Mol Neurobiol (2014) 49:1077–1086
DOI 10.1007/s12035-013-8582-8

Switching Responses: Spatial and Temporal Regulators of Axon Guidance

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Received: 16 September 2013 / Accepted: 31 October 2013 / Published online: 24 November 2013

Abstract The ability of the axonal growth cone to switch between attraction and repulsion in response to guidance cues in the extracellular environment during nervous system development is fundamental to the precise wiring of complex neural circuits. Regulation of cell-surface receptors by means of transcriptional control, local translation, trafficking and proteolytic processing are powerful mechanisms to regulate the response of the growth cone. Important work has also revealed how intracellular signalling pathways, including calcium and cyclic nucleotide signalling, can alter the directional response elicited by a particular cue. Here, we describe how these multiple regulatory mechanisms influence growth cone turning behaviour. We focus on recent evidence that suggests a significant role for 14-3-3 adaptor proteins in modifying growth cone turning behaviour and mediating directional polarity switches during development. Characterizing how 14-3-3 s regulate growth cone signalling will provide invaluable insight into nervous system development and may facilitate the identification of novel targets for promoting nerve regeneration following injury.

Keywords Axon guidance · 14-3-3 · Growth cone · Commissural neuron · Local translation

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Introduction

Precise regulation of growth cone responses is critical for the complex navigational decisions that axons face as they project over long distances to establish neuronal circuits [1–3]. Developmental switches in response to guidance cues are a common feature of nervous system development and a complex array of molecular mechanisms underlie these switches. The trajectory of commissural neurons, which read attractive and repulsive gradients to navigate towards the floor plate, cross the midline, then position themselves laterally and turn to grow along the longitudinal axis, beautifully exemplifies these dynamic responses [4]. Both intracellular and extracellular signalling molecules can interact to switch the polarity of the directional response elicited by guidance cues. Characterizing these pathways is of critical importance to our understanding of nervous system development and may contribute to the conception of therapeutic strategies to promote regeneration after injury. A number of recent articles can be consulted for critical insight into broad themes of molecular regulation of axon guidance [5–9]. This review covers specific mechanisms that have been discovered to switch the directional polarity of growth cone responses, including regulation of cell-surface receptors, intracellular calcium and cyclic nucleotides. We focus on recent studies that have identified a role for 14-3-3 adaptor proteins in regulating axon guidance and polarity switching in vitro and in vivo. These studies support the idea that 14-3-3 proteins function as molecular switches to

regulate growth cone turning responses during development and raise the interesting possibility that they may be targeted to promote nerve cell repair following injury.

Receptor Expression, Localization and Processing

The ability of the growth cone to direct axon extension in response to an extracellular cue depends on the binding of the ligand to a receptor expressed on the surface of the growth cone. Accordingly, a fundamental mechanism to regulate growth cone responses is by regulating the expression, processing and localization of guidance cue receptors within the plasma membrane of the growth cone (Fig. 1). The complement of guidance cue receptors expressed on a neuron is regulated at the expression level through both transcriptional and translational control while the cell surface availability is regulated through a variety of mechanisms including proteolytic cleavage, trafficking and endocytosis.

