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Ena/VASP: Proteins at the tip of the nervous system

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Summary

The emergence of neurites from a symmetrical cell body is an essential feature of nervous system development. Neurites are the precursors of axons and dendrites and are tipped by growth cones, motile structures that guide elongating axons in the developing nervous system. Growth cones steer the axon along a defined path to its appropriate target in response to guidance cues. This navigation involves the dynamic extension and withdrawal of actin-filled finger-like protrusions called filopodia that continuously sample their environment. Ena/VASP proteins, a conserved family of actin regulatory proteins, are critical for filopodia formation and function downstream of several guidance cues. Here we review recent findings into Ena/VASP function in neurite initiation, axon outgrowth and guidance.

Introduction

Growth cones, specialized fan-shaped structures at the ends of developing axons, steer growing axons to their targets. As an axon extends through the complex extracellular environment *in vivo*, its growth cone explores the local environment and responds to a variety of short- and long-range guidance cues. Although progress has been made in identifying the guidance cues and receptors [1], less is known about how environmental signals are converted into changes in the direction and rate of axonal outgrowth. Growth cone morphology and motility are determined by local dynamic changes in the actin and microtubule (MT) cytoskeleton, which are regulated by complex interactions downstream of second messenger signaling pathways [2]. Filopodia, rod-like projections composed of parallel F-actin bundles, extend beyond the dense actin meshwork of the lamellipodial veils at the growth cone periphery. Filopodia are characteristic for cells displaying exploratory behavior and are thought to sense guidance cues and steer the growth cone [3,4]. Ena/VASP proteins are implicated in integrating guidance signals into appropriate changes in cytoskeletal dynamics and are key regulators of filopodia formation and dynamics.

Ena/VASP proteins are a conserved family of actin-regulatory proteins concentrated at areas of dynamic actin remodeling and have a well-established role in filopodia formation and elongation [3,5]. The Ena/VASP family, which is conserved from Dictyostelium to vertebrates, has a domain structure consisting of an N-terminal Ena/VASP homology 1 (EVH1) domain, a central proline-rich region and a C-terminal EVH2 domain (Figure 1). Binding of the EVH1 domain to proteins that contain a proline-rich motif with the consensus (D/E)FPPPPX(D/E)(D/E) (abbreviated “FPPPP”) regulates subcellular localization of Ena/VASP proteins and mediates formation of complexes with receptors and signaling molecules. EVH1-binding

proteins include the axon guidance receptor Robo and Lamellipodin (Lpd), the mammalian orthologue of the *C.elegans* protein MIG-10 [6,7]. Exogenous expression of this FPPPP motif fused to a mitochondrial-targeting motif ("FPPPP-Mito") has been used in functional studies to neutralize Ena/VASP activity by sequestering them away from their subcellular sites of function [8]. This sequestration approach mimics the deletion of Ena/VASP in isolated cortical neurons [9] and phenocopies zygotic *Ena* mutations in the fly [7,10]. Conversely, directing Ena/VASP localization to the plasma membrane by fusing the FPPPP motif to a membrane targeting motif enhances Ena/VASP activity [11].

Ena/VASP proteins regulate cellular protrusions by modulating the geometry of actin filament network assembly [8]. Motifs in the EVH2 domain mediate direct binding to both monomeric (G-) and filamentous (F-) actin and, because they are tetramers, they can bundle actin filaments [12–14]. Ultrastructural analysis of actin filaments at the leading edge of fibroblasts and neuronal growth cones provided important clues to how Ena/VASP proteins influence cytoskeletal architecture. Depletion of Ena/VASP proteins from the cell edge in fibroblasts or growth cones promote formation of dense actin networks with short, highly branched filaments. In contrast, enrichment of Ena/VASP proteins at the plasma membrane results in sparse networks containing primarily long, unbranched filaments, which in growth cones coalesce into filopodia [8,15].

