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How does calcium interact with the cytoskeleton to regulate growth cone motility during axon pathfinding?

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ABSTRACT

The precision with which neurons form connections is crucial for the normal development and function of the nervous system. The development of neuronal circuitry in the nervous system is accomplished by axon pathfinding: a process where growth cones guide axons through the embryonic environment to connect with their appropriate synaptic partners to form functional circuits. Despite intense efforts over many years to understand how this process is regulated, the complete repertoire of molecular mechanisms that govern the growth cone cytoskeleton and hence motility, remain unresolved. A central tenet in the axon guidance field is that calcium signals regulate growth cone behaviours such as extension, turning and pausing by regulating rearrangements of the growth cone cytoskeleton. Here, we provide evidence that not only the amplitude of a calcium signal is critical for growth cone motility but also the source of calcium mobilisation. We provide an example of this idea by demonstrating that manipulation of calcium signalling via L-type voltage gated calcium channels can perturb sensory neuron motility towards a source of netrin-1. Understanding how calcium signals can be transduced to initiate cytoskeletal changes represents a significant gap in our current knowledge of the mechanisms that govern axon guidance, and consequently the formation of functional neural circuits in the developing nervous system.

1. Introduction

During embryogenesis, neurons connect with their synaptic partners to form functional circuits in a process called axon pathfinding. Growth cones, located at the distal tips of growing axons respond or "steer" axons towards substrate-bound and secreted guidance molecules present in the embryonic milieu, thereby connecting the functional neuronal circuitry. Growth cones comprise two key structures that are necessary for this motility: actin-rich antenna-like filopodia that sense guidance cues and membranous lamellipodia that protrude along filopodia to promote axon outgrowth (Fig. 1). These well described behaviours of filopodial extension and lamellipodial engorgement are essential for growth cone motility and are accomplished by cytoskeletal reorganisation *in vitro* and *in vivo* (Raper et al., 1983, 1984; Cohan and Kater, 1986; Wilson and Easter, 1991; Halloran and Kalil, 1994; Polinsky et al., 2000). Crucially, these behaviours are highly sensitive to changes in calcium concentration in the growth cone. For instance, global increases in the spatial distribution and amplitude of calcium control stopping, pausing and collapse (Kater and Mills, 1991; Wen et al., 2004; Tojima et al., 2011) whereas small, spatially localised elevations of calcium can effectively "steer" growth cones *in vitro* (Zheng, 2000). It is now generally accepted that spatial alterations in growth cone calcium concentration can direct axon growth and protrusion.

There is now good evidence that the amplitude, localisation and source of calcium signals, or calcium gradients generated across a growth cone, can regulate attraction and repulsion (Henley et al., 2004; Wen et al., 2004; Ooashi et al., 2005). What is not well understood however, is how the spatial localisation of calcium signals are regulated and ultimately transduced to the cytoskeleton to effect growth cone motility. Moreover, how are calcium signals amplified and sustained sufficiently over an appropriate timescale to orchestrate changes in

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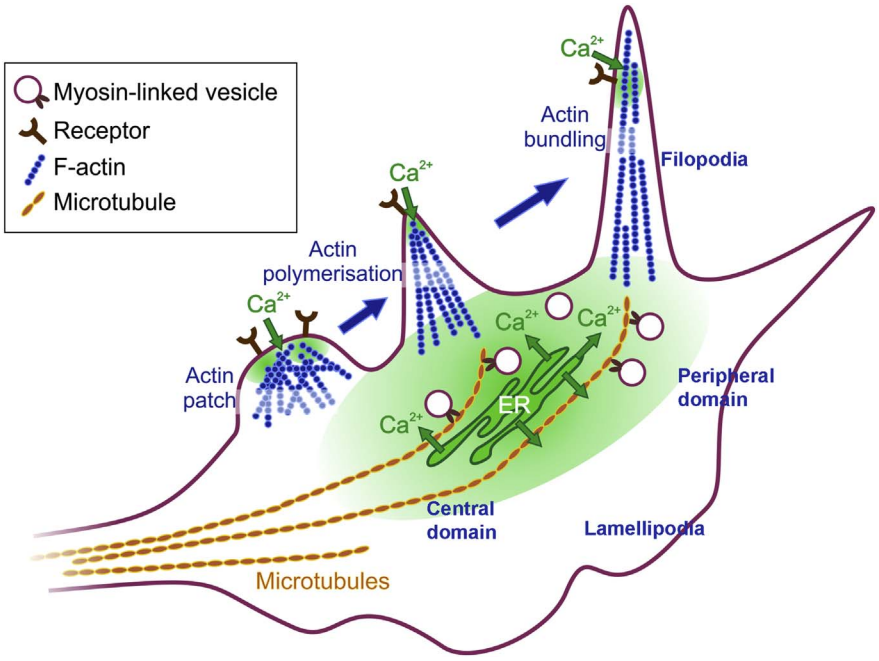