Receptor Expression

Axon guidance decisions are often predicated on the distinct expression pattern of receptors at the cell surface. The projection of commissural axons across the midline relies on the ability of pre-crossing commissural neurons to respond in an attractive manner towards the floor plate, while post-crossing axons must be repelled from the floor plate to prevent re-crossing. The switch in responsiveness to the floor plate relies in part on the attractive response of pre-crossing axons to Netrin and subsequent responsiveness of post-crossing axons to repellents such as Slit. Netrin attracts commissural axons to the midline by binding to its receptor, deleted in colorectal cancer (DCC) [10, 11]. The expression of DCC on pre-crossing commissural axons is critical for attraction. Subsequent to midline crossing, DCC-expressing commissural axons lose responsiveness to Netrin. In Xenopus, the formation of a complex between DCC and the slit receptor Roundabout (Robo) silences the attractive response to Netrin and permits a repulsive response to the midline repellent Slit2. This eliminates conflicting turning behaviour and facilitates definitive exit from the floor plate [12]. The regulation of Robo activity in commissural axons relies on the coordinated expression of three unique Robo genes, *Robo1*, *Robo2* and *Robo3*. A splice variant of Robo3 (Robo3.1) is required for midline crossing [13, 14]. Pre-crossing axons express both Robo3.1 and low levels of Robo1. Blocking Robo3.1 expression increases Slit responsiveness, however this has no effect on the level of Robo1 expression in pre-crossing axons, indicating that Robo3.1 serves to mediate growth towards the midline by suppressing Robo1-mediated Slit repulsion [13]. The precise spatial and temporal control of expression of multiple receptors is therefore critical for conveying guidance information to these axons. In neurons that express both the Netrin receptors DCC and Unc-5, such as trochlear motor neurons or cerebellar granule neurons, Netrin is interpreted as a repellent cue [15, 16]. Interestingly, expression of Unc-5 in DCC-expressing cells is sufficient to convert attraction to repulsion but the repulsion remains dependent on DCC expression [17]. Short-range Netrin repulsion can be mediated by Unc-5 alone, whereas repulsion by the DCC/Unc-5 complex occurs developmentally over longer distances [18]. More comprehensive overviews of transcriptional regulation in axon guidance have also been published recently [19, 20].

Recent work supports the idea that local translation also plays an important role in altering turning responses over time. The capacity of the growth cone to locally translate and traffic proteins to the cell surface was elegantly demonstrated in a study in which RNAs encoding fluorescent reporters and membrane-anchored alkaline phosphatase (AP) were introduced into isolated growth cones from retinal explants [21]. The detection of reporter fluorescence as well as AP reactivity in individual growth cones provided evidence for the presence of the machinery required for both translation and trafficking of proteins to the cell surface. Supporting the idea that local translation of guidance cue receptors has a function in axon guidance, the study further demonstrated an upregulation of the EphA2 receptor in the growth cones of spinal commissural axons upon midline crossing. Interestingly, the upregulation of EphA2 was shown to be dependent on the cytoplasmic polyadenylation element (CPE) within its 3′ UTR, suggesting that CPEs and CPE binding protein (CPEB) may be important regulators of local translation at the growth cone [21]. Recent studies have identified crucial roles for post-transcriptional regulation of receptor mRNAs in mediating switching events during axon guidance. In post-crossing spinal commissural axons, the extent of lateral repulsion away from the floor plate is locally tuned by nonsense-mediated decay (NMD) of the Robo3 splice isoform *Robo3.2* upon exposure to the floor plate. Mice deficient in NMD machinery have increased expression of Robo3.2 in post-crossing commissural axons and these axons are laterally repelled to a greater distance from the floor plate [22]. In precerebellar neurons, the RNA-binding protein Musashi1 has been identified as a factor that is critical for translation of *Robo3/Rig1* and is required for midline crossing [23]. While examples of local translation of receptor mRNAs in vivo are limited, it is reasonable to speculate that further research into this area will identify roles for local translation in mediating growth cone switching. Interestingly, laser-capture microdissection and microarray analysis have revealed age-dependent differential localization of mRNAs in pathfinding retinal ganglion cell (RGC) growth cones, suggesting a potential role for local translation in regulating axon guidance events [24].

