that excess DS in the synapses is linked to co-agonist-mediated signalling, ultimately leading to the internalisation of extrasynaptic GluN2B-containing NMDARs (Ferreira et al., 2017). This internalisation phenomenon, with its potential impact on EPSC responses, provides a critical perspective to consider when interpreting the effects of DS manipulation on NMDAR activity, potentially impacting EPSC responses.

Given these findings, in situations where there is an excess of co-agonists, it is hypothesised that there could be potential changes in the transcription or expression of different subunits of NMDARs. Therefore, it is crucial to investigate NMDAR subunit-specific effects on EPSCs in mice treated with selective subunit blockers. This involves isolating and studying the currents specifically from synaptic NMDARs, composed of GluN1 and GluN2A subunits, by blocking extra-synaptic NMDARs containing GluN1 and GluN2B subunits. Notably, CP-101,606 and Ro 25-6981 have demonstrated specificity in blocking GluN2B containing NMDARs (Fischer et al., 1997; Chazot et al., 2002). Similarly, NVP-AAM077 and TCN-201 demonstrate specificity in blocking GluN2A containing NMDARs (Berberich et al., 2005; Lundbye et al., 2018). By selectively investigating the synaptic NMDAR currents, insights into the specific modulation of this receptor subtype under conditions of altered co-agonist concentration could be achieved. Further, by applying these blockers to brain slices and monitoring NMDAR-dependent EPSCs,

a comparative analysis of the influence of ASCT1, SNAT1/2, or asc-1 transporters on GluN2A and GluN2B subunits becomes feasible. Given their distinct localisations at synapses and away from synapses, respectively, this approach provides a distinct perspective on their regulatory roles. These comprehensive studies promise valuable insights into ASCT1 and other transporter-mediated regulation of synaptic DS levels.

Considering the variability in endogenous DS concentrations in slice preparations, further research is needed to comprehensively understand the implication of saturation of GBS in the hippocampus. For a targeted examination of glycine and DS effects on NMDAR-mediated EPSCs, employing specific enzymatic breakdown tools such as glycine oxidase (Job et al., 2002) or DAAO (D amino acid oxidase; which selectively degrades D amino acids to corresponding imino acids; Pollegioni et al., 1992) presents a promising avenue. This could present a viable approach to metabolise the endogenous levels of glycine and DS respectively and minimise its influence on EPSC responses. The anticipated result of such an experiment would be more consistent baseline DS concentrations, free from the influence of endogenous DS (Pاناتier et al., 2006). Further, this controlled environment would allow for a more precise investigation of the effects of exogenous DS and the impact of DS transporters on the regulation of synaptic DS levels. In particular, by employing DAAO, manipulation of DS transporters is expected to have a more pronounced and discernible effect on the NMDAR currents.

Overall, future experiments could utilise physiological concentrations of DS (1-10 μ M) and prophylactic block of GluN2B receptors when recording any synaptic event-associated EPSC responses from the somas of CA1 pyramidal neurons. This approach will enhance the accuracy and relevance of the recorded data in the context of synaptic NMDAR activity.

5.3.7 Concluding remarks

The findings presented in this chapter shed light on the intricate regulatory mechanisms governing D-serine (DS) concentrations at the CA3-CA1 hippocampal excitatory synapses, specifically focusing on the involvement of the ASCT1 (*Slc1a4*) transporter. The results from this chapter revealed that neither additional DS nor L-serine impacted the EPSCs mediated by NMDAR at the hippocampal CA3-CA1 synapses. This observation suggests that, under *in-situ* physiological conditions, the glycine binding site is saturated, indicating a tightly regulated synaptic environment. This implies that any effect of ASCT1 in regulating DS concentrations will not immediately impact NMDAR activation under these conditions, considering that the glycine binding site is saturated. The functional nature of the glycine binding site was

demonstrated through inhibition by 60 μ M kynurenic acid, which led to a 39% reduction in NMDAR-mediated EPSCs, indicating the site's desaturation potential. Post desaturation of the glycine binding site, neither additional DS nor L-serine influenced the EPSCs recorded at the CA3-CA1 synapses, reinforcing the notion of tightly regulated glycine binding sites. The physiological levels of glycine and DS in the extracellular fluid of the brain are not well characterised, and they may vary depending on various factors, including the brain region, age, and physiological or pathological conditions. However, it is thought that the concentrations of glycine and DS in the synaptic spaces of the brain are in the micromolar range ($\sim$ 1-10 μ M). Extrapolating from desaturation experiments, the synaptic DS concentration in the CA3-CA1 synapses was estimated to be approximately 6 μ M, which is in line with the measured values in the extracellular fluid. This estimation provides valuable quantitative insight into the synaptic DS levels, a key parameter for understanding co-agonist dynamics. In addition, inhibiting SNAT1 or 2 (*Slc38a1* or *2*) or asc-1 (*Slc7a10*) transporter significantly reduced NMDAR-mediated EPSC responses, hinting at their involvement in governing synaptic DS concentrations. This highlights the complexity of synaptic regulation, involving multiple transporters in the maintenance of neurotransmitter homeostasis.

The chapter provides avenues for future research. 1. Exploring the saturation state of the glycine binding site under spontaneous activity by recording miniature EPSCs (mEPSCs) in hippocampal slices could provide insights into the receptor dynamics during natural synaptic events. 2. Investigate whether the glycine binding site is unsaturated during periods of high synaptic demand, due to increased synaptic DS and glutamate utilisation or clearance. An in-depth understanding of this saturation could allow for deciphering the precise role of ASCT1 and other transporters in regulating synaptic DS concentrations, offering a more nuanced understanding. 3. Investigating the effects of inhibiting Serine Racemase (SR), the enzyme responsible for converting L-serine to DS, on EPSC responses or utilising D-amino acid oxidase (DAAO), to metabolise DS, could unravel the influence of endogenous DS production on synaptic co-agonist dynamics.

In conclusion, the findings of this chapter not only enhance our knowledge of the intricate mechanisms governing synaptic DS concentrations but also suggest new avenues for exploring the roles of ASCT1, other DS transporters and enzymatic systems in maintaining synaptic DS homeostasis. This comprehensive knowledge is fundamental for unravelling the complexities of excitatory neurotransmission and holds implications for potential therapeutic interventions.

Chapter 6 General discussion

Despite ongoing investigations into D-serine (DS) and L-serine (LS) dynamics within the central nervous system, key regulatory mechanisms remain elusive. Unanswered questions persist involving DS clearance after NMDAR activation and the pathways facilitating LS transfer from astrocytes to support neuronal DS synthesis. This thesis specifically addresses these gaps by investigating the role of the astrocytic sodium-dependent alanine serine cysteine transporter (ASCT), particularly ASCT1 (*Slc1a4*), in governing serine dynamics at glutamatergic synapses. Utilising *in situ* patch clamp recordings in acutely isolated mouse brain slices, primarily in the hippocampal region, this study deciphered the mechanisms underlying DS and LS regulation, emphasising the astrocytic contribution. Pharmacological inhibition experiments utilising ASCT1 inhibitors, confirmed the functional expression of ASCT1 in astrocytes ensheathing excitatory synapses, highlighting its pivotal role in mediating DS and LS uptake. These findings suggest that astrocytic ASCT1 aids in the clearance of DS from the synaptic cleft. At the same time, due to its functioning as an exchanger, astrocytic ASCT1 has the potential to release LS in exchange for extracellular DS. This aligns with the expectation that astrocytic ASCT1 could mediate the LS shuttle transport, supporting DS synthesis in neurons.

The understanding of the role of ASCT1 in regulating DS / LS concentration and thereby the NMDAR activity was substantiated through a series of experiments in CA3-CA1 hippocampal synapses. The tightly regulated glycine binding site under *in situ* physiological conditions suggests a saturated environment, as inhibitors of ASCT1 showed no alteration in NMDAR-mediated excitatory postsynaptic current (EPSC) responses. The addition of exogenous DS or LS did not alter recorded EPSCs significantly under conditions of low synaptic activity. These findings highlight the need for a deeper understanding of the underlying regulatory mechanisms behind saturation of glycine binding site.

In summary, this thesis presents a comprehensive characterisation of the functional presence of astrocytic ASCT1, elucidating its role in orchestrating synaptic serine dynamics. These findings not only contribute to the fundamental understanding of serine regulation in the brain but also hold implications for potential therapeutic strategies in neurological disorders associated with serine dysregulation. For instance, inhibiting ASCT1 may emerge as a therapeutic strategy for conditions associated with DS hyperactivity, such as schizophrenia. Conversely, enhancing ASCT1 activity could be explored for increasing synaptic DS levels in disorders characterised by DS deficiency, like Alzheimer's disease. This final chapter places

the key findings in the context of the bigger picture of existing literature, suggesting several hypotheses for future exploration and a greater contribution to the field.

6.1 Pathological role of ASCT1 in the transport of D-serine

The implications of my findings on DS clearance via ASCT1 extend beyond the realm of fundamental neuroscience. DS dysregulation has been implicated in various neuropsychiatric and neurological disorders, underscoring its therapeutic relevance. The role of ASCT1 transporters in DS clearance opens new avenues for investigating potential therapeutic interventions that could modulate DS levels and also, by extension, influence synaptic function and neuronal excitability.

