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Balanced Vav2 GEF activity regulates neurite outgrowth and branching *in vitro* and *in vivo*

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Abstract

We have investigated the role of Vav2, a reported Rac1/Cdc42 GEF, on the development of *Xenopus* spinal neurons *in vitro* and *in vivo*. Both gain and loss of Vav2 function inhibited the rate neurite extension on laminin (LN), while only GFP-Vav2 over-expression enhanced process formation and branching. Vav2 over-expression protected neurons from RhoA-mediated growth cone collapse, similar to constitutively active Rac1, suggesting Vav2 activates Rac1 in spinal neurons. Enhanced branching on LN required both Vav2 GEF activity and N-terminal tyrosine residues, but protection from RhoA-mediated collapse only required GEF activity. Interestingly, wild-type spinal neurons exhibited increased branching on the cell adhesion molecule L1, which required Vav2 GEF function, but not N-terminal tyrosine residues. Finally, we find that Vav2 differentially affects Rohon-Beard peripheral and central process extension, but promotes neurite branching of commissural interneurons near the ventral midline. Together, we suggest that balanced Vav2 activity is necessary for optimal neurite outgrowth and promotes branching by targeting GEF activity to branch points.

Keywords

axon pathfinding; *Xenopus*; GEF; Rac1; L1; commissural interneuron; growth cone

Introduction

To generate neuronal connectivity during development, neurons undergo cell-specific morphological differentiation, such as process polarization, elongation, branching and arborization. A number of factors in the environment of developing neurons positively and negatively influence the extension and branching of axons and dendrites. For example, neurotrophin, Netrin, Wnt and Slit proteins have been found to promote axon branching, while Slits, Ephrins and Semaphorins can inhibit axon branching (Bodmer et al., 2009; Campbell et al., 2007; Dent et al., 2004; Ma and Tessier-Lavigne, 2007; Marler et al., 2008; Miyashita et al., 2004; Szebenyi et al., 2001; Wang et al., 1999; Yeo et al., 2004). In particular, Slits have been demonstrated to promote branching and arborization of peripheral sensory axons in mice and zebrafish (Ma and Tessier-Lavigne, 2007; Miyashita et al., 2004), although the precise molecular mechanisms responsible for branching are poorly understood.

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The Rho family of GTPases regulate the reorganization of the cytoskeleton to influence neuronal morphogenesis, as well as migration, adhesion, and proliferation. Of the twenty-two putative Rho GTPases identified in mammals, Cdc42, Rac1 and RhoA proteins are the best-characterized regulators of cell morphology and motility. Many studies have shown a significant role for Cdc42, Rac1 and RhoA in different aspects of neuronal development, including axon outgrowth and guidance, neuronal morphogenesis and synaptic formation (Dickson, 2001; Luo, 2002; Negishi and Katoh, 2002). In addition, cross-talk between Rho GTPases has been demonstrated to contribute to cellular responses (Iden and Collard, 2008). For example, Rac1 has an antagonistic effect on RhoA (Nimnual et al., 2003; Sander et al., 1999). However, the molecular basis for the complex linkage between Rho GTPases is largely unknown.

Rho GTPases act as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state. Activation of Rho GTPases is promoted by guanine nucleotide exchange factors (GEFs), which promote the GDP-GTP exchange. GEF proteins outnumber Rho GTPase proteins and show tissue-, developmental-, and sub-cellular location specific distribution, suggesting that cellular functions are specified at this level (Schmidt and Hall, 2002). Since Cdc42, Rac1 and RhoA play such a variety of essential roles in neural development, GEFs are likely to provide much of the specificity in their functions. Consistent with this notion, several GEFs have now been found to regulate Rho GTPase activities in the nervous system. For example, the Rac-specific GEFs, T lymphoma invasion and metastasis 1 (Tiam1) and its Drosophila homolog Still life (SIF), promote axon formation and dendritogenesis downstream of axon guidance cues (Kunda et al., 2001; Leeuwen et al., 1997; Matsuo et al., 2002; Miyamoto et al., 2006; Sone et al., 1997; Tanaka et al., 2004). GEFs are multi-domain proteins that often contain multiple accessory or regulatory elements that provide specific functions, some of which are independent of GEF activity. However, it is not clear how individual GEFs are regulated in neurons downstream of environmental factors to influence neuronal motility and morphogenesis.

Vav is a prototypical GEF that has diverse and still poorly understood cellular functions. Three members of mammalian Vav family proteins have been identified. Vav1 is exclusively expressed in hematopoietic cells, while Vav2 and Vav3 are ubiquitously expressed (Katzav et al., 1989; Movilla and Bustelo, 1999; Schuebel et al., 1996). Vav proteins contain several domains that may function in various signaling events; a calponin-homology (CH) domain, an acidic-rich (AR) region, Dbl homology (DH) and pleckstrin homology (PH) domains, a zinc finger domain and two SH3 regions flanking an SH2 domain. Interestingly, similar to Ephexin (Zhang et al., 2007), Vav proteins exhibit tyrosine-phosphorylation-dependent GEF activity (Lopez-Lago et al., 2000). Previous reports have shown that Vav1 is tyrosine phosphorylated (pY) by a variety of tyrosine kinases including SFKs in lymphocytes (Fujimoto et al., 1999; Michel et al., 1998) at residues in the AC region (pY142, 160, 174 in Vav1), which leads to an open configuration that promotes Vav1 GEF activity (Aghazadeh et al., 2000; Amarasinghe and Rosen, 2005; Crespo et al., 1997; Llorca et al., 2005).

