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Structural and functional plasticity of dendritic spines – root or result of behavior?

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Abstract

Dendritic spines are multifunctional integrative units of the nervous system and are highly diverse and dynamic in nature. Both internal and external stimuli influence dendritic spine density and morphology on the order of minutes. It is clear that the structural plasticity of dendritic spines is related to changes in synaptic efficacy, learning and memory, and other cognitive processes. However, it is currently unclear whether structural changes in dendritic spines are primary instigators of changes in specific behaviors, a consequence of behavioral changes, or both. In this review, we first review the basic structure and function of dendritic spines in the brain, as well as laboratory methods to characterize and quantify morphological changes in dendritic spines. We then discuss the existing literature on the temporal and functional relationship between changes in dendritic spines in specific brain regions and changes in specific behaviors mediated by those regions. Although technological advancements have allowed us to better understand the functional relevance of structural changes in dendritic spines that are influenced by environmental stimuli, the role of spine dynamics as an underlying driver or consequence of behavior still remains elusive. We conclude that while it is likely that structural changes in dendritic spines are both instigators and results of behavioral changes, improved research tools and methods are needed to experimentally and directly manipulate spine dynamics in order to more empirically delineate the relationship between spine structure and behavior.

Keywords

Dendritic spine; plasticity; morphology; glutamate; actin; cytoskeleton; behavior; Golgi; histology; dynamics

Introduction

Dendritic spines are key specialized structures of neuronal connectivity and signaling in the nervous system. Dendritic spines are also highly dynamic in nature, having the capacity to change their morphology, number, density, and motility within relatively short timeframes. For the purposes of this review, we utilize the term dendritic spine “dynamics” to refer to changes in one or more of the aforementioned structural properties, as opposed to

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subcellular phenomena such as biochemical signaling or dendritic mRNA translation. The dynamic nature of dendritic spines has recently become of great interest in the study of the neurobiological underpinnings of behavior. For example, synaptic plasticity, which is thought to underlie learning processes and the formation and consolidation of memory (Durand *et al.*, 1996; Fortin *et al.*, 2012; Oe *et al.*, 2013), involves de novo dendritic spine formation (spinogenesis) and remodeling of existing spines to sustain neuronal connections that result from input from the environment or other brain regions. Synaptic strengthening and long-term potentiation (LTP) are associated with increases in spine density and/or spine head diameter, while synaptic weakening induced by long-term depression (LTD) causes spine retraction (Alvarez & Sabatini, 2007; De Roo *et al.*, 2008a; Sala & Segal, 2014; Segal, 2005; van der Zee, 2015). However, it should be noted that synaptic overstimulation can also lead to spine shrinkage to prevent damage from excess calcium influx (Paulin *et al.*, 2016).

The involvement of spine dynamics in behavior and the impact of environmental stimuli on spines have become topics of interest across various fields of research. However, it remains unclear whether spine changes *precede* environmental input and/or behavioral output, or if environmental input and/or behavioral output in and of themselves *lead to* spine changes. In this review, we will examine the function of dendritic spines as well as technological advancements in the neuroanatomical methods used to analyze them. In doing so, we will discuss whether changes in spine morphology are an accurate correlate for changes in synaptic strength. We will then discuss structural changes in dendritic spines in relation to different types of environmental input or behavioral output including learning and memory processes, addiction-related behaviors, stress, depression, and aging. Finally, we will attempt to address the question: do changes in dendritic spine dynamics lead to behavioral changes, specifically when behavior becomes aberrant, or is behavior itself the root cause of changes in dendritic spines? In other words, do spine dynamics underlie pathological behaviors and therefore constitute a neurobiological substrate of certain brain disorders?

Structure and function of dendritic spines

Structure

Dendritic spines were discovered more than century ago by renowned Spanish anatomist and Nobel laureate Santiago Ramón y Cajal. In his investigations of the microarchitecture of the avian cerebellar cortex, Cajal noted that the “surface of the Purkinje cells' dendrites appear ruffled with thorns or short spines, which on the terminal dendrites look like light protrusions” (Ramón y Cajal, 1888). Cajal subsequently revealed dendritic spines to be present in numerous neuronal subtypes and brain regions, and also relatively consistent across various animal species (Ramón y Cajal, 1899). Incorporating these observations into his neuron doctrine, Cajal hypothesized that dendritic spines served to increase the surface area of dendrites to accommodate the vast complexity and number of neural connections in the brain (Ramón y Cajal, 1899; Yuste, 2015).

The subsequent development of high resolution microscopy techniques such as confocal microscopy, two-dimensional transmission electron microscopy, and three-dimensional reconstruction of serial confocal and electron microscopy images, has revealed substantial heterogeneity in dendritic spine morphology and associated metrics. In general, dendritic

spines are 1–3 μm in length, 0.01–0.8 μm^3 in volume, and typically consist of a rounded "head" (which receives primarily excitatory synaptic input) atop a thinner "neck" apparatus emanating from the dendritic shaft (Harris & Spacek, 2016; Yuste, 2010) (Fig. 1A). However, neurons exhibit many variations of dendritic spine subtypes based on their morphological and physiological properties, each with unique putative functions. Spine-containing ("spiny") neurons are found in numerous brain regions, the most widely studied being pyramidal cells of the cerebral cortex, medium spiny neurons of the dorsal and ventral striatum, and Purkinje cells of the cerebellum. Recently, advancements in cellular imaging techniques have allowed three-dimensional analyses of dendritic spines across different cell types, brain regions, and time. Importantly, it should be noted that although these advancements have yielded a wealth of information regarding the dynamic structure of dendritic spines, they have not yet fully defined the exact functions of changes in dendritic spine morphology, particularly with respect to behavior.

More than 100,000 dendritic spines can populate the dendritic arbor of a single neuron (Yuste, 2010). During the development of the nervous system, dendritic spines tend to have a long (>2 μm), "filopodia-like" morphology containing relatively few organelles and no discernible head apparatus. This type of dendritic spine is highly dynamic in its ability to retract or extend within minutes of chemical stimulation (Fischer *et al.*, 1998; Harris, 1999). However, its functionality in synaptic transmission, particularly in adulthood, appears limited, since it largely devoid of synaptic inputs and thus is often considered an "immature" or "transient" phenotype. Yet following increased synaptic input, immature dendritic spines can transform into more "mature" and "stable" phenotypes (Bourne & Harris, 2007).

Dendritic spines in adult neurons can have simple or branched morphologies (Fig. 1B), and over the years, investigators have developed various criteria for classifying spines into specific categories based on their overall morphology. However, as we caution below, such categorical approaches to examining spine dynamics may be inherently self-limiting and potentially flawed. Some spine types are sessile in nature, being devoid of any discernible neck apparatus and relatively short in length (<0.5 μm), which are often referred to as "stubby" spines (Harris & Spacek, 2016). Other mature types of spines are pedunculated, containing a head apparatus that is wider in diameter than that of the neck, often referred to as "mushroom" spines. Spines that retain this distinct head and neck apparatus but with much narrower diameters are often referred to as "thin" spines. Less frequently, mature spines can have two (bifurcated) or more (multi-branched) processes with fully functional heads, thus allowing multiple excitatory synaptic inputs onto a single spine. It should be noted that aside from these classical dendritic spine morphologies, other dendritic specializations for receiving inputs include more complicated architectures such as synaptic crests, claw-like glomerular endings, brush endings, and thorny and coralline excrescences. Yet the occurrence of these structures tends to be far less frequent than that of simple dendritic spines (Harris & Spacek, 2016).

