● INVITED REVIEW

On the road towards the global analysis of human synapses

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Abstract

Synapses are essential units for the flow of information in the brain. Over the last 70 years, synapses have been widely studied in multiple animal models including worms, fruit flies, and rodents. In comparison, the study of human synapses has evolved significantly slower, mainly because of technical limitations. However, three novel methods allowing the analysis of molecular, morphological, and functional properties of human synapses may expand our knowledge of the human brain. Here, we briefly describe these methods, and evaluate how the information provided by each unique approach may contribute to the functional and anatomical analysis of the synaptic component of human brain circuitries. In particular, using tissue from cryopreserved human brains, synaptic plasticity can be studied in isolated synaptosomes by fluorescence analysis of single-synapse long-term potentiation (FASS-LTP), and subpopulations of synapses can be thoroughly assessed in the ribbons of brain tissue by array tomography (AT). Currently, it is also possible to quantify synaptic density in the living human brain by positron emission tomography (PET), using a novel synaptic radio-ligand. Overall, data provided by FASS-LTP, AT, and PET may significantly contribute to the global understanding of synaptic structure and function in both healthy and diseased human brains, thus directly impacting translational research.

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Introduction

Processing, storage and retrieval of information in the brain rely on circuits of neurons connected by synapses, the "mode of nexus between neurons". Over the last 70 years, structural and functional properties of synapses have been studied in invertebrate animal models such as *C. elegans* and *D. melanogaster*, as well as in vertebrate models including mice, rats, cats, and non-human primates. In comparison, the study of human synapses has evolved significantly slower, mainly because of technical limitations. A better understanding of structural and functional dynamics of human synapses is both timely and critical, as synapse dysfunction is a major cause of most brain diseases, which are increasingly in prevalence in the fast-growing aged population (Selkoe, 2002; Grant, 2012; Morrison and Baxter, 2012).

Rapid technical progresses coupled with creative approaches developed by neuroscientists have opened up opportunities to study human synapses *in vitro* and *in vivo*. Here, we briefly describe three novel methods that allow the analysis of molecular, morphological, and functional properties of human synapses. First, we present a new method that allows the study of synaptic plasticity, specifically long term potentiation (LTP), from synaptosomes isolated from cryopreserved postmortem human brain tissue (Prieto et al., 2017). Second, we discuss how a detailed analysis of subpopulation of synapses can be derived by array tomography (AT), also in postmortem brains (Kay et al., 2013). Third, we discuss how *in vivo* quantification of synaptic density can now be studied by tracking a novel synaptic radio-ligand by positron emission tomography (PET) (Finnema et al., 2016). We conclude by evaluating of how the information provided by these methods may contribute to the global-functional and anatomical-analysis of synaptic populations in both healthy and diseased human brains.

Fluorescence Analysis of Single-Synapse Long-Term Potentiation (FASS-LTP)

A fundamental property of synapses is their ability to show long term change. In 1973, Bliss and Lomo first discovered that brief patterns of afferent activity can initiate a long lasting strengthening of synapse (Bliss and Lomo, 1973), a phenomena first called long-lasting potentiation and currently known as LTP (Bliss and Collingridge, 1993). LTP is commonly held to be a cellular mechanism serving learning and memory (Morris et al., 1986; Roman et al., 1987; Whitlock et al., 2006; Fedulov et al., 2007; Nabavi et al., 2014), and is a topic of intense study in many laboratories (Lynch et al., 2007).

LTP has been studied for decades, both *in vivo* and *in vitro*, using electrophysiological methods which deliver trains of electrical stimulation bursts to initiate LTP in intact neural circuitries (Huganir and Nicoll, 2013). A method to study LTP in human brain would extend studies on LTP from animal models to the human brain. Recently, we developed a method to study LTP in isolated synaptosomes, including synaptosomes isolated from cryopreserved postmortem human brains,

which we refer to as FASS-LTP (Prieto et al., 2017).

LTP reflects the insertion of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors (AMPAR) into the post-synaptic surface (Muller et al., 1988), the essential event for the potentiation of synaptic transmission. FASS-LTP, first described using mouse hippocampus (Prieto et al., 2015), evaluates LTP in isolated synaptosomes by focusing on the insertion of AMPAR into the post-synaptic surface following chemically induced LTP (cLTP). Flow cytometry analysis identifies synaptosomes on the basis of size-related parameters, and evaluates the activity-dependent increase in levels of surface GluA1-containing AMPARs, using immunofluorescence labeling of extracellular epitopes (**Figure 1A**). Simultaneous labeling for the presynaptic marker neurexin-1β (Nrx1β), which is stabilized at the membrane surface by synaptic activity (Fu and Huang, 2010) and captures postsynaptic surface GluA1 *via* PSD95 (de Wit et al., 2009; Mondin et al., 2011), further allows to focus on "snowman-shaped" synaptosomes that contain both pre- and post-synaptic elements (GluA1 and Nrx1β double-labeling). FASS-LTP has several unique strengths: synapses are examined directly, multiple samples can be tested in parallel, and minimal amounts of tissue are needed for each assay (**Table 1**).

