RPTPα is required for rigidity-dependent inhibition of extension and differentiation of hippocampal neurons

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Summary

Receptor-like protein tyrosine phosphatase α (RPTPα)-knockout mice have severe hippocampal abnormalities similar to knockouts of the Src family kinase Fyn. These enzymes are linked to the matrix-rigidity response in fibroblasts, but their function in neurons is unknown. The matrix-rigidity response of fibroblasts appears to differ from that of neuronal growth cones but it is unknown whether the rigidity detection mechanism or response pathway is altered. Here, we report that RPTPα is required for rigidity-dependent reinforcement of fibronectin (FN)-cytoskeleton bonds and the rigidity response in hippocampal neuron growth cones, like in fibroblasts. In control neurons, rigid FN surfaces inhibit neurite extension and neuron differentiation relative to soft surfaces. In RPTPα$^{-/-}$ neurons, no inhibition of extension and differentiation is found on both rigid and soft surfaces. The RPTPα-dependent rigidity response in neurons is FN-specific, and requires clustering of $\alpha_v\beta_6$ integrin at the leading edge of the growth cones. Further, RPTPα is necessary for the rigidity-dependent concentration of Fyn and p130Cas phosphorylation at the leading edge of the growth cone, like it is in fibroblasts. Although neurons respond to rigid FN surfaces in the opposite way to fibroblasts, we suggest that the mechanism of detecting FN rigidity is similar and involves rigidity-dependent RPTPα recruitment of Fyn.

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Key words: Rigidity response, Growth cone, RPTPα, Fibronectin

Introduction

The role of mechanosensing has long been underappreciated, but recent studies have revealed the importance of cellular responses to the mechanical properties of the environment (Giannone and Sheetz, 2006; Vogel and Sheetz, 2006). Hay and colleagues were the first to address the dependence of cell behavior on the matrix compliance. Their studies showed that morphology and migration of fibroblasts grown on deformable 3D-collagen gels were strikingly similar to the cells in host tissue, unlike fibroblasts plated on 2D-collagen matrices (Bard and Hay, 1975; Hay, 1982). The substrate-rigidity preferences that different cell types exhibit, have been correlated to the rigidities of their native tissues in vivo (Discher et al., 2005), but the rigidity response mechanisms remain unclear. In addition, recent studies have shown that matrix rigidity affects the gene expression and differentiation of the stem cells (Engler et al., 2006). The rigidity response is also altered in cancer (Paszek et al., 2005), indicating the relevance of correct mechanosensing to differentiation, development and invasion. Substrate rigidity is known to affect the response to growth factors (Opas and Dziak, 1990), cell morphology, cytoskeletal architecture and migration (Choquet et al., 1997; Friedl and Brocker, 2000; Lo et al., 2000; Peyton and Putnam, 2005; Yeung et al., 2005). A different approach, in which cells are cultured on chemically stable polyacrylamide gels of varying rigidities (Pelham, Jr and Wang, 1997), was used by our group and others to determine the effect of matrix rigidity on cellular behavior (Engler et al., 2004; Jiang et al., 2006; Kostic and Sheetz, 2006; Wang et al., 2000).

Several recent studies have shown that soft substrates stimulated neurite extension and branching, but inhibited glial cell growth (Balgude et al., 2001; Engler et al., 2004; Flanagan et al., 2002; Georges et al., 2006; Lamoureux et al., 2002; Strassman et al., 1973). However, mechanisms of this regulation are not clear. Despite the complexity of the regulation of neurite extension, the cytoskeletal structures involved resemble the ones that occur in fibroblasts and depend on actin polymerization (Forscher et al., 1992; Lin et al., 1994; Mitchison and Cramer, 1996). Growth cones form filopodia and lamelipodial veils, similar to the cytoskeletal structures observed at the leading edges of fibroblasts (Dent and Gertler, 2003; Lin et al., 1994), and mechanical tension is crucial in the process of extension (Lamoureux et al., 2002).