As alluded to above, many studies investigating the structural plasticity of dendritic spines in normal and diseased states have utilized categorical approaches to quantifying spine plasticity. For example, observed increases in the numbers of "mushroom-shaped" spines following a specific experimental manipulation, concomitant with decreases in the numbers

of “filopodia-like” spines, are often interpreted as being suggestive of increased spine maturation. We argue, however, that many metrics of spine morphology, including head and neck diameter and volume, neck length, ratio of head to neck diameter, etc., occur along various continuums. As a result, categorical approaches to assessing structural plasticity of dendritic spines may overlook subtle differences in spine morphology that do not meet pre-defined classifications (Arellano *et al.*, 2007). For example, putative thin and filopodia-like spines often have similar diameters, and the length of the spine that differentiates between these two subtypes is not universally agreed upon. Similarly, spines that have discernible yet relatively small head apparatuses located on spine necks with large diameters may be mistakenly classified as thin spines. The conundrum of discrete morphological spine classification is further exemplified by recent findings that indicate spine neck width and length are more indicative of synaptic efficacy and compartmentalization than spine head measurements (Tonnesen *et al.*, 2014). As suggested by others (Arellano *et al.*, 2007; Yuste & Majewska, 2001), we assert that dendritic spine dynamics are more accurately assessed by continuous numerical measures, such as spine head diameter and volume, neck width and length, and so forth, rather than discrete morphology-based classifications.

Hypothesized functions of dendritic spines

As noted earlier, Cajal hypothesized that dendritic spines served to increase the surface area of dendrites, thereby increasing their capacity to receive synaptic inputs (Ramón y Cajal, 1899). While this hypothesis continues to be supported today, recent advances in electrophysiology, microscopy, and biochemical techniques have allowed additional hypotheses to be formed regarding the function of dendritic spines. These functions include, but are not limited to, (1) a means for regulating the efficacy of single synapses at the pre- and post-synaptic levels; (2) expanding the computational capacity of neurons; and (3) compartmentalization of postsynaptic biochemical signaling and gene expression (Crick, 1982; Koch & Zador, 1993; Lee *et al.*, 2012; Rall, 1978; Shepherd, 1996; Yuste & Majewska, 2001). Indeed, all of these hypothesized functions are substantiated by a considerable body of empirical studies.

One of the most widely studied aspects of structural plasticity of dendritic spines is the influence of learning and memory related processes. Early studies showed that tetanic stimulation of hippocampal afferents, with parameters similar to those used for inducing long-term potentiation (LTP), resulted in increased spine density and enlarged spine heads and necks in granule cells of the dentate gyrus (Chang & Greenough, 1984; Desmond & Levy, 1986; Fifkova & Anderson, 1981; Fifkova & Van Harreveld, 1977; Van Harreveld & Fifkova, 1975). Since dendritic spine head diameter and volume have been positively correlated with synaptic strength (De Roo *et al.*, 2008a; Sala & Segal, 2014), it would seem reasonable to conclude that these morphological changes in dendritic spines are anatomical substrates of increased synaptic efficacy. Similarly, increases in dendritic spine density in the dentate gyrus have been observed following optogenetic activation of entorhinal inputs to these cells, paralleled by increases in membrane capacitance (Ryan *et al.*, 2015). On the other hand, chemically induced synaptic de-potentiation (e.g., LTD) can cause spine retraction and shrinkage (Nagerl *et al.*, 2004; Oh *et al.*, 2013; Wang *et al.*, 2007; Zhou *et al.*, 2004). It should be noted, however, that LTP- or LTD-induced changes in spine dynamics

are not necessarily uniform within a specific brain region, population of neurons, or even along the length of a single dendritic shaft, but rather appeared clustered to specific dendritic segments (Bosch & Hayashi, 2012; De Roo *et al.*, 2008a; Fortin *et al.*, 2012; Yuste & Bonhoeffer, 2001).

The ability of LTP to induce increases in spine density raises some interesting questions. For example, does LTP actually induce the de novo growth of dendritic spines (spinogenesis), or merely enlarge existing spines? Are these changes merely transient, or do they become stabilized and mature to establish new synaptic connections? The answers to these questions appear to be in the affirmative. It is now clear that de novo spinogenesis occurs under conditions of synaptic potentiation both in vitro (Engert & Bonhoeffer, 1999; Maletic-Savatic *et al.*, 1999; Toni *et al.*, 1999) and in vivo (Hayashi-Takagi *et al.*, 2015; Holtmaat *et al.*, 2005; Kopec *et al.*, 2006; Matsuzaki *et al.*, 2004; Ryan *et al.*, 2015; Trachtenberg *et al.*, 2002; Yang *et al.*, 2009). Glutamatergic signaling appears to be critical for this phenomenon (Fischer *et al.*, 2000; Kwon & Sabatini, 2011; Mattison *et al.*, 2014; Richards *et al.*, 2005; Ultanir *et al.*, 2007), although other neurotransmitter such as dopamine also likely play a role (Yagishita *et al.*, 2014). Ultrastructural studies have shown that at least some of these newly formed spines are capable of becoming mature and stabilized to form intact synapses, albeit hours to days following de novo spine growth (Knott *et al.*, 2006; Nagerl *et al.*, 2007; Toni *et al.*, 1999).

Another interesting question raised by these observations is whether dendritic spine growth, plasticity, or even elimination occurs on a regular basis in the absence of significant synaptic input. This appears to be the case especially during brain development, where spine turnover (protrusion, maturation, and elimination) occurs in as many as 10–15% of dendritic spines in a 24-hour period, whereas in adulthood this number declines to ~1–2% (Attardo *et al.*, 2015; Grutzendler *et al.*, 2002; Trachtenberg *et al.*, 2002; Zuo *et al.*, 2005). While many "stabilized" spines appear to remain plastic throughout the lifespan, only a small subset of dendritic spines actually undergo complete turnover. Interestingly, under pathological developmental conditions such as in Fragile X syndrome, the abolishment of the fragile X mental retardation protein (FMRP) impacts both the stability of spines (Cruz-Martin *et al.*, 2010; Pan *et al.*, 2010) and proper spine pruning during development, resulting in an excess of small diameter and longer length spines on pyramidal neuron apical dendrites within the cerebral cortex. For a more detailed summary of dendritic spine abnormalities associated with neurodevelopmental or other neuropsychiatric disorders, the reader is directed to several recent reviews (Glausier & Lewis, 2013; He & Portera-Cailliau, 2013; Leuner & Shors, 2013; van Spronsen & Hoogenraad, 2010). Although these observations in Fragile X syndrome highlight the potential importance of proper synaptic pruning and spine stabilization in normal brain development, care should be taken when interpreting dendritic spine function based solely on measurements such as head diameter and neck length, as these may lead to incorrect assumptions regarding spine function. Additional measurements such as receptor insertion, postsynaptic density quantification, or synaptic efficacy should be utilized, when possible, to confirm functionality of changes in spine dynamics. Finally, traditional classification of spines into different categories has recently been suggested to be obsolete, as a time lapse imaging study using high resolution stimulated emission depletion microscopy found that spines previously categorized as "stubby" are actually "mushroom"

shaped (Tonnesen *et al.*, 2014). This discrepancy is attributable to the limits of less evolved imaging technologies that measure spine neck diameter inaccurately. These findings also suggest that the proportion of putative "stubby" spines may be overestimated in earlier studies (Segal, 2016), which leads to a concern regarding the interpretation of spine function based on categorizations across different fields of research.

Molecular mechanisms of dendritic spine dynamics

The dynamic nature of dendritic spines suggests a substantial amount and sophistication of cellular machinery present locally in the dendrite that subserves the demands of synaptic homeostasis. In addition to the presence of polyribosomes for local mRNA translation in dendrites (Martin & Zukin, 2006), hundreds of different molecular entities have been identified as mediators of dendritic spine formation, structure, stability, motility, and elimination. These can be classified into various functional categories including: actin binding and cytoskeletal proteins (e.g., actin, myosin, profilin, cofilin, etc), small guanosine exchange factors and triphosphatases (GEFs and GTPases, respectively) and associated proteins (including RhoA, Kalrin-7, and Cdc42), cell surface receptors and adhesion molecules (e.g., glutamate receptors, integrins and cadherins), receptor tyrosine and other kinases (e.g., TrkB receptors, calcium/calmodulin-dependent protein kinase II, etc.), postsynaptic scaffold and adaptor proteins (e.g., Homer, Shank, PSD, etc.), microRNAs, microRNA binding proteins and transcription factors, and steroid hormones (e.g., estrogen and glucocorticoids). For an in depth discussion of the function of all of these classes of spine-associated proteins, the reader is directed to several other excellent recent reviews (Bellot *et al.*, 2014; Frankfurt & Luine, 2015; Murakoshi & Yasuda, 2012; Sala & Segal, 2014; Tada & Sheng, 2006; Uchoa *et al.*, 2014; Wang & Zhou, 2010).

