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Review

Dynamics of surface neurotransmitter receptors and transporters in glial cells: Single molecule insights

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ABSTRACT

The surface dynamics of neurotransmitter receptors and transporters, as well as ion channels, has been well-documented in neurons, revealing complex molecular behaviour and key physiological functions. However, our understanding of the membrane trafficking and dynamics of the signalling molecules located at the plasma membrane of glial cells is still in its infancy. Yet, recent breakthroughs in the field of glial cells have been obtained using combination of superresolution microscopy, single molecule imaging, and electrophysiological recordings. Here, we review our current knowledge on the surface dynamics of neurotransmitter receptors, transporters and ion channels, in glial cells. It has emerged that the brain cell network activity, synaptic activity, and calcium signalling, regulate the surface distribution and dynamics of these molecules. Remarkably, the dynamics of a given neurotransmitter receptor/transporter at the plasma membrane of a glial cell or neuron is unique, revealing the existence of cell-type specific regulatory pathways. Thus, investigating the dynamics of signalling proteins at the surface of glial cells will likely shed new light on our understanding of glial cell physiology and pathology.

1. Introduction

The lateral diffusion of proteins at the cell surface has been well-described and established over several decades. This process has now been demonstrated to occur in a wide variety of cells, including cells from the central nervous system such as neurons [1–6], astrocytes [7–10] and microglia [11]. Surface diffusion is a thermodynamic process by which proteins, inserted into the membrane by exocytosis, move in a Brownian manner and potentially interact with a vast number of other proteins and lipid rafts which may impede their diffusion in the membrane [12]. This impedance, through protein–protein interactions has been suggested to be the main mechanism by which neurotransmitter receptors are retained in the post-synaptic density (PSD) of synapses. Quite surprisingly, the turnover of surface receptors in stable structures, such as the PSD, appears to be higher than previously thought, with receptor dwell times in the order of minutes rather than the hours, days, and years believed necessary for memory retention [1]. This process of receptor lateral diffusion plays a pivotal role in basal synaptic transmission as well as forms of synaptic plasticity [13,14]. Furthermore, the surface diffusion of specific glutamate receptors is directly modulated by physiological challenges such as those associated with stress [15–17], suggesting that adaptations of the brain cell network under these conditions involve a fast redistribution of membrane receptors. Thus, there is a consensus that the surface diffusion of neurotransmitter receptors, together with the exo/endocytosis cycling, plays a key role in synaptic and network plasticity in the healthy brain. Despite our wide ranging knowledge of the conditions and constraints of surface diffusion of many different proteins in neurons, where fast

Abbreviations: P2 × 7, purinergic receptor P2 × 7; mGluR5, metabotropic glutamate receptor 5; DAT, dopamine transporter; AQP4, aquaporin-4; DOPE, 1,2-dioleoyl-sn-*glycero*-3-phosphoethanolamine; GLT-1, glutamate transporter-1; $Ca_v1.2$, L-type voltage-gated calcium channels; KCC2, potassium/chloride cotransporter 2; NMDAR, *N*-methyl-D-aspartate receptor; αNa,K-ATPase, sodium-potassium adenosine triphosphatase; D1R, dopamine receptor 1; $GABA_AR$, γ-aminobutyric acid receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; A7-AchR, α7 nicotinic acetylcholine receptors; GlyR, glycine receptor; Bkca, calcium-activated potassium channel; Eag1, ether-à-go-go 1 potassium channel; CB1R, cannabinoid receptor 1