Receptor-like protein tyrosine phosphatase α (RPTPα) has been implicated in the regulation of various signaling pathways including fibronectin (FN)-rigidity response (Jiang et al., 2006; Kostic and Sheetz, 2006). No soluble RPTPα ligands have been identified so far, but lateral activation of RPTPα by contactin and NCAM has previously been reported and these complexes are believed to regulate neurite extension (Bodrikov et al., 2005; Zeng et al., 1999). A regulatory role for other RPTPs in axon elongation both in *Drosophila* and vertebrates has been documented (Lorber et al., 2004; Stepanek et al., 2005). Several studies have shown that RPTPα forms a force-

transducing complex with $\alpha_v\beta_3$ integrins at the leading edge of fibroblasts (von Wichert et al., 2003). Our group and others showed that Src and Fyn are activated by RPTPα-mediated dephosphorylation both in fibroblasts (Ponniah et al., 1999; Su et al., 1999; von Wichert et al., 2003) and neurons (Bodrikov et al., 2005; Helmke et al., 1998; Zeng et al., 1999).

RPTPα is abundantly expressed in the brain (Petrone et al., 2003; Sap et al., 1990) and present in the growth cones (Helmke et al., 1998). Although the knockout mice are viable (Sap et al., 1990), various processes are affected by RPTPα ablation. It has been shown that the inside-out radial migration is preserved in RPTPα$^{-/-}$ mice, but the disorganization of the hippocampal layers indicates that RPTPα$^{-/-}$ neurons migrated at lower rates during initial soma translocation or along the radial glia at later developmental stages. Long-term potentiation (LTP) and spatial learning were also impaired in RPTPα-deficient mice (Petrone et al., 2003), and anxiety was reduced (Skelton et al., 2003). Interestingly, Fyn-knockout mice display similar phenotype (Grant et al., 1992; Kojima et al., 1997). RPTPα-deficient neurons also showed reduced Fyn activity upon NCAM stimulation (Bodrikov et al., 2005).

Our recent studies indicated that activation of Fyn and p130Cas phosphorylation mediated by the $\alpha_v\beta_3$-integrin–RPTPα complex is involved in the FN-rigidity response in fibroblasts (Jiang et al., 2006; Kostic and Sheetz, 2006). The stretching of p130Cas substrate domain in the periphery of contractile fibroblasts has been shown to be involved in signaling to the nucleus (Sawada et al., 2006). Although p130Cas is an indispensable component in the regulation of actin-cytoskeleton organization, focal-contact formation and migration of fibroblasts (Cary et al., 1998; Cho and Klemke, 2000; Honda et al., 1999), its role in neuronal motility is poorly understood.

In this study, we describe our findings that RPTPα mediates a FN-specific response to rigid matrix in hippocampal neurons that inhibits neurite extension and differentiation, whereas fibroblast spreading is increased. Although the response is different, it appears that a similar molecular mechanism of rigidity sensing occurs at the leading edges of growth cones and fibroblasts. Thus, the rigidity-sensing and rigidity-response pathways are differentiated for different cells.

Results

Reinforcement of integrin-cytoskeleton bonds is impaired in RPTPα$^{-/-}$ neurons interacting with fibronectin but not vitronectin

Extracellular matrix (ECM) proteins bind to specific integrin receptors on the cell surface (Giancotti and Ruoslahti, 1999), causing formation of integrin-cytoskeleton bonds that are reinforced by force-dependent recruitment of focal-contact proteins. Reinforcement of the integrin-cytoskeleton bonds is crucial for the response to the matrix rigidity (Choquet et al., 1997; Giannone et al., 2004; Kostic and Sheetz, 2006). Since the RPTPα$^{-/-}$ fibroblasts are defective in the reinforcement of the FN- and vitronectin (VN)-beads, we speculated that the reinforcement would also be impaired in RPTPα$^{-/-}$ neurons. To test this possibility, we used the laser-tweezer assay. Beads coated with FN and VN, were held by the optical trap at the leading edge of the growth cones to mimic interactions between advancing growth cones and the matrix. Since the defects in RPTPα$^{-/-}$ fibroblasts were most pronounced on FN, we asked whether reinforcement of the FN-coated beads would be deficient in neurons. The beads were placed at the edge of the growth cones with a laser tweezer. After 3-5 seconds the beads were released from the laser trap to check for binding. When the bead was bound, the laser trap was turned on and the rearward bead movement was recorded (Movies 1, 2).

