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# Surface trafficking of neurotransmitter receptors: from cultured neurons to intact brain preparations

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# Surface trafficking of neurotransmitter receptors: from cultured neurons to intact brain preparations

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**Abstract.** Over the last decade, developments in single molecule imaging have changed our vision of synaptic physiology. By providing high spatio-temporal resolution maps of the molecular actors of neurotransmissions, these techniques have revealed that pre- and post-synaptic proteins are not randomly distributed but precisely organized at the nanoscale, and that this specific organization is dynamically regulated. At the centre of synaptic transmissions, neurotransmitter receptors have been shown to form nanodomains at synapses and to dynamically move in and out of these confinement areas through lateral diffusion within the membrane plane on millisecond timescales, thereby directly contributing to the regulation of synaptic transmission and plasticity. Since the vast majority of these discoveries originated from observations made on dissociated neurons lacking several features of brain tissue (e.g. three-dimensional organization, tissue density), they were initially considered with caution. However, the recent implementation of single-particle tracking (SPT) approaches in cultured and acute brain preparations confirmed that early findings on the dynamic properties of receptors at the surface of neurons can be extended to more physiological conditions. Taking example of dopamine D1 and NMDA glutamate receptors we here review our current knowledge of the features of neurotransmitter receptor surface diffusion in intact brain tissue. Through detailed comparison with cultured neurons, we also discuss how these biophysical properties are influenced by the complexity of the extracellular environment.

## Introduction

Development, maintenance and adaptation of synapses in the brain require constant adjustments in the number of receptors mediating neurotransmissions. While initially thought to be firmly anchored within post-synaptic densities, groundbreaking discoveries at the end of the 1990s and beginning of the 2000s successively revealed that neurotransmitter receptors (i) are dynamically cycling between the plasma membrane and intracellular compartments through endo-exocytic events (reviewed in (Malinow and Malenka, 2002), and (ii) can laterally diffuse within the plasma membrane and exchange between synaptic and extrasynaptic sites on fast timescales following Brownian motion (for review, see (Choquet and Triller, 2013)). By finely regulating receptor numbers at synapses through a dynamic equilibrium between synaptic, extrasynaptic and intracellular compartments, this combination of intracellular and surface trafficking plays a central role in synaptic plasticity, which is generally considered as the cellular substrate for learning and memory. As an example, lateral redistributions of glutamate receptors at excitatory synapses were recently shown to be of major importance in the establishment of long-term potentiation and to contribute to the formation of associative memories in the hippocampus (Dupuis et al., 2014; Penn et al., 2017; Potier et al., 2015). Moreover, impairments in these redistribution processes were also associated with severe brain conditions including neurodegenerative and neuropsychiatric disorders (Jezequel et al., 2017; Jezequel et al., 2018; Mikasova et al., 2012; Porras et al., 2012). Therefore, studying the local organization and dynamics of neurotransmitter receptors at the surface of neurons within dendritic and synaptic compartments is of major interest and has captured an ever-growing attention in the neuroscience community over the past two decades. Our current understanding of neurotransmitter receptor surface trafficking essentially originates from *in vitro* single-molecule imaging investigations performed on dissociated rodent neurons. While cultured preparations were instrumental to dissect the regulation mechanisms governing receptor organization and mobility, they unfortunately lack key features of intact brain preparations (e.g. components of the extracellular space, three-dimensional geometry) which were shown to affect receptor trafficking and may thereby eventually influence neuronal activity and behavior (Ferreira et al., 2017; Frischknecht et al., 2009; Groc et al., 2007a; Michaluk et al., 2009; Papouin et al., 2012). Although single molecule studies in native brain preparations long remained technically out of reach, the recent development of single-particle tracking approaches in brain slices opens new perspectives to understand the contribution of receptor surface trafficking to the physiology of neuronal networks. Here, we review the technological transition of single molecule tracking from dissociated neurons to brain slices, and discuss some important new

features of neurotransmitter receptor surface trafficking recently enlightened by imaging native preparations.