In pathological scenarios, DS release from astrocytes has been observed, which deviates from its typical behaviour under normal conditions. Elevated levels of DS in the extracellular environment can intensify the neurotoxic effects of glutamate, particularly when excessive NMDAR activation leads to an influx of calcium ions and subsequent neuronal damage (Hardingham and Bading, 2010). For instance, in traumatic brain injury, astrocytes undergo a reactive state and upregulate the production of the enzyme serine racemase (SR), resulting in an increased concentration of DS inside these cells (Perez et al., 2017). ASCT1, being an exchanger capable of both importing and exporting amino acids, can facilitate the release of DS from astrocytes (Tapanes et al., 2022) when its cytoplasmic concentration rises. As a consequence, the source of DS shifts from primarily neuronal to astrocytic, contributing to synaptic damage (Perez et al., 2017). In such contexts, inhibiting ASCT1, either through the targeted deletion of ASCT1 in glial cells or by pharmacological means, has been demonstrated to protect synapses (Tapanes et al. 2022).

Moreover, in Alzheimer's disease, astrocytes exhibit a reactive and neurotoxic phenotype, characterised by upregulation of the enzyme SR and subsequent DS release (Balu et al., 2019; Folorunso et al., 2023). This contrasts with the typical age-related decline, where DS levels tend to decrease (Wu et al., 2022). This suggests the potential for DS to serve as a biomarker for Alzheimer's disease (Orzylowski et al., 2021; Piubelli et al., 2021). Notably, in a mouse model of Alzheimer's disease, inhibiting astrocytic DS production and release has been found to improve synaptic function and ameliorate the disease condition (Qi et al., 2019). This raises the intriguing possibility that targeting astrocytic DS production or ASCT1-mediated DS release could represent a novel therapeutic approach for Alzheimer's disease, diverging from the current strategies that predominantly focus on amyloid proteins (Karran and De Strooper, 2022).

Furthermore, in the context of neuropathic pain, the upregulation of SR in astrocytes within the spinal cord can lead to the release of DS, thereby sensitising pain pathways (Miraucourt et al., 2011; Lefèvre et al., 2015). This suggests that suppressing ASCT1-mediated DS release might offer therapeutic benefits in the management of neuropathic pain.

Understanding how astrocytes regulate extracellular DS concentrations through ASCT1 could have significant implications for identifying treatment strategies for schizophrenia. Recent models of schizophrenia have emphasised the reduced activity of glutamate at NMDARs, with diminished DS activity at these receptors potentially playing a central role in the development of the condition (Javitt and Kantrowitz, 2022). Supporting this theory, the reduction of SR activity has been found to produce schizophrenia-like characteristics in animal models (Basu et al., 2009; Labrie et al., 2009; Matveeva et al., 2019; Jami et al., 2021). Furthermore, genetic variations in the SR gene have been associated with schizophrenia in humans (Morita et al., 2007; Labrie et al., 2009).

In the realm of schizophrenia treatment, strategies such as the inhibition of DAAO or direct administration of DS, have shown some efficacy in managing schizophrenia (de Bartolomeis et al., 2022). However, the observation of reduced ASCT1 expression in schizophrenia (Weis et al. 2007), suggests that increasing SR activity or ASCT1 expression could be beneficial in this condition. By enhancing DS availability at NMDARs, such interventions might help restore normal glutamatergic NMDAR signalling and alleviate symptoms in individuals with schizophrenia. This avenue of research could hold promise for novel and potentially more effective treatments that target the underlying molecular mechanisms of schizophrenia.

6.2 Functional significance of L-serine in astrocytes and neurons

The observed uptake of LS into astrocytes (4.2.2, 4.2.4) aligns with the physiological requirement of these cells for this amino acid in the synthesis of important biomolecules, including amino acids and phospholipids. This demand is also evidenced by the high expression of the enzyme 3-phosphoglycerate dehydrogenase (3PGDH) in astrocytes, enabling them to produce LS (Yamasaki et al., 2001). The current observation of LS sequestration by astrocytes, coupled with their intrinsic capacity to synthesise LS, suggests that these cells either possess or have the potential to possess elevated LS levels. Therefore, it is hypothesised that this abundant astrocytic LS acts as a readily available supply, enabling the shuttle supply of LS to neighbouring neurons.

Determining the potential transporter mediating the astrocytic supply of LS to neighbouring neurons is a crucial aspect of this mechanism. Considering the observed inhibitory profile of LS uptake, the reported pattern of transporter expression, and the directionality of these transporters, astrocytic ASCT1 emerges as a likely candidate responsible for supplying LS to neighbouring neurons. The bidirectional capability of ASCT1 (Zerangue and Kavanaugh, 1996), positions it as an effective candidate for facilitating the shuttle of LS between astrocytes and neighbouring neurons (pre or post). For instance, when DS enters the astrocytes, it could trigger the exchange of another amino acid such as LS.

Research has shown that activating ASCT1 leads to the release of astrocytic LS (Kaplan et al. 2018; Foster et al. 2016). This finding suggests that transporting DS into astrocytes via ASCT1 could stimulate the release of LS. Since the release of DS from the neurons and its uptake by astrocytes depletes the neuronal DS supply, it is advantageous to facilitate the transfer of LS from astrocytes back to neurons. As an extension of the above-stated hypothesis, astrocytic ASCT1 could facilitate the exchange of DS for LS, which promotes the replenishment of DS in neurons. In contrast, the unidirectional nature of System A transporters (Reimer et al. 2000), makes them less likely candidates to reverse and release LS from astrocytes under physiological conditions.

Similarly, in the context of pyramidal neurons, determining the transporter responsible for LS uptake is also crucial for completing the serine shuttle mechanism. Based on the expression pattern and inhibition profile observed (Figure 4.7), SNAT1 and SNAT2 emerge as potential candidates for mediating LS uptake in these neurons.

The significance of LS in neuronal function and viability has been underscored by previous research. Studies have highlighted the significant impact of LS on the survival and neurite outgrowth of cultured neurons (Savoca et al., 1995). *In vitro* studies have further established the necessity of exogenous LS for the biosynthesis of crucial neuronal compounds like DS, phosphatidyl-L-serine and sphingolipids in hippocampal neurons (Mitoma et al., 1998a; Mitoma et al., 1998b). This aligns with the necessity of LS for DS biosynthesis in adult mice brains *in vivo* (Yang et al., 2010) and in humans (Murtas et al., 2020). Taken together, the facilitated uptake of LS is indispensable for maintaining neuronal function and viability, underlining the crucial role of astrocytic LS in supporting neuronal health and functionality.

The impairment in LS transport could influence the availability of DS, potentially affecting synaptic transmission and plasticity. Recent research by Le Douce et al. (2020) characterised this impairment in Alzheimer's disease (AD). In the early stages of AD, disturbances in the

brain's aerobic glycolysis are prominent. However, how these metabolic changes contribute to synaptic plasticity and behavioural impairments in AD remains unclear. This recent study investigated the LS biosynthesis pathway, a branch of glycolysis, and found this pathway to be dysfunctional in both young AD mice and AD patients. Given that LS is a precursor for DS, a key determinant of synaptic plasticity, AD mice exhibited reduced activity at the NMDAR, accompanied by synaptic and behavioural deficiencies. Interestingly, these deficits observed mirrored symptoms when the LS synthetic pathway is disrupted in astrocytes within the hippocampus, confirming the pivotal role of astrocytic LS. More interestingly, dietary LS supplementation effectively prevented both synaptic and behavioural impairments in AD mice. Collectively, these findings underscore the significance of astrocytic glycolysis, and LS shuttle in cognitive function and suggest the potential use of oral LS as a therapeutic approach for AD.

6.3 ASCT1 could regulate synaptic DS levels during periods of high synaptic demand

The synaptic DS concentration, estimated to be around 6 μ M using desaturation, raises intriguing questions about the regulation of DS levels during periods of heightened synaptic demand. Given that the ASCT1 could potentially be less efficient in clearing synaptic DS, compared to other *Slc1* members, which are highly efficient glutamate transporters, it is logical to propose that the astrocytic ASCT1 may act as a key regulator, during extended periods of synaptic activity, influencing synaptic DS concentrations via the LS shuttle mechanism.

During high periods of synaptic activity, there is low pre-synaptic ATP levels which leads to a reduction in functional presynaptic release sites and slowdown in vesicle recycling (Lucas et al., 2018; Li and Sheng, 2022). This can impede synaptic transmission and likely to cause a rundown of EPSC responses. This rundown of the NMDAR current could be linked to a major decrease in glutamate and a minor decrease in DS concentrations. This possibility of a simultaneous rundown of glutamate and DS during prolonged synaptic activity has not been extensively studied.

While the observation indicates that the glycine binding site (GBS) on NMDARs may reach saturation under *in situ* conditions used in the experiments, extended periods of synaptic activity can ultimately result in decreased synaptic DS, potentially impacting synaptic plasticity. This idea is backed up by experiments that showed enhancing the release of DS from neurons improved LTP in hippocampal slices from both mice and rats (Rosenberg et al., 2013). This aligns with the idea that the GBS might not be saturated during HFS, as HFS is used for the

induction of LTP. One explanation could be that during HFS/LTP conditions, the GBS site may experience transient unsaturation, allowing for enhanced DS binding and an associated impact on synaptic plasticity.

