THE IMPORTANCE OF ULTRASTRUCTURAL ANALYSIS OF MEMORY

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Abstract

Plasticity of glutamatergic synapses in the hippocampus is believed to underlie learning and memory processes. Surprisingly, very few studies report long-lasting structural changes of synapses induced by behavioral training. It remains, therefore, unclear which synaptic changes in the hippocampus contribute to memory storage. Here, we systematically compare how long-term potentiation of synaptic transmission (LTP) (a primary form of synaptic plasticity and cellular model of memory) and behavioral training affect hippocampal glutamatergic synapses at the ultrastructural level enabled by electron microscopy. The review of the literature indicates that while LTP induces growth of dendritic spines and post-synaptic densities (PSD), that represent postsynaptic part of a glutamatergic synapse, after behavioral training there is transient (< 6 hr) synaptogenesis and long-lasting (> 24 hr) increase in PSD volume (without a significant change of dendritic spine volume), indicating that training-induced PSD growth may reflect long-term enhancement of synaptic functions. Additionally, formation of multi-innervated spines (MIS), is associated with long-term memory in aged mice and LTP-deficient mutant mice. Since volume of PSD, as well as atypical synapses, can be reliably observed only with electron microscopy, we argue that the ultrastructural level of analysis is required to reveal synaptic changes that are associated with long-term storage of information in the brain.

1. Synaptic basis of memory

Each neuron in the brain is connected to other neuronal cells by approximately 7000 synapses (Drachman, 2005). Donald Hebb (1949) proposed that synapses are the locus of memory trace. He postulated that neurons undergo metabolic and synaptic changes that enhance their ability to communicate and create a neural network of experiences. The Hebbian learning assumes that the connections between neurons increase in efficacy in proportion to the degree of correlation between pre- and post-synaptic activity. The concept of a synaptic growth as the foundation of memory was also proposed by Jerzy Konorski (1948) who created the term neuroplasticity as the ability of the brain to continuously change connections between neurons throughout an individual's life. Since then the synaptic hypothesis of memory is one of the most discussed problems of neuroscience. It was particularly ignited by the startling discovery by Tere Lomo and Tim Bliss that high frequency electrical stimulation can evoke long-term potentiation of synaptic transmission (LTP) (Bliss and Lomo, 1973). LTP was discovered in the hippocampus, a brain region that is fundamentally important for declarative memory in humans (Corkin, 2002). Years after the discovery of LTP, long-term depression of synaptic transmission (LTD) became known. LTD was discovered first in the cerebellum by Masao Ito and implicated in cerebellar learning (Ito, 2001). Importantly, evidence both for an increase and decrease in the amplitude of evoked synaptic transmission during memory tasks was established in rodent hippocampus (Whitlock et al., 2006; Kemp and Manahan-Vaughan, 2007), supporting the role of LTP and LTD as memory mechanisms.

Given that such different types of functional synaptic changes have been linked with memory, it is of interest to understand what synaptic changes occur during learning and memory and what their precise role is. In this review we systematically compare structural changes of dendritic spines and glutamatergic synapses induced in the hippocampus by LTP and memory formation. We will discuss emerging principles of memory processes which have been obtained from ultrastructural analyses of synapses in the hippocampus.

2. Synapse diversity and its detection

The excitatory synapses in the mammalian central nervous system are chiefly located on small protrusions of dendrites – termed dendritic spines (Fig. 1B). Dendritic spines were first described, and beautifully drawn, by Santiago Ramón y Cajal, a Spanish neuroscientist who devoted his career to the microscopic observation of neurons (Cajal, 1896). Later, with the advent of electron microscopy (EM), the excitatory synapses in the mammalian nervous system were visualized on the heads of dendritic spines (Gray, 1959). It is important to note that only EM can undoubtedly resolve that a dendritic spine has actually a presynaptic input (Fig. 1C). For example, analysis of dendritic spine density with Golgi, or fluorescent staining, does not provide information whether a spine forms a synapse, as the presynaptic input is not detected. Further, fluorescence microscopy does not provide sufficient resolution to assure

that synapses are detected. A few exceptions to this limitation exist: fluorescence imaging with eGRASP reporters that interact with synaptic contact, and carefully validated super-resolution multiscale imaging workflows, such as SEQUIN (Choi et al., 2018; Sauerbeck et al., 2020). However, even this technique relies on identification of marker proteins, as PSD-95, which may not be present in all synapses, and additionally this approach cannot detect multi-synaptic dendritic spines. Thus, EM remains the gold standard to identify dendritic spines that have synaptic input and to identify multi-synapses, such as multi-spine boutons (MSBs) and multi-innervated dendritic spines (MIS).

