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Integrin Signaling Switches the Cytoskeletal and Exocytic Machinery that Drives Neuritogenesis

Stephanie L. Gupton[1] and Frank B. Gertler[1,*]
[1]The Koch Institute for Integrative Cancer Research at MIT, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
*Correspondence: fgertler@mit.edu
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SUMMARY

Neurons establish their unique morphology by elaborating multiple neurites that subsequently form axons and dendrites. Neurite initiation entails significant surface area expansion, necessitating addition to the plasma membrane. We report that regulated membrane delivery coordinated with the actin cytoskeleton is crucial for neuritogenesis and identify two independent pathways that use distinct exocytic and cytoskeletal machinery to drive neuritogenesis. One pathway uses Ena/VASP-regulated actin dynamics coordinated with VAMP2-mediated exocytosis and involves a novel role for Ena/VASP in exocytosis. A second mechanism occurs in the presence of laminin through integrin-dependent activation of FAK and src and uses coordinated activity of the Arp2/3 complex and VAMP7-mediated exocytosis. We conclude that neuritogenesis can be driven by two distinct pathways that differentially coordinate cytoskeletal dynamics and exocytosis. These regulated changes and coordination of cytoskeletal and exocytic machinery may be used in other physiological contexts involving cell motility and morphogenesis.

INTRODUCTION

During cortical development, neurons sprout multiple neurites, growth cone-tipped processes that are the precursors of axons and dendrites. In culture, cortical neurons progress through several morphological stages (Dotti et al., 1988), starting with the extension of F-actin-rich lamellipodia and filopodia (Dehmelt et al., 2003). Within 24 hr, neurons elaborate multiple neurites, a process that requires both F-actin and microtubule (MT) dynamics. Cortical neurons from mice lacking all three members of the Ena/VASP family of actin regulators fail to form axons in vivo (Kwiatkowski et al., 2007) due to a block in neuritogenesis (Dent et al., 2007; Kwiatkowski et al., 2007). Ena/VASP-deficient neurons lack filopodia, which are essential for cortical neuritogenesis (Dent et al., 2007; Lebrand et al., 2004). Filopodia induction by Ena/VASP-independent methods rescues neuritogenesis (Dent et al., 2007). Despite the striking neuritogenesis defect in the cortex of Ena/VASP null animals, neurons elsewhere in the developing embryo, such as those the retina and dorsal root ganglia, form neurites and axons (Dent et al., 2007; Kwiatkowski et al., 2007), indicating that neuritogenesis can also occur through Ena/VASP-independent mechanisms. Interestingly, even Ena/VASP-deficient cortical neurons can form neurites under the right conditions: a small fraction of Ena/VASP-deficient cortical neurons migrate out of the cortex, reach the pial membrane, and extend axons back into the cortex (Dent et al., 2007). Therefore, the Ena/VASP-independent neuritogenesis pathway appears to be triggered by non-cell-autonomous environmental factors such as the extracellular matrix (ECM).

The locations in which Ena/VASP-deficient neurons form neurites and axons contain the ECM component laminin (LN) (Dent et al., 2007); the cortex, however, contains only small amounts of LN. Since cortical neurons normally grow in an environment with little LN, they are usually cultured without LN. When cultured on LN, Ena/VASP-deficient cortical neurons form neurites, confirming that LN can trigger Ena/VASP-independent neuritogenesis. Attachment to LN is mediated by transmembrane receptors, including the integrin family (Buck and Horwitz, 1987). Integrin-ECM attachment activates signaling pathways that modulate cellular processes including cytoskeletal dynamics (Geiger et al., 2001). The mechanisms driving LN-dependent neuritogenesis, including the role of integrin activation and signaling, are unknown.

Neuritogenesis results in a rapid and large increase in surface area (Pfenninger, 2009) that requires rapid insertion of new membrane and proteins into the plasma membrane. Regulated delivery via exocytosis is required for other neuronal morphogenic events that involve increases in surface area (Futerman and Banker, 1996; Lanzetti, 2007; Martinez-Arca et al., 2000; Pfenninger, 2009; Tang, 2001; Tojima et al., 2007) though a role for regulated exocytosis in neuritogenesis has not been established. Exocytic vesicles move along the cytoskeleton to the cell periphery (Schroer, 1992; Tsaneva-Atanasova et al., 2009), where the exocyst complex tethers them to the membrane (EauClaire and Guo, 2003; Murthy et al., 2003; TerBush et al., 1996). Membrane fusion is mediated by a vesicle (*v*-SNARE) complexing with target SNAREs (t-SNAREs) in the destination membrane (Hong, 2005; Tang, 2001). Many SNARE proteins are brain enriched (Malsam et al., 2008) and several *v*-SNAREs have specific neuronal functions. vesicle-associated membrane protein 2 (VAMP2; synaptobrevin) is implicated in growth cone chemoattraction (Tojima et al., 2007) and synaptic function (Schoch et al., 2001; Wang and Tang, 2006), but not neurite elongation (Osen-Sand et al., 1996); VAMP7 [tetanus-insensitive VAMP (TI-VAMP)] is implicated in neurite elongation (Alberts et al., 2006; Martinez-Arca et al., 2000).

Here we focus on mechanisms underlying Ena/VASP- and LN-dependent neurite initiation and discover they are two mutually

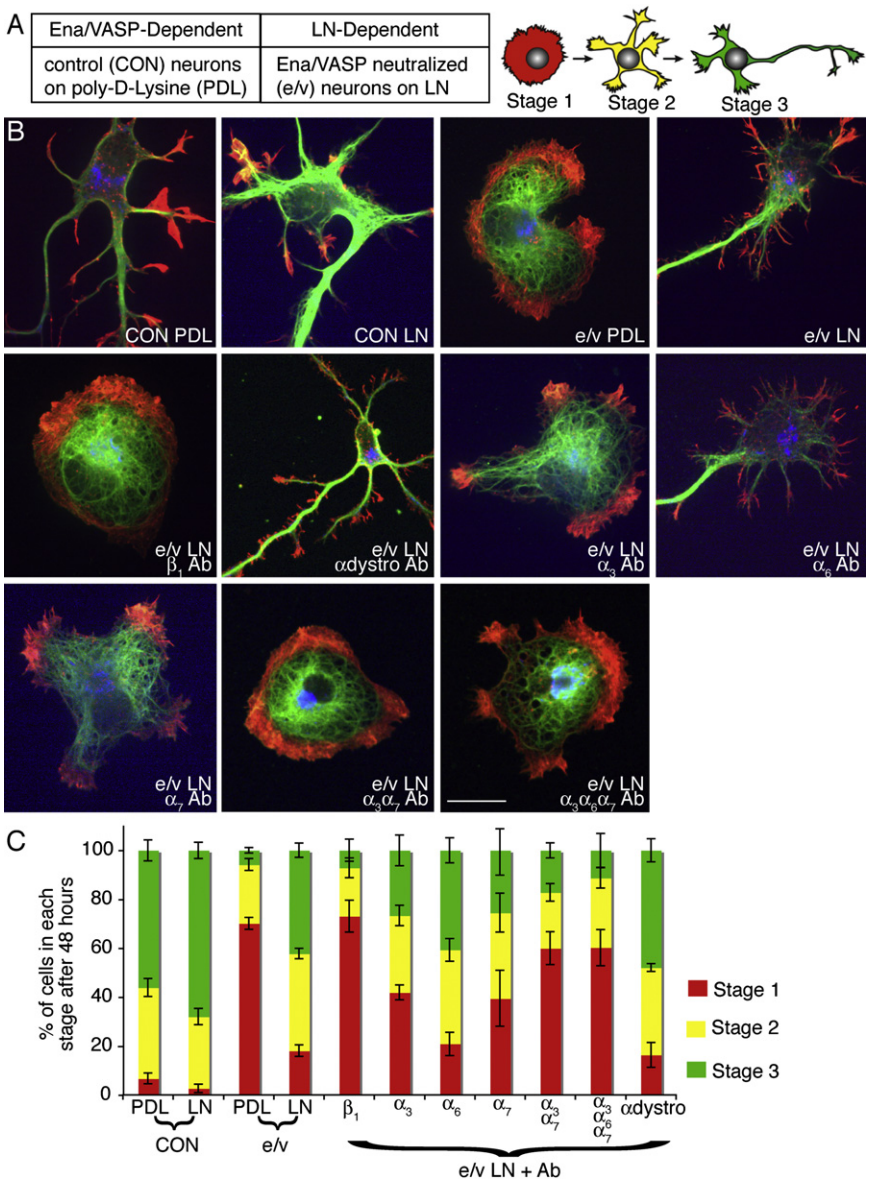


Figure 1. $\alpha_3\beta_1$ and $\alpha_7\beta_1$ Integrins Mediate LN-Dependent Neuritogenesis