Exploring the intricate role of ASCT1 in regulating synaptic DS levels during periods of heightened synaptic demand requires a comprehensive approach. To delve deeper into this, a HFS (20 Hz; Tani et al., 2014) experiments are essential in an ASCT1 KO mice model (Kaplan et al., 2018). If a rundown of NMDAR currents occurs in ASCT1 KO conditions during HFS, it would strongly suggest ASCT1's pivotal role as a mediator of synaptic DS levels under periods of high synaptic demand. Further, if the effect is reversed by the application of exogenous DS, this reversal not only supports the notion that the rundown is due to effects at the GBS but also rules out other factors such as glutamine handling and subsequent glutamate regulation as the primary contributors.

An intriguing avenue for future research involves investigating the simultaneous rundown of synaptic glutamate and DS levels during heightened synaptic demands. A future experiment employing microdialysis or using fluorescent dye measurements of glutamate (Marvin et al., 2018) and of DS (Vongsouthi et al., 2021) could measure the synaptic concentrations of both glutamate and DS before and after HFS conditions, contrasting these results with data from pre-and post-treatment with ASCT1 inhibitors. Such an approach will offer valuable insights into the interplay between these crucial neurotransmitters, enhancing our understanding of synaptic dynamics during periods of high activity.

6.4 Vesicular DS release and Fuelling DS pools with exogenous LS

The mechanisms underlying the storage and release of DS in the brain are not fully understood. While transporter-mediated DS release is supported by increasing evidence (Rosenberg et al., 2010; Rosenberg et al., 2013), the existence of a vesicle-mediated mechanism for DS storage and release remains contentious. Manipulating the DS transporters in the currents experiments resulted in relatively small or no change (5.2.5 and 5.2.6). This leads to compelling hypothesis that there could be vesicle mediated release that contributes to the baseline levels of synaptic DS. Some studies have suggested the involvement of vesicular serine transporters (VSerT, potentially PAT2 transporter) in packing DS into small stored Synaptobrevin2/cellubrevin-vesicles (Martineau et al., 2008; Martineau et al., 2013) or large vesicles (Kang et al., 2013). Additionally, the role of Ca^{2+} - SNARE fusion protein-dependent exocytosis and connexin43 (Cx43) hemichannels in DS release from astrocytes has been debated under physiological conditions (Mothet et al., 2005; Henneberger

et al., 2010; Meunier et al., 2017). Recent developments shed light on the longstanding question of whether astrocytes possess vesicle-release machinery. de Ceglia et al. (2023) provided compelling evidence by demonstrating the presence of an atypical subpopulation of specialised astrocytes in the adult brain expressing synaptic-like glutamate-release machinery. The presence of such specialised astrocytes demands further investigation to reconcile existing theories of vesicular DS release in the context of this recent evidence. As of now, there is no conclusive evidence for such DS release mechanisms, especially during periods of high co-agonist demand or altered states where transporter-mediated release might be insufficient or dysfunctional. In light of these uncertainties, a hypothetical mechanism is put forth where LS might contribute to fuelling the post-synaptic or astrocytic intracellular stores of DS. This hypothesis presents a potential avenue for future research to explore the intricate processes involved in DS storage and release in the brain (Figure 6.1).

Under basal conditions of synaptic transmission, the synaptic DS concentrations are saturating, leaving little room for observable changes in NMDAR-mediated EPSCs (depicted as normal EPSC response in Figure 6.1A). This saturated environment suggests sufficient readily releasable and the reserve pools of DS inside neurons or astrocytes or both. This process is hypothesised to be rate limited either by local DS metabolism and synthesis, or by the process of vesicular uptake by VSerTs (shown in Figure 6.1A).

Preliminary experiments supplementing LS during these basal conditions did not alter EPSC responses (Figure 5.2; depicted as normal EPSC in Figure 6.1B), suggesting that LS does not significantly contribute to the readily releasable pool of DS. However, supplying exogenous LS could still replenish the reserve DS stores in neurons or astrocytes (depicted as increased reserve DS pools in Figure 6.1B). In essence, post-synaptic neurons appear self-sufficient in producing adequate DS levels under these basal synaptic conditions.

While the proposed mechanism for DS release via a vesicle-mediated process adds complexity to our understanding, the current experimental conditions suggest that under basal states, LS's role in replenishing the DS pool may not significantly impact the observed saturation levels (Figure 5.2, 5.3). This implies that the additional DS supplied by LS does not induce observable changes when the DS system is already saturating, as seen in the present experiments. However, it is crucial to consider potential scenarios where the supply of DS might be limiting, introducing the significance of the LS shuttle. This could be particularly relevant under different physiological or pathophysiological conditions, such as at various stages of development, in different synapses, or under specific activity levels. Speculating on

these conditions opens the possibility that LS supplementation could manifest observable effects in situations where DS availability becomes a limiting factor.

Such condition where DS could be a limiting factor can be explored experimentally using a depletion strategy. To achieve selective depletion of intracellular DS stores, a hypothetical PAT2 inhibitor or conditional neuronal or astrocytic knockout of PAT2 or the traditional method of vesicle depletion like High-Frequency Stimulation (HFS; Figure 6.1C Tani et al., 2014; Marx et al., 2015) could be utilised. Once intracellular DS pools are selectively depleted (depicted as decreased DS reserve and readily releasable pools, and decreased EPSC response in Figure 6.1C), such conditions will enable further exploration of LS supplementation and its role in replenishing DS pools.

Post depletion of DS pools, if the supplementation of exogenous LS leads to an enhanced NMDAR-EPSC response, it would indicate LS dependence for maintaining readily releasable DS pools (depicted as increased reserve and readily releasable DS pools in Figure 6.1D). This observation could indirectly suggest the functionality of the serine shuttle under physiological conditions. It could imply that neurons depend on LS being transported from astrocytes to maintain their intracellular DS pools. Further using a hypothetical VSerT inhibitor or HFS, along with utilising specific inhibitors for ASCT1 or asc1 or SNAT1/2 transporters, would help clarify the mechanisms involved in DS release and its effects on NMDAR-mediated responses. This line of investigation could offer valuable insights into the interplay between LS, DS, and synaptic transmission, shedding light on the regulatory mechanisms underlying neurotransmission.

One limitation in supplementing LS or DS in the brain slices is that the potential contributions of endogenous DS pools, the serine-shuttle system, and exogenously applied DS or LS make the interpretation of experimental results complex. Additionally, accounting for the potential uptake of LS by astrocytes or neurons and its conversion into glycine highlights the intricate biochemical processes at play. This conversion is facilitated by serine hydroxy-methyl transferase (SHMT) enzyme, via a pyridoxal phosphate and tetrahydrofolate (THF) dependent reaction (Wang et al., 2013). This compensatory mechanism involving newly synthesised glycine could influence NMDAR-mediated EPSC responses, further emphasising the need for meticulous experimental design and interpretation. Navigating these complexities will undoubtedly enhance the depth of understanding, shedding light on the intricate interplay of DS, LS, and the various transport mechanisms within the brain's synaptic environment.