Dendritic spines have a neck and head, which can be thin or bulbous. Traditionally, dendritic spines are divided into three categories: thin, mushroom and stubby (Harris and Stevens, 1989). In reality they exist on a continuum of shapes with immature filopodia, often lacking a PSD, on one side, and mature mushroom-shaped spines on the other side of the spectrum. In CA1 stratum radiatum around 25% of dendritic spines display typical mushroom morphology with heads more than 600 nm in diameter, around 10% have typical filopodial or stubby shapes and the majority of dendritic spines falls on a continuum between these two categories (Bourne and Harris, 2007, 2011) (Fig. 1D). In the rodent brain, dendritic spine volumes range from 0.01 to around 1 μm^3 and their necks have lengths up to 3 µm and are between 50 and 500 nm thick (Harris and Stevens, 1989; Trommald and Hulleberg, 1997; Arellano et al., 2007). Since, even with the use of super-resolution techniques, it is not possible to distinguish structures smaller than 50 nm using conventional light microiscopy (LM) (Nägerl et al., 2008; Knott and Genoud, 2013), EM is the only technique which allows studying ultrastructural details of synapses, such as precise dimensions and shape of a post-synaptic density (PSD), representing a post-synaptic part of a synapse, or presence and shape of smooth endoplasmatic reticulum (SER) inside a dendritic spine (Fig. 1C) as well as the size and shape of an axonal bouton, and synaptic surroundings. It is of particular importance as the synaptic plasticity concerns, apart from a dendritic spine and presynaptic bouton, also the astroglial component (Perea, Navarrete, and Araque 2009). As the biological processes occur in three dimensions, two-dimensional data is mostly unsatisfactory. Traditionally, serial sections transmission electron microscopy (ssTEM) has been used for 3D reconstructions (Geinisman, 2000; Nikonenko et al., 2008). In 2004, Denk & Horstmann presented an alternative technique called serial block-face scanning electron microscopy (SBEM, formerly SBFSEM), which is based on installation of an ultramicrotome inside a scanning electron microscope (SEM) (Denk and Horstmann, 2004). SBEM allows for reliable collection of series of many aligned images, with eliminated section loss and damage. Recent years have brought other advancements in 3D EM including FIBSEM (Focussed Ion Beam milling combined with Scanning Electron Microscopy), where ultrathin slices are milled by a focused ion beam (Stokes et al., 2006). 3D EM analyses showed that some excitatory synapses do not have the classic one-input-one-output relationship. For example, there are multi-spine boutons (MSBs) (Fig. 1E), where a presynaptic bouton connects with two or more dendritic spines. MSBs can connect with spines from the same dendrite (sdMSBs) or with spines from different dendrites, possibly from different neurons. The converse of MSBs are multi-innervated spines

(MIS) (Fig. 1E), where two or more presynaptic boutons connect with a dendritic spine. Importantly, MIS and MSBs can only be unequivocally detected and characterised by 3D EM.

Although EM is required for an accurate structural analysis it has some drawbacks. First, EM does not allow for live imaging. This aspect is important as with neuronal activity dendritic spines may disappear or appear and change shape or size. It has been shown that in the hippocampus *in vivo*, within a month the rate of spine turnover approaches 100% (Attardo et al., 2015; Pfeiffer et al., 2018). To image dendritic spines changes *in vivo* two-photon and super-resolution light microscopy techniques provide state of the art approach to monitor dendritic spine plasticity in real time (Nagerl et al., 2008; Nair et al., 2013; Wegner et al., 2018). Secondly, even with the advent of 3D EM imaging systems and the progress made in image analysis, 3D EM experiments remain extremely laborious and time consuming. Thus, for projects intending on identification of synaptic contacts in bulk, for example across multiple brain regions, more error-prone but less time-consuming LM methods may be preferable (Sauerbeck et al., 2020). Additionally, super-resolution LM allows for imaging of a number of synaptic proteins at the same time (Heller et al., 2020). The fourth disadvantage is the disappearance of fluorescence in the course of EM staining. Here, however, several approaches may be used to overcome this obstacle. One is with immunohistochemical approach, that results in a dark precipitate formation (Nikonenko et al., 2008), and another one is correlative light-electron microscopy, where laser marks are used to overlay fluorescent and EM. Finally, eGRASP, an approach that provides fluorescence when synaptic contact is made, could be used to study synaptic changes between neurons activated during memory formation (Choi et al., 2018). Such specific labelling is not yet available at the EM level.