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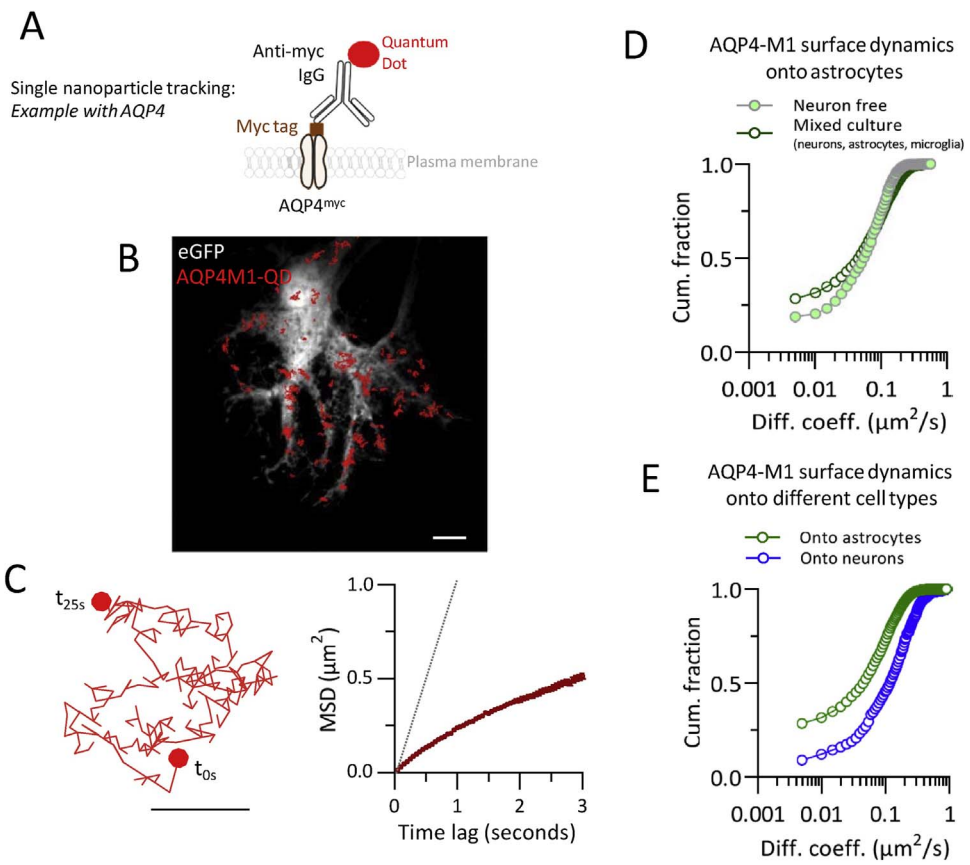

Fig. 1. Single-particle tracking Quantum Dot of surface over-expressed AQP4M1myc on astrocytes in mixed hippocampal culture cells. (A) Schematic description of the experimental procedure to track surface AQP4myc by spt-QD. (B) eGFP- expressing astrocyte with AQP4-QD trajectories (red). Scale bar 5 µm. (C) Left: representative of a 25-s-long single AQP4 trajectory surface diffusion. Scale bar, 500 nm. Right: mean square displacement of AQP4 in mixed hippocampal culture, shows a negative curve characteristic of confined movement. (D) Cumulative distribution of AQP4 diffusion coefficient in neuron-free or mixed culture. (E) Cumulative distribution of AQP4 diffusion coefficient on astrocytes or neurons in mixed culture, showing that AQP4 "artificially" expressed on neurons has a higher numbers of mobile proteins compared to overexpressed AQP4 on astrocytes. Even thought AQP4 is not naturally expressed on neurons, the presence of water channel could have induced shape modifications (swelling and shrinking). However no visible changes were found. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adaptations are key to normal function, our understanding of the surface diffusion occurring at the surface of glial cells is still in its infancy.

Astrocytes are highly ramified cells with extensive branching of fine processes throughout the neuropil. Each astrocyte process contains several microdomains, classically defined by highly localised calcium activity, which can function independently to the rest of the cell and influence nearby synapses and the vasculature. These microdomains on astrocyte processes are highly specialised in terms of structure and function. For instance, perivascular endfeet formed by astrocytic process are enriched with dystrophin associated protein complex (DAPC) and α-syntrophin that are now considered key factors for the molecular assembly stabilization, and concentration of receptors at the end-foot [18]. Another interesting feature of the astrocyte is its branching through the numerous fine-processes that surround synapses, it has been demonstrated that these processes can be tens of nanometers in size, well below the diffraction limit of light [19]. It has been suggested that these astrocytic process regulate several pre- and postsynaptic functions through, the release of gliotransmitters triggered by the local activation of astrocytic neurotransmitter receptors [20,21]. Although the mechanism underlying the anchoring of these receptors and transporters on astrocyte processes near synapses remains largely unknown, specific scaffold proteins as well as the shape of individual processes have been proposed to contribute to the stabilization of receptors/transporters [22–24]. As in neurons, transmembrane proteins located in the plasma membrane of astrocytes diffuse laterally and are dynamically regulated in different cell compartments [7,8,25]. Indeed, their diffusion has been demonstrated to be directly regulated by cell activity (neuronal as well as glial) in both physiological and pathological conditions [7,8,25,26]. Here, we review the literature describing the surface dynamics of neurotransmitter receptors and transporters located on glial cells, emphasizing the unanswered questions, technical advances, and challenges facing the field.

