Dissecting tripartite synapses with STED microscopy

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The concept of the tripartite synapse reflects the important role that astrocytic processes are thought to play in the function and regulation of neuronal synapses in the mammalian nervous system. However, many basic aspects regarding the dynamic interplay between pre- and postsynaptic neuronal structures and their astrocytic partners remain to be explored. A major experimental hurdle has been the small physical size of the relevant glial and synaptic structures, leaving them largely out of reach for conventional light microscopic approaches such as confocal and two-photon microscopy. Hence, most of what we know about the organization of the tripartite synapse is based on electron microscopy, which does not lend itself to investigating dynamic events and which cannot be carried out in parallel with functional assays. The development and application of superresolution microscopy for neuron–glia research is opening up exciting experimental opportunities in this regard. In this paper, we provide a basic explanation of the theory and operation of stimulated emission depletion (STED) microscopy, outlining the potential of this recent superresolution imaging modality for advancing our understanding of the morpho-functional interactions between astrocytes and neurons that regulate synaptic physiology.

1. Introduction

Originally described more than 150 years ago by the German anatomist Otto Deiters and aptly named for their star-shaped morphology by the Hungarian anatomist Mihály Lenhossek, astrocytes occupy a special position in brain parenchyma between blood vessels and neurons. They have long been known to be critical for providing structural and nutritional support for neurons [1,2], but because astrocytes cannot fire action potentials, their contribution to fast signal processing in the brain has been largely dismissed.

However, numerous studies over the past two decades indicate that astrocytes are not merely playing the role of sluggish supporters of brain function. Indeed, astrocytes are endowed with versatile and powerful mechanisms that actively implicate them in signalling and regulatory processes that have traditionally been considered the exclusive preserve of neurons.

The application of modern neurophysiological approaches such as Ca2+ imaging and patch-clamp electrophysiology to study astrocyte physiology has revealed that they are electrochemically excitable structures, capable of sensing synthetically released neurotransmitters [3–6], and, in turn, capable of regulating the efficacy of synaptic transmission through the release of gliotransmitters such as glutamate, ATP and D-serine [3,4,7–11].

Mounting evidence for bidirectional communication between synapses and astrocytes has led to the concept of the ‘tripartite synapse’, where astrocytes form an integral functional component of synapses on equivalent terms with the classic pre- and postsynaptic neuronal structures [12] (figure 1). For a long time, it was thought that astrocyte influence is appreciable only during sustained and intense bouts of synaptic activity. However, recent studies have shown that astrocytes can also detect and influence synaptic events locally at individual synapses during synaptic transmission induced

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by single presynaptic action potentials [13,14], indicating that the mechanisms that mediate astrocyte–neuron cross-talk at synapses are extremely sensitive and cover a wide dynamic range.

Astrocytes surely possess powerful mechanisms to influence synaptic function by way of: (i) the clearance of glutamate from the synaptic space by glutamate transporters, such as GLT-1, which are thought to be expressed at extremely high density (more than 5000 μm⁻²) at synapses [15]; (ii) the buffering of potassium ions in the extracellular environment [16]; (iii) the release of a panoply of gliotransmitters (3); (iv) physically shaping the diffusional space of neurotransmitters; and (v) triggering intercellular Ca²⁺ elevation (2). In turn, the astrocyte regulates the efficacy of transmission through the release of gliotransmitters (3).

2. Superresolution microscopy for imaging synaptic structures

(a) A veritable challenge for microscopy

There is a great need to develop new microscopy techniques that reconcile nanoscale spatial resolution with dynamic imaging and functional analyses in living brain tissue. Confocal and two-photon fluorescence microscopy have become the methods of choice for imaging neural morphology and protein dynamics in thick brain tissue (brain slices or in vivo), owing to much higher signal-to-noise ratios and better optical sectioning over regular wide-field imaging. Nevertheless, they are still conventional optical microscopy techniques in that they cannot be used for longitudinal studies of the dynamic behaviour of identified astrocytic processes, which are reportedly mobile [21–23].

The depicted synapse is formed by the two pre- and postsynaptic structural compartments (grey) and the astrocytic element (yellow). During synaptic transmission, glutamate released into the synaptic cleft activates postsynaptic receptors (AMPA and NMDA) as well as astrocytic receptors such as metabotropic glutamate receptors. This triggers intercellular Ca²⁺ elevation. In turn, the astrocyte regulates the efficacy of transmission through the release of gliotransmitters.