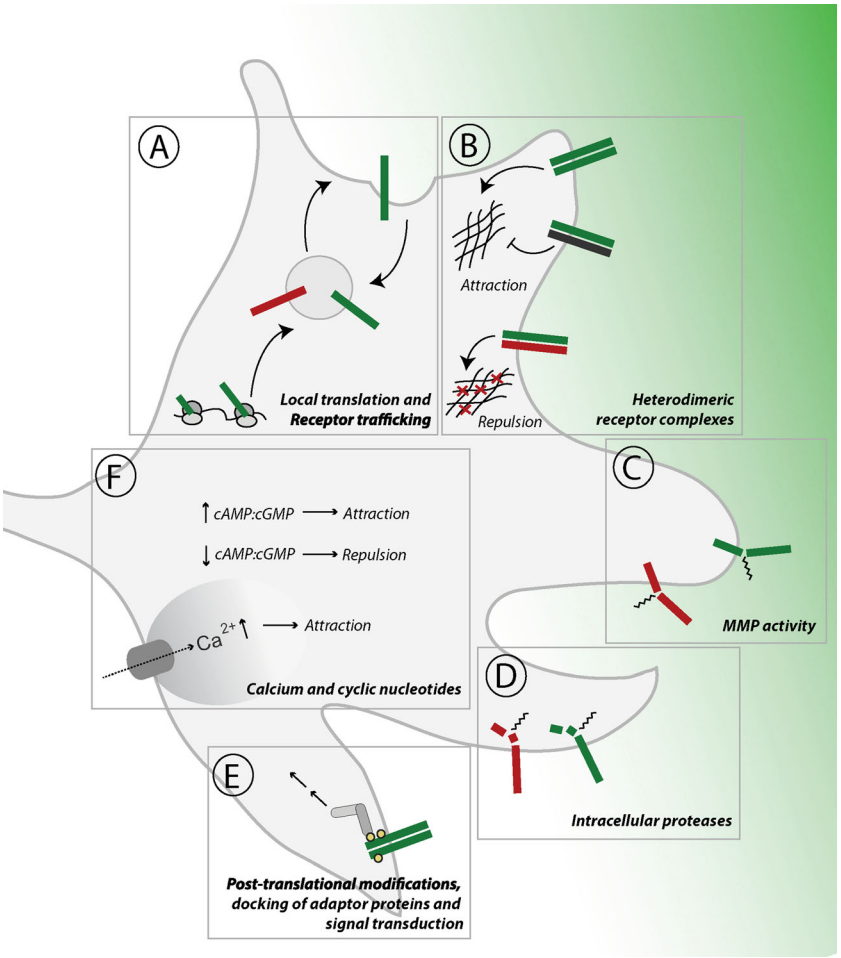

Fig. 1 Spatial and temporal regulation of receptors and intracellular signalling pathways impacts growth cone guidance. **a** Local translation and insertion of receptors into the plasma membrane can regulate the sensitivity of the growth cone to gradients of guidance cues in the environment. **b** Receptors that form heterodimeric complexes can be modulated by co-receptors that can either silence or reverse the direction of turning. **c** Extracellular processing of receptors by secreted and membrane-associated proteases such as MMPs can reduce receptor availability. **d** Intracellular processing by cytosolic proteases can silence intracellular signal transduction. **e** Post-translational modification (e.g., phosphorylation) of guidance receptors and docking of cytosolic adaptor proteins can influence signal transduction and receptor activity. **f** Calcium influx induced by some guidance cues generates an intracellular gradient that often promotes an attractive response. A high cAMP/cGMP ratio within the cell generally promotes attractive responses, while low cAMP levels and increased cGMP levels promote repulsion

Receptor Processing at the Cell Surface

Regulated expression of different combinations of guidance cue receptors provides important spatial and temporal sensitivity to extracellular ligands, but the level of precision required in regulating the signalling pathways downstream of these receptors often involves mechanisms that must act locally at the growth cone in short time frames. This can be achieved by proteolytic cleavage, endocytosis, and directed trafficking of receptors. These mechanisms can establish exquisite control of guidance signalling in individual growth cones.

The targeted enzymatic cleavage of receptors by proteases is a powerful regulatory mechanism that can have a wide variety of effects. Receptors can be cleared from the cell surface rendering a cell insensitive to the cognate ligand, or cleaved to generate unique extra- or intracellular signalling fragments that function as dominant negative receptor bodies or downstream effectors. Axon guidance defects in Drosophila mutants lacking the kuzbanian gene first suggested a role for proteases in axon guidance [25]. Kuzbanian or ADAM10 is an A Disintegrin And Metalloproteinase (ADAM) family metalloproteinase. Follow-up studies determined that Eph receptor binding to Ephrin2 initiates ADAM10-dependent cleavage of EphrinA2. EphrinA2 cleavage allows cellular detachment and converts an adhesive signal to a repulsive signal and subsequent neurite withdrawal [26, 27]. Vertebrate commissural neurons are also sensitive to the effects of proteases. Treatment of rat dorsal spinal cord explants with a metalloproteinase inhibitor potentiates the outgrowth-promoting effect of Netrin and increases DCC immunoreactivity, suggesting that metalloproteinase-