(A) Conditions used to study Ena/VASP-dependent and LN-dependent neuritogenesis and schematic of neuronal morphologies.

(B) Representative images of E14.5 cortical neurons cultured for 48 hr, expressing AP4Mito (CON) or FP4Mito (e/v) (blue) plated on PDL or LN. Neurons were stained for βIII-tubulin (green) and phalloidin (red). β_1-, α_3-, and α_7-integrin function-blocking antibodies (Ab; 1:1000) decreased neurite formation, while α_6-integrin (1:1000) and α-dystroglycan (α-dystro; 1:200) function-blocking antibodies did not. Scale bar represents 10 μm.

(C) Percentage of cells in each morphological stage ± SEM. n ≥ 3 independent experiments/treatment; n ≥ 25 neurons/experiment.

exclusive pathways. We find neuritogenesis requires both actin dynamics and exocytosis; however, the Ena/VASP- and LN-dependent modes use distinct pairs of an actin regulatory protein and a *v*-SNARE. On LN, integrin signaling triggers the concomitant switch in cytoskeletal and exocytic machinery driving neuritogenesis. Therefore, the ECM exerts a context-dependent influence over cell shape and behavior by inducing a coordinated switch in the cytoskeletal and exocytic machinery used to initiate neurite formation, a hallmark of nervous system development.

RESULTS

Integrin Activation Supports Ena/VASP-Independent Neuritogenesis

Neuritogenesis and axon outgrowth are blocked in cortical neurons genetically null for all three Ena/VASP proteins (Dent et al., 2007; Kwiatkowski et al., 2007). Due to the complexity of obtaining triple null embryos from timed pregnancies, we used a well-established method to inhibit Ena/VASP. This approach exploits the highly specific interaction of the EVH1 domain of Ena/VASP with the ligand motif DFPPPPXDE (FP4) attached to a mitochondrial targeting sequence (FP4Mito) to deplete Ena/VASP from sites of function and sequester Ena/VASP on the mitochondrial surface, blocking function (Bear et al., 2000). This phenocopies the defects observed in cells genetically null for Ena/VASP, including fibroblasts, neurons, and endothelial cells; a control form of the construct (AP4Mito) has no effect on Ena/VASP localization or phenotype (Bear et al., 2000, 2002; Dent et al., 2007; Furman et al., 2007; Lebrand et al., 2004). The strategy is also effective in vivo in *Drosophila* (Gates et al., 2007). To investigate LN-dependent (Ena/VASP-independent) neuritogenesis, we plated FP4Mito-expressing [Ena/VASP neutralized ("e/v")] cortical neurons on LN. Ena/VASP-dependent neuritogenesis was studied in AP4Mito expressing control (CON) neurons plated on poly-D-lysine (PDL; Figure 1A).

We first identified the LN receptor required for Ena/VASP-independent neurite initiation. Candidate receptors include $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_7\beta_1$ integrins and α-dystroglycan (Flanagan et al., 2006; Gorecki et al., 1994; Hynes, 2002; Ivins et al., 1998; Plantman et al., 2008). Due to the rapid kinetics of neurite formation and the postmitotic state of neurons, siRNA was not effective at depleting protein levels during neuritogenesis. Therefore, we used function-blocking antibodies to LN receptors. After 48 hr, the morphology of neurons was classified (Figure 1A) as lacking neurites (stage 1), having one or more neurites (stage 2), or having one neurite/axon at least twice as long as any other (stage 3). CON neurons formed neurites on both PDL and LN (Figure 1), while e/v neurons failed to form neurites on PDL

($p < 0.05$; Figure 1), equivalent to null neurons (Dent et al., 2007). As expected, this was rescued by LN ($p < 0.05$; Figure 1A). A β_1-integrin function-blocking antibody inhibited neurite formation by e/v neurons on LN ($p < 0.05$; Figure 1), while a function-blocking antibody against αdystroglycan did not. Blocking either β_1-integrin or α-dystroglycan had no effect on neurite initiation by CON neurons grown either on PDL or LN (see Figure S1A available online), indicating that loss of Ena/VASP exposes a requirement for integrin activation during neuritogenesis.

β_1-Integrin dimerizes with α_3, α_6, and α_7 integrins to form LN receptors (Hynes, 2002). Function-blocking antibodies against α_3 and α_7 integrins partially reduced neuritogenesis in e/v neurons on LN ($p < 0.05$; Figure 1), while an α_6-integrin function-blocking antibody did not. Using the α_3- and α_7-integrin antibodies together phenocopied β_1-integrin inhibition; addition of the α_6-integrin antibody did not reduce neuritogenesis further (Figure 1), indicating LN-dependent neuritogenesis was mediated by $\alpha_3\beta_1$- and $\alpha_7\beta_1$-integrin heterodimers.

Unlike LN, fibronectin (FN) and collagen do not rescue cortical neuritogenesis in the absence of Ena/VASP (Dent et al., 2007). Western blots of cortical lysates indicated cortical neurons expressed only trace amounts of α_5 and α_v integrins (data not shown), which dimerize with β_1 or β_3 integrins to form FN receptors (Hynes, 2002). Ectopic expression of α_5-integrin rescued neuritogenesis in e/v neurons on FN ($p < 0.05$) (Figure S1) but not on PDL; therefore, integrin-mediated neuritogenesis likely results from a general integrin-signaling pathway that depends on the integrin repertoire expressed by neurons and the local ECM composition.

FAK, src, and Rac Function in LN-Dependent Neuritogenesis

To identify signaling molecules regulating neuritogenesis, we used pharmacological perturbations or dominant-negative mutants (Figure 2). Surprisingly, inhibition of molecules implicated in axon specification and guidance, such as phosphoinositide 3 kinase (PI3K), phospholipase D, protein kinase A, and protein kinase C (Han et al., 2007; Shen et al., 2002; Wolf et al., 2008; Yoon et al., 2005), did not affect neuritogenesis in e/v neurons on LN (Figures 2A and 2B) or CON neurons (Figure S1). In contrast, inhibition of focal adhesion kinase (FAK) by its noncatalytic domain, FRNK (Schaller et al., 1993), or src with 50 nM PP2 (Ohnishi et al., 2001) decreased neurite initiation in e/v neurons on LN ($p < 0.05$; Figures 2A and 2B), but not in CON neurons on PDL (Figure S1A). Conversely, constitutively active src (srcY527F) rescued neuritogenesis in e/v neurons on PDL ($p < 0.05$; Figures 2A and 2B). Therefore, src and FAK were necessary for LN-dependent neuritogenesis, but dispensable for Ena/VASP-mediated neuritogenesis. In the absence of Ena/VASP, activated src was sufficient to support neuritogenesis and bypassed the requirement for LN.