The most rapid events in initiating structural plasticity in dendritic spines are changes in intracellular calcium levels (which occurs on a millisecond time scale), followed by activation of various kinases and GTPases (on the order of seconds), activation of GEFs, actin and other cytoskeletal reorganization, and insertion of various neurotransmitter receptors (on the order of minutes). Lastly, phosphorylation and nuclear translocation of various transcription factors to initiate gene expression (on the order of minutes to hours), culminates in spinogenesis or structural changes and accompanying synaptic potentiation or de-potentiation (Yasuda, 2016).

Since this review focuses on the structural alterations of dendritic spines, it is worth briefly mentioning the basic aspects of cytoskeletal dynamics. The cytoskeleton of dendritic spines is composed primarily of smaller globular (G) actin building blocks that assemble into paired and twisted filaments that constitute filamentous (F) actin. F-actin is the primary cytoskeletal component of the spine neck and latticework of the spine head (see Fig. 1A). Changes in spine morphology during cytoskeletal restructuring are thought to be accompanied by changes in AMPA receptor expression (Cingolani & Goda, 2008). During dendritic spine restructuring, F-actin can become depolymerized into G-actin by direct actin-binding molecules such as cofilin-1 and destrin. Alternatively, F-actin can be bound and either contracted or stabilized by members of the myosin family of molecular motors, such as non-muscle myosin IIB. Other actin binding protein that promote stabilization are drebrin

A, Ras, profilin, and gelsolin. Actin polymerization and de-polymerization are also influenced by proteins such as Shank, PSD, cortactin, Rac1, and various capping proteins. As a side note and as discussed below, there are recent reports of the development of a photoactivatable form of the GTPase Rac1 that allows for optogenetic induction of spine shrinkage (Hayashi-Takagi *et al.*, 2015). There is also recent evidence that microtubule-associated proteins also participate in dendritic spine dynamics, as do epigenetic phenomena (see below). For a more detailed description of actin and other proteins involved in dendritic spine morphogenesis and plasticity, see (Bellot *et al.*, 2014; Bhatt *et al.*, 2009; Bramham, 2008; Fortin *et al.*, 2012; Halpain, 2000; Hotulainen & Hoogenraad, 2010; Koleske, 2013; Lamprecht, 2014; Murakoshi & Yasuda, 2012; Sala & Segal, 2014; Shirao & Gonzalez-Billault, 2013; Tada & Sheng, 2006).

Methods for assessing dendritic spine morphology

Assessment of dendritic spine dynamics has traditionally been performed in postmortem brain tissue using various methods, most frequently using Golgi-Cox or fluorescent dye staining. However, these methods are inherently limited in their ability to reveal information about changes in dendritic spines over time and/or in conjunction with specific behaviors, in that they allow only a “snapshot” in time rather than real-time imaging of spine dynamics. In addition, they typically involve using fixed tissue to allow for between-subjects comparisons. Fortunately, technological advances in microscopy and genetic engineering have now made imaging dendritic spine dynamics in the living brain a distinct reality. However, as mentioned below, imaging spine dynamics in deep brain structures with sufficient resolution remains a challenge.

Golgi-Cox staining

The first method utilized for assessing the fine structure of cells of the nervous system was the Golgi stain. This method was developed by renowned anatomist Camillo Golgi (Golgi, 1873), utilized by Cajal in his seminal studies described earlier (Ramón y Cajal, 1888; Ramón y Cajal, 1899), and refined by Cox and other groups of investigators to produce what is now widely referred to as the modified Golgi-Cox stain (Cox, 1891; Gibb & Kolb, 1998; Glaser & Van der Loos, 1981). In this procedure, post-mortem brain tissue is cut into relatively thick (~100–300 μm) sections, and immersed in a solution containing potassium chromate and dichromate and chloride salts of heavy metals (silver or mercury) for several weeks. Following tissue dehydration, clearing, and mounting onto microscope slides, Golgi-impregnated tissue can be viewed under brightfield microscopy to reveal detailed dendritic arborizations as well as high resolution of individual dendritic spines (Fig. 2A), which can then be quantified manually or semi-automatically using various computer-based algorithms (Orlowski & Bjarkam, 2012). Advantages of the Golgi-Cox method for assessing dendritic spine dynamics are its relative low cost and resistance to fading or photobleaching over time. Often cited disadvantages of Golgi-Cox staining are the limited ability to determine the neurochemical phenotypes of impregnated neurons, overlapping and out-of-focus dendritic segments, and underestimation of spine numbers due to the two-dimensional nature of the obtained images. However, methods to minimize these disadvantages have been described, including the use of fluorescent Golgi stains (Koyama & Tohyama, 2013), confocal laser

scanning microscopy to view Golgi-impregnated neurons in reflected mode to allow for subsequent three-dimensional reconstruction of serial images (Tredici *et al.*, 1993), and introducing immunofluorescence procedures to allow for identification of labeled neurons (Pinto *et al.*, 2012; Spiga *et al.*, 2011). Despite these advances, Golgi-Cox staining yields comparatively less morphological detail than newer fluorescence-based methods.

Fluorescent dyes

Another approach to examining neuronal microarchitecture is the use of fluorescent dyes that fill the cell body and its processes to reveal the fine structure of dendritic spines. Following labeling with fluorescent dyes, serial sections of images are obtained on a confocal laser scanning microscope and processed for three-dimensional reconstruction and analysis of dendritic spine density and morphology. One such fluorescent dye is Lucifer Yellow (Belichenko & Dahlstrom, 1995) which produces robust spine labeling (Fig. 2B). A disadvantage of this dye, however, is that cells must be filled manually via intracellular injections. Yet this method allows for specific cell impregnation, and is useful in the analysis of spine morphology following electrophysiological recordings, thus permitting structure and function to be measured from the same cell rather than from cells from different experimental cohorts. Another advantage of Lucifer Yellow staining is that fewer cells within a tissue section are labeled, allowing for less interference from overlapping cells and improved imaging and analysis.

An alternative method to Lucifer Yellow is the use of carbocyanine dyes such as 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI; see Fig 2C), 3,3'-dioctadecyloxacarbocyanine (DiO), or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine (DiD). The lipophilic nature of these dyes makes them particularly suitable for the assessment of dendritic spine morphology, as they are easily incorporated into the lipid bilayer and diffuse laterally to fill the neuronal microarchitecture. Methods have been developed to inject these dyes into tissue sections via helium-pressured ballistic “gene guns”, which results in cell labeling efficiency roughly equivalent to that achieved by Golgi-Cox staining. In addition, various investigators have introduced immunohistochemical methods to identify fluorescently labeled neurons (Dunaevsky, 2013; Gan *et al.*, 2009; Gan *et al.*, 2000; O'Brien & Lummis, 2006; Seabold *et al.*, 2010; Staffend & Meisel, 2011a; Staffend & Meisel, 2011b). As with Golgi-Cox staining, computer-based algorithms can be used for semi-automatic detection and classification of dendritic spines (Mancuso *et al.*, 2013; Parekh & Ascoli, 2013; Shen *et al.*, 2008). However, like most methods employing fluorescent labels, Lucifer Yellow and carbocyanine dye are susceptible to fading and photobleaching with prolonged imaging, as well as loss of anchoring to the labeled cell, especially when stored for extended periods of time. Generally, in our experience, DiI-labeled cells can only be imaged for up to ~6 months following tissue preparation prior to the leaching of the fluorescent dye from the membranes.

Other methods for assessing dendritic spine morphology include immunohistochemical staining for markers of dendritic spines such as PSD. However, this approach is inherently limited by poor and non-uniform antibody penetration into thicker tissue sections (Mancuso *et al.*, 2013). Similar methods utilized in ultra-thin sections, such as array tomography

followed by three-dimensional reconstruction of serially collected images, have also been utilized and appear to yield improved results (Micheva *et al.*, 2010). Unfortunately, this method requires laborious sample preparation and image reconstruction methods, limiting its utility in larger data sets.