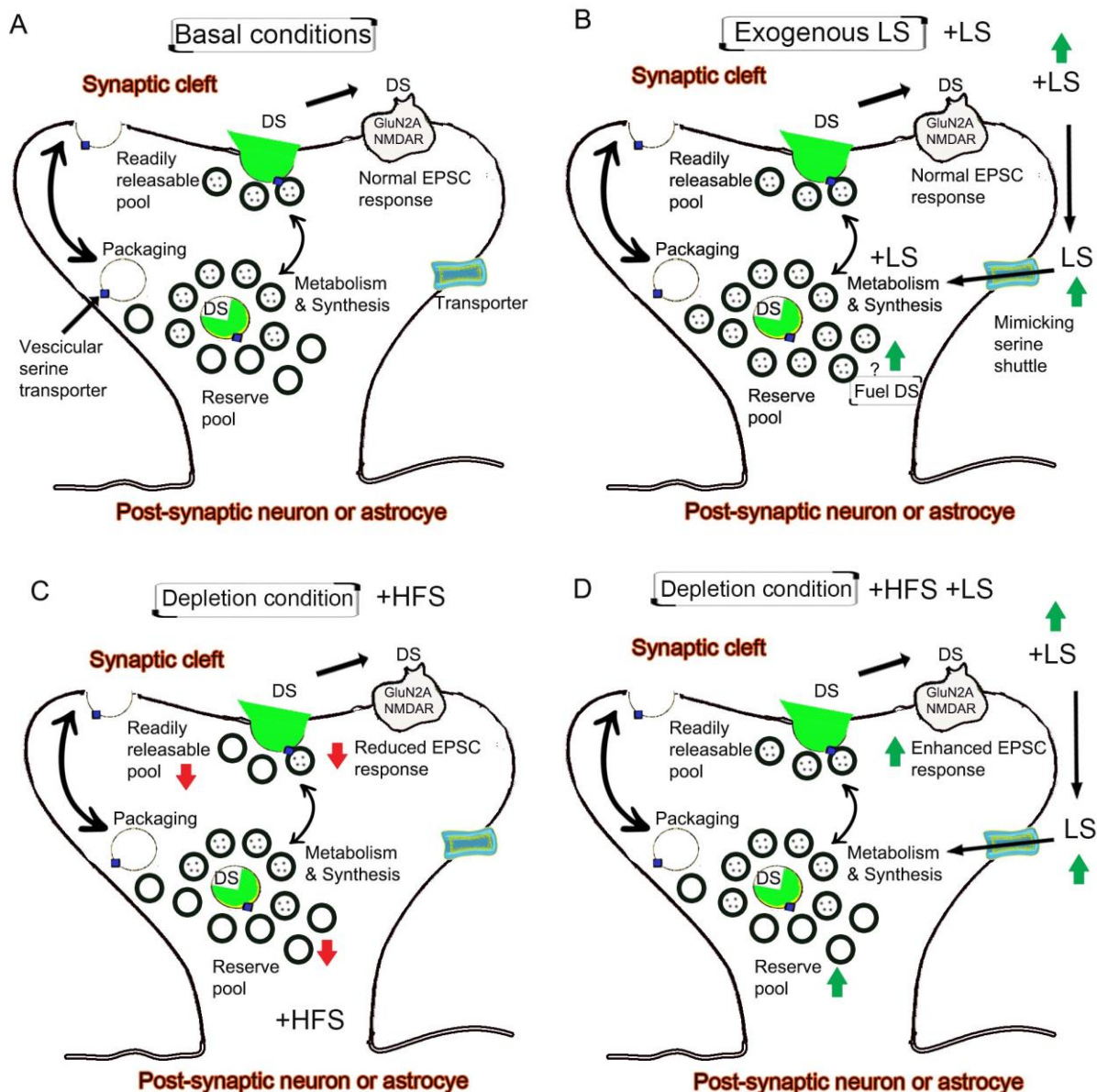


Figure 6.1: Hypothetical mechanism of DS release and LS fuelling DS pools in the postsynaptic neuron or astrocyte

(A) Shows that under basal conditions of synaptic transmission, the DS released from the post synaptic cells or astrocytes is sufficient to saturate the glycine binding site of NMDARs, and hence any changes to synaptic DS/LS does not alter the NMDAR mediated EPSC responses. (B) Shows that exogenous LS does not contribute to the readily releasable pool of DS but could fuel the synthesis of DS in the cells, without altering the synaptic DS levels. (C) Shows that depletion of intracellular DS by using a high frequency stimulation (HFS), could decrease the availability of DS at the synaptic cleft, thereby reducing the NMDAR mediated EPSCs. (D) Shows that post depletion of the intracellular DS stores with a HFS protocol, exogenous addition of LS could restore the DS stores. This consequently will increase the DS being released into the synaptic cleft, thereby rescuing the NMDAR mediated EPSCs.

6.5 Serine racemase holds the key to understanding DS regulation

The complex interplay between the enzymatic reactions and various DS transporters raises an important question: Which component/s of DS metabolism, including enzymatic processes mediated by SR, DAAO and DS / LS uptake systems such as ASCT1, asc-1, SNAT1 or 2 significantly regulate synaptic DS concentrations? Manipulating the DS / LS uptake systems alone, as demonstrated in present experiments, provided limited impact in regulating NMDAR activation in the CA3-CA1 synapses under basal conditions (Figure 5.9, Figure 5.10), indicating the need for a comprehensive approach that combines manipulating enzymatic systems along with uptake systems. This integration is crucial for fully understanding the primary contributors to synaptic DS dynamics and, by extension, NMDAR-mediated neurotransmission in these synapses.

The interplay between SR and NMDARs at glutamatergic synapses offers a multifaceted perspective on the regulation of DS metabolism and synaptic function. Over the years, researchers have probed the molecular intricacies of glutamatergic synapses, emphasising the regulatory role of SR in AMPAR and NMDAR-mediated signalling in hippocampal synapses (Balu and Coyle, 2012). Under normal synaptic conditions, GluN2A and GluN2B-containing NMDARs coordinate excitatory signals, with SR interacting intricately with PDZ-PSD95 scaffolding proteins (Figure 6.2A; Sans et al., 2000). The stability of NMDARs at synapses is suggested to be influenced by co-agonists, which impacts the internalisation and recycling of NMDARs (Nong et al., 2003; Burnet et al., 2011; Ferreira et al., 2017). Extending from this line of research, in elevated DS environments, SR is hypothesised to act as a central regulator potentially altering the surface expression and endocytotic trafficking of GluN2A, GluN2B-containing NMDARs, and AMPARs (Figure 6.2B; Papouin et al., 2012; Ferreira et al., 2017).

Beyond the NMDAR dynamics, a detailed analysis of SR's protein interactions and post-translational modifications sheds light on its multifaceted roles. Astrocytic and neuronal SR interacts with proteins like Glutamate Receptor Interacting Protein (GRIP1), Protein Interacting with C Kinase (PICK1), stargazin, and PSD95 (Kim et al., 2005; Fujii et al., 2006; Ma et al., 2013; Ma et al., 2014; Lin et al., 2016). GRIP1 activation of SR enhances DS release, influencing NMDAR activity and modulating cerebellar granule cell migration. PICK1 interaction suggests SR's involvement in fine-tuning surface expression and phosphorylation of AMPAR and NMDAR, potentially linking glutamatergic dopaminergic systems to schizophrenia (Torres et al., 2001). Disrupted in schizophrenia 1 (DISC1) interaction with SR in neurons and astrocytes is critical for SR stability, and disruption leads to SR degradation and behavioural abnormalities consistent with NMDAR hypofunction (Stark et al., 2006).

Furthermore, SR's interaction with phosphatidylinositol bisphosphate (PIP2) and mGluR5 activation highlights regulatory mechanisms beyond protein interactions (Mustafa et al., 2009).

In terms of post-translational modifications, NMDAR activation affects SR activity in a synaptic activity-dependent manner, with translocation to the plasma membrane and modulation by phosphorylation and palmitoylation (Balan et al., 2009). Nitric oxide (NO) modulation adds another layer of complexity, with S-nitrosylation at C113 inhibiting SR catalytic activity, providing a feedback mechanism for DS synthesis control (Mustafa et al., 2007). AMPAR or NMDAR activation has been thought to trigger the S-nitrosylation of SR, serving as an inhibitory feedback mechanism (Mustafa et al., 2007). This mechanism can ultimately control the synthesis of DS within the post-synaptic neuron.

Further calcium entry via NMDARs, along with calmodulin can activate nitric oxide synthetase in the post-synaptic neuron, which leads to NO production (Bredt and Snyder, 1989). The NO released from the post-synaptic neuron could act as a signalling molecule between the post-synaptic neuron and the neighbouring astrocytes. For instance, NO diffused into the astrocytes could induce S-nitrosylation of astrocytic SR, which ultimately inhibits the catalytic enzyme activity. This mechanism could further refine the regulatory orchestration of DS levels within the tripartite environment.

The localisation of SR in apical dendrites and post-synaptic densities holds physiological significance. Apical dendrites, rich in NMDARs, suggest SR's role in the breakdown of excess DS into pyruvate, potentially regulating local DS concentrations (Balu and Coyle, 2012). At the post-synaptic level, SR's interactions with stargazin and PSD95 modulate NMDAR and AMPAR recycling, desensitization, and deactivation, revealing its multifunctional impact on synaptic function (Jaffrey et al., 1998; Tomita et al., 2005). The comprehensive insights into SR's multifaceted roles underscore its significance in the intricate balance of DS metabolism and synaptic regulation.

Overall, research focussed on SR could help 1. In understanding the co-agonist occupancy at the GBS of GluN1 subunit 2. In understanding the post-translational modifications and protein interactions of SR could help in understanding the recycling, surface expression and desensitisation of NMDARs. 3. In determining the changes in subunit composition during periods of normal and prolonged synaptic activity. 4. In determining the trafficking of NMDARs which could ultimately affect LTP and LTD of synapses. To sum up, deciphering the multifaceted roles of SR not only advances our comprehension of co-agonist dynamics and

NMDAR trafficking but also provides crucial insights into the intricate interplay of neurotransmitter systems within the synaptic environment.

