Synaptic environment and extrasynaptic glutamate signals: the quest continues

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ABSTRACT:

Behaviour of the mammal relies on brain's excitatory circuits equipped with glutamatergic synapses. In most cases, glutamate escaping from the synaptic cleft is rapidly buffered and taken up by high-affinity transporters expressed by nearby perisynaptic astroglial processes (PAPs). The spatial relationship between glutamatergic synapses and PAPs thus plays a crucial role in understanding glutamate signalling actions, yet its intricate features can only be fully appreciated using methods that operate beyond the diffraction limit of light. Here, we examine principal aspects pertaining to the receptor actions of glutamate, inside and outside the synaptic cleft in the brain, where the organisation of synaptic micro-physiology and micro-environment play a critical part. In what conditions and how far glutamate can escape the synaptic cleft activating its target receptors outside the immediate synapse has long been the subject of debate. Evidence is also emerging that neuronal activity- and astroglia-dependent glutamate spillover actions could be important across the spectrum of cognitive functions.

KEY WORDS:

synaptic connections, synaptic cleft, dendritic spines, micro-physiology, perisynaptic astrocyte processes (PAP), glutamate spillover

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Introduction

Excitatory glutamatergic synapses are thought to mediate the bulk of information transfer, handling, and storage in the mammalian brain. They are therefore what we rely upon in our behaviour. That individual synaptic connections can provide a one-way signalling channel between two nerve cells is at the core of the theories explaining a computational modus operandi prevalent in the CNS. Indeed, computer chips incorporate millions of diode- and transistor-based circuits which, in their very basic features, imitate synaptic connections. One-to-one connectivity is there by default, and the computation logic of such circuits has long been established and benefited from in the rapid emergence of machine-learning applications.

Hence it is believed that glutamatergic connections must provide strictly one-to-one transmission, and any significant deviation from it should be considered a deficiency or pathology. In other words, glutamate released by a presynaptic axonal specialisation should act strictly on its receptors in the postsynaptic cell only, and probably within the synaptic cleft only. Understanding whether synaptic organisation is indeed conducive to this type of signalling has been a long-standing quest. The progress has been relatively slow, owing to the nanoscopic features of excitatory synapses which put them beyond the diffraction limit of conventional light microscopy. Thus, the main breakthroughs came from electron microscopy studies. It has long been appreciated that the majority of small excitatory synapses are surrounded, or at least approached by thin, leaf-like cell processes of astroglia (Spacek, 1985; Ventura and Harris, 1999a; Wenzel et al., 1991; Williams et al., 1980). In some areas of the brain, astroglia wrap around synaptic structures (or any remaining openings into the synaptic cleft) thus virtually isolating synapses from the surrounding tissue (Barbour, 1993; Borst and Soria van Hoeve, 2012; Grosche et al., 1999; Rollenhagen et al., 2007) whereas in others astroglia-free areas can be seen between synapses (Gavrilov et al., 2018; Ventura and Harris, 1999a; Witcher et al., 2007; Witcher et al., 2010). The perisynaptic astroglial processes (PAPs) processes are packed with high-affinity glutamate transporters that rapidly take up glutamate escaping the cleft (Danbolt, 2001; Lehre and Danbolt, 1998; Rothstein et al., 1994). Nonetheless, receptor actions of glutamate escaping the synaptic cleft had long been detected in what seem to be physiologically relevant circumstances. When and to what extent glutamate can escape the synaptic cleft, whether it could thus exert receptor actions on multiple targets, and what cellular mechanisms if any can control such actions remains therefore a subject of intense interest.

Thus here we will overview the structure of glutamate synapses and the various aspects found to affect release, diffusion, and receptor actions of glutamate, inside and outside the

synaptic cleft in the brain. We will discuss some of the findings showing how the organisation and micro-physiology of excitatory synapses and their environment could control glutamate escape. We will review reported associations between extrasynaptic glutamate escape and behaviour, albeit without dwelling on multiple molecular cascades that might underlie such actions.

Synaptic structure, active zones, synaptic clefts, and postsynaptic densities

Glutamatergic or excitatory synapses have a synaptic junction with an active zone in apposition to the more prominent post synaptic density (PSD) which may be 25-50nm in thickness. Figure1A is a schematic of a glutamate synapse while Figure1B is an electron micrograph showing an example of a synaptic bouton (PRE) with a prominent post-synaptic density (arrowhead- PSD) and spheroid vesicles; the bouton makes an asymmetric contact with a thin dendritic . synaptic junction of glutamate synapses is thus termed asymmetric. The opposing pre- and postsynaptic membranes are separated by a narrow cleft (~20-40nm) which provides conditions for neurotransmitter spill-over among multiple postsynaptic densities. A detailed biophysical model has suggested that the optimal height of the free-medium cleft, which would maximize postsynaptic current, falls between 12-20 nm (Savtchenko and Rusakov, 2007). The vesicles in the synaptic bouton are of spheroid character (normally containing glutamate). While glutamate is localized inside presynaptic vesicles there are glutamate receptors on the postsynaptic membrane of 2 main types, ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). Glutamatergic synapses can vary considerably in size and are dependent on their location and physiological state. Those in the dentate gyrus have a diameter of ~350 nm, whereas the giant excitatory connections between hippocampal mossy fibres (MFs) and CA3 pyramidal cells are very large ~ 0.5–2 μm, (Figure 1C) .