A crucial advantage of FASS-LTP over the classical electrophysiological recordings relies on the possibility of study LTP in the human brain. Indeed, using synaptosomes of Alzheimer's disease (AD) and control cases, FASS-LTP-derived data have provided the first direct evidence to support the idea that AD-diseased synapses are intrinsically defective in LTP (Prieto et al., 2017), thereby answering a long standing question in neuroscience. In addition, FASS-LTP have been also used to screen a drug library from over 40 hippocampal samples derived from AD cases in a single day, a novel and relevant application for identifying therapeutics. Thus, FASS-LTP could provide the basis for protocols to study LTP in humans, a previously unattainable goal.

AT

For decades, synapse structure analysis has been mostly based on electron microscopy (EM). Although EM is a powerful technique to visualize synapses, this method is time-consuming and has technical limitations for immunolabeling synaptic proteins, mainly due to high levels of non-specific staining, and because only a restricted number of markers can be simultaneously visualized. A similar disadvantage accompanies Golgi silver staining, a common method to estimate synaptic density but not suited for immunolabeling of synaptic proteins. In recent years, labeling of pre- and postsynaptic structures for counting and determining size have been significantly improved by AT, a fluorescence-based imaging method allowing single-synapse imaging analysis of diverse synapse populations (Micheva and Smith, 2007; Micheva et al., 2010). Originally used to study synapses in rodent brain, AT has been extended to study synapses in serial sections of human brain tissue (Koffie et al., 2009). One key advantage of the AT technique relies on imaging serial ribbons sections of 70-nm, since the axial (*z*)-resolution is adequate at this width for imaging synapses by light microscopy (**Figure 1B**), which
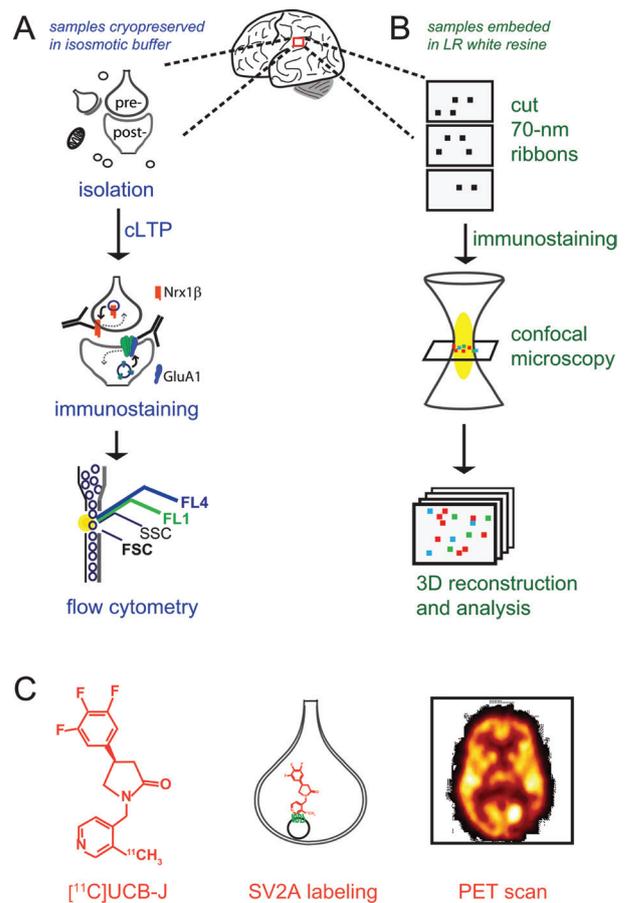
Figure 1 Novel approaches to study human synapses.
(A) Fluorescence analysis of single-synapse long-term potentiation (FASS-LTP) overview. To maintain functional synaptosomal responses, brain tissue (< 1 cm³) is cryopreserved in 320 mM sucrose, slowly frozen and maintained at –80°C. Isolation of crude synaptosomal fraction (P2), containing intact synaptic units (sealed pre- and post-synaptic compartments) along with presynaptic terminals, free mitochondria and cellular debris, is followed by chemically induced long term potentiation (cLTP) stimulation based on glycine treatment and KCl-mediated depolarization. After cLTP, immunolabeling of surface GluA1 and neurexin-1β (Nrx1β) identifies potentiated synapses. According to previous reports (see text), cLTP simultaneously orchestrates an upregulation of postsynaptic GluA1 levels while facilitating the expression of presynaptic Nrx1β at the synaptosomal surface (arrows in the model). In addition, reduced endocytosis (dashed arrows in the model) may also contribute to increase surface GluA1 and Nrx1β following cLTP. In the last step, flow cytometry identifies potentiated synapses by size and GluA1⁺Nrx1β⁺ double labeling. (B) Array tomography (AT) overview. Brain samples (~1 cm³) are fixed, dehydrated and embedded in LR White resin. Blocks of tissue are cut in straight ribbons of 70-nm serial sections. The ribbons are mounted in coverslips, immunostained, and imaged in a microscope. Stacking of images from the same location on each section in the ribbon is followed by counting and measuring synapses, and by assessing co-localization between channels. Although the resolution of confocal microscopes in the *z* plane is too large to accurately image synapses, AT overcomes this problem by physically sectioning the tissue at a resolution smaller than a single synapse. In the diagram the *z* plane resolution (~1,000 nm) is illustrated by the yellow oval. Note that samples from same brain regions can be analyzed in parallel by FASS-LTP and AT. (C) Synaptic vesicle glycoprotein 2A (SV2A)-PET overview. It is shown the structure of [¹¹C]UCB-J[(R)-1-((3-(¹¹C-methyl-¹¹C)pyridin-4-yl)methyl)-4-(3,4,5-trifluorophenyl)pyrrolidin-2-one] ([¹¹C]UCB-J), which binds to the SV2A. In human subjects, intravenous bolus injection of [¹¹C]UCB-J is followed by positron emission tomography (PET) scan and data analysis to evaluate synaptic density.

