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Surface Diffusion of the Astrocytic Glutamate Transporter GLT-1 Shapes Synaptic Transmission

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Soutenue le 6 Juin 2014

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in Science, Technology & Health specialising in Neuroscience

Presented on 6th of June 2014

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“Education is an admirable thing. But it is well to remember from time to time that nothing that is worth knowing can be taught”

Oscar Wilde

“A Few Maxims for the Instruction of the Over-Educated”

The Saturday Review of Politics, Literature, Science and Art

Nov. 17, 1894

Abstract

A classic understanding of neurotransmitter clearance at glutamatergic synapses is that, in order to ensure sufficient glutamate uptake on a fast timescale, it is necessary to have high numbers of glutamate transporters in the vicinity of release sites to compensate for their slow transport kinetics. Using a combination of single molecule imaging and electrophysiological approaches, we now challenge this view by first demonstrating that GLT-1 transporters are not static but highly mobile at the surface of astrocytes, and that their surface diffusion is dependent upon both neuronal and glial cell activities. In the vicinity of glutamate synapses, GLT-1 dynamics are strongly reduced favoring their retention within this strategic location. Remarkably, glutamate uncaging at synaptic sites instantaneously increases GLT-1 diffusion, displacing the glutamate-bound transporter away from this compartment. Functionally, impairment of the transporter lateral diffusion through an antibody-based surface cross linking, both *in vitro* and *in vivo*, significantly slows the kinetics of excitatory postsynaptic currents. Taken together, these data reveal the unexpected and major role of the astrocytic surface GLT-1 fast dynamics in shaping glutamatergic synaptic transmission.

Keywords:

Glutamate transporters, Glutamate uptake, lateral diffusion, single nanoparticle imaging, tripartite synapse, neuron-glia interactions

Résumé

Le glutamate est le principal neurotransmetteur excitateur du système nerveux central des vertébrés, et le codage de l'information cérébrale repose en partie sur des modulations de l'amplitude et de la fréquence des transmissions synaptiques glutamatergiques. De ce fait, la résolution spatiale et temporelle de ces transmissions nécessite un contrôle fin de la présence de glutamate dans la fente synaptique. Cette durée de vie du glutamate dans les synapses dépend directement de l'action de transporteurs spécifiques exprimés à la surface des astrocytes, en particulier les transporteurs de type GLT-1, qui retirent le neurotransmetteur et permettent ainsi de « nettoyer » la fente synaptique avant la survenue d'un nouvel épisode de neurotransmission.

Des études antérieures ont conclu qu'en raison d'un cycle de transport lent du glutamate, un large excès de transporteurs est nécessaire par rapport à la quantité de molécules de glutamate libérées dans la zone synaptique[1].Ces études ont suggéré que par la seule force du nombre, les transporteurs du glutamate peuvent ainsi lier efficacement toutes les molécules de glutamate et les transporter lentement à l'intérieur des astrocytes[1-3]. Ces travaux ont également conduit à l'hypothèse que les transporteurs du glutamate peuvent agir comme des filtres perméables à haute activité, ce qui signifie qu'une forte libération de glutamate à la synapse peut saturer ces transporteurs et conduire à l'activation des récepteurs extrasynaptiques, qui ont

de nombreux rôles physiologiques et pathologiques[4,5]. Récemment, ce point de vue a commencé à changer et de nouvelles découvertes ont suggéré que ce n'est pas seulement le niveau d'expression des transporteurs du glutamate, mais aussi leur localisation précise en particulier à proximité de la fente synaptique, qui permettent de contrôler la durée de l'activation des récepteurs neuronaux du glutamate[6-9].

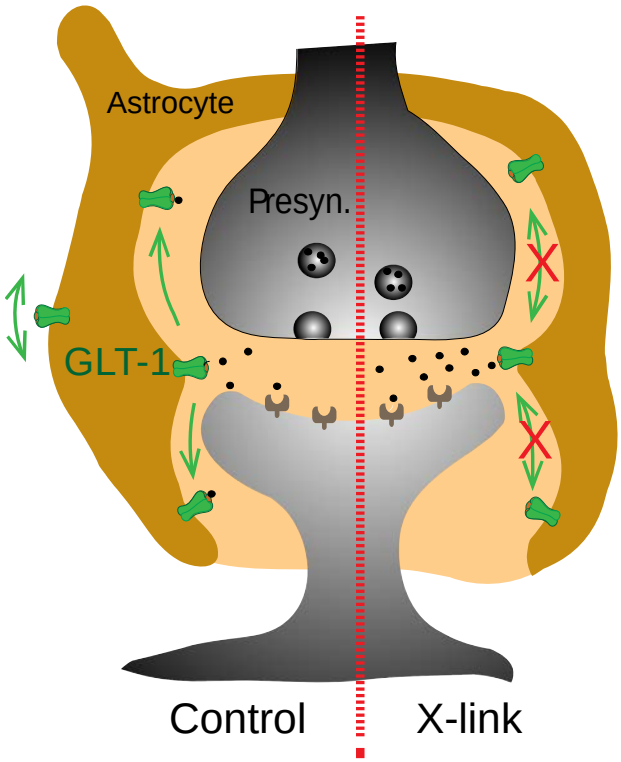
Ici, nous apportons des éléments nouveaux qui supportent cette hypothèse en démontrant que la mobilité latérale des transporteurs GLT-1 à la surface des astrocytes, plus que leur nombre, joue un rôle clé dans la clairance du glutamate de la fente synaptique sur des laps de temps courts. Pour parvenir à ce résultat, nous avons combiné l'utilisation de techniques de suivi de nanoparticules unique (*single nanoparticle tracking*), qui permettent de suivre la diffusion latérale des protéines de surface dans le plan membranaire dans et hors des zones synaptiques, et d'électrophysiologie afin de suivre l'activité synaptique.

Nos résultats suggèrent que c'est la diffusion de GLT-1 à la surface des astrocytes qui contribue essentiellement au tamponnage et à la recapture rapide du glutamate dans la fente synaptique. Nous avons montré que ces transporteurs ne sont pas fixes à la membrane, mais sont hautement dynamiques. Leur diffusion de surface est dépendante de l'activité, et est régulée à la fois par les cellules neuronales et gliales. Nous avons mimé la libération du glutamate neuronal par « décagage » de glutamate à proximité des synapses, et observé une augmentation de la diffusion de surface des GLT-1, entraînant le déplacement de ces transporteurs depuis la fente synaptique vers la zone extrasynaptique. Enfin, nous avons montré que

l'immobilisation artificielle des transporteurs GLT-1 par agrégation (« cross-linking ») a des conséquences fonctionnelles majeures, à la fois *in vitro* et *ex vivo* dans une préparation de tranches d'hippocampe, et entraîne un changement significatif de la cinétique des courants synaptiques. Des résultats similaires ont été obtenus en bloquant pharmacologiquement les transporteurs du glutamate. Ceci indique que la diffusion de surface des GLT-1 joue un rôle fonctionnel dans le contrôle de l'évolution temporelle du glutamate synaptique. Les changements cinétiques observés montrent une exposition prolongée des récepteurs neuronaux du glutamate au neurotransmetteur, soulignant ainsi le rôle essentiel de la diffusion de surface des GLT-1 dans le maintien de l'homéostasie synaptique du glutamate *in vitro* et *in vivo*. Ces données renforcent le concept de la synapse tripartite, qui veut que les astrocytes soient des partenaires actifs à la synapse et participent directement à la communication cérébrale.

Le trafic des récepteurs à la surface des neurones joue un rôle important dans le contrôle de leur distribution et de la signalisation synaptique, aussi bien au niveau physiologique que pathologique[10-12]. Il est donc possible que des altérations des mécanismes de régulation de la diffusion de surface des transporteurs du glutamate sur les astrocytes puissent aussi contribuer à l’apparition de pathologies neurologiques et psychiatriques. Il a précédemment été démontré que la perturbation des mécanismes de recapture du glutamate contribue à de nombreuses maladies neurodégénératives comme la sclérose latérale amyotrophique, l'épilepsie, la maladie d'Alzheimer ou la chorée de Huntington[13-16]. Ainsi, en plus d'améliorer notre compréhension de la contribution des transporteurs dans le contrôle du

glutamate à la synapse, ce mécanisme inattendu ouvre de nouvelles perspectives de recherche dans les domaines des troubles neurologiques et psychiatriques.



Représentation schématique du rôle de la diffusion latérale des transporteurs astrocytaires GLT-1 dans la recapture du glutamate synaptique. En condition normale (control, gauche), la diffusion latérale rapide des transporteurs GLT-1 à la surface des astrocytes facilite la recapture du glutamate et le nettoyage de la fente synaptique. En revanche, quand les transporteurs GLT-1 sont immobilisés (X-link, droite), la recapture du glutamate est plus lente, son temps de résidence synaptique est augmenté et la cinétique des courants synaptiques excitateurs est modifiée.

Mots clés:

Transporteurs du glutamate, recapture du glutamate, diffusion de surface, l'imagerie de nanoparticules unique, synapse tripartite, interactions neurone-glie

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7. Edu-GLIA mid-term review meeting, Slovenia, 24-27th August 2011.
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2. Murphy Royal C, Dupuis J, Pinson B, Baufreton J, Groc L, Oliet S, *Contribution of Astroglial GLT-1 surface dynamics to glutamatergic synaptic transmission*. Abstract and poster at the French society for neuroscience, Lyon, 21-24th May 2013.

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4. Murphy Royal C, Dupuis J, Pinson B, Baufreton J, Groc L, Oliet S, *Surface dynamics of the astrocytic glutamate transporter GLT-1*. Abstract and poster at the Society for Neuroscience, New Orleans, 13-17th October 2012.

5. Murphy Royal C, Groc L, Oliet SHR*. Surface trafficking of the astrocytic glutamate transporter GLT-1.* Abstract and poster at the 12th Doctoral school meeting of Bordeaux, Arcachon, 28th March 2012.

6. Murphy Royal C, Groc L, Oliet SHR*. Surface trafficking of the astrocytic glutamate transporter GLT-1.* Abstract and poster at ISN Satellite meeting 'Glial cells in (patho)physiology', Ljubljana, 24-27th August 2011.

7. Murphy Royal C, Groc L, Oliet SHR*. Surface trafficking of the astrocytic glutamate transporter GLT-1.* Abstract and poster at 10th meeting of the French society for Neuroscience, Marseille, 24-27th May 2011.

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Abbreviations

aCSF	artificial cerebrospinal fluid
ALS	amytrophic lateral sclerosis
AM dye	acetoxymethyl ester dye
AMPA (AMPAR)	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (receptor)
AP5	2-amino-5-phosphopentanoic acid
ATD	amino terminal domain
ATP	adenosine triphosphate
CA1-4	cornu ammonis regions 1-4 in hippocampus
Ca^{2+}	calcium
CaMKII	calcium/calmodulin kinase II
CNS	central nervous system
CTD	carboxy terminal domain
D	diffusion coefficient
d.p.m.	disintegrations per minute
DG	dentate gyrus
DIV	days *in vitro*
DNA	deoxyribonucleic acid
EAAT1-5	excitatory amino acid transporter (subtypes 1-5)
eGFP	enhanced green fluorescent protein
EM-CCD	electron multiplying charge coupled device
FITC	fluorescein isothiocyanate
GABA (GABAR)	gamma aminobutyric acid (receptor)
GAD	glutamate decarboxylase
GECI	genetically encoded calcium indicators
GLAST	glutamate aspartate transporter
Gln	glutamine
GLT-1	glutamate transporter-1
Glu	glutamate

GS	glutamine synthase
H^+	hydrogen (proton)
HBS	HEPES buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
K^+	potassium
KA (KAR)	Kainate (receptor)
LBD	ligand binding domain
LTD	long-term depression
LTP	long-term potentiation
MAP kinase	mitogen activated protein kinase
MCPG	α-methyl-4-carboxyphenylglycine
Mg^{2+}	magnesium
mGluR	metabotropic glutamate receptor
MSD	mean squared displacement
MSG	monosodium glutamate
Na^+	sodium
NBQX	2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonaminde
NMDA (NMDAR)	n-methyl-d-aspartate (receptor)
PAG	phosphate-activated glutaminase
P2X4	ligand gated purinoreceptor
PSD	postsynaptic density
QD	quantum dot
ROI	region of interest
SAT	system A transporter
Sc	Schafer collateral
sEPSC	spontaneous excitatory postsynaptic current
SN1	system N transporter
STP	short-term potentiation
T1R (1-3)	taste receptor (subtypes 1-3)
TBOA	threo-b-benzyloxyaspartic acid
TCA cycle	tricarboxylic acid cycle
TMD	transmembrane domain

TTX	tetrodotoxin
vGLUT	vesicular glutamate transporter

Introduction

1. Glutamate

Glutamate or glutamic acid is one of the 21 amino acids in the body. Its genetic codons in DNA are GAA and GAG. Glutamate is a non-essential amino acid, meaning that it can be synthesised by the body and therefore doesn't need to be provided in the diet. In 1953, Hans Adolf Krebs won the Nobel Prize in Physiology for his discovery that glutamate is a key compound in cellular metabolism serving as fuel for other biological processes. Catabolism of sugars, fats and amino acids, including glutamate, are all necessary for the production of energy in the citric acid cycle (also known as the TCA or Krebs cycle) which is the main form of aerobic energy production in the body (Krebs, 1935).

Glutamate, apart from being a vital amino acid in the body and brain, is also commonly used as a flavour enhancer in its salt form monosodium glutamate (MSG). This salt was discovered by Japanese scientist Kikunae Ikeda in 1908, upon tasting the salt he identified it as flavour found in many foods. A flavour he termed unami, translated as sweet or delicious. He then patented a method of mass-producing this salt for the food industry. Recently, it was discovered that the combination of T1R1 and T1R3 taste receptors in the tongue, which are distantly related to metabotropic glutamate receptors in the brain, are responsible for this flavour when glutamate is present in food (Nelson *et al.*, 2002). These receptors also responsible for recognising other amino acids in foods, this has been suggested to be a possible evolutionary advantage for animals, including humans, as we cannot produce all the necessary amino acids endogenously.

In the brain, glutamate plays a very important role as an excitatory neurotransmitter. Glutamate is stored in vesicles and can be released by electrical impulses which travel in neurons. This release of glutamate allows the transfer of information from one cell to another

and in this way glutamate can be involved in many processes in the brain such as learning and memory. However, glutamate in large concentrations can be toxic for neurons, therefore there are specific transporters to remove this glutamate from the extracellular space. These transporters are known as excitatory amino acid transporters (EAATs) of which there are several types, which have differential locations in the brain and are expressed in different cell types according to their functions. Other functions for glutamate in the brain include metabolism into a precursor for an inhibitory neurotransmitter gamma aminobutyric acid (GABA), by the protein glutamate decarboxylase (GAD).

1.1 Glutamate as a neurotransmitter

Arguably one of the most important roles of glutamate in the body is its role as a neurotransmitter in the brain. The first indications that glutamate may play a role as a neurotransmitter were observed in experiments whereby injection of glutamate into the brain or carotid arteries produced convulsions (Hayashi, 1954). Soon after, an independent laboratory found that glutamate was indeed a neurotransmitter and activated a wide range of neurons in the central nervous system (CNS) (Curtis *et al.*, 1960). Classically, for a substance to be accepted as a neurotransmitter it has to fulfil several criteria; first, this chemical has be present in the presynapse; second, it has to be released upon physiological stimulation of the cell; third, it has to have receptors on the target cell; and finally, there must be an innate mechanism to terminate the action of the transmitter. Glutamate falls comfortably into these criteria and is widely accepted to be the major excitatory neurotransmitter in the brain (Fonnum, 1984). This excitatory neurotransmitter plays roles in many physiological brain processes such as cognition, learning and memory. The concentration of glutamate is highly regulated to ensure that this amino acid can carry out its neurotransmitter function effectively. The brain contains a large amount of glutamate, estimated to be in the range of 5-15 mmol/kg (Danbolt, 2001). However, the concentration of

glutamate is not uniform in the brain, it has been demonstrated there are vast differences in its extracellular, cellular and sub-cellular locations. Extracellular levels glutamate are maintained at very low levels, with early reported values of around 4 μM (Hamberger and Nystrom, 1984). More recently, extracellular glutamate concentration in hippocampal slices has been estimated to be closer to 25 nM (Herman and Jahr, 2007). Indeed it is more plausible that the extracellular glutamate concentration is in the nM range, this concentration is low enough to avoid any significant glutamate receptor activation, while 4 μM would induce constitutive glutamate receptor activation and would likely have deleterious effects. Intracellular glutamate concentrations have been estimated at ~ 10 mM, a more than 10,000 fold difference (Burger *et al.*, 1989). It has also been suggested that glutamate transporters working under physiological conditions should be capable of establishing a concentration gradient of 10^6, equating to a glutamate concentration of 10 nM extracellularly and 10 mM inside the cell (Zerangue and Kavanaugh, 1996), this is in agreement with extracellular glutamate concentrations proposed by Herman and Jahr (2007). Sub-cellularly, glutamate has been estimated to be present at extremely high concentrations in synaptic vesicles, ~ 60 mM (Burger *et al.*, 1989; Shupliakov *et al.*, 1992).

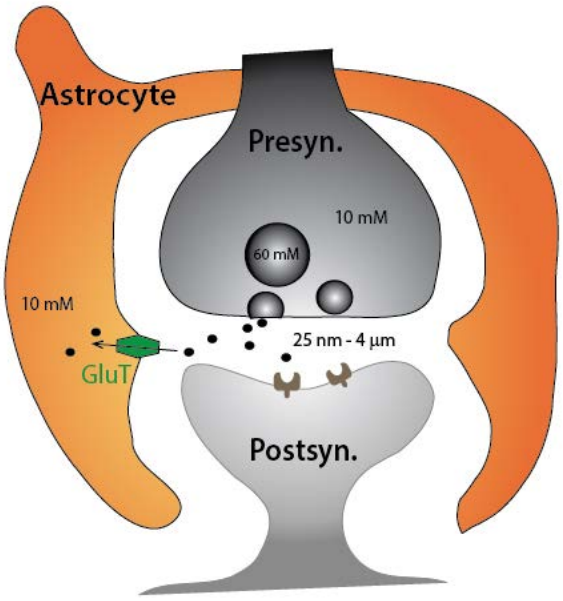


Figure 1.Extracellular, Intracellular and sub-cellular glutamate concentrations in the brain

Extracellular concentrations of glutamate are in the range of 25 nM – 4 μM (synaptic concentrations of glutamate can transiently rise to 1 mM for 1-2 ms following synaptic glutamate release), while intracellular concentrations are approximately 10 mM. Finally, synaptic vesicles, which express highly efficient vesicular glutamate transporters contain glutamate at concentrations up to 60 mM.

2. Glutamatergic neurotransmission

2.1 Glutamate receptors

Synaptic transmission is the basis of neuronal communication in the brain. Classically, neurons can transmit information in two ways, electrically and chemically. Beginning with the chemical signal, we observe the release of a neurotransmitter, such as glutamate, from the presynaptic cell. Glutamate then diffuses across the synaptic cleft where it acts upon glutamate receptors on the postsynaptic neuron. Glutamate binding to its specific receptors allows the influx of ions into the postsynaptic cell, resulting in depolarisation, i.e. a change in the cell's membrane potential towards more positive values. If this depolarisation is large enough, it may result in an action potential which is conducted through the neuron allowing information to pass from one cell to another. This is the classical view of neurotransmission. However, it has now been demonstrated that glutamate not only acts on postsynaptic receptors but can also carry out presynaptic functions, which can result in a change in the release probability of presynaptic vesicles (Oliet *et al.*, 2001).

This classical view of glutamate acting on receptors located solely on neurons has been updated, with recent evidence demonstrating that these receptors are not neuron-specific but are also expressed on glial cells (Gallo and Ghiani, 2000). Activation of glutamate receptors on astrocytes is still a topic of debate, with not only the existence of these receptors but also the consequence(s) of glutamate receptor activation on these cells remaining ambiguous. The neurotransmitter which is the focus of this thesis is glutamate, however there are many others such as GABA, dopamine and endocannabinoids which do not all follow the same rules as glutamate, regarding release, receptor activation and uptake/termination of action, and will not be mentioned further on.

As previously mentioned, one criterion for a substance to be accepted as a neurotransmitter is the presence of receptors on the target cell. Glutamate performs its role

as a signalling molecule by binding to receptors located on both neurons and glia. Glutamate receptors can be separated into two distinct families, ionotropic and metabotropic glutamate receptors.

2.1a. Metabotropic Glutamate Receptors

The first family of glutamate receptors I will describe are known as metabotropic glutamate receptors (mGluRs). These are G-protein coupled receptors of which there are 8 individual receptors (mGluR 1-8). mGluRs can be split into three main groups, which are based on their generalised location and functions (Niswender and Conn, 2010). Group I mGluRs, comprised of mGluR1 and 5, are coupled to G_q signalling. This group is predominantly expressed post-synaptically and activation results in phospholipase C, adenylyl cyclase as well as MAP kinase stimulation. Group II and III mGluRs are coupled to $G_{i/o}$ proteins involved in inhibitory signalling. Group II mGluRs, mGluR2 and 3, can be found both pre- and post-synaptically. Activation of Group II mGluRs results in inhibition of adenylyl cyclase, activation of K^+ channels and inhibition of Ca^{2+} channels. Finally Group III mGluRs, mGluR4 and 6-8, each expressed differentially in terms of synaptic location and brain region, result in inhibition of adenylyl cyclise activation of K^+ channels and inhibition of Ca^{2+} channels (see Table 1). It is widely accepted that astrocytes express both mGluR3 and mGluR5 (Arizono *et al.*, 2012; Panatier *et al.*, 2011; Sun *et al.*, 2013). Activation of these receptors by neuronal activity has been demonstrated to result in Ca^{2+} activity in astrocytes, therefore, these receptors are believed to play a functional role in neuron-glia communication (Panatier *et al.*, 2011).

Group	Receptor	CNS expression	Neuronal Localisation	Signalling pathways
Group I	mGluR1	Neuronal	Predominantly postsynaptic	• Stimulation of phospholipase C & adenylyl cyclase • MAP kinase phosphorylation
	mGluR5	Neuronal & Astrocytic		
Group II	mGluR2	Neuronal	Pre- & post-synaptic	• Inhibition of adenylyl cyclase • Activation of K^+ channels • Inhibition of Ca^{2+} channels
	mGluR3	Neuronal & Astrocytic		
Group III	mGluR4	Neuronal	Presynaptic	• Inhibition of adenylyl cyclase • Activation of K^+ channels • Inhibition of Ca^{2+} channels
	mGluR6	Retinal	Postsynaptic in ON-bipolar retinal cells	
	mGluR7	Neuronal	Presynaptic terminals	
	mGluR8	Neuronal	Presynaptic	

Table 1. Key features and differences between mGluRs. Adapted from (Niswender and Conn, 2010).

2.1b. Ionotropic Glutamate Receptors

There are 3 main ionotropic receptors, which mediate fast synaptic transmission, named after their affinities for exogenous agonists. It is possible to make some generalisations about this family of glutamate receptors; these receptors all form tetramers, thus four subunits are needed to combine to form a functional receptor; each subunit is composed of an extracellular amino-terminal domain (ATD) which participates in subtype specific receptor assembly, trafficking and modulation; a ligand binding domain (LBD); a

transmembrane domain (TMD) which forms the ion channel and a cytoplasmic carboxy-terminal domain (CTD) involved in receptor localisation and regulation (see Figure 1).

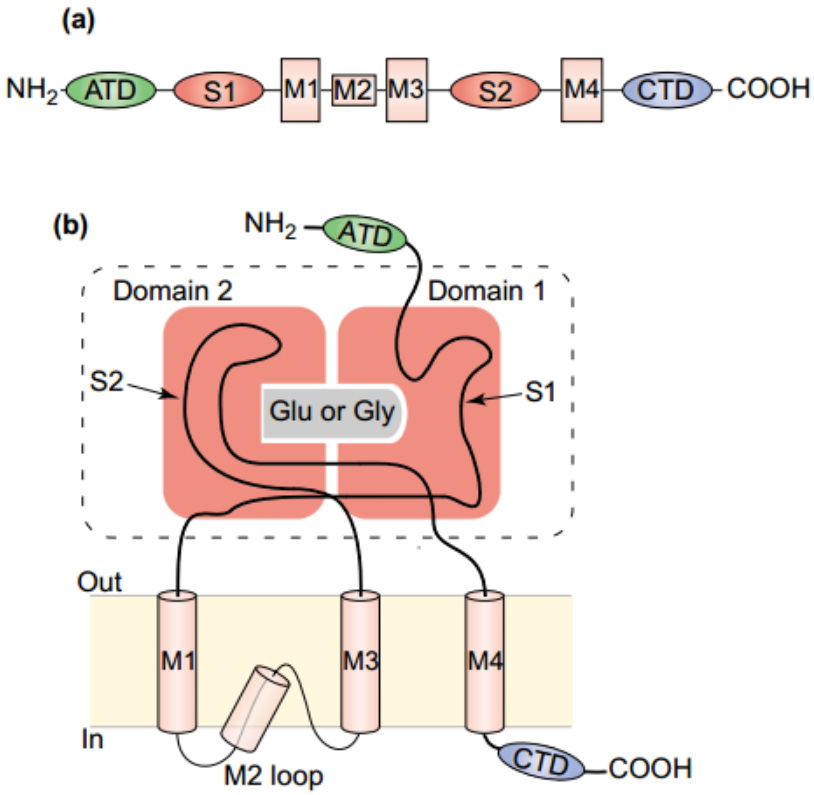


Figure 2. Generalisation of ionotropic glutamate receptor subunit

Adapted from (Wollmuth and Sobolevsky, 2004). **(a)** Overview of a glutamate receptor subunit. We can observe the N-terminal domain (ATD), S1 and S2 lobes forming the ligand/co-agonist binding domain (LBD), 4 hydrophobic domains M1-M4 and the C-terminal domain (CTD). **(b)** Membrane topology of a glutamate receptor subunit.

The first ionotropic glutamate receptor I will describe is the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) which is expressed on neurons and potentially on astrocytes. On neurons AMPARs are expressed post-synaptically. When activated, by pre-synaptically released glutamate, these receptors allow the influx of mainly Na^+ and K^+ (as well as Ca^{2+} to a lower extent) into the cell which may result in depolarisation

of the post-synaptic neuron. AMPARs are tetramers composed of subunits (GluA1-4), and generally functional AMPARs are composed of a dimer of dimers. Activation, deactivation and desensitisation of these receptors occurs on a millisecond timescale (Hansen *et al.*, 2007), thus these receptors are responsible for fast synaptic transmission on neurons.

On astrocytes, the expression and role of AMPARs is not so well defined. Evidence from studies of cultured hippocampal astrocytes has demonstrated that AMPARs on astrocytes have a much higher Ca^{2+} permeability (Fan et al., 1999). As such, it is believed that activation of these receptors on astrocytes leads to activation of Ca^{2+}-dependent processes such as release of 'gliotransmitters' (glutamate, ATP, D-serine etc.) and the resulting modification of synaptic transmission (Mothet *et al.*, 2005). These receptors could also act as a neuronal activity sensor, allowing high-speed communication between these different brain cells.

