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Review

Roles for neuronal and glial autophagy in synaptic pruning during development

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ABSTRACT

The dendritic protrusions known as spines represent the primary postsynaptic location for excitatory synapses. Dendritic spines are critical for many synaptic functions, and their formation, modification, and turnover are thought to be important for mechanisms of learning and memory. At many excitatory synapses, dendritic spines form during the early postnatal period, and while many spines are likely being formed and removed throughout life, the net number are often gradually "pruned" during adolescence to reach a stable level in the adult. In neurodevelopmental disorders, spine pruning is disrupted, emphasizing the importance of understanding its governing processes. Autophagy, a process through which cytosolic components and organelles are degraded, has recently been shown to control spine pruning in the mouse cortex, but the mechanisms through which autophagy acts remain obscure. Here, we draw on three widely studied prototypical synaptic pruning events to focus on two governing principles of spine pruning: *1) activity-dependent synaptic competition* and *2) non-neuronal contributions*. We briefly review what is known about autophagy in the central nervous system and its regulation by metabolic kinases. We propose a model in which autophagy in both neurons and non-neuronal cells contributes to spine pruning, and how other processes that regulate spine pruning could intersect with autophagy. We further outline future research directions to address outstanding questions on the role of autophagy in synaptic pruning.

1. Introduction

Neuronal networks are composed of balanced connections between excitatory, inhibitory, and modulatory neurons. These balances are disrupted in a range of neurodevelopmental and neurodegenerative diseases, including autism spectrum disorders (ASD), schizophrenia, drug abuse, Down syndrome and Alzheimer's disease. Understanding the basis of these "synaptopathies" promises deeper insight into pathophysiology of these diseases and improved therapies (Dölen and Bear, 2009).

Within the brain, excitatory glutamatergic neuronal connections often occur between presynaptic release sites located *en passant* along axons and a postsynaptic site on a dendritic shaft or spine. Mature dendritic spines are typically 0.5–5 μm long with a narrow neck and wider head, and feature a range of shapes due to variations in the size of the head and neck length (Peters and Kaiserman-Abramof, 1970). The spine shape is correlated with the synaptic strength (Matsuzaki et al., 2001), stability (Trachtenberg et al., 2002), and function, as the spine neck segregates biochemical and electrical events between the spine head, where the postsynaptic density and neurotransmitter receptors are located, and the dendritic shaft (Koch and Zador, 1993; Yuste and Denk, 1995; Yuste, 2013).

Each spine is a dynamic structure, and *in vivo*, spine shape and the presence of the spine itself can change over short (minutes) and long (days to weeks) time scales (Alvarez and Sabatini, 2007; Trachtenberg et al., 2002; Yang et al., 2009). Immature spines, known as filopodia, lack a head, are predominantly observed during synaptogenesis, and presumably develop into mature shapes in an activity-dependent manner (Vaughn, 1989; Yuste and Bonhoeffer, 2004; Ziv and Smith, 1996). This modulation of morphology has led neuroscientists over the past century to postulate that such synapses are critical to memory storage, and that changes in spine structure and synaptic strength underlie forms of learning (Dickstein et al., 2013). Thus, understanding the processes through which dendritic spines are removed and restructured should provide important insights into the formation and maintenance of memories.

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Neuronal connections begin to form prenatally and are continually refined throughout life (Huttenlocher and Dabholkar, 1997; LaMantia and Rakic, 1990; Rakic et al., 1986). From mid-embryonic development through adolescence, the number of synaptic contacts throughout the brain increase dramatically. During puberty and into adulthood, however, there is a net loss of synapses in many brain regions. This process, known as synaptic pruning, is critical for the establishment and function of mature neuronal networks. Synaptic pruning appears to occur through multiple mechanisms as discussed below, but generally involves the removal of both pre- and postsynaptic elements (Purves and Lichtman, 1980). The removal of the presynaptic component, however, has been more challenging to measure. Here, we will refer to *synaptic pruning* as the process in which both the pre-and postsynaptic elements are removed, and refer to *spine pruning* when only the postsynaptic structure has been measured experimentally.

Postnatal synaptic pruning was initially identified as "resorption" of neurites in Purkinje and granule cells by Ramon y Cajal (Yuste, 2015) and was reemphasized in studies of synapses and axons of the cortex almost thirty years ago (Huttenlocher, 1990; LaMantia and Rakic, 1990; Rakic et al., 1986). Significant insights have been made into mechanisms of synaptic pruning in the peripheral nervous system (neuromuscular junction; NMJ) (Purves and Lichtman, 1980; Sanes and Lichtman, 1999) and in the central nervous system (retinogeniculate synapses) (Huberman, 2007) and cerebellum (Hashimoto and Kano, 2013), and these provide a foundation for the future characterization of pruning in the cerebral cortex. Here we review the literature on synaptic pruning events in these systems and propose research to address the relationship between synaptic autophagy and pruning.

2. Developmental synaptic pruning

2.1. Neuromuscular Junction

The study of the development and maturation of the neuromuscular junction (NMJ) provided many of the discoveries of mechanisms that regulate synaptic pruning. At birth, muscles are innervated by multiple motor axon terminals (Fig. 1A), while the mature NMJ is composed of a *single* presynaptic input from a motor neuron that synapses on a postsynaptic specialization on the muscle surface. In rodents, "superfluous" axon terminals are removed over the first two postnatal weeks, illustrating fundamental principles of synaptic pruning including *activity-dependent synaptic competition* and *non-neuronal contributions*.

Clusters of postsynaptic acetylcholine receptor (AChR) form in response to the arrival of motor neuron axons (Frank and Fischbach, 1979; Ko et al., 1977). Motor neuron axon terminals mature when they reach the muscle by synthesis and positioning of the presynaptic machinery required for neurotransmitter release (Chow and Poo, 1985; Evers et al., 1989; Kidokoro and Yeh, 1982; Xie and Poo, 1986). The postsynaptic stabilization occurs in regions of the muscle directly opposite to axon terminals, while AChR distal from axon terminals are removed (Balice-Gordon and Lichtman, 1993; Lin et al., 2005; Misgeld et al., 2005). Numerous proteins that play a role in this process have been identified, with contributions from neuronal, muscle and glial cells required for the formation of the synapse (See (Sanes and Lichtman, 1999) for review). The synaptic area is limited by perisynaptic Schwann cells (PSC), the glial cells of the NMJ, which at this stage ensure that AChR clusters are selectively present at the site of contact with the axon (Yang et al., 2001).

Pruning of superfluous motor neuron axons during the first two postnatal weeks is dependent on neuronal activity (Thompson et al., 1979), and the axons with relatively "weaker" synapses are removed (Buffelli et al., 2003) (Fig. 1A). Differences in presynaptic strength related to altered presynaptic release probability are critical to the specification of the weaker inputs (Colman et al., 1997; Kopp et al., 2000). Alterations in the composition of the presynaptic terminal, including calcium channels (Urbano et al., 2002) and the organization of synaptic vesicles and active zone proteins (Chen et al., 2011; Fox et al., 2007; Umemori and Sanes, 2008) enable these changes in presynaptic strength. Intriguingly, the difference in synaptic strength alone is insufficient to drive synapse elimination, and asynchronous activity arising from disparate synaptic strengths between synaptic inputs is required (Buffelli et al., 2002; Favero et al., 2012; Thompson, 1983).

In addition to neuronal activity, survival signals such as brain-derived and glial-derived neurotrophic factors (BDNF and GDNF) are released from muscle and glia and contribute to the strengthening of ACh release (Henderson et al., 1994; Je et al., 2013, 2012). As the uptake of these factors by the axon is activity-dependent and they act to further enhance synaptic strength, they can initiate a feedforward cascade to strengthen a single axonal input (Snider and Lichtman, 1996). This provides an example of how non-neuronal cells contribute to selecting the "winning" axonal input via activity-dependent uptake of non-neuronally derived factors.

Regulatory signaling cascades contributing to presynaptic and postsynaptic maturation during this period also depend on intracellular calcium (Adams and Goldman, 1998; Dai and Peng, 1993) and protein kinase C (PKC) (Huang et al., 1992; Lanuza et al., 2002) (Fig. 1A).