2. How to track membrane transporters?

Several approaches have been used to monitor the lateral trafficking of proteins on neuronal membranes each with distinct advantages and limits. These methods were then applied to investigate the diffusion of receptors, transporters, and channels in astrocytic plasma membranes. There are two main procedures used to track diffusion of a protein: ensemble measurements and single particle/molecule tracking (SPT). Regarding the first method, a well-described approach is the fluorescent recovery after photobleaching (FRAP) [27]. This approach was used to determine the lateral mobility of numerous neurotransmitter receptors [28–37] and neuronal transporters such as GABA transporter GAT-1 [38]. This technique takes advantage of the fact that most fluorophores are irreversibly bleached by incident light of very high intensity. A defined region of the sample is photobleached with high intensity light and these molecules are subsequently replaced by non-bleached molecules over time. The proportions of fluorescent molecules that can participate in this exchange indicate the mobile fraction. The fraction of molecules that cannot exchange between bleached and non-bleached regions is called the immobile fraction. Fluorescence microscopy is often a question of compromise: FRAP technique allows the collection of extensive information regarding the dynamic processes occurring in our sample due to the fast acquisition of images, but at the same time delivering limited spatial information, due to the signal being obtained from a single layer of plasma membrane (~200 nm). This is a limitation because cells are not flat and have various shapes and contours, a limitation that will currently apply to all 2D-imaging approaches. Furthermore it only gives an estimation of the mobility of a group of proteins. Nevertheless, FRAP experiments are commonly performed on laser scanning microscopes, making it an accessible method for determining the diffusion of a population of surface proteins. Another way to measure the average mobility of a protein population is the fluorescence correlation spectroscopy (FCS), which is based on the analysis

of fluorescence intensity fluctuation produced when the tagged proteins enter and exit the light exited membrane domain [39,40]. The FCS represents a significant advantage for studying protein diffusion in dendritic spines because of the capacity to measure diffusion in small volumes. However only few studies have used the FCS to reveal neuronal membrane proteins diffusion [41,42].

The SPT approach is a single molecule detection-based method. For neurotransmitter receptors and transporters, the classical complex consisted of a nano-reporter coupled to an antibody directed against an extracellular epitope of a transmembrane protein of interest (Fig. 1A) [43,44]. The SPT allows tracking individual proteins conversely to FRAP where the diffusion value is an average of the movement of many molecules. Several nano-reporter types can be used, as for instance organic dyes and nanoparticle (e.g. quantum dots; QDs). Fluorescent organic dyes are small in size but they are short-lived and photobleach rapidly. QDs are nanometre-size semiconductor fluorescent particle with a point accuracy of 1–10 nm that also provide long observation times without photobleaching. As such, SPT is adept to demonstrate differences in the mobility of the same protein expressed in different cell types (Fig. 1E). The use of SPT-QD has revolutionized our understanding of neurotransmitter receptor and transporter trafficking, shedding new light on dynamic membrane dynamic organization in neurons (Fig. 2A, B). This approach allows direct visualisation of the movements of protein into and out of a defined domain, such as the PSD, and provided the first evidence that lateral diffusion is the major contributor to the exchange of receptors in and out of the postsynaptic site[45–52]. Furthermore, this strategy contributed to a profound discovery in synaptic physiology: at extrasynaptic sites proteins diffuse relatively freely whereas at synapses diffusion of proteins is greatly impacted by the presence of obstacles, binding to specific scaffolding proteins, cytoskeleton components, and interactions with other binding partners. This approach was used to reveal the surface trafficking of a wide variety of neuronal receptors such as AMPA, mGluR5, Glycine, $GABA_A$, NMDA, Cannabinoid 1, Dopamine D1, and Nicotinic α7 receptors [28,45,53–60], as well as Na+/K+ ATPase [61], channels and transporters such as Eag1, BKCa, $Ca_v1.2$ and KCC2 [35,62–64]. Specifically for astrocytes this list includes AQP4, mGluR5, DOPE, P2 × 7, GLT-1, DAT [7,25,65,66] (Fig. 2A, B). Even though SPT techniques can provide single-molecule tracking over long-durations, the capacity to simultaneously study a high number of individual molecules on a single cell is restrained by the diffraction limit, particularly if the protein of interest is expressed at a high density in a confined space. Interestingly, this limitation can be overcome by the combination of super-resolution methods such as photoactivated light microscopy (PALM) with SPT (spt-PALM) [67,68]. Spt-PALM is an approach used for mapping the trajectories of individual molecules in living cells at very high densities. Another limitation of SPT approach has been the use of cultured neuronal systems and not intact brain tissue. This was due to sufficient access to the surface of individual cells and the rapid binding of the antibody-QD complex to the protein of interest before internalization of the receptor-QD complex occurs, but also to a strong activation of the immune system after direct or intravenous injections. Recently, this barrier has been broken as SPT imaging was successfully performed in brain slices [69–71]. Varela et al. in particular used a unique means of *in vivo* delivery of fluorescent nanoparticles into the brain before acute brain slice preparation, thereby allowing the tracking of individual proteins in real time in a much more physiological preparation than ever seen before and without microglial activation. Overall surface trafficking of proteins can be studied using a wide-range of different microscopy and labelling techniques which seem to be growing more diverse and precise with novel methodological developments in microscopy, protein labelling, and tissue-preparation.