Next, I will introduce the N-methyl-D-aspartate receptor (NMDAR) which is also expressed on neurons and possibly on astrocytes. On neurons these receptors, like AMPARs, are located post-synaptically and are also responsible for fast excitatory neurotransmission. NMDARs are also composed of subunits which combine in hetero-tetramers to form a functional ion channel, these subunits are GluN1, GluN2A-D and GluN3A,B. Functional receptors are usually composed of two GluN1 and two GluN2 subunits. However, unlike AMPARs, NMDAR kinetics are much slower with activation and deactivation occurring on tens to hundreds of milliseconds (Forsythe and Westbrook, 1988). Furthermore, in contrast to AMPARs, NMDARs need not only glutamate to be activated but are also voltage-dependent, i.e. the cell needs to be depolarised before NMDAR activation occurs. This is due to the presence of a Mg^{2+} ion in the pore of NMDARs which blocks the ion channel at resting membrane potentials (-70 mV in neurons). Following activation of AMPARs, neurons can become depolarised, raising the membrane potential which can result in the removal of the Mg^{2+} ion from the NMDAR, if glutamate is present in these conditions this results in the influx of Ca^{2+} and Na^{+} ions through the NMDAR and into the

cell. Activation of NMDARs is further complicated by the necessity of simultaneous binding of glutamate as well as a co-agonist. GluN2 subunits bind glutamate, while GluN1 subunits are the locus for the co-agonist binding site. Both glycine and D-serine can bind to the co-agonist site and it has been recently shown that D-serine, provided by astrocytes (Mothet et al., 2005; Panatier et al., 2006), is the co-agonist at synaptic sites while glycine is predominant in extrasynaptic areas (Papouin *et al.*, 2012).

Due to the Mg^{2+} ion blocking the NMDAR pore, which can only be removed through depolarisation and the fact that astrocytes are not electrically excitable cells, it was believed that functional NMDARs could not be expressed on astrocytes. Only recently, this belief has been challenged, with evidence suggesting that not only are these receptors present but functional on astrocytes (Lalo *et al.*, 2006). Furthermore, this study by Lalo and colleagues (2006) demonstrated that NMDARs on astrocytes seem to lack sensitivity to Mg^{2+}, indicating that these receptors can be functional at astrocytic resting membrane potentials of -80 mV. The precise role and expression pattern of NMDARs on astrocytes in different brain regions remains unknown.

Finally, it is worth mentioning kainate receptors (KARs). These receptors resemble AMPARs however their function and contribution to glutamatergic signalling is less understood compared to its previously mentioned counterparts. There are 5 subunits of KARs (GluK1-5) which can combine to form tetramers. However, for the KAR to be functional it is necessary to include at least one of the GluK1-3 subunits. KARs were first believed to be solely involved in pathology such as epilepsy (Ben-Ari, 1985). It is now believed that KARs play a more physiological role in synaptic integration in specific hippocampal circuits (Pinheiro *et al.*, 2013). As the precise role of KARs are quite ambiguous, I will omit these receptors from further discussion in this thesis. Furthermore, the evidence for the expression of KARs on astrocytes has yet to be demonstrated.

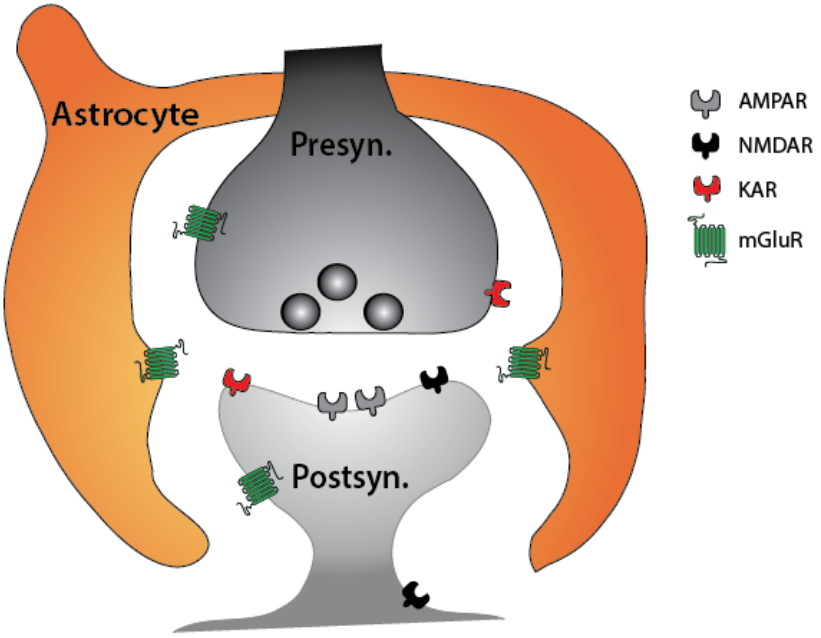


Figure 3. Locations of glutamate receptors on neurons and astrocytes

AMPARs, NMDARs and mGluRs have only been included on cell types where their function is known. Thus neurons express AMPA, NMDA, KA and mGlu receptors postsynaptically, while expressing KA and mGlu receptors presynaptically. On astrocytes only mGluRs are shown due to the controversial evidence and unknown function of AMPAR, NMDA and KA receptors on these cells.

2.2 Synaptic plasticity in the hippocampus

Synaptic transmission, as previously stated, is the chemical communication between two neurons across a synapse. This form of communication is highly dynamic, with changes in the strength of communication believed to be associated with many physiological processes such as learning and memory. These dynamic changes in the strength of transmission are known as synaptic plasticity. The idea of synaptic plasticity was proposed by Donald Hebb in 1949. Hebb proposed a coincidence detection rule whereby the synaptic communication between two neurons could be strengthened if both neurons are activated at the same time (Hebb, 1949). Hebb hypothesised that this form of synaptic plasticity could be

the substrate for learning and memory in the brain, this theory became known as Hebbian learning. It was not until many years later that Hebb's idea of synaptic plasticity was proven to be true *in vivo*. Several revolutionary papers were published in the 1970's demonstrating the existence of synaptic plasticity in the brain.

First, Eric Kandel's group published papers demonstrating synaptic correlates of simple learning paradigms, habituation and sensitisation, in the sea slug *Aplasia* (Castellucci *et al.*, 1970; Kupfermann *et al.*, 1970; Pinsker *et al.*, 1970). These findings were postulated to be the first evidence of synaptic plasticity in monosynaptic circuits. Swiftly following Kandel's group, two revolutionary papers from Tim Bliss's lab demonstrated the first evidence of long-term synaptic plasticity in anaesthetised (Bliss and Lomo, 1973) and un-anaesthetised (Bliss and Gardner-Medwin, 1973) rabbits. By stimulating neurons using a high frequency stimulation protocol Bliss and colleagues (1973) observed a long-term increase in the strength of the post-synaptic response which lasted for many hours. (On a side note, an anecdote from a review article by Bliss and colleagues (Bliss *et al.*, 2003), highlights the evolution of modern science which has become a race to get to the finish line. The experiments which lead to publication of the 1973 papers demonstrating LTP were carried out and completed several years earlier, in this almost forgotten era, scientists had the luxury of spending a lot more time pondering the relevance of these important scientific discoveries). Since this initial biological demonstration of synaptic plasticity many other forms of synaptic plasticity have been demonstrated, both *in vivo* and *in vitro*, these different forms of plasticity vary in terms of induction, duration and molecular mechanisms.

This section will focus only on hippocampal forms of synaptic plasticity although the mechanisms which induce and maintain these different forms of glutamatergic synaptic plasticity are rather ubiquitous in the brain. Synaptic plasticity has been extensively studied in the brain but no area has been subject to more scrutiny than the hippocampus. The hippocampus is one of the most extensively studied brain regions in contemporary neuroscience, as such, much is known about its circuitry and signalling (see Figure 4). The

high level of interest in this particular brain structure is due to the profound importance of the hippocampus for learning and memory. This was discovered, by chance, by the neurosurgeon William Scoville after he performed experimental surgery to alleviate epileptic seizures in a patient known by his initials H.M (Scoville and Milner, 1957). This surgery involved bilateral hippocampus removal, following this patient H.M. suffered from anterograde amnesia, meaning he could no longer form new memories. Further psychological testing demonstrated that not all forms of memory were affected by bilateral hippocampus removal; only semantic memory formation was abolished while patient H.M. was still capable of learning with respect to other forms of memory such as motor memory. Much of our current knowledge about the brain would not have been possible without the cooperation of patient H.M. over many years. Patient H.M. died in 2008 which lead to the release of his real name, Henry Molaison. Even after his death, Henry Molaison continues to add to our understanding of the brain. Recent work, which histologically sliced through the entirety of Molaison's post-mortem brain, indicates that a significant proportion of the hippocampus was intact in both hemispheres (Annese *et al.*, 2014). This finding raises the question of the functional viability of the remaining hippocampal tissue.

Although no experimental work has been carried out on synaptic plasticity during this thesis, we have done some electrophysiology, therefore I will briefly describe the three most exhaustively studied forms of synaptic plasticity which are; short-term potentiation, long-term potentiation and long-term depression without going into too much detail regarding these processes.

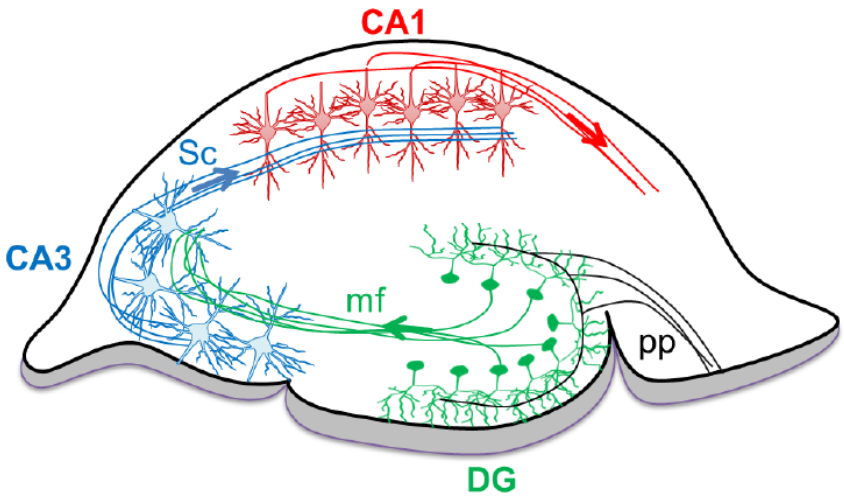


Figure 4. The hippocampal circuit.

This diagram indicates the unidirectional signalling of the hippocampus. Afferent innervation of the hippocampus arrives from the perforant pathway, into the dentate gyrus. This innervates cells in the CA3, which in turn innervates cells in the CA1. pp: perforant pathway. DG: dentate gyrus. mf: mossy fibres. CA; cornu ammonis. Sc: schafer collaterals. Adapted from (Hilal, 2013)

2.2a. Short-Term Plasticity

Short-term plasticity in the hippocampus is believed to be a presynaptic phenomenon. This form of plasticity involves modifications in the probability of release of a synaptic vesicle from the presynaptic bouton which lasts for minutes at most, hence the name short-term plasticity. When considering short-term plasticity, we must be familiar with the processes involved. Short-term plasticity is based on changes in the probability of neurotransmitter release (*p*). This probability of neurotransmitter release is also related to the amount of vesicles which can be release at any one time. The smallest synaptic event, which is known as a *quantal* event (*q*), is the equivalent of the release of a single vesicle. It is believed that there are several pools of vesicles in any given presynaptic bouton, these pools can be categorised in readily releasable pool and the reserve pool. Short-term

plasticity can affect the amount of vesicles in the readily releasable pool thereby modifying the potential amount of neurotransmitter which can be released following stimulation. Theoretical models of presynaptic release have been proposed, which explain the relationship between these different elements (Hennig, 2013). The following equation demonstrates that the amount of neurotransmitter (*T)* released is a function of the probability of release (*p)* and the amount of vesicles in the readily releasable pool (*N).* As changes in the probability of neurotransmitter release are generally activity-dependent, each component of this equation is expressed as a function of time (t);

$$T(t) = p(t) \cdot N(t)$$

For a more comprehensive model of presynaptic short term plasticity see Hennig, 2013. Thus, short-term plasticity can result from a change in the probability of neurotransmitter release due to high levels of intracellular Ca^{2+} needed for vesicle release, or even changes in the readily releasable pool of vesicles. A change in any of these factors, individually or in combination, will result in a change in the overall amount of neurotransmitter released (*T*).

2.2b. Long-Term Plasticity

Long-term plasticity, believed to be the cellular engram of learning and memory, resembles Donald Hebb's early predictions of synaptic plasticity. Hebb predicted that memories could be stored in a synapse when there is a long lasting change in the strength of communication between neuron A and neuron B (Hebb, 1949). As previously mentioned, proof of this principle *in vivo* was published in 1973 (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973) and since then countless papers have reproduced this synaptic phenomenon.

LTP is believed to be the cellular substrate for learning and memory throughout the brain. As such, LTP in the hippocampus, an area immensely important in learning and

contextual memory, has been one of the most widely studied phenomena in the brain. One of the major reasons why this is the case is owed to the extremely robust expression of this form of synaptic plasticity. It is quite simple to induce LTP *in vitro* and *in vivo*, using high frequency stimulation, thus many laboratories use this cellular phenomenon to compare the effects of drugs or disease states etc. (Bliss and Collingridge, 1993). In this review, Bliss and Collingridge (1993) aptly describe LTP as:

"Activity-dependent synaptic potentiation occurs within milliseconds and can persist for many hours in the anaesthetised animal or in the in vitro slice preparation, and for days when induced in the freely moving animal"

There are different mechanisms which can lead to LTP induction and the maintenance of this potentiation. Here I will mention two mechanisms which are NMDAR-dependent and –independent. Beginning with the NMDAR-dependent pathway, one can assume from the name that the activation of NMDA glutamate receptors is necessary to induce this form of LTP. As previously mentioned, NMDAR are peculiar receptors needing both agonist and co-agonist binding sites to be occupied as well as the simultaneous depolarisation of the cell in order to remove the Mg^{2+} molecule blocking the pore. It is through these stringent conditions that NMDARs are perfectly adapted as a coincidence detection receptor only allowing LTP to occur when there is simultaneous activation of both pre- and post-synaptic elements.

Induction protocols for LTP generally require high frequency stimulation which induces high levels of pre-synaptic glutamate release activating both AMPARs and NMDARs (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). Following the activation of NMDARs in these conditions, there is a great influx of Ca^{2+} into the post-synaptic cell through the NMDAR. This leads to the activation of intracellular signalling pathways, including activation of calcium/calmodulin-dependent kinase II (CaMKII) among other secondary messenger molecules producing changes in protein phosphorylation and gene

expression, resulting in a long-term potentiation of the postsynaptic response to stimulation (Lynch, 2004).

On the other hand, there is an NMDAR-independent pathway which involves the insertion of AMPARs into the postsynaptic density (Malinow and Malenka, 2002). It has been suggested that there are many synapses lacking AMPARs, these synapses, which still contain NMDARs are known as 'silent' synapses. It has been demonstrated that protocols used to induce LTP in experimental conditions are capable of inducing the trafficking of AMPARs to the synapse (Liao *et al.*, 1995). Therefore, synapses which were once silent can now respond to presynaptic glutamate release, resulting in synaptic transmission.

In comparison to LTP, long-term depression (LTD) is induced by low frequency stimulation resulting in low levels of Ca^{2+} entry into the postsynaptic cell. This low level of Ca^{2+} entry will bind to proteins which have a higher affinity for Ca^{2+} than CaMKII such as calcineurin, which signal in different pathways, compared to those involved in LTP, inducing a functional decrease in the postsynaptic response to further stimulation (Mulkey *et al.*, 1994). It has also been suggested that LTD works in the opposite manner of LTP, effectively reducing the synaptic content of AMPARs (Carroll *et al.*, 2001; Groc *et al.*, 2004). As a consequence of the removal of synaptic AMPARs, and other mechanisms involved in LTD, the postsynaptic response to further glutamate release will be attenuated.

2.3 Contribution of surface trafficking to synaptic transmission

An important point alluded to in the previous section is that there are activity-dependent changes in the surface content and localisation of receptors on neurons (Carroll *et al.*, 2001; Groc *et al.*, 2004; Heine *et al.*, 2008). It is taken for granted that un-silencing of synapses involves the insertion of AMPARs into the synaptic membrane (Liao *et al.*, 1995). It has been suggested that this occurs through exocytosis of the protein into the membrane but

it remained unclear whether this exocytosis occurred directly in the synapse or outside of the synapse. The post-synaptic density (PSD) is a protein rich area, there is an extremely high concentration of scaffold proteins, which bind and stabilise neurotransmitter receptors, as well as many different signalling proteins at the synapse. It has been demonstrated that indeed, as predicted, both exo- and endo-cytosis occur in specific domains located outside of the PSD (Kennedy *et al.*, 2010; Smith *et al.*, 2012). Smith and colleagues (2012), state that surface receptors can become stabilised in these endocytotic zones before internalisation. Conversely, Kennedy and colleagues (2010) focused on exocytosis, they stated that proteins are inserted into the membrane in specific domains outside of the PSD. Down-regulation of specific proteins involved in this receptor exocytosis resulted in an impairment of LTP revealing the role of receptor insertion in synaptic plasticity (Kennedy *et al.*, 2010). These findings are in agreement with earlier findings demonstrating the insertion/removal of receptors from the synapse contributing to synaptic plasticity. However, the reader is left wondering how these receptors move to the synapse from where they are inserted into the membrane and, *vice versa*, from the synapse to the endocytic zones.

Recent findings add another dimension of complexity to this process, demonstrating that many of these proteins, particularly receptors, are highly mobile on the surface of the cell. Through thermodynamic, Brownian diffusion these proteins move along the surface of the cell from one area to another, while experiencing varying levels of confinement in specialised areas, e.g. the synaptic area, where they can become transiently 'trapped'. Receptors such as AMPARs, NMDARs and GABARs are subject to this surface diffusion (Groc *et al.*, 2007; Heine *et al.*, 2008; Muir *et al.*, 2010). In recent years, a lot of effort has been devoted to uncover the molecular mechanisms by which proteins move (and stop) on the cell membrane. Lateral diffusion of receptors has been the primary focus of many laboratories for the past decade. Research from a handful of teams has divulged a wealth of knowledge regarding the regulation and consequence of surface diffusion of receptors on the surface of neurons. These teams have demonstrated that there are in fact very few

receptors that do not move on the surface of neurons and glia, including but not limited to AMPARs (Bats *et al.*, 2007; Groc *et al.*, 2008; Heine *et al.*, 2008), NMDARs (Bard *et al.*, 2010; Dupuis *et al.*, 2014; Groc *et al.*, 2007; Groc *et al.*, 2006), GABARs (Jacob *et al.*, 2005; Muir *et al.*, 2010; Smith *et al.*, 2012), mGluRs (Arizono *et al.*, 2012), P2X4 receptors (Toulme and Khakh, 2012) and dopamine receptors (Ladepeche *et al.*, 2013b; Porras *et al.*, 2012). Surface diffusion of these receptors is stringently regulated by many different factors such as binding with intracellular scaffold proteins (Bard *et al.*, 2010; Bats *et al.*, 2007; Porras *et al.*, 2012), interaction with other surface proteins and receptors (Ladepeche *et al.*, 2013a; Mikasova *et al.*, 2012; Muir *et al.*, 2010) as well as confinement due to extracellular matrix proteins (Groc *et al.*, 2007; Michaluk *et al.*, 2009). It is now known that surface diffusion of receptors is an activity-dependent process and plays a strong role in synaptic plasticity (Frischknecht *et al.*, 2009; Heine *et al.*, 2008; Krugers *et al.*, 2010). The checks and balances put in place to regulate surface diffusion of proteins are rather fragile and it has been observed that surface diffusion can be affected by physiological processes such as stress (Groc *et al.*, 2008). Furthermore, as our technology and understanding of the mechanisms regulating surface diffusion of receptors continues, it is becoming increasingly clear that these mechanisms go awry in certain pathological conditions (Mikasova *et al.*, 2012).

2.4 Astrocytes and synaptic transmission

The role played by astrocytes in synaptic transmission in the brain must not be overlooked. The astroglial biology field, which developed from a small cult following, is now one of the fastest growing fields in neuroscience as researchers realise the important contribution of these cells to neuronal activity. Astrocytes were first believed to serve only as support cells in the brain, providing a metabolic and structural scaffold upon which neurons could grow. These cells were overlooked for many years due to the difficulty in stimulating

and recording activity from these cells. Neuronal activity is electrical, thus quite easy to record whereas astrocytes communicate through changes in intracellular Ca^{2+} (Cornell-Bell *et al.*, 1990). Furthermore, until recently, neuroscientists were pouring the majority of their energy into the development of new technologies to investigate neuronal function. Thus, we find ourselves in a sort of predicament where we are using tools, developed to study neurons, in order to study a completely different cell type. Take for example one of the biggest technological advances of recent neuroscience, optogenetics (Aravanis *et al.*, 2007; Boyden *et al.*, 2005). This tool is being used to excite and well as inhibit neurons using genetically encoded light-sensitive ion channels. This technology has now been successfully transferred to astrocytes and is employed to induce intracellular Ca^{2+} release on a large scale (Gourine *et al.*, 2010). This technology will certainly increase our current understanding of astrocyte biology but whether this gross manipulation of intracellular Ca^{2+} is physiologically relevant in astrocytes remains to be seen.

One major difference in neuron versus glial activation is that neuronal firing is an all or none event, whereas astrocytes can have changes in intracellular Ca^{2+} levels which can occur independently in the fine processes as well as whole cell (Di Castro *et al.*, 2011; Panatier *et al.*, 2011). These intracellular Ca^{2+} increases can spread as calcium waves throughout the astrocytic syncytium (Charles *et al.*, 1991; Newman and Zahs, 1997). The precise role of these small Ca^{2+} transients as opposed to large Ca^{2+} waves which spread throughout the whole astrocyte is unknown. It is a fact that many stimuli, such as activation of excitatory glutamate receptors or even inhibitory GABA receptors on astrocytes leads to an increase in Ca^{2+} (Haustein *et al.*, 2014). This leads us to believe that astrocytes are unable to distinguish between excitatory and inhibitory inputs, which is very counterintuitive. One would assume that astrocytes, with each individual cell contacting hundreds of thousands of synapses in rodent brains and millions of synapses in the human brain, should be able to sense differences in GABAergic and glutamatergic signalling and respond accordingly. Therefore, it is possible that a lot of information is communicated in these Ca^{2+}

signals that we are not able to decipher using current techniques; perhaps the intensity and/or the propagation of the intracellular Ca^{2+} signal encodes information rather than a simple on/off switch with each event encoding the same message; it is also possible that Ca^{2+} signalling in astrocytes resembles a sort of LTP/LTD process which occurs in neurons, whereby small Ca^{2+} events may activate proteins which have a high affinity for Ca^{2+} whereas large Ca^{2+} transients could activate low affinity proteins resulting in the activation of distinct pathways. If one day we can listen to astrocytic Ca^{2+} activity and translate it into an intelligible 'language' of sorts, we will surely uncover a highly complex system of astrocytic communication and gather a wealth of information about astrocytic function in physiology and pathology. Research attempting to decode the information stored in astrocytic Ca^{2+} transients is well under way. A recent paper using genetically encoded calcium indicators (GECIs) highlights just how little we know about Ca^{2+} signalling in astrocytes and the current constraints in imaging astrocytic Ca^{2+} activity let alone determination of the functional consequences of this activity (Haustein *et al.*, 2014).

Following the initial discovery that astrocytes signal through changes in intracellular calcium, researchers set about exploiting this signalling mechanism to better understand these cells. A lot of progress has been made in the glial biology field, uncovering the role of these cells in many processes which were believed to be purely neuronal. It is now widely accepted by both astro- and neuro-centric researchers that astrocytes contribute strongly to synaptic transmission and plasticity. This important development came when it was demonstrated that; astrocytes are implicitly involved in the regulation of extracellular levels of glutamate (Oliet *et al.*, 2001; Rothstein *et al.*, 1996); astrocytes can release neurotransmitters such as ATP (Gordon *et al.*, 2005; Panatier *et al.*, 2011) and glutamate (Araque *et al.*, 2000; Jourdain *et al.*, 2007; Parpura and Haydon, 2000) thereby modifying neuronal activity; as well as the important observation the D-serine, the NMDA receptor co-agonist is released by astrocytes (Mothet *et al.*, 2000; Panatier *et al.*, 2006; Papouin *et al.*, 2012). Breakthrough papers demonstrated that LTP, the cellular substrate for learning and

memory in the brain, was dependent on astrocytic signalling, thus implicating these so-called supporting cells in synaptic plasticity (Henneberger *et al.*, 2010; Panatier *et al.*, 2006). These observations, among many others, fundamentally changed the field of neuroscience; the dogma that a synapse is composed of simply a pre- and post-synaptic element had to be revised and now the synapse is considered to be a tripartite structure. On a side note, there is increasing evidence that microglia, the immune cells of the brain, play a role in synaptic activity and development (Kettenmann *et al.*, 2013). Momentum is now growing for further revision of the concept of a synapse to include other glial cells into this structure. Convincing evidence has been put forward to include microglia in synaptic communication in what has been coined the 'quad-partite' synapse (Schafer *et al.*, 2013). A study, published this year, has demonstrated that myelin coverage of neurons by Oligodendrocytes in the cortex in not uniform, as formerly assumed, but more intermittent especially in higher cortical layers II/III, compared to deeper layers of the cortex (Tomassy *et al.*, 2014). The functional significance of these findings are unknown but this study calls into question the dynamic role of Oligodendrocytes in the control, or perhaps synchronisation, of neuronal activity as impulse propagation speeds are a function of myelin coverage of axons.

This tripartite structure is important for maintaining and synchronising communication between neurons and astrocytes. It has been demonstrated that astrocytic coverage of the synapse impacts upon presynaptic neurotransmitter release by affecting the level of presynaptic glutamate receptor activation (Oliet *et al.*, 2001). The majority of synapses are ensheathed by astrocytic processes; although the overall level of coverage is believed to vary between brain regions (Ventura and Harris, 1999). As suggested by Fonnum (1984), one other criterion for defining a substance as a neurotransmitter is that there must be an endogenous mechanism to terminate the action of the neurotransmitter. This mechanism in the mammalian brain is carried out by a family of glutamate transporters, which will be the focus of the remainder of the introduction.

3. Glutamate Transporters

As previously elucidated, the concentration of glutamate in the brain must be tightly regulated so that receptors do not become constitutively activated, which would lead to excitotoxic cell death. As there is no endogenous extracellular enzyme to degrade glutamate, this neurotransmitter is removed by a family of glutamate transporters in the brain. These transporters, known as Excitatory Amino Acid Transporters (EAATs) are located on neurons and glia, each has its own individual role depending on brain area and cellular location. It is believed that glutamate transporters on astrocytes play the most important role in removing extracellular glutamate (Danbolt, 2001; Rothstein *et al.*, 1996). Once glutamate is removed from the extracellular space by astrocytes, this glutamate can be converted into glutamine, in an energy-dependent process, by the glial specific enzyme glutamine synthetase (see Danbolt, 2001). System N glutamine transporters (SN1) located on the astrocytic membrane export this physiologically inactive amino acid into the extracellular space where it can be taken up by neurons which express the system A transporters (SAT1). Once inside the neurons it is believed that glutamine is reconverted to glutamate, in an energy-independent process by phosphate activated glutaminase (PAG). Finally, glutamate is packaged into vesicles in the presynapse but vesicular glutamate transporters (vGLUT) thus completing the glutamate – glutamine cycle (see Figure 5).

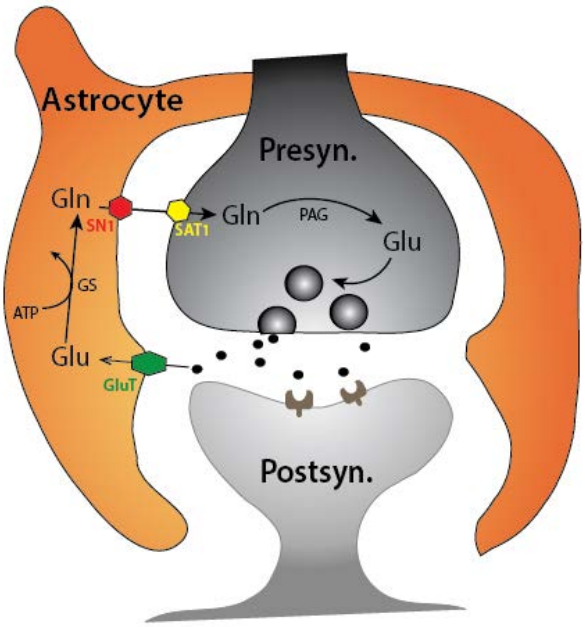


Figure 5. Role of astrocytic glutamate transporters in removal and recycling of synaptic glutamate.