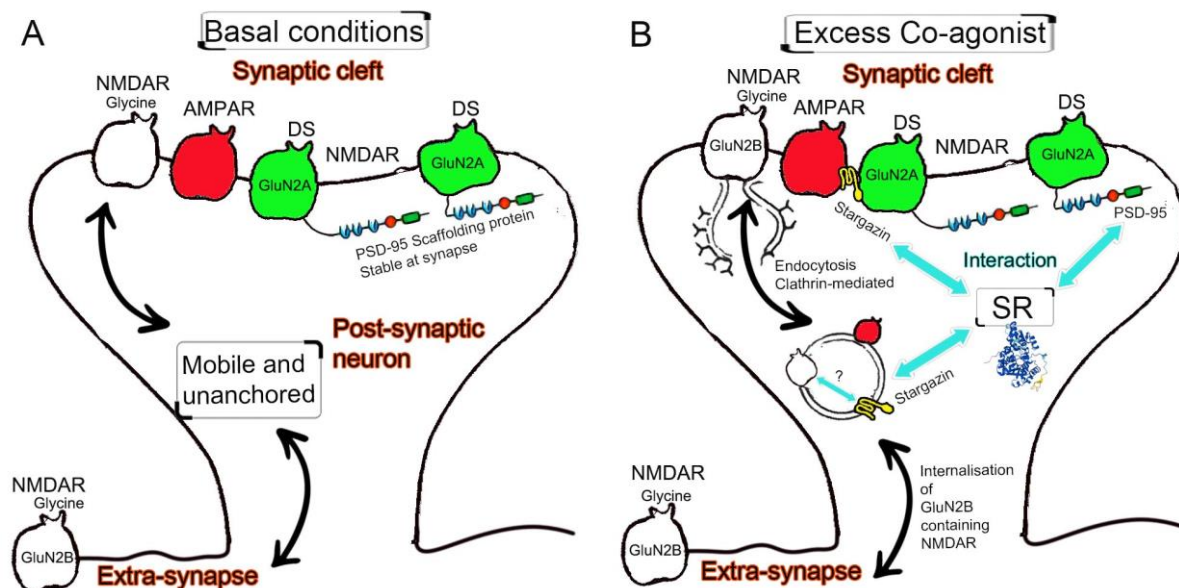


Figure 6.2: Serine racemase could be a key regulator of AMPAR and NMDAR mediated neurotransmission

(A) Shows that under normal conditions, the surface expression of GluN2A containing NMDARs is stable in the synaptic cleft owing to its interaction with PSD-95 scaffolding proteins, whereas the surface expression of GluN2B containing NMDARs is not anchored, changing between the synaptic cleft and the extra synaptic regions. (B) Shows that during presence of excess co-agonist at the synaptic cleft, the activity of post synaptic serine racemase governs the surface expression of NMDARs by modulating the receptor internalisation mechanisms (via interaction with stargazin and PSD 95).

6.6 Future Considerations

6.7 Age of the animal

My experiments on postnatal day P20-21 mice (early juvenile mice), P30 mice (late juvenile mice or early peripubertal; Figure 3.5A, B) and P6 (neonatal; Figure 3.5C, D), showed sensitivity to O-benzyl-L-serine, suggesting that the DS uptake mechanism remains consistent even as the mice transition from the critical neonatal stage. To obtain a more comprehensive understanding of DS regulation across different stages of life, it is imperative to conduct future studies involving adult mice, typically at around 2 to 3 months of age, as well as aged mice ranging from 18 months to 2 years (Bell, 2018). Given that DS levels tend to decrease with ageing (Billard, 2015), I hypothesise that the uptake of DS via astrocytic ASCT1 would be lower in older animals compared to younger ones. This could be due to the regulatory downregulation of ASCT1 in astrocytes, as observed by Sakai et al. 2003. Due to the lower DS levels, I hypothesise that the GBS on NMDARs would be less saturated in older animals, potentially impacting synaptic function.

Furthermore, conducting immunohistochemical assessments of ASCT1 expression across different age groups would provide valuable insights into the regulation of DS transport. During such investigations, it is crucial to consider the expression patterns of enzymes that could potentially influence ASCT1 expression. For example, SR levels have been observed to significantly decrease within the hippocampus, whereas the SR levels remain relatively stable in the cortex and cerebellum with age (Turpin et al., 2011; Wu et al., 2022). Conversely, the expression of DAAO may follow a distinct trajectory in the frontal cortex when compared to other brain regions like the brainstem, cerebellum, and striatum (Jagannath et al., 2017). While the study found a positive correlation between DAAO protein and its activator protein in all brain areas except frontal cortex, they observed that the DAAO mRNA levels may only increase early in life, and that there is no correlation between age and DAAO protein. Examining these factors will provide valuable insights into how DS transport and regulation take their course with age.

6.8 Brain areas and cell types

While my study focused on DS uptake in specific brain regions, including the hippocampus, somatosensory cortex, and cerebellum, the results indicate that DS uptake is a widespread phenomenon in astrocytes across these brain areas. In brain areas like the striatum, where circuits are primarily inhibitory in nature (Knowles et al., 2021; Ahmed et al., 2023), the role of DS and its uptake mechanisms may differ significantly, implying potentially distinct regulatory mechanisms. Therefore, future investigations should aim to explore DS uptake in brain regions characterised by predominantly inhibitory circuits. This research could shed light on the unique roles and regulatory pathways of DS in inhibitory neurotransmission, offering a more comprehensive understanding of its functional significance in different circuitry contexts.

Traditionally, regulation of DS in the excitatory synapses has been attributed to either the pyramidal neurons or the neighbouring astrocytes. While my study has primarily focused on astrocytes as key players in DS regulation, there is a need to explore the role of other cell types. For instance, neurons (Pre or Post), are also known to release and regulate DS (Rosenberg et al., 2013). Therefore, to obtain a comprehensive understanding of DS homeostasis within the brain, future research should explore the contributions of different cell types, including both excitatory and inhibitory neurons, to DS dynamics.

In my current observations, CA1 pyramidal neurons and interneurons did not exhibit DS currents (Figure 3.4). In the case of pyramidal neurons, the entry of DS could occur through electroneutral asc-1. Despite DS being observed in interneurons (Balu et al., 2014), the uptake

did not manifest as membrane currents in these cells either, suggesting a potential electroneutral uptake mechanism here too. However, research by (Miya et al., 2008) revealed a paradigm-shifting finding involving the expression of SR in GABAergic interneurons of the striatum. Intriguingly, DS within the interneurons has not been reported to be released along with GABA. Moreover, post-synaptic GABA receptors do not have a DS binding site. This leads to a compelling hypothesis that the DS within the GABAergic interneurons might modulate the activity of excitatory neurons, where NMDARs are prevalent. While this phenomenon could be region-specific, the robust expression of SR in GABAergic medium spiny neurons, especially those receiving dopaminergic input in the striatum (Takagi et al., 2022), raises intriguing possibilities of such a regulatory mechanism for DS in the hippocampus. In the CA1 region of rats, 10-15% of the total neuronal population comprises Parvalbumin (PV⁺) basket cells and bistratified cells (Pelkey et al., 2017), although only 25-40% of interneurons express SR (Balu et al., 2014). This interneuronal SR expression suggests a potential mechanism, that possibly could explain why interneurons stain for DS (Schell et al., 1997; Balu et al., 2014). Interneurons may release DS upon demand, although the precise signalling mechanisms remain elusive. Future studies investigating this phenomenon could have a concept-changing impact on our understanding of hippocampal synapses. Firstly, interneurons might govern the activity of NMDAR by releasing DS into the synapses, challenging conventional literature. Additionally, these findings could open avenues for a DS-centered perspective on how excitatory neurons communicate with inhibitory counterparts, potentially influencing brain circuit homeostasis. Given the hippocampus's susceptibility to recurrent excitation and epilepsy (Fujiwara-Tsukamoto et al., 2004), understanding how interneurons regulate excitatory neurotransmission could revolutionise epilepsy therapeutics, paving the way for novel treatment strategies.

6.9 Regional Variability and Generalisability of the Finding

It is crucial to acknowledge the limitations of my study, conducted exclusively in acute brain slices. While valuable for controlled drug application onto individual cells, these slices may not fully represent the complex *in vivo* environment of the mammalian brain. This raises an important question about the extent to which these results can be extrapolated to other species and, crucially, to the intact brain. Therefore, further research is needed to ascertain the generalisability of these findings to other brain areas, validate them in an *in vivo* context, and utilise other animal models for a more comprehensive understanding of DS regulation in the intact brain.

In line with this concept of generalisability, prior findings from Nuzzo et al. (2019), emphasise that the metabolism of DS in the basal ganglia of Parkinson's disease (PD) monkeys exhibits distinct region-specific changes compared to the brains of control monkeys. In PD monkeys with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; a neurotoxin) lesions, there was a trend towards increased DS levels in the post-commissural dorsal putamen. Additionally, there was a significant elevation of DS in the rostral putamen region. In contrast, no alterations in DS levels were observed in the subthalamic nucleus, lateral and medial globus pallidus. However, a substantial reduction in DS, coupled with a notable increase in DAAO mRNA and protein expression, was identified in the substantia nigra of MPTP-lesioned monkeys. These findings strongly emphasise the need for future research to delve into regional variations of DS in the brain.

6.9.1.1 MPTP-induced Parkinson's models

A recent study by Serra et al. (2023) employed two well-established models of PD: MPTP-treated macaques (*Macaca mulatta*) and MPTP-treated mice. The use of these models allowed them to link the findings of my study on ASCT1 to the key features of PD, particularly the hallmark severe dopaminergic denervation of the nigrostriatal pathway. I have summarised key points in this heading as they provided valuable insights into exploring the impact of dopaminergic degeneration on serine metabolism, shedding light on the potential role of astrocytes and offering a new avenue for therapeutic exploration.

6.9.1.2 Altered serine levels and regional specificity

Among the most compelling findings in the study by Serra et al. (2023) was the significant alteration in serine levels, both DS and LS, specifically within the posterior putamen of MPTP-treated monkeys. Importantly, these changes were not reflected in other basal ganglia regions such as the subthalamic nucleus and the medial and lateral globus pallidus. This observation is in line with the previous findings by Nuzzo et al. (2019) and this regional specificity suggests a potential link between dopaminergic denervation in the rostral putamen and changes in serine metabolism.