Together, these mechanisms lead to a synaptic competition that specifies the "winning" synaptic input that will remain and the "losers" to be eliminated. During this process, postsynaptic sites undergo morphological changes from a continuous plaque of membrane-inserted AChR to a "pretzel-shaped area" with segments that lack postsynaptic AChR (Balice-Gordon and Lichtman, 1993; Marques et al., 2000), and the axon terminals withdraw from areas lacking the receptors (Balice-Gordon and Lichtman, 1993). The withdrawal of one innervating axon leads to the expansion of remaining axon terminals at the same postsynaptic site (Walsh and Lichtman, 2003). This process remains dynamic, as the withdrawing axon terminal can reinnervate the synapse and "win" the competition if the original "winner" axon terminal is selectively ablated (Turney and Lichtman, 2012).

Perisynaptic Schwann cells (PSCs) contribute to axon withdrawal by actively separating axon terminals from the postsynaptic membrane (Smith et al., 2013). Whether Schwann cells are able to selectively separate the losing axon terminals from the muscle based on their weaker synaptic strength or act to separate axon terminals indiscriminately remains controversial (Darabid et al., 2013; Jahromi et al., 1992; Robitaille, 1998; Rochon et al., 2001; Smith et al., 2013; Todd et al., 2010). The withdrawing axon terminal then forms a retraction bulb ensheathed by Schwann cells (Smith et al., 2013), leaving behind "axosomes," which are filled with material from the presynaptic compartment including synaptic vesicle clusters and mitochondria (Bishop et al., 2004; Walsh and Lichtman, 2003) (Fig. 1A). These axonal components are then degraded within the Schwann cell (Song et al., 2008). Finally, these events yield singly innervated NMJs capable of triggering mature muscle activity (Fig. 1A).

2.2. Cerebellum

Synaptic pruning has also been well characterized in synapses of the cerebellum. The mature cerebellar circuit consists of a Purkinje cell (PC) that receives strong inputs from a single climbing fiber (CF) arising from the inferior olivary nucleus onto its proximal dendrites, and weak inputs from many parallel fibers (PF) that arise from cerebellar granule cells onto its distal dendrites (Fig. 1B). The PC is initially innervated by multiple weak CF inputs in the first postnatal week (Chedotal and Sotelo, 1993). During this period, CF inputs synapse onto the PC soma in a "pericellular nest" (Ito, 1984; Sugihara et al., 1999). By the end of this week, a single CF input is strengthened while others have become progressively weaker (Hashimoto and Kano, 2003). The principles of activity-dependent synaptic competition and non-neuronal contributions that regulate synaptic pruning at the NMJ are also illustrated at this synapse (Fig. 1B).

The selective synaptic strengthening is thought to arise from

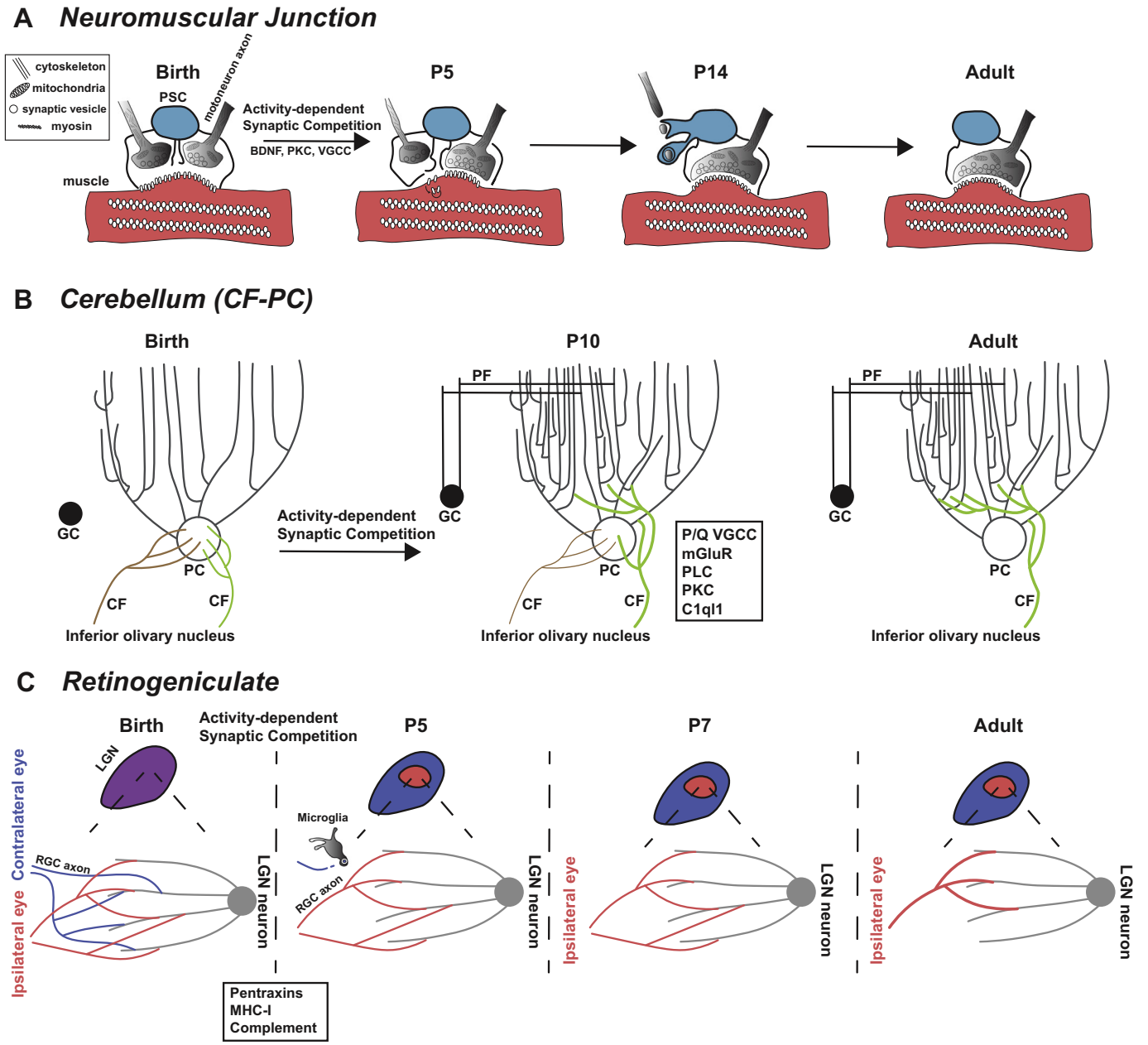

Fig. 1. Schematic representations of NMJ, cerebellar, and retinogeniculate synaptic pruning events. (A) At birth, individual muscles are innervated by multiple motor neuron axons. The NMJ is surrounded by a perisynaptic Schwann cell (PSC). By P5, selective strengthening of a single axon has occurred and pre- and post-synaptic elements are rearranged, including the beginning of axonal retraction and removal of nAChR from the muscle membrane. At P14, the "loser" axon retracts, leaving behind axosomes that are engulfed by PSCs and degraded. The "winner" axon expands its synaptic territory. In adulthood, all muscles are monoinnervated with a PSC delimiting the synaptic area. (B) At birth, cerebellar Purkinje cells (PCs) are innervated by multiple climbing fiber (CF) axons from the inferior olivary nucleus. At this time, CF axons synapse onto the PC soma. Over the first postnatal week, parallel fibers from cerebellar granule cells (GCs) synapse onto the distal dendrites of PCs. In addition, selective strengthening of a single CF input (green) occurs. The synapses of the stronger CF input begins to translocate onto the proximal dendrites of the PC. The weaker CF inputs (brown) are then withdrawn by adulthood. (C) At birth, retinal ganglion cell axons from both eyes innervate the lateral geniculate nucleus of the thalamus without any spatial segregation. During the first week postnatally, the RGC inputs segregate so that the ipsilateral RGC inputs innervate a smaller central area. Complement proteins coat synapses to be pruned from the contralateral eye (not shown). Microglia then phagocytose and degrade these synapses. Later stages of refinement include strengthening and further pruning of inputs from the ipsilateral eye.

enhanced presynaptic release due to increased probability of fusion of multiple synaptic vesicles and an increased number of active release sites, with no potentiation of quantal size (Hashimoto and Kano, 2003). This synaptic selection can be mimicked by a spike-timing dependent plasticity protocol in which the PC and a single CF input are coincidently activated. Interestingly, stimulating the stronger CF input elicits long-term potentiation, while activation of weaker CF inputs produces long-term depression (Bosman et al., 2008; Ohtsuki and Hirano, 2008). These findings emphasize the role of *activity-dependent synaptic competition* in synaptic maturation at this synapse.