We assumed that most of the binding events occurred through integrins, since the nonspecific binding of BSA-coated beads occurred at low levels (21.4±2.5% in controls and 17.1±1.1% in knockouts). Given that RPTPα is downstream of integrin activation by ECM, we expected that bead binding would not be affected by the absence of RPTPα. Indeed, no difference in binding frequencies of FN-coated beads was observed between RPTPα$^{+/+}$ and RPTPα$^{-/-}$ growth cones (65.5±6.6% and 64.4±3.4%, respectively) (Fig. 1C).

Further, we analyzed the rearward movement of bound beads and determined frequency of the reinforcement events. Two types of events were identified: some beads moved rearwards and, once they reached the edge of the trap, were pulled back into the center of the trap (designated as breaking events, Fig. 1A); others continued moving past the edge of the trap, towards the proximal part of the growth cone, and occasionally continued up the axon (designated as reinforcement, Fig. 1B; supplementary material Movies 1, 2). In previous studies, the fraction of breaking events was reciprocally proportional to the rigidity of the trap and a rigid trap was used in this study to cause the rigidity response (Jiang et al., 2003). As predicted, the number of breaking events was twice as high in RPTPα$^{-/-}$ neurons than in controls (Fig. 1C). Thus, we concluded that reinforcement of FN-clustered integrin-cytoskeleton bonds was impaired in RPTPα$^{-/-}$ growth cones.

Next, we quantified the bead diffusivity by calculating the mean square displacement (MSD), which is used as a measure of the stiffness of the bead-cytoskeleton link and is inversely proportional to the bead reinforcement (Choquet et al., 1997; Qian et al., 1991). Individual trajectories of the beads were generated (Fig. 1E,F), and MSD was determined as described previously (Qian et al., 1991). The average MSD of the beads moving rearwards was determined during the initial period of time after the beads moved outside of the trap. We found that diffusion rates (given by the slopes of MSD curves) are much higher during early rearward movement on RPTPα$^{-/-}$ growth cones compared with RPTPα$^{+/+}$ growth cones (Fig. 1G). Thus, the greater bead diffusion perpendicular to the path of movement in RPTPα$^{-/-}$ growth cones supports our hypothesis that RPTPα is required for reinforcement of integrin-cytoskeleton bonds.

Since VN binds strongly to some of the FN-binding integrins, we decided to test whether RPTPα was also involved in VN signaling. We performed the previously described experiments with VN-coated beads (Fig. 1D,I). To our surprise, no differences in reinforcement or binding frequencies were observed between knockout and wild type neurons. Hence, we speculate that RPTPα is involved in a signaling pathway upregulated by the activation of (a) FN-specific integrin(s).

FN-stimulated RPTPα signaling is activated through $\alpha_v\beta_6$ integrin

We asked which integrin was involved in the RPTPα-mediated reinforcement in neurons. The expression of a variety of FN-specific integrin subunits was reported in the hippocampus

(Pinkstaff et al., 1999), including $\alpha_v\beta_6$ integrins (Chan et al., 2003), which were previously believed to be limited to epithelial cells. Since $\alpha_v\beta_3$ integrin, previously implicated in RPTPα–signaling, was expressed at low levels in the brain, we speculated that different integrin subunits were responsible for RPTPα activation in the neuronal FN rigidity response. We tested several logical candidates by measuring the effect of blocking antibodies on bead reinforcement. Controls were performed with BSA-coated beads (data not shown). GPen is a cyclic peptide (GPenGRGDSPCA) which at a concentration of 0.5 mM acts as a selective, competitive inhibitor of the $\alpha_v\beta_3$-integrin that inhibits activation of intracellular signaling (Etienne-Manneville and Hall, 2001) and does not block $\alpha_5\beta_1$ integrins (Pierschbacher and Ruoslahti, 1987). Addition of GPen had no effect on FN-coated bead binding and reinforcement. Further, a blocking antibody for $\alpha_5\beta_1$ also had no influence.