Fig. 1. Asymmetric calcium signals drive lamellipodial and filopodial protrusion. The growth cone at the distal tip of extending axons is mainly comprised of filamentous actin (F-actin) meshworks in lamellipodia and bundles of F-actin in the filopodia, which may arise from actin patches in the lamellipodia. The central domain is rich with microtubules and organelles, such as the ER. Filopodia extend from the peripheral domain. A delicate balance of vesicle endo- and exocytosis regulate membrane addition and withdrawal, necessary for growth cone attraction and repulsion.

cytoskeletal dynamics? This review will focus on describing how calcium signals can alter the cytoskeleton to regulate growth cone motility, especially through the activation or inhibition of phosphorylation-mediated signalling cascades. We will present data obtained using a variety of axon guidance models, from the bag cell neurons of *Aplysia*, sensory neurons of chick and rodent dorsal root ganglia, and *Xenopus* spinal motor neurons. We will also outline recent data that suggests there is a direct link between the endoplasmic reticulum (ER) and the cytoskeleton, as we aim to understand how calcium signals influence the growth cone cytoskeleton and ultimately axon guidance.

2. Intracellular and extracellular calcium signals guide growth cone motility

Intracellular calcium is precisely regulated in growth cones. The complete repertoire of cytosolic buffers that control calcium concentration including sequestration into internal organelles such as the ER, mitochondria and by excretion to the external environment have been elegantly described recently in *Aplysia* (Groten et al., 2013). The integral of cytosolic calcium buffering creates an optimal level of calcium, or "set-point" that regulates growth cone motility (Kater and Mills, 1991; Davenport et al., 1996; Henley and Poo, 2004; Henley et al., 2004). The maintenance of basal calcium levels also relies on scaffold proteins such as homer proteins that control the basal activity of calcium influx channels. Homer proteins gate transient receptor potential channels (TRPC) and inositol-triphosphate receptors (IP_3R) to regulate calcium influx, thereby assisting in the maintenance of basal calcium (Yuan et al., 2003; Gasperini et al., 2009). For example, when homer1b/c expression is reduced, there is an increase in spontaneous calcium transient activity in rat sensory growth cones (Gasperini et al., 2009) which correlates with defects in axon guidance when homer function is perturbed *in vivo* (Foa et al., 2001).

Extracellular calcium entry into growth cones initiates and sustains instructional calcium signals in both the organelle-rich central zone, as well as at the peripheral filopodial tips of growth cones (Gomez et al., 2001; Ooashi et al., 2005; Gasperini et al., 2009; Shim et al., 2013). Extracellular calcium influx *via* voltage gated calcium channels (VGCCs) and TRPC channels is required for growth cone motility in response to a range of guidance cues, including netrin-1 and brain derived neurotrophic factor (BDNF) (Gomez et al., 1995; Shim et al., 2005; Wang and Poo, 2005; Gasperini et al., 2009; Mitchell et al., 2012). However, the complete repertoire of calcium channels activated in growth cones during axon guidance is likely cell and species specific. For example, netrin-1 initiates attraction of *Xenopus* spinal neuron growth cones *via* indirect activation of TRPC and L-type VGCCs (Wang and Poo, 2005). However, in rat sensory neurons, netrin-1 mediated growth cone attraction is less dependent on L-type VGCCs (Fig. 2). We found that inhibition of L-type VGCCs blunts, but does not abolish or reverse growth cone turning towards a gradient of netrin-1 or BDNF. Furthermore, activation of L-type VGCCs with the agonist Bay K8644, abolished turning towards both netrin-1 and BDNF (Fig. 2), suggesting that the source of calcium influx is important and that L-type VGCCs are