Genetic evidence shows that Vav proteins play essential roles in several developmental processes (Bustelo, 2000; Faccio et al., 2005; Sauzeau et al., 2006). However, most data concerning the Vav protein family emerge from studies in hematopoietic cells (Turner and Billadeau, 2002). While Vav function in developing neurons remains less clear, recent evidence does suggest that Vav has important roles in axon guidance (Cowan et al., 2005). For example, Vav2 mediates endocytosis-dependent growth cone collapse by activation of Rac1 in ephrin/Eph signaling during axon guidance. In addition, Vav2/3 deficient mice exhibited defects in the ipsilateral retinogeniculate projections.

To better understand the function of Vav2 in neuronal development, we examined spinal neuron morphogenesis *in vitro* and *in vivo* under conditions of Vav2 gain of function (GOF) and loss of function (LOF). We observed substratum-specific effects of Vav2 GOF and LOF *in vitro*. Over-expression of Vav2 promoted axon branching, but inhibited neurite outgrowth on LN, while branching on L1 is dependent on endogenous Vav2 function. Examination of Rohon-Beard (RB) sensory neurons shows a differential role for Vav2 in peripheral and central process axon outgrowth *in vivo*, as well as a specific axon branching phenotype by commissural interneurons (CIs) during midline crossing. Together our results suggest that Vav2 has an important role in neuronal morphogenesis that depends on the cellular environment.

Results

Vav2 over-expression slows neurite outgrowth and enhances neurite branching

Only two Vav family members have been cloned from *Xenopus laevis*. xVav1 (70% similarity to human Vav1) and xVav2 (81% similarity to human Vav2) were isolated from adult and from embryonic tissues, respectively (NIH *Xenopus* initiative project) (Klein et al., 2002). As Vav1 is exclusively expressed in hematopoietic cells, we examined more closely the expression pattern of Vav2. We found that Vav2 is uniformly expressed throughout early *Xenopus* embryonic development and is present in the spinal cord, as detected by western blot of total Vav2 (supplemental Fig. S1A). Moreover, using an anti-phosphotyrosine-Vav antibody (pY174-Vav) (Barfod et al., 2005), which recognizes a conserved regulatory site for all Vav family members (Amarasinghe and Rosen, 2005; Bustelo, 2000; Lopez-Lago et al., 2000; Miletic et al., 2006; Tamas et al., 2003), we found that phospho-Vav is expressed within growth cones and filopodia of isolated spinal neurons *in vitro* (supplemental Fig. S1B-G). The expression of Vav2 in the developing spinal cord suggests it may function in the morphological differentiation of spinal neurons.

To begin to better understand the function of Vav2 in spinal neuron development, we performed both gain and loss of function experiments. First, we examined the effects of over-expressing GFP-Vav2 wild type (WT) on dissociated spinal neurons cultured on laminin (LN) (Fig. 1A-D). Surprisingly, over-expression of GFP-Vav2 WT inhibited the rate of neurite outgrowth (Fig. 1F). Assuming that Vav2 over-expression increases active Rac1 and/or Cdc42 in growth cones, this result is consistent with the notion that balanced levels of Rho GTPase activity is necessary for optimal promotion of neurite outgrowth (Kuhn et al., 1998; Ruchhoeft et al., 1999; Woo and Gomez, 2006). Expression of GFP-Vav2 WT also altered the morphology of spinal neurons on LN (Fig. 1A-D). The majority of control spinal neurons on LN (78%) had a single process, whereas more than sixty percent of GFP-Vav2 WT neurons were multi-polar (Fig. 1G). In addition, GFP-Vav2 expressing neurons had increased branching by their primary neurites compared to control neurons (Fig. 1H, I). Consistent with Vav2 activating Rac1 over Cdc42, constitutively active (CA)-Rac1 had similar effects on neurite formation and branching, while CA Cdc42 had little effect on branching (Supplemental Fig. S2). Together, these findings suggest that Vav2 enhances neuronal morphological complexity through activation of Rac1.

To address whether Vav2 GEF activity is necessary for neurite formation and branching, we used a Vav2 DH mutant, where the L342/L343 residues were mutated to R342/S343 in the DH domain of Vav2 (Vav2L342R/L343S) (Fig. 1E). Unlike Vav2 WT, expressing Vav2L342R/L343S in neurons did not promote branching, rather eliminated all basal branching on LN (Fig. 1H, I), consistent with the dominant negative effects of this Vav2 DH mutant (Marignani and Carpenter, 2001). This result suggests that endogenous Vav2 functions to promote neurite formation and branching by spinal cord neurons on LN.