The synapse shown in Figure 1A, and B is an example where a synaptic bouton makes a single contact with a dendritic spine, an axodendritic synapse. Spines are the small twig-like protrusions from dendrites which were first identified by Camillo Golgi in 1873 and later detailed by Santiago Ramon y Cajal in 1893 (DeFelipe, 2006). Spines are found in different forms from stubby, to thin and then to large - so called mushroom shape (Figure.1D). These may be a continuum dependent on the functional state of the synaptic connection. The many types of synapses and forms have been reviewed by Peters (Peters et al., 1991) and 3-D structure was discussed by Harris and Weinberg (Harris and Weinberg, 2012).

The structural association between the PSD of a synaptic bouton and dendritic spine can be seen clearly in 3-dimensional reconstructions from serial ultrathin sections, viewed in the

transmission electron microscope as in Figure 2A. which is from 12 serial ultrathin serial sections of a dendritic segment of a granule cell in the dentate gyrus. The reconstruction in Figure 2A shows the entire spine and the synapse and synaptic vesicles, with the PSD in red. None of this fine detail would be visible without 3-D reconstruction.

Excitatory axospinous synapses are typical of synapses formed by the unmyelinated axons that predominate throughout the brain. In the dentate gyrus most of the presynaptic axons with excitatory synapses originate from the perforant path to the hippocampus contacting apical dendrites of the granule cells. The majority make a single synaptic contact, but a small proportion are multisynaptic boutons (MSBs) (Aziz et al., 2019), the formation of which is promoted by long term potentiation (LTP) and such augmentation is associated with an increase in contacts with mushroom spines. Structural changes in synaptic form, - synaptic plasticity, can be correlated to changes in function, as in long term potentiation (LTP), an increase in synaptic efficacy, or a decrease in synaptic efficacy long term depression (LTD). Changes can occur in the pre-synaptic component with increases or decreases in size, vesicle number and their distribution. A direct connection between functional efficacy of synapses and the type of dendritic spine is unclear, though suppression of synapse efficacy is accompanied with reversible retraction of thin spines and CA3 thorny excrescences during hibernation-arousal (Popov et al., 1992; Popov et al., 2008b). Recovery from stress reverses a volume decrease in thorny excrescences in CA3 while water maze training increases the surface area of the PSDs (Stewart et al., 2005a). Mushroom spines have been called "memory spines", typically exhibiting enhanced PSD complexity, and/or dimensions, after physiological stimulation (Bourne and Harris, 2007; Bourne and Harris, 2008). The PSDs of these spines will likely contain a higher concentration of glutamate receptors, more smooth endoplasmic reticulum and endosomes (Bourne and Harris, 2008).

How boutons and spines alter form is not completely clear but synaptic boutons have an extensive actin cytoskeleton and microtubules which would provide the underlying molecular basis for morphological plasticity. Synaptic stimulation increases F-actin in the pre- and postsynaptic terminals and presynaptic F-actin is assembled and/or recruited from synaptic and axonal pools of actin. Actin concentrates at the active synaptic zone while the spine head undergoes a lateral expansion and contracts toward the dendrite shaft in a process coordinated with the remodelling of presynaptic actin. In spines the actin cytoskeleton may be involved in preserving the morphology of dendritic spines after learning to maintain long-term memory (Basu and Lamprecht, 2018). An immediate early gene, Activity Regulated Cytoskeletal protein, Arc (Arg3.1), controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus *in vivo* (Messaoudi et al., 2007).

Arc mRNA is localized to activated synaptic sites in an NMDA receptor-dependent manner where the newly translated protein plays a crucial role in learning and memory-related molecular processes (Steward and Worley, 2001). Arc has been suggested as a potential "master regulator" of protein synthesis-dependent forms of synaptic plasticity and cognition (Bramham et al., 2010; Nikolaienko et al., 2017).

Spinules are prominent structures that arise from discontinuities in the PSD on the spine head, and evaginate from the presynaptic bouton (sp-Figure 2B) while endosomes (en - Figure 2B) can be identified at the electron microscope level primarily in a form termed as multivesicular bodies (or endosome-like structures) (Cooney et al., 2002; Spacek and Harris, 1997; Stewart et al., 2005a). Endosomes are involved with plasticity after synaptic stimulation and play a major role in the endocytotic route sending proteins and lipids to multiple destinations including the cell surface, Golgi complex, and lysosomes (Cooney et al., 2002; Murk et al., 2003; Popov et al., 2008a). Ehlers (Ehlers, 2000) demonstrated that AMPAR sorting occurs in early endosomes and is regulated by synaptic activity and activation of AMPA and NMDA receptors, and Blanpied et al. (Blanpied et al., 2002) showed that in dendritic spines, endocytotic zones lie lateral to the PSD where they develop and persist independent of synaptic activity, similar to the PSD itself. There is an increase in endosome-like structures following water maze training (Stewart et al., 2005a) and a decrease following restraint stress which is likely to reflect altered synaptic activity since endosomes are major sorting stations in the endocytotic route sending proteins and lipids to multiple destinations including the cell surface, Golgi complex, and lysosomes (Murk et al., 2003). Down regulation of endosomes retards recycling of GluR1 to the cell surface after NMDA stimulation. Clathrin-coated pits invaginate to form coated vesicles that become large vesicles (Stewart et al., 2005b) after the loss of the coat. Large vesicles merge into tubular endosomes and MVB-tubular complexes. A study by Sharma et al (Sharma et al., 2010) suggested that signalling endosomes could trigger synapse assembly with data that showed NGF-TrkA signalling endosomes travel from distal axons to cell bodies and dendrites where they promote PSD clustering.