Thus, while super-resolution LM is the state of the art in the imaging of fluorescently labelled synaptic proteins and allows the researcher to answer questions about their localization and quantity, 3D EM remains the gold-standard for collection of ultrastructural data and provides information about the size of synapses, which is a proxy for its strength, the precise dimensions of dendritic spines, which can inform about their electrical properties and the dimensions and presence of other structures at the same time. Overall, super-resolution light microscopy and EM methods can be considered complementary in structural synaptic plasticity research as they address distinct aspects of this phenomenon.

3. Hippocampal LTP in CA1 *stratum radiatum* and synaptic changes at the ultrastructural level

3.1 LTP increases dendritic spine volume

NMDA receptor-dependent LTP in the hippocampal CA1 *stratum radiatum* (Fig. 1A) is thought to be a memory mechanism (Martin et al., 2000; Matynia et al., 2002; Lisman, 2017). Therefore, it is of great interest to understand which structural changes at glutamatergic synapses occur after LTP induction. Here, we will review such changes and compare them with ultrastructural changes in CA1

stratum radiatum induced by behavioural training in memory tasks. Please note that the synaptic mechanisms underlying CA1 LTP may differ for adolescent and adult animals (Harris, 2020).

EM studies have detected changes in spine morphology during CA1 LTP. The general conclusion is that NMDAR-dependent chemical and electrical LTP results in increased dendritic spine volume without pronounced dendritic spine density changes at timepoints ranging between 5 min and 6 hours (Fig. 2A) (Sorra and Harris, 1998; Toni et al., 1999; Popov et al., 2004; Stewart et al., 2005; Bourne and Harris, 2011; Bell et al., 2014; Borczyk et al., 2019b). A body of research in the field exists that indicates that dendritic spines from the opposite sides of the size continuum exhibit different properties (Berry and Nedivi, 2016). This idea has been postulated in the early 90's of the 20th century based on the modelling studies showing that relatively small changes in buffer concentration and spine geometry can have a profound effect on dendritic spine calcium dynamics, possibly affecting the plasticity of its synapse (Holmes, 1990; Gold and Bear, 1994). Large, mushroom-shaped dendritic spines are long-lasting, more resistant to activity-independent random spine size fluctuations (Bourne and Harris, 2007; Yasumatsu et al., 2008; Chen and Sabatini, 2012), they show smaller calcium transients, and are less easily potentiated than the small and thin spines (Matsuzaki et al., 2004; Nimchinsky, 2004; Noguchi et al., 2005; Sobczyk, 2005).

3.2 LTP and PSD changes

Most dendritic spines are decorated by a protein supercomplex associated with the membrane, forming the post-synaptic part of a glutamatergic synapse and called post-synaptic density (PSD). The total mass of an average PSD is around 1 GDa, which equals to approximately 10 000 protein molecules (Chen et al., 2005). This high concentration of proteins makes PSD visible under an electron microscope as a dark disk (Fig 1C). Dendritic spines typically contain a type I PSD, which means that the thickening is easily visible and extends slightly into the dendritic spine head. This is characteristic of excitatory PSDs, whereas inhibitory synapses have no or little thickening and are termed type II PSD, although a more detailed 5-class classification has also been proposed (Gray, 1959; Klemann and Roubos, 2011).