As pointed out in the above descriptions, these techniques have limitations. Overall, one of the main critic and limitation is that most imaging and electrophysiological studies of surface diffusion have been performed in vitro. This critic has now been partly addressed as surface dynamics measurements, including single nanoparticle tracking, have been performed in brain slices [8,70,71]. These studies highlight the similarities in the membrane dynamics of receptor/transporter in either dissociated cells or cells from slices. Yet, *in vivo* imaging of membrane receptors and transporters at the single molecule level has not been reported as several major limitations still preclude such an experiment.

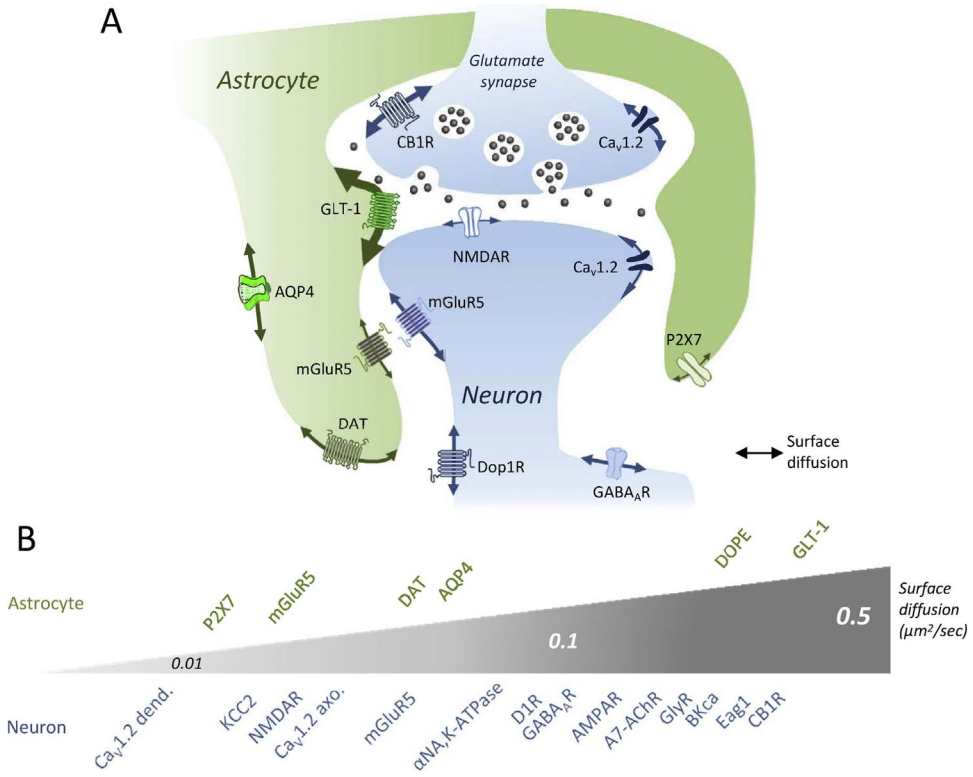

Fig. 2. Comparison of the surface trafficking of neurotransmitter receptors and transporters, as well as ion channels in neurons and astrocytes. (A) Schematic representation of a glutamate synapse with the neuronal and astrocytic components. Several membrane neurotransmitter receptors, transporters, and ion channels are displayed. Their relative surface diffusion is depicted using arrows, with high surface diffusion represented with thick arrows. (B) Comparison of neurotransmitter receptor, transporter, and ion channel surface diffusion in neurons (blue) and astrocytes (green). The surface diffusion was extracted from publications and expressed as average diffusion coefficient s in $\mu m^2/s$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Furthermore, new methodological developments are still needed to track single molecules both in live cells and tissues in order to obtain 3D trajectories, as our current knowledge is mostly based on 2D-projections.

3. Receptor dynamics in glial cells

Although less understood than in neurons, the dynamics of membrane receptors in glial cells is gaining more attention. The surface diffusion of P2 × 4 channels has been characterised in quiescent and activated microglial cells [11]. This study demonstrated a dynamic regulation of the ion channel by its agonist (ATP), as well as by 'activation' of these microglial cells induced by exposure to lipopolysaccharide. Although, this study was carried out in a microglial cell line, it demonstrated quite nicely the sensitivity of surface diffusion of receptors on a type of glial cell. However, whereas on neurons it is easy to assess the functional impact of these processes through measuring parameters such as synaptic content and postsynaptic potentials, the impact of changes in P2 × 4 receptor diffusion on microglial function remains unresolved. One well-characterised receptor in terms of surface diffusion on astrocytes is the metabotropic glutamate receptor (mGluR5) [25,66]. This receptor is known to play an important role in neuron-glia interactions serving as a sensor for