A molecular mechanism for the selective strengthening of strong inputs and weakening of weaker inputs has recently been proposed. C1ql1, a protein expressed at the CF axon terminal, interacts with Bai3, the C1ql1 receptor expressed by PCs. Bidirectionally modulating this interaction disrupted the selective strengthening and selection of a winner CF during postnatal cerebellum development (Kakegawa et al.,

2015). The downstream mechanisms underlying the role of C1ql1-Bai3 in CF-PC synapse maturation remain unexplored.

Following the differentiation of CF inputs, the strongest CF input translocates its synapses onto the proximal dendritic tree (Hashimoto and Kano, 2003) (Fig. 1B). This event is critical to determining winner and loser CFs, as the CF that translocated generally continues to innervate the PC, while the remaining CF inputs onto the PC soma are pruned (Carrillo et al., 2013) (Fig. 1B). An important contribution to the elimination of weak CF inputs, but not to the differentiation in strength between CF inputs, is the cerebellar granule cell parallel fiber (PF) to PC synapse. PF to PC synaptogenesis occurs during the second postnatal week (after CF input differentiation) (Altman, 1972). In mouse mutants with reduced PF to PC synapses or rodents whose cerebellum is irradiated in the first postnatal week to eliminate cerebellar granule cells, CF elimination is incomplete (Crepel et al., 1981; Hashimoto et al., 2001). In mice with reduced PF inputs and impaired CF elimination, CF inputs extend onto the distal PC dendrites and invade the space predominantly occupied by PF inputs in the “normal” cerebellum (Ichikawa et al., 2002).This demonstrates that heterosynaptic interactions, and perhaps competition, are responsible for the elimination of weak CF inputs to PCs.

Although CF input strengthening during the early phase of maturation in the first postnatal week occurs presynaptically (Hashimoto and Kano, 2003), several postsynaptic components in the PC have been implicated in controlling the second phase of CF input elimination during the second and third postnatal weeks (Hashimoto and Kano, 2013; Kano and Hashimoto, 2009) (Fig. 1B). CF to PC synapses in mice deficient in the type 1 metabotropic glutamate receptor (mGluR1) undergo reduced elimination in the second and third postnatal weeks (Kano et al., 1997). It is hypothesized that PC-expressed mGluR1, which is activated by functional PF to PC synapses, is critical for CF elimination, providing a mechanism for heterosynaptic involvement in the late phase of CF elimination (Hashimoto et al., 2009b). Furthermore, elements of the signaling cascade downstream of mGluR1, such as PKC gamma (Kano et al., 1995), phospholipase cβ4 (Kano et al., 1998), and Gαq (Offermanns et al., 1997), are critical for CF synapse elimination. Postsynaptic PC P/Q type voltage-gated calcium channels (VGCCs) are also critical for CF input elimination (Hashimoto et al., 2011), possibly by providing the intracellular calcium influx required for synaptic plasticity and selective synaptic strengthening during the early phases of CF elimination (Kano and Hashimoto, 2009).

The downstream effectors of these signaling cascades have recently begun to be elucidated. Arc/Arg3.1, a protein locally synthesized at the dendritic spine, has been shown to act downstream of P/Q type VGCCs to mediate CF elimination and weakening CF to PC synaptic strength (Mikuni et al., 2013). While the role of postsynaptic Arc is not clear, these findings may indicate a molecular basis for postnatal CF elimination.

2.3. Retinogeniculate pathway

The neural circuits underlying vision also undergo significant postnatal refinement. At this synaptic pruning event, the contribution of microglia has been well established and illustrates the principle of non-neuronal contributions to synaptic pruning.

Retinal ganglion cells (RGCs) project to the dorsal lateral geniculate nucleus (dLGN) of the thalamus, where information is relayed to the primary visual cortex. During the first postnatal week, RGC axons from both eyes are intermixed in the dLGN with terminals from both eyes synapsing onto single dLGN neurons (Fig. 1C). By the end of the first week, RGC axons segregate to ensure that each dLGN cell is innervated only by axons from one eye. Further refinement occurs over the next weeks, as 2–3 RGC inputs per cell are selectively strengthened and the remainder are pruned (Huberman, 2007) (Fig. 1C).

Similar to the CF to PC synapse in the cerebellum, RGC axon segregation is activity-dependent. Intriguingly, because eye opening has not occurred, the patterns of activity that induce synapse elimination differ from the CF to PC synapse. Nevertheless, blockade of spontaneous retinal activity in the first postnatal week with tetrodotoxin to block action potentials is sufficient to disrupt RGC axon segregation and synaptic maturation (Hooks and Chen, 2006; Shatz and Stryker, 1988). Experience-dependent activity, modified by dark rearing, does not affect this early stage of maturation but disrupts subsequent synaptic refinement (Hooks and Chen, 2006). Extensive research has focused on the characteristics of RGC spontaneous activity required for synaptic maturation in the early postnatal period, and is reviewed elsewhere (Butts et al., 2007; Penn et al., 1998; Stellwagen and Shatz, 2002; Torborg and Feller, 2005; Torborg et al., 2005). As discussed above for CF to PC synaptic elimination, this activity-dependence manifests through selective strengthening of individual inputs (Datwani et al., 2009; Ziburkus et al., 2009).

Efforts to address the mechanism of synaptic refinement that follows selective synaptic strengthening at the RGC to dLGN synapse have, surprisingly, identified proteins and processes associated with the immune system (Huberman, 2007). Early studies from Carla Shatz's laboratory identified neuronally expressed Class I major histocompatibility complex (MHC-I) as a mediator of dLGN refinement and eye-specific segregation (Huh et al., 2000) (Fig. 1C). Interestingly, MHCI appears to regulate synaptic plasticity, as MHC-I deficient mice showed enhanced long-term potentiation and disrupted LTD (Huh et al., 2000; Lee et al., 2014).

How might neuronal MHC-I molecules control synaptic plasticity? Upregulation of calcium-permeable AMPA receptors in the selective knockout mice biases synaptic plasticity toward long-term potentiation, possibly driving structural plasticity away from synapse elimination (Lee et al., 2014). The signaling pathways downstream of MHC-I that lead to alterations in AMPA receptor trafficking remain unknown.

Another study identified the neuronal pentraxins (NPs) as mediators of dLGN maturation (Bjartmar et al., 2006) (Fig. 1C). Pentraxins are synaptic homologs of a class of immune proteins that include acute phase proteins (Dodds et al., 1997). NPs were shown to be required for the segregation of eye-specific inputs to the dLGN in the early phase (spontaneous activity-dependent) of dLGN refinement (Bjartmar et al., 2006). The role of NPs in synaptic pruning appears to be independent of alterations in synaptic plasticity or neuronal firing patterns, but the precise mechanism remains unknown.

Recent studies by several groups further emphasize the role of the immune system in dLGN maturation. These studies demonstrate that synapse elimination is dependent on phagocytosis of synapses by microglia and astrocytes, suggesting that non-neuronal cells in the CNS may play an important role in developmental synaptic pruning and maturation. Pioneering work by Beth Stevens and Ben Barres demonstrated that mice lacking the critical complement cascade proteins C1 and C3 had reduced dLGN input segregation (Stevens et al., 2007) (Fig. 1C). The complement cascade is best known for its role in the clearance of pathogens and debris in the periphery (Gasque, 2004). An initiating protein, C1q, opsonizes a target leading to a proteolytic cascade (including the protein C3) and either phagocytosis by macrophages or microglia, or cell lysis via the membrane attack complex (Gasque, 2004). Barres and Stevens noted developmentally-regulated expression of C1q from RGCs from P5 to P30 (Stevens et al., 2007). Immunofluorescence analysis demonstrated coating of synapses in the dLGN by C1q and C3 and engulfment of presynaptic components by microglia and astrocytes (Schafer et al., 2012; Stevens et al., 2007). The receptor for C3 (CR3) is only expressed by microglia and astrocytes in the CNS, suggesting that synaptic coating by these proteins signals these cells (Gasque et al., 1998). C1q and C3 coating, and microglial engulfment, were activity dependent and appeared to occur on the relatively smaller synapses present in the dLGN (Schafer et al., 2012). These findings illustrate the role of *activity-dependent synaptic competition* in synaptic maturation.