Tyrosine phosphorylation of Vav2 is required for enhanced axon branching

Vav proteins are tyrosine phosphorylated in immune cells by Src and Syk family kinases in response to growth factor stimulation (Aghazadeh et al., 2000; Bustelo, 2000; Crespo et al., 1997; Marignani and Carpenter, 2001; Schuebel et al., 1998; Servitja et al., 2003; Tamas et al., 2003). In particular, phosphorylation of conserved tyrosine residues within the N-terminal acidic region of Vav proteins has been shown to relieve the autoinhibition of the Vav GEF domain (Aghazadeh et al., 2000; Llorca et al., 2005). Vav1Y174F exhibited elevated GEF activity compared to WT Vav1, suggesting Vav tyrosine mutants are constitutively active (Li et al., 2008). Moreover, several studies conclude that tyrosine phosphorylation within the AR domain regulates endogenous Vav function through protein-protein interactions (Kuhne et al., 2000; Miletic et al., 2006; Tamas et al., 2003; Tybulewicz et al., 2003). To examine the role of tyrosine phosphorylation of Vav2 in neurite outgrowth and branching, we tested the effects of Vav2Y159/172F expression in neurons (Tamas et al., 2003). Expression of Vav2Y159/172F inhibited neurite outgrowth similar to Vav2 WT (Fig. 1F), but did not significantly promote process formation or branching (Fig. 1G-I). However, Vav2Y159/172F did not reduce the basal level of axon branching on LN, suggesting it may not inhibit endogenous Vav2 function. To determine whether Src family kinase (SFK) activity is necessary upstream of Vav2 to promote branching, we examined the effects of PP2, a selective SFK inhibitor. Treatment of neurons with PP2 attenuated the increased process formation and branching induced by Vav2 WT, but also did not reduce basal branching on LN (Fig. 1G-I). PP3, an inactive PP2 analog, did not significantly change the outgrowth or branching of control neurons (data not shown). These data suggest that tyrosine phosphorylation of Vav2 at Y159 and Y172 by SFKs enhance neurite formation and branching, but are not necessary for basal branching on LN.

Vav2 suppresses LPA-induced neurite retraction

To determine whether Vav2 over-expression influences other Rho GTPase signaling pathways in neurons, we tested the effects of lysophosphatidic acid (LPA), a bioactive lipid that strongly activates RhoA to induce neurite retraction and chemorepulsive turning (Kozma et al., 1997; Kranenburg et al., 1999; Yuan et al., 2003). Since Rac1 and Cdc42 inhibit RhoA signaling through signal cross-talk (Nimnual et al., 2003; Sander et al., 1999; Yuan et al., 2003), Vav2 over-expression is predicted to dampen the effects of LPA if these neurons have elevated Rac1 and/or Cdc42 activity. Indeed, while the majority of control neurons (88%) retracted after a 10 min exposure to 100 nM LPA (Supplemental Movie 1), only 30% GFP-Vav2 WT expressing growth cones were affected by LPA (Fig. 2). Similar to Vav2 WT, expression of CA Rac1 strongly attenuated LPA-induced neurite retraction, while CA Cdc42 only partially blocked LPA-induced retraction (Fig. 2E). As expected, the GEF-inactive Vav2 DH mutant did not protect neurons from LPA-induced retraction (Fig. 2E). However, the inhibitory effect of LPA on neurite outgrowth was strongly dampened in neurons expressing Vav2Y159/172F (Fig. 2E), consistent with reports showing Rac1 is activated by AC domain tyrosine mutants (Lopez-Lago et al., 2000; Marignani and Carpenter, 2001; Miletic et al., 2006). Together, these results suggest that Vav2 activates Rac1 to inhibit RhoA signaling downstream of LPA.

Antisense knockdown of xVav2 inhibits branching

We examined the effects of xVav2 knockdown (KD) on neurite development by using a translation-blocking antisense morpholino oligonucleotide (MO). Injection of xVav2 MO into two blastomeres of four-cell stage embryos led to a 25% reduction in xVav2 protein expression by western blot densitometry (Fig. 3A,B). As no greater than one-half of the cells can be descended from xVav2 MO injected blastomeres, we can infer that Vav2 was reduced by at least 50% in KD cells. In contrast, control MO injection did not significantly alter Vav2 protein levels (data not shown). To identify xVav2 KD neurons in culture, we co-injected either GFP or mCherry-CAAX together with xVav2 MO. Since total Vav2 antibodies that work by

immunocytochemistry in *Xenopus* are not available, we tested if the levels of p-Vav were reduced in xVav2 KD growth cones. Phospho-Vav was modestly, but significantly reduced within Vav2 KD growth cones (Fig. 3C-K), possibly reflecting a compensatory increase in the proportion of remaining Vav2 that is tyrosine phosphorylated in KD neurons. However, despite only a partial KD of Vav2, loss of Vav2 function had dramatic effects on neuronal morphology (Fig. 3L-O). First, consistent with the need for balanced Rho GTPase activity for optimal neurite outgrowth, we found that xVav2 KD neurons had a reduced rate of neurite outgrowth compared to control MO injected neurons (Fig. 3L). Second, although spinal neurons are typically uni-polar and not highly branched on LN, the frequency of basal neurite branching by xVav2 KD spinal neurons was reduced even further (Fig. 3N, O), suggesting that endogenous Vav2 promotes neurite branching on LN.

L1 promotes neurite branching that requires Src and Vav2 GEF activity

L1 is a cell adhesion molecule (CAM) of the immunoglobulin super-family that promotes neurite outgrowth, branching, axon fasciculation and guidance (Castellani et al., 2000; Cheng et al., 2005; Dahme et al., 1997). In B35 cells, L1-dependent clustering led to tyrosine phosphorylation of Vav2 and increased GEF activity toward Rac1 (Schmid et al., 2004). To test whether L1 signals through Vav2 to regulate spinal neuron morphogenesis, we examined neurons cultured on an L1 substratum. Compared to neurons on LN, L1 increased the average number of processes and branches per neuron, but reduced the rate of primary neurite outgrowth (Fig. 4). To investigate the functional role of Vav2 in neurite formation and branching elicited by L1, we performed GOF and LOF experiments as described previously. Both Vav2 DH and xVav2 MO significantly suppressed neurite formation, branching and outgrowth on L1 (Fig. 4C-F), as did expression of dominant negative (DN) Rac1 (supplemental Fig. S3). Surprisingly, over-expression of either Vav2 WT or Vav2Y159/172F did not significantly alter neurite branching on L1 (Fig. 4C-F). Strong activation of endogenous Vav2 by L1 may account for little effect of Vav2 WT and Vav2Y159/172F. However, inhibition of SFKs with PP2, but not PP3 (data not shown), strongly inhibited neurite extension and basal branching on L1 (Fig. 4C-F). These results suggest that L1 promotes neurite formation and branching by activating Src and Vav2 GEF dependent activation of Rac1.