The majority of glutamate uptake in the hippocampus is accomplished by glutamate transporters (GluT) on astrocytes. Once inside the astrocytes, glutamate (Glu) can be converted in to glutamine (Gln), an ATP-dependent process involving glutamine synthase (GS). Glutamine is then transported out of the astrocyte by SN1 transporters and taken up by neurons by SAT1 transporters. Gln is then converted back into Glu and packaged into vesicles.

3.1 Glutamate transporter subtypes

Glutamate transporters were originally referred to as 'sodium-dependent high affinity transporters'. However, their affinities are not extraordinarily high, and depending on the brain preparation used, Km values have been estimated to be anywhere between 1 – 100 μM. Also, these transporters do not depend solely on sodium, but also use the energy stored in potassium gradients to facilitate transport (Wadiche *et al.*, 1995; Zerangue and Kavanaugh, 1996). Thus, these transporters are now referred to as either 'sodium/potassium coupled glutamate transporters' or 'excitatory amino acid transporters'. To date, five transporter subtypes have been identified, through cloning of the transporter from rodent, rabbit and human tissue. The 5 human transporters (and their rodent or rabbit homologues) are: EAAT1/GLAST (Storck *et al.*, 1992), EAAT2/GLT-1 (Pines *et al.*, 1992), EAAT3/EAAC1 (Kanai and Hediger, 1992), EAAT4 (Fairman *et al.*, 1995) and EAAT5 (Arriza *et al.*, 1997). Comparison of cDNA sequences for these human transporters has shown that they share over 90% sequence identity with their rodent/rabbit homologues, indicating that these

transporters are highly conserved between species (Arriza *et al.*, 1994). In addition to these 5 main transporters, splice variants of individual transporters have been identified, with at least 3 splice variants of GLT-1 being known. Nomenclature for these splice variants was not always agreed upon and this makes for a lot of confusion when reading the literature. GLT-1a, which is the original GLT-1 cloned by Pines and colleagues (1992) was later identified in rats by independent laboratories and named GLT-1a (Chen *et al.*, 2002) and GLT-1alpha (Reye *et al.*, 2002). GLT-1b, a C- terminal splice variant of the original GLT-1 was identified by Chen and colleagues (2002) as well as another laboratory which used the name GLT-1v for variant (Schmitt *et al.*, 2002). There exists a third splice variant of GLT-1, GLT-1c which is developmentally expressed in photoreceptor cells in the retina (Rauen *et al.*, 2004).

3.2 Mechanism of glutamate transport

Early studies showed that glutamate transporters use the energy stored in Na^+ and K^+ concentration gradients to move glutamate across the membrane (Kanner and Sharon, 1978). The Na^+ and K^+ concentration gradients are maintained by ATPase pumps so a lot of energy is used by the brain just to keep these gradients intact. Every glutamate molecule transported into the cell is associated with the co-transport of three Na^+ and one H^+ ion, followed by the counter-transport of one K^+ ion (Wadiche *et al.*, 1995; Zerangue and Kavanaugh, 1996). Therefore, with each transport cycle is associated with a net positive charge across the membrane. It should also be mentioned that transporters can work in the opposite direction. This can occur in pathologies such as hypoxia where there is not enough energy to maintain high ionic concentration gradients across the membrane and transporters begin to release glutamate as well as other ions (Swanson *et al.*, 1995).

The kinetics of glutamate transporters are quite complex, involving binding of several different molecules and conformational change of the transporter. One complete glutamate transport cycle has been estimated last about 70 ms (Otis and Jahr, 1998; Wadiche *et al.*,

1995) (see Figure 6). Each transport cycle commences with the binding of Na^+, H^+ and glutamate, followed by the conformational change in the transporter to an inward facing state. These transported molecules are then released before the binding of K^+, followed by structural reorganisation of the transporter to an outward facing state before K^+ is released into the extracellular space completing the cycle (Wadiche *et al.*, 1995).

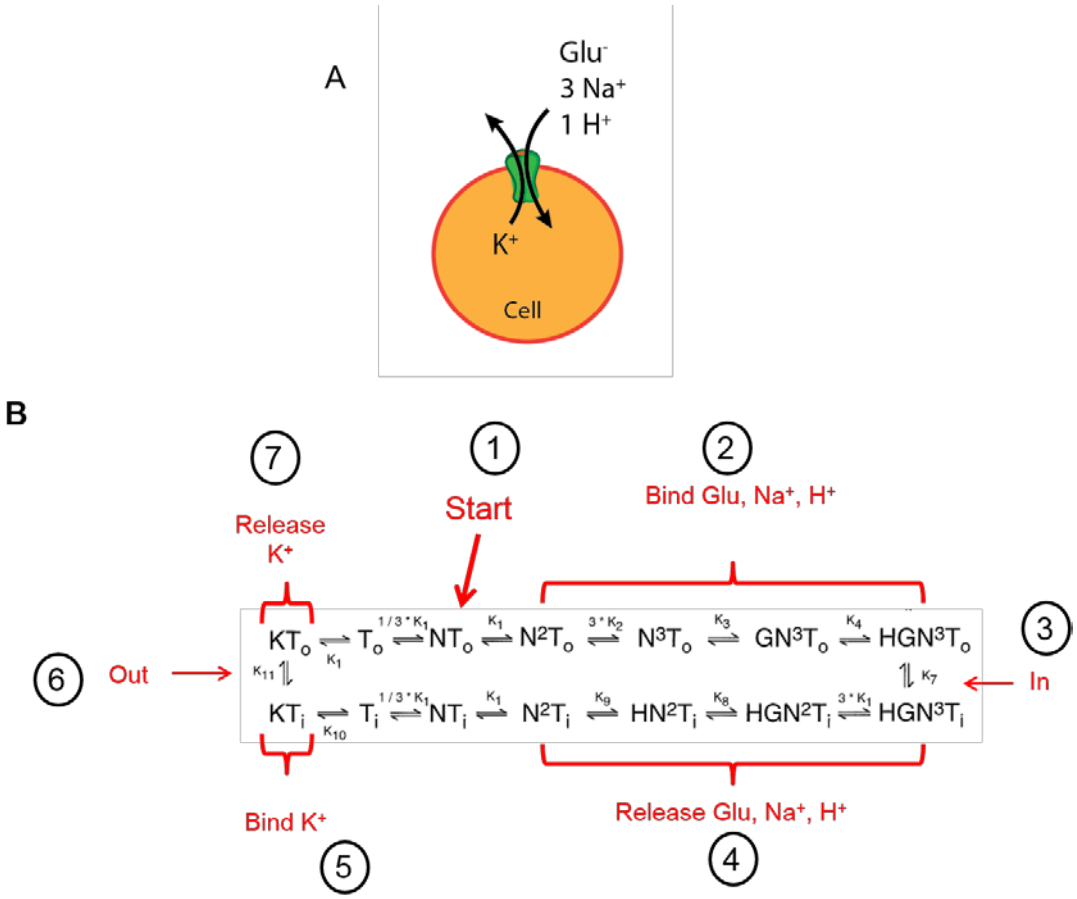


Figure 6. Stoichiometry and kinetics of glutamate transport

(A) Stoichiometry of glutamate transporters. Glutamate transport uses the energy stored in Na^+ and K^+ concentration gradients to remove glutamate from the extracellular space. **(B)** Kinetics of the human glutamate transporter (EAAT2) adapted from Wadiche *et al.* (1995). Transport cycle begins with empty transporter in outward facing state (1). If substrates are available the transporter will bind glutamate, Na^+ and H^+ (2) which leads to a conformational change whereby the transporter faces inward (3) to release these substrates into the intracellular medium (4). Following this the transporter is free to bind intracellular K^+ (5) which induces a conformational change to an outward facing state (6) facilitating the release of K^+ into the extracellular space (7).

3.3 Structure of glutamate transporters

The high level of conservation of structure between different transporters is thought to be due to the specialised function of these proteins. Comparison of amino acid sequences between members of the glutamate transporter family shows that they share roughly 50-60% homology. Until recently, structural studies of glutamate transporters have been lacking. In 2004 one group elucidated the structure of a bacterial homologue of GLT-1 from *Pyrococcus horikoshii*, known as Glt_{Ph} (Yernool *et al.*, 2004). This transporter shares around 34% homology with human EAAT2 and so it is reasonable to assume that transport mechanisms are conserved. At the moment Glt_{Ph} is one of the best tools we have to try to understand the structural changes which take place during the glutamate transport cycle.

It has been suggested for quite some time that glutamate transporters may function in multimers. This is due to the fact that although GLT-1 has a molecular weight of 64 kDa, immunoblotting for GLT-1 has resulted in protein bands roughly 2-3 times this size (Haugeto *et al.*, 1996). Yernool and colleagues (2004) provided direct evidence for multimerisation, at least for the bacterial transporter Glt_{Ph}, showing that this transporter was indeed in a trimer. However, we should not necessarily assume that these transporters exist solely in trimers and do not exist as functional monomers and dimers (see Figure 7). Interestingly, it was observed that trimers of Glt_{Ph} form a bowl-like structure in the middle which may facilitate access of glutamate and other ions to their respective binding sites. For GLT-1, it has been demonstrated that modifications in the protein sequence towards the C-terminal part of the protein, including transmembrane domains 7 and 8, have the biggest impact upon transporter function (Grunewald and Kanner, 2000). Again, results from Yernool and collaborators (2004) demonstrated that the C-terminal region was indeed the area responsible for translocation of ions and glutamate across the membrane. In fact their crystallised structure contained a bound aspartate molecule which is another amino acid

closely resembling glutamate and often used for functional assays of glutamate uptake by this family of transporters.

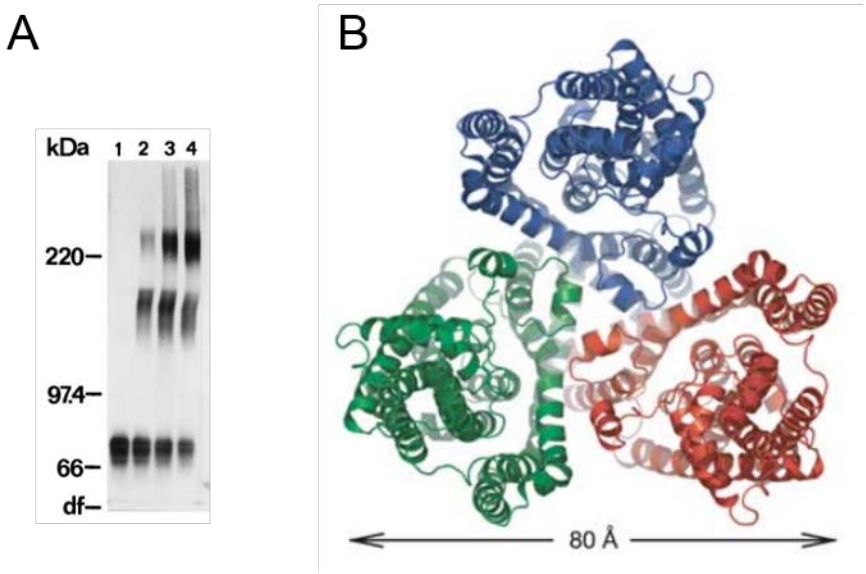


Figure 7. Evidence for multimerisation of glutamate transporters

(A) Immunoblot of GLT-1 from Huageto *et al.* (1996) showing 3 bands for GLT-1 which correspond to monomeric, dimeric and trimeric forms of GLT-1. **(B)** Crystal structure of bacterial glutamate transporter GltPh showing trimeric structure of this transporter from Yernool *et al.* (2004). Each monomer is coloured individually.

3.4 Location of glutamate transporters

Each member of the glutamate transporter family has a distinct location which is likely to be directly related to their function. The first differentiation we can make between transporters is between those located on glial cells, specifically on astrocytes, and others which are located on neurons. GLT-1 and GLAST have both been demonstrated to be astrocytic transporters (Chaudhry *et al.*, 1995; Furuta *et al.*, 1997b; Rothstein *et al.*, 1994) whereas EAAC1 and EAAT4 are located on neurons (Furuta *et al.*, 1997a; Rothstein *et al.*, 1994). It has been shown that expression of glutamate transporters is developmentally regulated (Furuta *et al.*, 1997b), it was suggested that EAAC1 is the most highly expressed

glutamate transporter in pre-natal rats. Post-natally however, GLT-1 and GLAST assume their roles as the predominant glutamate transporters in the brain and are the transporters most commonly found on astrocytic processes facing synapses (Chaudhry *et al.*, 1995), implying an important role for glial transporters in the control of synaptic glutamate concentrations. Expression levels of GLT-1 and GLAST were not found to be uniform in the adult brain, demonstrating transporter-specific, regional expression patterns. GLAST expression was found to dominate the cerebellum while GLT-1 expression was more pronounced in the forebrain areas particularly in the hippocampus (Chaudhry *et al.*, 1995; Furuta *et al.*, 1997b).

It has been estimated that GLT-1 accounts for 1% of the total brain protein (Danbolt, 2001; Lehre and Danbolt, 1998), if this is indeed the case, then it is certain that GLT-1 plays an extremely significant role in the brain. Total numbers of GLT-1 and GLAST protein in the have been estimated in the Danbolt lab. Their findings are high with average GLT-1 and GLAST densities on astrocytic membranes calculated to be 8500 and 2300 transporters/μm^2 in the stratum radiatum of the hippocampus respectively; and 740 and 4700 transporters/μm^2 in the cerebellar molecular layer respectively (Lehre and Danbolt, 1998). These high levels of protein expression may be overestimated due to the methods used in the study, cell homogenates were measured for protein content and the authors were not able to distinguish between surface and intracellular glutamate transporter expression.

Neuronal transporters EAAC1 and EAAT4 can be found throughout the brain, with EAAT4 acting as the predominant neuronal transporter in the cerebellum (Furuta *et al.*, 1997a). It should be noted that Furuta and colleagues (1997b) revealed that neuronal transporters were more likely to be found on the soma and dendrites of neurons, rather than in opposing synapses as is the case for glial transporters. This indicates a potential role for neuronal glutamate transporters in the control of extrasynaptic glutamate concentration.

EAAT5 has been demonstrated to be specifically located on retinal photoreceptor cells and may play a role in visual processing (Arriza *et al.*, 1997).

Importantly, the efficiency of glutamate transporters depends on their location. It has been estimated that each synaptic vesicle contains approximately 4000 glutamate molecules (Riveros *et al.*, 1986). Thus for each quantal event, several thousand glutamate molecules are estimated to be released into the synaptic cleft, raising the synaptic concentration to approximately 1 mM (Clements *et al.*, 1992). Clements and colleagues (1992) further suggested that glutamate is removed from the synaptic cleft in 1.2 ms, which is very fast when compared to the time scale of one transport cycle of a glutamate transporter (70 ms). In order for the glutamate uptake system to work efficiently, it has been hypothesised that there are more transporters than glutamate molecules at any given synapse (Diamond and Jahr, 1997; Lehre and Danbolt, 1998), thus the rapid removal of glutamate from the synapse can possibly be accounted for through buffering of glutamate by binding to transporters and not actual transport of glutamate across the astrocytic membrane. Due to the fact that the majority of glutamate transporters are located on astrocytes (Lehre and Danbolt, 1998) and that these astrocytic transporters play the most important role in removing glutamate from the synapse (Rothstein *et al.*, 1996; Tanaka *et al.*, 1997; Watase *et al.*, 1998), astrocytic coverage of the synapse will play a great role in controlling glutamate concentration and spill over from one synapse to another. The precise location of astrocytic processes has been demonstrated to directly affect glutamate uptake (Melone *et al.*, 2009; Oliet *et al.*, 2001; Omrani *et al.*, 2009; Pannasch *et al.*, 2014). Studies have shown that GLT-1-containing astrocytic processes are located approximately 0-400 nm from the synapse (Melone *et al.*, 2009; Omrani *et al.*, 2009), with further evidence suggesting that it is through close apposition of GLT-1-containing astrocytic processes to the synaptic area that permits astrocytes to control glutamatergic synaptic transmission (Pannasch *et al.*, 2014).

4. Glutamate transporters in physiology

Clearly, the single most important role for glutamate transporters is to maintain the extracellular concentration of glutamate at an extremely low level, this is particularly true in the synaptic cleft. The high level of protein expression and location of these transporters close to the synapse serves to highlight this point. As mentioned, synaptic glutamate release results in an excitatory postsynaptic current (EPSC), i.e. a flux of ions across the membrane, mediated by activation of postsynaptic glutamate receptors. The kinetics of these EPSCs are a product of two main factors; the concentration and timecourse of the neurotransmitter at the synapse and the properties of these postsynaptic receptors. First, it has been shown that under physiological conditions, the timecourse of glutamate at the synapse is rapid, around 1.2 ms (Clements *et al.*, 1992). Second, the properties of the receptors can play an important role in the timecourse of synaptic events as observed by measuring EPSCs. Glutamate receptors have been exhaustively characterised and we now know that desensitisation of both NMDARs and AMPARs can occur with prolonged exposure to glutamate (Colquhoun *et al.*, 1992; Hestrin, 1992; Mayer and Westbrook, 1985; Trussell *et al.*, 1993). It has been shown that NMDARs desensitise relatively slowly (hundreds of ms) (Forsythe and Westbrook, 1988), on the other hand AMPARs are believed to desensitise rapidly with some studies demonstrating that saturating concentrations of glutamate result in EPSCs of about 10 ms (Colquhoun *et al.*, 1992; Hestrin, 1992). Thus, it seems that receptor desensitisation does not play a major role in controlling the timecourse of postsynaptic receptor activation during low activity events, because the glutamate transient at the synapse is shorter than the maximum potential EPSC. Rather, it has been suggested that desensitisation of glutamate receptors curtails postsynaptic excitation during multivesicular release of glutamate (Trussell *et al.*, 1993) where the timecourse of glutamate at the synaptic cleft is prolonged. Originally, it was hypothesised that diffusion of neurotransmitter from the synapse could account for quick synaptic transients observed at the neuromuscular

junction (Eccles and Jaeger, 1958). Since then, evidence has come to light that diffusion alone cannot account for these quick synaptic transients and in the CNS studies implicated glutamate transporters in the regulation of glutamate timecourse at the synapse (Barbour *et al.*, 1994).

It is now beyond doubt that astrocytic glutamate transporters play a role in regulating the timecourse of synaptic glutamate as observed at both hippocampal (Bergles and Jahr, 1997) and cerebellar (Clark and Barbour, 1997) synapses. Many studies have provided evidence implicating glutamate transporters in synaptic transmission by pharmacologically blocking transporters, leading to a build-up of synaptic glutamate as witnessed by increased glutamate receptor activation (Barbour *et al.*, 1994; Mennerick and Zorumski, 1994; Overstreet *et al.*, 1999; Takahashi *et al.*, 1996; Tong and Jahr, 1994). However, the specific findings of these papers vary between studies and are likely to result from the brain preparation used, as astrocytic coverage of synapses is not uniform in the brain (Ventura and Harris, 1999). The most robust findings were observed in cerebellar synapses where astrocytes are believed to completely ensheath synapses. Barbour and colleagues (1994) specifically blocked glutamate transporters which resulted in increased AMPAR activation as witnessed by slower decay kinetics of AMPAR-mediated EPSCs. These findings were confirmed independently in the same preparation by Takahashi *et al.* (1996). Overstreet *et al* (1999) also observed prolonged AMPAR-mediated EPSCs in a distinct area of the cerebellum when glutamate uptake was blocked. Here the authors used the mossy fibre – granule cell glomerulus of the cerebellum and hinted towards a role for transporters in regulating glutamate spillover between synapses adjacent to the glomerulus during high activity. A paper by Tong and Jahr (1994), was one of the few studies in the literature which investigated the role of glutamate transporters in controlling the timecourse of synaptic glutamate at both room temperature (24°C) and near physiological temperature (34°C). Interestingly, while no effect of blocking glutamate transporters at 24°C was observed, they reported an increase in amplitude of AMPAR EPSCs at 34°C. This could suggest that most

studies carried out at room temperature have underestimated the role of transporters in the control of synaptic glutamate receptor activation.

In contrast to the cerebellum, astrocytic coverage of hippocampal synapses is not considered to be complete (Ventura and Harris, 1999), which could possibly indicate a physiological role of glutamate spillover in this brain region. As such, findings from studies in the hippocampus have been rather mixed, with some studies directly implicating transporters in controlling the timecourse of synaptic glutamate, reporting delayed kinetics of AMPAR EPSCs (Mennerick and Zorumski, 1994), similar to findings in the cerebellum. Whereas other studies have discounted the role of transporters in the hippocampus, first, because of incomplete astrocytic coverage of synapses in this brain region and second, due to the observation that glutamate can diffuse out of the synapse and act on adjacent synapses (Kullmann *et al.*, 1996). However, it was later reported that experimental conditions used by the former study in hippocampal slice preparation can dramatically influence the findings, underestimating the role of glutamate transporters in this brain region (Asztely *et al.*, 1997). Asztely and colleagues (1997) reported that spillover of neurotransmitter between synapses is greatly reduced at physiological temperatures since diffusion of neurotransmitter, receptor kinetics and neurotransmitter transport are temperature dependent processes.

Glutamate transporters can further control the timecourse of synaptic glutamate by directly competing with glutamate receptors for glutamate. This hypothesis was suggested following the demonstration that transporters have an affinity for glutamate comparable to that of the high-affinity NMDARs (around 1 μM) (Patneau and Mayer, 1990) with the K_m of glutamate transporters roughly 30 μM (Arriza *et al.*, 1994). Further evidence, observed by measuring transporter currents in astrocytes, indicated that these receptors can bind glutamate as rapidly as AMPARs (Bergles and Jahr, 1997; Wadiche *et al.*, 1995), suggesting a high level of competition between glutamate receptors and transporters for synaptically released glutamate.

The data presented above offer a very convincing hypothesis that glutamate transporters play an active part in synaptic transmission, controlling the timecourse of glutamate in the synaptic cleft thereby curtailing glutamate receptor activation. However, these studies did not address the question of exactly how transporters carry out this role taking into account that one complete transport cycle lasts around 70ms (Otis and Jahr, 1998; Wadiche *et al.*, 1995). It was first hypothesised that glutamate transporters are able to effectively remove glutamate on a synaptic time scale through binding glutamate, thus shielding receptors from this neurotransmitter (Diamond and Jahr, 1997). This hypothesis was strengthened by findings from an independent group who used multiple approaches, including manipulation of membrane potential of astrocytes which blocked transport but not binding of glutamate to the transporters to elucidate that glutamate translocation across the astrocyte membrane, as well as binding to the transporter, play a strong role in controlling the timecourse of synaptic glutamate (Mennerick *et al.*, 1999). These observations are in agreement with previous work on glutamate transporter kinetics which proposed that it is not the glutamate translocation but rather K^+ translocation which is the rate limiting step of the transport cycle (Otis and Jahr, 1998; Wadiche *et al.*, 1995).

Other studies further elucidated the role of transporters in synaptic transmission using more physiological preparations, taking advantage of the structural changes in astrocytic coverage of the synapse known to occur during lactation (Theodosis and Poulain, 1984). Using this preparation, Oliet and colleagues (2001) observed an increase in presynaptic mGluR activation when glutamate transporter function was reduced by morphological changes or blocked by drugs, thereby decreasing the probability of neurotransmitter release. This physiological model definitively showed that astrocytic coverage of synapses regulates synaptic efficacy, by controlling presynaptic glutamate receptor activation through glutamate uptake.

Strong evidence indicates that another primary role of glutamate transporters is to control spillover of glutamate between synapses maintaining the input specificity of individual

synapses. Some studies have suggested that glutamate transporters play a small role in glutamate uptake during single synapse/low activity events (Arnth-Jensen *et al.*, 2002; Marcaggi *et al.*, 2003). Interestingly, these studies noted that transporter activity is crucial during high activity/multi-synapse events to control glutamate receptor activation as well as preventing spillover to maintain input specificity between synapses, both in the hippocampus (Arnth-Jensen *et al.*, 2002) and in the cerebellum (Marcaggi *et al.*, 2003). Under certain conditions, spillover of glutamate can play a physiological role. In the mossy fibre glomerulus of the cerebellum, spillover from excitatory mossy fibre synapses onto inhibitory granule cell interneurons activates presynaptic mGluRs on these inhibitory interneurons resulting in a decrease of neurotransmitter release (Mitchell and Silver, 2000). Importantly, in this brain circuit, activation of mGluRs reduced GABAergic tone from these interneurons resulting in feedforward disinhibition, thereby increasing the efficacy of these excitatory fibres. The architecture of this mossy fibre glomerulus excludes a role for astrocytic glutamate transporters in this synaptic spillover inside the glomerulus, as astrocytes are only found on the periphery, rather astrocytes surround this structure limiting glutamate spillover onto extraglomerular synapses.

In the hippocampus, glutamate spillover is believed to play a physiological role in LTP expression, through activation of silent synapses, which exclusively express NMDARs until activated by spillover of glutamate from another synapse (Isaac *et al.*, 1995; Kullmann *et al.*, 1996). As previously mentioned spillover of glutamate in the hippocampus is tightly regulated by transporters in order to efficiently regulate heterosynaptic plasticity (Asztely *et al.*, 1997), therefore it is likely that high levels of activity are needed to induce this form of plasticity. Unsilencing of synapses is now understood to involve the recruitment of AMPARs to the synapse in an NMDAR-dependent mechanism (Malinow and Malenka, 2002).

Together, these experiments clearly show the important role played by glutamate transporters in controlling the timecourse of glutamate of the synapse. Blocking transporter function pharmacologically or in certain physiological conditions can have varied effects,

which could be due to several factors including differences in the structure of the synapse, astrocytic coverage of the synapse, the proximity different types of neurons (excitatory/inhibitory) which could be activated by glutamate spillover, as well as receptors present on these neurons.

5. Glutamate transporters in pathology

From the outset it seems quite paradoxical that glutamate, while being necessary for many vital processes in the brain, can also be toxic in high concentrations (Choi, 1985, 1987). Choi (1987) discovered that glutamate neurotoxicity (or excitotoxicity) is highly dependent on Ca^{2+} entry through both NMDARs as well as voltage-gated Ca^{2+} channels, opened during depolarisation. As elucidated in the previous section, NMDAR desensitisation is a very slow process, thus once NMDARs become activated, prolonged pathological glutamate exposure can lead to great flux of ions across the cell membrane. It is believed that the damaging effects exerted by high levels of intracellular Ca^{2+} are due to aberrant signalling which leads to further glutamate release from neurons as well as activation of apoptotic pathways (Orrenius *et al.*, 2003). This excitotoxicity highlights the complexity and importance of regulating extracellular glutamate concentrations in the CNS, with modest concentrations of glutamate activating receptors physiologically while high concentrations result in neuronal cell death. The importance of glutamate transporters in protecting against excitotoxicity was not observed until many years later, when specific glutamate transporter blockers became available. It has been repeatedly observed that blocking glutamate transporters pharmacologically is sufficient to increase extracellular glutamate to pathological levels resulting widespread neuronal cell death in culture (Robinson *et al.*, 1993) and organotypic slice cultures (Bonde *et al.*, 2003). Consistent with early reports, these studies have shown that glutamate toxicity observed in these conditions could be

significantly attenuated by addition of NMDAR antagonists (Bonde *et al.*, 2003; Robinson *et al.*, 1993).