Rho family GTPases are intermediates coordinating integrin signaling and cytoskeletal dynamics. Rac and Cdc42 in particular regulate neuronal morphogenesis and membrane protrusion (Brouns et al., 2001; Gallo et al., 2002; Miyashita et al., 2004; Nobes and Hall, 1995; Pommereit and Wouters, 2007; Schaefer et al., 2008). Expression of dominant-negative Cdc42N17 (Ridley and Hall, 1992; Ridley et al., 1992) caused only a slight reduction of neurite initiation in CON neurons on PDL ($p < 0.05$; Figures 2C and 2D), but did not affect e/v neurons on LN. Constitutively active Cdc42Q61L expression did not rescue neuritogenesis in e/v neurons on PDL, although it induced filopodia formation (Figures 2C and 2D); it is possible these filopodia were unable to support neuritogenesis due to confounding secondary effects.

RacN17 expression in CON neurons on PDL reduced neurite formation slightly ($p < 0.05$; Figure 2). In contrast, RacN17 expression or treatment with a Rac inhibitor (NSC-23766; Gao et al., 2004) reduced neuritogenesis substantially in e/v neurons on LN ($p < 0.05$; Figures 2C and 2D). RacQ61L, however, failed to rescue neuritogenesis in e/v neurons on PDL. These data suggest Cdc42 and Rac play minor roles in Ena/VASP-dependent neuritogenesis. In contrast, Rac activity was required for LN-dependent neuritogenesis, although unlike activated src, active Rac was insufficient for Ena/VASP-independent neuritogenesis.

LN Switches the Actin Regulators Mediating Neuritogenesis

We next asked whether other types of actin regulatory proteins compensated for the absence of Ena/VASP in LN-mediated neuritogenesis. Ena/VASP proteins promote F-actin polymerization by protecting the fast growing ends of F-actin from capping and can cluster the ends of actin filaments (Applewhite et al., 2007; Bachmann et al., 1999; Barzik et al., 2005; Bear et al., 2002; Breitsprecher et al., 2008; Schirenbeck et al., 2006) and mediate filopodia formation (Dent et al., 2007; Gupton and Gertler, 2007; Lebrand et al., 2004). Previously we found that ectopic expression of Myosin X or mDia2, proteins that drive Ena/VASP-independent filopodia formation, rescued neuritogenesis in e/v neurons; however, neither is detectably expressed in cortical neurons at this developmental stage (Dent et al., 2007). The Arp2/3 complex can be activated downstream of Rac, nucleates and branches F-actin, and has been implicated in axon guidance and neuronal morphology (Korobova and Svitkina, 2008; Strasser et al., 2004; Withee et al., 2004). We used the CA domain of the Arp2/3 activator N-WASP to reduce Arp2/3 activity during neuritogenesis. CA binds Arp2/3, blocks its activation in vitro (Rohatgi et al., 1999), and attenuates its activity in cells (May et al., 1999, 2000). CA expression in CON neurons on PDL did not reduce neurite formation, although it reduced the number of stage 3 neurons (Figure 3), indicating that Arp2/3 may play a role in axon specification. However, CA expression reduced neuritogenesis in e/v neurons on LN ($p < 0.05$; Figure 3). Therefore, Arp2/3 is required for LN-dependent but not Ena/VASP-dependent neurite initiation, revealing a novel switch in the roles of actin regulators or a requirement for specific types of F-actin supramolecular structures for LN-dependent neuritogenesis.

An Exocytic Switch on LN

We next examined the regulation of membrane delivery during the rapid increase in surface area that occurs during neuritogenesis (Pfenninger, 2009; Vega and Hsu, 2001); while exocytosis is likely involved in this process, it is not clear which of the *v*-SNAREs mediate vesicle fusion during neuritogenesis. The *v*-SNARE VAMP2 functions in growth cone chemoattraction (Tojima et al., 2007), but not neurite elongation (Osen-Sand

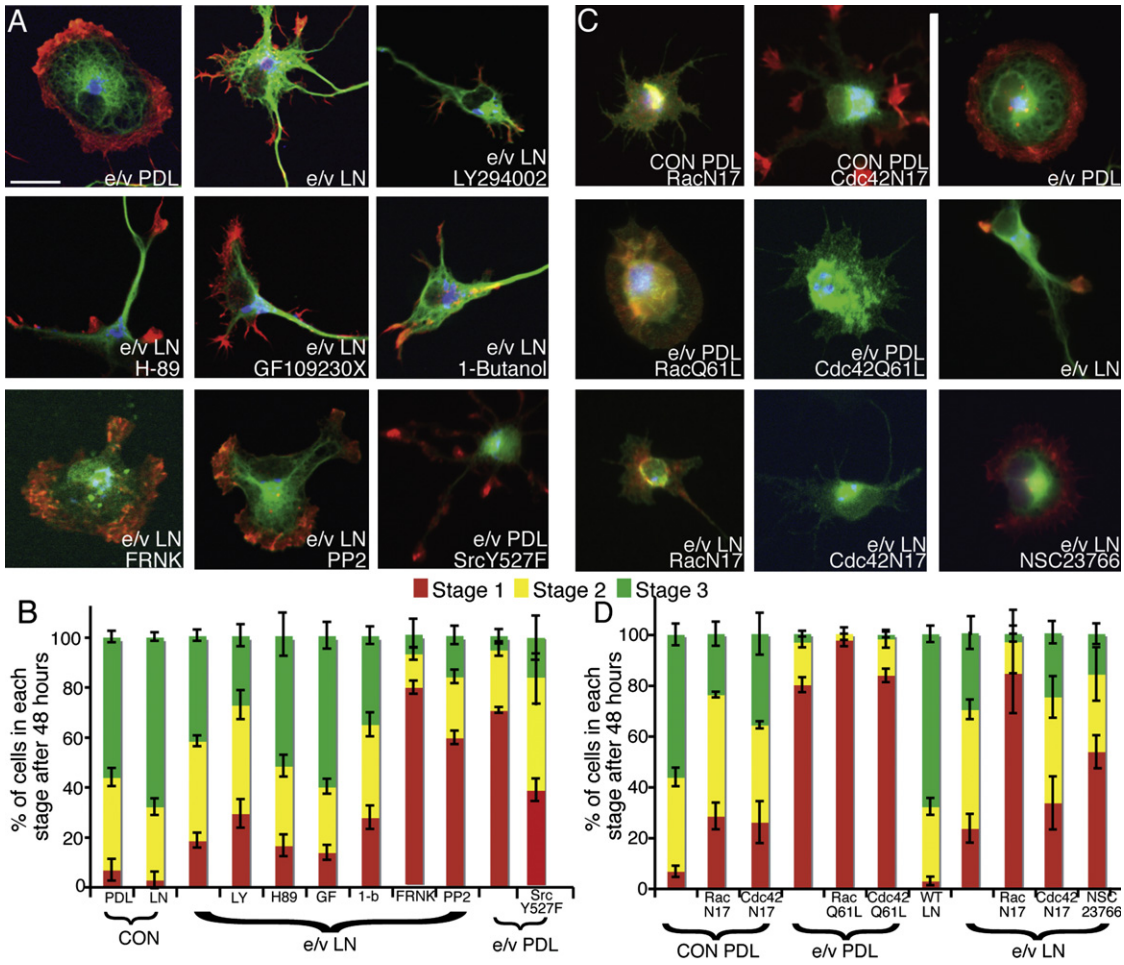


Figure 2. FAK, Src, and Rac Activity Are Necessary for LN-Dependent Neuritogenesis

(A) E14.5 e/v cortical neurons cultured 48 hr expressing FP4Mito (blue) and either the FAK inhibitor (FRNK; green) or constitutively active Src (Y527F; green) or treated with the indicated drugs, plated on PDL or LN. Neurons not expressing FRNK or SrcY527F were stained for βIII-tubulin (green) and phalloidin (red). Scale bar represents 10 μm.