Vav2 is required for development of RB axons

Next we examined whether Vav2 functions in the development of RB sensory axons *in vivo*. RB neurons are a transient population of primary sensory neurons within the embryonic spinal cord. These neurons have central axons that project unbranched processes rostrally and caudally within a dorsal longitudinal fascicle (DLF) of the spinal cord and highly branched peripheral processes that extend over or through the segmental myotomes to innervate the skin (Clarke et al., 1984; Taylor and Roberts, 1983). First we examined the RB central axons within the DLF. Microinjection of one blastomere of four or eight-cell stage embryos allowed us to compare the axon tracts of neurons of the injected half with the uninjected control side within the same spinal cord. All RB axons were identified by immunolabeling with anti-HNK-1, which also labels crossed commissural interneurons (CIs). In control embryos, the RB central axons form DLFs on each side of the spinal cord that are similar widths (DLF width injected side/uninjected side=1.02±0.07, p=0.10) (Fig. 5A,B,G,H,M). On the other hand, over-expression of GFP-Vav2 WT in RB neurons resulted in an enlarged DLF along the length of the spinal cord (DLF width injected side/uninjected side=1.31±0.05, P<0.001) (Fig. 5C,D,I,J,M). In contrast, KD of endogenous xVav2 in RB neurons resulted in thinner DLFs on the injected sides of the spinal cord compared to the uninjected sides (0.70±0.02, P<0.001) (Fig. 5E,F,K-M). As an independent measure, we found that the fluorescent intensity of HNK labeling within the DLF was elevated in Vav2 WT expressing embryos and reduced in xVav2 KD embryos compared to control DLFs (Fig. 5N). To assess whether changes in the number of RB neurons may account for differences in fascicle widths, we counted the number of RB

nuclei labeled with an antibody against islet1, a marker for RB and primary motoneuron nuclei (Inoue et al., 1994). We found no differences in the number of RB nuclei counted in dorsal spinal cords between labeled and unlabeled sides of embryos (data not shown). Together with our *in vitro* data, these findings suggest that Vav2 regulates the DLF width by increasing RB axon number or length, rather than by regulating neuronal survival, which could be due to both increased process formation and axon branching.

Next we assessed the effects of Vav2 over-expression and KD on RB peripheral processes within the dorsal skin. RB peripheral axons exit the spinal cord and project ventrally along the inner surface of the skin, where they fasciculate very little, but branch extensively during development (Clarke et al., 1984). We expected that Vav2 gain and loss of function might lead to branching defects by RB peripheral processes. However, we could not observe obvious defects in RB peripheral axon branching, possibly due to our inability to resolve individual processes by this method. On the other hand, we did observe that both increasing and decreasing Vav2 function reduced RB peripheral axon outgrowth (Fig. 6). We compared the maximum lengths and threshold area of peripheral RB skin projections from control and injected halves of embryos. Injection of control MO did not change the outgrowth of RB peripheral axons compared to the uninjected side (Fig. 6A,D,G,H). On the other hand, both Vav2 WT over-expression (Fig. 6B,E) and xVav2 KD (Fig. 6C,F) significantly decreased the area and maximum length of peripheral axons (Fig. 6G,H). These results suggest that balanced Vav2 activity promotes the outgrowth of RB peripheral axons in the skin.

Vav2 affects development of commissural interneurons (CIs)

Finally, we investigated the consequences of Vav2 gain and loss of function in midline crossing by spinal CIs. CI axons encounter a variety of guidance cues near the midline, which is an important intermediate choice point where growth cones undergo morphological and directional changes (Kaprielian et al., 2001). To measure CI growth cones and their trajectories across the midline, we imaged GFP fluorescent CIs of stage 25-26 whole mount embryos viewed ventrally along the spinal cord. Normally, CIs extend a single, unbranched process ventrally and cross the midline, followed by turning to grow longitudinally or branching in the contralateral marginal zone (Fig. 7A) (Moon and Gomez, 2005). CI growth cones typically remain morphologically simple near the midline, but become more complex after crossing to the contralateral side of the spinal cord (Moon and Gomez, 2005). Interestingly, growth cones of Vav2 WT-expressing CIs were larger, more lamellipodial and more complex than control CI growth cones (Fig. 7B,C). Over thirty percent of Vav2 expressing CIs had branched axons before reaching the contralateral ventral fascicle (Fig. 7B,D). These findings suggest that Vav2 WT-expressing CI growth cones are hypersensitive to cues near the midline that signal through Vav2. On the other hand, we did not observe significant differences in growth cone morphologies of xVav2 KD CI neurons (Fig. 7C,D), but these neurons did show stunted outgrowth and some guidance errors (Fig. 7E-I). To measure the extent of CI outgrowth we injected one blastomere with xVav2 MO + GFP RNA and the opposite side blastomere with control MO + mCherry-CAAX RNA. The total length of CI axons could then be compared between the control side (Fig. 7E) and the xVav2 KD side (Fig. 7F) of the same spinal cord within each embryo. Analysis of the average growth cone position (see Methods) within four quadrants defined along the anterior-posterior (A-P) axis of the spinal cord revealed that xVav2 KD CIs were significantly shorter than control CIs across the A-P axis of the spinal cord (Fig. 7H). In addition, the angle of midline crossing by CIs was categorized as anterior, medial or posterior (see Methods). By this analysis, the vast majority of CIs showed an anterior bias as they extend across the midline, whereas xVav2 KD CIs exhibited more random orientations (Fig. 7I).