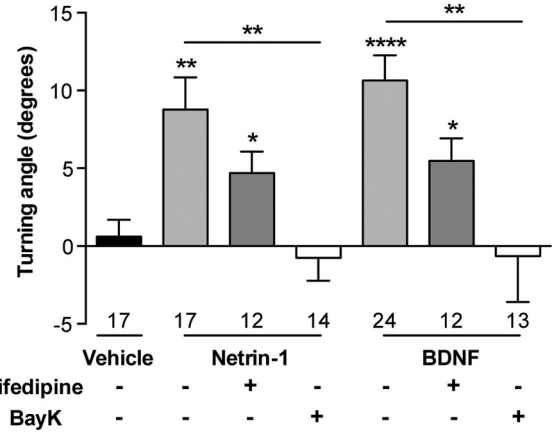

Fig. 2. Growth cone turning towards the guidance cues netrin-1 and BDNF does not require the activation of L-type voltage gated calcium channels (VGCC). Isolated rat sensory growth cones were exposed to a microgradient of soluble netrin-1 or BDNF *in vitro* for 30 min as described previously (Gasperini et al., 2009; Mitchell et al., 2012). The L-type VGCC inhibitor nifedipine or activator BayK8644 (BayK) were bath applied to the primary cultures prior to the start of the turning assay (both used at 5 μM, Alomone Labs, Jerusalem, Israel). The angle of turning was compared between zero and 30 min. A positive angle of turning represents growth cone attraction, a negative angle represents repulsion and an angle of zero represents random growth. The total numbers of growth cones imaged for each condition are shown below each bar (at least 5 different experiments, biological replicates). Error bars represent SEM *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$; compared to vehicle or as indicated with significance bars. Statistics: Mann-Whitney *U* test.

non-essential in rat sensory growth cone turning towards netrin-1 and BDNF. These data support the notion that the context, localisation and source of calcium signals are important for growth cone motility and that calcium channel activation can be cell or species specific. Additionally, the complete molecular repertoire of calcium signalling events may also exist on a developmental spectrum.

Intracellular calcium, particularly ER-derived calcium, is vital for growth cone motility. In response to activation by external guidance cues, an influx of extracellular calcium can trigger the release of calcium from the ER *via* activation of ryanodine receptors (RyRs) in a process known as calcium induced calcium release (CICR) (for review see Berridge, 1998). Transduction of guidance cues may also mobilise the second messenger inositol triphosphate (IP_3) and subsequent calcium release from the ER *via* IP_3 receptors in a process known as IP_3-induced calcium release (IICR) (reviewed in recently in Tojima et al., 2011). These mechanisms are important in determining growth cone turning responses towards netrin-1 and BDNF and in response to contact mediated cues (Gomez et al., 1995; Ooashi et al., 2005; Gasperini et al., 2009; Mitchell et al., 2012). The ER, however represents a finite store of calcium and the question of how calcium signals are sustained during motility remains largely unanswered.

Store operated calcium entry (SOCE) is necessary for axon guidance and neuronal circuit development (Venkiteswaran and Hasan, 2009; Mitchell et al., 2012; Shim et al., 2013). SOCE is activated after ER depletion, likely following CICR or IICR in growth cones (Mitchell et al., 2012). SOCE has been extensively characterised in non-neuronal cells, and is activated by the luminal calcium sensor, stromal interacting molecule 1 (STIM1). Upon ER calcium depletion, STIM1 translocates within the ER to sites close to the plasma membrane where it binds and activates Orai proteins. Upon activation by STIM1, Orai forms highly selective calcium release-activated calcium (CRAC) channels that drive influx of extracellular calcium (Hoth and Penner, 1992; Prakriya and Lewis, 2003; Liou et al., 2005, 2007; Luik et al., 2008). STIM1 is necessary for growth cone responses to guidance cues and interacts with TRP channels (Mitchell et al., 2012; Shim et al., 2013). TRPC1 and STIM1-mediated SOCE are also required for the sustained amplification of filopodial calcium signals in *Xenopus* growth cones *in vitro* and *in vivo* (Shim et al., 2013). These data illustrate the notion that that complex interactions between extracellular and intracellular sources produce finely localised, transient or sustained calcium signals, and that these are necessary to direct growth cone motility. However, there has been some discourse in the literature over whether the source and/or the amplitude of the calcium signal is the primary determinant of growth cone motility (Henley et al., 2004; Wen et al., 2004; Ooashi et al., 2005; Tojima et al., 2014).