The enormous variability of synaptic shapes and sizes precludes us from drawing on a clear and unambiguous association between their form and function. However, the shape of dendritic spines has long been related to their biophysical properties that control electric signal transfer (Araya et al., 2006; Cartailler et al., 2018; Harris and Stevens, 1989; Yuste et al., 2000), and significant evidence exists pointing to a strong correlation between AMPA receptor expression and spine head size (Matsuzaki et al., 2001), and between glutamate release probability and the dimensions of presynaptic boutons and active zones, at least in

small central synapses (Holderith et al., 2012; Matz et al., 2010; Schikorski and Stevens, 1997). Studies employing live super-resolution and glutamate imaging techniques should provide further insights into the key aspects of synaptic identities associated with their anatomy.

Astroglial synaptic microenvironment

Electron microscopy (EM) studies have found that, in the rodent cortex, synaptic neuropil contains on average 1.5-2.0 synapses per μm^3 (e.g., (Calverley et al., 1988; DeFelipe et al., 1999; Geinisman et al., 1996; Mayhew, 1979; Motta et al., 2019; Rusakov et al., 1998; Rusakov and Kullmann, 1998; Unal et al., 2002)). This value has been reported to fluctuate with development or specific learning tasks (Eyre et al., 2003; Nikolakopoulou et al., 2006; Sandi et al., 2003; Witcher et al., 2007) (although (Popov et al., 2004)). In humans, the numerical synaptic density appears slightly lower, around 1 μm^{-3} (Alonso-Nanclares et al., 2008; DeFelipe et al., 2002; Witcher et al., 2010), decreasing in pathologies such as Alzheimer's and temporal sclerosis (Scheff et al., 1990; Witcher et al., 2010). Thin astroglial protrusions, or leaflets, many of which occur near synapses, and thus termed perisynaptic astroglial processes (PAPs), seem omnipresent throughout the synaptic neuropil (Hama et al., 2004; Shigetomi et al., 2013). They occupy a 6-9% fraction of the tissue volume, with the values slightly decreasing towards the astrocyte periphery (Gavrilov et al., 2018; Lehre and Rusakov, 2002; Medvedev et al., 2014b; Patrushev et al., 2013; Savtchenko et al., 2018). On average, one astrocyte approaches 20,000- 120,000 synapses in the rodent cortex, and 0.2-2 million synapses in the human brain (where astroglial cells are several-fold larger than in rodents), with little spatial overlap between the territories of individual cells (Bushong et al., 2002; Oberheim et al., 2009; Oberheim et al., 2006).

A regular presence of PAPs near excitatory synapses has long been documented by EM studies (Spacek, 1985; Wenzel et al., 1991; Williams et al., 1980), and important spatial detail has more recently been provided by tri-dimensional (3D) EM reconstructions (e.g., (Grosche et al., 1999; Patrushev et al., 2013; Rollenhagen et al., 2007; Ventura and Harris, 1999a; Witcher et al., 2007; Witcher et al., 2010)). The synaptic proximity of PAPs can vary from the immediate contact to hundreds of nanometres (Lushnikova et al., 2009; Medvedev et al., 2014a; Pannasch et al., 2014; Patrushev et al., 2013; Spacek and Harris, 1998), with a fraction of synapses showing a close-contact astroglial coverage. This fraction varies across brain regions, from 29-56% om the rat neocortex (Bernardinelli et al., 2014a; Reichenbach et al., 2010), ~62% in the rat hippocampus (Ventura and Harris, 1999b; Witcher et al., 2007), or ~90% in layer IV of the somatosensory cortex of adult mice

(Bernardinelli et al., 2014a). Thus, in the hippocampus, a significant fraction of perisynaptic neuropil free of astroglia has been considered a feature conducive of extrasynaptic glutamate escape (Ventura and Harris, 1999a), although how strongly tissue geometry can curtail such escape remains incompletely understood (see below).

It appears that PAPs approach preferentially synapses that have complex, perforated postsynaptic densities (PSDs) (Witcher et al., 2007), synapses on immature, thinner dendritic spines (Medvedev et al., 2014b) while occurring more frequently on the postsynaptic side of the connection (Lehre and Rusakov, 2002). In area CA3 of the hippocampus or at parallel fibre connections in the cerebellum, astroglia wrap around synaptic structures thus shielding them tightly from the local neuropil (Grosche et al., 1999; Rollenhagen et al., 2007). A similar shield is present in the giant calyx of Held synapses where it covers spaces between pre- and postsynaptic calyceal structures (Borst and Soria van Hoeve, 2012) Another specialised circuit structure surrounded by astroglia is synaptic glomeruli, or spatial clusters of functionally related connections found within the sensory thalamus, the olfactory bulb, and the cerebellar cortex (Reichenbach et al., 2010). Because of such synaptic glomeruli, astroglia are often considered distinct functional units (Barbour et al., 1994; Mitchell and Silver, 2000; Reichenbach et al., 2010).

An unambiguous assessment of synaptic astroglial coverage is not straightforward, mainly because of the subjective nature of describing highly complex 3D geometry of the synapse synapses and PAPs, but also because of the inherent errors in existing 3D reconstruction methods. Figure 3 (a,b) shows the extent of astroglia coverage of a spine and synapse while Figure 3 (c,d) show 3D reconstructions from~100 serial sections in dentate gyrus, with Figure 3 (c) showing the astrocytic network while Figure 3 (d) shows the dendrites and axons only. Recent studies suggest a volumetric, i.e., shape-independent, measure of astroglial coverage: tissue volume fraction occupied by astroglial processes (Lehre and Rusakov, 2002), or the occurrence of the nearest glial surface (Medvedev et al., 2014b), plotted against the distance to the synapse (Gavrilov et al., 2018; Henneberger et al., 2020; Patrushev et al., 2013). Meanwhile, innovative methods of 3D EM, such as block-face scanning, array tomography, or focused ion beam milling or scanning (Denk and Horstmann, 2004; Knott et al., 2008; Micheva and Smith, 2007; Xu et al., 2017), promise a qualitative improvement in shape reconstruction of synaptic astroglial environment, at high resolution.