In vivo imaging of spine plasticity

All of the aforementioned methods are performed in post-mortem tissue, and thus lack the ability to assess one of the most critical aspects of dendritic spines with respect to their behavioral relevance: dynamic changes in spine number and morphology over time, particularly during specific behaviors. This limitation has only recently been overcome with advanced imaging methods involving two-photon microscopy in awake and behaving animals. In this approach, either viral vectors or transgenic animals are employed to express fluorescent proteins such as enhanced green fluorescent protein (EGFP) or yellow fluorescent protein (EYFP) under the control of a ubiquitous or cell-type specific promoter (Grutzendler & Gan, 2006; Grutzendler *et al.*, 2011; Hayashi-Takagi *et al.*, 2015; Majewska *et al.*, 2006; Matsuzaki *et al.*, 2004; Ryan *et al.*, 2015; Trachtenberg *et al.*, 2002; Yang *et al.*, 2009; Yasumatsu *et al.*, 2008). Fluorescence is then imaged *in vivo* using a thinned skull, cranial window, or microendoscopic camera connected to a two-photon microscope system (Isshiki & Okabe, 2014). A particular advantage of this approach is that not only can cellular morphology be determined in an intact living brain, but other experimental methods such as fluorescent calcium or other ionic dye indicators as well as pharmacological uncaging (e.g., glutamate) can be incorporated, as can near infrared excitation wavelengths that penetrate deeper into tissue with reduced light scattering and absorption (Holtmaat *et al.*, 2012; Murayama & Larkum, 2012; Swanger *et al.*, 2011; Yuste, 2010). A disadvantage of this approach, however, is that the quality of the image through a thinned skull, without the incorporation of other methods such as microendoscopy, degrades at distances deeper than 50 μm from the brain surface (Isshiki & Okabe, 2014).

Do changes in spine morphology have behavioral relevance?

Learning and Memory Processes

Dendritic spines receive synaptic contacts that can be altered following new experiences, and are necessary in the processes of learning and memory. The overarching hypotheses regarding the role of dendritic spines in learning and memory have changed over time, beginning with the notion that spine density correlates with learning, eventually changing to the idea that morphological changes within the spine itself are more important for new learning and memory formation than total spine number or density (van der Zee, 2015). Some have postulated that spines with small diameters and/or lengths are unstable, easily eliminated, and responsible for acquisition of memory, whereas spines with larger head diameters are more stable and necessary for long-term memory formation (Kasai *et al.*, 2010; Kasai *et al.*, 2003). In line with this notion, it has been postulated that these spines with large head diameters arise from the maturation of thinner spines with small or absent head apparatuses (Bourne & Harris, 2007).

However, there is not universal agreement on the function of dendritic spines and their morphology with respect to learning and memory, particularly when discordant results are reported in studies that have utilized similar model systems of learning and memory. For example, increases in dendritic spine density in apical and basilar dendrites of hippocampal neurons have been observed following trace eyeblink conditioning (Leuner *et al.*, 2003), while others have failed to observe such changes on apical dendrites of hippocampal neurons (Geinisman *et al.*, 2000), though this latter study did report increases in total PSD area. A subsequent study by Geinisman and colleagues found that trace conditioning induced increases in the formation of multi-synapse boutons (MSBs), in which two or more postsynaptic elements are associated with a single presynaptic element (Geinisman *et al.*, 2001). These examples illustrate that discordant reports on effects of learning on spine dynamics, even those utilizing similar learning paradigms, may be a reflection of different experimental approaches (Golgi staining vs. spine ultrastructure via electron microscopy), different dendritic domains analyzed (apical vs. apical and basilar dendrites), or even different dependent measures (spine density vs. PSD area and number of MSBs). In light of this example, it is clear why it is difficult to come to a consensus regarding cause-and-effect relationships between spine dynamics and learning and memory.

Fortunately, more recent studies utilizing *in vivo* spine imaging have increased our understanding of this complex relationship. In one such study, Yang and colleagues (Yang *et al.*, 2009) utilized EYFP-expressing mice to examine spine dynamics in the primary motor cortex following a motor (rotarod) learning task, or in the barrel cortex following sensory experience (environmental enrichment). Experience-induced de novo spine formation was observed within two 2 days of task performance, and a small percentage of these newly formed spines persisted for months, while other spines were eliminated by subsequent exposure to novel experiences. Interestingly, in this study, control experiments demonstrated that spinogenesis was not a result of non-specific motor activity or sensory experience. These findings strongly suggest that sensory experiences result in long-lasting synaptic alterations that underlie persistent sensory or motor memories. However, since spine dynamics were not directly manipulated in this study, it is difficult to determine precisely whether spinogenesis and its persistence were induced by environmental input, or were a result of specific behavior output.

A more recent study (Hayashi-Takagi *et al.*, 2015) used direct experimental manipulations of spine dynamics to examine their role in learning and memory processes. In this study, the authors developed a photoactivatable form of the GTPase Rac1, which under conditions of prolonged activation results in spine shrinkage. The expression of this photoactivatable form of Rac1 (PaRac1) was targeted to recently activated synapses (AS-PaRac1) via use of the promoter for the activity-dependent immediate early gene Arc/Arg3.1. Using this technology, the authors demonstrated that motor learning in a rotarod task resulted in de novo spinogenesis and enlargement of existing spines in the primary motor cortex. Upon photoactivation of AS-PaRac1, which resulted in shrinkage of previously potentiated spines (estimated to be ~410,000 spines localized to ~4,700 neurons), the authors were able to disrupt (“erase”) learning in the rotarod task during a critical time period in the experiment. These highly novel findings suggest that not only are dendritic spines potentiated by learning experiences, but their disruption can adversely impact motor learning ability. Thus, changes

in dendritic spines can not only result from learning experiences, but also regulate behavioral output that is indicative of learning.

During early life development, when neuronal plasticity is arguably at its peak, it is thought that dendritic spines and synaptic contacts are formed or pruned via mechanisms that rely on an interplay between learning-based environmental input and behavioral output. Learning-related patterns of neuronal activity induce LTP and lead to stabilization and localization of spines (De Roo *et al.*, 2008a; De Roo *et al.*, 2008b). In very young animals (postnatal day 15 rats), a time of immense synaptogenesis, it has been demonstrated that theta burst stimulation (TBS)-induced LTP activates existing synapses and leads to the formation of new spines, illustrating robust plasticity of neuronal networks during development (Watson *et al.*, 2016). Interestingly, hippocampal synaptic growth and pruning during the early life postnatal period induces remodeling of neuronal networks which is regulated by activity. In one recent study, the impact of synaptic adhesion molecules on synaptic maturation and survival was assessed in 7 to 9 week old mice (Krzisch *et al.*, 2016). In this study, it was demonstrated that overexpression of the synaptic adhesion molecule SynCAM1 increased maturation of dendritic spines in the hippocampus, whereas overexpression of another adhesion molecule Neuroligin-1B increased spine density in the hippocampus. In contrast, overexpression of Neuroligin-2A increased spine density and GABAergic innervation, which resulted in significantly higher levels of neuronal survival. In addition, mice overexpressing Neuroligin-2A specifically in new neurons, showed impaired spatial learning performance in the Morris water maze. Although higher levels of spine survival appear to be linked to impaired performance in this particular task, suggesting that overall spine density may not be the most important neurobiological substrate for enhancing cognitive ability, it should be noted that the role of each adhesion molecule was assessed by cell-autonomous overexpression which may have led to other compensatory mechanisms and/or neuronal organization that resulted in altered cognitive performance. Regardless, these results show a link between synaptic maturation, increased neuronal survival during a period of high levels of synaptic reorganization, and learning processes.