### 1. Early studies on cultured neurons

Much of the early evidence for surface diffusion of neurotransmitter receptors arose from ensemble approaches such as patch-clamp recordings and diffraction-limited fluorescence imaging, both of which yielded comparable results. In the first case, electrophysiological monitoring of the functional recovery of synaptic currents after irreversible blockade of a subpopulation of receptors was used as a proxy to estimate the average surface mobility of glutamate AMPA and NMDA receptors (Adesnik et al., 2005; Tovar and Westbrook, 2002) as well as GABA receptors (Thomas et al., 2005). However, a major limitation of this strategy is that it is only applicable to ionotropic receptors and cannot allow investigations on the surface behavior of other types of transmembrane proteins (e.g. metabotropic receptors). This limitation can be overcome using live imaging methods of fluorophore-tagged proteins such as fluorescence recovery after photobleaching (FRAP), which consists in photobleaching a subregion of the fluorescent surface staining by a brief and focused laser illumination and monitoring the replenishment of fluorescence within the bleached area that occurs through lateral diffusion from the surrounding surface of molecules harboring intact fluorophores (Axelrod et al., 1976b; Poo, 1982; Poo and Cone, 1974; Thomas et al., 2005); **Figure 1a**). Developed in the 1970s, this strategy was successfully used to measure the average mobility of nicotinic acetylcholine, AMPA glutamate and GABA<sub>A</sub> ionotropic receptors (Ashby et al., 2006; Axelrod et al., 1976a; Axelrod et al., 1976b; Jacob et al., 2005; Rasse et al., 2005; Schlessinger et al., 1976; Thomas et al., 2005), but also of metabotropic receptors such as serotonin 5-HT<sub>1A</sub>, dopamine D1 or human Mu opioid (hMOP) receptors (Pucadyil et al., 2004; Sauliere-Nzeh Ndong et al., 2010; Scott et al., 2006). While these approaches provide precious information about average behaviors of populations of molecules (i.e. mobile fraction and diffusion coefficient), they do not allow monitoring subgroups of receptors which may fulfill specific functions in synaptic physiology. The parallel development of single-molecule imaging revolutionized the field by allowing detailed exploration of the distribution and diffusive properties of individual surface receptors. A variety of techniques have been developed to follow the movements of separate surface molecules, all of which rely on decorrelating fluorescence emission of the labelled proteins of interest as a function of time in order to reach nanometric resolution of single-emitting objects. This can be achieved either by stochastically lighting up a limited subset of fluorophores at a given time (e.g. photo-activated localization microscopy, PALM; (Betzig et al., 2006), or by sparse labelling with photobleachable probes

(e.g. universal Point Accumulation for Imaging in Nanoscale Topography, uPAINT; (Giannone et al., 2010) or photostable nanoparticles (single-particle tracking, SPT; (Dahan et al., 2003). Choosing one or another of these approaches usually depends on the nature of the biological question to be addressed. While PALM and uPAINT allow to record from a high number of surface proteins within a single acquisition and to efficiently monitor their surface behavior in confined compartments such as synapses due to the small size (3-4 nm) and rapid bleaching (few seconds) of the organic fluorophores they are based on, SPT provides poorer access to confined areas because of the larger size (up to 20 nm) of the nanoparticles used as labels but allows much longer recordings thanks to their high photostability (virtually unbleachable) which opens the door to individual molecule monitoring over several minutes. SPT and uPAINT also provide the notable advantage of allowing investigations on endogenous proteins when PALM requires the expression of genetically engineered recombinant proteins incorporating organic photo-switchable probes. Single molecule imaging approaches confirmed that all neurotransmitter receptors examined so far are mobile within the membrane plane and follow a Brownian diffusion regime driven by thermal agitation (for review, see (Groc and Choquet, 2008)). They also revealed that while diffusion speeds and mobile fractions may vary significantly from one receptor to another - ranging from 0.05  $\mu\text{m}^2/\text{s}$  and 40 % mobile for glutamate NMDA receptors (Groc et al., 2004) up to almost 1  $\mu\text{m}^2/\text{s}$  and 90 % mobile for dopamine D1 receptors (Ladepêche et al., 2013b; Scott et al., 2006) - common features can be observed. In particular, prolonged SPT recordings unveiled that within a few minutes, a single receptor can alternately transition from freely diffusive to confined, corralled diffusive or stationary states (Choquet and Triller, 2003, 2013). A multitude of factors have been identified that may account for these changes in diffusion regime. Those include interactions with (i) intracellular scaffolding or cytoskeleton-coupled proteins which help stabilize receptors at synaptic or extrasynaptic sites (Bard et al., 2010; Bats et al., 2007; Hausrat et al., 2015; Jacob et al., 2005; Mukherjee et al., 2011), (ii) transmembrane partners such as receptors or cell-adhesion proteins (Ladepêche et al., 2013a; Mikasova et al., 2012; Mondin et al., 2011) or (iii) proteins of the extracellular matrix (Frischknecht et al., 2009; Groc et al., 2007a; Michaluk et al., 2009). Surface mobility can also be affected by the activity status of neurons or of the receptor itself (Bannai et al., 2009; Borgdorff and Choquet, 2002; Constals et al., 2015; Groc et al., 2004; Levi et al., 2008; Tardin et al., 2003), by the presence of extracellular ligands (Ferreira et al., 2017; Gouzer et al., 2014; Groc et al., 2008; Jezequel et al., 2017; Mikasova et al., 2012; Mikasova et al., 2017; Mukherjee et al., 2017; Papouin et al., 2012; Potier et al., 2015; Sarabdjitsingh et al., 2014; Sarabdjitsingh et al., 2016), or by post-translational modifications resulting from the activation of intracellular signaling pathways (Bannai et al., 2015; Dupuis et al., 2014; Gerrow and Triller, 2014;