The radius of PSD disc-like structure in the forebrain falls between 200 to 526 nm (Cohen and Siekevitz, 1978; Chen et al., 2005) and a strong positive correlation between PSD area and dendritic spine volumes is a major assumption in the field (Tønnesen and Nägerl, 2016). However, some indications exist that this relationship is not set in stone. These include the fact that dendritic spine growth has been shown to exceed PSD enlargement in glutamate uncaging experiments (Bosch et al., 2014; Meyer et al., 2014). In our research we observed that the correlation strength between PSD area and dendritic spine volume is distinct for various dendritic spine sizes (Fig. 2A). Namely, small and large dendritic spines maintain a tight relationship between their volume and their synapse size (surface area and volume), while for medium-sized dendritic spines volume is a poor predictor of PSD area (Borczyk et al 2019). Moreover, the ratio of dendritic spine and PSD volume (and PSD surface area) is

not a constant value and the correlation between these variables is tightened after induction of NMDAR-dependent LTP (Borczyk et al., 2019b) (Fig. 2A). Interestingly, the precise reasons for alignment in PSD and dendritic spine size remain unknown (Berry and Nedivi, 2017). It has been postulated that this correlation allows for optimal calcium dynamics (O'Donnell et al., 2011).

Proteomic studies show that up to 1000 different proteins are present in the PSD (Husi et al. 2000). Most abundant proteins of the PSD include: PSD-95 (Postsynaptic Density Protein 95; in around 300 copies), CaMKII (Calcium and Calmodulin-dependent protein kinase II), SynGAP (Synaptic GTPase-activating protein) and Actin (Cho et al., 1992; Walsh and Kuruc, 1992; Walikonis et al., 2000; Chen et al., 2005). PSD has a laminar organisation. Ion channels, cell adhesion molecules and receptors are membrane-bound. Further into the spine head lumen there are auxiliary subunits associated with AMPA receptors, such as Stargazin, and Membrane Associated Guanylate Kinase (MAGUK) scaffolding proteins, such as PSD-95 or SAP-97 (Dosemeci et al., 2001); then enzymes, for example CaMKII, and secondary scaffolds like Shank and Homer and finally the cytoskeleton and its associated proteins (Valtschanoff and Weinberg, 2001; Petralia et al., 2005; Tao-Cheng, 2014; Dosemeci et al., 2016).

The main actors of excitatory synapses are the NMDA and AMPA-type glutamate receptors. NMDA receptor is present in approximately 20 copies per synapse (Sheng and Hoogenraad, 2007) and AMPA receptor was estimated at 15 tetrameric receptors per PSD (Cheng et al., 2006). Using EM jointed with gold immunolabeling it has been shown that PSD area is proportional to the overall number of AMPA and NMDA receptors, however the relationship for AMPA receptor subunit GluR1 has been reported to be supralinear (Takumi et al., 1999; Ganeshina et al., 2004a; Sinohara and Hirase, 2009). Moreover, small PSDs (< 180 nm in diameter) typically have no AMPA receptors and can be considered silent synapses (Takumi et al., 1999). Thus, PSD area can be treated as a readout of synaptic strength. From the experiments using EM a general conclusion is that LTP induction results in accumulation of AMPA receptors and increased PSD surface area (Toni et al., 1999; Popov et al., 2004; Stewart et al., 2005; Bourne et al., 2013; Bell et al., 2014; Borczyk et al., 2019) (Fig. 2A).

The extension of the PSD is called pallium and the main thickening is termed the core of PSD (Dosemeci et al. 2016). Data gathered by our group and others indicate that chemical LTP increases the thickness of the PSD (Dosemeci et al., 2001; Borczyk et al., 2019) (Fig. 2A). This thickening is the result of protein accumulation. It has been shown that CaMKII translocates from F-actin-bound state to PSD-95 bound state upon stimulation of NMDARs. Under the EM this is seen as visible darkening of the pallium (Dosemeci et al., 2016). This is precluded in autophosphorylation-deficient mutants of CaMKII (T286A) (Strack et al., 1997; Shen and Meyer, 1999).

Discontinuities called perforations are an important aspect of the planar organisation of the PSDs. When PSD is totally partitioned it is called segmented; PSD without perforations is called macular. Perforated synapses are usually large, have more glutamate receptors and are located on

dendritic spines with SER (Borczyk et al., 2019) and higher content of kalirin (actin regulatory protein), than non-perforated (macular) PSDs (Nicholson et al., 2012). They typically belong to mushroom dendritic spines, whereas macular PSDs are associated with smaller and thinner spines (Ganeshina et al., 2004a, 2004b; Nicholson et al., 2006). The frequency of perforated PSDs is increased during LTP (Toni et al., 1999, 2001; Stewart et al., 2005; Borczyk et al., 2019) (Fig. 2A). Some researchers support the idea that, as more than 80% of large dendritic spines have perforated PSDs, perforations are just a function of synaptic size (Nieto-Sampedro et al., 1982; Stuart et al., 2016). However, it was also proposed that, as perforations increase the area of PSD edge, they facilitate PSD expansion by adding new molecules from peri-synaptic areas (Stewart et al., 2005; Stuart et al., 2016). This is further corroborated by EM immunostaining with gold nanoparticles which shows that perforated PSDs contain more AMPA glutamate receptors than their size would suggest (Takumi et al., 1999; Ganeshima et al., 2004a, 2004b).