Discussion

Previous studies have linked Vav2 to Eph receptor endocytosis in retinal neurons (Cowan et al., 2005) and CD47-mediated dendritic branching of hippocampal neurons (Murata et al., 2006), but Vav2 function in the development of spinal axons has not been explored. Here we used Vav2 GOF and LOF to study the role of Vav2 in spinal neuron morphogenesis *in vitro* and *in vivo*. We found that Vav2 over-expression reduced spinal axon outgrowth, while increasing neurite formation and branching on LN. In contrast, xVav2 KD with antisense MO decreased both neurite outgrowth and branching. Vav2-mediated branching was strongly promoted by L1 *in vitro*, which required Vav2 GEF activity, as well as Rac1 and Src function. Manipulation of Vav2 function in spinal neurons *in vivo* resulted in cell-type specific defects in outgrowth and branching. For example, the DLF, which is composed of RB central processes, expanded or contracted in response to Vav2 GOF or LOF, respectively. On the other hand, RB peripheral process extension was inhibited by both treatments. Similar to our *in vitro* studies, CI axons exhibited abnormal branching with Vav2 over-expression and reduced outgrowth with Vav2 KD. Our findings suggest that balanced Vav2 activity is necessary for optimal neurite outgrowth and branching via GEF and tyrosine phosphorylation dependent pathways.

Mammalian Vav2 was originally reported to act as a GEF toward RhoA, RhoB and RhoG GTPases (Schubel et al., 1998). However, more recent studies have shown that Vav2 activates Cdc42 and Rac1 (Abe et al., 2000; Liu and Burrige, 2000; Marignani and Carpenter, 2001). In agreement with these recent studies, our data suggest that Vav2 functions as a Rac1/Cdc42 GEF, rather than a RhoA GEF in spinal neurons, since Vav2 over-expression prevented neurite retraction induced by LPA (Fig. 2). As LPA is known to strongly activate RhoA and that Rho GTPases can exhibit signal cross-talk (Kozma et al., 1997; Kranenburg et al., 1999; Yuan et al., 2003), our results suggest that Vav2 activation of Rac1/Cdc42 likely inhibits RhoA signaling in growth cones. Further, our observation that CA Rac1 most closely phenocopies Vav2 over-expression (Figs. 2, S2), also supports previous studies showing that Vav2 specifically activates Rac1 over Cdc42 (Kawakatsu et al., 2005; Marignani and Carpenter, 2001).

Our studies suggest that Vav2 activates Rac1 in spinal neurons to promote neurite outgrowth, process formation and axon branching. Rac1 and several Rac1 GEFs have been shown to play a crucial role in various aspects of neuronal morphogenesis including axon extension, branching, and guidance (de Curtis, 2008). For example, many studies using Rac1 mutants reported that Rac1 promotes dendritic and axonal branching, as well as neurite outgrowth (Albertinazzi et al., 1998; Li et al., 2000; Luo, 2002; Luo et al., 1994; Ng et al., 2002; Ruchhoeft et al., 1999; Threadgill et al., 1997). In the present study, we find that expression of exogenous Vav2 resulted in excess process formation and branching (Fig. 1), while expression of DN Vav2 (DH mutant) and xVav2 KD reduced process formation and branching on both LN and L1 (Figs. 1, 3, 4). While we cannot distinguish axons from dendrites *in vitro*, over-expressing Vav2 clearly increased branching by CI axons *in vivo*. Similar effects were observed with CA Rac1 expression, suggesting that Vav2 promotes axon branching through Rac1. The effectors of Rac1 that are responsible for axon branching are unknown, but p21-activating kinase (PAK) is one Rac1 target that has been implicated in neurite branching (Bokoch, 2003; Hayashi et al., 2007; Ng et al., 2002). Alternatively, it is possible that Vav2-induced branching could be due to inhibition of RhoA signaling, as our data suggests that active RhoA is reduced in neurons expressing Vav2 (Fig 2). RhoA signaling through ROCK and myosin II has been linked to branch destabilization in several systems (Billuart et al., 2001; Rico et al., 2004).

One question that our study raises is why activation of Rac1 by Vav2 over-expression inhibits neurite outgrowth. It is generally believed that Rac1 and Cdc42 promote neurite outgrowth,

while RhoA inhibits outgrowth (Luo, 2002). However, we have previously shown that both CA and DN Rac1 mutants reduce of axon outgrowth on LN (Kuhn et al., 1998; Woo and Gomez, 2006), suggesting that balanced Rac1 activity within growth cones is necessary for optimal axon outgrowth. As Rac1 is known to control neurite advance through the regulation of integrin-dependent adhesive point contacts (Woo and Gomez, 2006; Woo et al., 2009), it is possible that imbalanced Vav2 function inhibits axon outgrowth by disrupting proper assembly and disassembly of point contacts that is necessary for rapid neurite outgrowth. In addition, as Vav2 over-expressing neurons have reduced basal RhoA signaling, it is possible that imbalanced RhoA inhibits axon outgrowth.