Groc et al., 2004; Hannan et al., 2016; Ladepeche et al., 2013a). Furthermore, surface mobility is also locally influenced by the lipidic composition of the membrane (Renner et al., 2009) or the geometry (Ashby et al., 2006) and limited by non-specific corralling phenomenon originating from the compartmentalization of the plasma membrane by intracellular cytoskeleton elements (e.g. actin filaments) which create obstacles (“pickets” and “fences”) and confine receptor movements to restricted subdomains (Choquet and Triller, 2013). This complex combination of molecular events is specific to each type of receptor and defines its residence time in surface subcompartments. Thus, lateral diffusion emerges as a highly regulated process dynamically influencing the distribution of neurotransmitter receptors at the neuronal surface, and its potential implications on the spatio-temporal control of synaptic strength has attracted continuous attention over the past decade.

Early insights on the contribution of receptor mobility to synaptic physiology arose from investigations on short-term synaptic plasticity at glutamatergic synapses where lateral movements of AMPA receptors in and out of synapses on millisecond timescales were shown to impact synaptic transmission fidelity: by allowing a fast exchange between desensitized and naïve receptors, diffusion facilitates recovery from frequency-dependent depression of synaptic responses (Heine et al., 2008; Frischknecht et al., 2009; Constals et al., 2015). Activity-dependent long-term changes in synaptic strength, which rely on rapid changes in receptor types/numbers and are believed to be the cellular substrate for learning and memory, were also shown to require critical steps of receptor redistribution. As an example, long-term potentiation involves diffusion-dependent changes in NMDAR composition and AMPAR accumulation at excitatory synapses, and preventing these redistribution processes impairs temporal and contextual memory formation (Dupuis et al., 2014; Makino and Malinow, 2009; Penn et al., 2017; Potier et al., 2015). Finally, accumulating evidence suggests that impairments in receptor surface distribution and diffusion could be associated with neurodegenerative and neuropsychiatric diseases. Indeed, exposure to antibodies from patients with autoimmune anti-NMDA encephalitis or schizophrenia triggers severe alterations of NMDAR surface dynamics, and changes in D1 receptor surface trafficking could contribute to levodopa-induced dyskinesia, a detrimental consequence of L-DOPA administration as a treatment for Parkinson’s disease (Jezequel et al., 2017; Jezequel et al., 2018; Mikasova et al., 2012; Porrás et al., 2012). Altogether, these elements support a critical role of neurotransmitter receptor surface diffusion in cerebral functions and urge the need to study this new feature of neuronal physiology in native brain preparations.

## **2. Imaging receptor dynamics in brain slices**

A critical step to further understand the contributions of receptor surface trafficking to cognitive functions is to access molecular dynamics within the context of the brain. Unfortunately, expanding live single-molecule studies from dissociated neurons to native preparations preserving the network architecture and molecular environment complexity of the brain long remained technically challenging. Indeed, adapting total internal reflection fluorescence or oblique illumination required to achieve sufficient axial resolution for PALM and uPAINT imaging deep into three-dimensional tissue is highly complicated. Moreover, strong autofluorescence background in the short-wavelength spectrum and light scattering significantly complicate the detection of fluorophores in brain samples with spatio-temporal resolutions compatible with single-molecule imaging. Once again, the first demonstration of neurotransmitter receptor surface diffusion in native preparations originated from ensemble imaging of nicotinic acetylcholine receptors (nAChR) at the neuromuscular junction, which confirmed the dynamic behavior of these receptors at the cell surface *in vivo* (Akaaboune et al., 1999). Comparable strategies based on fluorescence imaging were further successfully applied to characterize the mobility of D1 and NMDA receptors in cultured or acute brain slices (Dupuis et al., 2014; Scott et al., 2006), and to monitor redistributions of AMPA receptors *in vivo* at the neuromuscular junction of *Drosophila* larvae along synaptogenesis and in the mouse somatosensory cortex during experience-dependent synaptic plasticity (Rasse et al., 2005). While these studies reported average diffusion properties that were close to those initially observed in dissociated cultures (e.g. similar mobile fractions for D1 and NMDAR; see next section for detailed discussion), they could however not provide detailed information on the behavior of individual surface receptors.