MAGUKs are important organisers of the PSD, as loss of PSD-95 leads to the loss of vertical filaments of PSD-core structure resulting in PSD perforations (Chen et al., 2005). Furthermore, after triple knock-down of PSD-95, PSD-93 and SAP-102 PSD areas are greatly decreased (Chen et al., 2015). On the other hand, overexpression of synaptic scaffold proteins PSD-95 and SAP97, both *in vitro* and *in vivo*, results in dramatic enlargement of dendritic spines and PSDs (Nikonenko et al., 2008; Poglia et al., 2010; Ziółkowska et al., 2020). Formation of the perforations also requires CaMKII activity (Toni et al., 1999).

In recent years additional information about the planar organization of the PSD has been discovered. This is thanks to super-resolution imaging that allows for localization of single protein molecules. Before, PSD face was considered rather uniform, with random distribution of proteins. However, it seems that it is organized into nanoclusters (also called nanodomains) that contain glutamate receptors and scaffolding proteins. Each PSD has between 1 and 4 of such domains (Opazo et al., 2012; Nair et al., 2013; Compans et al., 2016). These postsynaptic nanodomains align with places of glutamate release on the presynaptic side creating trans-synaptic nanocolumns (Tang et al., 2016). Thus, the PSD is organized not only linearly, but also planarly. These nanocolumns may correspond to vertical filaments of the PSDs observed under the EM as knocking-down MAGUKs leads to loss of these filaments (Chen et al., 2011; Tang et al., 2016).

3.3. LTP and changes of resources in dendritic spines

In the neuronal soma the rough endoplasmic reticulum (RER) is prominent, but in the dendrites and dendritic spines smooth endoplasmic reticulum (SER) is present (Spacek and Harris, 1997). SER in dendrites forms a network that extends its tubules into dendritic spines. Some mature dendritic spines contain single tubules of SER, or its specialization in the form of stacks, called spine apparatus (SA) (Fig. 1C). SER-containing spines are larger and more mushroom-shaped than those without SER

(Holbro et al., 2009; Segal et al., 2010; Borczyk et al., 2019). Interestingly, the role of SER in dendritic spines is still debated. It is considered to store and release calcium ions and play a role in protein and lipid trafficking to the plasma membrane (Korkotian and Segal, 1999; Verkhratsky, 2005). What is more, as SA labels positively for glutamate receptor subunits, they may play a role in AMPA and NMDA receptor trafficking to or from the postsynaptic membrane (Nusser et al., 1998; Racca et al., 2000). In addition, a recent study showed that SER prevents runaway potentiation of synapses, keeping most of them at intermediate strength levels from which both LTP and LTD are possible (Perez-Alvarez et al., 2020).

LTP increases the frequency of dendritic spines with SER (Borczyk et al., 2019). Moreover, dendritic spines with SER tend to increase their volume and synaptic size (both surface area and thickness of PSDs) during LTP, while for the spines without SER just median size of PSD is increased and median spine volume remains constant (Borczyk et al., 2019; Chirillo et al., 2019). We also observed that LTP increases correlation between PSD volume and dendritic spine volume specifically for the spines with SER (Borczyk et al 2019).

Synaptic resources also have an important role in learning and memory formation. The crucial role of dendritic spine SER has been confirmed by experiments showing that mice lacking Synaptopodin have no SA and are impaired in learning and synaptic strengthening (Deller et al., 2003; Segal et al., 2010). On the other hand, our recent study showed that spatial long-term memory in Intellicages associates with an increase in PSD volume both in spines without and with SER and spine apparatus (Sliwinska et al., 2020). More studies are needed to characterize memory-associated changes in resources at the post-, but also pre-synapse.