Ena/VASP promotes filopodia formation by 1) binding and clustering actin filaments barbed ends, 2) shielding elongating filaments from capping protein (3) and decreasing filament branching. Ena/VASP proteins are stably anchored within filopodial tips [16] through interactions with elongating actin filaments and binding to EVH-1 ligands such as Lpd [6]. *In vitro* actin polymerization assays and, more recently, direct visualization of growing fluorescently-labeled actin filaments show that Ena/VASP proteins capture filament barbed ends, antagonize barbed end capping and enhance filament elongation; this anti-capping activity is enhanced by direct binding to profilin-actin complexes [12,17]. However, the mechanism underlying Ena/VASP function in decreasing filament branching remains to be elucidated.

Ena/VASP proteins are likely to function during multiple steps in nervous system development. Roles for Ena/VASP in neurulation [18,19], neuronal migration [20–22], dendritic morphology [23,24], and synapse formation [25,26] have been demonstrated. In addition, recent evidence suggests that both Ena/VASP and MIG-10/Lpd play a role in axon regeneration in *C.elegans* [27]. Here we focus on two aspects of nervous system development that require Ena/VASP: neuritogenesis and axon guidance.

Ena/VASP proteins in Neurite Initiation

Recent analysis of the cortex of mice lacking all three vertebrate paralogs (Mena, EVL and VASP) reveal an unexpected requirement for Ena/VASP proteins in neurite initiation. Complete loss of Ena/VASP blocks axon fiber tract formation in the cortex in a cell autonomous manner. Analysis of cultured cortical neurons indicates that this defect results from the failure of cortical neurons to produce neurites [22]. This neuritogenesis defect arises from a failure to form actin bundles and filopodia. Neuritogenesis in Ena/VASP deficient neurons can be rescued by ectopic expression of mDia2 and myosinX [9], factors that can induce filopodia independently of Ena/VASP [28–30]. Dynamic MTs are also required to form neurites [9]. MT behavior is altered in the absence of Ena/VASP as a result of a lack of bundled F-actin to help guide MTs into filopodia. This is consistent with observations that F-actin bundles underlying filopodia act as guides for MTs [31] in growth cones. During axon outgrowth, MTs invade and dynamically explore filopodia [32,33], where they stabilize elongating axons and likely support

transport of proteins and membrane out to the tip; it is likely that MT: F-actin interactions function in a similar manner to support neurite initiation.

Interestingly, although Ena/VASP proteins were required for neuritogenesis within the cortex, cortical neurons that had aberrantly migrated outside of the pial membrane in Ena/VASP deficient mice formed axons, as did other neuronal types, such as retinal ganglia, hippocampus and dorsal root ganglia. This suggests that signals absent from the cortex but present in structures that form axons promote Ena/VASP-independent neurite initiation. One such factor is the extracellular matrix protein laminin which is largely absent from the cortex but found in the areas where Ena/VASP-independent neuritogenesis occurs. Neuritogenisis is rescued by plating Ena/VASP deficient primary cortical neurons on laminin but not fibronectin or collagen [9]. The existence of extrinsic (such as laminin) or intrinsic (such as mDia2 and myosinX) mechanisms that bypass the requirement for Ena/VASP in neuritogenesis may also explain why this defect is not observed in mutants of the invertebrate Ena/VASP orthologues.

Ena/VASP proteins in Axon Guidance

Drosophila and *C.elegans* each have a single Ena/VASP ortholog, Enabled (Ena) and UNC-34, respectively. Loss of Ena/VASP function in invertebrates leads to subtle defects in axon guidance. These phenotypes are primarily observed in sensitized genetic backgrounds. Interestingly, Ena/VASP appears to function downstream of both attractive and repulsive guidance cues, sometimes within the same cell. In worms, for example, UNC-34 functions downstream of UNC-40/DCC and UNC-5, the two Netrin receptors in *C.elegans* [34,35]. Loss of UNC-34 partially suppresses the morphological phenotypes induced by a gain-of-function mutation in UNC-40/DCC [35], as well as axon repulsion induced by ectopic expression of UNC-5 [34]. Genetic evidence also implicates *Drosphila* Ena in Netrin mediated guidance [36]. In addition, Ena functions downstream of the repulsive guidance receptor Robo/Sax3 and can bind directly to Robo [7,37,38].