Technological advances in genetic manipulation, have allowed researchers to specifically ablate individual glutamate transporters, thus elucidating roles of different transporters in the brain. These studies further confirmed the importance of astrocytic transporters in removing extracellular glutamate (Rothstein *et al.*, 1996; Tanaka *et al.*, 1997; Watase *et al.*, 1998). On the other hand, knock-down of the neuronal transporter EAAC1, did not provoke any phenotype nor neuronal cell death in the mice, indicating that these transporters are not vital for life (Rothstein *et al.*, 1996). Knock-out of GLAST, an astrocytic glutamate transporter, did not produce any dramatic phenotype (Watase *et al.*, 1998), these genetically modified mice seemed to develop normally and have normal life expectancies. However, this study noted a significant reduction in motor coordination and an increased susceptibility to injury in the cerebellum. These findings are consistent with the localisation of GLAST as the predominant glutamate transporter in the cerebellum (Chaudhry *et al.*, 1995; Furuta *et al.*, 1997b), a brain region important for motor coordination. It has been demonstrated, using pharmacological and genetic manipulations, that GLT-1 is in fact the most important glutamate transporter in the forebrain. In contrast to animals lacking the GLAST transporter (Watase *et al.*, 1998) which presented a mild phenotype, animals lacking GLT-1 suffered from excitotoxicity which lead to cell death and eventually death of the animals through epileptic seizures (Rothstein *et al.*, 1996; Tanaka *et al.*, 1997). Consistent with GLAST knock-out animals, GLT-1 knock-out mice also experienced an increased susceptibility to brain injury (Tanaka *et al.*, 1997). Thus, it is now beyond doubt that this family of amino acid transporters play a vital role in glutamate homeostasis, one could already suggest that dysregulation of these transporters could be deleterious and have widespread damaging effects in the CNS. This is indeed the case as we will see in the final paragraphs of this introduction discussing the role of glutamate transporters in pathology.

Among the first reports suggesting that glutamate transporters may be directly implicated in pathology discovered that the cerebrospinal fluid from patients with amyotrophic lateral sclerosis (ALS) contained 100-200% more glutamate and aspartate compared to healthy individuals (Rothstein *et al.*, 1990). ALS is the most common motor neuron disease in adults with a prevalence of 2-3 per 100,000 people. Most of the cases of this disease are sporadic in origin with the greatest risk factor (like most neurodegenerative diseases) being age, however, a small percentage of cases (< 10%) are genetically inherited and linked to a gain function in the superoxide dismutase (SOD1) enzyme (Cleveland and Rothstein, 2001). Further work by Rothstein and colleagues (1992), observed that there was dramatic decrease in glutamate uptake, up to 90% reduction in some cases, in tissue from patients with ALS. Later studies concluded that this loss in the capacity to remove glutamate from the extracellular space in ALS is due to selective loss of GLT-1, with no modification in EAAC1 or GLAST (Rothstein *et al.*, 1995). As mentioned above dysregulation of GLT-1, which leads to excitotoxicity and cell death (Rothstein *et al.*, 1996; Tanaka *et al.*, 1997), is one of the primary factors in the progression of this disease.

Dysregulation of glutamate uptake function is now implicated in a wide range of diseases in the CNS, particularly in pathologies where excitotoxicity plays a strong role in disease development, such as epilepsy (Molinari *et al.*, 2012; Tanaka *et al.*, 1997). It has been demonstrated that spillover of glutamate is not solely a physiological process but can also occur in certain epilepsy-like pathologies, in which a failure of uptake by transporters leads to the activation of postsynaptic group I and II mGluRs. Activation of postsynaptic mGluRs, as opposed to presynaptic mGluRs, increases excitation, allowing postsynaptic neurons to remain hyperpolarised during prolonged periods of time resulting in burst/epileptiform activity (Molinari *et al.*, 2012). Furthermore, spillover of glutamate in pathological conditions is likely to result not only in the activation of postsynaptic mGluRs but also the activation of extrasynaptic NMDARs. These extrasynaptic NMDARS, as opposed to

synaptic NMDARs, have been shown to be implicated in cell death pathways (Hardingham *et al.*, 2002).

Increasing evidence suggests that malfunctions in the glutamate uptake system are implicated in neurodegenerative diseases which have not previously been considered to be associated with disrupted glutamate uptake such as Alzheimer's (Lauderback *et al.*, 2001; Lauderback *et al.*, 1999; Scimemi *et al.*, 2013) and Huntington's (Arzberger *et al.*, 1997; Lievens *et al.*, 2001) disease. Alzheimer's disease has been associated with a decrease in glutamate transporter expression and function both in human brain tissue (Masliah *et al.*, 1996) as well as in animal models of this disease (Masliah *et al.*, 2000). Moreover it has been demonstrated that the amyloid-beta (Aβ) plaques, one classical hallmark of Alzheimer's disease, can effectively inhibit glutamate uptake (Harris *et al.*, 1996; Lauderback *et al.*, 1999). Some potential mechanisms have now been elucidated with one study pointing towards oxidation of GLT-1 leading to inactivation and dysfunction of the transporter (Lauderback *et al.*, 2001) while another study suggests that Aβ acts to increase the lifetime of synaptically release glutamate by reducing GLT-1 surface expression on astrocytes (Scimemi *et al.*, 2013). Interestingly the latter study indicated that the drug Trolox, a vitamin E derivative, prevented the Aβ induced reduction of GLT-1 expression. Thus proposing a novel treatment strategy directed towards glutamate transporters to alleviate the symptoms of Alzheimer's disease. Malfunction in glutamate uptake has also been implicated in Huntington's disease, a genetic neurodegenerative disease caused by an expansion of CAG codons (coding for glutamine) resulting in a mutant form of huntingtin protein. Studies have reported a specific reduction in EAAT2/GLT-1 glutamate transporter expression in human brain tissue (Arzberger *et al.*, 1997) and in animal models of this disease (Lievens *et al.*, 2001). In Huntington's disease, it is sure that glutamate transporter dysfunction does not play a causal role in the disease but likely exacerbates the neurodegeneration observed across multiple brain areas. Whether GLT-1 dysregulation is a cause or effect of the formerly mentioned diseases, particularly ALS and epilepsy, is unclear. What is certain is that

glutamate uptake, both at the synapse and extrasynaptically, is a highly important process in both physiology and pathology in the brain.

6. Aim of thesis project

Since the initial observations that neurotransmitter receptors are capable of lateral diffusion on the surface of the cells on which they are expressed, this lateral diffusion has consistently been shown to impact upon the efficacy of synaptic transmission (Dupuis *et al.*, 2014; Heine *et al.*, 2008). Furthermore, surface diffusion of receptors has been demonstrated to be highly dynamic and can be modified by many physiological stimuli such as stress (Groc *et al.*, 2008; Krugers *et al.*, 2010). These observations lead to the idea that perhaps glutamate transporters, responsible for removing glutamate from the extracellular cleft, could also be subject to surface diffusion. This mechanism could shed light onto how transporters, with their slow cycling time, are capable of rapidly remove glutamate from the synapse. Integrating our current knowledge regarding the importance of astrocytic glutamate transporters at the synapse, as well as what we know about the surface diffusion of receptors, the next logical step forward is to consider the possibility that glutamate transporters may too diffuse on the surface of astrocytes which could possibly impact upon neuronal synaptic transmission.

We could further speculate that through this surface diffusion, glutamate transporters may be capable of buffering glutamate in a manner which has been suggested in previous studies (Diamond and Jahr, 1997; Mennerick et al., 1999). The hypothesis being that glutamate transporters, having upmost importance in removing synaptic glutamate, may diffuse along the surface of the cell from non-synaptic regions to areas where there are high levels of glutamate release, such as synapses. Once the individual transporters bind a molecule of glutamate, beginning the slow transport cycle, these transporters may move away from the synapse allowing a naïve unbound transporter to move into the confined synaptic space which binds another molecule of glutamate resulting in the effective buffering and removal of synaptic glutamate. Moreover, diffusion of transporters is likely to increase in importance with high glutamate release activity, by moving glutamate-bound transporters

away from the synapse allowing unbound transporters to enter this confined space. Through this mechanism, the effects of saturation of transporters by high concentrations of glutamate are likely to be minimised. This process, in physiological conditions may indeed play a role in shaping the timecourse of glutamate at the synapse as well as ensuring input specificity of synaptic connections by limiting glutamate spillover between synapses. In the long-term, implication of surface diffusion of glutamate transporters in buffering and removing glutamate from the synapse, could provide a novel target in alleviating symptoms of certain diseases.

Methods

1. Hippocampal Cell Culture

Mixed cultures of hippocampal glia and neurons were prepared from E18 Sprague-Dawley rats (Janvier) based on a protocol developed by Banker and colleagues (Banker and Cowan, 1977) following previously described methods (Mikasova *et al.*, 2012). Briefly, the hippocampi were removed from the brain and dissociated by trypsin and mechanical treatments and cells were plated onto poly-L-lysine-treated glass cover-slips at a density of 60 x 10^3 cells per ml. Cultures were kept in neurobasal medium (Invitrogen) with 3% horse serum (Invitrogen) for several days before changing to serum-free neurobasal medium (Invitrogen). Cultures were maintained at 37°C in 5% CO_2 for 20 days *in vitro* (DIV) at maximum.

'Neuron-free' cultures were prepared with the same dissection protocol described above. However, these cultures differ in the medium used and treatment of cells once plated on culture dishes. For 'neuron-free' cultures cells were maintained in MEM medium (Invitrogen) containing 10% horse serum, again at 37°C in 5% CO_2 until experimentation. Furthermore, these dishes were subjected to intense agitation after 3 DIV which was repeated twice per week when changing culture medium. Combining the medium, which does not promote neuronal growth, as well as intense agitation of culture dishes, which removes weakly attached cells, microglia and neurons, we achieved a 'neuron-free' culture. It is possible that these culture dishes contained a low amount of neurons and perhaps microglia, these parameters were not quantified. However, while imaging these cultures we never observed any neurons.

2. COS cell culture

COS-7 cells were plated in 12 well plates at a density of 25,000 cells/well in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 1% glutamax (Gibco), 1% sodium pyruvate (Sigma-Aldrich), 10% Fetal Bovine Serum (Invitrogen). After 1 day, cells were transfected or not with GLT-1^{flag} using lipofectamine 2000 (Invitrogen) and left under humidified 5% CO_2 atmosphere (37°C) for 2 days before experimentation.

3. Protein expression

Cells were transfected at 7-10 DIV using Effectene transfection reagent (Qiagen) following the manufacturer's protocol before experimentation between 10-16 DIV. For live imaging cells were transfected with GLT-1 transporter with a flag tag (DYKDDDDK) inserted into an extracellular loop of the protein (between Pro^{199} and Pro^{200}; Figure 5; a gift from M. Rattray) (Peacey *et al.*, 2009), as well as enhanced Green Fluorescent Protein (eGFP), this approach allowed us to determine the cell type which has been transfected. For live imaging of GLT-1^{flag} diffusion around the synapse, cells were electroporated with Homer 1c-dsRed before plating. Electroporation was carried out using the Nucleofector device (Lonza) following the manufacturer's protocol. For immunostaining, cells were transfected with GLT-1^{flag} as well as the synaptic marker Homer 1c-dsRed to quantify the colocalisation of GLT-1^{flag} at the synapse.

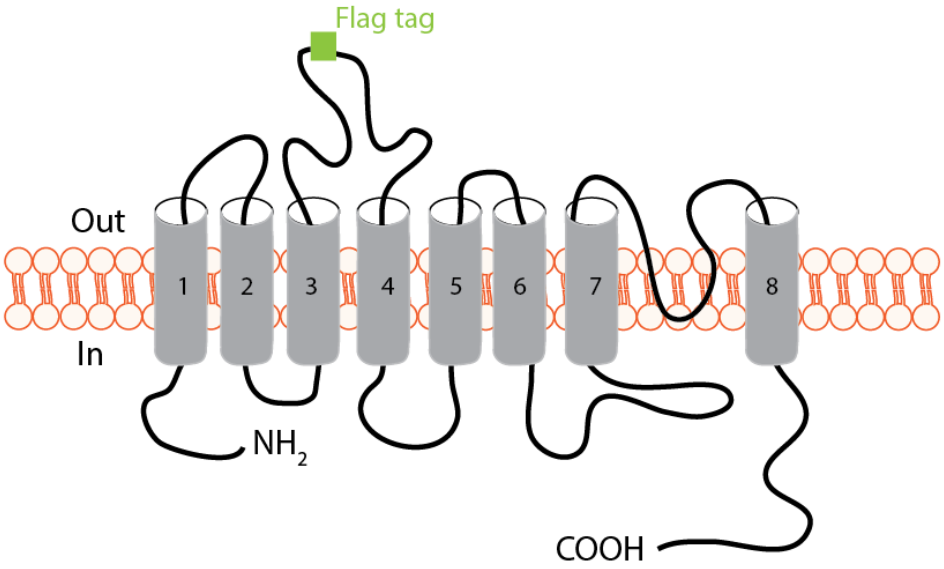


Figure 8. Structure of GLT-1^{flag}.

GLT-1 is an eight transmembrane domain protein with both NH_2 and COOH termini located on the intracellular side of the cell. For live QD imaging it was necessary to have an extracellular tag, thus we utilised a GLT-1 transporter expressing a flag tag in its second extracellular loop between Pro^{199} and Pro^{200}.

4. Immunocytochemistry

For immunostaining, surface GLT-1^{flag} was stained using a monoclonal anti-flag antibody (1 µg/ml; Stratagene) for 30 min on live mixed cultures at 37°C and 5% CO_2. Cells were then fixed with 4% paraformaldehyde for 15 min, washed and then incubated with appropriate secondary antibodies. For synapse labelling experiments we used secondary anti-mouse Alexa-568 (1 µg/ml, 1 hr.; Molecular probes) for GLT-1^{flag} and to label shank, neurons were permeabilised using 0.1% Triton X-100, incubated with a primary rabbit polyclonal anti-shank antibody (1 µg/ml, 1 hr; Abcam), and finally incubated with secondary antibody anti-rabbit Alexa-488 antibodies (2 µg/ml, 30 min; Molecular Probes). For quantification of synaptic content, cells were transfected with Homer 1c-dsRed before immunostaining for GLT-1^{flag} using a monoclonal anti-flag antibody (1 µg/ml; Stratagene) for

30 min followed by a secondary FITC anti-mouse (3 µg/ml; Invitrogen) for 1 hr. Cells were washed and mounted and preparations were kept at 4°C until imaging. Fluorescent specimens were examined under a confocal microscope (Leica) equipped with appropriate excitation/emission filters. Images were analyzed in Metamorph software (Universal Imaging Corp.).

5. Single Particle (QD) Tracking and Surface Diffusion Calculation

Single particle (Quantum dot) labelling and microscopy was performed as previously described (Mikasova *et al.*, 2012). Hippocampal primary cultures were incubated for 10 min (37˚C) with monoclonal antibodies against flag epitope (10 µg/ml; Stratagene) to tag GLT-1^{flag}. Cells were then washed and incubated for 10 min (37˚C) with quantum dots 655 goat F(ab')2 anti-mouse IgG (1 µg/ml; Invitrogen). Non-specific binding was blocked by the addition of 1% casein or 1% BSA (Vector Laboratories) to the quantum dots 15 min before use. Cells were again rinsed and mounted in an aluminium chamber containing aCSF solution (containing 140 mM NaCl, 2 mM KCl, 2 mM $CaCl_2.2H_2O$, 2 mM $MgCl_2.6H_2O$, 10 mM HEPES and 10 mM D-glucose; 250-260 mOsm; pH 7.4, 37˚C) on a Nikon microscope (NIKON Eclipse TE2000-U) with the stage heated to 37˚C using an air bower (World Precision Instruments) and an objective heater (Bioptechs). Quantum dots were detected using a mercury lamp and appropriate excitation/emission filters. Images were obtained with and acquisition time of 50 ms for 1000 consecutive frames. Signals were detected using an EM-CCD camera (Quantem, Roper Scientific). Quantum dots were followed on randomly selected astrocytes expressing both GFP and GLT-1^{flag}. Quantum dot recording sessions were processed with the MetaMorph software (Universal Imaging Corp.). The instantaneous diffusion coefficient (D) was calculated for each trajectory, from linear fits of the first four points of the mean-square-displacement versus time function using $MSD(t) = \langle r^2 \rangle (t) = 4Dt$. The 2D trajectories of single molecules in the plane of focus were constructed by

correlation analysis between consecutive images using a Vogel algorithm. Synaptic dwell time was calculated for exchanging transporters and defined as the mean time spent within an annulus of 320 nm around the synaptic area. The 2D trajectories of single molecules in the plane of focus were constructed by correlation analysis between consecutive images using a Vogel algorithm.

Although this epifluorescent microscope, equipped with a high sensitivity EM-CCD camera, is not a super-resolution microscope, we still have a high sensitivity with regards to pointing accuracy, when tracking a single QD-tagged protein. From analysis of a single QD we can determine the centre point and create a Gaussian curve (see Figure 9). Using the coordinates of the half-width full maximum point of this Gaussian curve we can detect in a very precise manner the movement around this centre point thus we can tell if a single protein is moving on a ~40 nm scale. By tracking the movements of a single protein we can define characteristics of this protein, i.e. speed of diffusion as well as determining the confinement (see Figure 9).

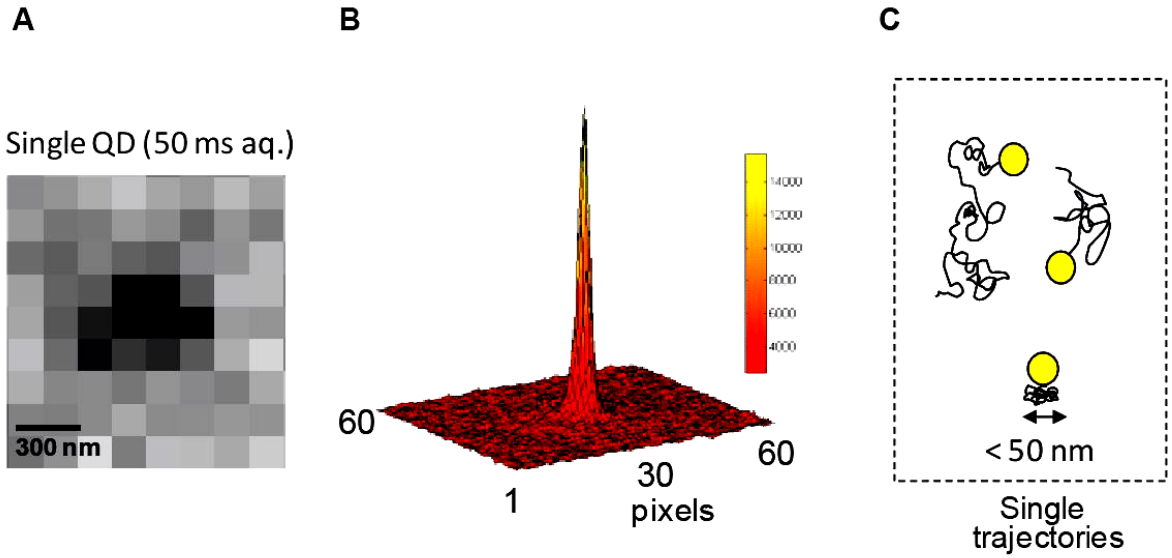


Figure 9. High-sensitivity tracking of a single Quantum Dot-tagged molecule.

(A) High magnification image of a single QD-tagged protein (black pixels). **(B)** Gaussian curve from (A) from this curve we can find the full height at half-width and track one single QD from frame to frame with high precision. **(C)** After following several QD-tagged proteins

we can map their movement by reconstructing their trajectories over time. These trajectories can vary, with some displaying high levels of confinement compared to others which can be very freely-diffusing.

Drugs were added into the bath, in different experimental conditions, to observe potential effects on GLT-1 surface diffusion. The drugs applied were; L-glutamate (100 µM; Sigma-Aldrich); TBOA (DL-threo-benzyloxyaspartate, 30 µM; Tocris); TTX (Tetrodotoxin, 1 µM; Tocris); The glutamate receptor antagonist cocktail comprised AP5 (D-(-)-2-Amino-5-phosphonopentanoic acid, 10 µM; Tocris), NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydr-obenzo[*f*]quinoxaline-7-sulfonamide, 10 µM; Tocris) and MCPG ((*RS*)-α-Methyl-4-carboxyphenylglycine, 500 µM; Tocris).

6. MNI-caged-L-Glutamate Uncaging

Uncaging experiments were carried out in mixed hippocampal cultures between 14-16 DIV, cells were transfected with GLT-1^{flag} 24 hours before uncaging experiments. On the day of experimentation, cells were incubated with primary and secondary antibodies for QD tracking before 30 second incubation with mitotracker-green (1/2000) to mark mitochondria-rich synapses. Coverslips were imaged in aCSF solution (containing 140 mM NaCl, 2 mM KCl, 2 mM $CaCl_2.2H_2O$, 2 mM $MgCl_2.6H_2O$, 10 mM HEPES and 10 mM D-glucose; Osmolarity was 250-260 Osm/L; pH 7.4) with 5 mM MNI-caged-L-glutamate. Uncaging experiments were carried out on a Nikon Ti-Eclipse inverted microscope equipped with a stage heater at 37˚C (World Precision Instruments), a 100X objective and 405, 491, 561 and 642 nm lasers. Signals were detected using an EM-CCD camera (Quantem, Roper Scientific). Regions of interest (ROIs) were chosen arbitrarily at synapses in close apposition to GLT-1^{flag} transfected astrocytes. Uncaging was performed using a single laser pulse at 405 nm on ROIs directly beside synapses, with a pulse duration of 2 ms. Trajectories of

GLT-1^{flag} tagged with QDs were analyzed as described above for a duration of 30 seconds before uncaging and 60 seconds directly after uncaging.

7. Stereotaxic Injections

Surgical procedures were done in accordance with the guidelines of the ethical committee for animal research in Bordeaux University. Sprague-Dawley rats (P16-18) were anaesthetized with isoflurane and mounted on a Kopf stereotaxic frame. The heads were placed in a surgical mask to maintain the skull stable. A constant flux of an isoflurane / air mixture was applied inside the surgical mask. 500-1000 nl of either rabbit polyclonal antibodies directed against GLT-1 (NBP1-20136, Novus Biologicals) or goat anti-rabbit IgG (Invitrogen) (0.4 µg / µl) were dissolved in a PBS (0.1 M, pH 7.4) and infused into the dorsal hippocampus (coordinates relative to bregma, AP: -4.5 mm, ML: ±2.2 mm, DV: -2.5 mm at P17) using borosilicate micropipettes (GC150F-10, Harvard Apparatus) prepared with a vertical micropipette puller (PC-10, Narishige). The solution was injected in the hippocampus at approximately 250 nl/min. After injection, the needle was left *in situ* for few minutes to reduce reflux up the needle. The incision was both mechanically and chemically sutured. Rats were then allowed to recover before being used for slice electrophysiology experiments.

8. Electrophysiology

Cell Culture: Spontaneous excitatory postsynaptic currents (sEPSC) were recorded in whole-cell patch-clamp (Vhold = -70 mV) from hippocampal neurons at 14-16 days *in vitro* located in the close proximity of GFP/GLT-1^{flag} transfected astrocytes. All experiments were conducted under continuous perfusion of extracellular medium containing (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 10 D-glucose, 2 $MgCl_2$, 2 $CaCl_2$, adjusted to pH 7.4 with NaOH.

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. The bath was heated to 32°C using a temperature control system (Badcontroller V, Luigs & Neumann) and was supplemented in $GABA_A$ and $GABA_B$ receptors antagonists SR95531 (10 µM) and CGP55845 (5 µM; Tocris), respectively, in order to block inhibitory neurotransmission. Whole-cell recordings were performed using 1.5 mm external diameter borosilicate pipettes (GC150F-10, Harvard Apparatus) prepared with a micropipette puller (P97, Sutter Instruments). Electrodes (3.5-5 MΩ) were filled with a solution containing (in mM): 125 $CsCH_3SO_3$, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES, 4 Na_2-ATP, 0.4 Na_3-GTP, 5 QX-314, adjusted to pH 7.25 with CsOH. Alexa-568 was added to the internal solution for further immunodetection.

Recordings were performed using a Multiclamp 700A amplifier and a Digidata 1322A interface controlled by Clampex 10.1 (Molecular Devices). Signals were sampled at 20 kHz and low-pass filtered at 2 kHz, respectively. sEPSC detection and analysis was performed using an in-house software (Detection Mini, Michel Goillandeau). Access resistance and leak currents were monitored continuously and experiments were discarded if these parameters changed by more than 15% during recording.

Brain slices: P16-18 Sprague-Dawley rats anesthetized with isoflurane and parasagittal brain slices (350 µm-thick) were prepared in an ice-cold sucrose buffer solution containing (in mM): 250 sucrose, 2 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 1.15 NaH_2PO_4, 11 glucose, and 26 $NaHCO_3$ (gassed with 95% O2 / 5% CO2). Slices were then incubated for 30 min at 33°C and subsequently stored at room temperature in an artificial CSF (aCSF) solution containing (in mM): 126 NaCl, 3.5 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 12.1 glucose (gassed with 95% O2 / 5% CO2; pH 7.35). Whole-cell voltage clamp recordings of CA1 pyramidal cells were performed using infrared differential interference contrast microscopy under continuous perfusion of heated ACSF (32°C) saturated with 95% O2 / 5% CO2. Electrodes (4–5 MΩ) were prepared from borosilicate pipettes (GC150T-10, Harvard

Apparatus) with a vertical micropipette puller (PC-10, Narishige) and filled with a solution containing (in mM): 120 cesium methanesulfonate, 4 NaCl, 4 MgCl2, 10 HEPES, 0.2 EGTA, 4 Na_2ATP, 0.33 Na_3GTP, and 5 phosphocreatine adjusted to pH 7.3 with CsOH. sEPSC were recorded using an EPC10 USB amplifier (HEKA Elektronik) at -70 mV in the presence of bicuculline (20 µM) in order to block GABAA receptors. Signals were sampled at 20 kHz and low-pass filtered at 2 kHz, respectively. sEPSC detection and analysis was performed using an in-house software (Detection Mini, Michel Goillandeau). Access resistance and leak currents were monitored continuously and experiments were discarded if these parameters changed by more than 15% during recording.

9. ^{3}H-glutamate Uptake

^{3}H-glutamate uptake assays were performed on both COS-7 cell and mixed hippocampal cell cultures (astrocytes and neurons). Incubations with radiolabelled glutamate were performed in HEPES-buffered saline (HBS) solution (5 mM Tris base, pH 7.4, 10 mM HEPES, 140 mM NaCl, 2.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM K_2HPO_4, 10 mM glucose) supplemented with 50 µM L-glutamate. We found it was vital to add glutamate to the medium in the initial washes. Glutamate is a negatively charged amino acid and binds to the positively charged polylysine used to promote cell adhesion in culture dishes. Thus, if we failed to add non-radioactive glutamate in our initial wash medium we observed a lot of false positive ^{3}H-glutamate uptake.

Cells were washed twice with Na^+-free HBS solution (prepared by equimolar replacement of Na^+ with choline) at 37°C then incubated with normal HBS (containing 1 µCi/ml ^{3}H-glutamate) at 37°C for 6 min. Assays were stopped by aspiration of HBS (containing ^{3}H-glutamate) followed by two washes with ice-cold Na^+-free HBS and dishes were also placed on ice. Mixed cultures were then lysed in 0.1 M NaOH and accumulated radioactivity measured by liquid scintillation counting.