Apart from SER, dendritic spines contain other structures. These include: polyribosomes, vesicles (including endosomes) and multi-vesicular bodies (MVBs) (Spacek and Harris, 1997; Sheng and Hoogenraad, 2007). All these structures provide resources for synapses as they aid in production and modification of proteins. Experiments with electrical stimulation have shown that more polyribosomes occur in spines with larger PSDs 2 hours post-stimulation (Bourne and Harris, 2007; Bourne et al., 2013). What is more, dendritic spines containing polyribosomes preferentially enlarge their synapses as compared with other dendritic spines (Chirillo et al., 2019). Recycling endosomes were found to be elevated specifically in small spines after LTP induction, suggesting a high load of local protein and membrane trafficking in these spines (Kulik et al., 2019). Overall, although the data is still limited, SER and other resources seem to increase the probability of a synapse to be potentiated and are also signs of ongoing synaptic remodelling. So far no data is available linking dendritic spines with polyribosomes, vesicles and MVBs with memory processes.

3.4 LTP and generation of atypical synapses

Atypical synapses have either more than one input or more than one output. MSBs are synapses with one input and several outputs: presynaptic terminal contacts several dendritic spines. In the hippocampus the vast majority of MSBs consist of one pre-synaptic bouton contacting two dendritic spines. Sometimes (<20%) MSBs contain three or four dendritic spines. Amazingly, in hippocampal CA1 *stratum radiatum* about 10 to 30% of all excitatory boutons are MSBs (Xu X, Kraev I and Giese KP, unpublished data) (Zhan et al., 2014). It is important to note that MSBs do not result from splitting of post-synaptic spines (Fiala et al., 2002; Medvedev et al., 2014). Instead, either a newly generated post-synaptic dendritic spine or one from neighbouring synapses connects with the pre-synaptic bouton of an existing synapse.

MIS are the converse of MSBs. They have two or more inputs and only one output. MIS are less abundant than MSBs in the hippocampus, and it is estimated that there are 0.5% of all synapses in the dorsal CA1 area of a young-adult mouse (Radwanska et al., 2011). Their abundance is highest in adolescence, declines with adulthood, but increases again with ageing (Fiala et al., 1998; Aziz et al., 2019). In the hippocampus the vast majority (>90%) of MIS are two-excitatory-input-spines, and sometimes they have three inputs. MIS are generated by the addition of an additional pre-synaptic input onto an existing synapse (Nikonenko et al., 2008). Most MIS are likely to connect three, or sometimes four, neurons, although it remains possible that both pre-synaptic boutons derive from different axonal branches of the same pre-synaptic neuron.

In vitro and *in vivo* LTP stimulation in the hippocampus leads to the generation of MSBs, which appear to be mainly sdMSBs (Toni et al., 1999; Fiala et al., 2002; Medvedev et al., 2014), and MIS (Nikonenko et al., 2003). Moreover, overexpression of PSD-95 results in high frequency of MIS, and this process requires NO signalling (Nikonenko et al., 2008). It is important to note that MSB and MIS generation are not underlying LTP, as LTP is a strengthening of existing synapses. Rather, electric stimulations that induce LTP also lead to the generation of MSBs and MIS, which then in turn change the connectivity between neurons.

4. Synaptic changes in CA1 *stratum radiatum* after training in a memory task

4.1 Transient synaptogenesis and memory formation

Several EM studies also studied morphology and density of dendritic spines in the rodent hippocampus after training in memory tasks, such as the water maze, passive avoidance paradigm, trace eyeblink conditioning, contextual fear conditioning and spatial learning in IntelliCages (O'Malley et al., 1998, 2000; Geinisman et al., 2001; Eyre et al., 2003; Radwanska et al., 2011; Scully et al., 2012; Aziz et al., 2019; Śliwińska et al., 2020). The consensus from these studies is that there is evidence for transient increase in excitatory synapse numbers, which is detectable 2-6, but not 24 hours, after training (O'Malley et al., 1998, 2000; Eyre et al., 2003; Radwanska et al., 2011; Scully et al., 2012) (Fig. 2B).