Labelling surface receptors with bright nanoparticles coupled to antibodies or engineered probes recently emerged as an efficient alternative strategy to access their spatiotemporal dynamics in native conditions (**Figure 1b**). Quantum Dots (QD) - i.e. semiconductor nano-crystals typically composed of a core of CdSe beneath a shell of ZnS - are particularly well suited for this purpose with their relatively small size (10 nm), wide excitation but narrow emission spectra, and outstanding brightness and photostability and have been used to pioneer investigations in brain slices (Michalet et al., 2005). Using spinning disk confocal microscopy, Biermann and collaborators first demonstrated the feasibility of measuring the surface dynamics of lipids (glycosylphosphatidylinositol, GPI) and cell adhesion molecules (neuroligin 1, Nlg1;  $\beta$ -neurexin) in three dimensions (3D) in organotypic brain slices using antibodies coupled to red light-emitting QDs (QD605 nm / QD655 nm) (Biermann et al., 2014; Sokoll et al., 2015). Although observation was limited in depth to the first 60  $\mu$ m of the samples due to light scattering and while lateral localization accuracy obviously dropped because of a poorer signal-to-noise ratio, single-

particle tracking could be efficiently achieved and the diffusion parameters reported for GPI and Nlg1 were very similar to those observed in culture. GPI was found to be more mobile than Nlg1, which is consistent with previous observations in cultures and confirms that surface trafficking of molecules in slices is not a random but rather a molecule-specific and regulated process. Importantly, one should note that trajectories recorded in slices were shorter compared to cultures, possibly because of the 3D geometry, and that confinement was higher and seemed to increase in a depth-dependent manner, showing a clear influence of tissue specificities on surface mobility patterns. This approach was soon after extended to acute brain preparations and applied to the tracking of neurotransmitter receptors, i.e. dopamine D1 and glutamate NMDA receptors, with similar success and conclusions (**Figure 1c**) (Varela et al., 2016a; Varela et al., 2016b) (see next section). Furthermore, imaging of GABA<sub>A</sub> receptor diffusion at the surface of neurons or GLT-1 glutamate transporter diffusion at the surface of astrocytes was also successfully demonstrated in brain slices (Al Awabdh et al., 2016; Modi et al., 2018). Thus, the diffusion properties appear to follow common rules *in vitro* and *ex vivo*, although tissue-specific features may impact the mobility of proteins at the surface of neurons.

### **3. Comparing receptor dynamics in primary cultures and brain slices: what did we learn?**

One obvious question that emerged from the discovery of neurotransmitter receptor membrane dynamics was whether this fast trafficking was simply an “artifact” of cultured brain cells in which the low density of cellular elements would favor membrane protein dynamics. As described above, proteins move passively and randomly within the plasma membrane through the thermal motion of surrounding molecules (e.g. lipids). Thus, the diffusion of a given plasma membrane protein should not, in theory, differ between an isolated cell and a cell embedded within a network. Among the forces that shape the diffusion of a protein, the viscosity of the plasma membrane is one of the most influential as mass, in comparison, is negligible (Groc and Choquet, 2008). Furthermore, the diffusion of a protein weakly depends on its radius, explaining for instance why receptor clusters can similarly diffuse as individual receptors (Groc and Choquet, 2008). Yet, the diffusion values of membrane receptors are lower than those expected from artificial lipid bilayers. This is mostly due to the presence of obstacles and interacting partners (extracellular, within or beneath the membrane) (Axelrod et al., 1976b; Tardin et al., 2003), setting the theoretical framework in which the diffusion of a membrane neurotransmitter receptor should be observed both *in vitro* and *in vivo*, with putative differences related to the presence of various obstacles and interacting partners.

The first report of neurotransmitter receptor lateral diffusion came from cultured myotubes in which acetylcholine receptors (AChR) were tagged with fluorescent bungarotoxin and diffusion estimated by FRAP imaging (Axelrod et al., 1976a; Axelrod et al., 1976b; Poo, 1982; Schlessinger et al., 1976; Young and Poo, 1983). These pioneer observations were later confirmed by *in vivo* imaging investigation (Akaaboune et al., 1999), strengthening the model of receptor trapping within cellular junctions. Testing this experimentally in neurons of the central nervous system was however a technological challenge due, in part, to the small size of synapses (e.g. ~100 times smaller than neuromuscular junctions). As described in the above section, this limitation was recently circumvented and the diffusion of membrane neurotransmitter receptors and transporters was successfully measured. Here, we focus our discussion on diffusion data from D1 dopamine and NMDA glutamate receptors collected from *in vitro* and *ex vivo* hippocampal preparations. At first glance, trajectories of single surface D1R imaged in acute hippocampal slices and cultured hippocampal networks were rather similar, exhibiting a wide range of behaviors with immobile, slowly and highly mobile receptors. In addition, D1R diffusion properties in brain slices depend on the neuronal compartment (e.g. dendritic spines *versus* shafts) and the presence of receptor agonists, as previously observed *in vitro* (Ladepeche et al., 2013a; Ladepeche et al., 2013b; Varela et al., 2016a). Consistently, the comparison of D1R dynamics in slices and cultured cells indicates comparable instantaneous diffusion coefficients (ranging 0.06-0.1  $\mu\text{m}^2/\text{s}$ ) and fraction of mobile receptors (~90%) (Ladepeche et al., 2013a; Ladepeche et al., 2013b; Varela et al., 2016a). However, the MSD curves of D1R trajectories, which reflect the pattern of diffusion and confinement, differed considerably between the two models. Indeed, D1R diffusion was five-fold more confined in acute hippocampal slices than in cultured networks, suggesting the presence of additional obstacles and/or interacting partners in the intact brain tissue (**Figure 2**). Collectively, these specific studies unveil that one of the main characteristics of a single neurotransmitter receptor surface dynamics in intact brain tissue is its higher confinement, with similar diffusion, when compared to primary cultured neurons. Several points should be discussed. First, neuronal networks in slices are organized in a 3D-fashion which may impact some of the characteristics of the dopamine and NMDA receptor surface diffusion, such as the length of the trajectories (shorter in brain slices as single nanoparticle-receptor complex get off the focus plane more frequently as in 2D-cultured neurons). We previously reported comparable diffusion values for GluN2B subunit-containing NMDA receptor comparing cultured neurons and organotypic slices using the FRAP approach (Dupuis et al., 2014). While the nature of NMDAR subunits tracked is unlikely to account for this apparent discrepancy between studies, this suggests that careful consideration must be taken when comparing diffusion measurements evaluated with ensemble and