By contrast, both anti-$\alpha_v\beta_6$ and anti-α_v integrin blocking antibodies caused a 50% decrease in the binding frequencies compared with antibody-free controls (Fig. 2A), to levels only slightly higher than the nonspecific binding levels determined for BSA-coated beads (24.2±1.9% for anti-α_v and 31.8±4.8% for anti-$\alpha_v\beta_6$, compared with 21.4±2.5% for BSA-coated beads). The reinforcement frequency was also significantly reduced (33.7±5.3% for anti-α_v and 35.7±10.0% for anti-$\alpha_v\beta_6$) and corresponded to the reinforcement levels in RPTPα$^{-/-}$ neurons (35.1±3.2%) (Fig. 2B). Thus, we propose that $\alpha_v\beta_6$ integrins are involved in RPTPα-mediated reinforcement of FN-cytoskeleton bonds in the growth cones of hippocampal neurons.

Next, we looked at the effect of ECM on $\alpha_v\beta_6$ integrin localization. We plated neurons on laminin (LN)-coated and FN-coated glass for 48 hours, and then visualized $\alpha_v\beta_6$ integrins by immunofluorescence. Next, intensity of the fluorescence signals at the leading edge was quantified and normalized with respect to the signal in the perinuclear area. On FN, $\alpha_v\beta_6$ integrins were enriched at the edge of the growth cone (2E). By contrast, on LN, $\alpha_v\beta_6$ was concentrated at the cell body with low levels along the axon and in the growth cones (2F). Interestingly, RPTPα was present in the growth cones of neurons plated both on FN and LN. (Fig. 2C,D). The quantification of fluorescence signal intensities confirmed our observations (Fig. 2G). Thus, we speculate that an increased fraction of $\alpha_v\beta_6$ integrins accumulated at the edge of the growth cone upon interaction with FN, which caused activation of RPTPα-mediated rigidity-response pathway.

Soft FN surfaces cause increased neurite extention and differentiation of hippocampal neurons