Liquid scintillation counting is a technique which incorporates a radiolabelled analyte with a liquid medium capable of converting the kinetic energy of nuclear emissions into light energy. Briefly, after lysis of cells, the lysate was mixed with a flow detection cocktail ULTIMA-Flo (Pearson-Elmer), which is a mixture of a solvent and solute. In this cocktail the energy released by beta particles during the decay of ^{3}H-glutamate, excites the solvent molecule which emits UV light, this UV light the excites solute molecules, which are fluorophores and emit blue light flashes which are detected by photon multipliers. Thus the more ^{3}H-glutamate molecules in the cell lysate, the more radioactive decays we will observe and thus it is possible to quantify the amount of glutamate taken up in each condition by measuring the nuclear disintegrations per minute (d.p.m.). All values are displayed in d.p.m.

10. Acid Wash

To investigate how long GLT-1^{flag} stays at the surface of a cell we used an acid wash protocol (Tardin *et al.*, 2003). For acid wash experiment we used a low pH (pH 2) aCSF of the same composition as for live QD imaging at 4°C. Mixed hippocampal cultures, transfected with GLT-1^{flag} were incubated for 10 min (37˚C) with monoclonal antibodies against FLAG epitope (10 µg/ml; Stratagene) to tag GLT-1^{flag}. Cells were then washed and incubated for 10 min (37˚C) with quantum dots 655 goat F(ab')2 anti-mouse IgG (1 µg/ml; Invitrogen). Following this step, cells were imaged in exactly the same manner as for QD imaging (1000 frames, 50ms/frame acquisition) and cells were left for 10, 15, 20 and 30 min intervals before a 1 min 'acid wash' (incubation with a low pH, cold aCSF; pH 2, 4°C) to break antibody-protein bonds and so the only transporters with QDs still attached are the ones that have been internalised. This acidic aCSF was then removed and replaced with normal aCSF (pH 7.4, 37°C) and another QD imaging series was acquired.

Total numbers of trajectories were compared in before and after acid wash at different time points to assess the internalisation of GLT-1^{flag}-QD complexes during these specific time periods.

11. Data and Statistical Analysis

For imaging data, statistical values are given as mean ±s.e.m. or median ±interquartile range, defined as the interval between 25 to 75% percentile. Statistical significance was tested using Prism 4.0 (GraphPad). Normally distributed data sets were compared using the paired Student's t-test and unpaired Student's t-test. Statistical significance between more than two normally distributed data sets was tested by one-way analysis of variance test, followed by a Newman-Keuls test to compare individual pairs of data. Non-Gaussian data sets were tested by non-parametric Mann-Whitney test. Indications of significance correspond to *p*-values $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Results

1. Diffusion characteristics of GLT-1

We set out to determine if GLT-1 displays surface trafficking properties on the surface of astrocytes, using tools which had been developed to investigate the surface diffusion of proteins on neurons. This transfer of technology from neurons to astrocyte was straightforward due to the specificity of antibodies for this modified GLT-1 (GLT-1^{flag}), thus we were certain that our QD-tagging of GLT-1^{flag} was specific. Astrocytes were transfected with both GFP and GLT-1^{flag}. We then randomly selected astrocytes which were double transfected and recorded GLT-1 trajectories from these cells under basal conditions (Figures 10A-C). We observed that GLT-1 does indeed diffuse on the surface of astrocytes (Figures 10C and D) and its instantaneous diffusion coefficient (D) is very fast (D = 2.3 x 10^{-1} $\mu m^2/s$ ± 1.2-3.6 x 10^{-1} $\mu m^2/s$; *n* = 720 trajectories; Figure 10E), compared to what has been reported in the literature for neurons (Groc *et al.*, 2004), astrocytes (Arizono *et al.*, 2012) and microglia (Toulme and Khakh, 2012). Furthermore, we observed that the immobile fraction (D < 0.005 $\mu m^2/s$) of the total GLT-1 population was low at only 6% (Figure 10F). Even though these transporters are moving very fast, they are still subject to confinement (Figure 10G). This suggests that there may be some regulatory mechanism controlling the diffusion of GLT-1 on the surface of astrocytes.

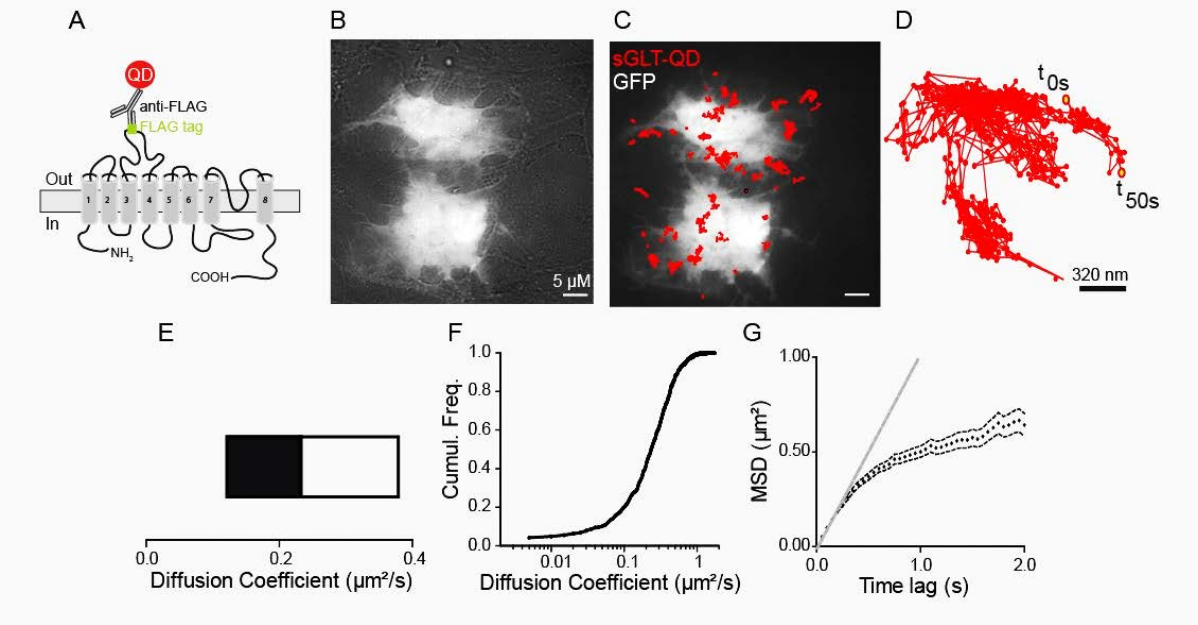


Figure 10.Characteristics of GLT-1 diffusion.

(A) Schematic representation of GLT-1 that has been modified to express a flag (DYKDDDK) domain on the extracellular loop, between Pro^{199} and Pro^{200}, allowing surface quantum dot labelling. **(B)** Overlay of eGFP expressing astrocyte on DIC image taken from hippocampal culture showing both astrocytes and neurons in the same environment. **(C)** eGFP expressing astrocyte with 50s long QD trajectories overlaid (1000 frames, 50 ms/frame acquisition).**(D)** A single, enlarged, trajectory of $GLT\text{-}1^{flag}$ moving over 50 s. **(E)** The instantaneous diffusion coefficient distribution of GLT-1 (median ±25-75% IQR): 2.3 x 10^{-1} $\mu m^2/s$ ±1.2-3.6 x 10^{-1} $\mu m^2/s$; n = 720 trajectories. **(F)** The cumulative frequency curve versus membrane diffusion ($\mu m^2/s$) for GLT-1 shows the distribution of the transporters. Note the first point at 0.005 $\mu m^2/s$, anything below this point is considered immobile. Therefore only 6% of GLT-1 is immobile in control conditions. **(G)** Plot of the mean squared displacement (MSD) versus time for GLT-1. The curve exhibits a negative curvature characteristic of confined behaviour.

2. Activity-dependent surface trafficking of GLT-1

2.1 Neuron-free

Following the discovery that GLT-1 moves on the surface of astrocytes, we were intrigued as to whether this diffusion could be regulated in some manner. The existence of a regulatory mechanism is quite logical seeing as the presence of GLT-1 in close proximity to the synapse is extremely important for synaptic transmission (Oliet *et al.*, 2001). We began this line of enquiry by investigating what happens to GLT-1 surface diffusion when there are no neurons in culture. Thus, we prepared a 'neuron-fee' culture (see Materials & Methods). These cultures, primarily astrocytes, were transfected as before with eGFP and GLT-1^{flag}. In these experimental conditions it was particularly important to transfect cells with GLT-1^{flag} as it has been reported that astrocytes in primary cultures lacking neurons, express little or no endogenous GLT-1 (Yang *et al.*, 2009). When searching for transfected astrocytes, before the QD recordings it was clear to us that the morphology of these astrocytes was different to that of astrocytes in mixed cultures of neurons and glia, it appeared that they did not have many processes (Figure 11A). This observation was not quantified. This difference did not end with the morphology, we saw that individual trajectories were smaller and we also observed that the diffusion coefficient of GLT-1 in neuron-free cultures was a lot lower than that of GLT-1 in mixed cultures i.e. Control conditions ($D = 2.9 \times 10^{-4}$ $\mu m^2/s \pm 1.1\text{-}16 \times 10^{-4}$ $\mu m^2/s$, n = 333 trajectories; Figures 11B-D).

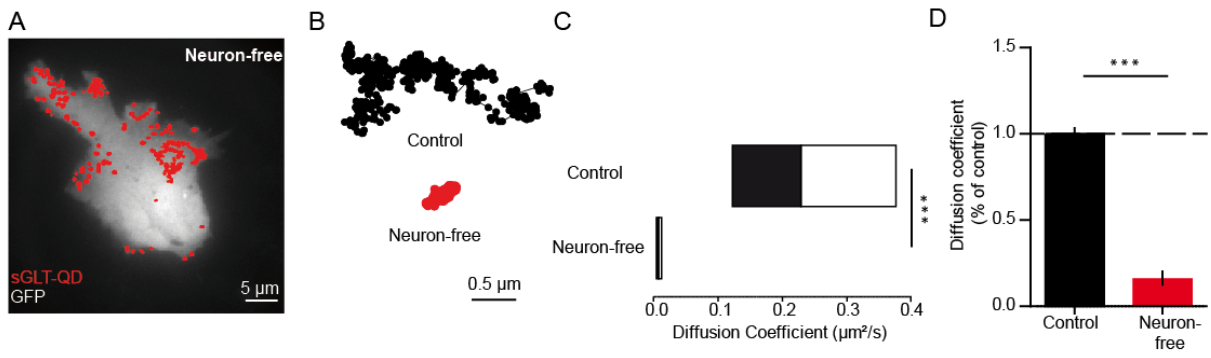


Figure 11. GLT-1 diffuses slower in neuron-free conditions.

(A) eGFP expressing astrocytes with 50s long GLT-1^{flag} trajectories overlaid in neuron-free condition. **(B)** Representative single trajectories of GLT-1^{flag} surface diffusion in control and neuron-free conditions. **(C)** Instantaneous diffusion coefficient (median ±25-75% IQR) of GLT-1 was significantly affected by removal of the majority of neurons in culture. Control: 2.3 ±1.2-3.6 x 10^{-1} µm^2/s; n = 720 trajectories. Neuron-free: 2.9 x 10^{-4} µm^2/s ± 1.1-16 x 10^{-4} µm^2/s, n = 333 trajectories. $P < 0.001$. **(D)** Normalised data expressing the decrease in the diffusion coefficient between control and neuron-free conditions (84% decrease compared to control, $p < 0.001$).

2.2 Decreased neuronal firing

After elucidating the effect of the absence of neurons upon GLT-1 surface diffusion, we investigated whether this effect was due to having no neurons in culture or simply the lack of neuronal activity. We employed TTX, a potent blocker of voltage gated Na^+ channels, which effectively blocks action potentials in neurons to investigate whether absence of neuronal firing could have an impact on GLT-1 surface diffusion. The effect of this drug is highly specific because neurons are the only brain cells which communicate by action potential firing. Thus, we incubated mixed hippocampal cultures, containing neurons and glia, with TTX (1 µM) for a maximum of 20 min, acquiring images (1000 frames, 50ms/frame acquisition) before TTX application and after 10, 15 and 20 min incubation with TTX. We

saw that after only 20 min there was a significant reduction in the diffusion coefficient of GLT-1 (Figures 12A-D).

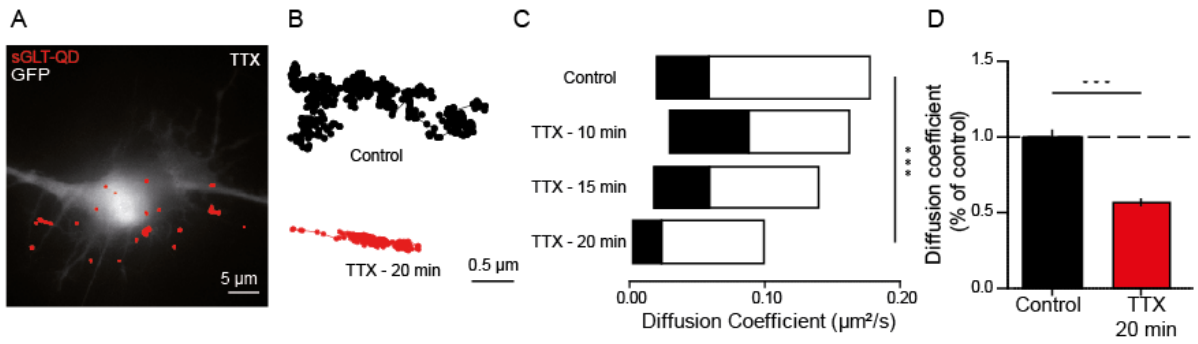


Figure 12. Blocking neuronal firing reduces GLT-1 surface diffusion on astrocytes.

(A) eGFP expressing astrocytes with 50s long GLT-1^{flag} trajectories overlaid after 20 min TTX application. **(B)** Representative single trajectories of GLT-1^{flag} surface diffusion in control and TTX (1 µM, 20 min) conditions. **(C)** Instantaneous diffusion coefficient (median ±25-75% IQR) of GLT-1 was significantly reduced after 20 min TTX application. Control: 5.9 ±2.1-18.1 x 10^2 µm^2/s; n = 1891 trajectories. TTX - 10 min: 8.8 x 10^2 µm^2/s ±3.1-16 x 10^2 µm^2/s, n = 2199 trajectories. TTX - 15 min: 5.9 x 10^2 µm^2/s ±1.8-14 x 10^2 µm^2/s, n = 2113 trajectories. TTX - 20 min: 2.4 x 10^2 µm^2/s ±0.3-9.9 x 10^2 µm^2/s, n = 2199 trajectories. $p < 0.001$. **(D)** Normalised data expressing the decrease in the diffusion coefficient between control and TTX (20 min) conditions (42.5% decrease compared to control, $p < 0.001$).

2.3 Glutamate transport blocker TBOA

We have determined without doubt that the presence and activity of neurons have effects on GLT-1 diffusion, thus we decided to investigate whether the activity of the transporter itself could affect its surface diffusion. We began by blocking the activity of the transporter using a general blocker of glutamate transporters TBOA (30 µM). TBOA was added to the bath in the same manner as TTX, a general bath application with images acquired (1000 frames, 50 ms/frame acquisition) before and after 0, 2, 5 and 15 min incubation with TBOA. We observed a significant decrease in GLT-1 surface diffusion after 5 minute TBOA incubation which remained after 15 minutes (Figures 13A-D).

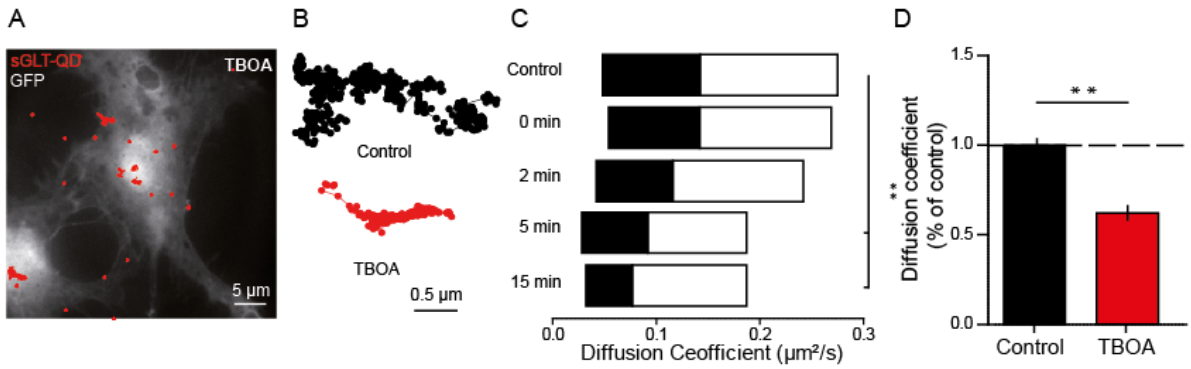


Figure 13. GLT-1 surface diffusion is reduced when transporter is blocked.

(A) eGFP expressing astrocytes with 50s long GLT-1^{flag} trajectories overlaid after 15 min TBOA application. **(B)** Representative single trajectories of GLT-1^{flag} surface diffusion in control and TBOA (30 µM, 15 min) conditions. **(C)** Instantaneous diffusion coefficient (median ±25-75% IQR) of GLT-1 was significantly reduced after 5 min TBOA application. Control: 1.4 ±0.49-2.7 x 10^1 µm^2/s; n = 655 trajectories. TBOA - 0 min: 1.4 ±0.55-2.7 x 10^1 µm^2/s, n = 601 trajectories. TBOA - 2 min: 1.1 ±0.43-2.4 x 10^1 µm^2/s, n = 535 trajectories. TTX - 5 min: 9.1 ±2.9-18.4 x 10^2 µm^2/s, n = 554 trajectories. $p < 0.01$. TTX - 15 min: 7.7 ±3.3-18.54 x 10^2 µm^2/s, n = 432 trajectories. $p < 0.01$. **(D)** Normalised data expressing the decrease in the diffusion coefficient between control and TBOA (15 min) conditions (36% decrease compared to control, $p < 0.01$).

2.4 Glutamate, the substrate of GLT-1 transporters

We predicted that glutamate may have a strong effect on GLT-1 surface diffusion due to the fact that the primary role of GLT-1 it to remove glutamate from the extracellular synapse. Thus we applied glutamate to the bath at a non-toxic concentration (100 µM). As for previous experiments we used mixed hippocampal cultures, placed them under the microscope and acquired one QD image (1000 frames, 50 ms/frame acquisition) before and after adding the drug, taking images at several time points. In this case, we observed that the effect of glutamate was be transient, increasing as long as glutamate is present and then

going back to control conditions when the levels of glutamate go back to baseline. Thus we acquired images at 2, 5 and 10 minute intervals (Figure 14A-D).

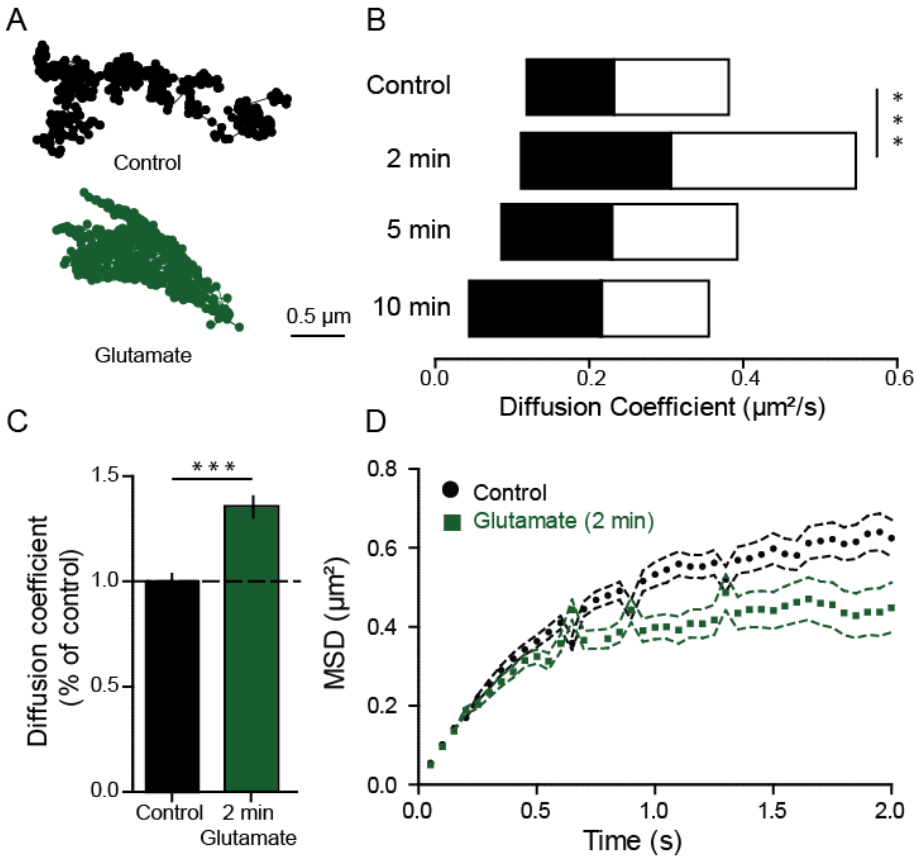


Figure 14. Glutamate transiently increases the surface diffusion of GLT-1.

(A) Representative single trajectories of GLT-1^{flag} surface diffusion in control and Glutamate (100 µM, 2 min) conditions. **(B)** Instantaneous diffusion coefficient (median ±25-75% IQR) of GLT-1 after 2, 5 and 10 min glutamate application. Control: 2.3 x 10^{-1} µm^2/s ± 1.2-3.6 x 10^{-1} µm^2/s; n = 720 trajectories. Glutamate-2min: 3.0 x 10^{-1} µm^2/s ± 1.1-5.4 x 10^{-1} µm^2/s; n = 855 trajectories; $p < 0.01$. Glutamate-5min: 2.7 x 10^{-1} µm^2/s ± 1.4-4.4 x 10^{-1} µm^2/s; n = 511 trajectories; $p > 0.05$. Glutamate-10min: 2.3 x 10^{-1} µm^2/s ± 0.7-3.9 x 10^{-1} µm^2/s. n = 394 trajectories. $p > 0.05$. **(C)** Normalised data expressing the increase in the diffusion coefficient between control and Glutamate conditions (36% increase compared to control, $p < 0.001$). **(D)** Plot of the mean squared displacement (MSD) versus time showing that glutamate induces increase in confinement of transporters.

2.5 Does glutamate act directly on GLT-1 or indirectly through glutamate receptors?

The previous findings, that glutamate increases GLT-1 surface diffusion, is quite ambiguous as glutamate can act on receptors as well as binding to transporters. We investigated whether the effect of glutamate on GLT-1 surface diffusion was due to glutamate acting directly on the transporter or glutamate. To do this, we applied a cocktail of glutamate receptor antagonists (GluR antag.) comprising AP5 (10 µM), NBQX (10 µM) and MCPG (500 µM) to inhibit NMDARs, AMPARs and mGluRs respectively. We found that simply adding this antagonist cocktail alone reduced GLT-1 surface diffusion (Figures 15A-D). This draws attention to the fact that GLT-1 is a highly sensitive to the activity of glutamate receptors.

We then examined whether glutamate could still impact on the surface diffusion of GLT-1in the presence of the GluR antagonist cocktail. Thus, we incubated cells in the GluR antagonist cocktail for 5 minutes, as in the previous experiment, and then added glutamate at 100 µM to elucidate the effects of glutamate directly on the transporter. We observed that the effect of glutamate was conserved. However, this increase was to a lower extent than that of glutamate alone (Figures 15C and D); glutamate alone increased GLT-1 surface diffusion by 36% while glutamate in the presence of GluR antagonists resulted in a 14% increase in the diffusion coefficient.

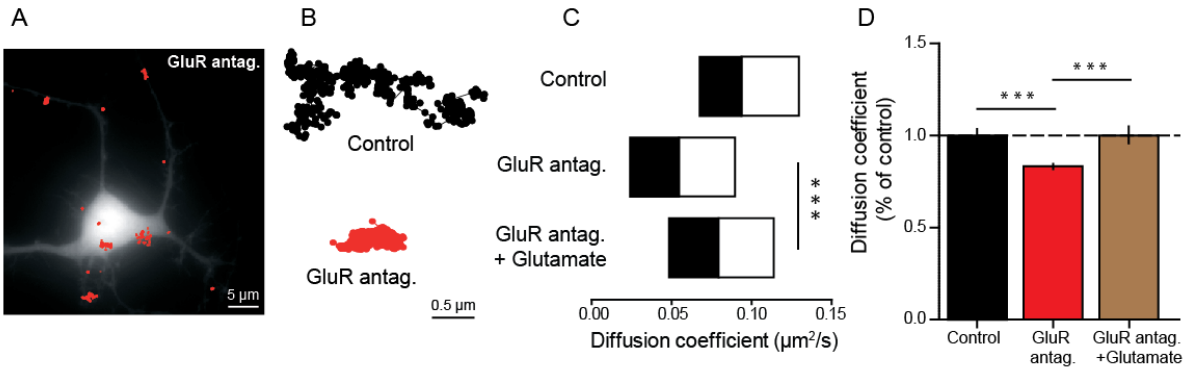


Figure 15. GLT-1 is highly sensitive to activity of glutamate receptors.

(A) eGFP expressing astrocytes with 50s long GLT-1^{flag} trajectories overlaid in glutamate receptor antagonist (GluR antag.) condition. **(B)** Representative single trajectories of GLT-1^{flag} surface diffusion in control and GluR antag. conditions. **(C)** Instantaneous diffusion coefficient (median ±25-75% IQR) of GLT-1 was significantly affected by removal of the majority of neurons in culture. Control: 8.9 x 10^{-2} µm²/s ± 5.3-12.3 x 10^{-2} µm²/s; n = 2100 trajectories. GluR antag.: 6.3 x 10^{-2} µm²/s ± 2.9-9.8 x 10^{-2} µm²/s, n = 1986 trajectories, $p < 0.001$ GluR antag. +Glutamate: 7.1 x 10^{-2} µm²/s ± 4.2-10.7 x 10^{-2} µm²/s. n = 2226 trajectories, $p < 0.001$. **(D)** Normalised data expressing changes in the diffusion coefficient between control and GluR antag. conditions (17% decrease compared to control, $p < 0.001$) and control and GluR antag. +Glutamate conditions (14% increase compared to GluR antag., $p < 0.001$).

3. Compartmentalisation of GLT-1

3.1 Soma versus process

We tested the hypothesis that the surface diffusion of GLT-1 may be compartmentalised, i.e. have differential diffusion properties depending on its location on the surface of astrocytes. We began by investigating whether there was a difference in the diffusion coefficient between the soma and the processes of the astrocytes, in mixed hippocampal cultures control conditions. However, first we needed to confirm that astrocytes in our culture model have processes distinct from the soma of the cell. Cultures were transfected with GLT-1^{flag}, as for QD experiments. Cells were then fixed and stained for surface GLT-1^{flag} using anti-flag antibodies and appropriate secondary antibodies. Cells were then imaged using confocal microscopy. We observed that astrocytes do indeed have processes and that GLT-1 forms clusters on the surface of the cell at the level of the processes (Figures 16 A and B). These findings convinced us that our culture system retained some physiological structures found in more intact systems and we were confident to proceed to live imaging of GLT-1 in these distinct compartments.

We recorded trajectories from GLT-1^{flag} and during the analysis separated trajectories on the soma from those on the processes. We observed that GLT-1 moved slower in the processes compared to the cell body (Figures 16C and D).