3. How are the spatial localisation, amplitude and source of calcium integrated to determine growth cone motility?

The spatial regulation of calcium signals in growth cones remains an intense topic of research and it is likely that the volume of structures in growth cones alters the spatial concentration of calcium. Growth cones are highly dynamic, which might underpin the formation of micro-signalling domains, where interactions between calcium and cytoskeletal regulatory proteins play out (Augustine et al., 2003; Robles et al., 2003). Filopodia are an example of such micro-signalling domains. Filopodia extend and retract to sample extracellular guidance cues during axon pathfinding *in vitro* and *in vivo* (Tosney and Landmesser, 1985; Gomez and Letourneau, 1994; Polinsky et al., 2000). The observation of high concentrations of guidance receptors and ion channels along filopodial shaft and tips (Goodhill and Urbach, 1999; Gomez et al., 2001), supports the notion that amplification of calcium transient signals in filopodia results from their physical volume. For example, filopodial calcium transients generated by non-voltage dependent ion channels regulate growth cone steering in response to substrate-bound guidance cues (Gomez et al., 2001). The exquisite control that the spatial localisation of calcium signals can have over specific growth cone behaviour, was elegantly demonstrated using focal laser induced photolysis (FLIP) to generate microdomains of calcium in *Xenopus* growth cones (Zheng, 2000). Asymmetric activation of calcium on one side of the growth cone robustly induced reorientation of growth cones, mimicking guidance cue-induced attraction. Conversely, in the absence of extracellular calcium, this response was converted into repulsion. These seminal experiments demonstrate that both spatial localisation and amplitude of calcium signalling is instructional for growth cone motility.

The hypothesis that the amplitude of cytosolic calcium is crucial in determining growth cone motility is further supported by the exacting work of Henley et al. (2004). Using precise titration of extracellular calcium combined with ionomycin to alter cytosolic calcium levels, they demonstrated that a tight threshold of calcium can regulate growth cone motility, with high calcium (200 nM) promoting attraction, low calcium (75 nM) promoting repulsion and mid-range (135 nM) causing random growth. These data confirmed the long-standing “set point hypothesis” for growth cone motility (Kater and Mills, 1991). Similarly, Wen et al. (2004) demonstrated that a relatively large increase in intracellular calcium on one side of the growth cone activates calcium-calmodulin-dependent protein kinase II (CaMKII) and subsequent growth cone attraction towards the activated side, while a modest calcium signal activates calcineurin (CaN) and protein-phosphatase 1 (PP1) results in growth cone repulsion. The notion that calcium-binding proteins can detect and respond to differing amplitudes in calcium signals has been recently confirmed in rat hippocampal dendritic spines (Fujii et al., 2013). Using optical uncaging of glutamate at either low (5 Hz) or high (20 Hz) frequency coupled with simultaneous imaging of CaN and CaMKII activity, Fujii and colleagues elegantly showed that CaMKII is only activated when the input frequency of the calcium signal is high. Conversely, CaN is responsive, regardless of frequency, activating readily in response to low frequency transients. The authors described CaMKII as an input frequency/number decoder, and that this decoding role means that CaMKII responds to the overall amplitude of the calcium signal. Conversely, CaN was simply an input number counter, responding to the integral of the overall calcium signal. While this work was performed in dendritic spines, it correlates well with the work of Wen et al. (2004), and supports the concept that CaMKII and CaN respond to defined levels of calcium (Forbes et al., 2012) in restricted domains of growth cone filopodia.