single-particle imaging methods. In addition, the potential limitation of 2D-single nanoparticle tracking in a 3D-environment (brain slices) likely underestimates the population of highly mobile receptors (Varela et al., 2016a; Varela et al., 2016b). Second, the geometrical organization of neurite compartments, which is likely more complex in brain slices, may also influence the estimation of receptor surface dynamics (Renner et al., 2011). Third, the surface trafficking of membrane receptors is currently limited to genetically-expressed receptors since endogenous membrane receptor tracking proved unsatisfactory. As previously described, over-expressed NMDAR are slightly more mobile than endogenous ones (Groc et al., 2007b), calling for great control of the experimental design in such studies (e.g. controlling the level of overexpression). Overall, if single nanoparticle tracking in brain tissue is still in its infancy, it remains a very powerful tool to investigate the membrane trafficking of receptors in complex environments, and recent technical developments should further extend and improve its applications.

### **Technical challenges, future directions and perspectives**

Tracking individual neuronal proteins embedded into their native networks in live conditions has long been a dream for cellular neurobiologists. Recent technological breakthroughs have opened the possibility to perform real time imaging of neurotransmitter receptors at the plasma membrane of neurons in preserved tissue preparations. As the field is moving toward more complex systems such as acute brain slices and live brain imaging in rodents, several technical issues (e.g. tissue penetration, synapse accessibility, overexpression of recombinant proteins, compensation of movements resulting from heart contractions) still require further developments to be circumvented.

#### *Smaller probes to image deeper and with better accuracy*

Significant improvements have been recently made to reduce the size of the labels used to track receptors. Replacing antibodies (approximately 12 nm of length) by smaller probes with comparable (or better) affinities such as monomeric streptavidin, nanobodies or even aptamers shows encouraging promises and should ease the labelling of receptors in dense 3D tissular environments as well as in confined surface compartments such as synapses (Chamma et al., 2016; Chamma et al., 2017; Modi et al., 2018). In addition to a reduction in size (from ~12 nm IgG to ~4 nm nanobody), the use of monovalent tools will also avoid the risk of target cross-linking that needs to be thoroughly calibrated for each experimental setting. Furthermore, the parallel emergence of tiny photostable fluorophores with high quantum yield and red or near-infrared photoluminescence properties - e.g. small QDs or

single-walled carbon nanotubes - easily combinable with these small probes also converges in the same direction (**Figure 1b**) (Danne et al., 2018; Lee et al., 2017). As an example, the recent development of squaraine derivatives such as SeTau-647 may substantially improve our ability to track surface proteins with nanometric resolution in brain tissue (Podgorski et al., 2012; Tsunoyama et al., 2018). Such organic dyes recapitulate the properties ideally suited to perform single-molecule imaging in complex three-dimensional environments: they are small (thus easily diffusible in tissue), bright and highly photostable, allowing prolonged super-resolution imaging (up to several minutes) of the proteins of interest which is of utmost importance to monitor complete surface trafficking patterns (Tsunoyama et al., 2018). Another challenge that has to be faced is ensuring our ability to distinguish between surface and internal movements of proteins of interest, both of which may contribute in different ways to produce adapted cellular responses to external stimuli. This issue was already a concern in the early days of surface trafficking and diffusion studies (Axelrod et al., 1976b), which was addressed by engineering recombinant transmembrane proteins fused to pH-sensitive organic fluorophores (e.g. super-ecliptic pHluorin; Miesenböck et al., 1998) allowing to visualize surface proteins only while internalized ones are quenched at acidic intracellular pH (Jacob et al., 2005; Ashby et al., 2006). The same limitation is faced when conducting single particle tracking experiments with classical organic or inorganic dyes. In this case, experimental conditions are generally adapted to lower as much as possible the occurrence of endocytic events (e.g. by limiting acquisition durations to few minutes after surface staining), and distinction between surface and internalized proteins can be performed based on offline analysis of diffusion regimes. Indeed, surface movements are usually believed to alternate between classical Brownian and confined motion, while internalized proteins are generally actively transported by the intracellular microtubule machinery and show directed motion (Varela et al., 2016a). However, the recent description of cytoskeleton-independent directed motion of EphrinB2 receptors within the plasma membrane of neurons suggests that surface proteins may also occasionally follow this type of mobility, and calls for adapted tools such as pH-sensitive quantum dots allowing to monitor these phenomena (Taylor et al., 2018).