mediated processing may reduce the amount of available DCC. In support of this hypothesis, the presence of a DCC fragment corresponding to the molecular weight of the ectodomain was detected in the medium from dissociated dorsal spinal cord cultures, but was absent in cultures that had been treated with a metalloproteinase inhibitor [28]. A role for proteolytic processing in mediating growth cone response switches was recently highlighted by a study which showed that pre-crossing commissural axons can resist the repulsive effects of the midline repellent Sema3B through intracellular calpain-mediated cleavage of the Sema3B co-receptor, PlexinA1. Following midline crossing, sensitivity to Sema3B is acquired by suppression of calpain activity mediated by midline factors including glial cell-derived neurotrophic factor (GDNF) and neuronal cell adhesion molecule (NrCAM) [29, 30].

Responsiveness to guidance cues can be dampened by targeted endocytosis and internalization of specific receptors. For example, stimulation of dorsal root ganglion neurons with Sema3A induces the endocytosis of the Plexin and Neuropilin1 receptors [31]. The internalization of these receptor complexes is mediated, in part, by L1, a member of the IgG superfamily of cell adhesion molecules (IgSFCAM) [32]. The regulation of L1 by other IgSFCAMs in various axon guidance contexts can lead to rapid desensitization or conversion to attractive responses to Sema3A by enhancing or blocking the endocytosis response [33]. Netrin-dependent repulsion can also be converted to attraction through protein kinase C alpha-dependent endocytosis of Unc-5A in mouse cerebellar granule neurons [34]. Precise regulation of cell surface distribution and stability of guidance cue receptors at the growth cone therefore represent crucial mechanisms to mediate developmental switches in axon guidance.

Calcium Signalling

While guidance cue receptors are common targets for regulation of growth cone responses, the direction turned by the growth cone upon ligand binding also depends, in large part, on the state of diverse intracellular signalling mechanisms. Changes in intracellular calcium ion concentration, ($[Ca^{2+}]_I$), occur in many cellular contexts and provide a means to convey rapid, frequency based signals globally or in highly localized micro- or nano-domains. $[Ca^{2+}]_I$ was identified as a strong candidate for regulating dynamic axon guidance responses when temporal $[Ca^{2+}]_I$ fluctuations were shown to have important effects on the rate of axon outgrowth and growth cone motility [35]. Ratiometric imaging of Ca^{2+} sensitive fluorophores in growth cones of Xenopus spinal neurons revealed that Netrin-1 gradients induce attractive turning and a corresponding $[Ca^{2+}]_I$ gradient, with high $[Ca^{2+}]_I$ on the side of the growth cone exposed to Netrin-1 [36]. This $[Ca^{2+}]_I$ gradient is necessary for the turning response and can be abolished by blocking membrane Ca^{2+} channels or depleting intracellular Ca^{2+} stores. Further studies demonstrated that a $[Ca^{2+}]_I$ gradient was also sufficient to induce growth cone turning. Use of focal laser induced photolysis (FLIP) to asymmetrically uncage Ca^{2+} in the growth cone, local and transient increases in $[Ca^{2+}]_I$ were shown to drive turning in the direction of the increased $[Ca^{2+}]_I$ [37]. Further, altering $[Ca^{2+}]_I$ signalling leads to changes in turning responses. Reducing available Ca^{2+}, either by blocking L-type voltage dependent calcium channels (L-VDCC) or closing ryanodine receptors (RyR), responsible for Ca^{2+} induced Ca^{2+} release (CICR), switches the Netrin-1 turning response from attraction to repulsion [36]. Furthermore, reducing resting levels of $[Ca^{2+}]_I$ converted FLIP-induced attractive turning to repulsion [37] and high frequency $[Ca^{2+}]_I$ transients in growth cone filopodia also induce repellent turning [38]. These findings suggested a model whereby binding of a guidance cue induces a localized change in $[Ca^{2+}]_I$ towards the side of ligand binding, but that the direction of the resulting turn is determined by the amplitude of the $[Ca^{2+}]_I$ signal, with low amplitude $[Ca^{2+}]_I$ changes resulting in repulsion and high amplitude $[Ca^{2+}]_I$ changes inducing attraction. This model is supported by experiments examining the bi-directional turning response to gradients of myelin-associated glycoprotein (MAG). Normally, repellent responses to MAG induce a $[Ca^{2+}]_I$ gradient through CICR from intracellular stores; however, depolarizing the cell, which drives up CICR and $[Ca^{2+}]_I$, converts MAG turning to attraction [39]. Furthermore, asymmetric $[Ca^{2+}]_I$ induced by a gradient of ionomycin is sufficient to induce growth cone turning. The direction of turning can be controlled by increasing or decreasing the concentration of Ca^{2+} in the medium to achieve attraction or repulsion, respectively [39].