(B) Quantification of morphologies, as in Figure 1. Targeted molecules include PI3K [50 μM LY294002 (LY)], protein kinase A (20 μM H-89), protein kinase C [1 μM GF109230X (GF)], src (50 nM PP2), and phospholipase D [0.3% 1-butanol (1-b)]. n ≥ 3 independent experiments/treatment; n ≥ 25 neurons/experiment. Scale bar represents 10 μm.

(C) E14.5 neurons cultured 48 hr, expressing AP4Mito (CON) or FP4Mito (e/v) (blue), plated on PDL or LN. Red indicates phalloidin and green indicates either anti-myc (for Rac mutants), GFP (for Cdc42 mutants), or βIII-tubulin (in neurons not expressing a mutant).

(D) Quantification of morphologies ± SEM. n ≥ 3 independent experiments/treatment; n ≥ 25 neurons/experiment.

et al., 1996). We used tetanus neurotoxin (TeNT) to cleave and inhibit exocytosis mediated by VAMP1, VAMP2, and VAMP3 (Figure S2) (Sikorra et al., 2008; Verderio et al., 1999). Treatment with TeNT blocked neuritogenesis in CON neurons on PDL ($p < 0.05$; Figure 4). This block was reversible (Figure S2) and, as previously reported, TeNT treatment did not affect outgrowth once neurites had formed (Figures S2E and S2F) (Osen-Sand et al., 1996). VAMP2 is expressed highly in cortical neurons at this stage (Figure S2), while closely related *v*-SNAREs VAMP1 and VAMP3 are not detectable (Schoch et al., 2001), suggesting VAMP2-mediated exocytosis is essential for Ena/VASP-dependent neuritogenesis.

Surprisingly, TeNT did not block neuritogenesis on LN, indicating that attachment to LN also bypasses the necessity for VAMP2 during neuritogenesis. This suggested LN triggered neurons to use another *v*-SNARE. VAMP7 was a logical candidate: it is present in the cortex (Malsam et al., 2008) (Figure S2) and is insensitive to TeNT (Galli et al., 1998). The N-terminal fragment of VAMP7 blocks VAMP7 SNARE complex formation (Martinez-Arca et al., 2000) and, as expected, its expression reduced VAMP7-mediated, but not VAMP2-mediated, exocytosis (Figure S2G). VAMP7 inhibition blocked neurite formation in e/v neurons on LN ($p < 0.05$; Figure 4) but not CON neurons on PDL (Figure S1A). Together these data indicate that exocytosis is required for neuritogenesis and that LN induced a switch in the key *v*-SNARE mediating exocytosis: VAMP2 was required for Ena/VASP-dependent neuritogenesis; VAMP7 was necessary for LN-dependent neuritogenesis.

Since VAMP2 inhibition phenocopied loss of Ena/VASP activity, we wondered if the neuritogenesis defect in e/v neurons

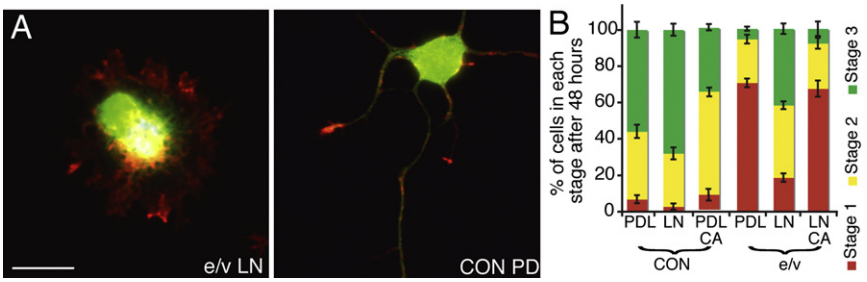


Figure 3. The Arp2/3 Complex Functions in LN-Dependent Neuritogenesis

(A) E14.5 neurons cultured 48 hr, expressing mCherry-CA (green) and either AP4Mito (CON) or FP4Mito (e/v) (blue), plated on PDL or LN. Phalloidin staining is red. Scale bar represents 10 μm.

(B) Quantification of morphologies ± SEM. n ≥ 3 independent experiments/treatment; n ≥ 25 neurons/experiment.

involved reduced exocytosis. Exo70 is a component of the exocyst complex that when overexpressed drives filopodia formation, membrane protrusion, vesicle tethering, and secretion (Liu et al., 2009; Zuo et al., 2006). Exo70 overexpression restored neuritogenesis ($p < 0.05$; Figure 4), as did VAMP7 ($p < 0.05$; Figure 4), whereas overexpression of VAMP2 or VAMP3 failed to rescue neuritogenesis of e/v neurons (Figure 4). These data indicate that the Ena/VASP-dependent defect in neuritogenesis may result from reduced exocytosis. Coexpression of the inhibitory VAMP7 NH_2 fragment impaired the ability of exogenous Exo70 to drive neuritogenesis in e/v neurons ($p < 0.05$; Figure 4), indicating that Exo70 rescued neuritogenesis through a mechanism partially dependent on VAMP7. Furthermore, in this system, VAMP7 function was necessary and sufficient for Ena/VASP-independent neuritogenesis.

Vesicle Dynamics and Exocytosis Are Altered on LN

VAMP2 and VAMP7 localize to distinct vesicle populations in cortical neurons (Figure S3). Since the ECM can switch both the *v*-SNAREs and F-actin regulators required for neuritogenesis, we hypothesized that the dynamics of *v*-SNARE-containing vesicles would be sensitive to substrate as well. To characterize vesicle dynamics, we performed live cell total internal reflection fluorescence microscopy (TIR-FM; Axelrod et al., 1983) of GFP-tagged *v*-SNAREs expressed at low levels (Figures 5A–5D), which localize with endogenous proteins (Figure S3). In CON neurons on PDL, VAMP2-containing vesicles dynamically explored the cell periphery and filopodia, while on LN most vesicles remained static in central regions. VAMP7-containing vesicles exhibited inverse dynamics, visiting the periphery frequently on LN (Movie S1). Quantification of velocity showed no change on PDL or LN (Figure 5C). However, VAMP2-containing vesicles moved more directionally on PDL than LN, while VAMP7-containing vesicles were more directional on LN (t tests, $p < 0.05$; Figure 5C). Dual spectral imaging of VAMP2 and VAMP7 confirmed these distinct dynamics (Figure 5D; Movie S2). Therefore, directional vesicle movement occurs for a *v*-SNARE when it is required for neurite initiation.

The observations described above led us to hypothesize that Ena/VASP affects vesicle dynamics. Ena/VASP inhibition did not affect the velocities of VAMP2- or VAMP7-containing vesicles on PDL or LN. However, on PDL, Ena/VASP inhibition caused VAMP2-containing vesicles to no longer pause at the periphery. In contrast, VAMP7-containing vesicle dynamics appeared unaffected by Ena/VASP inhibition (Movie S3), suggesting Ena/VASP or associated F-actin may be important for dynamics or docking of VAMP2- but not VAMP7-containing vesicles.