The notion that calcium signal amplitude determines growth cone motility contrasts with research performed by Kamiguchi and colleagues who found that the primary determinant of growth cone steering, was the source of the calcium (Ooashi et al., 2005; Akiyama and Kamiguchi, 2013). One of the first pieces of evidence to suggest that the source of calcium is crucial in determining growth cone motility has come from exploiting growth cone steering in response to contact-mediated guidance cues in neurons from RyR knock-out mice (Ooashi et al., 2005). Activation of RyR-mediated CICR from the ER was required for attractive responses to calcium and cyclic nucleotide-dependent guidance cues N-cadherin or L1, while repulsion in response to contact with laminin resulted when there was a relatively small rise in intracellular calcium in the absence of CICR. This work demonstrated that release of calcium from the ER acts as a molecular switch. The concept of ER as a source of calcium mobilisation that determines growth cone steering is consistent with the demonstration that SOCE sustains calcium signals (Mitchell et al., 2012; Shim et al., 2013). While a tubular network of ER traversing the periphery of growth cones was reported more than three decades ago (Dailey and Bridgman, 1989, 1991), we still do not fully understand how calcium release from the ER is spatially regulated during growth cone motility. Calcium release from the ER and subsequent SOCE increases the amplitude and duration of receptor-mediated calcium signals (Gasperini et al., 2009; Mitchell et al., 2012), suggesting that perhaps source and amplitude of calcium signals are difficult to separate (Tojima et al., 2014) and that growth

cone motility is an integrated response to a multiplicity of calcium fluxes. Ultimately, growth cone steering results from interplay between cytoskeletal dynamics and the spatiotemporal kinetics of calcium ions.

4. Calcium controls the growth cone cytoskeleton by regulating phosphorylation signalling cascades

Early work demonstrated that both actin and microtubules are sensitive to changes in calcium activity. Electron microscopic studies (Lankford and Letourneau, 1989) revealed the close association between intracellular calcium and the actin cytoskeleton where inhibition of neurite extension with calcium ionophores caused actin filament disassembly. These data suggested that calcium-mediated neurite extension is underpinned by a balance between F-actin assembly and disassembly and we now know that directed growth cone motility is initiated by the protrusion and stabilisation of F-actin enriched filopodia (Dailey and Bridgman, 1991; Dent and Kalil, 2001; Steketee and Tosney, 2002; Svitkina et al., 2003). Similarly, calcium dependent regulation of microtubule polymerisation has been shown to be a crucial regulator of growth cone motility in response to guidance cues (Williamson et al., 1996; Challacombe et al., 1997; Buck and Zheng, 2002; Suter et al., 2004). During growth cone navigation, coordinated microtubule polymerisation is necessary to support and stabilise filopodia (Buck and Zheng, 2002), and could act as scaffolds for the transport of calcium signalling machinery such as ER into filopodia (Waterman-Storer and Salmon, 1998). Exactly how calcium regulates actin and microtubules during growth cone steering is still to be fully elucidated.

The key link between calcium and the growth cone cytoskeleton is the regulation of phosphorylation by calcium. Local filopodial calcium transients in *Xenopus* spinal neurons for example, activate the calcium-dependent protease, calpain, to regulate the balance of kinase and phosphatase activity, ultimately stabilising filopodia (Robles et al., 2003). Proteases regulate a wide range of phosphorylation events by decreasing the activity of protein tyrosine kinases such as protein kinase C (PKC), focal adhesion kinases (FAKs) and increasing CaN activity (Kerstein et al., 2017). Cofilin, an important modifier of actin is inactivated by phosphorylation at ser-3 by multiple kinases including LIMK1 (Arber et al., 2000) and reactivated by the phosphatase slingshot (Endo et al., 2003). Exactly how calcium interacts with cofilin was elucidated with *in vitro* experiments in HeLa cells that showed slingshot is activated when dephosphorylated by CaN (Wang et al., 2005). Furthermore, calcium activation of CaMKIV in N2a cells was found to activate LIMK1 (Takemura et al., 2009). Taken together, this work provides a mechanistic link between calcium signals, cofilin function and actin remodelling. Such a model where CaN and CaMKinase interact with cofilin *via* slingshot and LIMK1 (respectively) would help explain how changes in calcium amplitude and the CaMKII-CaN molecular switch (Wen et al., 2004) could regulate growth cone attraction or repulsion.