Importantly, accessing targets located deep inside a tissue remains a critical parameter to perform live *ex vivo* imaging. Indeed, in contrast with immunohistochemical staining on fixed samples, time is an important parameter when performing live SPT as the experimenter needs to control the amount of surface versus internalized labelling. To date, it appears that most of the SPT imaging in brain tissue has been performed in rather “superficial” layers (i.e. from 0 to 50  $\mu\text{m}$  depth). Accessing deeper layers is obviously a challenge that needs to be tackled in order to i) confirm that SPT of membrane proteins in

superficial layers of brain slices is not biased by mechanical/chemical reorganization of this superficial tissue when compared to deeper, more preserved layers, and ii) depict the dynamics of membrane proteins in specific brain regions that have not been explored so far. So far, the strategy of delivery of antibody/QD complexes has mostly relied on passive diffusion (Biermann et al., 2014; Varela et al., 2016a,b; Modi et al., 2018), which is likely not applicable to deeper layers because of diffusibility and time limitations. Instead, one may envision alternative methods such as local delivery of antibody/QD complexes into deeper structures through micro-infusion systems.

#### *Tracking endogenous and recombinant surface proteins expressed at native levels ex vivo*

A main limitation to our current knowledge of receptor dynamics in brain slices is that it comes essentially from overexpressed genetically-modified receptors. With the recent development of CRISPR-based genome-editing methodologies through which one can express tagged receptors at native levels (Mikuni et al., 2016), it is now possible to track down endogenously synthesized proteins at the surface of selected brain cell types (e.g. pyramidal cells, interneurons, astrocytes). Indeed, precise genome editing of proteins that are key actors of synaptic plasticity (e.g. insertion of an HA tag on CAMKII) has been successfully reported (Nishiyama et al. 2017). Using similar means, inserting mutations or specific tags (e.g. HA, Myc) in neurotransmitter receptors in genetically-selected subpopulations of neurons is now fully in our grasp and should allow further imaging applications such as SPT of membrane receptors expressed at endogenous levels to study cell-type specific regulations of receptor surface dynamics *ex vivo* and eventually *in vivo* (Peng et al., 2018).

Furthermore, another obstacle to our understanding of the regulation of receptor surface dynamics *in* and *ex vivo* is our poor ability to visualize endogenous actors of pre- and post-synaptic compartments, which is a key prerequisite to explore the local organization of the synaptic machinery and the dynamics of neurotransmitter receptors within synaptic compartments. While labelling synapses can be artificially achieved using recombinant proteins bearing fluorescent labels (e.g. green fluorescent protein (GFP)-tagged post-synaptic scaffolding proteins such as PSD-95 or Homer1c; Biermann et al., 2014; Modi et al., 2018) as in cultured neurons through electroporation, viral infection, or knockin approaches, this useful strategy does not avoid the usual pitfalls of introducing possible artefacts through the over-expression of genetically-engineered proteins. A morphology-based proxy to monitor the behavior of receptors in the vicinity of synapses in brain slices is to track their movements within dendritic spines (Biermann et al., 2014; Varela et al., 2016a), but this does not allow accurate measurements of receptor trapping at post-synaptic densities. The development of GFP-fused Fibronectin intrabodies generated with mRNA display (FingRs; Gross et al., 2013) - i.e. disulfide-free antibody-like proteins (intrabodies) that bind to

endogenous target proteins and are transcriptionally regulated by these target protein - recently brought an elegant solution to this problem and now allows labelling of endogenous inhibitory (e.g. gephyrin) or excitatory (e.g. PSD-95) pre- or post-synaptic markers with a high affinity and a transcriptional control which ensures preservation of neuronal function. Combining this technology with SPT will undoubtedly expand our ability to explore the regulation mechanisms governing receptor surface trafficking and stabilization at synapses in brain tissue.