Ena mutations in *Drosophila* also result in defects in motor axon pathways in which the ISNb motor axons fail to branch and instead bypass their muscle target [39]. Deletions of the receptor tyrosine phosphatase Dlar cause a similar phenotype, and both Dlar and Ena antagonize the function of the tyrosine kinase D-Abl in this pathway. Ena suppresses D-Abl dependent phenotypes, at least in part by reducing formation of ectopic F-actin spikes formed in D-Abl mutants [40]. Since *Drosophila* Ena is a direct target of both Dlar and D-Abl [41], these three proteins may define a tyrosine phosphorylation state-dependent switch controlling growth cone behavior. Interestingly, however, a role for Abl in axon guidance has not been observed in vertebrates [42].

The broad, overlapping expression patterns of the three vertebrate Ena/VASP paralogs requires combined deletions or inhibition of two or all three to reveal their functions in cell and developmental biology. Mice lacking Mena exhibit subtle defects in forebrain commissure [18] and optic nerve formation [19]. Mena/VASP double mutants display defects in the formation of several axon fiber tracts in the central and peripheral nervous systems, including the defects in all of the major forebrain commissures [19].

Development of the optic nerves is perturbed in the absence of Ena/VASP function. However, different phenotypes have been reported in mice and *Xenopus* [9,19,43]. In mice, Mena/VASP/EVL triple mutants exhibit stunted optic nerves that extend into the brain but fail to form the optic chiasm [9]. In contrast, inhibition of Ena/VASP function in *Xenopus* retina by transfection of the FPPPP-Mito construct did not affect axon guidance, but did reduce elongation rates and terminal arborization [43]. Differences in the severity of the phenotypes may reflect variations in the experimental approaches. While growth cones expressing FPPPP-Mito show an expected reduction or absence of filopodia, not all axons express the construct. Therefore, some pioneer

axons are preserved and, because they elongate at a faster rate, extend in front of the axons with reduced Ena/VASP function and could therefore guide them by adhesive mechanisms. Additionally, accumulation of sufficient levels of the transfected FPPPP-Mito construct to inhibit Ena/VASP may only occur after axon outgrowth initiation.

Many of the axonal defects seen in Ena/VASP knockout mice involve midline guidance decisions, a major choice point in the developing nervous system, where axons must choose to cross to the contralateral side or remain ipsilateral. Formation of the forebrain commissures and the optic nerves is orchestrated by an array of short and long range guidance cues, including Slit and Netrin [44,45], suggesting that Ena/VASP functions downstream of receptors for these guidance factors as is the case in invertebrates. It is important to note that since Mena and VASP are expressed in glial cells structures required for commissure formation [19], it is also possible that non-cell autonomous defects contribute to the observed guidance phenotypes. A more thorough understanding of Ena/VASP function in axon guidance in vertebrates will require use of conditional knockouts to distinguish roles intrinsic to axons as well as to bypass the neuritogenesis defect in the cortex, which precludes analysis of complete Ena/VASP deficiency in axon guidance in the developing brain.

Growth Cone Filopodia in Guidance Sensing

Growth cone filopodia function as sensors for guidance signals and can initiate the growth cone turning response [46,47]. DCC is enriched in filopodial tips on growth cones, supporting the idea that filopodia act as remote sensors for guidance cues [48]. Netrin stimulation of dissociated hippocampal neurons in culture drives rapid increases the number and length of filopodia in an Ena/VASP-mediated manner [15]. Elimination of growth cone filopodia by cytochalasin B treatment, a drug that blocks actin polymerization, increases pathfinding defects [49] and eliminates growth cone turning responses [50,51]. Thus, Ena/VASP-deficient neurons may fail to respond to guidance cues because they have lost their filopodia. Therefore their ability to sample the environment is compromised. When presented with an attractant stimulus, growth cone filopodia re-orient towards the guidance cue [51,52]. Not all axon guidance decisions require filopodia. For example, *unc-34* mutants form axonal growth cones that lack filopodia but still migrate towards Netrin, apparently through lamellipodial guidance [35]. The requirement for filopodia (and Ena/VASP) in guidance responses might be species or system specific. For instance, filopodia may be much more important in vertebrate guidance since axons must extend much longer distances and navigate through much more complicated environments than their counterparts in *C.elegans* or *Drosophila*.