is perfectly suited for multi-parameter fluorescence analysis.

In the AT method, after staining by standard immunofluorescence protocols, images taken of serial 70-nm sections are used for 3D reconstruction. Reconstructed 3D images show tissue in great detail (resolution in *z*-direction is equivalent to that in EM), thus allowing precise calculations of synapse size and density (**Figure 1B**). Because AT allows stripping off antibodies and re-staining, AT data sets contain multi-parameter information about protein signatures at the synaptic level. In addition, AT allows high-throughput imaging of thousands of synapses. A detailed protocol for studying synapses in human brain by AT has been recently described (Kay et al., 2013). Using the AT technique in brains of AD cases, researchers have found that synapses loss around senile plaques in AD correlates with the burden of oligomeric amyloid-β. Notably, it was also found that carriers of apolipoprotein E (APOE)-4 allele, the most potent genetic risk factor for AD, have a higher oligomeric amyloid-β burden coupled to exacerbated synapse loss around plaques compared to APOE3 carriers (Koffie et al., 2009). Overall, AT-derived data on AD brains provide proof of concept that AT can be used to imaging human synapses in neurodegenerative diseases, therefore, this method could be further extended to other synaptic-related pathological conditions such as autism and schizophrenia.

FASS-LTP and AT relies on availability and quality of human brain tissue. When using postmortem tissue, cryopreservation is crucial to the preservation of the functional response evaluated by FASS-LTP (Prieto et al., 2017). For AT, a detailed protocol for tissue collection has been described, where low temperatures and orientation of samples are crucial parameters (Kay et al., 2013). While there are a number of challenges with using postmortem human brain tissue in research, with one of the most significant being the postmortem interval (PMI), several reports indicate that basic biochemical reactions remain well-preserved in the postmortem human brain. For instance, protein-protein interactions in human PSD fractions (Hahn et al., 2009), glycine-dependent NMDAR activation (Hahn et al., 2006), and insulin signaling (Talbot et al., 2012) are relatively insensitive to variation in PMI (< 15 hours) or age (relatively constant from 70-90 years), suggesting that dynamic and anatomical properties evaluated by FASS-LTP and AT, respectively, are resistant to some variables associated with a postmortem approach.