Several lines of evidence suggest that Src-dependent tyrosine phosphorylation of Vav2 modulates process branching under some circumstances. First, we find that enhanced process branching by WT Vav2 over-expression is prevented by treatment with PP2. Second, we find that process branching is not promoted by Vav2Y159/Y172F mutant expression. Third, increased branching of neurons on L1 is prevented by PP2 treatment. However, Src-dependent branching on L1 does not appear to require tyrosine phosphorylation of Vav2 at Y159/Y172, but does require Vav2 GEF activity. It is important to note that the Vav2Y159/172F mutant is likely GEF active, since Vav2Y159/172F expression protected neurons from LPA-induced collapse and reduced the rate of neurite outgrowth similar to Vav2 WT (Figs. 1, 2). Previous reports showed that Vav1 is tyrosine phosphorylated by SFKs in lymphocytes (Fujimoto et al., 1999; Michel et al., 1998) at residues in the AC region (Y142, 160, 174 in Vav1), which leads to an open configuration that promotes Vav1 GEF activity (Amarasinghe and Rosen, 2005; Crespo et al., 1997; Llorca et al., 2005). In contrast, while Vav2 in several cell types is tyrosine phosphorylated in response to growth factor stimulation (Liu and Burridge, 2000; Tamas et al., 2003), the level of GTP exchange activity does not correlate with Vav2 tyrosine phosphorylation (Tamas et al., 2003), suggesting that phosphorylation serves other functions for Vav2. Interestingly, several recent reports find that SFK activity can stimulate process branching downstream of diverse adhesion molecules and soluble guidance factors (Morita et al., 2006; Murata et al., 2006; Paveliev et al., 2007; Rajasekharan et al., 2009). We hypothesize tyrosine phosphorylation of Vav2 by SFKs downstream of axon guidance cues modulates branching on LN, but L1 signals through a parallel pathway to promote Vav2-dependent branching. It is noteworthy, that tyrosine phosphorylation of Vav1 Y160 is required for Vav1 recruitment to integrin adhesion complexes during $\alpha\beta3$ -mediated adhesion of hematopoietic cells, consistent with a role for Vav tyrosine phosphorylation in integrin-mediated cell functions (Gao et al., 2005). The precise mechanism how Vav2 tyrosine phosphorylation stimulates branching is unknown, but may involve SH2-domain dependent targeting of either active Rac1 or its effectors to sites of branch formation.

Our findings suggest that specific axon guidance cues may promote axon branching by targeting Vav2 GEF activity. To date, only CD47 and EphrinA1 have been shown to influence neuronal morphology by activating Vav2 (Cowan et al., 2005). However, several other axon guidance molecules such as FGFs, Netrin, BDNF and Slit have been shown to function as branch-promoting factors (Yamamoto et al., 2002). For example, BDNF promotes branching by several distinct classes of neurons (Alsina et al., 2001; Horch and Katz, 2002; Lom and Cohen-Cory, 1999). Interestingly, we find here that phosphorylation levels of Vav2 were increased in growth cones in response to BDNF (data not shown), suggesting that Vav2 may be a downstream effector of BDNF signaling. While the role of BDNF for spinal neuron axon guidance is uncertain, Netrin has a well-known function in midline crossing by CIs. Consistent with the regulation of midline crossing by Vav2, we find that Vav2 KD reduces CI axon outgrowth, while Vav2 over-expression enhances branching by CIs near the midline, suggesting that these neurons are hyper-sensitive to Netrin or another midline-associated molecule.

Experimental Methods

Xenopus embryo injection and neuron culture

Human Vav2 WT and Vav2Y159/172F mutant constructs fused to GFP were provided by Dr. Laszlo Buday (Semmelweis University, Budapest, Hungary). Vav2 DH mutant (Vav2L342R/L343S) obtained by Dr. Greenberg (Harvard Medical School, Boston, MA) was subcloned into pCSEGFPDest plasmid using Gateway cloning system (Invitrogen). One or two blastomeres of four-cell stage *Xenopus* embryos were injected with 50-200 pg of DNA. For xVav2 morpholino experiments, antisense MO (xVav2 MO: 5'-CTGCCGCCAGCCCTCAGTCATGTTG-3'; GenBank accession no. BC077868) and the standard control MO (Ctrl-MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3') were purchased from Gene Tools (Philomoth, OR). *Xenopus* embryos were injected with 8-16 ng MOs together with *in vitro* transcribed GFP or mCherry-CAAX mRNA (mMessage machine, Ambion) into one-pigmented blastomere at the eight-cell stage to target RB neurons and CIs (Jacobson and Hirose, 1981). Embryos that were positive for fluorescence were screened for further experiments. Neural tubes were dissected from one-day old embryos and dissociated spinal neurons or spinal cord explants were cultured as previously described (Gomez et al., 2003). Cells were plated on glass coverslips coated with 10 µg/ml laminin (LN, Sigma) or L1Fc (R&D) and maintained in 1XMR medium with or without 10 nM PP2 (Calbiochem). Cultures were imaged or fixed 16-24 hrs after plating.