Signaling Downstream of Guidance Receptors

In invertebrates, genetic studies indicate that Ena/VASP proteins are targets of signaling cascades downstream of guidance receptors and that their activity is modulated by second messenger signaling pathways. In addition to the D-Abl/D-Lar pathways, Ena mutants also interact genetically with Trio, which contains two guanine-nucleotide exchange domains specific for Rac and Rho [53]. Genetic and biochemical evidence links Ena/VASP to MIG-10/Lpd function [54,55]. MIG-10/Lpd in turn interact with phosphoinositides and Ras superfamily proteins [6,54], suggesting that these signaling pathways could also modulate Ena/VASP function. However, it is clear that MIG-10/Lpd and Ena/VASP also have independent functions since *mig-10/unc-34* double mutants have a more severe phenotype than either single mutant [54,55]. Interestingly, MIG-10 is implicated in the very earliest steps of neuronal polarization towards Netrin [55,56].

In vertebrates, signaling through various guidance factors can be modulated by cyclic nucleotides and the ratio of cAMP to cGMP present in a given cell can convert them from attraction to repulsion and vice versa [57]. Vertebrate Ena/VASP proteins are targets of both

the cyclic-nucleotide activated kinases PKA and PKG [58-60]. Since they are known to function downstream of attractive and repulsive guidance factors, Ena/VASP proteins are perfectly positioned to relay this switch into cytoskeletal and migratory changes (Figure 2). Indeed, Netrin stimulation or pharmacological activation of PKA both cause a rapid increase in filopodia number and length in an Ena/VASP dependent manner along with a concomitant increase in Mena phosphorylation [15]. Though it is not known whether repulsive guidance signaling affects the phosphorylation status of Ena/VASP proteins in neurons, *in vitro* phosphorylation at the preferred PKG site decreases VASP's affinity for actin filaments and abolishes its anti-capping and filament bundling activities [12]. Thus, modulation of Ena/VASP activity by PKA might promote axon turning by supporting filopodia formation and elongation towards a guidance cue. Conversely, repulsive guidance cues might inhibit Ena/VASP function locally, thereby suppressing filopodia formation in the direction of the cue in favor of movement in the opposite direction. It is important to note, however, that while PKA activity can regulate the sensitivity of axons to Netrin-1, a direct role for PKA in guidance conversion has not been observed [61]. In addition, Netrin-1 stimulation does not activate PKA [62]. It seems likely that PKA phosphorylation of Ena/VASP may be triggered by other cues, for example changes in the extracellular matrix [63]. It is well established that growth cone responses to guidance factors changes depending on environmental context; PKA- and PKG mediated phosphorylation of Ena/VASP may facilitate such context-dependent guidance conversion in concert with other cyclic-nucleotide mediated signaling events [64]. Interestingly, this PKA/PKG regulation of Ena/VASP is not conserved between vertebrates and invertebrates, since the invertebrate orthologs lack the corresponding phosphorylation sites. How phosphorylation modulates Ena/VASP activity in vertebrate neurons, and how this contributes to cytoskeletal reorganization during axon guidance remains to be elucidated.

Conclusions

Since their discovery in *Drosophila*, Ena/VASP proteins have been implicated in the proper development of the nervous system [65]. In vertebrates, Ena/VASP plays a pivotal role in the initial establishment of cortical neuronal morphology and is required elsewhere for proper axon guidance; both neuritogenesis and guidance depend on filopodia, highlighting the importance of understanding the mechanisms that govern filopodial dynamics. However, Ena/VASP proteins appear to be involved in different aspects of neuronal development depending on the organisms, experimental conditions and cell types studied. This variability indicates that while Ena/VASP proteins universally play an important role in the developing nervous system, different organisms and neuronal cell types have evolved distinct mechanisms utilizing Ena/VASP proteins for outgrowth and guidance. Although progress towards understanding the mechanism underlying Ena/VASP regulation of actin dynamics and filopodia formation has been made, many questions remain. A major challenge will be to understand how Ena/VASP is regulated during axon guidance. Further characterization of filopodial tip complexes, monitoring of growth cones with altered Ena/VASP activity *in vitro*, and biochemical studies to determine the formation of differential protein complexes in response to guidance cues will help elucidate how Ena/VASP protein facilitate accurate axon guidance.