A role for microglia in developmental synaptic pruning has been

expanded to regions of the hippocampus (Paolicelli et al., 2011) and cortex (Chu et al., 2010), and recently to neurodegenerative and neuropsychiatric disease (Hong et al., 2016; Sekar et al., 2016; Stephan et al., 2012; Vasek et al., 2016), expanding on original observations by Peter and Edith McGeer (McGeer et al., 1989).

3. Neuronal autophagy

We have recently reported that macroautophagy may play fundamental roles in synaptic pruning in the cortex (Tang et al., 2014). Here we review evidence suggesting roles for macroautophagy in synaptic pruning, speculate about the mechanism underlying this phenomenon, propose specific directions to answer major outstanding questions on the role of macroautophagy in synaptic pruning, and propose a "unified model" for the roles of neuronal and nonneuronal mechanisms in synaptic pruning based in part on the three well-studied synaptic pruning paradigms described above.

Since the original description (and naming) of the lysosome, autophagy, and phagocytosis by Christian de Duve (Ohsumi, 2014; Sabatini and Adesnik, 2013), and the identification of the lysosome as an organelle by de Duve's collaborator Alexander Novikoff (Novikoff et al., 1956), significant work has been conducted to understand the mechanisms underlying these processes. Macroautophagy (hereafter autophagy) is a multi-step catabolic cellular process through which cytosolic proteins and organelles are degraded. Autophagy begins when substrates are sequestered into double membrane bound vesicles, known as autophagosomes. Autophagosomes then undergo retrograde trafficking toward the lysosome. En route, autophagosomes can fuse with endosomes to form amphisomes. Amphisomes or autophagosomes then fuse with the lysosome to form autolysosomes, in which the autophagosome cargo is degraded by lysosomal proteases (Fig. 2).

Autophagy is dependent on many genes, most of which were originally described in brewer's yeast. These genes are important for autophagosome membrane formation, cargo recognition and autophagic flux. Mouse models have been developed to provide for the conditional deletion of two genes encoding rate-limiting components of the autophagy machinery, Atg5 and Atg7 (Hara et al., 2006; Komatsu et al., 2006). The relative levels of the lipid-conjugated form (LC3-II) of the Atg8 homolog, LC3-I, and the cellular distribution of fluorophore tagged LC3 are often used to indicate the relative state of autophagic flux in neuronal systems (Klionsky et al., 2016). These tools have enabled the analysis of autophagy in the mammalian nervous system (Fig. 2).

Given the wide interest in neuronal autophagy at present and its control of a wide range of neuronal function and dysfunction, it may seem surprising that for many years, autophagy was widely considered not to occur in neurons at all, despite some very early morphological reports in Huntington's disease autopsy (Roizin et al., 1979). The presence of autophagosomes in axons was essentially rediscovered by Peter Hollenbeck (Hollenbeck, 1993) and was gradually acknowledged in the field (Larsen and Sulzer, 2002) following research from multiple groups.

Although autophagy has been implicated in several cell biological processes in neurons, we focus here on the role for autophagy in synaptic pruning and neuronal development. For background on other aspects of neuronal autophagy, please see other reviews in this edition.

In neurons, autophagosome formation has primarily been studied in the distal axon and soma (Hollenbeck, 1993; Maday and Holzbaur, 2016, 2014; Maday et al., 2012). Some reports have demonstrated autophagosome formation in dendrites, especially in response to particular stimuli (Hernandez et al., 2012; Shehata et al., 2012). Neurons also form autophagosomes in the cell body that can be exceedingly long lived, apparently through the lifetime of the organism, as seen by the accumulation of the neuronal aging pigments, lipofuscin and neuromelanin within autophagic organelles (Sulzer et al., 2008).

In axons, autophagosomes are trafficked retrogradely to the soma via dynein motors (Fu et al., 2014; Maday et al., 2014, 2012). During retrograde trafficking, autophagosome maturation occurs, as observed by increased acidification. Some argue that fusion with lysosomes occurs in neurites as opposed to solely in the cell body: this interpretation is largely dependent on the definition of the lysosome, which was originally defined by de Duve and Novikoff by the presence of acid hydrolases (Novikoff et al., 1956; Sabatini and Adesnik, 2013). Finally, in the soma, autophagosomes can fuse with conventional lysosomes where their contents are degraded (Fig. 2).

In dividing cells, autophagy is a critical response to starvation and nutrient deprivation. Numerous signaling cascades converge to control autophagy by coordinating multiple aspects of autophagosome formation, maturation and lysosomal fusion (Dunlop and Tee, 2014; Jung et al., 2010). Autophagy initiation is controlled by the activity of the serine/threonine kinase ULK1 (homolog of the yeast Atg1) (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009; Russell et al., 2013) and the lipid kinase, Vps34 (Backer, 2008). ULK1 activity is negatively regulated by mammalian target of rapamycin (mTOR)-mediated activation of Rheb and AMBRA1 (Jung et al., 2009; Russell et al., 2013). ULK1 activity is also positively regulated by AMPK signaling to promote autophagy (Kim et al., 2011). Together these signaling cascades ensure that autophagy activity is tightly controlled (Fig. 2). Nearly all labs that have studied these processes find that nutrient deprivation or serum starvation are relatively weak promoters of autophagy in neurons, and the molecular induction steps are likely to be somewhat different than other paradigmatic cell types that have been more thoroughly characterized.

4. Control of neuronal autophagy by mTOR

mTOR participates in two protein complexes, mTORC1 and mTORC2, that are integral to cellular signaling and can be distinguished by their sensitivity to the mTOR inhibitor, rapamycin. Although mTORC2 is critical for nutrient sensing in the periphery (Saxton and Sabatini, 2017) and is involved in plasticity in multiple brain circuits (Bockaert and Marin, 2015; Dadalko et al., 2015), mTORC1 has been most directly linked to the regulation of autophagy (Dunlop and Tee, 2014).

The role for mTOR regulation of *neuronal* autophagy has remained somewhat controversial, largely due to a variance in findings using rapamycin and its derivatives. In ventral midbrain dopamine neurons, mTOR activity appears to negatively regulate autophagy, as rapamycin induces LC3 puncta formation and conjugation of LC3-I to the lipidated LC3-II (Hernandez et al., 2012). Furthermore, genetic hyperactivation of mTOR following deletion of the mTORC1 inhibitors TSC1 and TSC2 (Tee et al., 2003, 2002; Zhang et al., 2003) in excitatory neurons in the cortex reduces LC3-II levels and increases p62, and decreases the number of LC3 puncta in the soma of primary cultured cortical neurons. This reduction in autophagy is rescued following pharmacological inhibition of mTOR with rapamycin both *in vivo* with an 8 day treatment regimen, and in culture, in an Atg7-dependent manner (Tang et al., 2014). Rapamycin treatment of wild-type mice *in vivo* and of wild-type primary cortical neurons was sufficient to elicit a small but significant increase in LC3-II or GFP-LC3 puncta, respectively (Tang et al., 2014) (Fig. 2). Autophagy activity in TSC1/2 null cells, however, may be complicated by a compensatory increase in AMPK signaling (Di Nardo et al., 2014), suggesting a more complex relationship between autophagy and this protein complex.

Other reports have not observed a basal inhibition of autophagy by mTOR in the CNS (Fox et al., 2010; Maday and Holzbaur, 2016; Tsvetkov et al., 2010). Inhibition of mTOR with everolimus, a rapamycin-like compound, following a 6–8 week treatment paradigm failed to elicit an increased in LC3-II conjugation *in vivo*, but did engage other downstream signaling pathways of mTOR such as protein synthesis (Fox et al., 2010). mTOR inhibition with torin-1 failed to elicit autophagy activation in hippocampal cultures (Maday and Holzbaur, 2016).