Figure 1. Schematic of the tripartite synapse. The concept of the tripartite synapse sees astrocytes as functionally integral components of synapses. The depicted synapse is formed by the two pre- and postsynaptic structural compartments (grey) and the astrocytic element (yellow). During synaptic transmission, glutamate released into the synaptic cleft activates postsynaptic receptors (AMPA and NMDA) as well as astrocytic receptors such as metabotropic glutamate receptors (1), triggering intercellular Ca²⁺ elevation (2). In turn, the astrocyte regulates the efficacy of transmission through the release of gliotransmitters (3).
infinitiesmally small point because its wave-like nature forces it to disperse behind obstacles (such as field stops and objective lens apertures), which is called diffraction. As a result, even with perfect optical components and alignment, any optical system will still produce only a blurry spot, whose physical dimensions can be calculated by the point-spread function (PSF).

Because the NA of objectives typically ranges between 1.0 and 1.5 and because the wavelengths for fluorescence excitation are in the visible range of the electromagnetic spectrum, the value of $\Delta$ is fundamentally limited to around 200–300 nm laterally (i.e. in the $x$–$y$ plane) and to more than 500 nm axially (i.e. in the optical $z$-direction). This three-dimensional volume roughly corresponds to the PSF of the microscope and is called the diffraction zone, which defines the spatial resolution of the microscope.

**3. STED microscopy: nanoscale imaging of the tripartite synapse**

(a) The STED microscopy principle

Like confocal or two-photon microscopy, STED microscopy uses a scanning laser to excite the sample. In addition, a second laser beam (the STED laser), shaped like a doughnut featuring a deep intensity minimum in the central region, is superimposed precisely onto the excitation laser. The STED laser quenches the fluorescence everywhere except in the central region, thereby dramatically reducing the size of the original fluorescence spot, which translates directly into higher spatial resolution (figure 2).

The molecules are de-excited by the process of stimulated emission induced by the STED laser, which is tuned to the long wavelength tail of the fluorescence emission spectrum, where it causes little excitation and can be readily separated from the fluorescence, but also still efficiently de-excite the molecules (figure 2a,d). The average time the fluorophore spends in the excited (ON) state (the fluorescence lifetime, which is on the order of a few nanoseconds) defines the time window of action for the STED laser. Hence, synchronized subnanosecond pulsed laser sources are preferably used to maximize the rate of de-excitation to the ground (OFF) state by stimulated emission within the lifetime of the dye molecule (figure 2b). The more completely the excited state gets de-populated, or depleted, by stimulated emission (hence the acronym), the tighter becomes the nozzle or doughnut around the central region ($\Delta_{x,y}$) from where fluorescence can still occur.

$$\Delta_{x,y} = \frac{\lambda}{2\pi \cdot \sin \alpha \sqrt{1 + (I_{\text{sat}}/I_{\text{sat}})},}$$

where $I_{\text{sat}}$ denotes the peak power of the STED laser and $I_{\text{sat}}$ a dye-specific constant on the order of tens to hundreds of MW cm$^{-2}$.

Because it is a laser-scanning confocal technique, STED microscopy can be quite fast (milliseconds per line in line scan mode, seconds per full frame, depending on size of the scan field) and it lends itself to imaging deep inside biological tissue. The combination of two-photon excitation and STED microscopy was recently shown to provide a spatial resolution of approximately 50 nm laterally more than 50 μm below the surface of acute brain slices using a water dipping objective with a long working distance on an upright microscope [35,36]. A similar depth penetration was demonstrated with a glycerol immersion objective lens equipped with a correction collar on an inverted microscope [37].