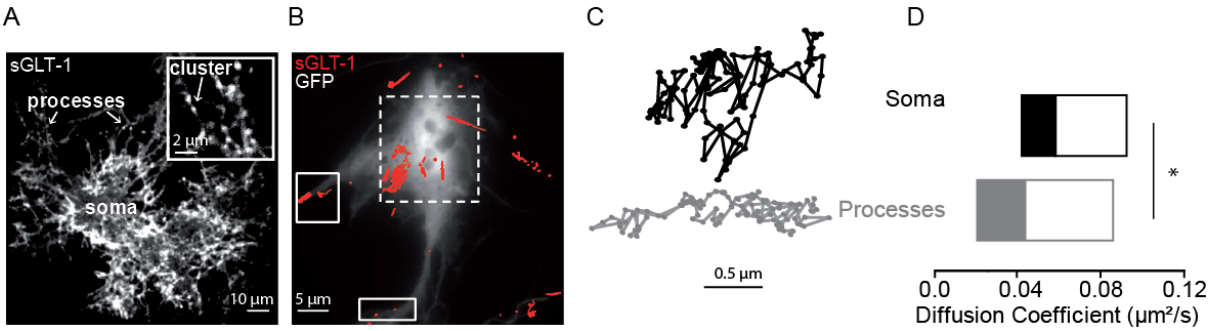


Figure 16. GLT-1 surface diffusion differs between soma and processes of astrocytes.

(A) Surface staining for GLT-1 to confirm presence of distinct processes and soma in culture. **(B)** eGFP expressing astrocyte with 50s long GLT-1^{flag} trajectories overlaid. Boxes with broken lines denotes processes compared to box with whole line, which denotes the soma. **(C)** Representative single trajectories of GLT-1^{flag} surface diffusion on the soma and processes. **(D)** Instantaneous diffusion coefficient (median ±25-75% IQR) of GLT-1 is lower in the processes compared to the soma. Soma: 5.9 x 10^{-2} µm^2/s ± 4.2-9.0 x 10^{-2} µm^2/s; n = 1601 trajectories. Processes: 4.4 x 10^{-2} µm^2/s ± 2.1-8.3 x 10^{-2} µm^2/s, n = 416 trajectories, $p < 0.05$.

3.2 Synaptic compartment

Following the observation that GLT-1 diffuses slower on the processes compared to on the cell body, we further investigated whether this reduction of GLT-1 surface diffusion at the processes could be due to the possibility that astrocytic processes are closely apposed to synapses. We hypothesised that due to the importance of GLT-1 for the removal of synaptically released glutamate, there must exist some mechanism to favour GLT-1 retention at the astrocyte membrane close to synapses. We began by verifying that our culture model had glutamatergic synapses which were surrounded by GLT-1. This was achieved by fixing cultures and staining for surface GLT-1^{flag}, cells were then permeabilised and stained for Shank, a postsynaptic scaffold protein found in glutamatergic synapses. Imaging was carried out using confocal microscopy. Following a line scan analysis of the acquired images, clusters of GLT-1 were found to bedirectly colocalised or very close to

synaptic shank staining (Figure 17A). At higher magnification it was sometimes possible to see shank completely surrounded by GLT-1 (Figure 17B). This further validated our culture model as these finding reflect observations reported in more intact brain preparations.

After confirming the presence of GLT-1 transporters around glutamatergic synapses, we examined the surface diffusion properties of GLT-1 at the synapse. Mixed hippocampal cultures were electroporated with Homer 1c-dsRed, to label synapses in live cultures, before plating (see Material & Methods). Astrocytes were then transfected with GLT-1^{flag} 24 hours before imaging session. Neurons expressing Homer 1c-dsRed were imaged before acquiring QD trajectories of GLT-1^{flag}. During the analysis the synaptic area was defined as the total synaptic staining with an annulus of 320 nm. GLT-1 is not a neuronal synaptic protein, thus we do not expect to find it directly colocalised with synapses but rather on the surface of astrocytes very close to synapses. Nevertheless, we often observed colocalisation between GLT-1 and synaptic markers (Figures 17A-C). This is likely due to the small size of astrocyte processes, their proximity to synapses and the resolution of our microscope. During our QD imaging we often saw trajectories that went from outside the synapse into the synaptic area (Figure 17C). Interestingly we saw that the surface diffusion of GLT-1 was greatly reduced in the synaptic area compared to the synapse (Figure 17D).

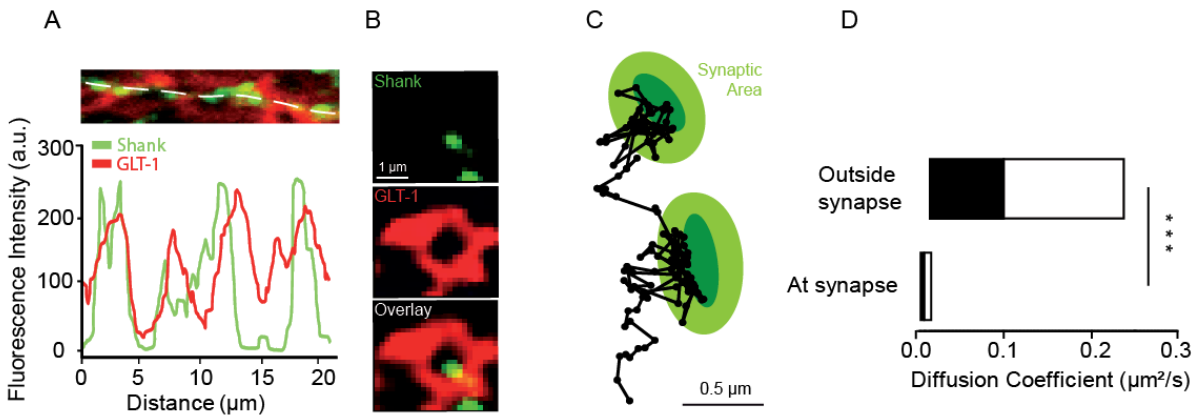


Figure 17. GLT-1 surface diffusion is greatly reduced inside the synaptic area.

(A) Surface staining for GLT-1 and postsynaptic protein Shank, confirms presence of GLT-1 at the synapse. **(B)** High magnification confocal image showing GLT-1 completely surrounding the synapse. **(C)** Representative single trajectory of GLT-1^{flag} moving from outside to inside the synaptic area and then going to a different synapse. **(D)** Instantaneous diffusion coefficient (median ±25-75% IQR) of GLT-1 is reduced in the synaptic area. Outside synapse: 8.9 x 10^{-2} µm^2/s; IQR ± 0.8-22 x 10-2 µm2/s; n = 444 trajectories. At synapse: 1.3 x 10^{-3} µm^2/s; IQR ± 0.014-4.6 x 10^{-3} µm^2/s, n = 61 trajectories, $p < 0.001$.

3.3 Mimicking synaptic activity using caged glutamate

The fact that GLT-1 diffusion is reduced in the vicinity of synapses and that for some unknown reason this transporter also moves away from these synapses was very intriguing. We hypothesised that GLT-1 moves to synaptic areas where it is stabilised and remains until a signal, possibly glutamate binding to the transporter, allows GLT-1 to overcome the forces which anchor the transporter to the synapse allowing GLT-1 to displace away from the synapse leaving space for a non-bound transporter to move into the confines of the synapse to further bind and buffer glutamate.

One method of testing this hypothesis was through glutamate uncaging experiments. In this way we can control the exact moment when GLT-1 transporters are exposed to glutamate. We began by transfecting astrocytes with GLT-1^{flag}, as in previous experiments,

and marking all synapses with Mitotracker-green an AM dye which stains mitochondria-rich areas such as the synapse. Mitotracker was used as a synaptic marker due to its high level of staining, the chance probability of finding a GLT-1^{flag} transfected astrocyte and a Homer 1c-dsRed transfected neuron in close apposition is quite low resulting in low-yield experiments. When using caged-glutamate it is important to maximise data yield thus, mitotracker was optimal for this purpose staining practically all synapses in culture dishes. As opposed to previous QD imaging experiments, here we needed to use an imaging system equipped with a 405 nm laser in order to uncage glutamate. We observed that uncaging close to synapses where GLT-1 was present, induced a significant increase in GLT-1 surface diffusion (Figures 18A and B). This was not the case for experimental conditions where glutamate was uncaged close to GLT-1 at non-synaptic sites and importantly no change in diffusion coefficient was observed in the absence of caged glutamate i.e. 405 nm laser alone (Figures 18A and B). Furthermore, we found that uncaging at synaptic sites resulted in a decrease in the synaptic dwell time for GLT-1 (Figure 18C) indicating that not only does exposure to glutamate increase GLT-1 diffusion, the transporter also moves away from the synapses as predicted.

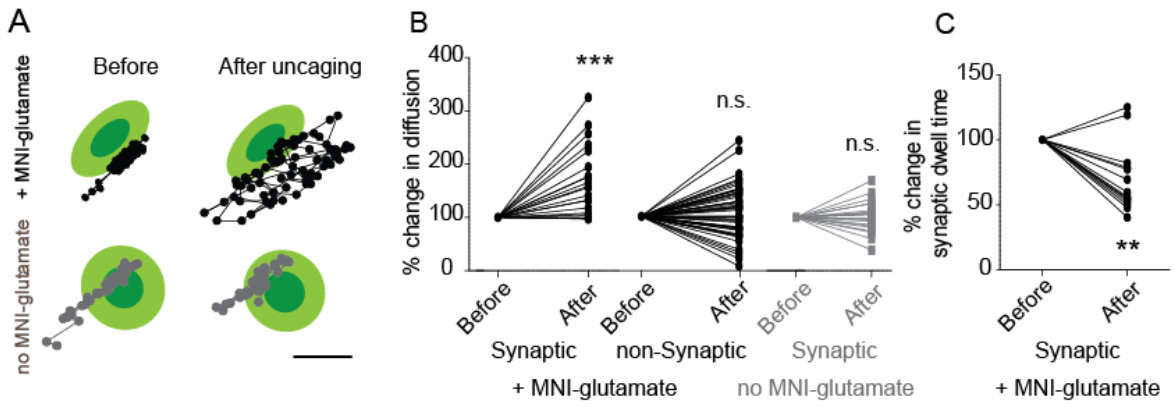


Figure 18. Synaptic glutamate uncaging increases GLT-1 diffusion and displaces transporters from the synapse.

(A) Representative trajectories of a single GLT-1^{flag} at a synapse, before and after uncaging in the presence (top) or absence (bottom) of MNI-caged-glutamate (Scale bar 0.32 µm). **(B)** % change in GLT-1 surface diffusion before and after uncaging laser at the synapse (56% increase after uncaging, n = 21, p< 0.001), at non-synaptic sites (0% change, n = 53, p> 0.05) and absence of MNI-caged-L-glutamate (2% increase, n = 22, p>0.05). **(C)** Significant decrease in synaptic dwell time for GLT-1 transporters after uncaging (p< 0.01).

4. Computational model of GLT-1 diffusion

Together, these data lead us to hypothesise that surface diffusion of GLT-1 could be important for the buffering and physical removal of glutamate from the synapse on a synaptic timescale. We tested this hypothesis using a computer simulation with parameters based on previous findings, in which i) GLT-1 density was estimated to be roughly 8500/μm^2 on the surface of hippocampal astrocytes (Lehre and Danbolt, 1998), ii) around 4000 glutamate molecules are released during one synaptic event, relating to the content of a single vesicle (Clements *et al.*, 1992) and iii) the surface area of astrocyte surrounding glutamate synapse was 30 nm x 1 µm (see Figure 19A). GLT-1 transporters were allowed to move in and out of the synaptic area, with a diffusion coefficient ranging from D = 0 $\mu m^2/s$ (immobile) up to D = 0.23 $\mu m^2/s$ whereas glutamate molecules remained in the cleft until clearance by transporters (70 ms per molecule per transporter; see Figure 19B). Consistent with the experimental data, the clearance rate of glutamate from the synaptic area was strongly dependent on the diffusion coefficient of the transporter (Figure 19C), with a high GLT-1 dynamics associated with a fast clearance of glutamate, supporting our experimentally-based claim that the surface diffusion of transporters play a key role in the buffering and removal of glutamate from the synapse.

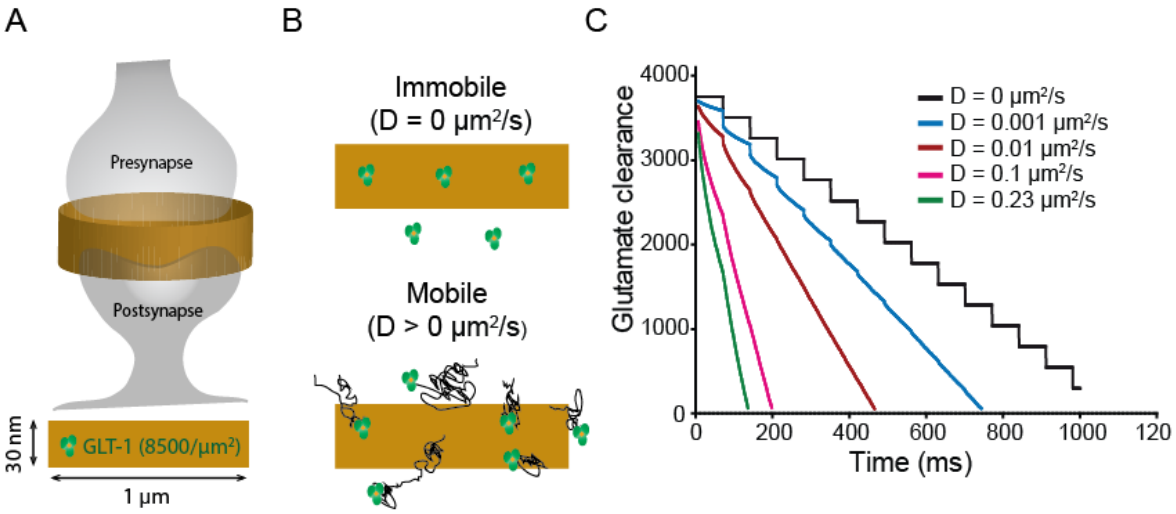


Figure 19. Computational model of the effects of diffusion of GLT-1 on glutamate uptake.

(A) Schematic representation of our computational model. We defined the total surface area of astrocytic synaptic coverage as a 2D box of 30 nm x 1 µm (bottom) in a total space of 1µm^2 (not shown). **(B)** Representation of two different scenarios corresponding to static and dynamic receptors, binding a glutamate molecule while they are in the astrocytic surface in contact with the synaptic cleft. **(C)** Brownian motion of GLT-1 transporters clearing glutamate was numerically simulated and the effect of clearance was analysed for different diffusion coefficients (see Materials and Methods). The clearance rate of available glutamate was found to be strongly dependent on the receptors' diffusion coefficient.

5. Physiological role for GLT-1 surface diffusion

Following our very promising findings in the computational simulation of whether surface diffusion aids GLT-1 in removing glutamate from the confines of the synapse, we moved into a more biologically relevant system. Beginning in cultured cells before finally demonstrating the importance of GLT-1 diffusion in a more intact system, the hippocampal brain slice preparation.

5.1 Immobilisation of GLT-1

One means to investigate whether the surface diffusion of GLT-1 holds any physiological importance is to stop GLT-1 from moving along the surface of the astrocytes and study the consequences of this alteration. To directly tackle this, we took advantage of a previously established strategy to immobilise proteins on the surface of the cell, a procedure known as cross-linking (X-link; see Figure 20A) (Heine *et al.*, 2008). First, it was necessary to establish the concentration of antibodies necessary to effectively reduce the surface diffusion of GLT-1. Our control concentration of primary (anti-flag) antibodies for QD experiments using GLT-1^{flag} transfected into astrocytes was 10 µg/ml, thus we tested several relatively high concentrations of primary antibodies to see which concentration had the greatest effect on GLT-1 surface dynamics. We found that with increasing concentrations of primary antibodies there was an increasing reduction in the diffusion coefficient of GLT-1 (Figure 20B) and decided to continue with the highest antibody concentration that we tested, 200 µg/ml (1/5). This concentration effectively reduced GLT-1 diffusion speed as well as increasing the mobile fraction (Figure 20D) and the confinement of the protein (Figure 20C and E).

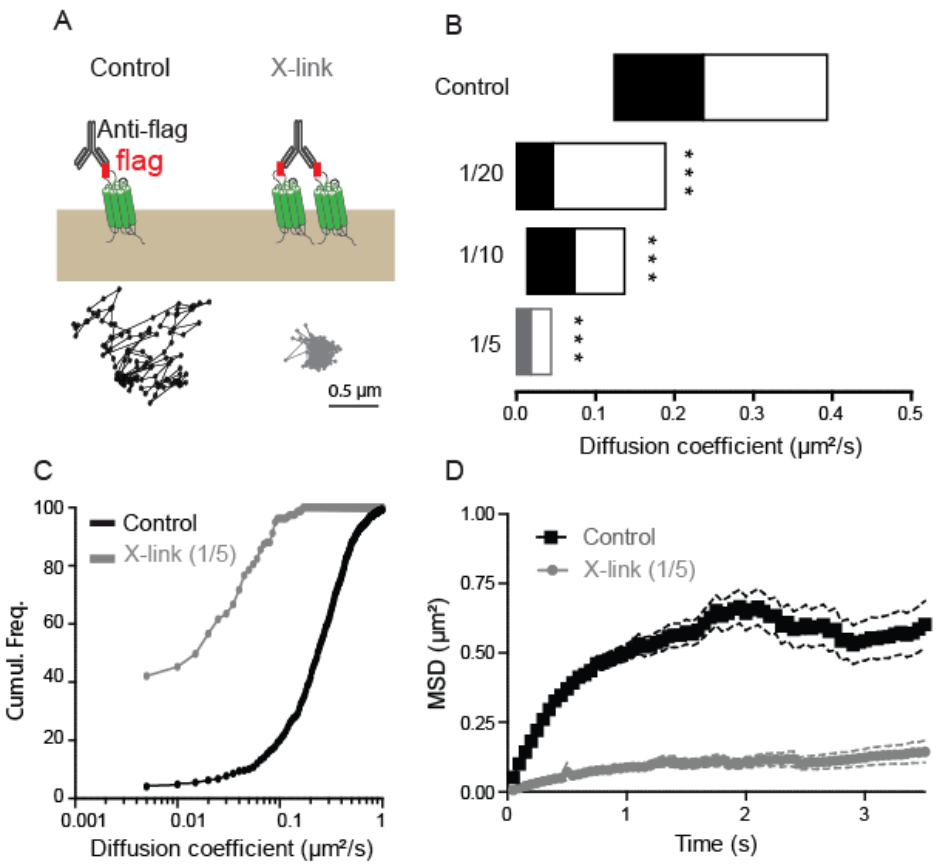


Figure 20. Establishing X-link protocol for GLT-1.

(A) Schematic depicting X-link protocol with representative single trajectories of GLT-1^{flag} in control and X-link (1/5) conditions below. **(B)** Instantaneous diffusion coefficient (median ±25-75% IQR) of GLT-1 is reduced with increasing primary antibody concentration. Control (1/100): 2.3 x 10^{-1} µm^2/s ± 1.2-3.6 x 10^{-1} µm^2/s; n = 720 trajectories. 1/20 antibody: 4.6 x 10^{-2} µm^2/s; IQR ± 0.02-18 x 10^{-2} µm^2/s, n = 362 trajectories, $p < 0.001$. 1/10 antibody: 7.4 x 10^{-2} µm^2/s; IQR ± 1.4-13.6 x 10^{-2} µm^2/s, n = 2010 trajectories, $p < 0.001$. 1/5 antibody: 1.8 x 10^{-2} µm^2/s; IQR ± 0.09-4.4 x 10^{-2} µm^2/s, n = 325 trajectories, $p < 0.001$. **(C)** Cumulative frequency curve distribution of GLT-1 in control and X-link conditions, highlighting the increase in immobile fraction from 6% in control to 42% in X-link. **(D)** Plot of the mean squared displacement (MSD) versus time showing that X-link dramatically increases confinement of GLT-1.

5.2 Secondary effects of GLT-1 X-link

Before carrying out further experiments, it occurred to us that it would be very important to verify that the X-link did not have any other effects on GLT-1, such as transport function or GLT-1 content at the synaptic area. We began by looking at the effect of the X-link on transporter function. This was achieved by measuring radiolabelled glutamate (^{3}H-glutamate) uptake in COS-7 cells, we chose this cell line because there is no endogenous glutamate transport. We started with two conditions, COS-7 cells transfected or not with GLT-1^{flag}, this has already been reported in the literature (Peacey *et al.*, 2009), nevertheless we wanted to be sure that we were doing the experiment correctly. We found that cells transfected with GLT-1^{flag} had a great uptake capacity compared to non-transfected cells (Figure 9A). Next we tested whether X-link has an effect on GLT-1 uptake capacity. COS-7 cells were transfected with GLT-1^{flag} and the chosen X-link concentration 1/5 primary antibody was incubated during 10 min, before the start of the ^{3}H-glutamate uptake experiment. We observed no significant difference between control antibody concentrations and X-link concentrations (Figure 21A).

Next we investigated the possibility that the X-link might affect the GLT-1 content at the synapse. To this end we used immunostaining for surface GLT-1 in control and X-link conditions and measured synaptic colocalisation between these conditions. As in previous experiments, the synaptic area was defined at the synaptic staining plus an annulus of 320 nm. Mixed hippocampal cultures were transfected with Homer 1c-dsRed one day before transfection with GLT-1^{flag}. 48 hours later, antibodies at control and X-link concentrations were incubated for 10 min, as in QD experiments, before fixation in 4 % PFA and incubation with appropriate secondary antibodies for GLT-1^{flag}. Using confocal microscopy, we revealed that there is no difference in the level of synaptic co-localisation between control and X-link conditions (Figures 21B and C).

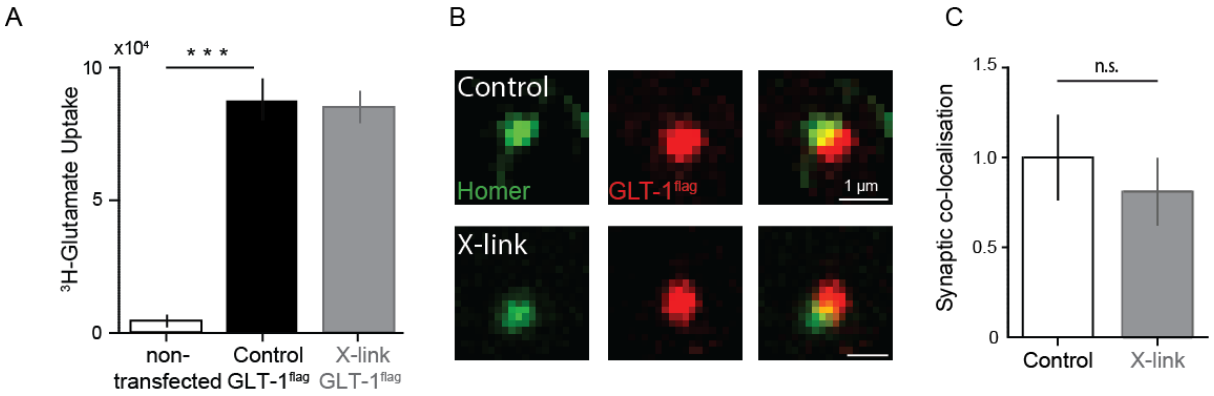


Figure 21. No secondary effects of X-link on GLT-1

(A) COS-7 cells transfected with GLT-1flag had greatly increased glutamate uptake capacities, $p < 0.001$. This uptake capacity was not affected by X-link. Control: 87173 ± 7562 d.p.m. versus X-link: 85211 ± 5719 d.p.m. $p > 0.05$. **(B)** High magnification immunostaining of GLT-1 and Homer 1c-dsRed. **(C)** Quantification of immunostaining revealed no difference in synaptic co-localisation of GLT-1 and Homer 1c-dsRed in X-link condition, $p > 0.05$.

5.3 Effect of GLT-1 X-link on neuronal activity

After finding the concentration of primary antibody necessary for an effective X-link of GLT-1 and then verifying that this X-link had no adverse secondary effects, we investigated the effect of the GLT-1 X-link on neuronal activity. These experiments were carried out in mixed hippocampal cultures at 14-16 days *in vitro*, transfected with GLT-1^{flag} and GFP 24-48 hours before experimentation. Just before experimentation, cultures were incubated with X-link antibody or a control, non-specific antibody. Spontaneous excitatory postsynaptic currents (sEPSC) were recorded in whole-cell patch clamp from neurons in close proximity of GFP/GLT1^{flag} transfected astrocytes (see Figure 22A). We found no difference in the frequency or amplitude of these spontaneous events (Figure 22B). However, we noted a change in the kinetics of single events after X-link (Figure 22C). Further analysis revealed that both rise time and decay were increased (Figures 22D and E), indicating an increase in neuronal excitability due to an increase in the glutamate available to bind to these receptors.

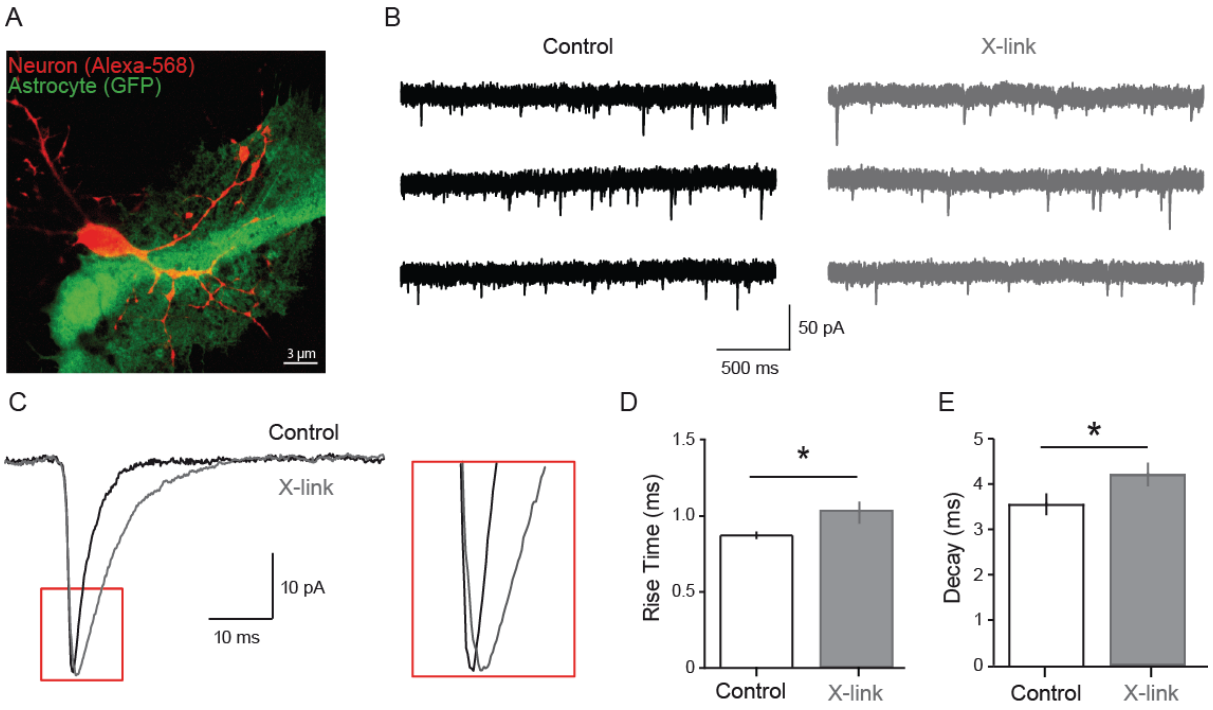


Figure 22. X-link of GLT-1 increases neuronal excitability

(A) Example of Alexa-568-filled neuron recorded in whole-cell patch-clamp in the close proximity of a GFP/GLT-1^{flag}-transfected astrocyte (green). **(B)** Representative sample traces of spontaneous EPSCs recorded from hippocampal neurons in the presence of secondary anti-mouse antibodies (control) or primary anti-GLT-1 antibodies (X-link). **(C)** Superimposition of sEPSCs recorded from hippocampal neurons in the presence of Control (black trace) and X-link (grey trace) antibodies. Inset: magnification of peak currents in Control (black trace) and X-link (grey trace) conditions. **(D and E)** Bar charts showing the increase in rise time and decay of sEPSCs in the presence of X-link compared to Control. $p < 0.05$.