glutamatergic synaptic transmission and allowing astrocytes to dynamically modulate neuronal activity through the release of adenosine [72] as well as a multitude of different roles such as neurovascular coupling [73,74]. Furthermore, the surface diffusion of mGluR5 on astrocytes depend on the sub-cellular location, i.e. soma versus processes [25]. Indeed, the presence of a diffusion barrier impacts the receptor, whereby receptors do not diffuse from soma to the processes or *vice versa*. Interestingly, this barrier was mGluR5-specific and could be overcome by overexpression of the receptor, potentially overwhelming the endogenous regulatory machinery [25]. Also noteworthy is that the diffusion of the purinergic ionotropic P2 × 7 receptor and the phospholipid DOPE (1,2-dioleoyl-sn-*glycero*-3-phosphoethanolamine) in astrocytes were not different between compartments, in comparison to mGluR5 [25], further supporting the hypothesis that mGluR5 membrane dynamics in astrocytes are highly regulated. More recently, diffusion of mGluR5 was also explored in more pathological conditions [26]. It has been reported that β-amyloid oligomers bind to the membrane of neurons and hinder diffusion of membrane proteins [75]. When examined in astrocytes, the pathological Aβ oligomers severely impact the surface diffusion of mGluR5, trapping and clustering these receptors [76]. This diffusional trapping leads, in turn, to an increased release of ATP from astrocytes.

4. Transporters and channels dynamics in glial cells

The first studies utilising the single-particle tracking (SPT) technique to study astrocyte-specific proteins were carried out on the water channel aquaporin-4 (AQP4) [65,77]. Based on transcriptome data, this protein is one of the most highly expressed in astrocytes, with the RNA levels that are well above other proteins such as glutamate transporters [78]. This water channel has been demonstrated to play strong physiological role in controlling intracellular as well as extracellular ion concentrations [79]. This study reported a remarkable difference in the diffusion of the two main protein isoforms in astrocyte cultures. The surface AQP4-M1 appears highly mobile whereas the AQP4-M23 is mostly immobile [65,77]. This difference is likely dependent on the fact that these two isoforms differ in 23 amino acids, a sequence missing in the M23 isoform. Interestingly, the surface dynamics of surface AQP4-M1 on astrocytes co-cultured with neurons (unpublished data; Fig. 1D) is different from astrocyte-pure preparations [65,77]. The decreased number of mobile AQP4-M1 in presence of neurons (Fig. 1D) suggests that neurons can modulate astrocytic AQP4 surface distribution and dynamics, likely through induction of functional neuron-glia interactions at the level of the astrocyte process surrounding the synapse.