Unlike the single-molecule-based superresolution techniques, STED microscopy does not place any special constraints on the fluorophores that can be used, as long as they are sufficiently bright and photostable. This flexibility greatly facilitates live-cell imaging, because common vital stains (e.g. GFP and Alexa dyes) and standard labelling strategies (transgenic expression or single cell dye filling) can be used. Even though...
Figure 2. STED microscopy principle and design. (a) Jablonski diagram. After the absorption of a photon, the molecule is elevated to the first excited state (S1; blue arrow) and goes back to the ground state (S0) spontaneously by the emission of fluorescence (green arrow). Alternatively, before the emission of fluorescence, the molecule can be forced to go back to the ground state by a process called stimulated emission (orange arrow). (b) Temporal scheme of pulsed excitation and stimulated emission. A laser diode is used to generate brief pulses of excitation light (90 ps, blue). The fluorescence (green dotted line) decays according to the natural lifetime ($t_{fl}$) of the fluorophore, or gets quenched by a brief STED laser pulse (300 ps, orange). (c) Light intensity distributions, or point-spread functions (PSF) of the excitation (blue) and STED (orange) lasers in the focal plane ($xy$). The STED laser quenches the fluorescence around the outer edge of the excitation PSF, allowing the emission (green) only to occur at the centre of the doughnut, resulting in a smaller PSF, and hence improved spatial resolution. (d) Scheme of two-colour home-built STED microscope. It is constructed around a glycerol objective on an inverted microscope stand and based on pulsed excitation at 485 nm and pulsed quenching at 592 nm. The depletion laser is derived from an optical parametric oscillator (OPO) pumped by a Ti : Sa femtosecond laser (Ti : Sa). Lasers for excitation and depletion are synchronized (small black arrow) and the STED beam is shaped by a phase mask to feature a central intensity zero. Emitted fluorescence from the sample is de-scanned and split into two detection channels. Alexa Fluor 488 and YFP signals are spectrally separated using a 514 nm long-pass dichroic mirror and detected with two avalanche photodiodes. A second Ti : Sa laser is routed into the microscope and co-aligned with the excitation/depletion beams, which is used for two-photon FRAP, imaging or glutamate uncaging experiments [34].
the STED laser complicates multi-colour imaging, several solutions for imaging in two or three colours exist, including a straightforward approach based on spectral detection and linear unmixing of green–yellow fluorophores (figure 2f) [38].

In addition to de-excitation by stimulated emission, other switching mechanisms can be used to achieve spatial resolution beyond the diffraction limit [39,40]. These other schemes are referred to as reversible saturable optical fluorescence transitions microscopy [41], where the molecular ON state typically has a much longer lifetime than fluorescence, and hence can be switched off with much lower peak intensities. On the downside, this approach currently requires special photo-switchable proteins, which can be tough to express inside cells and are not very bright and/or photostable.

STED time-lapse imaging can be readily combined on the same microscope with other modern biophysical or neurophysiological approaches such as two-photon glutamate uncaging, FRAP experiments and patch-clamp electrophysiology to study the structure–function relationship of neural compartments at the nanoscale (figure 2d) [34].

(b) STED imaging of astrocyte morphology
In contrast to other glial cells, the morphology of mature astrocytes is ‘spongiform’; it is characterized by stubby main processes and dense ramifications with irregular shapes [42,43]. Astrocytes do not overlap, each occupying an exclusive territory, called domain, that is thought to cover more than 100 000 synapses [43]. Thus, a given synapse will not be in contact with multiple astrocytes (inside the domain).

Our first step was to confirm that astrocytes in organotypic hippocampal slices have characteristics similar to those reported for acute brain slices. In order to carry out our analysis of the tripartite synapse at a mature stage (as opposed to during development), we checked the electrophysiological properties of astrocytes. After five weeks in culture, astrocytes in the *stratum radiatum* of CA1 appear to be electrophysiologically similar to those in acute slices (figure 3a) [16]. Astrocytes are characterized by (i) hyperpolarized resting membrane potential (∼−80 mV), (ii) a low input resistance (∼4–20 MΩ), (iii) a linear current–voltage relationship in voltage clamp as well as (iv) absence of spiking behaviour (figure 3a). Furthermore, as astrocytes are coupled with each other via gap junctions, dye molecules introduced into the astrocyte through the patch pipette diffuse to its astrocytic neighbours.

To visualize astrocyte morphology, standard labelling strategies can be used: loading the astrocyte with a cell-impermeable dye (Alexa Fluor 488) through a patch pipette, or transgenic labelling with GFP expressed under a specific astrocytic promoter, such as the glial fibrillary acidic protein (GFAP). Either approach makes it possible to visualize the spongiform and hyperfine morphology of the astrocytes by STED microscopy (figure 3c,d): thin processes can be seen extending from the main branches into different directions, forming secondary and tertiary branches, with diameters ranging between 110 and 439 nm (figure 3d). In EM micrographs [17], even thinner structures can be seen, which is probably due to the relatively poor z-axis resolution of our current STED approach, which is still confocal (approx. 600 nm).