The function of transient synaptogenesis after training remains unknown. It could serve the purpose to cluster synaptic inputs along dendrites to more efficiently excite the post-synaptic neuron. Consistent with this idea it seems that memory-encoding engram cells have higher synaptic connectivity than non-engram cells (Choi et al., 2018). Further, elevated dendritic spine density on engram cells has been suggested to enable memory retrieval (Ryan et al., 2015). Currently, it is also not understood which processes induce synapse loss after training-induced synaptogenesis. As NMDAR-dependent LTP is not associated with synaptogenesis it is clear that behavioural training in hippocampal memory tasks induces synaptic plasticity that is independent of LTP. Thus, this observation does not allow us to conclude that LTP is induced. To reach a conclusion about the induction of structural LTP the morphology of the dendritic spines and PSDs have to be considered.

4.2. Memory and changes in dendritic spines

EM studies after training in memory tasks have also detected a long-lasting growth of the PSDs and change in PSD morphology, without a significant change in dendritic spines volumes (Fig. 2B). The first study to detect such a memory-associated PSD change was by Geinisman et al, 2000. They found that 24 hours after trace eyeblink conditioning the PSD area of non-perforated synapses was increased in CA1 *stratum radiatum* in comparison with pseudo-conditioned controls. After contextual fear conditioning, there is a long-lasting shift from macular to non-macular PSDs and increase in frequency of large PSDs, at the expense of small ones (Aziz et al., 2019). Interestingly, this change in PSD morphology was not observed in aged mice, which could acquire memory equally as young-adult mice, suggesting that global synaptic strengthening may not be induced by contextual fear conditioning in older age (Aziz et al., 2019). Also, spatial long-term memory acquired in an IntelliCage is linked with an increase in PSD volume (Śliwińska et al., 2020). Interestingly, the persistent PSDs growth is not associated with dendritic spines growth, resulting in increased PSD/dendritic spine volume ratio. After spatial training in young adult alphaCaMKII-T286A mice, which cannot learn (Giese et al., 1998), the change in PSD volume is absent, meaning that learning-related growth of PSDs relies on autophosphorylation and, presumably, accumulation of CaMKII (Śliwińska et al., 2020). Finally, the training-induced change of PSD/ dendritic spine volume ratio is absent in old age (Śliwińska et al., 2020), suggesting that alternative synaptic processes accompany spatial memory formation in the aged brain. Thus, in addition to accumulation of AMPA receptors (Matsuo et al., 2008; Ryan et al., 2015), accumulation of signalling molecules in PSD, that results in PSD growth, may be an alternative mechanism to enhance synaptic function in order to enable memory storage.

Taken together, these studies show that long-term memory is associated with structural changes of PSD which are likely to enhance synaptic transmission, and they resemble PSD changes observed during the early phase of LTP (0.5-2 hr). This is also consistent with electrophysiological recordings showing CA1 LTP induction after training in a memory task (Gruart et al., 2006; Whitlock et al., 2006).

4.3 Memory and generation of atypical synapses

Even though memory storage unlikely results from a hippocampal increase in synapse number, a long-lasting increase in atypical synapses may contribute (Fig. 2B). This was shown first for MSBs in the CA1 *stratum radiatum*, which are increased 24 hours after trace eyeblink conditioning (Geinisman et al., 2001). It is not known whether the memory-associated MSB increase is due to elevated sdMSB generation, as shown for cerebellar memory (Lee et al., 2005), or whether the MSBs involve different post-synaptic neurons. The former case would strengthen the activity between one pre-synaptic neuron and one post-synaptic neuron, whereas the latter case would link activity of more than one post-synaptic neuron with a pre-synaptic neuron. Importantly, recent data suggests that MSBs affect functional brain connectivity (Zhan et al., 2014).

A long-lasting MIS generation has also been linked to hippocampal memory. This was found first in alphaCaMKII T286A knockin mice that lack NMDAR-dependent LTP in CA1 stratum radiatum (Radwanska et al., 2011). Recently, this was shown to be also the case in aged mice under conditions when they learn as well as young-adult mice (Aziz et al., 2019). This not only suggests that the synaptic basis of hippocampal memory storage changes with age, it also indicates that deficits in CA1 LTP enable MIS generation.

The findings that long-term memory can be associated with lasting generation of MSBs and MIS suggests that establishing a connectivity between three, sometimes more neurons is a memory coding principle. Such memory coding principle is different from LTP which strengthens the connection between two neurons. More ultrastructural analyses are needed to assure that establishing a connectivity between groups of three, or more neurons is involved in memory storage.