6. Surface diffusion of endogenous GLT-1

At the beginning of this project there were no commercially available antibodies which specifically bind to an extracellular epitope of GLT-1. To carry out QD surface trafficking experiments it is necessary to have an extracellular target due to the fact we are tracking molecules moving on the surface of the cell in real time. For this reason we have used GLT-1^{flag}, which has a flag domain one of the extracellular loop and allows for highly specific conjugation with QDs. Recently, we became aware that some other researchers

were working with antibodies targeting extracellular domains of GLT-1. We found that there was finally a commercially available antibody directed towards the same extracellular loop as where we put our flag domain in the modified GLT-1. Thus we began QD experiments to investigate first of all, whether endogenous GLT-1 (GLT-1^{endo}) diffuses on the surface of astrocytes and secondly, whether this diffusion is similar to what we have previously seen with GLT-1^{flag}.

To first characterise the surface diffusion properties of GLT-1^{endo} we needed to find the appropriate control concentration of primary antibody, while avoiding undesired side-effects such as X-link of GLT-1^{endo}. We tested several concentrations of antibodies and found that the most effective was 0.2 mg/ml, concentrations higher than this affected the diffusion coefficient of GLT-1 (Figure 23A). Comparison with GLT-1^{flag} revealed that endogenous GLT-1^{endo} diffuses slower compared to our transfected GLT-1^{flag} (Figure 23B). Following this observation, we removed all the immobile trajectories (below 0.005 $\mu m^2/s$) and found that there was still a significant reduction (Figure 23B), however this reduction was less pronounced. When plotted on a cumulative frequency curve we observed a very slight left shift in GLT-1^{endo} (Figure 23C). We analysed the MSD of the mobile fraction of GLT-1^{endo} and GLT-1^{flag} and found a higher degree of confinement in GLT-1^{endo} (Figure 23D). As in GLT-1^{flag} conditions it was necessary to verify that the transport function of GLT-1^{endo} was not compromised in X-link conditions. Uptake of radiolabelled glutamate was carried out in mixed cultures with important controls, Na^+ free and TBOA conditions, where uptake should be non-existent, as well as a condition where no antibodies or drugs are added to be compare with our antibody conjugated transporters. We observed that indeed in Na^+ free and TBOA conditions there was no uptake of glutamate, furthermore we observed no difference between, the control condition (No Ab), GLT-1^{flag}, GLT-1^{endo} control or GLT-1^{endo} X-link (Figure 24).

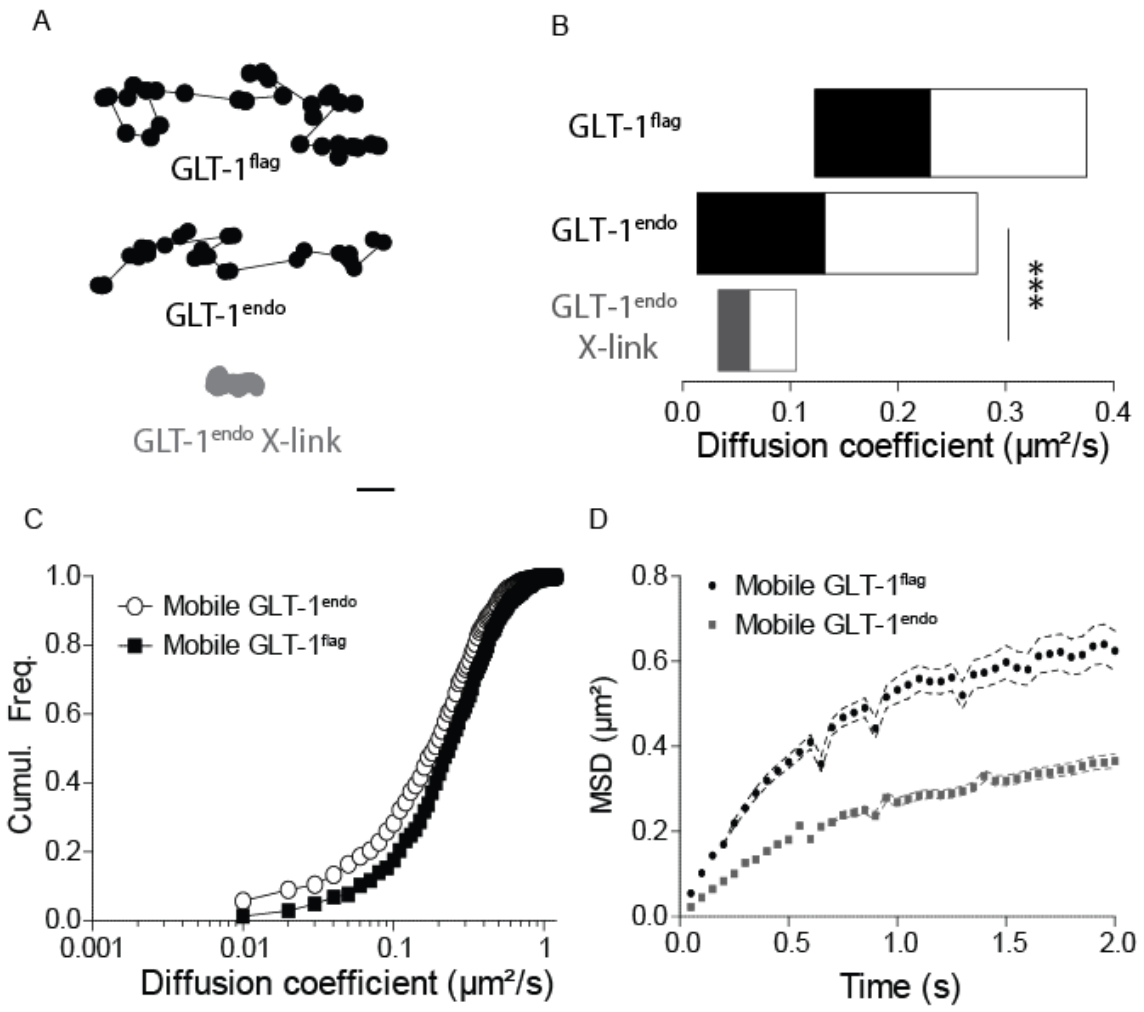


Figure 23. Endogenous GLT-1 surface diffusion

(A) Representative trajectories of GLT-1^{flag} in control conditions and GLT-1^{endo} in control and X-link conditions (scale bar, 0.5 µm). **(B)** Instantaneous diffusion coefficients of GLT-1^{flag}, compared to the GLT-1^{endo} at 1/500 (Diff. Coeff. - 0.13 µm^2/s; IQR ±0.014-0.273 µm^2/s, n = 668 trajectories) and X-link (Diff. Coeff. –0.06 µm^2/s; IQR ±0.033-0.105 µm^2/s, n = 326 trajectories) concentrations ($p < 0.001$). **(C)** Cumulative distribution of the mobile fraction of GLT-1^{flag} and GLT-1^{endo} in control conditions. **(D)** Plot of the mean squared displacement (MSD) versus time showing that the mobile fraction of GLT-1^{endo} is more confined than that of mobile GLT-1^{flag}.

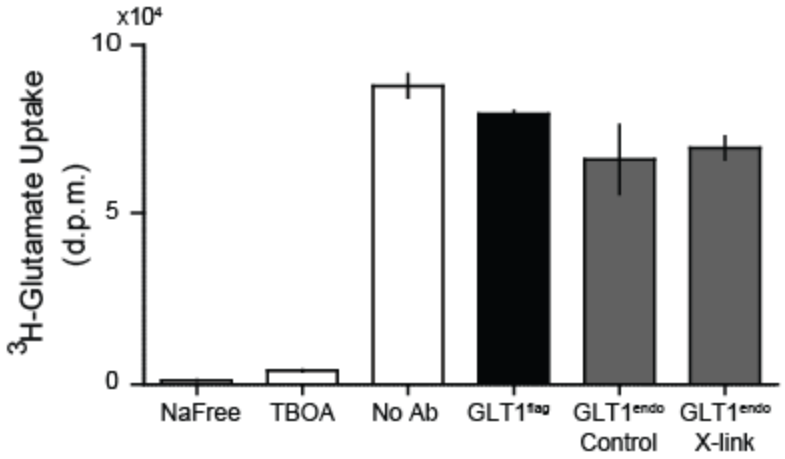


Figure 24. Antibodies do not affect glutamate uptake properties of GLT-1^{endo}

While lack of Na^+ and TBOA blocked glutamate uptake, antibodies has no significant effect on the uptake function of GLT-1^{endo}.

6.1 X-link of GLT-1^{endo} in hippocampal slices

Knowing that we could faithfully track GLT-1^{endo} in hippocampal cultures as well as being able to induce a X-link of GLT-1^{endo}, similar to that of GLT-1^{flag,} we proceeded to investigate whether surface trafficking of GLT-1 could also be important in a more physiologically intact preparation, the hippocampal brain slice.

To induce a X-link of GLT-1 in hippocampal slices it was first necessary to inject the anti-GLT-1 antibody into the hippocampus by *in vivo* stereotaxic injection in anaesthetised animals. Following injection of antibodies into the brain, animals were allowed to recover for 1 hour before sacrifice and slice preparation. Total time between injection and electrophysiological recordings was 3 hours (Figure 25A). We then recorded sEPSCs exactly as in culture conditions and observed the same change in kinetics as was previously observed, i.e. no change in amplitude or frequency of these events (Figure 25B) but rather a change in kinetics with increases in both rise time and decay of sEPSCs (Figures 25C-E).

This finding reinforces all of our *in vitro* data and proves that surface diffusion of GLT-1 is important in intact brain tissue.

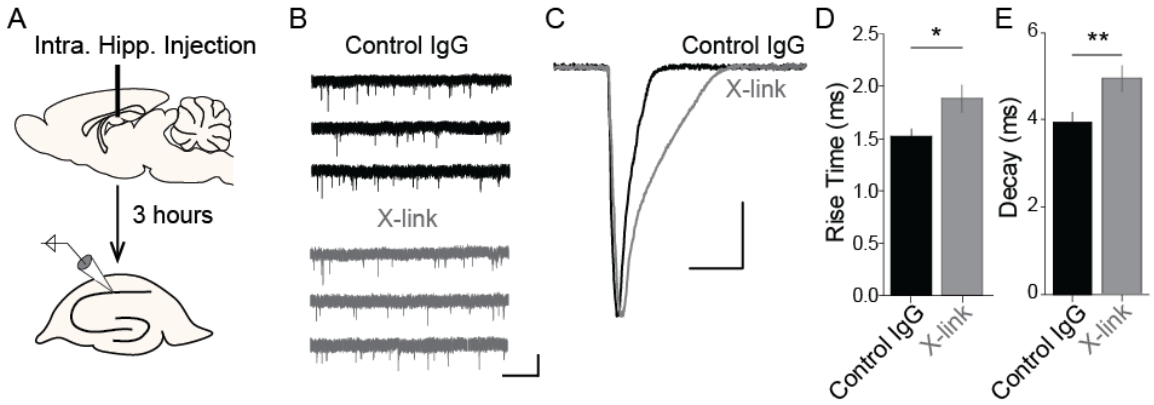


Figure 25. Endogenous GLT-1 surface diffusion

(A) Schematic indicating precise location of stereotaxic injection site, and slice preparation, in the hippocampus of the endogenous GLT-1 antibody used to induce X-link in acute hippocampal brain slices. **(B)** Representative sEPSCs recorded from hippocampal neurons in control and X-link conditions (scale bars 50 pA and 500 ms). **(C)** Superimposition of sEPSC recorded in control (black) and X-link (grey) conditions (scale bars 10 pA and 10 ms). **(D** and **E)** X-link of GLT-1 increased the rise and decay time of AMPAR sEPSCs.

Discussion

1. GLT-1 is highly mobile on the surface of astrocytes

We first demonstrated that GLT-1 diffuses on the surface of astrocytes. When we began, this was a novel finding because there were no studies in the literature demonstrating the existence of surface diffusion of any protein, receptor or transporter, on the surface of astrocytes. Since then a paper has been published in an independent laboratory showing that the metabotropic glutamate receptor 5 (mGluR5) diffuses on the surface of astrocytes (Arizono *et al.*, 2012), as well as this there have been other such publications demonstrating P2X4 receptor trafficking on the surface of microglia (Toulme and Khakh, 2012). One of the most prominent findings of our study is the high diffusion coefficient and low immobile fraction of GLT-1. We have demonstrated that GLT-1 diffuses on the surface of astrocytes 4-5 times faster than that of glutamate receptors, on neurons and astrocytes (Arizono *et al.*, 2012; Heine *et al.*, 2008). We also verified that this was not a phenomenon of all transporters in astrocytes by transfecting the dopamine transporter (DAT), data from DAT more closely resembled that of glutamate receptors from previous studies with slower diffusion coefficient and a higher level of confinement compared to GLT-1. Furthermore the immobile fraction is dramatically lower than what has been reported for glutamate receptors with only 6% of GLT-1 being immobile. This suggests that surface diffusion of GLT-1 is one of a kind, with the majority of transporters moving rapidly. This may imply an important functional role for fast movement of transporters from areas of low synaptic activity towards area of high activity, or *vice versa*, regarding glutamate uptake mechanisms. It is logical to think that if surface diffusion of GLT-1 plays a role in removing glutamate from the synapse through binding/buffering glutamate and then moving out of the

synaptic area, this diffusion would need to be very fast. Thus our primary observation, that GLT-1 is mobile, indeed matches our initial hypothesis.

It is important for us to consider the evidence suggesting that glutamate transporters may indeed form multimers, as witnessed by immunoblotting assays (Haugeto *et al.*, 1996) as well as in crystallographic studies (Yernool *et al.*, 2004). From the more recent study by Yernool and colleagues (2004) it seems that glutamate transporters are homotrimeric, which appears to be the case for the bacterial glutamate transporter (Glt_{Ph}) in their study. Taking into account the fact that there is a high level of homology between the glutamate transporter family, even between species, and evidence from immunoblotting of GLT-1, it is not far-fetched to speculate that GLT-1 is a homotrimer. This implies that, although the general dogma in single nanoparticle imaging studies is that there is a one to one ratio of target protein to quantum dot, it is possible that each quantum dot we are following is in fact attached to one individual GLT-1 in a trimer. This consideration does not diminish the importance of our findings. It is already accepted that glutamate transporters are one of the most highly expressed families of transporters in the brain (Lehre and Danbolt, 1998), therefore evidence suggesting that these transporters group together into multimers does not impact on the potential role of surface diffusion of these transporters in the regulation of synaptic glutamate concentration. To date, there is no quantitative evidence demonstrating which form, monomer or multimer, is the most common in intact brain tissue. Furthermore, it is completely unknown whether multimerisation will help or hinder GLT-1 transport function.

2. Surface diffusion of GLT-1 is activity-dependent

Although this transporter is moving very fast, with very few immobile transporters, we observed that GLT-1 diffuses in a confined manner. This led us to believe that there may exist some form of regulation for this transporter. Indeed, if the surface diffusion of GLT-1 is important for maintaining low levels of synaptic glutamate, it seems logical that there are regulatory mechanisms in place to ensure that sufficient numbers of GLT-1 are distributed at glutamatergic synapses. Consistent with this hypothesis, it would also be necessary to have a readily available pool of transporters sitting in the astrocytic membrane opposing synapses, even at less active synapses, so that following the release of glutamate there are ample numbers of transporters on hand to remove this glutamate from the extracellular space.

We focused on the effects of low activity, both neuronal and glial, on GLT-1 surface diffusion. Due to the importance of these transporters for synaptic glutamate uptake, we decided to investigate the possible role of neurons in the regulation of GLT-1. Previously, it has been reported that astrocytes cultured without neurons exhibit reduced expression of GLT-1 (Swanson *et al.*, 1997; Yang *et al.*, 2009). This is consistent with the fact that the primary role of GLT-1 is to remove glutamate from the extracellular space and neurons are the major contributors of glutamate release in the brain. Furthermore, neurons are most sensitive to the effects of excessive levels of ambient glutamate (Choi, 1985, 1987; Robinson *et al.*, 1993). However, it should be noted that neurons are not the only cells which release glutamate into the extracellular space. Astrocytes are also believed to be capable of glutamate release through mechanisms, vesicular or otherwise, which are subject to fierce debate in the field (Araque *et al.*, 2000; Parpura and Haydon, 2000; Woo *et al.*, 2012).

Crudely removing neurons from our mixed cultures was achieved by regular agitation and removal of cells in suspension before experimentation. Thus, these neuron-free cultures are not ‘pure’ astrocyte cultures but these cultures have a strong reduction in the number of

neurons in the culture dish, which was not quantified. The results from these neuron-free cultures were quite dramatic. We observed that GLT-1 was almost immobile in neuron-free conditions. This finding highlights two very important facts; first, our crude neuron-free culture was quite successful in disrupting neuron-glia communication and second, communication between neurons and astrocytes is of upmost importance concerning GLT-1 surface diffusion. These observations are in agreement with previous studies, which have shown that lack of neurons, results in an absence of GLT-1 expression in astrocytes (Swanson *et al.*, 1997).

Continuing our investigation on the impact of low activity on GLT-1 diffusion, blocking neuronal firing using TTX resulted in a strong reduction in GLT-1 surface diffusion. This observation is in agreement with previous studies which reported that expression of GLT-1 on astrocytes is highly dependent on neuronal activity. Studies in mixed hippocampal cultures found that TTX application over several days can lead to a decrease in the protein expression of GLT-1 (Perego *et al.*, 2000; Yang *et al.*, 2009). Benediktsson and colleagues (2012), working in organotypic slice cultures, found that application of TTX reduced the size and density of GLT-1 clusters on the astrocytic processes as well as reducing the expression of GLT-1 opposing synapses. Finally, Genoud *et al.* (2006) reported that astrocytic coverage of synapses and glutamate transporter expression in the barrel cortex is highly plastic, with expression of GLT-1 highly correlated with neuronal activity *in vivo*. Our findings further underline the highly sensitive nature of GLT-1 surface diffusion. GLT-1 is ideally located to sense and respond to changes in neuronal activity. In our experiments, TTX application was quite short, 20 minutes, nevertheless we observed a significant reduction in the surface diffusion of GLT-1. Thus it seems very likely that GLT-1 may possess the unique capacity to sense neuronal activity and rapidly respond to this by modifying its surface dynamics so that it can stay in line with neuronal release of glutamate and ensure sufficient uptake of this neurotransmitter on a short time scale. Changes in expression levels of glutamate transporters as witnessed by other studies (Perego *et al.*, 2000; Yang *et al.*, 2009) are likely

to be important in the medium- to long-term for preventing excess excitation which in pathology may lead to cell death.

After demonstrating that the diffusion of GLT-1 is highly dependent on neuronal activity, we investigated whether GLT-1 diffusion can be modified by changes in glial activity. The most obvious and pertinent activity which we decided to investigate was the activity of the transporter itself. This was achieved using a non-transportable blocker of glutamate transporters, TBOA. It has been demonstrated that application of TBOA can cause widespread cell death, which can be attenuated by the addition of NMDA and AMPA receptor antagonists (Bonde *et al.*, 2003). Impairing glutamate transporter function, genetically, pharmacologically or in pathology, has been shown to increase epileptiform activity and lead to neuronal cell death by excitotoxicity (Bonde *et al.*, 2003; Robinson *et al.*, 1993; Rothstein *et al.*, 1996; Tanaka *et al.*, 1997).

We found that acute application of TBOA can affect the surface diffusion of GLT-1. Following a relatively short incubation with TBOA, GLT-1 surface diffusion was significantly reduced. This may be due to a direct effect of the drug on the transporter or it may be due to the fact that GLT-1 is no longer functional therefore the surface diffusion is reduced. This finding indicates that the activity of GLT-1 itself can modify its surface diffusion properties. A general trend seems to be clear, when GLT-1 is inactive, as is the case in neuron-free, TTX and TBOA conditions, the surface dynamics of this transporter are reduced. Alterations in the speed of surface diffusion may be important physiologically, ensuring that the transporter remains where it may be needed in the future or moving away from a glutamate-rich area once it has bound free glutamate. If asked to speculate on the mechanism by which TBOA affects the surface diffusion of GLT-1, we could surmise that this change in surface dynamics is due to the conformational change of GLT-1 when TBOA is bound (Boudker *et al.*, 2007). TBOA, which binds to the glutamate binding site without being transported and changes the transporter's conformation, may fix GLT-1 into a conformation which no longer supports fast diffusion dynamics of the transporter.

Conversely we pursued a line of investigation into whether GLT-1 surface diffusion may also be sensitive to increases in neuronal and glial activities. We observed that the application of glutamate, at non-toxic concentrations, induced an immediate increase in the surface diffusion of GLT-1. This is intriguing as one would assume that GLT-1, the transporter responsible for the majority of synaptic glutamate uptake in the hippocampus (Lehre and Danbolt, 1998; Tanaka *et al.*, 1997), should slow down in a glutamate-rich area, i.e. where there is synaptic glutamate release, to ensure that the maximum number of transporters are trapped at the synapse and can remove glutamate on a fast timescale. The observation that glutamate effectively increases transporter diffusion forces us to reassess this hypothesis. We can speculate that GLT-1 moves faster after exposure to glutamate so that synaptic transporters which have bound glutamate move away from the confines of the synapse, thereby opening a space for naïve, unbound transporters to enter the synaptic area to further remove glutamate.

The observation that glutamate increases GLT-1 diffusion is rather ambiguous. The question still remains as to whether the effect of glutamate application is due to glutamate acting directly on the transporter or through glutamate receptors, neuronal and/or astrocytic. To answer this question, we applied a glutamate receptor antagonist cocktail blocking NMDARs, AMPARs and mGluR. Surprisingly, we found that GLT-1 surface diffusion was decreased in the presence of this cocktail alone, suggesting communication between neurons and glia *via* glutamate receptors. The limitation of this experiment is that we cannot distinguish glutamate receptors on neurons from receptors located on astrocytes due to the fact that these antagonists are not cell-specific. Although it is surprising that by blocking glutamate receptors we reduce the surface diffusion of GLT-1, it is not completely unexpected. It has already been demonstrated that inhibition of glutamate receptors can reduce the expression of GLT-1 (Yang *et al.*, 2009). Yang and colleagues (2009), observed that this effect was primarily due to AMPA receptor and group I mGluR (most likely mGluR5) antagonism. The authors proposed that synaptic communication between neurons and

astrocytes induces an upregulation in GLT-1 expression. It is worth noting that Yang and colleagues (2009) incubated slice cultures with glutamate receptor antagonists for 7 days while the effects seen by us on surface diffusion were seen following 5 minute incubation. This, once again, highlights the importance of GLT-1 surface diffusion in the immediate, short-term regulation of glutamate homeostasis.

Continuing on this line of enquiry, we repeated the previous experiment, i.e. adding a glutamate receptor antagonist cocktail, and then bath applied glutamate to observe any potential direct effects of glutamate on GLT-1 itself. We discovered that the increase in surface diffusion following glutamate application was preserved even in the presence of receptor antagonists, albeit to a lower level than glutamate alone. This suggests that glutamate does indeed have a direct effect on GLT-1. However, the effect we see when adding glutamate without glutamate receptor antagonists may be a composite of the glutamate activating glutamate receptors as well as acting on glutamate transporters. Together, these data reinforce the idea that neurons and glia constantly communicate to ensure the proper functioning of these brain cells.

We could further speculate that the effect of glutamate we observe is due to a combination of glutamate binding to astrocytic glutamate receptors, as well as binding to GLT-1. This seems logical with respect to the timescale of the effect. If glutamate was acting on neuronal receptors, resulting in downstream signalling pathways which affect the surface diffusion of GLT-1 we would expect to see a longer latency to increase in surface diffusion. The rapid increase in GLT-1 surface diffusion observed in response to bath application of glutamate suggest that glutamate is more likely to be acting on glutamate receptors on astrocytes. Potentially increasing intracellular Ca^{2+} in the astrocyte which may contribute to the observed change in GLT-1 surface trafficking. It has been well documented that astrocytes signal through changes in intracellular Ca^{2+} (Henneberger *et al.*, 2010; Mulligan and MacVicar, 2004; Panatier *et al.*, 2006) and that these waves are more common in the fine processes that in the cell body (Panatier *et al.*, 2011). Thus, local changes in Ca^{2+}

concentration would be an efficient mechanism to regulate the precise location of transporters in compartments of astrocytes close to active synapses.

3. Diffusion of GLT-1 varies according to its surface location

Previous studies have demonstrated that quite often, proteins diffusing on the cell membrane are subject to confinement, due to the extracellular matrix (Groc *et al.*, 2007), interactions with other proteins (Bats *et al.*, 2007) or by unknown mechanisms. In nature there are generally mechanisms to keep proteins in a specific location for functional reasons. Here, we decided to test whether GLT-1 may be subject to some sort of confinement or differential regulation according to its location. We began by focusing on the differences in surface diffusion properties of GLT-1 located on the processes compared to the cell body. First, it was pertinent to verify that our culture model contained astrocytes with processes as has been previously described in the literature (Benediktsson *et al.*, 2012). Immunostaining for surface GLT-1^{flag} revealed the presence of astrocytic processes in our culture system, closer inspection revealed GLT-1 clusters on the fine processes of the astrocyte. This was reassuring, astrocytes in our culture model indeed retain some features which can be found in more physiological preparations.

Moving on we carried out single molecule trafficking experiments and during post-acquisition analyses we separated trajectories located on cell bodies from those located on the processes using the GFP image of the astrocyte. We observed that trajectories in the processes were moving slower compared to those on the cell body. This could be for several reasons; perhaps GLT-1 moves slower on the processes because this is a more confined space and there is less room to move or maybe GLT-1 on astrocyte processes are located closer to synapses, as it is well documented that astrocytes extend their processes to wrap around synapses (Lehre and Rusakov, 2002; Ventura and Harris, 1999) and there is possibly a retention mechanism to ensure adequate numbers of GLT-1 per synapse for sufficient glutamate uptake on a synaptic time scale.

This finding, contrasts to that of Arizono and colleagues (2012) who demonstrated that mGluR5 moves faster in the processes compared to the soma. As previously

suggested, there may be some role for mGluR5, to locally modulate Ca^{2+} concentration in the fine processes of astrocytes which may in turn signal GLT-1 to change its surface dynamics. Before arriving at any conclusions regarding the potential mechanisms regulating fast moving mGluR5 and slow moving GLT-1 on the processes it is necessary to examine the absolute values regarding the speed of this diffusion. Although we observed that surface diffusion of GLT-1 is slower in the processes compared to the cell body, the absolute speed of GLT-1 in both compartments is still higher than that of mGluR5. So the relative effect of changes in the speed of mGluR5 surface diffusion is unlikely to share a similar mechanism to that of GLT-1 surface diffusion. These two mechanisms are likely to be independent of one another and indeed be linked to their individual roles on the surface of astrocytes.

We delved deeper into the possible compartmentalisation of GLT-1 and carried out experiments to decipher whether GLT-1 is retained at the synapse. Following immunostaining in fixed cultures, which verified that synapses were in close contact with GLT-1 expressing astrocyte processes, we examined how GLT-1 moves when inside a defined synaptic area. The synaptic area was defined with parameters normally used for studying surface trafficking of neuronal receptors; in our case the astrocytic synaptic area incorporated the synaptic staining plus the perisynaptic area (2 pixels around the synapse or 320 nm). We observed that GLT-1 surface diffusion was greatly reduced when inside the synaptic area, which implies that GLT-1 is highly regulated in the vicinity of the synapse. Interestingly, stabilisation of GLT-1 at the synapse was a transient event and transporters readily left the confines of the synapse where they recovered normal diffusion parameters. From these data one may suggest that glutamate release from the presynaptic terminal ‘unleashes’ these transporters from the synapse allowing naïve un-bound transporters to take their place thus facilitating buffering and removal of glutamate from the synaptic cleft.