The mechanisms by which the growth cone differentiates between a high amplitude and low amplitude $[Ca^{2+}]_I$ signal to either turn towards or away from an external cue remain poorly understood, but a few downstream effectors have been characterized. One model is that low amplitude $[Ca^{2+}]_I$ signals activate a calcineurin/protein phosphatase 1 axis that is required for repulsive guidance, while high amplitude signals preferentially activate calmodulin-dependent kinase II (CaMKII) and attractive turning [40]. This idea is supported by the fact that Calcineurin activation is known to require much lower $[Ca^{2+}]_I$ than CaMKII. More recent studies have focused on the impact of direct manipulation of $[Ca^{2+}]_I$ and second messengers on growth cone membrane organization through the regulation of vesicle trafficking. Processes which have been explored include vesicle associated membrane protein 2 (VAMP2)-dependent exocytosis and clathrin-mediated endocytosis [41]. While direct links to axon guidance events in vivo remain to be established, the ability of $[Ca^{2+}]_I$ signalling to establish bi-directional turning responses

by controlling the balance of exocytosis and endocytosis is a potentially powerful mechanistic basis for these complex signalling events. The introduction of optogenetics and two-photon microscopy should open the door to linking our understanding of $[Ca^{2+}]_I$ signalling to the temporal and spatial regulation of axon guidance decisions that occur in vivo.

Cyclic Nucleotides and Crosstalk

One of the first indications that the turning response of a growth cone to a gradient of an extracellular cue is subject to regulation by the state of intracellular signalling mechanisms came from efforts to understand downstream signalling cascades that differentiate attractive turning from repellent turning. Studies using gradients of attractive cues such as brain-derived neurotrophic factor (BDNF) and Netrin-1 [42, 43], as well as repellents such as MAG and Sema3 [44], applied to Xenopus spinal neurons, revealed that attractive cues can be converted to repellent ones by blocking the activity of cyclic nucleotide signalling and conversely repellent cues can induce attractive responses if cyclic nucleotide signalling activity is elevated. These early studies established a simple grouping of guidance cues. Type I cues are those subject to regulation by the activity of cyclic adenosine monophosphate (cAMP) and its effectors, such as cAMP-dependent PKA, and type II cues are those regulated by cyclic guanine monophosphate (cGMP) and its effectors such as cGMP-dependent protein kinase (PKG) [44–46]. It has now been well established that cyclic nucleotide regulation of guidance cue responses provides a critical mechanism for axon guidance decisions in vivo. RGCs are initially attracted to Netrin at the optic nerve head; however, as they continue to project along the optic nerve, this initial attraction switches to repulsion and this is mediated by a drop in cAMP levels which is both intrinsic [47] and driven by the increased presence of laminin-1 [48].