An important group of proteins that transmit phosphorylation activity to the cytoskeleton are the Rho GTPase proteins Rho, Rac and cell division cycle 42 (Cdc42). The Rho GTPases are activated by a range of proteins including the calcium-activated phosphorylating enzymes CaMKII and PKC (Jin et al., 2005). Early studies have demonstrated that Rho and Rac proteins regulate actin polymerisation necessary for the formation of stress fibers and lamellipodia respectively, while Cdc42 functions in filopodia formation (Ridley and Hall, 1992; Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995). At the growth cone, activation of Rac and Cdc42 is thought to promote axon extension and stabilisation, while RhoA activity favours growth cone retraction (Hall, 1998; Jin et al., 2005). Localised elevations of calcium can activate Cdc42/Rac, while inactivating RhoA in a transient manner to provide directional cues for cytoskeletal reorganisation in response to guidance cues (Jin et al., 2005). Interestingly, Zhang and Forscher (2009) demonstrated that Rac1 can promote ER and microtubule protrusion into peripheral areas of growth cones, which are important sites for extending filopodia and guidance cue detection. This Rac1 activity was in parallel to guidance cue-activated IICR, suggesting a mechanism whereby Rac1 regulates calcium signal localisation (Zhang and Forscher, 2009). The transduction of GTPase activity to the cytoskeleton involves further phosphorylating enzymes, such as Src and p21-activated kinases (PAK). PAK is described as a cytoskeletal regulator that can transduce Src and Cdc42 signals into increased actin polymerisation (Robles et al., 2005). Collectively, these studies describe a complex array of calcium-activated phosphorylation events that converge to regulate the cytoskeleton during growth cone motility (for a comprehensive review, see Gonzalez-Billault et al., 2012).

5. Calcium activated phosphorylation regulates plasma membrane remodelling

Dynamic remodelling of the plasma membrane as growth cones navigate the cellular environment underpins motility. Motility requires the coordinated assembly of cytoskeletal scaffolds within the growth cone along which membrane protrusion and expansion (Tosney and Landmesser, 1985; Steketee and Tosney, 1999; Polinsky et al., 2000). Directed motility therefore, can be thought of as the integral of highly regulated calcium-dependent signalling events in the cytosol that drive a balance of membrane protrusion and retraction. Asymmetric CICR promotes attraction (Ooashi et al., 2005) and regulates the exocytosis of vesicle associated membrane protein 2 (VAMP2) from the growth cone peripheral domain in a microtubule-dependent manner (Tojima et al., 2007). In a similar way, the protrusion of microtubules into the growth cone peripheral zone, thus facilitating vesicle exocytosis, appears to be a common downstream mechanism of both IICR and CICR, where calcium activates phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3K), a key enzyme that converts phosphatidylinositol 4,5-bisphosphate (PiP_2) to phosphatidylinositol-3,4,5-triphosphate (PiP_3). Exactly how PiP_3 regulates microtubule dynamics is not well understood, but through the regulation of kinases such as Akt (protein kinase B), it is believed to be an important regulator of neuronal orientation and cell polarity (Akiyama and Kamiguchi, 2010). Interestingly, microtubules are not the only substrates for PI3K and PiP_3 involved in growth cone extension. PI3K and PiP_3 are also implicated in actin regulation and the formation of new branches in navigating axons. Nerve growth factor (NGF) has been shown to activate PI3K, which is thought to recruit the actin nucleating Arp2/3 complex, to generate new actin filaments, and initiation of filopodia from actin patches in growing axons (Fig. 1) (Spillane et al., 2011). The mechanisms that govern the specificity of action for enzymes such as PI3K are yet to be determined, but may be important in the spatial localisation of calcium signals activated by external cues such as NGF.

Growth cone motility requires a balance of vesicle exocytosis for growth cone attraction and endocytosis for growth cone repulsion (Tojima et al., 2007, 2010, 2014). Semaphorin-3a (sema-3a) is an important repulsive guidance cue that elicits small changes in intracellular calcium and subsequent growth cone repulsion (Togashi et al., 2008). The molecular switch model proposed by Wen et al. (2004) suggests that sema-3a activates CaN to mediate repulsion. Work by Tojima et al. (2010) confirmed this notion by showing that sema-3a repulsion requires clathrin and dynamin-dependent endocytosis that can be blocked with CaN inhibition. Furthermore, inhibition of clathrin-mediated endocytosis reversed growth cone steering, resulting in attraction to sema-3a (Tojima et al., 2010). These experiments suggest that the balance between calcium-mediated membrane addition and withdrawal is a conserved process that alters bidirectional turning of growth cones. Consistent with this idea, the repulsive guidance cue myelin associated glycoprotein (MAG) also induces calcium-mediated, clathrin-dependent endocytosis in *Xenopus* spinal neurons (Hines et al., 2010). Endocytic vesicles containing β1-integrin receptors, the key component of focal adhesions which physically connect the cytoskeleton with extracellular