Fear conditioning is a form of associative learning in which subjects exposed to an aversive stimulus associate a neutral stimulus with defense responses, after which this neutral stimulus thus becomes a conditioned stimulus which elicits a conditioned response (Blair *et al.*, 2001). In the preclinical literature, the neurobiological mechanisms of fear conditioning have been studied in detail. Alterations in spine morphology in the amygdala have been shown to occur following fear conditioning, such that profilin, an actin polymerization regulatory protein, impregnates dendritic spines which then undergo post-synaptic density enlargement (Lamprecht *et al.*, 2006). Interestingly, one study found that recruitment of calcium-permeable (GluA2-lacking) AMPA receptors was specific to mushroom-type spines in hippocampal CA1 neurons after fear conditioning (Matsuo *et al.*, 2008). Specifically, transgenic mice showed enhanced GFP-GluA1 recruitment into spines with large head diameters 24 hours after fear conditioning, which then reversed by 72 hours post-conditioning. This reversal may indicate replacement of AMPA receptor subunits to others such as GluA2, which are more stable and calcium impermeable. Here, dynamic changes in spine morphology seem to have functional relevance and occur as a result of a learning mechanism. Alterations in spine morphology have also been found in the lateral amygdala

(LA) in a fear conditioning paradigm (Radley *et al.*, 2006a), as conditioned and unconditioned stimuli activate neurons within this region, and damage to the LA inhibits acquisition of fear conditioning (Quirk *et al.*, 1997; Romanski *et al.*, 1993; Schafe *et al.*, 2001). Specifically, fear conditioning increased spine density measured via spinophilin-immunoreactivity in spine heads (Radley *et al.*, 2006a). These studies suggest that processes of learning and memory induce alterations in spine morphology, specifically with respect to changes underlying acquisition of Pavlovian associations.

In another study, Ryan and colleagues found that both optogenetic activation of entorhinal cortex as well as contextual fear conditioning increased dendritic spine densities in putative "engram" cells in the dentate gyrus, which encode context-dependent memories (Ryan *et al.*, 2015). The increases in spine density were paralleled by increases in membrane capacitance, and were abolished by administration of anisomycin, indicating the necessity of de novo protein synthesis for this phenomenon to occur. These findings were among the first to identify structural spine plasticity in optogenetically and behaviorally defined memory engram cells, and that structural plasticity in these specific cell populations was required for memory to occur. These findings support the notion that contextual conditioning drives spine changes in specific cell populations, rather than being induced by the behavior itself.

Finally, there is a wealth of evidence that maladaptive spine dynamics may contribute to, and/or be a result of, brain disease processes. In the case of Alzheimer's disease (AD), evidence suggests that the loss of dendritic spines is one of the first structural changes that occurs in neurons of AD patients. This dendritic spine loss is directly correlated with the loss of synaptic function, and is likely a result of β-amyloid accumulation, tau hyperphosphorylation, excitotoxicity, neuroinflammation, dysfunction of intracellular cytoskeletal regulators, or a combination of these factors (Dorostkar *et al.*, 2015; Fiala *et al.*, 2002; Knobloch & Mansuy, 2008). Most research efforts on slowing the progress of AD have focused on amyloid clearance, neuroprotection, and delay of cognitive decline. However, new evidence suggests that promoting synaptogenesis and/or spinogenesis, perhaps via epigenetic mechanisms, may be of potential benefit. This was demonstrated recently in mouse models if AD, where histone deacetylase inhibitors were found to promote synaptogenesis, dendritic sprouting, and regaining of long-term memories especially when combined environmental enrichment (Fischer *et al.*, 2007). Thus, both intrinsic and extrinsic factors affecting spine dynamics may provide more effective avenues for treating age-related neurodegenerative diseases.

Addiction

Exposure to drugs of abuse such as nicotine (Brown & Kolb, 2001), cocaine and amphetamines (Robinson & Kolb, 1999a), alcohol (Carpenter-Hyland & Chandler, 2006; Spiga *et al.*, 2014a; Spiga *et al.*, 2014b), cannabis (Spiga *et al.*, 2010) and opiates (Robinson & Kolb, 1999b) can alter dendritic spine morphology (such as spine density or size) in various brain regions including the prefrontal cortex (PFC) and NA (Russo *et al.*, 2010). As a specific example, exposure to cocaine leads to differential changes in NA spine dynamics when measured proximally or distally from the cell soma, illustrating that spine morphology varies according to location on the dendrite (Dumitriu *et al.*, 2012). In many of these studies,

animals received experimenter-delivered injections of various drugs of abuse, and dendritic spine structure was characterized after a period of withdrawal, indicating drug-induced alterations in constitutive spine morphology. In addition to experimenter-delivered exposure, however, dendritic spines have been shown to be important in behaviors motivated by drugs of abuse. The development of addiction and relapse vulnerability involves complex learning and memory processes, as well as associations with constellations of environmental stimuli that become powerful cues in initiating craving and relapse behaviors (Beckmann *et al.*, 2011; Gipson *et al.*, 2014; Marchant *et al.*, 2015). Addiction is a complex, progressing disorder involving learning and memory processes (Hyman *et al.*, 2006; Nestler, 2001). Animal studies have long established that previously neutral environmental stimuli consistently and contiguously paired with drugs of abuse begin to take on an important associative meaning. These cues enhance drug self-administration thus illustrating the cue-dependency of drug use (Caggiula *et al.*, 2001; Schenk & Partridge, 2001). Once these stimuli become conditioned or discriminative stimuli predicting drug presentation, they consistently produce drug-seeking in animal models of cue-induced relapse (Bossert *et al.*, 2013; Crombag *et al.*, 2008; See, 2002; Shaham *et al.*, 2003) and can also elicit structural changes in dendritic spines within the nucleus accumbens (NA). Specifically, exposure to an amphetamine-paired environmental context elicited an increase in spine density and frequency in activated (c-fos positive) cells in the NA compared to non-paired controls (Singer *et al.*, 2016).

Interestingly, exposure to different drug classes can induce differential changes in spine morphology (Dumitriu *et al.*, 2012; Gipson *et al.*, 2014; Gipson *et al.*, 2013a; Shen *et al.*, 2011), however there are also overlapping patterns of change that occur as well (Mulholland *et al.*, 2016; Scofield *et al.*, 2016). Drug-induced spine changes are also highly dynamic and governed by glutamatergic and dopaminergic input from mesocorticolimbic circuit afferents (Shen *et al.*, 2014; Yagishita *et al.*, 2014). For example, Shen and colleagues (Shen *et al.*, 2009) demonstrated that acute passive cocaine exposure increases NA spine density accompanied by increases in excitatory transmission as well as levels of proteins involved in actin regulation, protein catabolism, and glutamatergic transmission on the order of minutes to hours. These changes appear to return to pre-drug levels by approximately by approximately 24 hours following cocaine exposure. However, in animals with a history or repeated cocaine exposure, such changes in protein levels follow a different time course and yield bi-direction changes in spine head diameters, with enlarged spine heads followed by reductions in spine head diameters (Shen *et al.*, 2009). These findings suggest a considerable degree of metaplasticity of dendritic spine dynamics in the NA that depend on the prior history of drug exposure. While such drug-induced changes in NA dynamics are largely attributable to alterations in excitatory glutamatergic transmission, recent optogenetic studies have revealed that synaptically released dopamine also contributes to NA spine dynamics (specifically, spine enlargement), although only within a narrow time window that follows glutamate-induced changes (Yagishita *et al.*, 2014).

These results highlight important mechanisms underlying spine dynamics, specifically the role of dopaminergic innervation of NA MSNs. Older studies revealed that dopamine depletion via 6-hydroxydopamine lesions of the NAcore or shell induced region-specific structural changes such as reduced dendritic length and spine density, and increased

dendritic tortuosities (Meredith et al., 1995). More recent findings indicate that subpopulations of NA MSNs, specifically those expressing dopamine D1 or D2 receptors, are differentially altered during cocaine-seeking (Heinsbroek *et al.*, 2014). Thus, the consequential changes in NA spines following exposure to drugs of abuse likely involve a complex and time-dependent interplay between glutamatergic and dopaminergic transmission. On an anatomical level, individual dendritic spines of NA MSNs often receive converging inputs, with glutamatergic afferents primarily forming synaptic contacts on spine heads and dopaminergic inputs forming synapses on spine necks (Sesack *et al.*, 2003). It is currently unclear what alterations in this convergent synaptic architecture occur across different classes of abused drugs, drug history, and phases of the addiction cycle, and subsequently influence addiction-related behaviors.