VAMP2- and VAMP7-mediated exocytosis were measured in stage 1 neurons using TIR-FM of pH-sensitive pHluorin-tagged VAMP2 and VAMP7 (Alberts et al., 2006; Miesenbock et al., 1998; Figures 5E and 5F; Movie S4). VAMP2-mediated exocytosis occurred 3-fold more frequently in CON neurons on PDL than on LN ($p < 0.05$). In contrast, VAMP7-mediated exocytosis was more frequent on LN ($p < 0.05$; Figure 5F; Movie S5). The high frequency of VAMP2- and VAMP7-mediated exocytosis on PDL and LN, respectively, correlated with their increased directionality and requirement in context-dependent neuritogenesis, indicating that attachment to LN switches the dynamics and function of exocytic machinery.

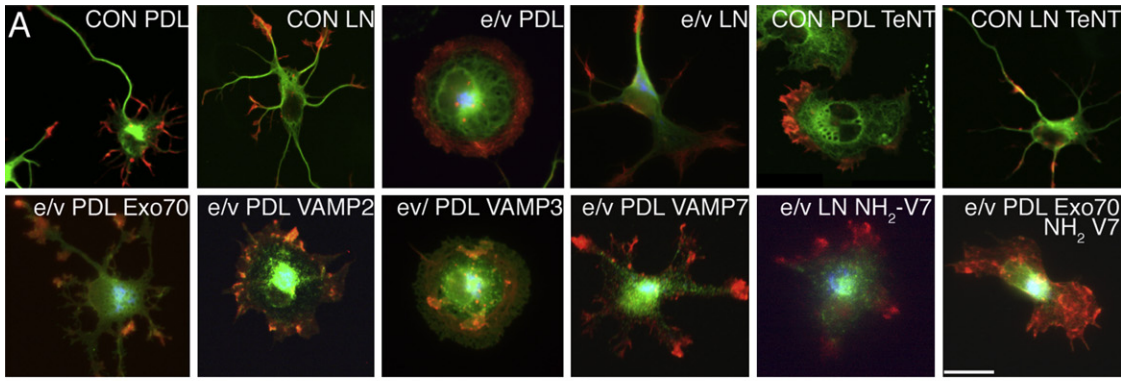
Exocytic Fusion and the Cytoskeleton

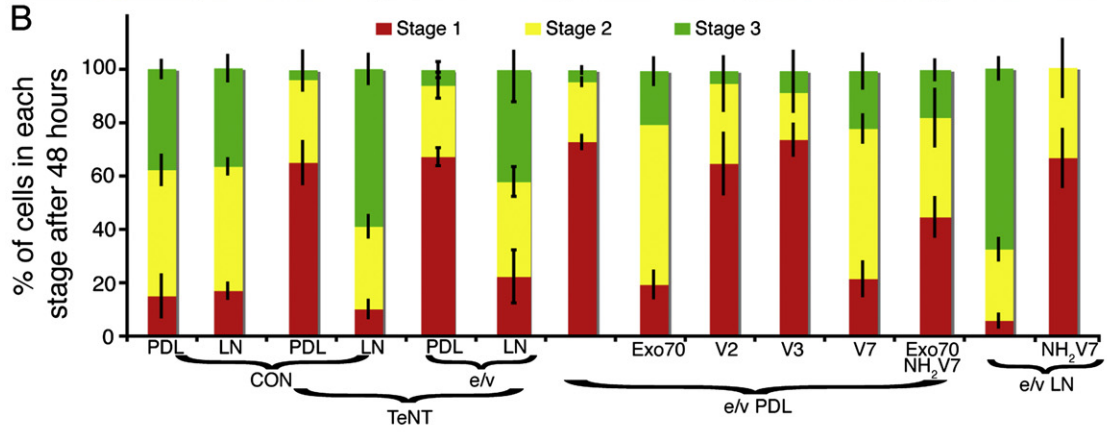
Since the relationship between cytoskeletal dynamics and exocytosis is complex (Becker and Hart, 1999; Sokac and Bement, 2006; Valentijn et al., 2000) and exocytosis and cytoskeletal dynamics are crucial for neuritogenesis in cortical neurons (Dehmelt et al., 2003; Dent et al., 2007), we hypothesized that they were coordinated. To determine how dampening of actin dynamics affects exocytosis, we treated CON neurons with the F-actin capping drug cytochalasin D (CD) at a concentration that blocks filopodia and neurite formation, but does not affect growth cone dynamics once neurites/axons have formed (Dent and Kalil, 2001; Dent et al., 2007). Since VAMP2 is required on PDL and VAMP7 is required for LN-dependent neuritogenesis, we analyzed vesicle fusion on these respective substrates. The frequencies of VAMP2- and VAMP7-mediated exocytosis were significantly decreased by long-term and acute CD treatment ($p < 0.05$) (Figure 6B and Figure S4), indicating that exocytosis mediated by these *v*-SNAREs requires proper F-actin dynamics.

To determine if MT dynamics function in exocytosis, we treated CON neurons with nocodazole at a concentration that dampens MT dynamics and blocks neuritogenesis but leaves polymer levels intact (Dent et al., 2007). Neither VAMP2- nor VAMP7-mediated fusion were significantly affected by long-term treatment (Movie S6; Figure 6B); however, both were slightly reduced by acute treatment (Movie S7 and Figure S4), indicating a complex relationship between MT dynamics and exocytosis exists.

The Cytoskeletal and Exocytic Switches Are Coordinated and Mutually Exclusive

We hypothesized that Ena/VASP or Arp2/3 activity is required for context-dependent (i.e., Ena/VASP versus LN) exocytosis. We imaged VAMP2-phluorin and VAMP7-phluorin CON neurons





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Molecule	Necessary for neurites formed by		Method of Inhibition
	Ena/VASP	LN	
$\alpha_3\beta_1$ integrin	No	Yes	function blocking Abs
$\alpha_7\beta_1$ integrin	No	Yes	function blocking Abs
PI3K	No	No	10 μm LY294002
PKA	No	No	20 μM H89
PKC	No	No	1μM GF109203X
PLD	No	No	0.3% 1-Butanol
src	No	Yes	50 nM PP2
FAK	No	Yes	FRNK
Cdc42	Slight	Yes	N17 mutant
Rac	Slight	Yes	N17 mutant
Ena/VASP	Yes	No	FP4MITO
Arp2/3	No	Yes	CA
VAMP2	Yes	No	TeNT
VAMP7	No	Yes	NH_2-VAMP7

Figure 4. VAMP2 and VAMP7 Are Required for Ena/VASP-Dependent and LN-Dependent, Ena/VASP-Independent Neuritogenesis, Respectively

(A) Neurons expressing AP4Mito (CON) or FP4Mito (e/v) (blue) plated on PDL or LN and cultured 48 hr.

(B) Quantification of neuronal morphologies ± SEM. n ≥ 3 independent experiments/treatment; n ≥ 25 neurons per experiment. 50 nM TeNT and expression of the NH_2 domain of VAMP7 (NH_2-V7) were used to block VAMP2- and VAMP7-mediated exocytosis, respectively. Overexpression of Exo70 or VAMP7 but not VAMP2 nor VAMP3 rescued neuritogenesis in e/v neurons on PDL. VAMP expression is green and phalloidin is red; neurons not expressing VAMP were stained for βIII-tubulin (green). Scale bar represents 10 μm.

(C) Summary of molecules implicated in Ena/VASP- or LN-dependent neuritogenesis.