To provide some insight into this hypothesis we carried out further experiments using caged glutamate. These experiments allowed us to precisely control the time at which these synaptic GLT-1 were exposed to glutamate. We observed that that GLT-1 surface diffusion

was increased following glutamate uncaging close to the synapse. Furthermore, these transporters moved away from the synapse, which was observed by a decrease in the synaptic dwell time. Importantly, we observed no change in surface diffusion of GLT-1 before and after glutamate uncaging at non-synaptic sites. This indicates the existence of a synapse-specific mechanism whereby GLT-1 diffusion is reduced which can be rapidly reversed following synaptic glutamate release.

Unfortunately, the exact mechanism by which GLT-1 surface diffusion is reduced in the vicinity of the synapse remains unknown. If we borrow some ideas from what has been shown in neurons, we could speculate that GLT-1 surface diffusion is reduced close to synapses through one or a combination of the following mechanisms; protein-protein interactions with other astrocytic proteins important at the synapse; interaction with an intracellular 'scaffold-like' protein in astrocytes; interactions with transmembrane proteins which span the void between neurons and astrocytes; or even lipid rafts which may be rich in other proteins therefore hindering the passage of GLT-1 through this confined area.

Of all the proposed mechanisms mentioned above, one of the more likely mechanisms by which GLT-1 may be transiently stabilised at the synapse is protein-protein interactions. To date there have been no studies showing a dense, protein rich area in astrocyte processes facing the synaptic cleft. If this structure did exist it would likely have been observed using electron microscopy. Thus, the mechanism by which GLT-1 is stabilised at the synapse is more likely to involve interactions between other surface proteins. There is not a great wealth of information available regarding the known binding partners of GLT-1, I believe that this is not due to lack of interest but rather a result of the complexity of experiments needed to demonstrate these interactions. It is highly likely that interactions between GLT-1 and its binding partners are transient in order to stabilise the transporter in specific place for a limited time. Furthermore, we could speculate that these protein-protein interactions are quite weak, which would be important to allow GLT-1 to move away from the synapse rapidly following exposure to glutamate. Several potential

binding partners of GLT-1 have been identified; the water channel Aquaporin 4 (Nielsen *et al.*, 1997; Zeng *et al.*, 2007); N^+/K^+ ATPase (Rose *et al.*, 2009); and mitochondria (Genda *et al.*, 2011). One could imagine that GLT-1 'prefers' to stay in close contact with N^+/K^+ ATPase and mitochondria due to their role in providing local conditions conducive to glutamate transport. The ion gradients needed for glutamate transport are created by the ATPase pump and energy is provided to this pump by mitochondria. Furthermore, Kir4.1 an inward rectifying potassium channel, demonstrated to colocalise with Aquaporin 4 (Nagelhus *et al.*, 1999), maintains a negative membrane potential which supports electrogenic glutamate transport. Moreover, it has been demonstrated that disruption of Kir4.1 expression reduces total glutamate uptake from the extracellular space (Djukic *et al.*, 2007; Kucheryavykh *et al.*, 2007).

Many studies have proposed potential intracellular binding partners for GLT-1. However, evidence for an astrocytic 'PSD-like' intracellular accumulation of scaffolding proteins, facing synapses, is quite weak. If high accumulations of scaffolding proteins in astrocytes existed, it is likely that they would have already been observed using electron microscopy. We can count several proteins which could possibly act as intracellular scaffold proteins for GLT-1, such as Ajuba (Marie *et al.*, 2002), MAGI-1 (Zou *et al.*, 2011), PKCa (Gonzalez *et al.*, 2005) as well as PICK1 which has been shown to interact with GLT-1b (Bassan *et al.*, 2008), an isoform of GLT-1 located distally from synapses (Sullivan *et al.*, 2004) with very low expression in the hippocampus (Holmseth *et al.*, 2009). To date, there has been no conclusive evidence for a direct intracellular binding partner for GLT-1, which could potentially stabilise this transporter near glutamatergic synapses. It is quite possible that it is GLT-1 itself which is stabilising itself. Evidence suggesting that GLT-1 forms multimers could hold to the key to how GLT-1 is stabilised at the synapse. We can imagine that individual GLT-1 form multimers close to the synaptic cleft and effectively cross-linking each other in this area. However, this opens the question as to why this occurs at the

synapse and not at non-synaptic sites? This area of the glutamate transporter field remains completely unexplored.

Another important question to ponder is how do astrocyte processes become stabilised at synapses? Regarding our findings, this is a very important question indeed. We observed that GLT-1 diffusion was reduced at synapses, which we postulate is due to interactions with other surface proteins but how does the astrocyte know the location of the synapse is and project its GLT-1-expressing processes accordingly? This question remains unresolved. If indeed there is a transient accumulation of different transporters, pumps and receptors on the glial membrane opposing neuronal synapses, then there must be a mechanism by which astrocytes sense synaptic activity, extend their processes and anchor themselves to this specialised region. One potential mechanism could be that could anchor astrocyte processes to synapses could involve transmembrane proteins signalling between neurons and glia. We could hypothesise that astrocytic processes scan the brain for activity until they find synapses where they can anchor themselves. Once anchored in place through transmembrane protein interactions, the astrocytic process begins to form a transient accumulation of proteins, highly adapted for sensing and controlling synaptic activity. If I was to speculate on a specific family of proteins responsible for astrocytic stabilisation at the the synapse, I would suggest the ephrin family of proteins. Indeed ephrin signalling may be a good target to investigate this question as it has already been demonstrated that neuron-glia signalling between neuronal EphA4 and astrocytic EphrinA3 controls glutamate transporter expression on astrocytes and dendritic spine morphology in the hippocampus (Carmona *et al.*, 2009; Filosa *et al.*, 2009). This is of course pure speculation and the discovery these mechanisms will no doubt arrive piece by piece. Over time it just might be possible to piece this puzzle together and understand exactly how and why astrocytes approach a synapse and carry out their vital functions in the brain. If we understand how this process occurs in normal, physiological conditions this will provide insight into how and why this process goes awry and its implication in pathology.

4. Simulation of GLT-1 surface diffusion

Before investigating surface diffusion in the physiological regulation of glutamate uptake at the synapse in a biological system, we created a computational model of the synapse to see if our hypothesis could stand up to a non-biased test. This model measured the efficacy of glutamate uptake from the synapse with differing GLT-1 diffusion coefficients from immobile to mobile. Our computer model of GLT-1 diffusion showed a correlation between the speed of GLT-1 surface diffusion and the efficiency of glutamate uptake from the synapse. It is clear that faster moving transporters are capable of removing more glutamate from the synaptic area compared to slower moving or immobile transporters. However, our model is rather limited in terms of faithfully representing our observations *in vitro* due to the complexity of this system. Two important parameters observed in our study but omitted from this model, due to their complexity, were; i) the reduction in surface diffusion of GLT-1 in the synaptic area compared to non-synaptic areas and ii) the increase in GLT-1 surface diffusion when exposed to glutamate. It is highly likely that if we were able to include these parameters, our simulation would show improved efficiency in glutamate removal from the synaptic area in conditions where diffusion is permitted.

Another important factor that we do not consider in our model is diffusion of glutamate away from the synapse. There have been reports that glutamate diffuses quite fast in the extracellular space in the brain, ~ 0.4 $\mu m^2/s$ (Zheng *et al.*, 2008), thus we can imagine that a lot of the synaptically released glutamate will diffuse away without the need for the intervention of synaptically localised glutamate transporters to remove glutamate. Another valid point is that we do not know the exact proportion of the synapse that is ensheathed by glial processes, in our model we took for granted that the synapse was completely ensheathed by the astrocyte thus giving us an astrocytic synaptic domain of 30 nm x 1 μm. It has been suggested that glial processes cover around 40% of each individual synapse in the hippocampus (Lehre and Rusakov, 2002; Ventura and Harris, 1999).

Ensheathment of synapses by glia is brain region specific and it has been estimated by electron microscopy that Purkinje cell synapses of the cerebellum are almost completely ensheathed by the Bergmann glia of the cerebellum (a specialised astrocyte specific to this area) (Xu-Friedman *et al.*, 2001). As a result of this high level of ensheathment, diffusion of glutamate out of the synapse is almost non-existent thus there is a high sensitivity to drugs which block the uptake of glutamate from the synapse by transporters (Barbour *et al.*, 1994; Overstreet *et al.*, 1999; Takahashi *et al.*, 1995; Tong and Jahr, 1994). Perhaps there is a functional role for the variation in astrocytic ensheathment of synapses between brain regions. It is known that the plasticity of astrocytic coverage of glutamatergic synapses in the supraoptic nucleus of the hypothalamus plays an important physiological role in lactation (Theodosis and Poulain, 1984). Thus it is not implausible that the differences in astrocytic coverage of hippocampal versus cerebellar synapses are linked to a physiological function. It has been suggested that glutamate spill-over at hippocampal synapses and activation of glutamate receptors on adjacent synapses, serves a physiological role (Isaac *et al.*, 1995; Kullmann *et al.*, 1996). It must be added, however, that we did not set out to successfully reproduce a physiological model of glutamate uptake at the synapse, we simply wanted to uncover whether mobile transporters were more efficient at removing glutamate from a confined synaptic cleft than static transporters. To this end our model was a success.

5. Physiological role of GLT-1 surface diffusion

If our hypothesis that surface diffusion of GLT-1 is important for the removal of glutamate from the synapse is true, experimental manipulation of GLT-1 surface diffusion should have an impact on synaptic transmission. To this end we used a previously described technique (Heine *et al.*, 2008) to effectively immobilise proteins on the surface of the cell, a procedure known as cross linking (X-link). This X-link successfully immobilised GLT-1 without affecting any other properties such as transport function or access to the synaptic area. We found no effect of antibody/quantum dot conjugation, necessary for surface trafficking experiments, on GLT-1 uptake function at any concentration used. Furthermore, our observation that X-link did not change the content of GLT-1 at the synapse indicates that the antibodies are not so big that they obstruct access of GLT-1 to the synapse.

Here, we propose that by binding two separate GLT-1 transporters together, we can effectively reduce their diffusion. It is important to consider the implications of this statement when, as previously mentioned, glutamate transporters have been suggested to form multimers. Our X-link, must therefore bind two GLT-1 transporters in distinct mulitmers. This is a reasonable assumption because if the primary antibody was binding individual GLT-1 in the same multimer, there would be no hindrance of the transporter's diffusion in X-link conditions. If indeed GLT-1 is moving on the surface of astrocytes in a multimer, this could potentially increase the efficacy of our X-link. Instead of one antibody being able to immobile two GLT-1 transporters, one single antibody would have the potential to immobilise six GLT-1 molecules, the equivalent of two trimers.

After verifying these important controls, we investigated whether surface diffusion of GLT-1 plays a role in removing glutamate from the synapse. To do this we searched cultures for astrocytes which were double transfected with GFP/GLT-1^{flag} and then recorded from spontaneous EPSCs from neurons situated inside the transfected astrocyte's domain. We observed no difference in amplitude or the frequency of firing between control and X-link

conditions. We did, however, observe slower kinetics of single events, with both rise time and decay of EPSPs increased in GLT-1X-link conditions.

These *in vitro* observations were further strengthened by our work in the acute hippocampal slice preparation. Of course, any biological observation *in vitro* must be interpreted with caution, we should always strive to integrate our findings and test our hypotheses in the most intact and physiologically relevant preparation possible. Recently, our lab developed a novel technique by which proteins diffusing on the surface of neuronal membranes in intact brains can be immobilised and then neuronal activity recorded following acute brain slice preparation (Dupuis *et al.*, 2014). This technique was readily transferred to our study in order in test our hypothesis in a more intact brain preparation. This was also made possible due to the fact that an antibody targeting an extracellular loop of GLT-1 was made commercially available, prior to this the only antibodies targeting endogenous GLT-1 recognised C- and N- termini thus were useless for live single nanoparticle imaging.

We characterised the diffusion characteristics of endogenous GLT-1 (GLT-1^{endo}) and compared to those of GLT-1^{flag}. We observed that while the mobile fraction of GLT-1^{endo} was rather similar to GLT-1^{flag}, there was a much higher population of slow moving and immobile transporters in the GLT-1^{endo} group. There are several possible explanations for this phenomenon; starting with the most critical, one could imagine that by transfecting astrocytes with GLT-1^{flag} we are overloading the regulatory mechanisms which are involved in the surface trafficking of GLT-1 and this is why we are seeing transfected transporters moving faster. This is a plausible explanation, however one could also imagine that there is limited machinery in the cell for the manufacture of proteins and insertion of these proteins into the membrane. Cells do not continue manufacturing proteins until they explode. There are many regulatory hurdles to pass before a protein is expressed on the surface of the cell. Overexpression of exogenous proteins is widely used and well accepted technology in modern neuroscience; take for example recent advances in optogenetics (Boyden *et al.* 2005) as well as GECIs such as GCaMP6 (Haustein *et al.* 2014), both techniques require

the expression of exogenous light-sensitive ion channels and calcium-sensitive fluorescent proteins respectively. Another piece of evidence against this 'overloading' hypothesis is the similarity in total distribution of endogenous and transfected GLT-1.The absolute diffusion speeds for the fastest and slowest moving transporters is exactly the same. Furthermore, the average speed of total GLT-1^{endo} (mobile & immobile) is slower than that of total GLT-1^{flag}, this observation may be due to the specificity of the antibody we are using. To track GLT-1^{flag} transporters we are using an anti-flag antibody, this flag sequence of proteins (DYKDDDDK) does not occur in nature therefore there will be very little non-specific tagging. However, the antibody we are using against an extracellular loop of endogenous GLT-1 may be less specific. Polyclonal antibodies can vary from batch to batch and may indeed recognise this amino acid sequence if it is shared with other proteins. This could account for the higher proportion of slower moving and immobile trajectories that we are seeing. Finally, another plausible reason as to why GLT-1^{endo} moves slower than GLT-1^{flag} is that there are at least 3 known splice variants of GLT-1 endogenously; a, b and c (Chen *et al.*, 2002; Pines *et al.*, 1992; Rauen *et al.*, 2004; Sullivan *et al.*, 2004). These splice variants all differ in the N- and C- termini, therefore our antibody against endogenous GLT-1 will recognise all three variants of GLT-1. It is also possible that this commercial antibody, which recognises the extracellular loop between transmembrane domain 3 and 4 (amino acids 143-238), may bind the GLAST transporter. Although the amino acid sequence identity between these two proteins on this extracellular loop is ~ 37% (see Figure 26A) it is still possible that the antibody might recognise GLAST. Most commercially available antibodies bind to the N- or C- terminus of the protein, which have no sequence homology (see Figure 26B).

A

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55.7% identity in 465 residues overlap; Score: 1260.0; Gap frequency: 0.9%

GLAST,        45 LFRNAFVLLTVSAVIVGTILGFALRPYKMSYREV-KYFSFPGELLMRMLQMLVLPLIISS
GLT-1,        41 LGKNLLLSLTVFGVILGAVCGGLLRLAAPIHPDVVMLIAFPGDILMRMLKMLILPLIISS
                  *  *    ***  ** *   *  **        *     ***  ***** ** *******

GLAST,       104 LVTGMAALDSKASGKMGMRAVVYYMTTTIIAVVIGIIIVIIIIPGKGTKENMYREGKIV-
GLT-1,       101 LITGLSGLDAKASGRLGTRAMVYYMSTTIIAAVLGVILVLAIIPGNPKLKKQLGPGKKND
                  * **   ** ****  * ** **** ***** * * * *  * ***          **

GLAST,       163 QVTAADAFLDLIRNMFPPNLVEACFKQFKTSYEKRSFKVPIQANETLLGAVINNVSEAME
GLT-1,       161 EVSSLDAFLDLIRNLFPENLVQACFQQIQT-VTKKVLVAPPSEEANTTKAVISLLNETMN
                  *   ********* ** *** *** *  *   *     *         ***    * *

GLAST,       223 TLTRIREEMVPVPGSV-NGVNALGLVVFSMCFGFVIGNMKEQGQALREFFDSLNEAIMRL
GLT-1,       220 EAPEETKIVIKKGLEFKDGMNVLGLIGFFIAFGIAMGKMGEQAKLMVEFFNILNEIVMKL
                             * * ***  *   **   * * **     ***  ***  * *

GLAST,       282 VAVIMWYAPLGILFLIAGKILEMEDMGVIGGQLAMYTVTVIVGLLIHAVIVLPLLYFLVT
GLT-1,       280 VIMIMWYSPLGIACLICGKIIAIKDLEVVARQLGMYMITVIVGLIIHGGIFLPLIYFVVT
                  *  **** ****  ** ***    *  *   ** **  ****** **  * *** ** **

GLAST,       342 RKNPWVFIGGLLQALITALGTSSSSATLPITFKCLEENNGVDKRITRFVLPVGATINMDG
GLT-1,       340 RKNPFSFFAGIFQAWITALGTASSAGTLPVTFRCLEDNLGIDKRVTRFVLPVGATINMDG
                  ****  *  *  ** ****** **  *** ** *** * * *** ***************

GLAST,       402 TALYEALAAIFIAQVNNFDLNFGQIITISITATAASIGAAGIPQAGLVTMVIVLTSVGLP
GLT-1,       400 TALYEAVAAIFIAQMNGVILDGGQIVTVSLTATLASIGAASIPSAGLVTMLLILTAVGLP
                  ****** ******* *   *  *** * * *** ****** ** ******   ** ****

GLAST,       462 TDDITLIIAVDWFLDRLRTTTNVLGDSLGAGIVEHLSRHELKNRD
GLT-1,       460 TEDISLLVAVDWLLDRMRTSVNVVGDSFGAGIVYHLSKSELDTID
                  * ** *  **** *** **  ** *** ***** ***  **   *
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B

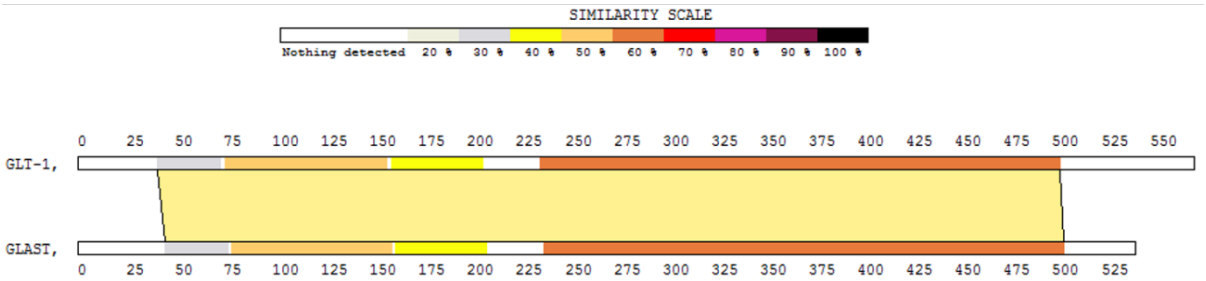


Figure 26. Alignment of GLAST and GLT-1 amino acid sequences

(A) Sequences highlighted in red denote the amino acid sequence in the extracellular loop between transmembrane domains 3 and 4. Note high level of sequence identity in the extracellular loop targeted by anti-GLT-1 antibody. Stars denote identical amino acids. **(B)** Similarity between GLT-1 and GLAST. Colour between the two sequences denotes a 55 % homology. Sequences were aligned using SIM alignment tool (http://web.expasy.org/sim/). Graphic for part b was made using LALNVIEW bioinformatics software.

Following the characterisation of GLT-1^{endo}, we were convinced that this antibody was relatively specific, at least for glutamate transporters. We carried out further experiments and found that X-link of GLT-1, i.e. a reduction in the surface diffusion without any effect on the transport function was also possible. Satisfied with these *in vitro* data, we proceeded to investigate the effect of GLT-1^{endo} X-link in hippocampal brain slices by stereotaxic injection of anti-GLT-1 antibodies into the hippocampus of the anaesthetised animal. As in culture we carried out exactly the same experiment, recording spontaneous EPSCs in neurons from the CA1 region of the hippocampus. We failed to observe any difference in frequency or amplitude of spontaneous EPSCs following X-link of GLT-1 in slices but again, exactly as in culture, we noted significantly slower kinetics with both rise time and decay increased in X-link conditions. These data further implicate surface diffusion of GLT-1 in the efficient buffering and removal of glutamate from the synapse, this time in an *ex vivo* brain slice preparation.

Many studies which pharmacologically blocked glutamate transporters have reported similar findings ours, i.e. blocking glutamate uptake increases the timecourse of synaptic glutamate, evidenced by slowed kinetics of EPSCs (Barbour *et al.*, 1994; Mennerick and Zorumski, 1994; Overstreet *et al.*, 1999; Takahashi *et al.*, 1996; Tong and Jahr, 1994). However, we did not pharmacologically block transporters in X-link conditions. Transporters in X-link conditions were fully functional. The only parameter modified in this experiment was the speed of diffusion of GLT-1 on the surface of astrocytes. These data suggest that surface diffusion of GLT-1 plays an important role in controlling the timecourse of synaptic glutamate in the hippocampus.

Changes in AMPAR EPSC kinetics can be directly linked to the concentration and timecourse of glutamate at the synapse as well as the properties of the postsynaptic receptors activated. The decay rate of EPSCs is known to be a function of the properties of the receptors activated by glutamate. In our experiments the membrane potential was held at -70 mV, thus we can be certain that the spontaneous EPSCs recorded come from

activation of postsynaptic AMPARs. As mentioned in the introduction, AMPARs are fast-desensitising receptors, so the duration of AMPAR EPSCs are tightly regulated by the desensitisation rate. We observed slower kinetics following X-link of GLT-1, which we believe are due to a reduction in glutamate uptake by transporters leading to prolonged exposure of AMPARs to glutamate. As we did not alter the properties of postsynaptic glutamate receptors in our experimental conditions, we can rule out any modifications in the properties of receptors which may influence the kinetic changes from our results. Therefore the only possible conclusion from our data is that GLT-1 surface diffusion plays a role in controlling the concentration and timecourse of glutamate, as observed by increased AMPAR activation in conditions where GLT-1 is immobilised.

We also observed an increase in the rise time of spontaneous EPSCs. This is quite interesting and has not been previously demonstrated by other laboratories. We could speculate that other studies have failed to observe this increased rise time due to the experimental conditions used. The majority of studies, which observed changes in the decay of EPSCs, were carried out at room temperature (21-24°C). It is now understood that glutamate transporter function, diffusion of glutamate and kinetics of glutamate receptors are temperature dependent synapses (Asztely *et al.*, 1997). The few studies which carried out experiments near physiological temperature (34°C) have reported reduced spillover at hippocampal synapses (Asztely *et al.*, 1997) and more dramatic effects on AMPAR EPSCs at cerebellar synapses in the presence of glutamate transport blockers (Tong and Jahr, 1994). Therefore, it is possible that many studies which observed changes in the decay of EPSCs in the presence of glutamate uptake blockers have, through experimentation in non-physiological conditions, underestimated the role that transporters play in regulating the timecourse of glutamate at the synapse.

In addition to increasing the efficacy of glutamate uptake by transporters, recording at physiological temperature also affects the diffusion of the neurotransmitter glutamate. This could further explain our observed increase in rise time of EPSCs. As hippocampal

synapses are not fully ensheathed by astrocytes, reducing glutamate uptake by X-link, is likely to result in increased glutamate spillover between synapses. Thus a delayed rise time could be explained by activation of AMPARs on adjacent synapses. At physiological temperatures glutamate, as well as other molecules, diffuse faster than at room temperature, therefore any impairment of the uptake system will lead to increased diffusion from the synaptic cleft. Activation of AMPARs on adjacent synapses would occur slightly later than activation of AMPARs in the original synapse therefore slowing the rise time kinetics.

The observed increase in rise time can be further explained by recent research which suggests that AMPARs are not distributed homogenously on the postsynaptic density, but are rather organised into nanodomains of receptors on the PSD (Nair *et al.*, 2013). This study by Nair and colleagues (2013) demonstrated that groups of AMPA receptors which are more than 150 nm apart can be functionally separated, depending on the precise location of single vesicle release. They calculated that if these nanodomains are separated by > 150 nm it is possible that single vesicle release of transmitter will act upon one nanodomain and not the neighbouring nanodomain. So if we consider the X-link conditions in this study, we know that glutamate uptake is impaired leading to an increase in timecourse and diffusion of glutamate at the synapse. Thus, we can hypothesises that X-link of GLT-1 allows monosynaptic spillover between nanodomains of AMPARs thereby increasing the rise time of EPSCs.

Conclusion

Here we have identified a novel mechanism for glutamate removal from the synapse on a fast timescale, through surface diffusion of GLT-1. Previous studies have come to the conclusion that, due to the slow transport cycle of glutamate transporters, there are more transporters than glutamate molecules at the synaptic area and it is by a sheer force of numbers that glutamate transporters can effectively bind all the glutamate molecules and slowly transport them on a synaptic scale (Attwell and Gibb, 2005; Diamond and Jahr, 1997; Lehre and Danbolt, 1998). These laboratories also hypothesised glutamate transporters may act as a high pass filter, meaning that high frequency synaptic glutamate release may saturate these transporters and lead to the activation of extrasynaptic receptors and/or adjacent synapses which have many physiological and pathological roles (Hardingham *et al.*, 2002; Kullmann *et al.*, 1996). Recently this point of view began to change direction with new evidence suggesting that it is not only the level of glutamate transporter expression but also their precise location, particularly close to the synaptic cleft, which can control neuronal glutamate receptor activation (Melone *et al.*, 2009; Oliet *et al.*, 2001; Omrani *et al.*, 2009; Pannasch *et al.*, 2014).

Here, we further update this hypothesis, challenging the supposed necessity for a large number of transporters at the synapse. Our findings infer that it is surface diffusion of GLT-1 which contributes to the buffering and removal of glutamate from the synaptic cleft on a fast timescale. We have shown that these transporters are not fixed in the membrane but are highly dynamic and that this surface diffusion is activity-dependent, regulated by both neuronal and glial cell activity. We observed that mimicking neuronal glutamate release by uncaging glutamate close to the synapse, increases GLT-1 surface diffusion resulting in displacement of these transporters from the confines of the synapse. Functional consequences of immobilising GLT-1 *in vitro* as well as *ex vivo* in a hippocampal slice preparation were observed through strong changes in kinetics of spontaneous EPSCs.

Similar findings have been demonstrated by pharmacologically blocking glutamate transporters indicating that surface diffusion of GLT-1 serves a functional role in controlling the timecourse of synaptic glutamate. The observed changes in kinetics indicate prolonged exposure of neuronal glutamate receptors to glutamate, highlighting the vital role of GLT-1 surface diffusion in maintaining synaptic glutamate homeostasis both *in vitro* and *in vivo*. Taken together these data further reinforce the concept of the tripartite synapse, which proposes that astrocytes are active partners at chemical synapses and directly participate in cerebral communication.

Trafficking of neurotransmitter receptors at the surface of neurons plays important roles in controlling receptor distribution and synaptic signaling in physiological as well as pathological processes (Gerrow and Triller, 2010; Krugers *et al.*, 2010; Mikasova *et al.*, 2012). Consequently, it is not far-fetched to consider the possibility of a failure in the regulatory mechanisms controlling glutamate transporter surface diffusion under certain pathological conditions. It has previously been demonstrated that disruption of this vital neurotransmitter clearance mechanism contributes to many neurodegenerative diseases including ALS, epilepsy, Alzheimer's and Huntington's disease (Lievens et al., 2001; Rothstein et al., 1995; Scimemi et al., 2013; Tanaka et al., 1997). Thus, in addition to improving our understanding of the contribution of transporters in controlling glutamate at the synapse, this unexpected mechanism opens new avenues of research for neurological and psychiatric disorders involving a dysfunction of glutamate transport.

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