PAPs are packed with a variety of important signalling proteins, receptors, channels and transporters, which roles have been reviewed in detail elsewhere (Bazargani and Attwell, 2016; Heller and Rusakov, 2015; Verkhratsky and Nedergaard, 2018). In the present context, the most important functional role of PAPs is that they buffer and take up glutamate

released by local synapses. The plasma membrane of PAPs is densely populated with high-affinity transporters, mainly GLT-1 and GLAST type, which are thought to be responsible for the removal of >90% glutamate from the extracellular space (Furness et al., 2008; Henneberger et al., 2020; Huang and Bordey, 2004; Lehre and Danbolt, 1998; Michaluk et al., 2021; Rothstein et al., 1994; Tanaka et al., 1997). The transporter enriched PAPs thus confine glutamate actions largely to the synaptic cleft, at least following individual discharges (Bergles et al., 1999; Bergles and Jahr, 1997; Diamond, 2001; Diamond and Jahr, 1997; Otis et al., 1996; Tong and Jahr, 1994). Both 'glial' GLT-1 and neuronal EAAC1 transporters are also expressed in nerve cells, albeit apparently at a much lower density (Chen et al., 2004; Furness et al., 2008; Holmseth et al., 2012; Zhou et al., 2019), and their role in controlling extrasynaptic glutamate actions is less clear. Nonetheless, extrasynaptic glutamate escape, or 'spillover', does not appear uncommon during intense synaptic activity, which could have a significant physiological impact, as discussed below.

Observations of spillover-dependent basal transmission

In cultured hippocampal neurons, fluorescence imaging of the optical glutamate sensor SuperGluSnFR during burst spiking has detected functionally significant glutamate spillover lasting for hundreds of milliseconds (Hires et al., 2008). In the prefrontal cortex, glutamate spillover has been causally related to the recruitment of extrasynaptic NMDA receptors, thus contributing to the initiation of dendritic NMDA spikes, a key plasticity mechanism mobilising intracellular Ca2+ (Chalifoux and Carter, 2011). Several independent observations have reported a functional role of glutamate escape in the hippocampus. Short trains of discharges at CA3-CA1 synapses activated extrasynaptic NR2B and NR2D-containing NMDA receptors, thus decelerating the postsynaptic current decay (Lozovaya et al., 2004). This type of inter-synaptic co-operation in hippocampal neuropil varies with temperature-dependent glutamate uptake (Arnth-Jensen et al., 2002; Asztely et al., 1997) and depends on the density of activated synapses (Scimemi et al., 2004). In the CA3 pyramidal cell network, glutamate escaping the cleft of mossy fibre synapses (at physiological temperatures) activated presynaptic metabotropic receptors at neighbouring synapses during brief tetanic activation (Vogt and Nicoll, 1999) although less so during low-frequency activity (Scanziani et al., 1997). In the olfactory bulb, mitral cell dendrites form glutamatergic synapses with dendrites of inhibitory granule cells whereas excitatory transmission among mitral cells functions solely due to glutamate spillover (Isaacson, 1999). In the cerebellum, glutamate released from climbing fibres diffuses through the neuropil activating AMPA and NMDA receptors on molecular layer interneurons (Szapiro and Barbour, 2007). This form of

glutamatergic volume transmission could be triggered in a spatially constrained manner by spontaneous bursts of synchronous climbing fibre activity (Szapiro and Barbour, 2007), which could functionally segregate molecular layer interneurons, depending on their location relative to release sites (Coddington et al., 2013). Spillover of glutamate was implicated in the AMPA receptor-dependent modulation of presynaptic Ca^{2+} entry at basket cell terminals synapsing on Purkinje cells (Rusakov et al., 2005). At the synapses between cerebellar parallel fibres and stellate cells, short trains of stimuli were found to generate a sustained and widespread glutamate spillover signal that can evoke large and prolonged EPSCs mediated by ionotropic glutamate receptors (Carter and Regehr, 2000). These and related observations indicate that excitatory signals transmitted by glutamate escaping the synaptic cleft could have a significant impact on signal propagation and integration in neural circuits of the brain.

Functional plasticity of astroglial environment

Because PAPs are critically important for constraining extrasynaptic glutamate escape, their activity-dependent changes could represent an adaptive mechanism to control glutamate spillover-mediated actions. EM studies have long focused on changes in PAPs during the induction of long-term potentiation (LTP), a basic form of synaptic memory. For up to 8 hours after high-frequency LTP induction in the dentate gyrus *in vivo*, there was a higher numerical density, but smaller volume of PAPs, which were also found to occur closer to synaptic clefts (Wenzel et al., 1991). LTP induced in organotypic hippocampal slices by theta-burst stimulation or by glucose deprivation was accompanied by increased astroglial coverage of synaptic structures (Lushnikova et al., 2009). Enhanced PAP motility and synaptic coverage was reported to follow LTP induction in organotypic slices and in vivo using a variety of protocols that mimic intense synaptic activation (Bernardinelli et al., 2014b). Interestingly, in animals reared in complex environment, the surface density of astrocytic membrane in direct apposition to synapses increased within the visual cortex compared with control groups of animals (Jones and Greenough, 1996). On the other hand, a threat-conditioning task led to a transient increase in the density of synapses in the lateral amygdala with no PAP contact whereas synapses with astrocytic contacts appeared smaller (Ostroff et al., 2014). In the rat hypothalamic supraoptic nucleus, lactation was causally associated with a lower degree of astrocytic coverage and, as a consequence, increased glutamate concentration and diffusion in the extracellular space (Oliet et al., 2001).