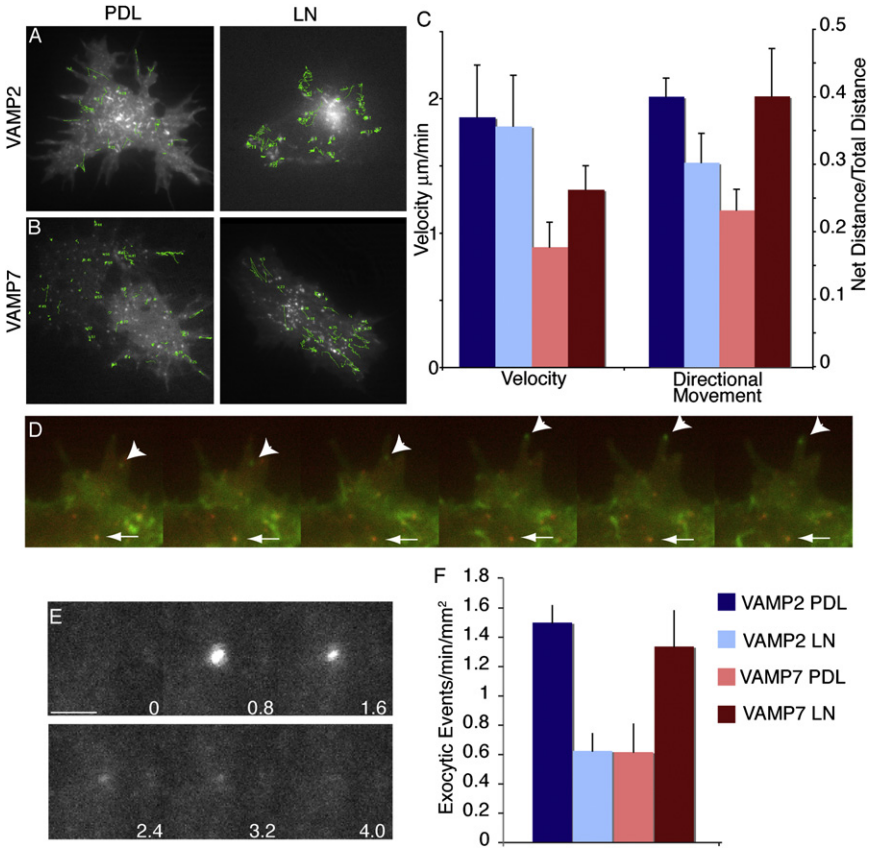

Figure 5. VAMP2- and VAMP7-Containing Vesicles Display Distinct Dynamics and Fusion on PDL and LN

Frames from time lapse of E14.5 CON cortical neurons expressing either GFP-VAMP2 (A) or GFP-VAMP7 (B) plated on PDL or LN, imaged 6 hr after plating. Green tracks indicate vesicle position over time.

(C) Quantification of vesicle speed and directionality of vesicle movement ± SEM. Directionality was calculated by dividing the net distance traveled by the total distance traveled by each vesicle; a higher ratio indicates more directional vesicle movement. $n \geq 5$ cells/condition and $n \geq 30$ vesicles/cell imaged in ≥ 5 consecutive frames. Scale bar represents 5 μm.

(D) Frames from a time lapse of E14.5 CON neurons expressing GFP-VAMP2 and mcherry-VAMP7. Arrowhead indicates a VAMP2-containg vesicle moving into a filopodia, while the arrow indicates a VAMP7-containing vesicle remaining stationary in a more central region.

(E and F) E14.5 CON neurons expressing VAMP2-pHluroin or VAMP7-pHluorin were plated on PDL or LN. After 6 hr, vesicle fusion events on the basal cell membrane were imaged by TIR-FM.

(E) Representative images from a time-lapse demonstrate a vesicle fusion event. The fluorescence intensity of pHluorin is low when it is within the vesicle lumen (t = 0 s). Upon fusion with the plasma membrane, pHluorin is exposed to the higher cytosolic pH, and there is a rapid increase in fluorescence intensity (t = 0.8 s), followed by a rapid diffusion of fluorescence as the molecules disperse within the plasma membrane (t = 1.6–2.4 s). The frequency of vesicle fusion ± SEM is shown in (F). $n \geq 15$ cells/ condition. Scale bar represents 1 μm.

following Ena/VASP or Arp2/3 inhibition. VAMP2-mediated exocytosis on PDL was attenuated significantly by Ena/VASP but not Arp2/3 inhibition ($p < 0.05$) (Figure 6B; Movie S8). In contrast, VAMP7-mediated fusion on LN was unaffected by Ena/VASP inhibition, but reduced by CA expression. Therefore, VAMP2- and VAMP7-mediated exocytosis requires Ena/VASP and Arp2/3 activity, respectively, and/or the type of F-actin structures formed by these two actin regulators. In addition, the switches in cytoskeletal and exocytic machinery following attachment to LN are coordinated.

mDia2 nucleates linear F-actin filaments, drives filopodia formation (Copeland et al., 2004; Harris et al., 2006), and rescues filopodia formation and neuritogenesis in the absence of Ena/VASP (Dent et al., 2007). Expression of mDia2 in e/v neurons also rescued the frequency of VAMP2-mediated vesicle fusion ($p < 0.05$) (Figure 6B; Movie S8), suggesting that F-actin bundles and/or filopodia, structures produced by either mDia2 or Ena/VASP, are required for VAMP2-mediated exocytosis and that mDia2 may support neurite initiation in e/v neurons by rescuing VAMP2-mediated exocytosis.

VAMP7-Mediated Exocytosis Occurs Downstream of LN-dependent Signaling

Our data suggest that (1) specific F-actin structures or remodeling proteins may specify or facilitate the specific v-SNARE driving neuritogenesis and (2) activation of integrin-dependent signaling pathways stimulates VAMP7-mediated vesicle fusion. We hypothesized that VAMP7 overexpression might stimulate exocytosis and neuritogenesis independently of the signaling pathway, as in e/v neurons on PDL, perhaps through a mechanism similar to that of Exo70 overexpression. We overexpressed VAMP7 in e/v neurons on LN when molecules required for LN-dependent neurite initiation were inhibited (Figures 7A and 7B). VAMP7 overexpression rescued neuritogenesis blocked by inhibition of FAK, src, Rac, and Arp2/3 (t tests, $p < 0.05$), suggesting that VAMP7 functions in LN-dependent neuritogenesis downstream of FAK, src, Rac, and Arp2/3. However, VAMP7 was unable to rescue neuritogenesis blocked by CD treatment (Figure S5), indicating that intact actin dynamics mediated by Arp2/3 and likely another actin regulator such as cofilin, DAAM1, or Cordon blue (Ahuja et al., 2007; Aizawa et al., 2001; Chen et al., 2006; Matusek et al., 2008) may be involved.

To assess directly whether VAMP7 functions downstream of integrin signaling, we imaged exocytic events on LN and found FAK or src inhibition (both of which block LN-dependent neuritogenesis) decreased VAMP7-mediated exocytosis ($p < 0.05$) (Figure 7C; Figure S4 and Movie S9), indicating that they regulate VAMP7-mediated exocytosis in this context.

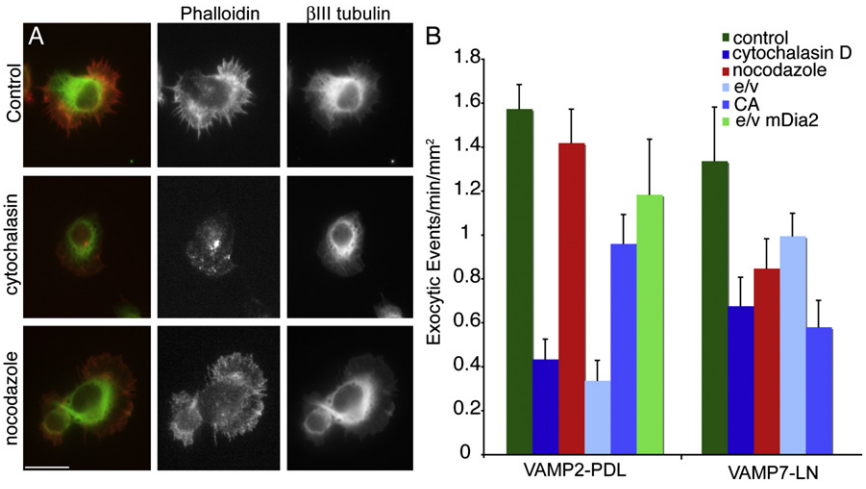

Figure 6. Cytoskeletal Dependence of VAMP-Mediated Vesicle Fusion

(A) Control, CD, and nocodazole-treated E14.5 CON neurons stained with phalloidin (red) and βIII-tubulin (green) 6 hr after plating. Scale bar represents 5 μm.