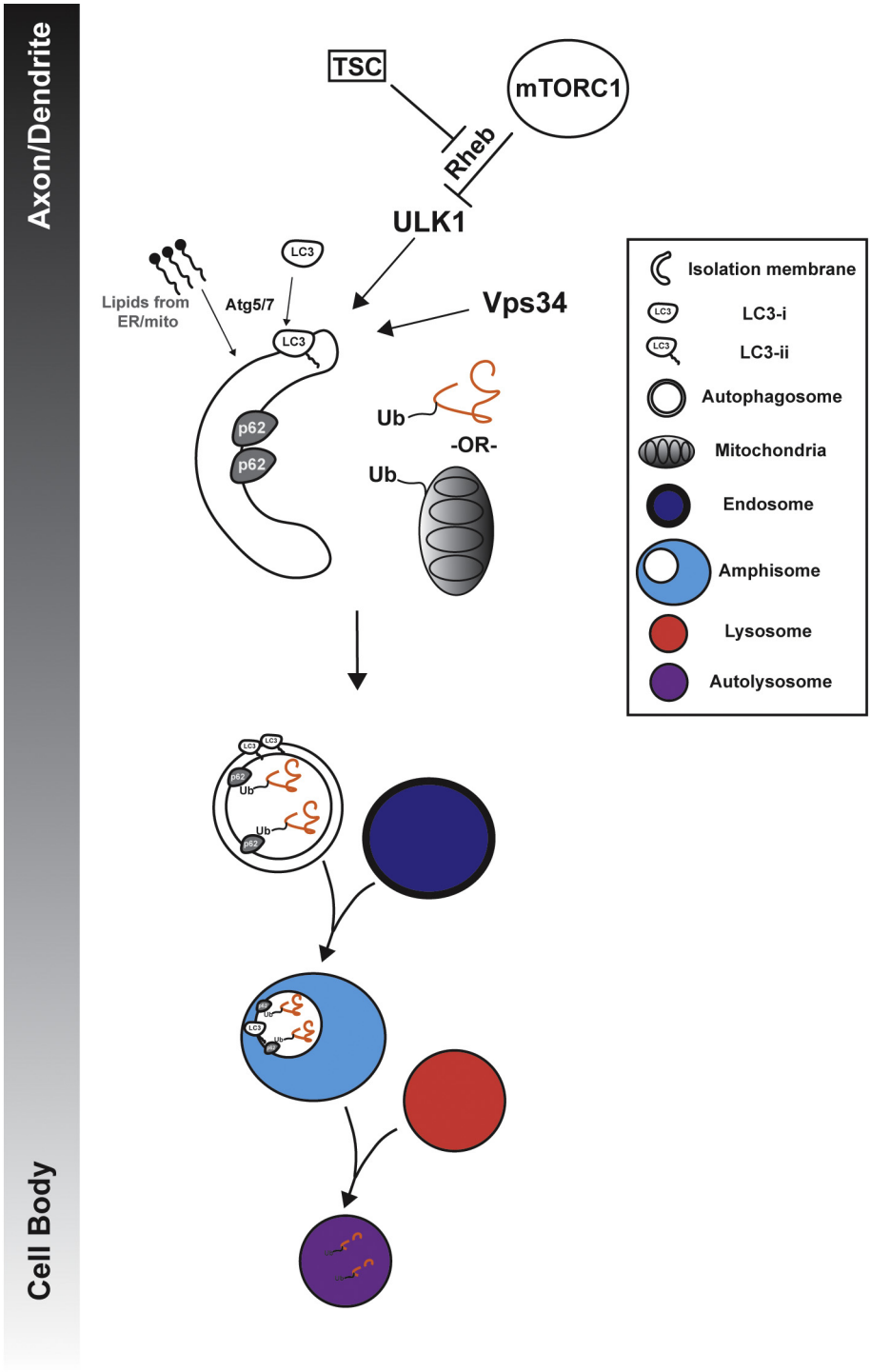

Fig. 2. Control of neuronal autophagy by TSC and mTORC1. mTORC1 and TSC control autophagy via regulation of ULK1, which controls formation of the nascent autophagosome (isolation membrane). The isolation membrane expands following addition of lipids from the endoplasmic reticulum or mitochondria. Autophagosome formation is dependent on many Atg genes, including Atg5 and Atg7, and requires lipid conjugation of the Atg8 homolog LC3. Ubiquitinated cytosolic proteins or organelles bind to adapters on the inner membrane of the growing autophagosome, such as p62. In neurons, autophagosome formation may occur most often in axons or dendrites, but also occurs in the cell body. Subsequently, autophagosomes traffic retrogradely toward the cell body, and en route fuse with endosomes to form amphisomes. Amphisomes and autophagosomes then fuse with lysosomes, where autophagic cargo is degraded by luminal proteases.

These results are intriguing given that, while the effect of rapamycin induced autophagy activation has been questioned, torin-1 mediated inhibition is reported to exert profound effects on autophagy (Thoreen et al., 2009). Differences in treatment paradigm, including the type, timing and concentration of mTORC1 inhibitor, and age may underlie these divergent conclusions about regulation of autophagy by mTORC1 in the CNS. Furthermore, differences in the regulation of autophagy between peripheral or dividing cells and neurons in the CNS have been clearly demonstrated *in vivo*. Starvation in the rodent induces autophagy in organs such as the kidney and liver, but fails to do so in the brain (Mizushima et al., 2004). It is further possible that starvation-induced autophagy may not be mediated by LC3, and that measurements of GFP-LC3 are not representative of starvation-induced autophagy in the brain. Nevertheless, the possible regulation of autophagy upstream kinases, such as mTOR, suggest that autophagic dysfunction may contribute to neurodevelopmental disorders in which these kinases are hyperactivated, such as ASD.

5. Contribution of neuronal mTOR-dependent autophagy to dendritic spine pruning

Alterations in dendritic spine density have been proposed to play a role in the pathophysiology of ASDs. In ASD, human postmortem tissue demonstrates elevated spine densities in the temporal cortex (Hutsler

and Zhang, 2010; Tang et al., 2014). Furthermore, in other neurodevelopmental disorders, such as Down, Angelman's, Rett, schizophrenia, and Fragile X syndrome, changes in spine density or morphology are observed in postmortem studies and in animals models (Phillips and Pozzo-Miller, 2015). To address the underlying mechanism for increased spine density in human ASD cases, Tang et al. measured the spine density in postmortem samples of temporal cortex from cases at different ages and found significantly reduced spine pruning in ASD brains compared to controls. Interestingly, this was inversely correlated with levels of the autophagy marker LC3-II, suggesting that impaired autophagy, downstream of mTOR hyperactivation seen in ASD (Auerbach et al., 2011; Tang et al., 2014), may be responsible for the reduced synaptic pruning.

This hypothesis was addressed in a mouse model of tuberous sclerosis complex (TSC), a developmental disorder characterized by intellectual disability, epilepsy, and the presence of cortical tubers in addition to other peripheral dysfunction (Crino et al., 2006). TSC arises from loss-of-function mutations in the genes TSC1 (tuberin) and TSC2 (hamartin) (European Chromosome 16 Tuberous Sclerosis Consortium, 1993; Kandt et al., 1992; Van Slegtenhorst et al., 1997). TSC1 and TSC2 inhibit mTOR signaling (Tee et al., 2002; Zhang et al., 2003) via direct inhibition of the Ras homolog enriched in brain, Rheb (Tee et al., 2003). Multiple studies have attempted to identify changes in dendritic spine density in mice carrying loss of function alleles in TSC1 and 2 with mixed results. Sabatini and colleagues reported deficits in both excitatory and inhibitory synaptic transmission in TSC-null mice but did not measure differences in spine density *in vivo* (Bateup et al., 2013, 2011). They however reported increased spine length, decreased spine density and disruption in soma size in primary neuronal culture (Tavazoie et al., 2005). Examination of hippocampal granule cells identified no effect of TSC1on spine density (Goorden et al., 2007). Interestingly, an increased spine density was observed in cerebellar Purkinje cells upon loss of TSC1 in 4 week old mice, following the classical period of synaptic reorganization seen postnatally (Tsai et al., 2012). Future work may elucidate the mechanism through which spine density is elevated in these TSC mutants. For example, excess spines may result from multiply innervated Purkinje cells (Hashimoto et al., 2009a) or spines may be present ectopically, for example on the soma where no spines are present in adulthood (Hashimoto and Kano, 2013). Finally, Meikle et al. demonstrate a reduction in spine density on the apical dendrite of cortical neurons in TSC1 knockout mice (Meikle et al., 2008).

In contrast, Tang et al. showed an increased spine density in cortical neurons from an excitatory neuronal specific TSC1 conditional knockout and TSC2 heterozygous mice by P30. Interestingly, spine density was unchanged at P20, suggesting that spinogenesis was intact but spine pruning was specifically deficient. Furthermore, this deficit in spine pruning was normalized by rapamycin (Tang et al., 2014). Differences in brain region, genetic background and mouse age may underlie the disparate conclusions on the role for TSC1/2 and mTOR signaling in controlling spine density.