One unexpected discovery was the similarity in protein levels of SR and phosphoglycerate dehydrogenase (PHGDH) in the rostral putamen between MPTP-treated and control monkeys. This raises intriguing questions about the mechanisms underlying the observed alterations in serine levels. It is possible that disturbances in serine metabolism, particularly in aerobic

glycolysis, might be responsible for these changes, given previous research implicating abnormal glucose metabolism in PD (Pellerin and Magistretti, 1994).

6.9.1.3 Astrocyte involvement, ASCT1 levels and neuroadaptive mechanisms

Another significant aspect of the study by Serra et al. (2023) points towards the involvement of astrocytes in serine metabolism. They imply that the astrocytes, known for their role in LS synthesis, might become activated in response to dopaminergic denervation, leading to elevated LS levels. Furthermore, the reduction in ASCT1 levels in the rostral putamen of MPTP-treated monkeys suggests that astrocyte-neuron interactions, specifically involving DS transport, could be disrupted in the context of PD. It's worth considering that the observed changes in serine levels may represent neuroadaptive responses aimed at counteracting the loss of dopaminergic input. These adaptations might serve to balance abnormal glutamate receptor activation in the midbrain while promoting dopamine release in the striatum, potentially contributing to the overall pathophysiology of PD.

6.9.1.4 Therapeutic implications of serine supplementation require further research

The idea of serine supplementation as a treatment for PD is particularly promising (Serra et al., 2023), especially given the documented positive effects of NMDAR co-agonists in PD treatment (Nava-Gómez et al., 2022). Nevertheless, further research, particularly in different PD models and at various disease stages, is warranted to assess the effectiveness and safety of serine supplementation as a potential therapy. While these findings are promising, they underscore the need for further research to validate and expand upon these preliminary discoveries and, ultimately, to better understand the clinical relevance of ASCT1.

6.10 Conclusion

In the vast boundaries of neuroscience, a wide array of molecular actors orchestrate the intricacies of synaptic signalling. A molecule of utmost importance emerges within this symphony – D-serine (DS). Functioning as a co-agonist at NMDA-type of glutamatergic receptors, DS plays a pivotal role in normal excitatory neurotransmission within the mammalian brain. However, the precise origin of DS and the mechanisms governing its fate remain enigmatic. With profound influences on synaptic plasticity, learning, and memory (Collingridge and Bliss, 1987; Panatier et al., 2006), DS draws scientific inquiry.

"The serine shuttle model", as proposed by Wolosker (2011), posits that neurons predominantly serve as the primary source of synaptic DS, with astrocytes playing a supportive yet dynamic role by supplying L-serine (LS) to neurons. However, this model encounters challenges in reconciling with findings by Henneberger et al. (2010), who suggest that astrocytes may directly provide the gliotransmitter DS to neurons. Consequently, a thorough investigation into the distinct roles of astrocytes and neurons in DS/LS regulation holds promise for gaining a deeper understanding of serine dynamics within glutamatergic synapses. This thesis aims to explore the mechanisms regulating serine within the dynamic milieu of the mammalian brain.

The sodium-dependent alanine serine cysteine transporter ASCT 1 and 2 (*Slc1a4* and *Slc1a5*) and the sodium-independent neutral amino acid transporter asc-1 (*Slc7a10*) have been extensively studied in oocyte expression systems and in astrocyte/neuronal cultures to mediate transport of several small and neutral amino acids, including DS and LS across the plasma membrane (Broer et al., 1999; Broer et al., 2000; Ribeiro et al., 2002; Foster et al., 2016). Both asc-1 and ASCT transporters function as obligatory exchangers of neutral amino acids across the membrane. ASCT1 is abundantly expressed in the astrocytes across different brain areas, whereas the ASCT1 expression in neurons is downregulated after birth (Sakai et al., 2003; Jeon et al., 2009). In contrast, ASCT2 is absent in astrocytes and is only minimally expressed in the dendrites of pyramidal neurons (Gliddon et al., 2009). Additionally, the previously unrecognised transport of DS by SNAT1/2 (Bodner et al., 2020), adds a further potential pathway for neuronal DS uptake.

The findings from chapter 3 contribute to a comprehensive understanding of the regulatory mechanisms influencing DS concentrations within distinct brain regions. Through direct electrophysiological recordings of transporters within their natural environment, the study revealed the electrogenic uptake of DS into glial cells. Specifically, the ASCT emerges as a

key player in mediating DS uptake into astrocytes adjacent to synapses in the CA1 hippocampal region. The ASCT1 isoform (*Slc1a4*) plays a predominant role, as evidenced by its inhibition with trans-4-hydroxy-L-proline. This pivotal role of ASCT extends to the cortex and Bergmann glia of the cerebellum, underscoring its central involvement in influencing extracellular DS levels and contributing to the metabolic breakdown of DS. These findings align with existing knowledge, emphasising the significance of astrocytes in regulating synaptic function and plasticity.

The ASCTs exhibit a sodium-dependent transport mechanism for their amino acid cycling, which could be accompanied by an uncoupled anionic leak or conductance during the transport cycle. This anionic leak follows a permeability sequence, with $\text{SCN}^- > \text{NO}_3^- > \text{Cl}^-$, and is reminiscent of the behaviour observed in their familial counterparts, the Excitatory Amino Acid Transporters (EAATs; Arriza et al. in 1993). However, the current findings indicate that substituting internal SCN^- for Cl^- does not have the expected effect, and there is no current reversal at positive membrane potentials. These findings align with activation of coupled transport currents rather than the activation of an uncoupled anion conductance. This is supported by previous research demonstrating that serine acts as an inhibitor rather than an activator of the anion conductance in ASCT1 (Wang et al., 2022). The present data from ASCT1 align with the mechanism previously reported on electrogenic coupled amino acid transport via ASCT2 (Scalise et al., 2014; Mazza et al., 2021).

The fourth chapter extends our understanding of LS regulation within the mammalian brain, emphasising the intricate astrocyte-neuron signalling. The electrogenic transport of LS by astrocytes, mediated by ASCT1, indicates its crucial role in the shuttle of LS between astrocytes and neurons. This bidirectional capability positions ASCT1 as a likely candidate in astrocytes for the exchange of extracellular DS for intracellular LS, a process vital for promoting the replenishment of DS in neurons.

The clearance of synaptic DS by ASCT1 and its subsequent metabolic degradation is hypothesised to decrease its extracellular availability, thereby affecting NMDAR activation. Since ASCT1 is expected to aid in DS clearance and mediate the LS shuttle transport, supplying LS to neurons, the physiological role of ASCT1 requires careful investigation. Furthermore, the findings provide crucial insights into the bidirectional capability of ASCT1 in modulating the synaptic effects of glutamate under normal physiological conditions. Importantly, in pathological contexts associated with alterations in DS levels, ASCT1's role

becomes pivotal, suggesting its potential contribution to various neurological disorders such as traumatic brain injury, Alzheimer's disease, neuropathic pain, and Schizophrenia.

Other amino acid transporters such as the system A transporters (alanine-preferring) SNAT1 and SNAT2 (*Slc38a1* and *Slc38a2*), have been less explored in transporting DS, but capable of LS transport in an electrogenic manner (Chaudhry et al., 2002; Bodner et al., 2020). SNAT1 expression has been observed in the glutamatergic pyramidal neurons and the GABA-releasing interneurons (Melone et al., 2004). Although SNAT1 expression has been observed in cultured astrocytes, its expression was not detected in the hippocampal astrocytes in adult rats (Brookes, 1988; Mackenzie et al., 2003). On the other hand, SNAT2 expression has been observed in the glutamatergic neurons and astrocytes across several brain areas (González-González et al., 2005). The identification of electrogenic LS uptake mediated by SNAT1 or SNAT2 in CA1 pyramidal neurons, evident from the inhibition by 2-(methylamino)isobutyric acid (MeAIB), underscores the complexity of astrocyte-neuron signalling essential for intercellular communication.

Dysfunctional LS biosynthesis pathways observed in Alzheimer's disease highlight the pivotal role of astrocytic LS in synaptic plasticity and cognitive function. Targeting ASCT1 or system A transporter-mediated LS uptake could offer as a prospective avenue for therapeutic interventions, offering novel perspectives for preventing synaptic and behavioural impairments in conditions like Alzheimer's disease.

Furthermore, the exploration of candidate serine transporters, particularly those exhibiting electroneutrality, requires further investigation. Among the two electroneutral system N transporters, SNAT3 and SNAT5 (*Slc38a3* and *Slc38a5*), SNAT5 has been observed to mediate LS transport but not SNAT3 (Cubelos et al., 2005; Todd et al., 2017). Within the CNS, they are exclusively located in the astrocytes and not neurons (Boulland et al., 2003; Cubelos et al., 2005). Two major transporters belonging to system L, LAT1 and LAT2 have been studied to be involved in the mediated transport of branched-chain amino acids like leucine, valine and phenylalanine (Kanai et al., 1998; Pineda et al., 1999). Although the expression of LAT 1 and LAT 2 has been observed in the rat blood-brain barrier, its cellular distribution remains unexplored (Boado et al., 1999; Segawa et al., 1999). Among the four isoforms of the family of transporters, only LAT2 (encoded by the *Slc7a8* gene) has been reported to be capable of serine transport (Lewis et al., 2007). Therefore, the specificity of LAT transporters to mediate DS or LS transport in the brain needs further exploration.