Because EM protocols cannot monitor PAPs in time, and might be prone to morphological distortions during fixation (Korogod et al., 2015), recent studies have used confocal or two-

photon excitation (2PE) fluorescence microscopy to register fine changes in PAPs in the course of network activity or after induced synaptic plasticity. In hippocampal slice cultures, changes in PAPs were coordinated with changes in local dendritic spines, and astrocyte-spine interactions appeared more stable at larger spines (Haber et al., 2006). Significant motility and morphological plasticity of PAPs were reported in acutely isolated brainstem slices (Hirrlinger et al., 2004). In an elegant combination of in vivo and in situ methods, changes in PAP motility and morphology were recorded upon LTP induction in the hippocampus and upon whisker stimulation in the somatosensory cortex (Bernardinelli et al., 2014b). Likewise, electrical stimuli inducing hippocampal LTP boosted the motility of synapse-associated PAPs, triggering structural remodelling of synaptic environment (Perez-Alvarez et al., 2014).

A recent work has combined multiple methods of sub-diffraction microscopy and optical glutamate sensor imaging to conclude that LTP, at least in its classical form, prompts withdrawal of PAPs leading to increase in glutamate spillover (Henneberger et al., 2020). Furthermore, several studies have shown that certain patterns of neuronal activity, across different brain regions, can affect various aspects of glutamate homeostasis including the its uptake rate (Armbruster et al., 2016; Pinky et al., 2018), or lateral mobility and recycling of its astroglial transporters (Al Awabdh et al., 2016; Michaluk et al., 2021; Murphy-Royal et al., 2017). Clearly, the complexity of morphological astroglial changes on the nanoscale, and the sheer variety of experimental or endogenous signals that can induce synaptic plasticity suggest that astroglial coverage could play a multitude of roles in regulating extrasynaptic actions of glutamate.

Biophysical aspects of glutamate release, escape, and receptor activation

In what conditions and how far glutamate can escape the synaptic cleft activating its target receptors outside the immediate synapse has long been the subject of debate. Because it is not technically feasible to measure fast synaptic or perisynaptic diffusion of glutamate directly, its quantitative assessment through biophysical modelling has long been a tool of choice.

Historically, glutamate release, diffusion and receptor activation inside the synaptic cleft was explored theoretically using analytical solutions (Kleinle et al., 1996; Uteshev and Pennefather, 1996) or Monte Carlo simulations (Bartol et al., 1991; Clements, 1996; Kruk et al., 1997; Trommershauser et al., 1999; Wahl et al., 1996) modelling the inside of the synaptic cleft. Since the earlier models it has been clear that, in addition to the cleft structure and glutamate release properties, a key parameter that determines the occupancy of

glutamate receptors post-release is the local glutamate diffusion coefficient (Barbour, 2001; Clements, 1996; Raghavachari and Lisman, 2004; Rusakov and Kullmann, 1998; Savtchenko et al., 2013). Experimental manipulations with extracellular medium viscosity confirmed that slowing down glutamate diffusion could significantly boost activation intra- and extrasynaptic glutamate receptors (Min et al., 1998; Nielsen et al., 2004; Zheng et al., 2008). However, how fast glutamate diffuses inside the cleft has long remained unknown. Only recently, a study employed time-resolved fluorescence-anisotropy 2PE imaging of chromatically separated soluble fluorophores, one intracellular and one extracellular, to gauge nanoscale diffusivity of small molecules inside and outside the synaptic cleft (Zheng et al., 2017). These measurements indicated that in brain interstitial gaps molecular mobility is ~30% lower than in a free medium, inside neuronal dendrites this retardation is ~70% whereas between pre- and postsynaptic membranes free diffusion of small molecules is decelerated by ~46% (Zheng et al., 2017). It turned out that, with ~2800 glutamate molecules released per vesicle at common small synapses (Savtchenko et al., 2013), decelerating glutamate diffusion inside the cleft by 46% provides a strong boost to AMPA receptor activation compared with the case of free-medium diffusion (Figure 4a).

Incorporating transporter-enriched PAPs in the simulated synaptic environment has provided important detail pertaining to the relationship between synaptic astroglial coverage and the extend of extrasynaptic glutamate actions. In a special case of synaptic structures tightly wrapped by astroglial processes, such as synaptic glomeruli, calyceal or giant mossy fibre synapses, biophysical models could readily explain experimental observations pointing to significant cross-talk among local, intrasynaptic release sites (Barbour et al., 1994; Nielsen et al., 2004; Renden et al., 2005; Vergnano et al., 2014). As for the common small synapses, models of the (multi-)synaptic neuropil suggested that single-vesicle release of glutamate could lead to only limited glutamate receptor activation outside the immediate synaptic microenvironment, as tested with either a cubic grid representing the tortuous extracellular space (Franks et al., 2002; Zheng and Rusakov, 2015), or with hybrid models incorporating average tissue porosity and known distributions of glutamate transporters (Armbruster et al., 2020; Zheng et al., 2008). However, once synaptic activity intensifies, glutamate spillover actions are predicted to become physiologically significant. One important conclusion of this theoretical exploration was that it is rapid binding to high-affinity transporters, rather than geometrical obstacles per se, which provides the principal mechanism constraining glutamate escape outside the synaptic cleft (Figure 4b). This notion comes from the simple fact that the 'spilling over' glutamate molecules do not behave like a liquid. Instead, they diffuse in a Brownian fashion, in all possible directions, encountering multiple nanoscopic obstacles millions of times. Thus, the likelihood is that the escaping glutamate molecules will

experience a collision with the transporter-enriched cell walls of astroglia even when the free diffusion paths are relatively wide (Figure 4b).