In vitro neurite outgrowth and branching assay

Fluorescent and DIC images of cultured neurons were acquired using an Olympus Fluoview 500 laser-scanning confocal system mounted on an AX-70 upright microscope equipped with 40×, NA 0.85 and 60×, NA 1.45 objectives or a CoolSnap camera connected to a Nikon Eclipse TE2000-E inverted microscope equipped with 20×, NA 0.5 and 40×, NA 1.30 objectives. The rates of neurite outgrowth were measured from 15 min time-lapse image sequences using either Metamorph (Universal Imaging Cooperation) or Image J (W. Rasband, NIH) software. The number of primary processes per neuron, the percentage of neurons with a branched processes and the total number of branch points (terminal process at least 10 µm) was quantified from 3-4 independent experiments. The effects of lysophosphatidic acid (LPA, Sigma) were analyzed after a 10 min exposure to 100 nM LPA. Statistical significance was determined using Mann-Whitney t-tests and variance reported as ±standard error of the mean (SEM) using KaleidaGraph software.

Immunoblotting and immunocytochemistry

Immunoblotting was performed as described (Robles et al., 2005). Total proteins were extracted from stage 25-26 (Nieuwkoop and Faber, 1994) uninjected control embryos or embryos that had two blastomeres at the four-cell stage injected with xVav2 MO. Vav2 (H-200) (Santa Cruz biotechnology) and β-actin (Abcam) antibodies were used at 1:250 and 1:2500. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz biotechnology) were used at 1:5000 and the blots were visualized by enhanced chemiluminescence (Amersham). ImageJ software was used to quantify western blots in three independent experiments.

Spinal neuron cultures were fixed in 4% paraformaldehyde/4% sucrose in calcium- and magnesium-free phosphate buffered saline (CMF-PBS), permeabilized with 0.1% Triton X-100, and blocked in 0.5% Fish gelatin in CMF-PBS. The phospho-Vav (pY174) antibody (Santa cruz) was used at 1:200 in block. Cells were then labeled with corresponding Alexa-fluorophore conjugated secondary antibodies (1:300, Molecular Probes, Eugene, OR). F-actin was detected with Alexa-546 phalloidin (1:100, Molecular Probes). The average intensity of fluorescence within growth cones was measured using MetaMorph software.

Whole-mount immunohistochemistry and image analysis

Whole mount immunostaining was performed as described previously (Moon and Gomez, 2005). RB peripheral axons were visualized in stage 25–26 embryos by isolating the dorsal skin and spinal cord by removing the lateral somites. Embryos were incubated with anti-HNK-1 antibody (Sigma, 1:250) for RB sensory neurons. Alexa 647-conjugated secondary antibody (Molecular Probes) was used at 1:300. Confocal images of skin and spinal cord preparations were viewed using 20×, or 40×/60× water immersion objectives, respectively. Confocal z-stacks were collected at 2–10 μm steps and displayed as maximum intensity projections using ImageJ software (W. Rasband, NIH). Image analysis was performed using either MetaMorph or ImageJ software. Reconstructed images were thresholded based on intensity to highlight labeled axons. Measurement of maximum dorsal fascicle width was made at 100 μm intervals along the entire length of spinal cord. For analysis of RB peripheral axon outgrowth, the threshold area and the length of the longest RB axon were measured within 100 μm segments and compared between the injected and uninjected sides of the skin. The length of CI axons was estimated by scoring the position of CI growth cones into one of four locations along their pathway: 1. positioned before the ipsilateral ventral longitudinal fascicle (VLF); 2. between the ipsilateral VLF to ventral midline; 3. between the midline to contralateral VLF; 4. positioned beyond the contralateral VLF. The average position of CI growth cones was measured within equal size quadrants along the A-P axis of the spinal cord. The orientation of all post-crossing CIs was scored as posterior ($>95^\circ$), anterior ($<85^\circ$) or medial ($90\pm5^\circ$). Adobe Photoshop (Adobe systems incorporated) was used to create final figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Vav2 over-expression slows neurite outgrowth, but enhances process formation and branching

A-D. GFP fluorescence and DIC images of neurons expressing GFP (A-B) or GFP-Vav2 WT (C-D). Note that the control spinal neuron extends a single, unbranched process, while the Vav2 WT over-expressing neuron has multiple branched processes. Scale, 25 μm . **E.** Schematic domain structure of Vav2 full length and localization of observed mutations. Mutations of two tyrosine (Y) residues in AR domain (Vav2Y159/172F) and leucine (L) residues in DH domains (Vav2L342R/L343S) are abbreviated Vav2 Y2F and Vav2 DH, respectively, in all figures. **F-I.** Quantification of the rate of neurite outgrowth (F), number of primary processes formed per neuron (G), percentage of neurons with neurite branching (H) and the average number of branch points per neuron (I) analyzed in neurons under indicated conditions. Over-expression of Vav2 WT reduces the rate of neurite outgrowth, but increases neurons having neurite branching compared to control. * $P < 0.05$, ** $P < 0.01$. $N > 15$ growth cones in at least 3 individual cultures for (F-I).

Figure 2. Vav2 WT suppresses LPA-induced neurite retraction

A-D. GFP fluorescence and DIC images of an image field containing both a GFP-Vav2 WT expressing growth cone (arrows) and a non-expressing control growth cone (arrowheads) before (A, B) and after (C, D) treatment with 100 nM lysophosphatidic acid (LPA) for 10 min. Note that as the control neuron retracts in response to LPA, it pulls back the right process of the branched GFP-Vav2 WT expressing neuron (arrow). **E.** The percentage of neurite retraction after a 10 min treatment with LPA for neurons expressing various constructs. Scale, 25 μ m. ** $P < 0.01$, *** $P < 0.0001$. $N > 22$ growth cones for each condition.