The fifth chapter delves into the investigation of synaptic DS concentrations at the CA3-CA1 hippocampal excitatory synapses, with a particular focus on ASCT1 transporters, providing valuable insights. Martina et al. (2003) demonstrated that exogenous DS potentiated the NMDA component of synaptic responses evoked by *Schaffer collateral* stimulation in rat hippocampal slices. However, in the current experiments utilising low-frequency stimulation at *Schaffer collaterals*, both DS and LS failed to enhance the amplitude of NMDAR-mediated EPSCs recorded from CA1 pyramidal neurons. While the precise reason for this discrepancy remains unclear, the current findings align with previous studies that reported addition of DS did not lead to an increase in the amplitude of synaptic and extra-synaptic NMDAR-mediated responses in mouse CA1 pyramidal neurons (Ballard et al., 2002; Scimemi et al., 2004; Le Meur et al., 2007). This collective body of evidence suggests a consistent pattern across different investigations and reinforces the notion that the effects of DS on NMDAR-mediated responses may be influenced by specific experimental conditions or cellular contexts. The tightly regulated glycine binding site under physiological conditions suggests a saturated environment, as inhibitors of ASCT1 showed no alteration in recorded EPSCs. Desaturation experiments and estimation of synaptic DS concentration provide quantitative insights into co-agonist dynamics, supporting saturation of NMDARs. Furthermore, the experiments involving the manipulation of SNAT1, SNAT2, and asc-1 transporters in governing synaptic DS or glutamine concentrations add complexity to synaptic regulation. Finally, the study suggests ASCT1's potential role in regulating synaptic DS levels during periods of heightened synaptic demand, proposes several hypotheses for future research, highlighting the need to explore NMDAR dynamics, synaptic DS demand at extended periods of synaptic activity, and the influence of endogenous DS production on co-agonist dynamics. In conclusion, these comprehensive findings not only advance our knowledge of intricate synaptic DS concentration mechanisms but also suggest promising avenues for therapeutic interventions. The roles of ASCT1, other DS transporters, and enzymatic systems in maintaining synaptic DS homeostasis are crucial for unravelling the complexities of excitatory neurotransmission, holding implications for potential therapeutic strategies.

In conclusion, this thesis significantly contributes to our understanding of serine regulation, offering new perspectives on the role of astrocytic ASCT1 in orchestrating synaptic DS dynamics and opening avenues for further exploration into excitatory neurotransmission modulation.

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Appendix

R codes with an example output of each script.

Scripts used in R-studio are written in blue.
Relevant comparisons are highlighted in yellow.
Significant p-values are highlighted in red.

Analysis for D-serine current amplitude - Different Conditions - Using Linear Mixed Effect (LME) Model

```
# Load libraries
```

```
> library(nlme)
```

```
# Load data
```

```
> data <- read.csv("source.csv")
```

```
> View(data)
```

```
> str(data)
```

```
'data.frame': 139 obs. of 3 variables:
 $ Cell      : Factor w/ 89 levels "03062020cell1",...: 8 9 89 82 22 23 28
29 24 25 ...
 $ Current   : num 25.7 17.9 16.3 15.1 11.3 ...
 $ Condition: Factor w/ 15 levels "NaCl","NaSCN",...: 2 2 2 2 1 1 1 1 1 1
...
```

```
# Releveling
```

This code will change the level of the Condition variable in the data frame to "NaSCN", which will become the new reference level. All other levels will be recoded relative to this new reference level. For example, if the original levels of the Condition variable were "Control", "Treatment A", and "Treatment B", and "NaSCN" was set as the reference level, the new levels would be "NaSCN", "Control", and "Treatment A", respectively.

```
> data$Condition = relevel (data$Condition, "NaSCN")
```

```
# Define LME model
```

```
> our.answer = lme(Current ~ Condition, random =~1|Cell, data = data)
```

```
> summary(our.answer)
```

Linear mixed-effects model fit by REML

```
Data: data
      AIC      BIC    logLik
793.7372 841.682 -379.8686
```

Random effects:

```
Formula: ~1 | Cell
(Intercept) Residual
StdDev:      2.110053 4.186175
```

Fixed effects: Current ~ Condition

	value	Std.Error	DF	t-value
p-value				
(Intercept)	18.774954	0.5684911	87	33.02594
0.0000				
ConditionNaCl	-3.026036	1.6628298	87	-1.81981
0.0722				
ConditionNaSCN (Cerebellum)	-1.752384	2.4119036	37	-0.72656
0.4721				
ConditionNaSCN(Cortex)	1.293515	2.4119036	37	0.53630
0.5950				
ConditionNaSCN(LS)	14.862810	2.4119036	37	6.16227
0.0000				

ConditionNaSCN(LS)+Benzyl serine 0.0000	-13.942393	2.4119036	37	-5.78066
ConditionNaSCN+10 mM MeAIB 0.6023	-1.229751	2.3396769	37	-0.52561
ConditionNaSCN+10mM SMLC 0.2356	2.534914	2.1024675	37	1.20569
ConditionNaSCN+30mM MeAIB 0.8876	-0.332998	2.3396769	37	-0.14233
ConditionNaSCN+5mM BCH 0.3843	-1.483826	1.6851683	37	-0.88052
ConditionNaSCN+5mM SMLC 0.8630	-0.365242	2.1024675	37	-0.17372
ConditionNaSCN+Benzyl serine 0.0000	-16.024987	2.1024675	37	-7.62199
ConditionNaSCN+Benzyl serine(Cerebellum) 0.0000	-17.500245	2.4119036	37	-7.25578
ConditionNaSCN+Benzyl serine(Cortex) 0.0000	-12.028696	2.4119036	37	-4.98722
ConditionNaSCN+T-Pro 0.0000	-14.734128	1.7933545	37	-8.21596
Correlation:	(Intr) CndtNC CndtnNSCN(Crb)			
CndtnNSCN(Crt) CNSCN(L				
ConditionNaCl	-0.342			
ConditionNaSCN (Cerebellum)	-0.236	0.081		
ConditionNaSCN(Cortex)	-0.236	0.081	0.056	
ConditionNaSCN(LS) 0.056	-0.236	0.081	0.056	
ConditionNaSCN(LS)+Benzyl serine 0.056	-0.236	0.081	0.056	
ConditionNaSCN+10 mM MeAIB 0.046	-0.194	0.066	0.046	
ConditionNaSCN+10mM SMLC 0.051	-0.216	0.074	0.051	
ConditionNaSCN+30mM MeAIB 0.046	-0.194	0.066	0.046	
ConditionNaSCN+5mM BCH 0.063	-0.269	0.092	0.063	
ConditionNaSCN+5mM SMLC 0.051	-0.216	0.074	0.051	
ConditionNaSCN+Benzyl serine 0.051	-0.216	0.074	0.051	
ConditionNaSCN+Benzyl serine(Cerebellum) 0.056	-0.236	0.081	0.247	
ConditionNaSCN+Benzyl serine(Cortex) 0.247	-0.236	0.081	0.056	
ConditionNaSCN+T-Pro 0.060	-0.253	0.086	0.060	
CNSCNB CNSCN+5S	CNSCN(S CNSCM CNSCN+1S CNSCNM			
ConditionNaCl				
ConditionNaSCN (Cerebellum)				
ConditionNaSCN(Cortex)				
ConditionNaSCN(LS)				
ConditionNaSCN(LS)+Benzyl serine	0.046			
ConditionNaSCN+10 mM MeAIB	0.051	0.042		
ConditionNaSCN+10mM SMLC	0.046	0.038	0.042	
ConditionNaSCN+30mM MeAIB	0.063	0.052	0.058	0.052
ConditionNaSCN+5mM BCH	0.051	0.042	0.046	0.042
ConditionNaSCN+5mM SMLC 0.058	0.051	0.042	0.046	0.042
ConditionNaSCN+Benzyl serine 0.058	0.056	0.046	0.051	0.046
ConditionNaSCN+Benzyl serine(Cerebellum) 0.063	0.056	0.046	0.051	0.046
ConditionNaSCN+Benzyl serine(Cortex) 0.063	0.056	0.046	0.051	0.046

Condition	0.060	0.049	0.055	0.049
ConditionNaSCN+T-Pro				
0.068 0.055				
CndtnNSCN+Bsrn(Crt)				
ConditionNaCl				
ConditionNaSCN (Cerebellum)				
ConditionNaSCN(Cortex)				
ConditionNaSCN(LS)				
ConditionNaSCN(LS)+Benzyl serine				
ConditionNaSCN+10 mM MeAIB				
ConditionNaSCN+10mM SMLC				
ConditionNaSCN+30mM MeAIB				
ConditionNaSCN+5mM BCH				
ConditionNaSCN+5mM SMLC				
ConditionNaSCN+Benzyl serine				
ConditionNaSCN+Benzyl serine(Cerebellum)	0.051			
ConditionNaSCN+Benzyl serine(Cortex)	0.051	0.056		
ConditionNaSCN+T-Pro	0.055	0.060		
0.060				