Variable glutamate spillover and altered behaviour

The extent to which extrasynaptic glutamate escape can vary has recently been attributed to conditions associated with pathological changes in behaviour. Tanaka and co-workers (Tanaka et al., 2013) found that genetically targeted attenuation of astroglial Ca^{2+} signalling reduce astrocytic coverage of asymmetric synapses in area CA1 of the mouse hippocampus. This led to facilitation of glutamate spillover reported by changes in glutamate uptake currents and increased cross-synaptic activation of NMDA receptors. The animals showed impairments in spatial reference memory and remote contextual fear memory, in which hippocampal circuits are involved (Tanaka et al., 2013). Similarly, reduced astroglial glutamate uptake degraded the pathway specificity of LTP in the neural circuitry of amygdala which was directly involved in fear conditioning (Tsvetkov et al., 2004). Studies using animal models of drug addiction detected a marked increase in the extrasynaptic glutamate in the core subcompartment of the nucleus accumbens during reinstated drug seeking (Gass et al., 2011; Gipson et al., 2013; McFarland et al., 2003). Through the analyses of glutamate uptake and NMDA receptor-mediated currents, it was shown that heroin self-administration produced long-lasting downregulation of glutamate uptake associated with increased spillover of synaptic glutamate to extrasynaptic NMDA receptors (Shen et al., 2014), which was associated with an enduring reduction in synaptic proximity by astroglia (Kruyer et al., 2019). In ageing, the glutamate uptake modulator riluzole can protect against synaptic alterations in hippocampus that are linked to age-related memory loss in rats (Pereira et al., 2014). Riluzole increases glutamate uptake and thus decreases glutamate spillover to extrasynaptic NMDA receptors while apparently boosting synaptic activity. Aged rats treated with riluzole were protected against age-related cognitive decline displayed in nontreated aged animals (Pereira et al., 2014). Astroglia-dependent glutamate spillover is likely to be important across the spectrum of cognitive functions since it can be controlled by the induction of the elementary synaptic memory form, long-term potentiation (Henneberger et al., 2020).

Concluding remarks

Synaptic circuits operating glutamatergic excitatory transmission are at the core of information processing and storage in the mammalian brain. It has long been assumed that

this type of transmission is strictly one-to-one, 'wired' type, akin to wired electrical connections in computer chips. There is however a growing body of evidence suggesting that this may not always be the case, and that in certain circumstances glutamate may escape the immediate synaptic cleft, activating target receptors in a volume-transmitted manner. Here, we have reviewed existing evidence to discuss whether and how structural features of excitatory synapses and their microenvironment could be related to the prevalence and degree of glutamate spillover-mediated actions. Whilst the structural, physiological, and biophysical modelling evidence provides us with a multi-faceted picture in this context, the emerging novel methods of super-resolution microscopy and genetically encoded glutamate sensor imaging promise answers to important and intriguing questions that remain.

AKNOWLEDGEMENTS

This work was supported by the Wellcome Trust Principal Fellowship 101896-212251, ERC Advanced Grant 323113, and EU CSA 811011 (D.A.R.).