Figure 3. Morpholino-mediated knock-down of xVav2 inhibits neurite outgrowth and branching
A. Western blot of xVav2 from total protein extracts of control embryos and embryos injected (2×4 blastomeres) with xVav2 MO. Blot was re-probed for β -actin as a loading control. **B.** Densitometric quantification of western blot signals of xVav2 and β -actin ($N=3$). Blastomere injection of xVav2 MO into two of four blastomeres reduced xVav2 expression by 25%. Therefore, we can infer that the cells that descended from the targeted blastomere had their xVav2 expression reduced by 50%. **C-J.** Representative images of growth cones without (C, E, G, I) or with (D, F, H, J) xVav2 MO (red) labeled for p-Vav (green) and F-actin (phalloidin; blue). **K.** Quantification of p-Vav immunofluorescence intensity within growth cones of xVav2 MO neurons normalized into the control growth cones in the same culture. $N > 89$ neurons in 2 experiments. Scale, 10 μ m. **L-O.** xVav2 MO reduces neurite outgrowth and branching. * $P < 0.05$, ** $P < 0.01$. $N > 68$ neurons in 3 cultures for (L-O).

Figure 4. L1 stimulates neurite formation and branching via Vav2 GEF-dependent pathway
A-B. Dissociated spinal neurons cultured on either LN (A) or L1 (B) and labeled with Alexa488-phalloidin. Note that spinal neurons on LN extend one or two long, unbranched processes while neurons on L1 have multiple, short and branched neurites. Scale, 40 μm . **C-F.** Quantification of the rate of neurite outgrowth (C), number of primary processes formed per neuron (D), percentage of neurite branching (E) and the number of branch points per neuron (F) analyzed in neurons cultured on either LN or L1 under indicated conditions. Both Vav2 DH and xVav2 KD significantly inhibited L1-mediated neurite formation and branching, while other treatments had more modest effects. * $P < 0.05$, *** $P < 0.001$. $N > 10$ growth cones in 3 cultures for (C) and $N > 18$ neurons in 3 cultures for (D-F).

Figure 5. Vav2 promotes in RB central process formation

A-L. Lateral views of whole mount stage 25 neural tubes immunolabeled for HNK-1 (pseudocolored blue in A-F), which marks RB sensory neurons and CI axons that have crossed the midline. GFP-Vav2 WT DNA, Ctrl MO or xVav2 MO with GFP mRNA was injected into one blastomere of 4-cell stage or 8-cell stage embryos. GFP fluorescent dorsal spinal neurons (green) indicate the injected sides (A, C, E) of neural tubes in the merged images in (A-F). HNK-1 labeling only is shown in (G-L). **G'-L'.** High magnification images of boxed regions in (G-L). Note that the dorsal fascicle is enlarged relative to the control side in Vav2 over-expressing embryos (compare I' to J') and is reduced relative to the control side in the Vav2 KD embryo (compare K' to L'). **M-N.** Quantification of the width of the thickest fascicle and HNK intensity between injected and uninjected side. Note that the HNK-1 labeled dorsal fascicles in Vav2 WT injected spinal cord is thicker than in uninjected side, while xVav2 MO injected side shows thinner fascicles (M). *P<0.05, **P<0.01. ***P<0.001. N>11 embryos for each condition. Scale bars, 100 μ m in (A-L) and 10 μ m in (G'-L').

Figure 6. Vav2 disfunction reduces RB peripheral axon outgrowth

A-F. Whole mount skin preparations labeled with HNK-1 antibody showing RB peripheral axon projections into the skin of control MO (A, D), GFP-Vav2 WT (B, E), and xVav2 MO (C, F) injected embryos. The injected side is above the dashed line and anterior is to the right in all preparations. Merged images of GFP (green) and HNK (blue) fluorescence are shown in (A-C). HNK-1 labeling only of RB peripheral processes are shown in (D-F). Note that both Vav2 over-expression and knockdown reduce neurite extension into the skin as determined by arbor area (G) and maximum average axon length (H) measured in 200 μ m segments (see Methods). * $P < 0.05$. $N > 11$ embryos for each condition. Scale bar, 100 μ m.

Figure 7. Vav2 dysfunction alters CI morphology and midline crossing

A-B. GFP fluorescent commissural interneurons (CIs) were visualized from ventral views of stage 25 neural tubes. GFP-Vav2 WT DNA, Ctrl MO or xVav2 MO with GFP mRNA was injected into one blastomere of 4-cell stage or 8-cell stage embryos. The injected side is above the midline (dashed line) and anterior is to the left. Note that the Vav2 WT expressing CIs have abnormal growth cones and exhibit axon branching (B-D). * $P < 0.05$. $N > 7$ embryos for each condition. **E-I.** Ventral views of a stage 25 neural tube labeled on one side with mCherry-CAAX plus a control MO (E) and the opposite side with GFP plus xVav2 MO (F). Merged image of mCherry-CAAX (red) and GFP (green) labeled CI axons (G). The A-P axis was divided into 100 μ m quadrants (dashed yellow lines in G) for analysis in (H). **H-I.** xVav2 KD

delayed CI axon outgrowth and altered the trajectory of crossing CIs at the midline. N>13 embryos. Scale bar, 40 μ m.