(B) Frequency of vesicle fusion ± SEM in E14.5 neurons expressing either VAMP2-pHluroin or VAMP7-pHluorin plated on PDL or LN, respectively, as in Figure 5. VAMP2 and VAMP7 fusion events were significantly reduced by CD treatment ($p < 0.05$), but not nocodazole treatment, although VAMP7 events showed an insignificant reduction (t test, $p = 0.1$). VAMP2-mediated fusion, but not VAMP7-mediated fusion, was reduced in e/v neurons. Inhibition of the Arp2/3 complex (CA) significantly reduced VAMP7-mediated fusion but not VAMP2-mediated fusion. The low frequency of VAMP2-mediated exocytic fusion in e/v neurons was rescued by expression of mDia2 (e/v mDia2). $n \geq 15$ cells/condition.

DISCUSSION

We have identified two mutually exclusive, parallel pathways that mediate neuritogenesis. Both pathways require specific coordinated sets of F-actin and exocytic machinery. We found that exocytosis is required for neurite initiation and is mediated by a specific pairing of *v*-SNARE and F-actin regulator. Cortical neurons normally require the activities of Ena/VASP (Dent et al., 2007) and VAMP2. Attachment to LN rendered Ena/VASP and VAMP2 dispensable and revealed a requirement for Arp2/3 and VAMP7 downstream of integrin, FAK, src, and Rac (Figure 4). Interestingly, VAMP2-mediated exocytosis required Ena/VASP while VAMP7-mediated exocytosis required Arp2/3. This may indicate a preference of *v*-SNARE-containing vesicles for specific F-actin structures—linear and/or bundled versus branched F-actin. These changes occurred downstream of integrin signaling, raising the possibility that similar conversion mechanisms may be used for neurite initiation by other neuronal types, for other steps in neuronal development, such as axon outgrowth and chemoattraction, or in shape changes and motility of non-neuronal cells.

An Unexpected Role for Ena/VASP in Exocytosis

Our data indicate that Ena/VASP proteins or associated F-actin structures function in VAMP2-mediated exocytosis and that an exocytosis defect contributed to a neuritogenesis block in e/v neurons. Both Ena/VASP and VAMP2 inhibition blocked neuritogenesis and e/v neurons had reduced VAMP2 exocytic fusion. Neuritogenesis in e/v neurons was rescued by overexpression of Exo70 or VAMP7, but not VAMP2, indicating that VAMP2 function requires Ena/VASP or the type of structures produced by Ena/VASP. Introducing mDia2, which promotes F-actin polymerization and filopodia formation, rescued the frequency of VAMP2-mediated exocytosis in e/v cells, correlating with the ability of mDia2 to rescue e/v neuritogenesis (Dent et al., 2007). Together these results lead us to propose that VAMP2-mediated exocytosis requires F-actin structures typically formed by Ena/VASP. Thus, a critical role of Ena/VASP in neuritogenesis involves VAMP2-mediated exocytosis. In contrast to VAMP2, VAMP7-mediated exocytosis required Arp2/3 but not Ena/VASP, suggesting VAMP7 may rely on branched F-actin for vesicle fusion. VAMP2 has been implicated in growth cone chemoattraction (Tojima et al., 2007), while VAMP7 is required for axon elongation (Martinez-Arca et al., 2000). It is possible that a mechanism similar to the regulated switch in *v*-SNAREs and actin regulatory proteins that we identified here may also be used at other times during neuronal morphogenesis to regulate exocytosis and therefore membrane extension and movement.

Integrin Activation Switches the Actin and Exocytic Machinery Driving Shape Change

Overexpression of α_5-integrin and attachment to FN rescued neuritogenesis in e/v neurons. Activated src, a common target of integrin signaling, also rescued neuritogenesis in e/v neurons, indicating the changes in cytoskeletal and exocytic machinery may be triggered by a canonical integrin signaling pathway, leading to neurite initiation or other morphological changes, depending on the ECM composition and integrin repertoire present.

In the cortex, neurons are packed tightly and not in contact with significant amounts of LN or many other types of common ECM components that activate integrin signaling—likely the reason why neurons lacking Ena/VASP exhibit a cortex-specific neuritogenesis defect in vivo. In fact, the relevant substrate for neurons undergoing neuritogenesis in the cortex remains unknown. However, Ena/VASP-deficient neurons elsewhere in the brain and body are in contact with substantial amounts of integrin ligands including the ectopic cortical mutant neurons that reach the pial membrane, dorsal root ganglia, and retinal neurons and form neurites in the absence of Ena/VASP. The context-dependent, mutually exclusive pathways may therefore be used to regulate distinct cell behaviors in different environments in both physiological and pathological conditions.

As a result of a canonical integrin-signaling pathway, we found an unexpected switch in exocytic and cytoskeletal machinery. On LN, Arp2/3 is likely activated downstream of integrin, src, FAK, and Rac. We anticipate this alters the role of Arp2/3 in