For time-lapse STED imaging, very bright labelling of the astrocytes is essential. As GFAP promoter activity is decreased in adult as compared with young mice, the fluorescence intensity is relatively low to begin with and photobleaching diminishes the GFP signal after just a few z-stacks. The cultures with overly bright GFP signal are to be avoided, because it is indicative of abnormal GFAP upregulation, which is often observed in pathological conditions. In contrast to GFP labelling, it is possible to vary the Alexa concentration to suit the requirements of the experiment. However, because patching astrocytes is quite challenging and invasive, the development of transgenic labelling approaches with brighter and more photostable fluorescent proteins is awaited.

(c) Astrocyte synapse morphological interactions at the nanoscale
As it is possible to visualize astrocyte morphology using STED microscopy, the next step is to investigate the interactions of astrocytic processes with the two neuronal elements of the synapse: presynaptic boutons and postsynaptic dendritic spines. For now, we have visualized only two elements at a time: the astrocytic process and either the pre- or the postsynaptic element. To this end, the dye (Alexa Fluor 488) is introduced through a patch pipette into the astrocyte in hippocampal organotypic slices prepared from transgenic mice (Thy1-YFP) where neurons are sparsely labelled with YFP (figure 4).

Confirming reports by conventional light microscopy and EM [21–23], astrocytic processes can be seen in close apposition with synaptic neuronal elements, such as dendritic spines (figure 4). We observed that the morphological interactions between astrocytic processes and spines are quite variable: depending on the size of the spines, a single spine head can be contacted in different places by a single process. Interestingly, astrocytic processes forming ‘O-ring’-like structures can also be frequently observed (figures 3d and 4). The reasons and functional relevance of these shapes and relationships are unclear.

4. Perspectives
Increasingly, progress in neuroscience hinges on a holistic approach that more fully and seamlessly takes into account both neuronal and glial contributions to brain physiology across the different scales of analysis. The ‘tripartite synapse’ is a powerful concept that has generated clear and meaningful hypotheses to guide experimental research. However, figuring out this ‘three-body problem’ is a tricky challenge and will require mashing-up a wide array of experimental approaches.

The combination of multi-colour STED imaging with modern neurophysiological and biophysical approaches in brain slices [34], supported by new molecular tools for visualizing and manipulating the structure and function of tripartite compartments, is an important methodological step in this direction. The development of STED microscopy in vivo represents a promising methodological advance [44] to study the tripartite synapse in the context of the living animal.

How variable and dynamic are tripartite synapses? How are they affected by synaptic remodelling during brain development, neural plasticity or brain diseases? What are the functional consequences when their size or shape changes? Questions such as these are increasingly coming into view thanks to these recent methodological advances.
In addition to nanoscale imaging of neural morphology, it will be important to develop tools to map out the distribution and to track the dynamics of astrocytic proteins. These are likely to be critical for regulating neuron–astrocyte crosstalk, much like the molecular dynamics of postsynaptic receptors were shown to be important for regulating synaptic transmission and plasticity [45,46]. For instance, metabotropic glutamate receptors, which have been implicated in detecting synaptic activity [14], may be highly mobile on the surface membrane of astrocytes [47]. The development of PALM/STORM in living brain slices holds great promise in this regard.

5. Conclusion
As the role of astrocytes in the regulation of synaptic function has gained recognition, an important question now is how...
Figure 4. Spine–astrocyte morphological interactions. An astrocyte (CA1 stratum radiatum) in organotypic hippocampal slices derived from Thy1-YFP mouse, was filled with Alexa Fluor 488 via a patch pipette. A small region of interest (xyz: 10 × 10 × 0.166 μm, four planes) within the territory of the labelled astrocyte was chosen and two STED images (each corresponding to two detection channels) capturing the fine structure of the neuronal elements and astrocytic processes were obtained. Then, these images were deconvolved using Huygens Professional (SVI) software and spectrally unmixed with IMAGEJ software (NIH). Two-colour STED imaging reveals that astrocytic processes (green) interact closely with neuronal synaptic elements (red; dendritic spines, arrows). Asterisks indicate the presence of O-ring structures.
this regulation takes place locally at the single synapse level. The advent of superresolution microscopy offers new opportunities to rigorously explore this issue under increasingly realistic experimental conditions in the healthy and diseased brain.

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diseased brain.