Much of the work investigating the manner in which cyclic nucleotides can induce a switch in turning polarity has focused on the crosstalk between cyclic nucleotides and other intracellular signals such as $[Ca^{2+}]_I$ and the Rho GTPases. The formation of $[Ca^{2+}]_I$ gradients in the growth cone through CICR, is dependent on the activation of RyRs and down-regulating PKA activity is sufficient to block this $[Ca^{2+}]_I$ signal [36]. This finding led to the suggestion that cAMP switching could be mediated through altering CICR. Further pharmacological studies in Xenopus spinal neurons revealed that both cAMP and cGMP pathways could regulate $[Ca^{2+}]_I$ signals [39, 49] through a variety of mechanisms including direct modulation of L-type VDCCs [49], cAMP positive regulation [50] or cGMP negative regulation [51] of RyRs, and effecting membrane potential through gating ion channels [52, 53]. These results indicate that rather than two types of guidance cues, one being responsive to cAMP and the other to cGMP, both pathways are involved in turning, and the critical factor for determining the response polarity is the cAMP/cGMP ratio. A high ratio appears to amplify $[Ca^{2+}]_I$ signals and lead to attraction and a low ratio dampens $[Ca^{2+}]_I$ signals and favours repellent turning [41, 49]. Interactions between cAMP and cGMP in neurons [54] and the antagonistic relationship they appear to have in growth cone signalling [49] has led to the proposal that cAMP and cGMP gradients may cross-repress each other across the growth cone, to amplify the initial asymmetric binding of a guidance cue, thereby defining protruding and retracting domains of the growth cone [41]. Using optogenetic manipulations of $[Ca^{2+}]_I$ and cAMP, one study found that cAMP signals in response to Netrin-1 are transient and precede changes in $[Ca^{2+}]_I$ signals. Interestingly, while cAMP transients occur throughout the growth cone, only those confined to the filopodia of the growth cone periphery are able to induce attractive turning [55].

The importance of investigating the role of cyclic nucleotide signalling in growth cones is highlighted by studies demonstrating that the elevated levels of cAMP in younger neurons underlie their ability to grow on normally inhibitory substrates such as MAG or myelin. Treatments to elevate cAMP in older neurons, and activate PKA, result in increased axon growth on inhibitory substrates and regeneration of axons in the spinal cord after injury [56, 57] as well as increased functional recovery [58]. Further work on this critical regulatory pathway can build on the promise of these early findings.

14-3-3s: An Emerging Mediator of Growth Cone Switching

A number of recent studies have revealed a role for 14-3-3 adaptor proteins in regulating growth cone responses to guidance cues. The 14-3-3 s are an abundant, highly conserved, family of adaptor proteins expressed in all eukaryotic organisms. They bind to serine and threonine –phosphorylated proteins and have been shown to regulate many aspects of cell signalling involved in diverse processes, including metabolism, cell cycle control, apoptosis, trafficking and cytoskeletal organization [59]. Their involvement in such a wide array of cellular processes has led to the extensive study of these proteins, with a more recent focus on their roles in axon guidance.

14-3-3 Proteins

In mammals, the 14-3-3 adaptor protein family consists of seven isoforms, (β/α,ζ/δ,ε,γ,η,τ/Φ,and σ) encoded by unique genes [60], with α and δ being identified as the

phosphorylated forms of β and ζ, respectively [61]. Structural studies have shown that 14-3-3 s readily form homo- and heterodimers, with each monomeric unit consisting of a bundle of nine α-helices [62, 63]. These helices are organized to form a highly conserved concave, amphipathic ligand-binding groove, as well as a surface region critical for dimerization [64]. Binding of 14-3-3 s to their targets is conferred by both a primary and secondary interaction. The primary interaction consists of binding to the phosphopeptide within the amphipathic groove [64]. Phosphorylation-independent binding of 14-3-3 s to targets has also been reported to occur at glutamic acid and cysteine-rich sequences [65]. While a number of 14-3-3 interactions have been shown to occur with equal affinity amongst several isoforms [66], there is increasing evidence, both biochemically and in vivo, that isoform specificity is functionally important, allowing for the integration of different targets into signalling complexes [67].