The trafficking of the major astrocytic glutamate transporter, GLT-1, has also been investigated. GLT-1 plays an essential role in the brain as its knock-out from astrocytes lead to severe impairment in synaptic function and eventually premature death of the animals [80]. Transcriptome analysis has also shown that GLT-1 mRNA is highly present in adult astrocytes [78]. One early study which used live-cell imaging to investigate the precise location of GLT-1 in different physiological conditions used a genetically modified GFP-tagged GLT-1 [81]. This construct allowed visualisation of GLT-1 clusters on the surface of astrocyte, directly opposed to synaptic clefts in an organotypic slice culture preparation. It was shown that GLT-1 clusters on the surface of astrocytes underwent rapid, dynamic regulation in response to pharmacological manipulation of synaptic activity. The size of GLT-1 clusters was found to be positively correlated with synaptic activity and underwent remodelling on the minute timescale [81]. In agreement, it was later demonstrated that similar GLT-1 clustering and localisation was observed near synapses using immunostaining in mixed neuronal-astrocyte cultures [7]. The diffusion of individual GLT-1 transporters on astrocyte membranes was then investigated near synapses (i.e. glutamate release sites), as well as the putative role of this diffusion on synaptic transmission. It clearly emerged that GLT-1 surface diffusion is greatly reduced close to the synapse, however, once exposed to experimentally released glutamate through photo-uncaging of MNI-glutamate, GLT-1 transporters mobility increased, resulting in their exit from synaptically opposed zones on the astrocyte membrane. If GLT-1 are experimentally immobilised with their transport function preserved, the basal synaptic response was prolonged. This experiment resulted in an increase in rise time and decay of individual spontaneous synaptic events, suggesting that surface diffusion of this transporter impacts strongly the clearance of glutamate from the synapse, with reduced diffusion leading to prolonged glutamate in the cleft. Consistent with this claim, blocking glutamate uptake has little effect on AMPAR-mediated synaptic currents [82,83]. Recently, the diffusion of the two main isoforms of GLT-1 (GLT-1a and GLT-1b) and the impact of neuronal activity on their diffusion properties was thoroughly investigated [8]. This study used both FRAP and SPT-QD techniques to reveal that both isoforms of GLT-1 are clustered at synapses and could be 'released' with exposure to glutamate as well as synaptic activity by electrical stimulation. This study provided the first evidence of that GLT-1 laterally diffuse on astrocytes in a hippocampal slice preparation [8]. The authors found that while overall diffusion was slower than in primary cultures, the trends remained the same with GLT-1 diffusion slowed, forming clusters opposing synapses and becoming more mobile with increased synaptic activity. Finally, the diffusion of a tagged dopamine transporter (DAT) was also investigated in astrocytes, revealing a slower diffusion coefficient when compared to GLT-1, suggesting difference between neurotransmitter transporters in cultured astrocytes [7,25].

5. Comparison of membrane dynamics between neurons and astrocytes

The studies mentioned above indicate that the surface diffusion of proteins embedded at the plasma membrane of cultured glial cells is fast, similar to that of neurons. How similar are the dynamics of a given receptor, transporter, or ion channel between a glial cell and neuron? The tracking of the same protein AQP4, mGluR5 or $Ca_v1.2$ in different cell types revealed interesting differences in their surface dynamics (Figs. 1E and 2) [9,35,54,66]. Several factors can be proposed to influence the lateral diffusion and explain these differences between neurons and astrocytes. On neurons, the surface distribution of neurotransmitters receptors is not uniform. Single particle tracking revealed that the mobility of receptors on neurons depends on their location: the synaptic fraction is slower than the extrasynaptic [45–52]. Intracellular scaffold proteins, cytoskeleton and extracellular matrix (ECM) molecules have been proposed to contribute to the observed heterogeneous