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Figures and legends

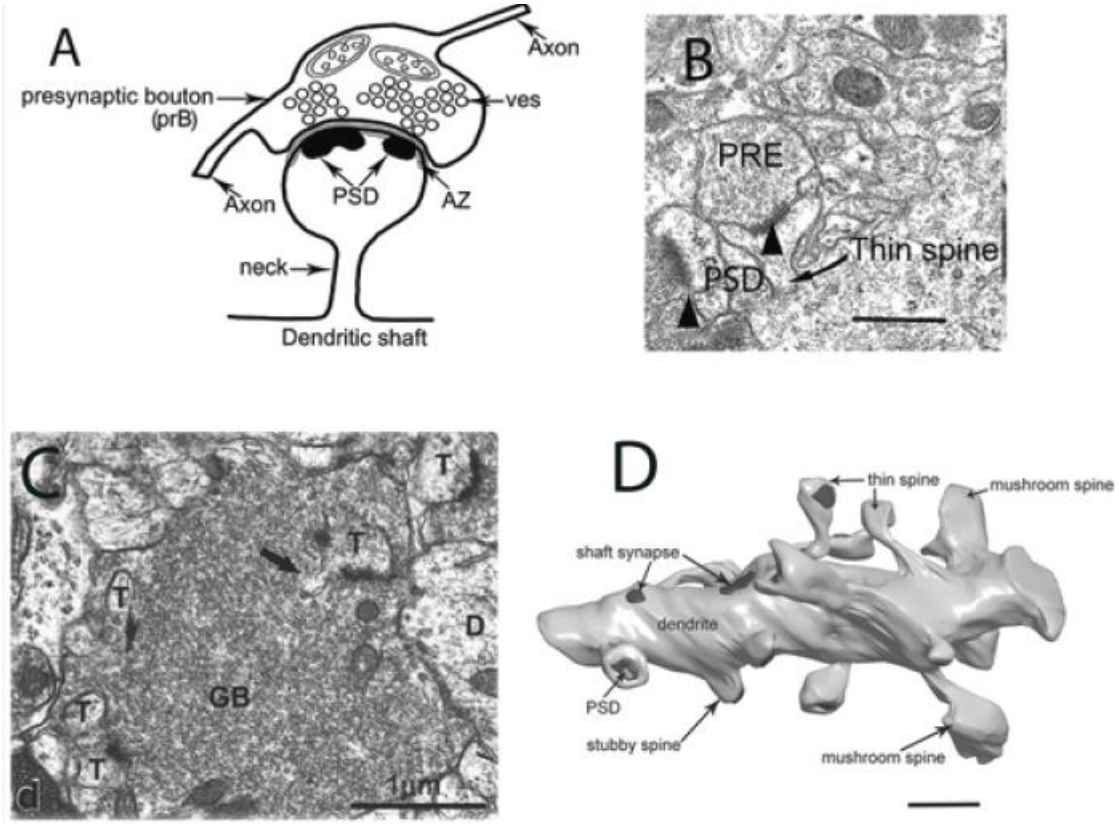

Figure 1A, B

Figure A. Schematic of a glutamatergic synapse showing a pre-synaptic bouton (prB) which contacts a large spine head (mushroom in type). The post-synaptic density (PSD) is shown with arrows and there is a perforation between the 2 parts of this PSD. The synaptic apposition zone (AZ) is greater in size than the PSD and its limits are shown on the diagram. There is a thin spine neck which leads to a dendritic shaft; (adapted from Stewart and Popov, 2012).

Figure B. Electron micrograph showing an example of a synaptic bouton (PRE) with a prominent post-synaptic density (arrowhead- PSD) and spheroid vesicles; the bouton makes an asymmetric contact with a thin dendritic spine. An adjacent asymmetric synapse with a prominent PSD is to the left. Scale bar is 0.3 □m.

Figure C. Electron micrograph of an excitatory connections between hippocampal mossy fibres which form giant boutons on CA3 pyramidal cells ~ 0.5–2 μm.

Figure D. 3-dimensional (3-D) reconstructions of a dendritic segment (~10□m) showing a number of spine types: Msp, mushroom spines; Tps- thin spines, stubby spines, and shaft synapses where the synapse directly contacts the dendritic shaft. PSD, post synaptic density) (see also Figure 2A). Scale bar = 0.5 μm.

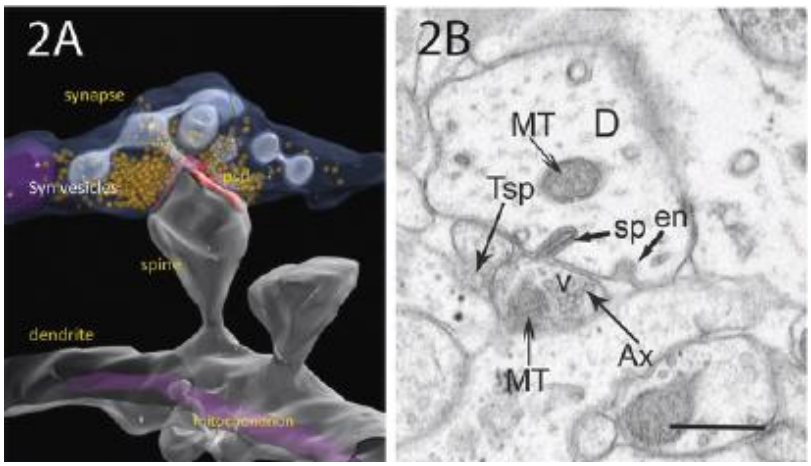

Figure 2 A, Fine detail is shown in 3-D reconstruction of over 100 serial sections of a glutamatergic synapse which contacts a mushroom dendritic spine. Note the synaptic vesicles and the post synaptic density (psd). The 3-D reconstruction shows that the mitochondrion runs as an uninterrupted entity through the dendrite. Reconstruction courtesy of Dr. Vadim Rogachevsk, Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Moscow region, 142290

Figure 2B Electron micrograph from dentate gyrus showing a dendrite (D) from a granule cell contacted by a small synapse (Ax—or axon terminal). A thin spine (Tsp) is also labelled. A spinule (sp) is clearly present and an endosome (en) is present at the side of the axon terminal. MT mitochondrion. Scale bar = 1.0 □m.