What is the downstream effector for TSC1/2 that controls spine density? As described above, TSC1/2 loss of function leads to mTOR hyperactivation (Tee et al., 2003, 2002; Zhang et al., 2003). Downstream from mTOR, protein synthesis is disrupted in TSC null cells via mTOR-dependent regulation of the S6 kinase and 4E-BP. However, mTORC1 hyperactivity also inhibits autophagy in TSC null neurons (Tang et al., 2014). To address whether the loss of autophagy was the cause of spine pruning deficits in TSC1/2 null mice, Tang et al. took two strategies. First, they showed that conditional deletion of Atg7, a required component of the autophagosome biogenesis machinery, in forebrain excitatory neurons was sufficient to phenocopy the spine pruning deficits observed in TSC1 null mice. Furthermore, both conditional knockouts for Atg7 and TSC1 null mice demonstrated social interaction deficits, thus providing face validity to these models for ASD. Tang et al. then tested whether autophagy was necessary for the rescue of TSC spine pruning deficits by rapamycin. They generated mice harboring heterozygous TSC2 alleles and conditional deletion of Atg7 in forebrain excitatory neurons. Rapamycin treatment failed to normalize spine densities at P30 in these mice. These results indicate both a necessity and sufficiency of neuronal autophagy for mediating spine pruning in cortical pyramidal neurons. Additional studies will be required to provide insights on the role of additional genes involved in autophagy in controlling dendritic morphology and function.

Intriguingly, peripheral inflammatory insults, which have been implicated in development of diseases such as autism (Malkova et al., 2012; Osokine and Erlebacher, 2017) and schizophrenia (Depino, 2017; Miller and Goldsmith, 2017), also inhibit autophagic function in the brain (François et al., 2014). Furthermore, neonatal exposure to drugs of abuse can lead to neuropsychiatric phenotypes and have previously been shown to regulate autophagy (Cubells et al., 1994; Larsen and Sulzer, 2002; Larsen et al., 2002; Plessinger, 1998). This suggests that autophagic dysfunction, due to either inflammation or genetic insults, may underlie dysfunction in synaptogenesis in neurodevelopmental disorders.

6. Suggested mechanisms for neuronal autophagy in autism and synaptic pruning

6.1. Mechanism of autophagic dysfunction in autism

Autophagic dysfunction in neurodevelopmental disorders can occur at different steps of the autophagy pathway (Fig. 2). In many neurodevelopmental disorders where autophagy has been suggested to be deficient, mTOR is hyperactive. These include disorders with mutations in TSC1, TSC2, PTEN, and NF1. In these syndromes, hyperactive mTOR could lead to disrupted autophagosome biogenesis due to decreased ULK1 activity (Ganley et al., 2009; Jung et al., 2009; Tang et al., 2014). Alternatively, a recent report suggests a deficit in autophagosome-lysosome fusion during the process of mitophagy (Ebrahimi-Fakhari et al., 2016). Future studies will be required to specifically identify the steps in autophagy that are disrupted in these disorders and lead to synaptic dysfunction.

Genetic lesions associated with two other syndromes, Vici and Beta-propeller protein-associated neurodegeneration, provide insight into the steps in autophagy that can be disrupted in neuropsychiatric disease. In Vici syndrome, EPG5, a Rab7 effector which is required for autophagosome-lysosome fusion, is mutated (Wang et al., 2016). In Beta-propeller protein-associated neurodegeneration, the Wdr45 gene is disrupted, yielding inefficient autophagosome membrane elongation and the buildup of early autophagic structures (Saitsu et al., 2013). These syndromes are both associated with neurodevelopmental delay amongst other sequelae, although associated changes in spine density or synaptic function have yet to be described. Thus, autophagy can go awry at several different steps to yield developmental delay or intellectual disability.

6.2. Synaptic plasticity

During prototypical synaptic pruning events, synaptic plasticity establishes differential synaptic strengthening to specify the synapse that remains (Piochon et al., 2016). For example, changes in synaptic strength precede synaptic pruning and refinement in the NMJ (Buffelli et al., 2003; Colman et al., 1997; Kopp et al., 2000), the CF to PC synapse (Bosman et al., 2008; Ohtsuki and Hirano, 2008), and the RGC to LGN synapse (Datwani et al., 2009; Ziburkus et al., 2009). Furthermore, induction of LTD is sufficient to remove specific spines (Becker et al., 2008; Zhou et al., 2004).

Autophagy may be required for changes in synaptic strength during synaptic pruning events. Recently, autophagic function has been implicated in controlling synaptic strength and plasticity. Autophagy is important for GABA and glutamate receptor turnover (Rowland et al.,

2006; Shehata et al., 2012), a process critical to changing synaptic strength during synaptic plasticity (Anggono and Huganir, 2012). Furthermore, autophagic activity is required for brain-derived neurotrophic factor (BDNF) mediated synaptic plasticity in the hippocampus (Nikoletopoulou et al., 2017). TSC1/2 deficiency, which reduces neuronal autophagy (Ebrahimi-Fakhari et al., 2016; Tang et al., 2014), leads to disruptions in synaptic plasticity in the hippocampus (Auerbach et al., 2011; Bateup et al., 2011; Chévere-Torres et al., 2012). Finally, presynaptic plasticity is controlled by autophagy in dopaminergic axons (Hernandez et al., 2012). Thus, autophagic activity, both pre-and postsynaptically, may contribute to synaptic plasticity, which is in turn required for selective synaptic strengthening.

A role for autophagy in the synaptic plasticity is further suggested because signaling cascades that control synaptic plasticity at these synapses also regulate autophagy. PKC signaling is required for CF to PC synaptic pruning (Kano et al., 1995) and NMJ maturation (Lanuza et al., 2002), and PKC activity can inhibit autophagy (Jiang et al., 2010; Wei et al., 2016). Bai3 (Kakegawa et al., 2015), another protein required for maturation of the CF to PC synapse, is an inhibitor of autophagy (Lipinski et al., 2010). In the absence of Bai3, the selective strengthening and weakening of CF inputs is lost, suggesting a role in the pruning of the CF to PC synapse (Bosman et al., 2008; Ohtsuki and Hirano, 2008).

The semaphorins are a family of secreted and transmembrane proteins critical for axon guidance and other developmental processes (Yazdani and Terman, 2006). In the cerebellum, Semaphorin 3A and its receptor, PlexinA4, is required for strengthening of CF inputs during the early phase of CF to PC synaptic development (Uesaka et al., 2014). The late phase of pruning at this synapse is dependent on Semaphorin 7A/PlexinC1 (Uesaka et al., 2014). This interaction is required downstream of mGluR1 signaling required for CF pruning (see above). Furthermore, Semaphorin 3F and its receptors, Plexin A3/A4 and Nrp2, are required for the selective removal of corticospinal tract axons projecting from the visual cortex during postnatal development (Low et al., 2008). Semaphorin 3F and Nrp2 inhibit mTOR signaling and activate autophagy (Stanton et al., 2013), suggesting that activation of autophagy may be required for selective axon pruning in the visual system. Semaphorin 3A may also signal through Nrp2 (Cariboni et al., 2011), implying that Semaphorin/Nrp2 activation of autophagy may be important in the cerebellum. Finally, P/Q type calcium channels are required for CF to PC pruning and may locally increase neuronal calcium levels (Hashimoto et al., 2011). Increased intracellular calcium can activate autophagy (Høyer-Hansen et al., 2007), providing an additional route for dynamic regulation of autophagy during postnatal cerebellar development.

Of note, cerebellar pathology is prominent in mice lacking neuronal autophagy (Hara et al., 2006; Komatsu et al., 2006). Autophagy may thus play a critical role in synaptic refinement downstream of these molecular pathways during postnatal development.

The findings that autophagy regulates synaptic activity via receptor trafficking and synaptic vesicle homeostasis, and that multiple cellular pathways are implicated in controlling synaptic strength during synaptic pruning events, suggests that autophagy may play a role in the first principle of developmental synaptic pruning we describe above: *activity-dependent synaptic competition*.