Thus, a vast repertoire of genetic, molecular and microscopy tools are now available to explore the surface dynamics of neurotransmitter receptors and transporters and its role in physiological and pathological brain functions. In this perspective, the emergence of 3D multicolor SPT now allows simultaneous imaging of multiple proteins which opens the door to real-time investigations on the occurrence, location and dynamics of homo- and/or heteromerization events between surface receptors (e.g. (Low-Nam et al., 2011; Modi et al., 2018)). This technological breakthrough is likely to change our view of neuronal integration as oligomerization between neurotransmitter receptors of different families is now known to mutually affect their signaling properties. Performing multicolor single-molecule imaging will allow us to explore these interactions live and understand how they contribute to neuronal physiology. The development of automated SPT (Yasui et al., 2018) will also undoubtedly pave the way to high-throughput single-molecule screening of (i) diffusion impairments in pathological situations, and (ii) drugs potentially acting on the surface trafficking of neurotransmitter receptors to correct these deficits.

## Figure Legends

**Figure 1: Imaging surface dynamics of neurotransmitter receptors in dissociated neurons and brain slices.** **(a)** Fluorescence recovery after photobleaching (FRAP) is an ensemble imaging approach that can be performed on cells either labelled for the endogenous receptors of interest with antibodies coupled with an organic fluorophore (e.g. GFP), or expressing the recombinant receptors of interest fused with an organic fluorophore. FRAP consists in photobleaching a subregion of the fluorescent surface staining (e.g. spine bleached at 0 s within the red circle, upper panel) by a brief and focused laser illumination and monitoring over time the replenishment of fluorescence within the bleached area that occurs through lateral diffusion from the surrounding surface of molecules harboring intact fluorophores (see bottom panel, 140 s). **(b)** Multiple labelling strategies can be applied to perform single-molecule imaging of endogenous or recombinant receptors at the surface of neurons. The incomplete list described here includes antigen-binding fragment (Fab)-functionalized quantum dots (QD) and organic fluorophores (e.g. GFP) coupled to antibodies, QDs and organic fluorophores functionalized with streptavidin to recognize biotinylated receptors, QDs and organic fluorophores functionalized with nanobodies against endogenous targets or recombinant tags (e.g. GFP), and fluorescent nanobodies. Choosing one label or another depends on the nature of the question that needs to be addressed. Typically, QDs (~20 nm for big QDs vs ~10 nm for small QDs) coupled with antibodies (~10 nm) are much larger probes than organic dyes (~4 nm) coupled to nanobodies (~2 nm) and have a lower ability to enter confined compartments such as synapses, but show much higher brightness and photostability which makes them the fluorophores of choice for long-lasting slice imaging applications. **(c)** Single-particle tracking of neurotransmitter receptors in cultured neurons, organotypic and acute brain slices. Labelling of recombinant receptors can be achieved either by directly incubating dissociated neurons or organotypic slices with QD-antibody complexes, or by injecting these complexes live into the cerebral ventricles of rodents (ICV, intra-cerebro-ventricular) and allowing them to diffuse within the tissue before slicing (acute preparations). Lower panels: trajectories of QD-labelled super ecliptic pHluorin (SEP)-tagged NMDAR (20 Hz acquisition rate, 30 s duration) at the surface of a transfected hippocampal neuron in culture (right panel) and of an electroporated CA1 pyramidal cell in an organotypic slice (left panel).

**Figure 2: Comparison of the neurotransmitter receptor surface diffusion at the surface of neurons from cultured brain cells and acute brain slices.** *Upper box*, Relative comparison of the MSD and diffusion coefficient of neurotransmitter receptors from cultured neurons (magenta) and acute brain

slices (green). This relative comparison is based on the values obtained for dopamine D1 and NMDA receptors (see text for details). Note that the confinement of neurotransmitter receptors is approximately 5 fold increased in acute slices versus cultured networks, whereas the diffusion coefficient is “only” 1-2 fold lower in acute slices versus cultured networks. *Lower scheme*, Schematic representation of the surface dynamics of neurotransmitter receptors in primary cultured brain cells and acute brain slices, highlighting that receptors diffuse in both situations with a higher confinement in the intact brain tissue.