Dendritic spine dynamics may also be influences by changes in dopamine and glutamatergic transmission that persist into withdrawal, such as hyperglutamatergic transmission during alcohol withdrawal (Holmes *et al.*, 2013), and hypoactivity of both glutamatergic and dopaminergic transmission following chronic exposure to cocaine (Baker *et al.*, 2003; Diana, 2011; Weiss *et al.*, 1992). In turn, glutamatergic and dopaminergic transmission in the NA become dysregulated during the reinstatement of cocaine-seeking behavior (McFarland *et al.*, 2003; Neisewander *et al.*, 1996). These drug-dependent fluctuations in both dopamine and glutamate signaling likely induce numerous transient or lasting changes in NA spine dynamics.

With regard to other drugs of abuse such as alcohol, proteomic analyses have revealed that changes in PSD-associated proteins are associated with alcohol-induced changes in synaptic morphology within the NA (Uys *et al.*, 2016). This study found that exposure to and withdrawal from chronic intermittent ethanol (CIE) induced an increase in NA spine density, dendritic shaft diameter, as well as spine head diameter. Additionally, this study found significant alterations in the expression of a multitude of scaffolding proteins, glycoproteins, kinases, neuropeptides, among many others within the NA after CIE. While alterations in spine dynamics occurred following exposure to ethanol, it is unclear if these changes are important for or relevant to ethanol-related motivated behavior.

Various investigators have postulated that drug-induced spine changes create a neurobiological environment that is more plastic compared to drug-naïve conditions, and may lead to aberrant motivation to seek the drug (Mulholland & Chandler, 2007; Nyberg, 2014; Spiga *et al.*, 2014a; Young *et al.*, 2014; Young *et al.*, 2016). While most studies supporting this theory have examined changes in spine dynamics in mesocorticolimbic circuits, an eloquent series of studies by Young and colleagues have recently expanded this to include the amygdala, a key regulator of the emotional and associative components of addiction. Utilizing place conditioning procedures, it was demonstrated that methamphetamine increased the plasticity of spines in the amygdala, and that inhibition of actin polymerization with latrunculin A in this region (which inhibits actin depolymerization and thus promotes spine stability) disrupted the retrieval of methamphetamine-associated contextual memories (Young *et al.*, 2014). These effects appeared to be specific methamphetamine, as they were not observed following contextual conditioning with saline, food, or footshock. The ability of spine stabilization to inhibit methamphetamine-associated

contextual memory were also independent of the strength of contextual training, and were observed following context-induced reinstatement in an operant paradigm. Unfortunately, due to its ubiquitous expression in numerous cell types throughout the brain and body, inhibition of actin polymerization with latrunculin A may not selectively modulate dendritic spines *per se*. To address this issue, Young and colleagues also demonstrated that intra-amygdala infusions of Blebbistatin, an inhibitor of the synaptically enriched actin polymerization promoter non-muscle myosin IIB, produces similar inhibitory effects on methamphetamine-induced contextual memories (Young *et al.*, 2014; Young *et al.*, 2016). Finally, Young *et al.* showed that systemic administration of Blebbistatin selectively disrupted the storage methamphetamine- but not fear-associated contextual memories, and also reversed the ability of methamphetamine-associated memories to increase spine density in the amygdala (Young *et al.*, 2016). Thus, specific targeting of synaptic actin dynamics may represent a novel approach to treating pathological memories associated with abused drugs.

Within dendritic spines, cell surface proteins such as AMPA and NMDA receptors are inserted or internalized regularly (Kopec & Malinow, 2006). Following extended periods of withdrawal from cocaine self-administration, craving for cocaine can “incubate”, and this has been associated with an increase in calcium-permeable (GluA2-lacking) AMPA receptors (Conrad *et al.*, 2008). Following extinction of cocaine self-administration, baseline increases in spine head diameter in the NA have been found (Gipson *et al.*, 2013a; Shen *et al.*, 2009), supporting the notion that cocaine causes NA spines to exist in a relatively potentiated state to increase surface area for protein insertion. It should be noted, however, that withdrawal from heroin self-administration results in an LTD-like state of NA core neurons, in which spines show reductions in head diameter compared to those of drug-naïve animals (Shen *et al.*, 2011). In addition, following heroin self-administration, there is an upregulation of NMDA receptors that has functional relevance in heroin-seeking behavior (Shen *et al.*, 2011). Interestingly, a hypothesis has recently emerged that the brain returns to more developmental, immature-like conditions following chronic exposure to drugs of abuse (Dong & Nestler, 2014). In this “neural rejuvenation” hypothesis, exposure to drugs of abuse lead to alterations in brain physiology that are comparable to developmental conditions at the molecular, cellular, and circuitry levels. In doing so, drugs of abuse open a window of plasticity that is not found in the adult brain, which may underlie habitual and pathological drug seeking behaviors. This notion is supported by the associated increase of calcium-permeable AMPA receptors in the NA as well as expression of the GluN2B subunit of NMDA receptors and silent synapses after withdrawal from different drugs of abuse (Conrad *et al.*, 2008; Dong, 2015; Gipson *et al.*, 2013b; Lee & Dong, 2011; Shen *et al.*, 2011). Interestingly, silent synapses, which lack AMPA receptors and thus are unable to remove the magnesium block of NMDA receptors, exist during development as well as after cocaine exposure. Although the behavioral relevance of these silent synapses in addiction processes is still not fully characterized, they have been associated with incubation of cocaine craving (Lee *et al.*, 2013). As well, it was recently found that re-silencing these synapses via optogenetic removal of calcium-permeable AMPA receptors, allowing environmental enrichment (EE; previously found to have anti-relapse beneficial effects (Thiel *et al.*, 2009))

to lead to insertion of calcium-impermeable AMPA receptors which inhibit relapse-like behaviors (Ma *et al.*, 2016).

In addition to baseline changes in dendritic spines after drug exposure and periods of withdrawal, drug-seeking behavior has been associated with altered dendritic spine morphology (Gipson *et al.*, 2013a; Gipson *et al.*, 2013b; Shen *et al.*, 2014; Stefanik *et al.*, 2016). Specifically, conditioned stimuli associated with self-administration of drugs of abuse such as cocaine and nicotine can elicit motivated behavior to seek the drug. These stimuli are also associated with rapid, transient increases in spine head diameters of NA core medium spiny neurons (MSNs). In the reinstatement model of drug relapse, exposure to drug-associated cues was associated with the rapid and transient growth of dendritic spines (within 15 min), with no alterations in dendritic spine density during this timeframe. Importantly, these changes in spines (as well alterations in the ratio of AMPA to NMDA receptor-mediated excitatory post-synaptic currents, a functional correlate of synaptic plasticity) were positively correlated with magnitude of cue reinstated drug seeking (Gipson *et al.*, 2013a). These rapid, transient changes in spine dynamics were found to be specific to drug seeking behavior rather than behavior motivated by food. However, since some overlap has been found between the neurobiological substrates of obesity and drug abuse (Brown *et al.*, 2015; Kenny, 2011a; Kenny, 2011b), it is thus possible that in a model of diet-induced obesity, synaptic plasticity may occur during food seeking behavior similar to that induced by drugs of abuse. It is also possible that in drug naïve animals, food seeking behavior (particularly in food restricted animals) induces rapid, transient plasticity on a much shorter timescale, thus going undetected at the 15 min time point assessed for drug-induced changes. In the drug-exposed system, the rapid, transient increase in dendritic spine head diameter found after 15 min of cue-induced cocaine seeking occurred specifically in dopamine D1-expressing (but not D2-expressing) MSNs (Heinsbroek *et al.*, 2014). These results illustrate cell-type specificity of reinstatement-associated spine dynamics. It still remains unclear, however, if drugs of abuse lead to these changes which then impact behavior, or if behavior itself (drug-seeking) drives these changes.