6.3. Mitochondrial function

Autophagy may also control synaptic pruning via selective degradation of mitochondria. In addition to the role of mitochondria in ongoing synaptic function and plasticity (Cameron et al., 1991; Kang et al., 2008; Levy et al., 2003; Sun et al., 2013; Tang and Zucker, 1997; Weeber et al., 2002), proper mitochondrial function is critical to the formation (Courchet et al., 2013; Kimura and Murakami, 2014; Lee and Peng, 2008) and maintenance of synaptic contacts and strength (Li et al., 2004). In both the axon and the dendrite, mitochondrial localization is precisely regulated and localized to active synaptic contacts (Chada and Hollenbeck, 2004). Furthermore, mitochondrial function and content is coupled to synaptic activity (Bindokas et al., 1998; Hevner and Wong-Riley, 1993; Wong-Riley and Welt, 1980). These data suggest that the presence of functional mitochondria may strengthen a synaptic contact and preclude its pruning. In support of this hypothesis, mitochondrial content is sensitive to neuronal activity during critical periods in synaptic development (Tieman, 1984; Wong-Riley and Welt, 1980), and mitochondrial motility is more dynamic during synaptic maturation than during adulthood (Lewis et al., 2016; Smit-Rigter et al., 2016).

Autophagy plays a critical role in the clearance of damaged or dysfunctional mitochondria through mitophagy (Youle and Narendra, 2011). Mitophagy is a process through which damaged mitochondria are tagged and selectively degraded. In neurons, this can occur to mitochondria in axons (Ashrafi et al., 2014; Berthet et al., 2014) and in the somatodendritic region (Cai et al., 2012; Joselin et al., 2012). As mitophagy occurs in response to the loss of mitochondrial membrane potential, and mitochondrial membrane potential is sensitive to neuronal activity, it is possible that mitophagy locally controls mitochondrial content in response to neuronal activity (see above). Furthermore, local mitochondrial content can contribute to dendritic spine pruning and synaptic maintenance, suggesting the possibility that autophagy and mitophagy contribute to synaptic pruning via targeted degradation of mitochondria. In support of this, mitochondrial abnormalities are present in TSC1-deficient neurons (Ebrahimi-Fakhari et al., 2016), which demonstrate deficient synaptic pruning (Tang et al., 2014), and in ASD brain (Tang et al., 2013).

Thus, the regulation of mitochondrial quality by autophagy may provide a cellular mechanism for the control of synaptic activity and, in turn, *synaptic competition* during developmental spine pruning by autophagy.

7. Non-neuronal autophagy contributes to spine pruning

Does autophagy play a role in the second principle of developmental synaptic pruning, *non-neuronal contributions*? Recently, work from the Yoon laboratory has addressed the role of non-neuronal autophagy in developmental spine pruning (Kim et al., 2017). Kim et al. argue that the reduction in autophagy inferred from postmortem tissue of ASD cases (Tang et al., 2014) may arise from non-neuronal cells such as microglia or astrocytes. Given the role of microglia and astrocytes in normal synaptic pruning (see above), the authors tested whether microglial autophagy was required for synaptic pruning. The authors used the same conditional allele of Atg7 (Komatsu et al., 2006) that Tang et al. used, but used the Lyz2-Cre line to generate Atg7-deficient microglia. This Cre line is expressed throughout the myeloid lineage, giving rise to Atg7-deficient macrophages and other peripheral cells in these mice (Clausen et al., 1999). A caveat to these findings is this also produces a loss of autophagy in peripheral cells, and recent evidence has emphasized a connection between peripheral immune cell function, the microbiome, and neurodevelopment (Vuong and Hsiao, 2017) that could be governed by host cell autophagy (Chu et al., 2016). Nevertheless, Kim et al. found that in these conditional knockout mice, cortical pyramidal cells in the somatosensory cortex (the same brain regions that Tang et al. examined) show increased spine density. In contrast to Tang et al., Kim et al. observe this difference at P15, prior to the classical window of spine pruning observed in the cortex, and at a time in which Tang et al. observed no requirement for neuronal autophagy. It is it possible that non-neuronal and neuronal autophagy contribute differentially at different stages of synaptic development. Alternatively, the results of Kim et al. may reflect increased spinogenesis rather than deficient synaptic pruning. The second possibility would be consistent with an increase in dendritic filopodia, the precursors of mature spines (Vaughn, 1989; Yuste and Bonhoeffer, 2004; Ziv and Smith, 1996), in neurons cocultured with microglia deficient

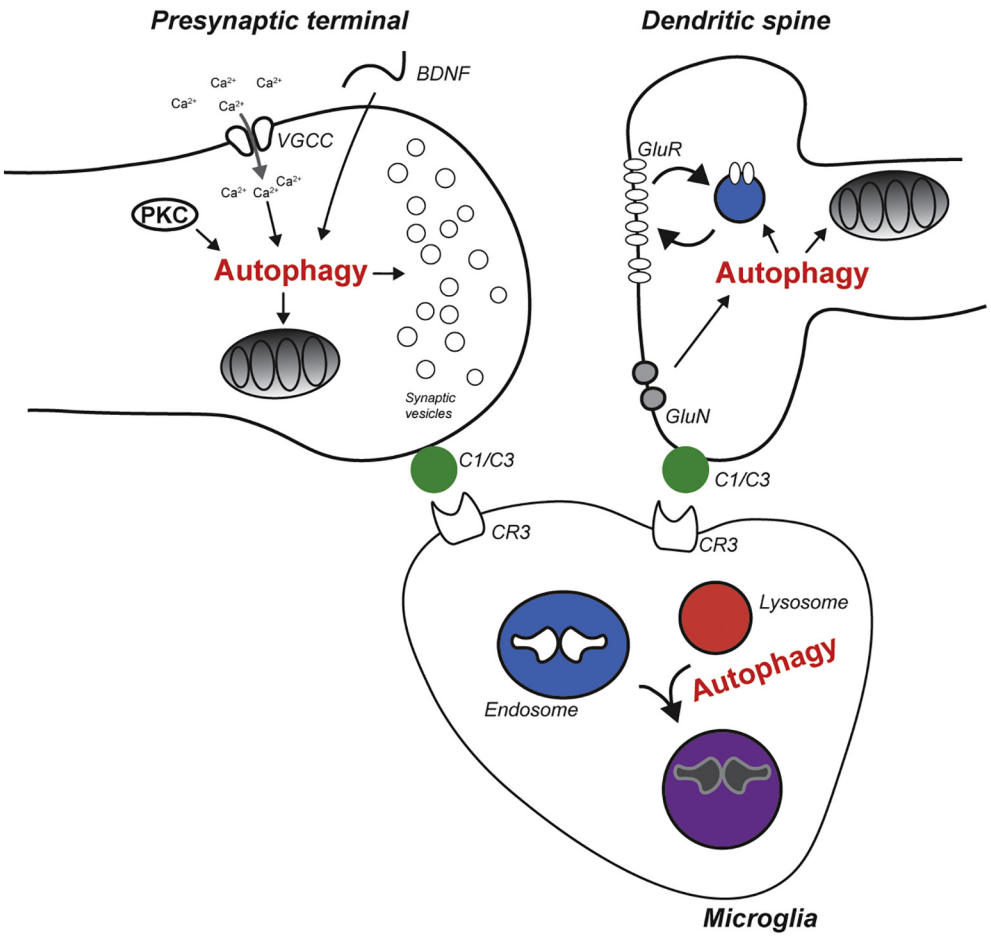

Fig. 3. Neuronal and microglial autophagy contribute to synaptic pruning. Presynaptic autophagy may be activated by BDNF, cytosolic calcium or PKC to modulate mitochondrial homeostasis and synaptic vesicle release. Postsynaptic autophagy is activated by NMDA receptor (GluN) signaling and controls both mitochondrial homeostasis and AMPA receptor (GluR1) trafficking. Weaker synaptic inputs are opsonized by C1/C3 during developmental synaptic pruning events. Opsonized synaptic contacts are phagocytosed and degraded in an autophagy-dependent fashion by microglia.

for autophagy (Kim et al., 2017).

Why might microglial autophagy be required for proper spine density? Using a culture system, Kim et al. showed that Atg7-deficient microglia are less efficient at degrading exogenous, purified synaptosomes. Interestingly, synaptosomal phagocytosis continued to be effective, but the presence of synaptosomal material in the degradative compartment was reduced. These findings suggest a role for microglia autophagy, seemingly downstream from phagocytosis, in regulating spine density. Why is spine density elevated if microglial phagocytosis is intact? It may be that impaired degradation of phagocytosed material backs up the phagocytic system, even if phagocytosis *per se* is intact.