5. Conclusions

Synaptic changes are believed to underlie learning and memory. Surprisingly, a very limited number of studies reports long-lasting synaptic alterations associated with behavioral training. Here, we argue that memory-related synaptic changes can only be understood if synapse diversity is adequately studied. Only ultrastructural studies offer sufficient resolution to unequivocally identify synapses and their structural characteristics. Thus, ultrastructural studies of memory-associated synaptic changes are fundamentally important to characterise synaptic changes taking place when memories are formed, stored and retrieved. Here, we have compared ultrastructural changes at glutamatergic synapses in the hippocampus after LTP induction and after training in memory tasks. This comparison allows to ask the question whether LTP could be induced after training in a memory task, and whether additional types of synaptic plasticity underlie learning and memory. Our review illustrates that after training there is transient synaptogenesis in the hippocampus, the phenomenon that was not observed during LTP. This

transient synaptogenesis may lead to re-wiring between neurons, but its function for memory processes warrants further investigation. Moreover, there is evidence that indeed a long-lasting increase in PSD volume, that is also characteristic for LTP, occurs after training in a memory task. Interestingly, this morphological change of PSD occurs without significant growth of dendritic spines, further strengthening the importance of EM ultrastructural analysis of neurons. From ultrastructural studies it has also emerged that atypical synapses, such as MSBs and MIS, can be generated. The generation of such synapses may lead to enhanced connectivity between three or more neurons, but more studies are needed to establish this. Further, future studies will need to address the functions of MSB and MIS generation for memory; are these multi-synapses required for retrieval because they connect multiple neurons, or do they enable memory storage due to reduced turnover (Fauth et al., 2015). Taken together, over recent years we have learned from ultrastructural studies that synapses and their changes are more complex than widely thought and that we have to take this into consideration to adequately appreciate memory processes in the brain. Still it remains to be established whether behavioral training is associated with such ultrastructural changes observed during LTP as increased frequency of dendritic spines with SER and polyribosomes or increased correlation of PSD and dendritic spine volumes (Borczyk et al., 2019; Chirillo et al., 2019). Finally, it remains unexplored what are the long-term ultrastructural consequences of the training resulting in LTD (Goh and Manahan-Vaughan, 2013).

Author statement

All authors concepted and designed the organization of the MS, and wrote the review. Gosia Borczyk designed the figures**.**

Declaration of Competing Interest

The authors report no declarations of interest.

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Figure Legends

Figure 1. Dendritic spines and their synapses can be studied with 3D SBEM. (A) Low magnification SBEM microphotography of the CA1 field of the hippocampus; **(B)** Fragments of mouse CA1 *stratum radiatum* dendrites. PSDs are reconstructed in red; 3D cube is 1×1×1μm. **(C)** High magnification SBEM microphotographs and 3D reconstruction of a dendritic spine and bouton. PSD - post-synaptic density, SER - smooth endoplasmic reticulum, Mito.- mitochondrium; 3D cube is 0.5×0.5×0.5μm. **(D)** 3D reconstructions of dendritic spines and PSDs showing their diversity. For each dendritic spine its volume, PSD surface area, PSD area/spine vol. correlation (P value for Spearman corr.), PSD core volume and PSD vol./spine vol. correlations (P value for Spearman corr.) are shown. 3D cubes are 30×30×30 nm. (Borczyk et al., 2019a). **(E)** 3D reconstructions of dendritic spines, PSDs and boutons. Dendritic spine 1 is a multi-innervated dendritic spine (MIS). Bouton 2 is a multi-spine bouton (MSB). 3D cube is 0.3×0.3×0.3μm. Image sources: **A-C, E** – unpublished images, provided by prof. radwanska's laboratory, **D** – Borczyk et al., 2019.

Figure 2. Findings on the ultrastructural plasticity of the CA1 area described in the review.

A. Two to six hours post LTP induction in the CA1 area multiple synaptic processes were observed using EM, including increased dendritic spines and PSDs volumes, increased frequency of MISs, MSBs, perforated PSDs, dendritic spines with SER and polyribosomes, and increases correlation of PSD/ dendritic spine volumes. **B.** After hippocampus-dependent training there is transient (< 6 hr) synaptogenesis in the CA1 area, that is followed by long-lasting (> 26 hr) increase in PSD thickness and increased frequency of perforated PSDs, MSBs and MISs (in aged mice and αCaMKII-T286A mutant mice).

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