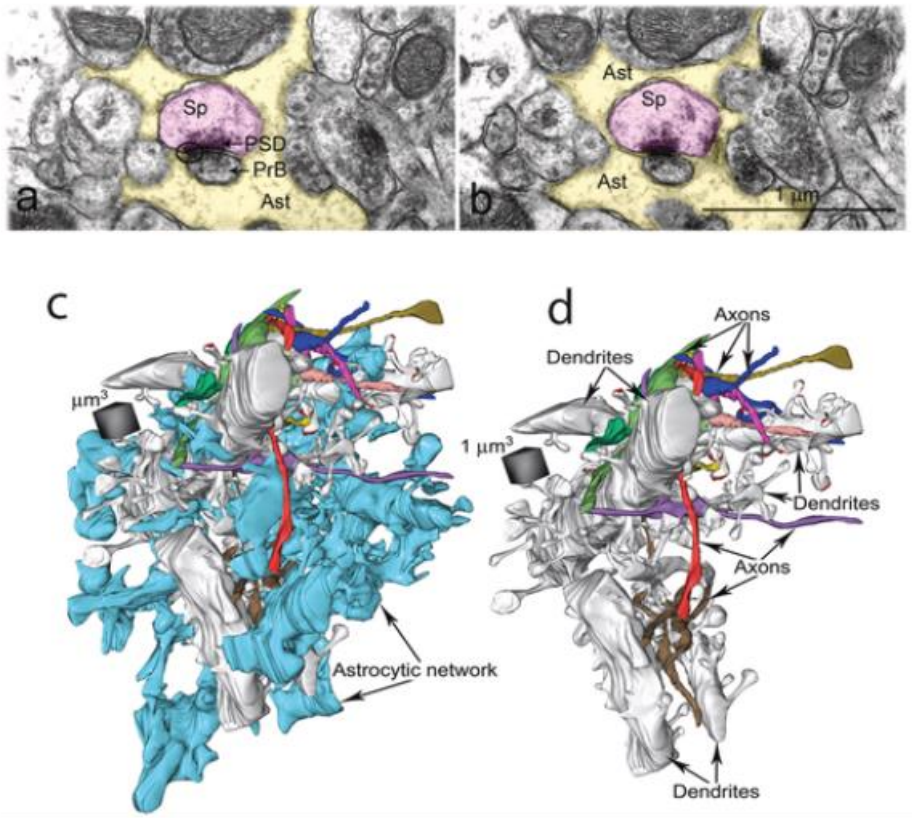

Figure 3 Two serial electron micrographs in dentate gyrus (a, b) show the extent of astroglia (Ast) coverage of a spine (Sp) and synapse with PSD, on a presynaptic bouton (PrB) scale bar= 1 µm, while Figure 3 (c, d) show 3D reconstructions from~100 serial sections in dentate gyrus, with Figure 3 (c) showing the extensive astrocytic network while Figure 3 (d) shows the dendrites and axons alone.

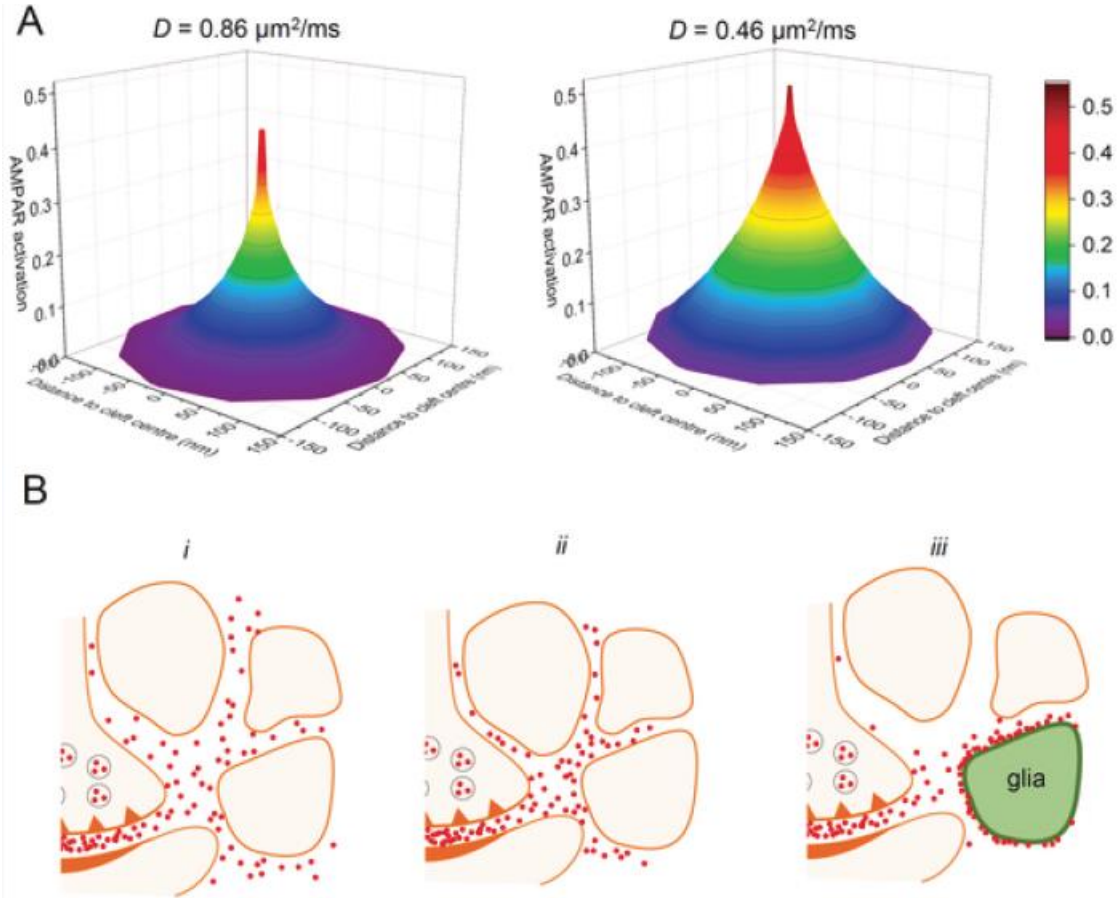

Figure 4**.** Basic features of glutamate diffusion inside and outside the cleft.

A: Computed 3D profiles of local AMPAR receptor activation (peak percentage of open receptors, false color scale) upon instantaneous release of 2800 glutamate molecules (Savtchenko et al., 2013) into the synaptic cleft centre, at two glutamate diffusion coefficients, as indicated: one in a free-medium ACSF (0.86 µm^2/ms, left) and one between pre- and postsynaptic membranes (0.46 µm^2/ms, right) measured using two-photon excitation TR-FAIM (Zheng et al., 2017). The data represent Monte Carlo simulations of the ~300 nm wide ~15 nm tall synaptic cleft of CA3-CA1 synapses (Rusakov and Kullmann, 1998) facing a porous neuropil (Savtchenko et al., 2013).

B: Diagram, qualitative summary of biophysical modelling; escape of glutamate molecules (red dots) from the synaptic cleft (shown) can be decelerated by the surrounding neuronal elements (*i*), with narrower interstitial gaps restricting the escape further (*ii*), but PAPs (glia)

enriched with high-affinity glutamate transporters act as a 'sticky' obstacle binding escaping glutamate molecules with much greater efficiency (*iii*).

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