Lysosomal degradation, possibly mediated by autophagy, in the Schwann cell is also critical in the transition from multi- to mono-innervation at the NMJ (Sanes and Lichtman, 1999; Song et al., 2008). By labeling cells with LysoTracker, Song et al. showed selectively and transiently enlarged lysosomal compartments proximal to and within motor axons receding from the NMJ, as well as in Bergmann glia surrounding receding cerebellar climbing fibers (Song et al., 2008). Using a mouse expressing GFP-tagged LC3, they further showed that autophagosomal structures form in receding motor neuron axons at the NMJ. Both GFP-LC3 puncta and LysoTracker-positive organelles then formed in Schwann cells to digest axonal material. Finally, disruption of autophagic flux and lysosomal storage by mutation of the membrane trafficking protein CLN3 resulted in the persistence of more retreating axons, as well as slower rates of recession. In summary, the integrity of autophagic-lysosomal flux in motor axons and their surrounding glial populations appears crucial for the proper timing and degree of synaptic pruning in multiple brain structures.

8. Possible mechanisms for microglial autophagy in synaptic pruning

The importance of microglial autophagy to cortical synaptic refinement emphasizes that autophagy may contribute to both principles of synaptic pruning, including the contribution of non-neuronal cells (Fig. 2). How may microglial autophagy control spine pruning? Microglial-dependent removal of synapses requires (1) active microglial migration, (2) phagocytosis of “tagged” material, (3) intracellular degradation of the phagocytosed components. The culture system used by Kim et al. suggests that engulfment of synaptic material is intact in microglia lacking autophagy (Kim et al., 2017). Indeed, the levels of engulfed synaptic markers (PSD-95 and synaptophysin) are elevated in microglia deficient for autophagy, as expected. These data suggest that microglial phagocytosis and migration to regions containing synapses that need to be pruned may remain intact without autophagy. Additional research is required to confirm the intact migration in the absence of microglial autophagy, as some signaling molecules involved in the control of microglial chemotaxis have been proposed to interact with autophagy machinery (Coly et al., 2017).

It may be that microglial autophagy's primary role during synaptic pruning may be to degrade phagocytosed components. In support of this hypothesis, Song et al. demonstrate that the lysosomal compartment, and presumably its degradative capacity, increases during synaptic pruning at the NMJ in Schwann cells (Song et al., 2008). This suggests that autophagy and lysosomal activity can be recruited on demand during synaptic pruning events to degrade phagocytosed material.

How may microglial autophagy be activated during synaptic pruning? Autophagy can be upregulated via signaling downstream of

Toll-like receptors/CR3 signaling (Sanjuan et al., 2007), the receptors required for microglial phagocytosis of synaptic material (Stephan et al., 2012). The role of autophagy proteins downstream of TLR/CR3 activation is complex, as both canonical autophagy and non-canonical LC3-associated phagocytosis (LAP) are regulated by these receptors (Sanjuan et al., 2007). LAP is a process in which canonical autophagy proteins such as LC3, Atg7, and Beclin-1 contribute to phagocytosis and promote efficient degradation of phagocytosed material independently of *de novo* membrane formation and sequestration of cytosolic components. Indeed, the deficit observed by Kim et al. is strikingly similar to that observed when LAP is disrupted. For example, in LAP, LC3, Atg7, and Beclin-1 are required for proper degradation of phagocytosed material: while LAP is required for full degradation, phagocytosis and internalization is not dependent on LAP function (Sanjuan et al., 2007). Thus, in concordance with the findings of Kim et al., the requirement for Atg7 in microglia-mediated synaptic pruning may be via LAP, in contrast to conventional autophagy. Further, both CR3-dependent phagocytosis, which is required for synaptic pruning by microglia, and LAP are independent of MyD88 signaling, suggesting a connection between these two processes (Hajishengallis et al., 2009; Sanjuan et al., 2007). Additional research is required to distinguish between LAP and canonical autophagy by addressing the role for specific autophagy-associated proteins, such as Rubicon, that contribute exclusively to either autophagy or LAP in microglia-mediated synaptic pruning (Martinez et al., 2015).

The temporal and functional connection between neuronal and microglial autophagy in spine pruning is an important future focus (Fig. 3). In the synaptic pruning principles described above (Fig. 1), the contribution of non-neuronal cells occurs after activity-dependent synaptic competition. It is tempting to hypothesize that neuronal autophagy and other mechanisms of spine pruning lead to a marking of synapses for opsonization by complement, followed by subsequent phagocytosis and digestion of the labelled synaptic material. Indeed, there is a temporally specific transition in the localization of lysosomal upregulation from the axon to the apposed microglia during developmental pruning at the NMJ (Song et al., 2008).

Alternatively, these processes may act independently. Microglia may select synapses to be pruned based on firing patterns and synaptic strength, as described for the NMJ and retinogeniculate synapse (Schafer et al., 2012; Smith et al., 2013; Tremblay et al., 2010) and require autophagy for the degradation of this material, whereas neuronal autophagy may be required for intrinsic neuronal mechanisms of structural plasticity (Cingolani and Goda, 2008; Piochon et al., 2016). Future research addressing the function of microglia in mice deficient in neuronal autophagy will be useful to define these mechanisms.

9. Future directions and conclusions

We have described evidence supporting roles for neuronal and non-neuronal autophagy in synaptic pruning, and their regulation by activity-dependent synaptic competition and the actions of non-neuronal cells. Here, we suggest experimental approaches to define how autophagy contributes to synaptic pruning.

Changes in presynaptic neuronal activity underlie synaptic pruning at the NMJ (Colman et al., 1997; Kopp et al., 2000), CF to PC synapse (Hashimoto and Kano, 2003), and the retinogeniculate synapse (Hooks and Chen, 2006). *Could autophagy act presynaptically to mediate synaptic pruning*? One possibility is that autophagy directly controls presynaptic strength. This is supported by the observation that loss of autophagy increases presynaptic dopamine release in adult mice (Hernandez et al., 2012). Furthermore, *de novo* autophagosome formation has been best described in the distal axon and contributes to mitochondrial homeostasis in this neuronal compartment (Ashrafi et al., 2014; Hollenbeck, 1993; Maday and Holzbaur, 2016). The importance of mitochondria for presynaptic plasticity (Sun et al., 2013) supports the possibility that autophagy regulates changes in presynaptic strength. Future experiments should focus on the direct observation of on-demand synthesis of autophagosomes, or changes in autophagosome content, in maturing axons undergoing changes in synaptic strength (Fig. 3).

Alternatively, changes in presynaptic strength by other mechanisms could activate autophagy to remodel axonal terminals. Direct modulation of presynaptic autophagy by selectively changing synaptic strength, for example by tetrodotoxin (Shatz and Stryker, 1988), has not been demonstrated but would test this. Determining the changes during prototypical synaptic pruning events in mice lacking autophagy specifically in the presynaptic compartment would address whether autophagy acts upstream or downstream of changes in presynaptic strength that might trigger synaptic pruning.

Autophagy may also act by mediating synaptic competition. The role of autophagy in controlling membrane trafficking has been clearly demonstrated in dividing and migrating cells (Coly et al., 2017; Sharifi et al., 2016). Thus, autophagy may contribute to changes in synaptic morphology such as axon retraction at the NMJ (Sanes and Lichtman, 1999) or translocation of CF inputs from the PC soma to the proximal dendrites (Hashimoto and Kano, 2013) that contribute to the *competition between synapses* during pruning events. Experiments demonstrating an absence of changes in axonal morphology during these events in the absence of autophagy, or the induction of neuronal autophagy during CF translocation, would provide evidence for this hypothesis.

Finally, microglial autophagy can contribute to the non-neuronal component of synaptic pruning (Kim et al., 2017). Defining the temporal relationship between contributions of neuronal and non-neuronal autophagy may be important for understanding respective roles in synaptic pruning. We suggest the use of a paradigm in which neuronal autophagy alters selective synaptic strengthening or synaptic competition, which may alter the tagging of synapses by complement proteins and phagocytosis by microglia, and may in turn indicate a requirement for autophagy in the proper degradation of synaptic material (Fig. 3). This temporal pattern could be demonstrated by testing whether synaptic strengthening fails to occur while superfluous synapses remain in mice lacking microglial autophagy or complement proteins. An absence of complement receptor coating of synapses in mice lacking neuronal autophagy would support this hypothesis.

In conclusion, the study of autophagy's role in synaptic pruning is at an early stage. However, the decades-long effort to understand the mechanism of synaptic pruning described here can be harnessed to focus these investigations.

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