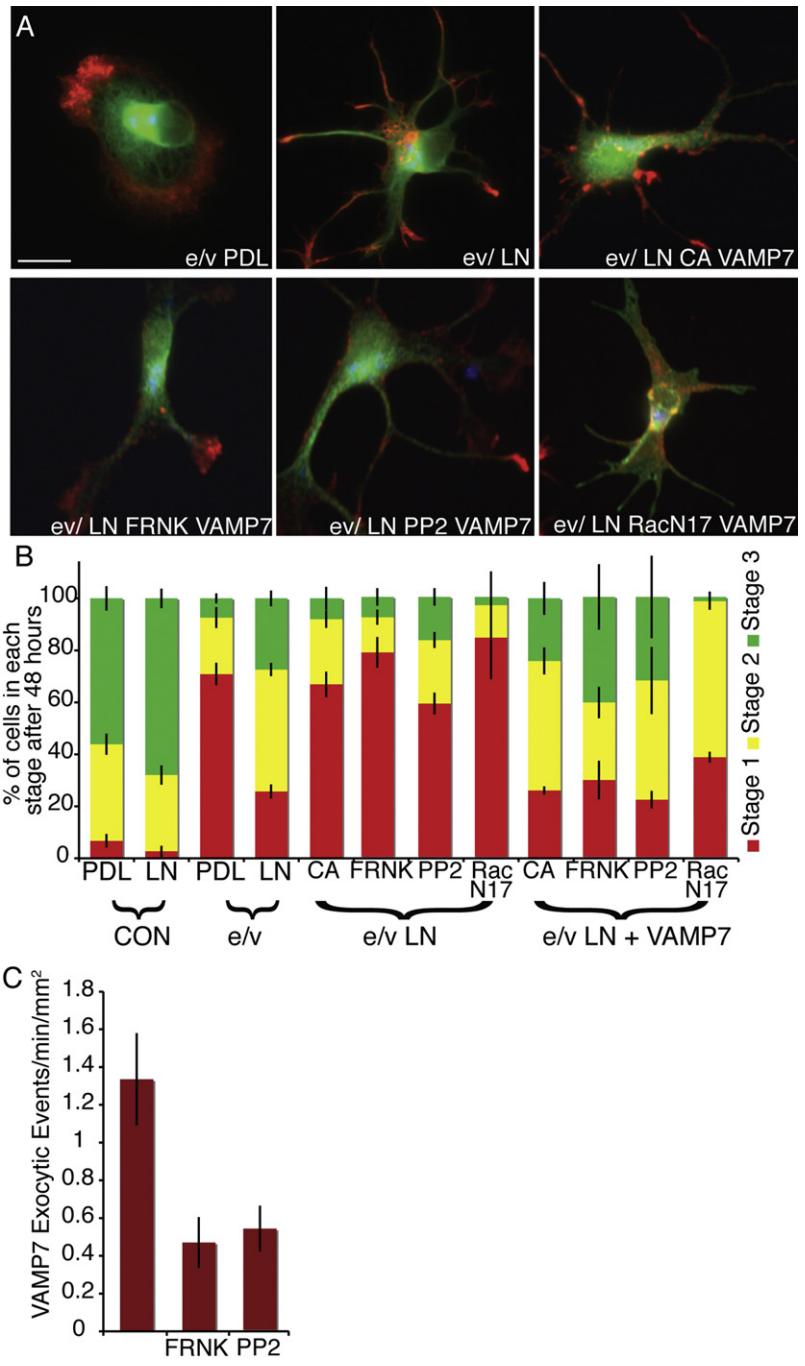

Figure 7. VAMP7 Rescues Neuritogenesis Downstream of FAK, Src, Rac, and Arp2/3 Activity

(A) E14.5 neurons cultured 48 hr expressing FP4Mito (e/v) (blue) ± GFP-VAMP7 (green) were plated on PDL or LN. Phalloidin staining is shown in red. Neurons not expressing GFP-VAMP7 are stained for βIII-tubulin (green). Scale bar represents 10 μm.

(B) Quantification of morphologies, as in Figure 1. n ≥ 3 independent experiments/treatment and n ≥ 25 neurons per experiment. VAMP7 overexpression rescues neuritogenesis of e/v neurons on LN with inhibited FAK (FRNK), Arp2/3 (CA), Rac (RacN17), or src (PP2).

(C) Frequency of VAMP7-mediated vesicle fusion ± SEM in CON E14.5 neurons expressing VAMP7-pHluorin and either FRNK or treated with 50 nM PP2 plated on LN.

neuronal morphology seen previously (Korobova and Svitkina, 2008; Pinyol et al., 2007; Strasser et al., 2004). Src, FAK, Rac, and Arp2/3 share several binding and activity partners, including paxillin, cortactin, p130Cas, p190RhoGAP, Nck, and Grb2 (Brouns et al., 2001; Brown et al., 2005; Brunton et al., 2004; Rohatgi et al., 2001; Tehrani et al., 2007; Vuori and Ruoslahti, 1995), which could coordinate FAK and src activity with Rac and Arp2/3 activation (Rohatgi et al., 2001; Tehrani et al., 2007).

VAMP7-mediated exocytosis was stimulated by LN and dampened by src, FAK, or Arp2/3 inhibition. This may involve interaction between Arp2/3 and Exo70 (Liu et al., 2009; Zuo et al., 2006). Exo70 has been found to activate or coactivate Arp2/3-mediated actin polymerization (Liu et al., 2009), is involved in SNARE complex assembly and stabilization (Wiederkehr et al., 2004), and is a component of the exocyst complex, which is integral in vesicle trafficking during neuronal morphogenesis (Murthy et al., 2003). Exo70 overexpression rescued neuritogenesis in e/v neurons on PDL, similar to VAMP7 overexpression, and Exo70-mediated neuritogenesis was dependent on VAMP7 activity. VAMP7 overexpression also rescued neuritogenesis in e/v neurons on LN following FAK, src, Rac, or Arp2/3 inhibition; inhibition of these same molecules decreased the frequency of VAMP7 exocytic events. We propose that VAMP7 is downstream of these molecules in the LN-dependent neuritogenesis pathway.

While the entire signaling pathway is not yet elucidated, the small GTPase Ral is an intriguing candidate molecule that may connect Arp2/3, the exocyst complex, and VAMP7 to integrin signaling. Exocyst-driven filopodia formation occurs downstream of RalA activity, which binds to Sec5 of the exocyst complex (Sugihara et al., 2002). Ral is activated by a number of molecules, including PI3K, which we find does not function in LN-dependent neuritogenesis; PDK1 (Tian et al., 2002); and BCAR3, which forms a complex with Cas, Src, and FAK (Gotoh et al., 2000), some of which we have implicated in neuritogenesis. Ral is also regulated by direct phosphorylation by an unknown kinase whose activity is negatively regulated by the tumor suppressor phosphatase PP2A (Sablina et al., 2007). PP2A associates with β_1-integrin and is involved in bidirectional integrin regulation. Future work will be needed to understand the entire regulatory pathways that link ECM to neuritogenesis. Such information may make it possible to manipulate the pathway to drive neuritogenesis in a spatiotemporally controlled manner and therefore be of value in designing therapies to rebuild a cortex damaged by injury or disease.

EXPERIMENTAL PROCEDURES

Cortical Neuron Culture and Transfection

All mouse procedures were approved by the Massachusetts Institute of Technology Committee on Animal Care.

Cortical neuron cultures were prepared from embryonic day (E) 14.5 mice, as previously described (Dent et al., 2007; Kwiatkowski et al., 2007). Standard methods were used for western blotting, fixation, and immunocytochemistry (Dent et al., 2007). Additional details are available in the Supplemental Experimental Procedures.

Microscopy

Immunofluorescence images were acquired on an ORCA-ER CCD camera (Hamamatsu) using a 60 × 1.4 NA Plan Apo (DIC-fluor) Nikon objective on an inverted TE300 microscope (Nikon) equipped with dual Ludl filter wheels for excitation and emission. Time-lapse images of neurons were acquired on an inverted Nikon TE2000U microscope modified to allow for through-the-objective multispectral TIR-FM using a 100 × 1.45 NA objective. The wavelength and intensity of the 2W multi-line laser (Coherent) were controlled with an AOTF. Laser light was focused at the aperture plane and directed to the coverslip by a dichromatic mirror (Chroma), and the laser angle was adjusted manually with a micrometer. Since neurons were imaged only 6 hr after plating and were not tightly adhered to the coverslips, a thick TIRF illumination field of approximately 200 nm was used. Fluorescence emission was controlled with a filter wheel device containing narrow bandpass emission filters (Sutter Instruments). Images were acquired on a CoolSnap HQ2 CCD camera (Photometrics). Neurons were imaged in Neurobasal media supplemented with B27 and kept at 37°C and 5% CO_2 in an incubation chamber (Solent) fitted for the microscope. Vesicle dynamics were imaged at 3 s intervals for 5 min. Exocytic fusion events were imaged at 0.8 s intervals for 5–8 min.

Image Analysis and Statistics

All images were collected, measured, and compiled with Metamorph imaging software (Molecular Devices). Morphological stage was quantified after 48 hr in culture; neurites were considered any narrow and consolidated extension proximal to cell body. Stage 1 neurons lack neurites, stage 2 have one or more minor neurites, and stage 3 have one neurite at least twice as long as any other. Vesicle dynamics were tracked using the "Track Points" function in Metamorph. Vesicle fusion events were specified by the appearance of a vesicle, a bright increase in fluorescence intensity (>3-fold over background), followed by a rapid diffusion (<2 s). All statistical tests were performed with AnalyzeIT software. ANOVA with a Tukey post-hoc test were used to determine significance, unless otherwise noted in cases of a standard t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, nine movies, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2010.02.017.

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