The Role of 14-3-3s in Growth Cone Signalling

The importance of 14-3-3 proteins in neural development was initially highlighted by the finding that 14-3-3 ε knockout mice have severe defects in cortical layer formation, closely resembling the pathology seen in lissencephaly, or "smooth-brain" disease [68]. The link between 14-3-3 ε and cortical development is also supported in genetic studies of humans suffering from Miller–Dieker syndrome (MDS), a severe form of lissencephaly, which is characterized by deletions in chromosome 17p13.3, a region that encodes 14-3-3 ε [69]. Further molecular characterization of the role of 14-3-3 ε in MDS revealed direct binding to phosphorylated nuclear distribution protein nudE-like 1 (NUDEL) and the formation of a complex consisting of NUDEL/LIS1/14-3-3ε, whose proper localization to the growth cone of developing axons is dependant on 14-3-3ε expression [68]. The co-localization of the complex with end-binding protein 1 (EB1) at the plus end of microtubules is an indication that this complex may have a role in regulating the growth cone cytoskeleton. Recently, 14-3-3 ζ has also been implicated in interacting with this molecular complex in combination with disrupted in schizophrenia 1 (DISC1) [70]. Mice that are deficient in 14-3-3 ζ were shown to have hippocampal lamination defects, aberrant mossy fibre tracts and exhibit behavioural and cognitive abnormalities [70]. These mice had no apparent defects in cortical layer formation, suggesting a differential requirement of one or more 14-3-3 isoforms, perhaps as specific homo- or heterodimers, for proper neuronal migration depending on the brain region.

Many studies have undertaken a systematic approach to identify molecules present in the growth cone at both the protein [71–73] and mRNA levels [24, 74]. While these studies have utilized growth cones from a variety of species, developmental stages and cell types, all have identified multiple 14-3-3 isoforms as constituents of the growth cone. The functional significance of 14-3-3 abundance in the growth cone was established through a series of loss-of-function studies using the R18 peptide, which competitively inhibits 14-3-3 binding to targets [75]. Strikingly, expression of R18 in E13 chick and P5 rat dorsal root ganglion (DRG) neurons converted nerve growth factor (NGF)-induced repellent growth cone turning to attractive turning, mimicking the turning response of earlier stage DRG axons, which are initially attracted by NGF. Knockdown of individual 14-3-3 isoforms, β, γ and ε, also converted NGF-dependent repulsion to attraction, indicating that specific 14-3-3 isoforms are required for conferring repellent responses to an otherwise attractive guidance cue [72]. This polarity reversal invoked a potential involvement of cyclic nucleotides; however, pharmacological studies have indicated that while the switch was not dependent on cAMP levels, it was abolished by inhibition of the cAMP-dependent protein kinase A (PKA). Together with the finding that 14-3-3 γ and ε bind to and antagonize PKA, these data support a model whereby increased 14-3-3 activity in later stage growth cones serves to down-regulate PKA activity and establish repellent turning [72].

The in vivo relevance of this model was established in a study investigating the bi-functional role of the morphogen sonic hedgehog (Shh) at the midline in the developing spinal cord [76]. During development, spinal commissural axons are attracted to the ventral floor plate, in part by a gradient of Shh, and upon crossing the floor plate, undergo a series of switches to ensure proper exit and turning in the anterior direction. Following floor plate exit, the previously attractive cue Shh becomes repellent, to guide axons down a posterior-high, anterior-low gradient [76]. Intriguingly, the axon turning responses of cultured commissural neurons recapitulates this switch in a time-dependent manner and this coincides with selective increases in the expression of 14-3-3 isoforms and reduced PKA activity. Loss-of-function experiments revealed that 14-3-3 activity is necessary both in vitro and in vivo for commissural axons to switch from attraction to repulsion in response to Shh. Inhibition of 14-3-3 s with the R18 peptide, as well as knockdown of 14-3-3 β and γ in cultured commissural neurons abolished the temporal switch from attraction to repulsion. Strikingly, disruption of 14-3-3 s with R18 in the developing neural tube randomized commissural axon trajectories upon floor plate exit, with turning in both posterior and anterior directions, while overexpression of 14-3-3 β and γ in pre-crossing axons induced premature repulsion away from the midline, indicating that 14-3-3 activity is capable of establishing a switch in turning response [76]. Another study recently demonstrated developmental regulation of 14-3-3 expression levels in RGC growth cones from Xenopus embryos, with peak expression coinciding with the phase of rapid extension of the retinotectal projection [77]. Disruption of 14-3-3 s with R18 resulted in a decreased RGC axon elongation

rate and a concomitant reduction in the level of inactive phosphorylated cofilin, consistent with previous studies which have shown that 14-3-3 binds to and stabilizes phosphocofilin [78], suggesting that 14-3-3 s may regulate axon outgrowth by regulating cofilin activity [77]. Precisely how 14-3-3 expression patterns are temporally and spatially regulated and the molecular links between 14-3-3 s and the cytoskeleton in the context of developmental axonal polarity switches remain open questions; however, it is now becoming clear that modulation of 14-3-3 activity is a mechanism to confer developmental switches in axon turning.