Stress, Depression, and Aging

Acute or repeated exposure to stressors such as restraint or social defeat can remodel dendritic spine morphology in multiple brain regions. Although different types of stressors likely impact dendritic spine dynamics in different ways, preclinical modeling of stress has evolved in an attempt to capture criterion, construct, and translational validity of human stress exposure. Stress exposure can impact numerous neurobiological systems, all of which can vary based on a number of factors: the type and duration of stressor exposure (Capriles & Cancela, 1999), developmental stage of the organism (Biala *et al.*, 2011; Romeo *et al.*, 2006; Romeo & McEwen, 2006), genetic and epigenetic factors (McEwen *et al.*, 2012; McEwen *et al.*, 2016; Pillai *et al.*, 2012), sex and hormones (McLaughlin *et al.*, 2009; McLaughlin *et al.*, 2005; Weinstock, 2011), anxiety levels (Adamec *et al.*, 2012; McEwen *et al.*, 2012), among others (Leuner & Shors, 2013). Because stress is a complex phenomenon and involves both external stimuli and internal physiological responses to them, experimental models of stress are highly varied. Commonly used models include restraint (Esparza *et al.*, 2012; Platt & Stone, 1982), footshock (Long & Fanselow, 2012), or social

defeat stress (Covington *et al.*, 2005) among others such as chronic variable or unpredictable stress (Lopes *et al.*, 2016). Although the translational validity of these models remains an important topic, these are typically intended to model aspects of anxiety, post-traumatic stress disorder (PTSD), and/or depression. Interestingly, acute restraint stress has been postulated to induce some neurobiological and behavioral alterations akin to that induced by stress disorders such as PTSD in humans. Specifically, one study found increased NA core dendritic spine density 3 weeks after one 2-hr session of restraint stress in rats (Garcia-Keller *et al.*, 2015). This acute stress exposure also increased acquisition of cocaine self-administration, suggesting stress-induced vulnerability to addiction-related behaviors. Stress and cocaine have been shown to differentially alter cell-type specific spine morphology when measured with two-photon laser scanning microscopy (Khibnik *et al.*, 2015). Specifically, animals given repeated cocaine injections showed decreased synaptic strength in spines localized to D1-expressing MSNs and increased synaptic responses in D2-expressing MSNs. In contrast, however, this study revealed that animals exposed to chronic social defeat stress, showed increased synaptic strength in D1-expressing and reduced synaptic strength in D2-expressing MSNs. These opposing results illustrate the importance of not only duration of stress exposure but also cell-type specificity in the effects of stress exposure on spine dynamics.

While the mechanisms underlying the impact of acute or chronic stress on spine dynamics is currently a subject of intense study, some important advancements has been made. As noted above, it remains an issue in the addiction field to determine whether spine changes occur prior to drug-induced behaviors, leading to increased relapse vulnerability, or if these changes occur as a result of motivated behavior. A significant issue with solving this question is that teasing apart spine dynamics from behavior is difficult within the context of addiction as a complex biobehavioral disorder involving learning components as well as circuitry, pharmacological, and multi-level neurobiological mechanisms that lead to drug-seeking and intake. In the stress literature, however, alterations in spine dynamics has been studied independently of actual stress exposure by passive administration of corticosterone, the predominant stress steroid released in rodents following exposure to a stressor. Passive administration of corticosterone itself can induce dendritic spine remodeling in the hippocampus by impacting cytoskeletal protein expression (Cereseto *et al.*, 2006). Further, it has been demonstrated that repeated exposure to corticosterone over the course of 3 weeks induced remodeling of dendritic spines in the medial prefrontal cortex (mPFC) (Anderson *et al.*, 2016; Gourley *et al.*, 2013; Radley *et al.*, 2008). Specifically, animals chronically exposed to corticosterone showed a decrease in spine volume that persisted for 3 weeks following cessation of treatment (although these effects were dependent on brain region; see (Gourley *et al.*, 2013)). These results demonstrate that spine remodeling that results from a biological response associated with stress (elevated corticosterone levels) that can be separated from the stress exposure itself. As of yet, however, it has been difficult to examine addiction-related changes in spine dynamics in isolation from the behavioral, learning and memory, and/or environmental components of the addiction process. The results reviewed above suggest that some aspects of stress-induced dendritic remodeling can be simulated by chronic, steady exposure to corticosterone. This has implications for determining not only mechanisms underlying some aspects of stress-induced alterations in spine dynamics, but

also if spine remodeling is sufficient and/or necessary to occur before or after a behavioral stress response. Although this is an important advancement in our understanding of the influence of stress-related hormones on spine dynamics, it is unclear if such spine alterations actually mimic those that occur in response to an actual stressor, and if such approaches have translational validity for examining the neurobiological substrates of stress disorders such as PTSD. As noted above, stress is complex and leads to various biological responses. Thus, mimicking the biological response of elevated corticosterone in rodents may only reveal some of the processes that occur in response to a more complex stress experience.

Some preclinical stress models (e.g., chronic restraint, social defeat) have been used to model various aspects of depression (Qiao *et al.*, 2016; Shimamoto *et al.*, 2011). Dendritic atrophy has been found both in the PFC and hippocampus following chronic restraint stress (Conrad, 2006; Conrad *et al.*, 1999; Magarinos & McEwen, 1995; McEwen *et al.*, 1997; Radley *et al.*, 2006b; Stewart *et al.*, 2005), paralleled by reductions in dendritic spine density (reviewed in (Duman & Duman, 2015; Licznerski & Duman, 2013). These findings are similar to those from postmortem examinations of brain tissue from depressed human subjects, where reduced spine density, overall synapse number on dendritic spines, as well as reduced synaptic protein levels have been observed in the dorsolateral prefrontal cortex and hippocampus, and opposite changes observed in the amygdala (Kang *et al.*, 2012; Licznerski & Duman, 2013). Thus, spine and synapse remodeling has been proposed to be a novel approach to treating depressive disorders (Duman & Duman, 2015; Licznerski & Duman, 2013). Other brain regions where spine morphology has been shown to be affected by chronic stress is the NA, where increases in the density of shorter spines with smaller PSDs have been observed, paralleled by increases in functional glutamatergic synapses (Christoffel *et al.*, 2011). It is unclear, however, if similar alterations exist in the NA of depressed human subjects.

Spine loss is also associated with brain aging, and there is some evidence of overlap between the mechanisms of spine loss in neurodegenerative diseases such as AD and those induced by chronic stress, namely a critical role for tau proteins (Lopes *et al.*, 2016; Sotiropoulos & Sousa, 2016). Thus, models of chronic stress may have cross-validity for other models of neurodegenerative diseases with significant cognitive impairment. Changes in dendritic spine morphology parallels age-related cognitive decline (Morrison & Hof, 1997), as neural pathways vulnerable in aging are comprised of glutamatergic PFC and hippocampal spiny pyramidal neurons (Dumitriu *et al.*, 2010; Morrison & Baxter, 2012; von Bohlen und Halbach *et al.*, 2006). In line with these findings, aged female rats show a decline in object recognition memory as well as PFC spine density (Wallace *et al.*, 2007). Formation of new dendritic spines occurs in clusters around activated synapses (De Roo *et al.*, 2008b) both in cell culture as well as *in vivo* following motoric learning (Fu *et al.*, 2012). Since clusters of dendritic spines that are important in fostering synaptic strength appear to be impacted by age-related decline, it is important to note that a recent study found that administration of riluzole, an activator of glutamate uptake that promotes synaptic over extrasynaptic NMDA receptor, rescued these clusters (Pereira *et al.*, 2014). Riluzole-treated animals also showed a positive correlation between memory-related behavior and density of spines on apical dendrites in the CA1 region of the hippocampus, suggesting that rescuing dendritic spine density in this area is important in enhancing memory processes.