substrates, were crucial in overall plasma membrane protrusion. An asymmetric gradient of MAG was sufficient to induce endocytosis and spatial redistribution of β1-integrin. This redistribution of β1-integrin was necessary for repulsive growth cone turning away from the source of MAG (Hines et al., 2010). MAG was subsequently shown to activate the phosphatase and tensin homologue (PTEN), resulting in decreased PiP_3 levels, and β1-integrin adhesions (Henle et al., 2013). This work, along with that of Akiyama and Kamiguchi (2010) would suggest that in growth cones, there exists a finely-tuned balance between the activity of PI3K and PTEN to regulate the activity of the signalling lipids.

The phosphorylation studies outlined in this review have provided insights into an important set of downstream mechanisms that regulate directed growth cone steering, however it is likely that the complete repertoire of calcium-dependent phosphorylation events is much more complex. Using chick sensory neuron growth cones, Kamiguchi and colleagues examined the role of phosphatidylinositol 4-phosphate 5-kinase type-1γ (PIPKIγ90), CaMKII and cyclin dependent kinase 5 (Cdk5) in growth cone motility (Tojima et al., 2014). All three proteins are highly conserved across species and are important in neuronal development and synaptic function. In these experiments, spatially restricted photolysis of caged calcium activated the dephosphorylation of PIPKIγ90 by CaN and promoted clathrin-mediated endocytosis and growth cone repulsion. Conversely, spatially restricted CICR provided attractive calcium signals that activated CaMKII, which phosphorylated Cdk5 and subsequently inhibited PIPKIγ90, thus promoting VAMP2-mediated exocytosis (Tojima et al., 2014). By manipulating attractive and repulsive calcium signals and regulating phosphorylation activity *via* PIPKIγ90, CaMKII or Cdk5, the authors demonstrated that a crucial balance between calcium-dependent membrane endocytosis and exocytosis regulates growth cone motility (Fig. 1).

More recently, Kamiguchi and colleagues extended their work on vesicle exocytosis to VAMP7, another vesicle membrane associated protein (Akiyama et al., 2016). These experiments suggest a regulation between membrane exocytosis, the cytoskeleton and the cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Cyclic nucleotides have long been regarded as key second messenger signals in growth cone motility (Lohof et al., 1992) (for an excellent early review see Song and Poo, 1999). While early studies suggested that cyclic nucleotides regulate bidirectional growth cone turning with attractive cues activating cAMP and repulsive cues activating cGMP (Song et al., 1997), the demonstration of a direct interaction between cyclic nucleotides and the cytoskeleton has been elusive. Akiyama et al. (2016) demonstrated that microtubules induce lamellipodial protrusion in a VAMP7-dependent manner, which was differentially regulated by cAMP and cGMP. The protrusion of microtubules and their VAMP7 cargo into the growth cone peripheral domain was promoted by cAMP and repressed by cGMP. Interestingly, they found that cyclic nucleotide signals, but not direct activation of CICR could modulate VAMP7 transport to the periphery. In contrast, VAMP2 was transported in response to CICR, but not cAMP activation, suggesting that distinct downstream cytoskeletal machinery is activated in response to calcium and cyclic nucleotide signals (Akiyama et al., 2016). It is now apparent from these studies that we are still some way off understanding how cyclic nucleotides and their downstream kinases interact with calcium signals to regulate the cytoskeleton during motility.

6. The ER and SOCE: connecting calcium to the cytoskeleton?

The ER and regulators of SOCE may link calcium to the growth cone cytoskeleton. The ER is thought to be a continuous network of tubules throughout neurons, forming contacts with organelles and the plasma membrane (Wu et al., 2017). Recently, Kamiguchi and colleagues extended their work on vesicle exocytosis, by demonstrating a more direct link between exocytosis and calcium release from the ER in chick growth cones (Wada et al., 2016). Consistent with a role for microtubule motor proteins in trafficking the ER to dendritic spines (Wagner et al., 2010), myosin-Va was shown to simultaneously bind VAMP2 and ryanodine receptors on the ER (Wada et al., 2016). This interaction resulted in the tethering of membrane vesicles to the ER. The activation of CICR caused myosin-VA to dissociate from RyRs and release VAMP2, promoting exocytosis. Importantly, vesicles were released on the side of the growth cone closest to CICR activation. This spatial localisation promotes membrane exocytosis and is necessary for growth cones growing towards attractive cues (Wada et al., 2016). These data provide evidence for a direct interaction between the ER and the cytoskeleton in the regulation of membrane recycling, necessary for growth cone motility.