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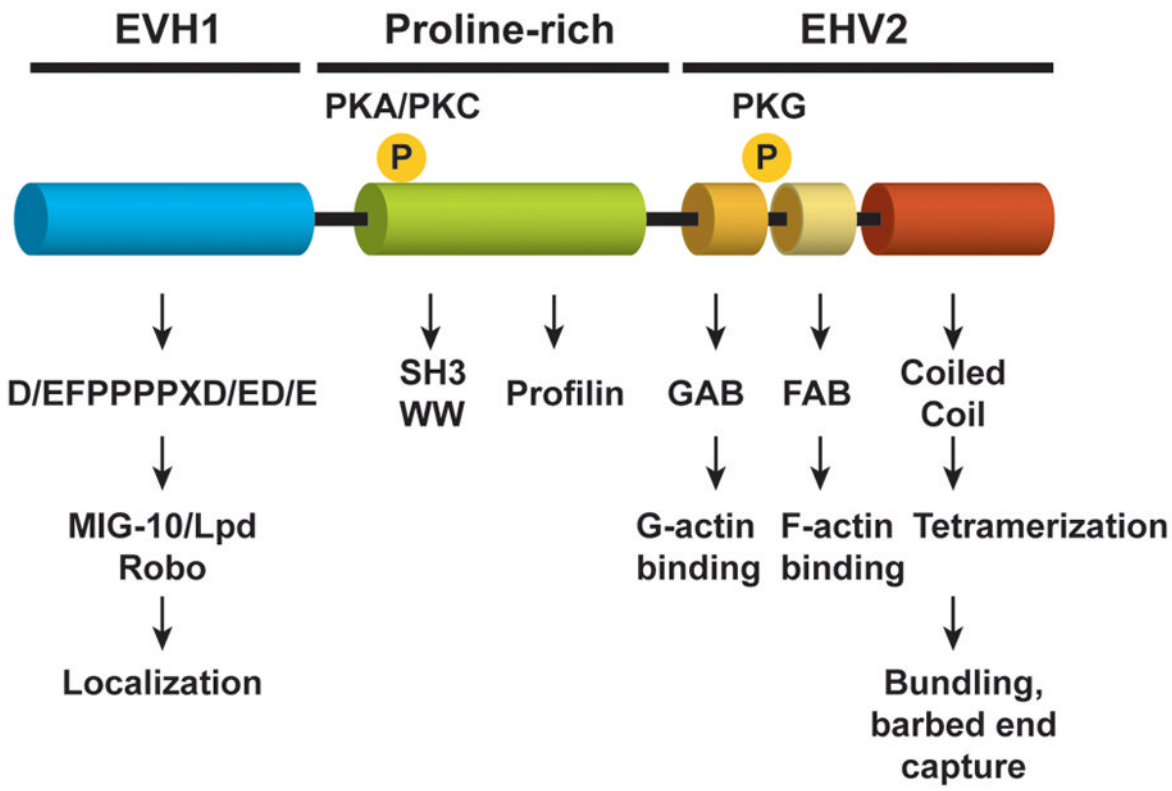

Figure 1.
Organization of Ena/VASP proteins. The domains and protein interaction sites are shown in the schematic. The EVH-1 binding partners Lpd and Robo are shown; other ligands not known to function in axon outgrowth and guidance are not indicated. Phosphorylation sites shared between Mena and VASP are also indicated. The amino terminal site can be phosphorylated by either PKA or PKC. The carboxy-terminal PKG site is not conserved in EVL. Note that the invertebrate Ena/VASP proteins lack these phosphorylation sites.