membrane dynamics [1,84–86]. Astrocytes are, in contrast, generally considered to lack such intracellular scaffold densities near synapses, as evidenced by electron microscopy of brain tissue [87]. However, the perivascular end-feet are endowed with dystrophin associated protein complex, α-syntrophin, b1-integrin and utrophin [88] proteins with the capacity to anchor diffusing channels such as AQP4 and Kir4.1. Thus, one can propose the existence of specialised domains in the astrocytic perisynaptic membrane. Indeed, GLT-1 and mGluR5 surface diffusion onto astrocytes is markedly reduced in fine processes and near synapses [7,25]. A molecular cross-talk of surface receptors could also modulate their surface dynamics between cell types. In neurons, it has been showed that NMDAR and dopamine receptor D1 form dynamic clusters at the vicinity of glutamatergic synapses and the disruption of such interaction, by the use of competing peptide, increase the receptor's lateral dynamics [89]. In this line, the stabilization of astrocytic GLT-1 near synapses could be proposed to rely on its interaction with other proteins such as AQP4, Na+/K+ ATP-ase and mGluR5, [90–94]. Another modulator of surface trafficking could be the physical cross-talk with tyrosine kinase receptors of Ephrin. The Eph receptor/ligand signalling is associated with synapse and spine formation, partly through its interaction with the NMDAR [95]. Interestingly, Ephrin receptors and their ephrin ligands have been suggested to also regulate neuron-glia interactions. The interaction between neuronal EphA4 and glial ephrin-A3 regulate the glial glutamate transporter expression, which in turn regulates synaptic glutamate concentration, postsynaptic depolarization and ultimately modulating synaptic plasticity [96,97]. Finally, the shape of the membrane (e.g. curvature and contours) and/or lipid composition could also influence the receptor lateral trafficking. For instance, the diffusion of the glutamate AMPAR is reduced at the neck of dendritic spines [30], likely through the strong curvature of the compartment. There is no experimental evidence for such an effect in astrocytes, probably due to the difficulty in detecting their complex spatial morphology [98]. The morphological changes of astrocyte over time could regulate the diffusion of membrane proteins. Future technical development in cellular imaging will surely shed light on these possibilities.

6. Concluding remarks and perspectives

It is becoming clear that the relationship between neurons and glia (e.g. astrocytes) at the synaptic cleft, is a highly complex and dynamic interaction. Astrocytes not only remodel their processes in response to neuronal activity [99] but their membrane proteins, involved in neurotransmission, are also dynamically regulated by network activity [100]. These studies have wide implications for our understanding of neuro- and glio-transmission. If we consider the potential role of glutamate transporters as 'sodium channels' (reviewed in [101]), then the dynamic diffusion of this transporter, which may act as an activity sensor, around synapses will greatly impact intracellular astrocyte signalling and in turn directly impact the release of gliotransmitters, impacting upon other pivotal functions of astrocytes such as neurovascular coupling. Among key questions, it is of great interest to decrypt how surface proteins are dynamically regulated. The mobility of neuronal receptors can be regulated by interactions with the ECM, cytoskeleton and scaffolding proteins. Whether these molecular and cellular pathways apply to astrocyte is an open question. For instance, how is GLT-1 confined near synapses? One can propose that distinct basic properties of the membrane, cell surface-associated proteins (e.g. Ephrin receptor tyrosine kinases) [96,97], scaffold PDZ-containing proteins and direct membrane interactors (AQP4, mGluR5, K+ channels [90–94]) control GLT1 surface dynamics and retention near glutamate release sites. There is also strong evidence of GLT-1 co-localisation with mitochondria [102,103]. Mitochondria become immobilised in areas of high calcium, which has been repeatedly demonstrated in astrocytic processes near synapses. This has been shown to be due to calcium-dependent detachment of motor proteins in mitochondria [104]. As glutamate transport is a secondary-active process, dependent on ionic concentration gradients set up by ATPase pumps, it is highly logical that there exists transient macromolecular complexes of proteins working in concert to maintain and regulate proper synaptic transmission, evidence for this hypothesis has already been presented [105]. One could envision that a functional astrocytic perisynaptic process should include multiple functional units including; receptors and ion channels to sense activity, transporters to remove neurotransmitters, ATPase pumps to maintain ionic concentration gradients, and mitochondria which provide the energy for the ATPase pumps. Whether these complexes exist in astrocyte remain unknown.

To conclude, the studies highlighted in this review begin to reveal the role and importance of surface diffusion of proteins at the surface of glial cells. It is tempting to speculate that with the development of cutting-edge imaging approaches we will discover new functions of channel, receptor, and transporter membrane dynamics, emphasizing that the quantity of a given protein is not sufficient to understand its physiological role. The ability to track a protein of interest at the single molecule level, over several microns, in three dimensions, and in a live neuronal network, has become a reality, thanks to implementations in the real-time single-particle-tracking [106]. This opens new avenues of research to understand the physiology, as well as pathology, of astrocytes.

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