The actin cytoskeleton may also directly interact with the ER *via* drebrin, a key actin-binding protein implicated in growth cone motility. Drebrin potently induces bundling of F-actin in a variety of cells (Dun and Chilton, 2010), as well as remodelling growth cone morphology and directing neuronal migration (Geraldo et al., 2008; Dun et al., 2012). The immunosuppressant agent 3,5-(bis(trifluoromethyl)pyrazole) (BTP) inhibits store-operated calcium release and binds drebrin, inhibiting its effects on actin reorganisation. These actions are likely to be linked since the loss of drebrin expression or treatment with BTP both result in decreased release of stored calcium in immune cells (Mercer et al., 2010). Interestingly, the authors suggest that drebrin regulates SOCE through its reported binding to homer proteins (Shiraishi et al., 2003) and TRPC channels (Goel et al., 2005), both of which facilitate calcium influx and have been linked to store-operated calcium entry in growth cones. Both Homer and TRPCs are required for growth cone steering in a variety of neurons (Foa et al., 2001; Wang and Poo, 2005; Gasperini et al., 2009), supporting the idea that drebrin may provide a direct link between calcium signalling and the actin cytoskeleton in growth cones.

A role for drebrin and store-operated calcium signalling might be further supported due to the known interaction between drebrin and the microtubule-end binding protein 3 (EB3) (Geraldo et al., 2008). The drebrin-EB3 interaction is crucial for filopodial initiation and in this role, drebrin is thought to act as a mediator between the actin and microtubule cytoskeleton (Ketschek et al., 2016). Drebrin is required for microtubule advance into filopodia, a mechanism regulated by a direct interaction between EB3-bound microtubules and drebrin-bound actin filaments (Geraldo et al., 2008; Ketschek et al., 2016). This interaction, which couples microtubules and actin filaments through EB3-drebrin binding, is not only vital for appropriate filopodial dynamics during development, but also mediates microtubule insertion into dendritic spines in a calcium dependent manner (Merriam et al., 2013; Gordon-Weeks, 2016). Interestingly, STIM1, a key regulator of store-operated calcium entry in growth cones also binds EB3 (Grigoriev et al., 2008) and hence a potential link between drebrin-EB3-STIM1 may provide a further direct link between calcium signalling and the cytoskeleton. Such a mechanism would be consistent with the idea that the ER is a crucial source of calcium in growth cone steering (Ooashi et al., 2005; Akiyama and Kamiguchi, 2010). Additional interactions between SOCE and the cytoskeleton have been described in non-neuronal cells. For example, STIM1 and Orai colocalise with cortactin, a regulator of actin, in membrane ruffles at the leading edge of mesenchymal cells (Lopez-Guerrero et al., 2017) and STIM1 has been shown to regulate focal adhesion kinases in migrating fibroblasts and HEK293 cells (Yang et al., 2009; Schäfer et al., 2012).

7. Summary

One of the most consistent and highly conserved signalling mechanisms in axon guidance described so far is the crucial role that cytosolic calcium plays in growth cone motility. However, the complete repertoire of biophysical mechanisms responsible for the unerring precision and fidelity of axon guidance have yet to be fully elucidated. Second messengers such as calcium effectively transduce guidance cues

to the cytoskeleton by regulating the delicate interplay of opposing physical forces such as membrane exocytosis and endocytosis to drive growth cone motility. Many of the downstream phosphorylation events that activate cytoskeletal rearrangements have been elucidated, although the final links with actin and microtubules are still unclear. Interactions between the cytoskeleton and the ER suggest that more direct links between calcium signals and the cytoskeleton may regulate motility, however since much of this work has been done in non-neuronal cells, the significance of these mechanisms in neuronal growth cones remains unclear. Understanding how calcium interacts with the growth cone cytoskeleton will move us an important step closer to understanding the factors that govern circuit formation and nervous system plasticity.

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