Additional studies have described roles for 14-3-3 s in regulating axon guidance events that may affect how receptors locally generate signals within the growth cone. Loss of D14-3-3ε in Drosophila larvae resulted in highly penetrant misrouting and defasciculation of pathfinding motor axons [79]. These defects were similar to mutants in which Sema1A/PlexinA signalling had been enhanced, and genetic interaction experiments suggested that 14-3-3ε plays a negative regulatory role in the Sema1A/PlexinA pathway. Subsequently, 14-3-3ε was shown to interact with the GTPase activating protein (GAP) domain of the PlexinA cytoplasmic region upon phosphorylation by PKA. This interaction was shown to interfere with the Ras GAP activity of PlexinA, thereby restoring Ras GTPase signalling. Functionally, this mechanism suppresses Sema1A-dependent repulsion and allows for integrin-mediated adhesion and outgrowth [79]. In a similar manner, the docking of 14-3-3 s or 14-3-3 –containing complexes onto the cytoplasmic region of cell adhesion molecules, including neural cell adhesion molecule (NCAM) and L1, has been shown to regulate neurite outgrowth and act as a molecular switch to balance positive and negative regulation of outgrowth mediated by these receptors [80, 81]. These studies implicate the direct regulation of receptor activity as another means by which 14-3-3 s can establish control over growth cone navigation.

Modulation of G protein activity by 14-3-3-mediated regulation of guanine exchange factors (GEFs), which activate G proteins by facilitating the exchange of GDP for GTP, and GAPs, which inactivate G proteins by promoting GTP hydrolysis, has been shown to play important roles in signal transduction and motile cell responses [82–85]. A recent study has identified a novel mode of 14-3-3-mediated regulation of G protein activity [86]. This work demonstrated that 14-3-3 s bind to Rnd proteins, a constitutively GTP-bound sub-family of the Rho GTPases. Binding of 14-3-3 s to Rnd3 was shown to induce its translocation from its site of action at the membrane to the cytosol, functioning in a similar manner to Rho guanine dissociation inhibitors (RhoGDIs). Overexpression of 14-3-3β blocked Rnd3-dependent cell rounding and membrane protrusions and this effect was dependent on binding between the two proteins [86]. Intriguingly, both farnesylated and geranylgeranylated Rnd3 were shown to interact with 14-3-3, suggesting that 14-3-3 s may be capable of interacting with a wide range of membrane-anchored prenylated proteins. This novel function for 14-3-3 s in modulating G protein activity raises the interesting possibility that 14-3-3 proteins may play a role in locally regulating Rho family GTPases and subsequent cytoskeletal rearrangements in response to guidance cues.

Conclusion

The chemotactic response of the growth cone is dynamically regulated in space and time. A variety of molecular strategies have been described to coordinate axonal responses to the environment. 14-3-3 proteins are emerging as an important family of proteins for spatial and temporal control in a complex environment of axon guidance cues. The presence of multiple 14-3-3 isoforms in growth cones of different cell types and developmental stages, along with the recent reports of 14-3-3 involvement in axon guidance and growth cone switching suggests that 14-3-3 s may have a pervasive role in regulating growth cone signalling that is only beginning to become understood. As we extend our understanding of the spatial and temporal regulation of growth cone turning responses, the study of 14-3-3 proteins will yield many insights into the molecular mechanisms by which motile structures can interact with the extracellular and intracellular environments to make complex navigational choices and may provide promise to the identification of novel targets for therapeutic interventions after injury to the nervous system.

Acknowledgements Work performed in the A.E.F and F.C. laboratories and presented in this study was supported by grants to A.E.F. and F.C. from the Canadian Institutes of Health Research (CIHR) and by the McGill Neuroengineering program.

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