Standardized within-Group Residuals:

Min	Q1	Med	Q3	Max
-2.68244127	-0.51254959	-0.01703691	0.52283710	2.15598685

Number of Observations: 139

Number of Groups: 89

**# Analysis for D-serine current amplitude – across voltage
– Using Linear Fixed Effect Model:**

```
# Define library
> library(nlme)
```

```
# Load data
> data <- read.csv("source.csv")
> View(data)
> str(data)
```

```
# Define LM model
> our.answer = lm(Voltage ~ Current, data = data)
> summary(our.answer)
```

Perform one-sample t-test to estimate if mean currents differ significantly from 0:

```
# Your data
> data <- c(-1.448781145, -1.77182523, 0.979815954, -2.296322249,
2.616213226, 2.024700809, -1.135603577)
```

```
# Perform one-sample t-test
> result <- t.test(data, mu = 0)
```

```
# Output
> print(result)
```

```
data: data
t = -0.19683, df = 6, p-value = 0.8505
alternative hypothesis: true mean is not equal to 0
95 percent confidence interval:
-1.979784 1.684983
sample estimates:
mean of x
-0.1474003
```

```

# Analysis for peak 1 amplitude - Different Conditions
- Using Linear Mixed Effect (LME) Model

# Load libraries
> library(nlme)

# Load data
> Data1 <- read.csv("source.csv")
> View(Data1)
> str(Data1)
'data.frame': 120 obs. of 3 variables:
 $ Cell      : Factor w/ 60 levels "2022_02_15_0022, 2022_02_15_0023,
2022_02_15_0024",...: 9 9 10 10 11 11 12 12 47 47 ...
 $ Current   : num 30.9 28.4 52.6 43.3 37.3 ...
 $ Condition: Factor w/ 13 levels "Kyn A","NBQX",...: 2 1 2 1 2 1 2 1 2 1
...
> Data1$Condition.1 = relevel (Data1$Condition, "NBQX")

# Define LME model
> our.answer = lme(Current ~ Condition.1, random =~1|Cell, data = Data1)
> summary(our.answer)
Linear mixed-effects model fit by REML
Data: Data1
      AIC      BIC    logLik
919.967 959.1938 -444.9835

Random effects:
Formula: ~1 | Cell
(Intercept) Residual
StdDev:      19.92967 9.909737

Fixed effects: Current ~ Condition.1
              Value Std.Error DF   t-value p-value
(Intercept)  47.38897   3.261859 56 14.528206  0.0000
Condition.1Kyn A    -7.15673   6.682276 45 -1.071002  0.2899
Condition.1NBQX+Benzyl serine -1.20472   5.468812 45 -0.220288  0.8266
Condition.1NBQX+DS   -6.85056   6.682276 45 -1.025183  0.3108
Condition.1NBQX+KynA    0.74408   8.518454 45  0.087349  0.9308
Condition.1NBQX+KynA(4) -18.62008   4.639744 45 -4.013170  0.0002
Condition.1NBQX+KynA(4)+DS -24.48217   7.132032 45 -3.432707  0.0013
Condition.1NBQX+KynA+DS    7.95104   9.264689 45  0.858210  0.3953
Condition.1NBQX+KynA+LS    1.72227  10.460703 45  0.164642  0.8700
Condition.1NBQX+LS      -8.75506   5.468812 45 -1.600908  0.1164
Condition.1NBQX+MeAIB   -17.87306   5.468812 45 -3.268179  0.0021
Condition.1NBQX+SMLC   -14.51838   5.983800 45 -2.426281  0.0193
Condition.1NBQX+T-Pro    -0.64491   5.468812 45 -0.117925  0.9067

Correlation:
              (Intr) Cn.1KA C.1NBs C.1NBQX+D Cn.1NBQX+KA
Cn.1NBQX+KA(4)
Condition.1Kyn A      -0.097
Condition.1NBQX+Benzyl serine -0.118  0.011
Condition.1NBQX+DS     -0.097  0.009  0.011
Condition.1NBQX+KynA   -0.383  0.037  0.045  0.037
Condition.1NBQX+KynA(4) -0.254  0.025  0.030  0.025  0.097
Condition.1NBQX+KynA(4)+DS -0.223  0.022  0.026  0.022  0.085
0.545
Condition.1NBQX+KynA+DS -0.352  0.034  0.042  0.034  0.764
0.089
Condition.1NBQX+KynA+LS -0.312  0.030  0.037  0.030  0.677
0.079
Condition.1NBQX+LS     -0.118  0.011  0.014  0.011  0.045
0.030
Condition.1NBQX+MeAIB  -0.118  0.011  0.014  0.011  0.045
0.030
Condition.1NBQX+SMLC   -0.108  0.010  0.013  0.010  0.041
0.027
Condition.1NBQX+T-Pro  -0.118  0.011  0.014  0.011  0.045
0.030

```

	C.1NBQX+KA(4)+	C.1NBQX+KA+D	C.1NBQX+KA+L
C.1NBQX+L			
C.1NBQX+M			
Condition.1kyn A			
Condition.1NBQX+Benzyl serine			
Condition.1NBQX+DS			
Condition.1NBQX+KynA			
Condition.1NBQX+KynA(4)			
Condition.1NBQX+KynA(4)+DS			
Condition.1NBQX+KynA+DS	0.079		
Condition.1NBQX+KynA+LS	0.070	0.521	
Condition.1NBQX+LS	0.026	0.042	0.037
Condition.1NBQX+MeAIB	0.026	0.042	0.037
0.014			
Condition.1NBQX+SMLC	0.024	0.038	0.034
0.013	0.013		
Condition.1NBQX+T-Pro	0.026	0.042	0.037
0.014	0.014		
C.1NBQX+S			
Condition.1kyn A			
Condition.1NBQX+Benzyl serine			
Condition.1NBQX+DS			
Condition.1NBQX+KynA			
Condition.1NBQX+KynA(4)			
Condition.1NBQX+KynA(4)+DS			
Condition.1NBQX+KynA+DS			
Condition.1NBQX+KynA+LS			
Condition.1NBQX+LS			
Condition.1NBQX+MeAIB			
Condition.1NBQX+SMLC			
Condition.1NBQX+T-Pro	0.013		

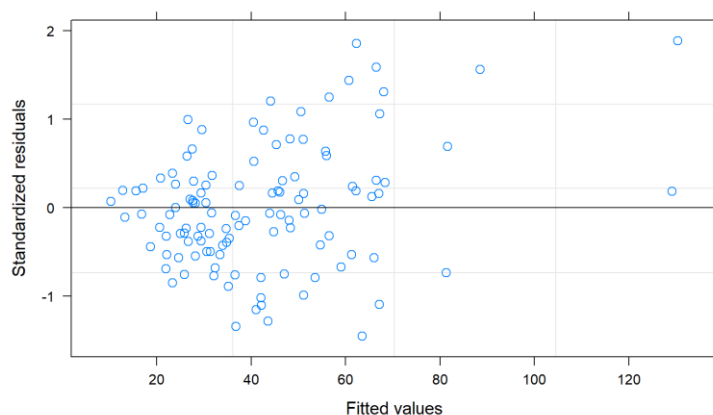
Standardized within-Group Residuals:

Min	Q1	Med	Q3	Max
-1.4544073	-0.4412890	-0.0632473	0.3029296	1.8866335

Number of Observations: 114
Number of Groups: 57

check model assumptions

> plot(our.answer)



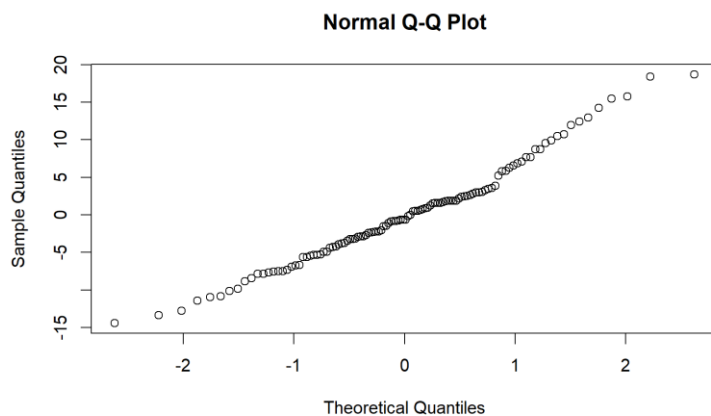
This code generated a plot to check the assumptions of a linear mixed effects model fit using the lme function. The plot included the residuals of the model, which were the differences between the observed data and the model predictions.

By examining the residual plots, I assessed whether the assumptions of the model were met and determined if the model was a good fit for the data.

From the residual plots, I checked the assumptions of the model, including:

1. Normality: The residuals were approximately normally distributed. As the given data was normally distributed, I considered the model a good fit for the data.
2. Independence: The residuals were independent, meaning that they were not correlated with each other. If the residuals were correlated, it might indicate that the model was not a good fit for the data.

```
> qqnorm(resid(our.answer))
```



QQ plot (or quantile-quantile plot) shows the correlation between the given sample and the normal distribution. A 45-degree reference line is also plotted. It was used to visually check the normality of the data.