Discussion and Conclusions

Dendritic spine plasticity appears to be critical not only for normal brain development, function, and aging, but also underlies the pathophysiology of several psychiatric, neurodevelopmental, and neurodegenerative disorders. Although newer advanced techniques have been developed to measure spine changes in three dimensions at high resolution, significant issues remain with measuring spine dynamics from one or more brain structures during behavior. Measuring dendritic spines in real time requires highly advanced imaging techniques which are more difficult when examining deep structures *in vivo*. In addition, much research has relied on categorization of the structure of dendritic spines based on distinct morphologies, yet often these categorizations are not accompanied by evidence for functional divergence between different spine types. The lack of analysis of both structure *and* function within a study may lead to inaccurate conclusions regarding the importance of dendritic spine dynamics in behaviors and/or cognitive processes. As well, given recent evidence that accurate spine categorization may suffer from limitations of currently and frequently used imaging tools (Tonnesen *et al.*, 2014), we suggest that categorizations based on structure alone be avoided without functional correlates. Rather, the distributions of various spine characteristics (e.g., neck width and length, head diameter) be analyzed and compared between groups (or within-subject when possible).

Importantly, it should be mentioned that the function of dendritic spines has been examined in various studies using the correlate of AMPA to NMDA post-synaptic excitatory current ratios recorded using whole cell patch clamp electrophysiology from the soma (Gipson *et al.*, 2013a; Shen *et al.*, 2014; Stefanik *et al.*, 2015). Although this yields an important amount of information and is a window into the potential summation of spine function across an entire neuron, it is imperative to differentiate whole cell function from individual spine function, especially when examining nuanced changes in spine structural characteristics and categorization. Technological advances are needed to image individual spine changes *in vivo* in awake, unrestrained, and behaving animals in deep structures to better characterize the underlying functional impact of dendritic spine dynamics on behavior.

Finally, the original question posed at the beginning of this review remains: do changes in dendritic spines lead to changes in behavior, or does behavior itself lead to changes in spine morphology? In this review, we have attempted to highlight potential answers to this question within the existing literature, depending on the type of environmental input or behavioral output under examination. It appears that in the case of stress, some biological responses such as alterations in spine dynamics can be mimicked using pharmacological manipulation (e.g., corticosterone administration). This contribution is important, as it opens the possibility that spine dynamics occur in *response* to a biological process associated with exposure to an environmental stimulus. However, interpretations of such results may reflect only a small part of larger and more complex stress responses. With regard to learning and memory and drug addiction (specifically, relapse-related behaviors), the ability to separate spine dynamics from behavior remains difficult and elusive. Thus, without more advanced and accessible *in vivo* imaging techniques, this may not be possible.

Along these lines, we argue that there is a great need to develop an experimental toolbox to selectively modulate dendrite spine dynamics, in order to observe direct effects on behavior. For example, several groups of investigators have utilized localized infusions of latrunculin A, an actin polymerization inhibitor (Coue *et al.*, 1987), to promote spine stabilization and investigate the effects of spine stabilization on drug-related behavior (Esparza *et al.*, 2012; Toda *et al.*, 2010; Toda *et al.*, 2006; Young *et al.*, 2014). However, such approaches lack cell-type specificity, and would potentially affect all local cytoskeletal cycling. With this limitation, other investigators have explored targeting more upstream regulators of actin cycling, such as non-muscle myosin IIB (Young *et al.*, 2016). These avenues of research, as well as the development of other approaches (e.g., optogenetic activation of GTPases or other modulators of spine morphology, chemogenetic, chemical uncaging), are critical for advancing our knowledge of the functional contributions of dendritic spine dynamics on behavior, and vice versa.

The most likely answer to our overall question is that dendritic spine dynamics are both a cause *and* consequence of behavior. We argue that it is most likely that environmental stimuli lead to constitutive changes in dendritic spine morphology, and these alterations create a neural environment in which an organism is rendered more likely to engage in certain behaviors. However, non-specific pharmacological manipulation of spine dynamics will likely not be a viable therapeutic option for treatment of brain disorders with underlying dendritic spine pathologies. Such an approach would also affect normal synaptic plasticity and likely lead to various adverse effects on normal cognition and learning and memory. A more viable option would be modulating dendritic spine dynamics via environmental influences, such as environmental enrichment (Gipson *et al.*, 2011; Rojas *et al.*, 2013; Thiel *et al.*, 2012; Turner *et al.*, 2003; Yang *et al.*, 2009) or approaches directed at engaging specific neural circuits to strengthen synapses that promote cognitive health or weaken synapses that promote maladaptive behaviors.

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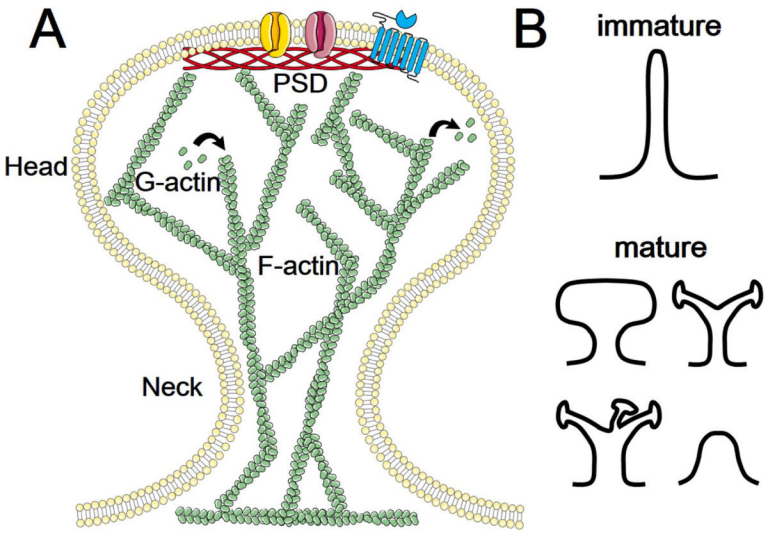

Figure 1.

(A) Illustration of a dendritic spine. Spines typically have neck and head compartments, whose structure is largely built on an actin cytoskeleton. G-actin polymerizes into F-actin to build the primary cytoskeletal structure, and thus remodels the spine during synaptic plasticity. This process continually reshapes brain circuitry and synaptic connections. At the PSD, membrane-bound proteins are continually inserted or retracted from the membrane, including ionotropic and metabotropic receptors. **(B)** Dendritic spines are often categorized into different subtypes, some of which are thought to be immature and filopodium-like, and others are thought to be mature with increased head diameters relative to that of the neck, as well as bifurcated and/or multi-branched morphologies. See text for a discussion of the limitations of spine categorization based on overall morphology. Abbreviations: PSD = post-synaptic density; G-actin = globular (monomeric) actin, F-actin = filamentous actin.

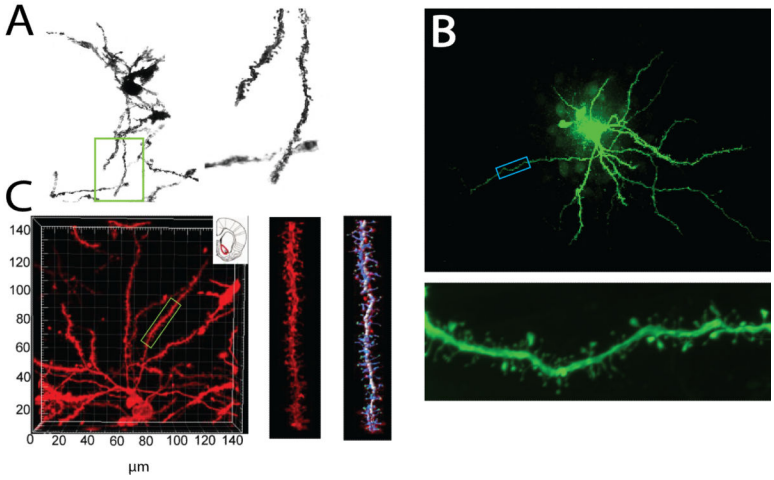

Figure 2.

(A) Image of a Golgi-Cox stained striatal medium spiny neuron and a higher magnification of the selected area showing dendritic spines. **(B)** A high resolution three-dimensional image of a Lucifer-Yellow filled neuron in the NA. The magnification of the boxed region is 63×, and a 3-D segment with clearly defined spines is reconstructed and analyzed. **(C)** Example of a DiI-filled MSN in the NA. The boxed region is magnified to 63× and analyzed using Imaris software (Bitplane).