Synapse Density Analysis by PET

FASS-LTP and AT are powerful techniques for dissecting plastic and structural properties of human synapses. However, these approaches cannot be used for early diagnosis or therapeutic monitoring, as these methods have only been validated in brain tissue obtained from autopsy. Recently, a first-in-human study showed that synaptic density can be monitored noninvasively by PET (Finnema et al., 2016), a well-established technique for detecting a wide range of brain molecules including receptors, transporters and enzymes. Quantification of human synaptic density by PET is based on the detection of the novel synaptic-specific radio-ligand [^{11}C]UCB-J, (chemical name: [^{11}C]UCB-J[(R)-1-((3-(^{11}C-methyl-^{11}C)pyridin-4-yl)methyl)-4-(3,4,5-trifluorophenyl)

pyrrolidin-2-one]), which labels the synaptic vesicle glycoprotein 2A (SV2A) (Finnema et al., 2016) (**Figure 1C**). The synaptic marker SV2A, ubiquitously present in synapses across the brain, is an integral membrane protein located in presynaptic vesicles membranes. PET studies on synaptic density were first validated in baboons. Validation of [^{11}C]UCB-J as a synaptic radio-ligand included a PET analysis of putative SV2A regional densities relative to signal obtained with synaptophysin, a bona fide synaptic marker. Importantly, the authors confirmed that [^{11}C]UCB-J binds specifically to SV2A by pharmacological and biochemical assays (Finnema et al., 2016). Using [^{11}C]UCB-J, SV2A-PET imaging allowed the quantification of synaptic density in over 10 healthy humans and, remarkably, this method provided *in vivo* evidence of synaptic loss in patients with temporal lobe epilepsy (Finnema et al., 2016). Thus, SV2A-PET analysis may be used to monitor changes in synaptic density non-invasively in a living brain.

Striving towards the Big Picture by Combining Functional and Anatomical Analysis of Human Synapses

Based on classic methods, preparations and molecular tools in neuroscience (*e.g.*, EM, synaptosomes, and radio-ligands), FASS-LTP, AT, and SV2A-PET offer a new scientific avenue for studying the human brain, in particular, for the detailed characterization of the synaptic component of brain circuitries. FASS-LTP, AT and SV2A-PET have been validated in animal models, and subsequently tested in the human brain. Also, all three novel approaches have proven useful for detecting synaptic changes in brains from patients, affected either by neurodegenerative (FASS-LTP and AT in AD cases) or neurological disorders (SV2A-PET in epilepsy). **Table 1** compares the advantages and capabilities of FASS-LTP, AT, and SV2A-PET. It is evident that SV2A-PET has the unique advantage of being an *in vivo* and minimally invasive method, but this approach is not suited for single-synapse or multi-parameter analysis. In contrast, *via in vitro* procedures in postmortem tissue, FASS-LTP and AT allow detailed multi-parametric analysis of subpopulation of synapses (*e.g.*, excitatory *vs.* inhibitory), at the single-event level. It is noteworthy that dye development is undergoing exponential technological expansion (*e.g.*, novel fluorescent dyes, quantum dots and metal tags) which in the future could increase the analytical power of both FASS-LTP and AT. In addition, FASS-LTP and AT can be scaled for high throughput analysis, and some steps on these methods could be automated (*e.g.*, automated flow cytometry). FASS-LTP is an unique approach as it tests a major functional response of the synapse: its plasticity. In contrast to the analysis of physically isolated synapses by FASS-LTP, AT analyzes molecular signatures of synapses in their native circuitry, thus preserving valuable anatomical information, at the 3D level. Thus, FASS-LTP and AT data from same tissue sample may provide an unprecedented set of functional and anatomical information at both cellular and molecular levels. Whether combining the information provided by FASS-LTP, AT and SV2A-PET will facilitate the global analysis of human synapses deserves further research.

Table 1 Comparison of current approaches for the analysis of human synapses.

	FASS-LTP	AT	SV2A-PET
Single-synapse resolution	√	√	×
High-throughput analysis	√	√	×
Multi-parameter analysis	√	√	×
Functional evaluation	√	×	×
Circuitry preservation	×	√	√
In vivo method	×	×	√
Minimal amount of tissue needed/assay	~0.1 g	~1.0 g	NA
Time/assay (experimental)	~5 hours	~3 days	~2 hour*
Time/assay (data analysis)	~1 hour	~3–4 weeks	~1 day*
TOTAL time/assay	~6 hours	~1 month	~1 day*
Cost	$	$$	$$$*

*Times for PET acquisition and analysis, as well as PET cost were estimated based on standard PET scan information. FASS-LTP: Fluorescence analysis of single-synapse long-term potentiation; AT: array tomography; PET: positron emission tomography; SV2A: synaptic vesicle glycoprotein 2A.

We envision that new functional and anatomical data on human synapses, the essential building blocks of the brain, may significantly contribute to several ongoing projects for brain mapping (Glasser et al., 2016). Also, this new information of human synapses may set the basis to the human synaptome, and lead to detailed studies on the role of major age-related factors that compromise cognition such as amyloid-β, oxidative damage, and inflammation. The information provided by these studies may offer a global perspective of synaptic function in diseased human brains, thus directly impacting translational research.

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