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**Figure 1**  
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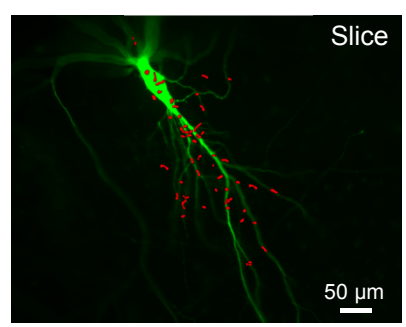
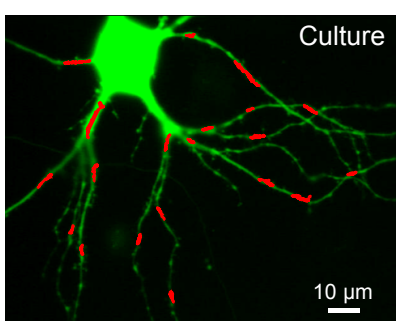
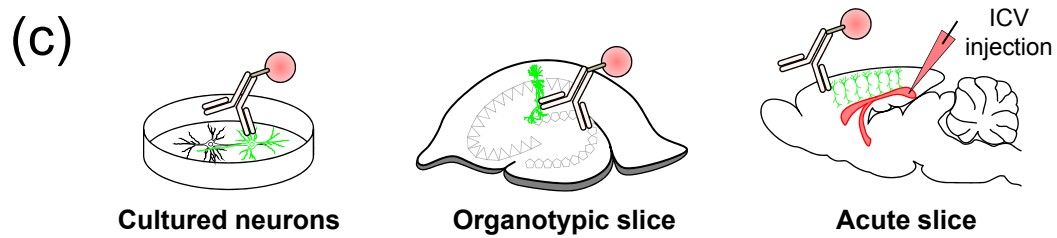
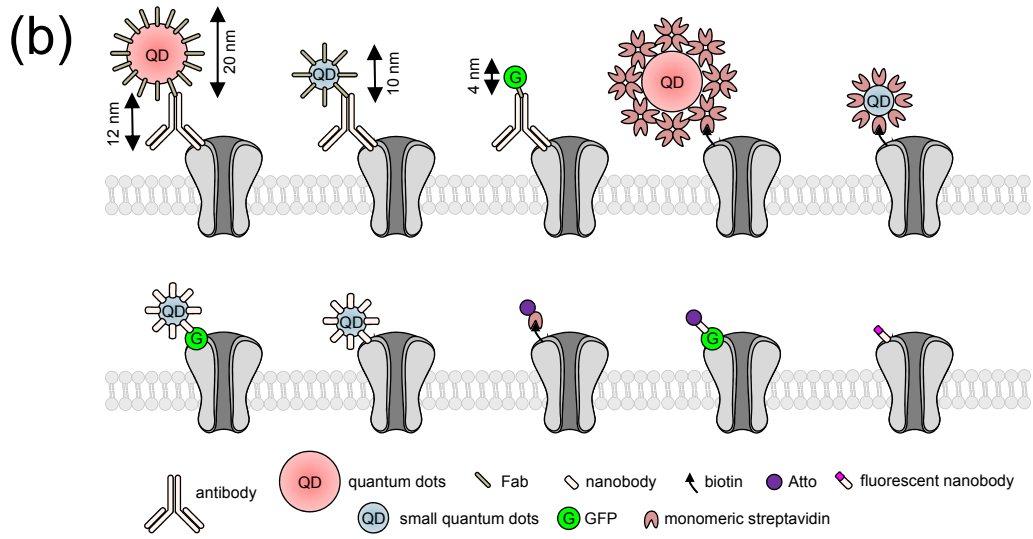
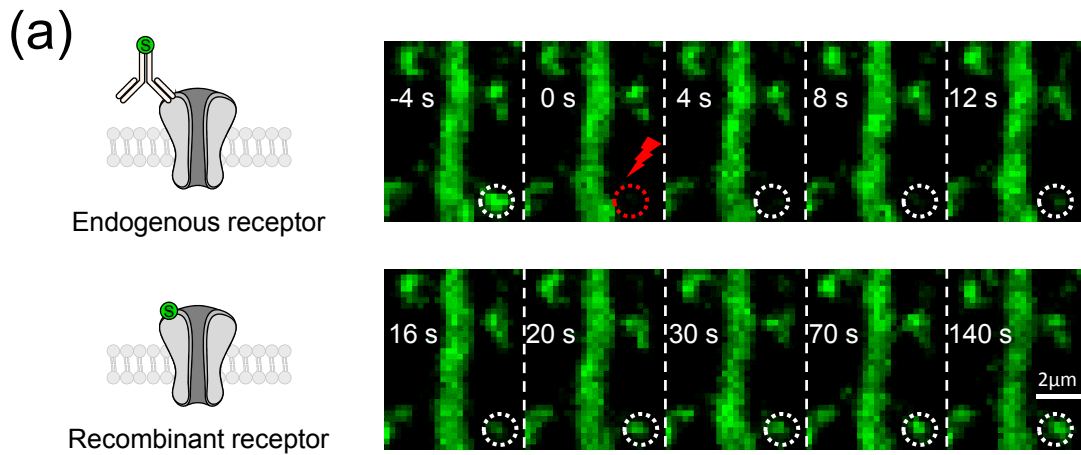


Figure 2  
Dupuis and Groc