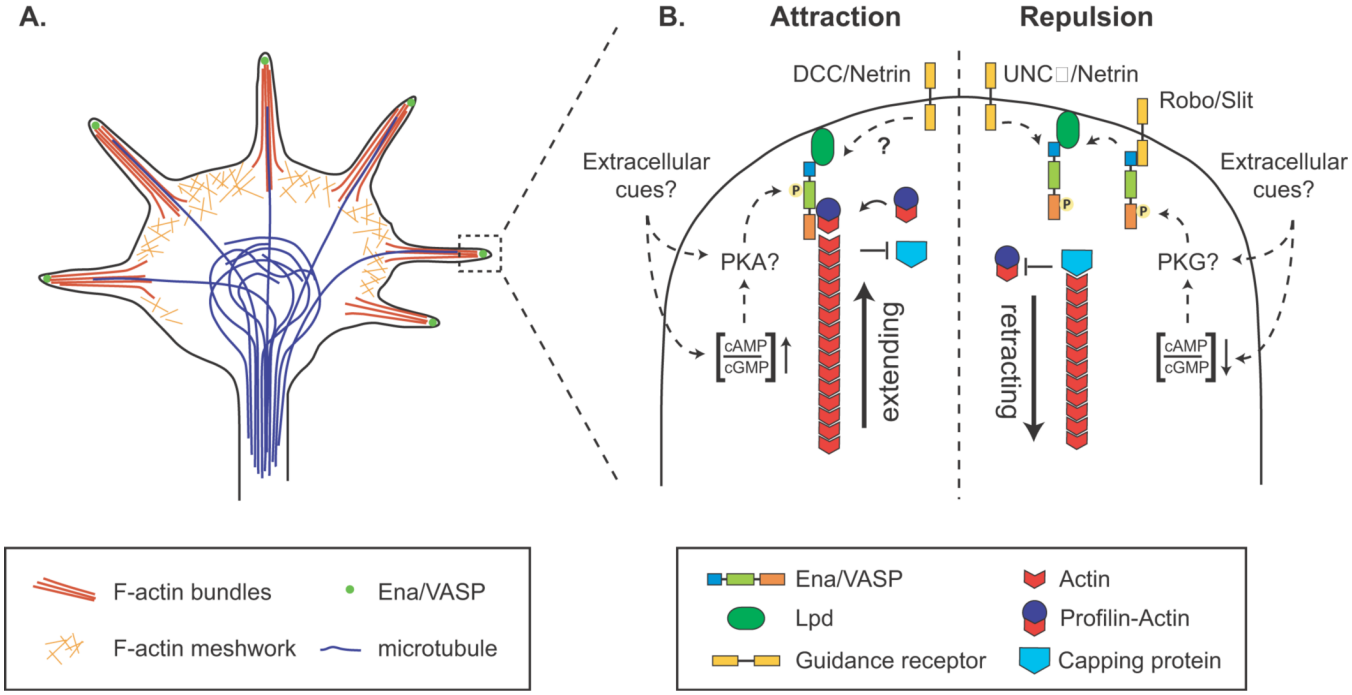

Figure 2.

A. Localization of Ena/VASP proteins in growth cones. Growth cones contain a meshwork of actin filaments in the lamellipodium, actin filament bundles that penetrate filopodia, bundled stable microtubules in the center, and dynamic microtubules that extend into the periphery, associating with actin filaments. Ena/VASP proteins are clustered at the tips of filopodia. B. Model for regulation of Ena/VASP activity by guidance cues. Activation of Ena/VASP proteins downstream of attractive guidance cues and/or their phosphorylation by PKA might result in the protection of actin filaments from capping protein and promote addition of actin-profilin and filament elongation. In contrast, repulsive guidance cues might inhibit Ena/VASP function, potentially through phosphorylation by PKG, leading to filament capping and retraction of filopodia. It is also possible that Ena/VASP-dependent filopodial dynamics are important to sense repulsive cues initially; subsequent inhibition/inactivation of Ena/VASP may then contribute to filopodial retraction.