Since RPTPα appeared to be required for the

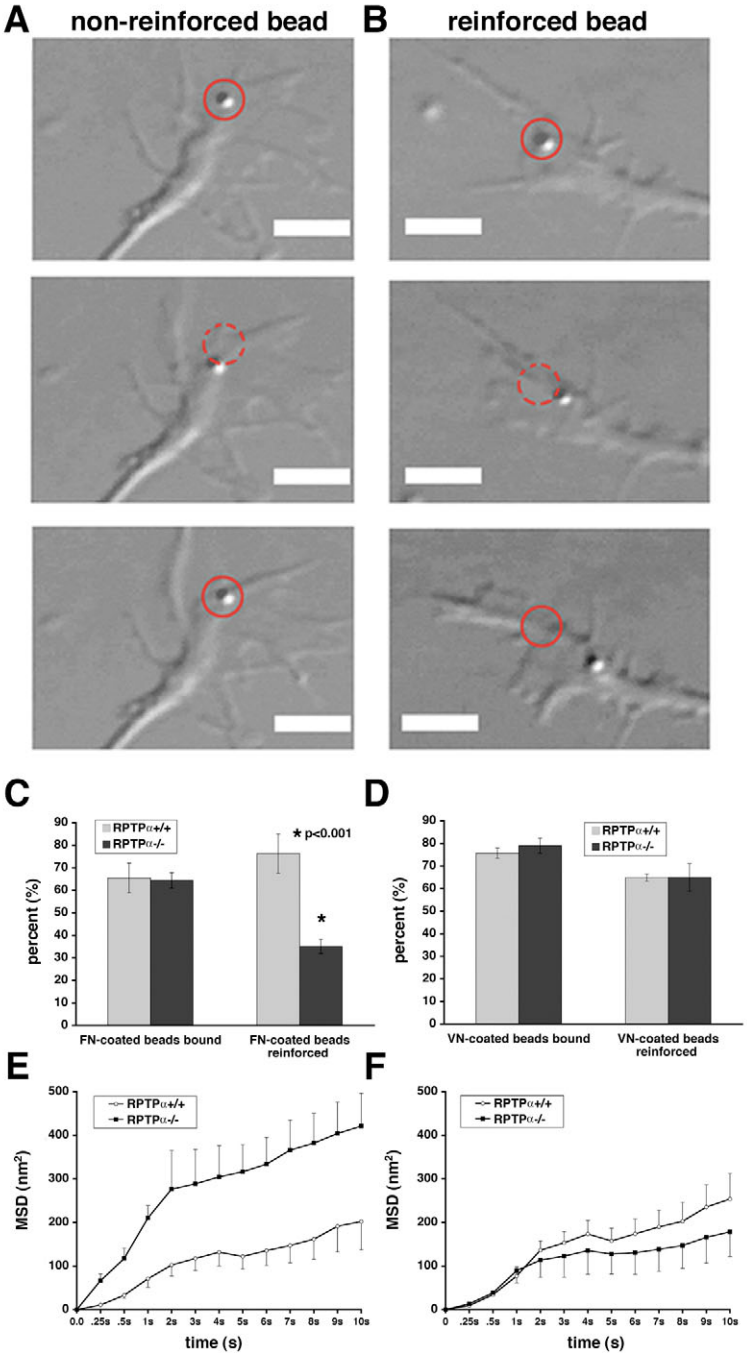

Fig. 1. RPTPα is required for the reinforcement of FN-specific integrin-cytoskeleton bonds in neuronal growth cones. A laser tweezer (represented by red circle) was used to place FN-VN-coated beads at the edge of the growth cones. (A,B) In non-reinforced beads (A), the linkage between the bead and the cytoskeleton was broken by the trap force and the beads were pulled back into the trap after initial rearward movement (breaking event). If reinforcement occurred, the linkage was not broken and beads moved out of the optical trap towards the axon hillock (B). Bars, 4 μm. (C,D) FN-bead reinforcement was decreased by half but bead binding was unaffected in RPTPα$^{-/-}$ neurons relative to controls. By contrast, VN-bead reinforcement and binding were unaltered on RPTPα$^{-/-}$ neurons (C,D). Mean square displacement (MSD) was calculated for the initial 10 seconds of rearward movement for each condition. The results (mean ± s.e.) were statistically significant (P<0.01). (E,F) On average, MSD was higher for FN-coated beads bound to RPTPα$^{-/-}$ growth cones compared with RPTPα$^{+/+}$ growth cones (E); no significant difference was observed for VN-coated beads (F).

reinforcement of the FN-cytoskeleton in neurons, we tested the effect of FN-coated substrate rigidity on neurite extension in hippocampal neurons. First, we determined whether rigidity affected the stages of differentiation (Dotti et al., 1988): stage 1 was characterized by the absence of neurites; at stage 2, neurites of approximately equal lengths were extended (Fig. 3A); and at stage 3, one significantly longer axon emerged (Fig. 3B). Neurons were isolated from the brains of P0 mice and plated on FN-coated polyacrylamide gels of varying rigidities. Immunofluorescence of the axonal marker Tau1 was used to identify axons. After 48 hours of incubation, the lengths of neurites were measured and the differentiation stages determined (Fig. 3C). As expected, RPTPα$^{+/+}$ neurons differentiated faster on soft than on rigid substrates (38.7±4.8% neurons at stage 3 on rigid substrates, 39.5±3.7% on intermediate substrates and 62.7±2.3% on soft substrates). The soft FN substrates stimulated neurite extension of RPTPα$^{+/+}$ neurons with on average a 30% increase in length compared with the stiffer substrates. The FN rigidity response in neurite extension is opposite to the motility response of fibroblasts that spread further on rigid than on soft surfaces.

RPTPα$^{-/-}$ neurons are deficient in FN-specific rigidity response

In contrast to the controls, RPTPα$^{-/-}$ neurons showed no preference for soft substrates and differentiated at a rate similar to control neurons plated on soft gels (62.0±6.5% neurons at stage 3 on rigid, 64.1±6.6% on intermediate, and 66.3±5.6% on soft). The average lengths of the neurites (both axons and dendrites) were also reflective of an impaired FN rigidity response in the absence of RPTPα; the neurites of RPTPα$^{-/-}$ neurons were on average longer and wavier than those of RPTPα$^{+/+}$ neurons (Fig. 3D,E,F). Thus, the inhibition of differentiation and neurite extension caused by rigid FN substrates was lost in RPTPα$^{-/-}$ neurons.

We next asked whether the absence of a rigidity response in RPTPα$^{-/-}$ neurons is limited to FN-coated substrates. On LN-coated substrates of different rigidities, RPTPα$^{-/-}$ neurons showed a normal rigidity response (Fig. 4). In both control and RPTPα$^{-/-}$ neurons, the increasing rigidities of LN-coated

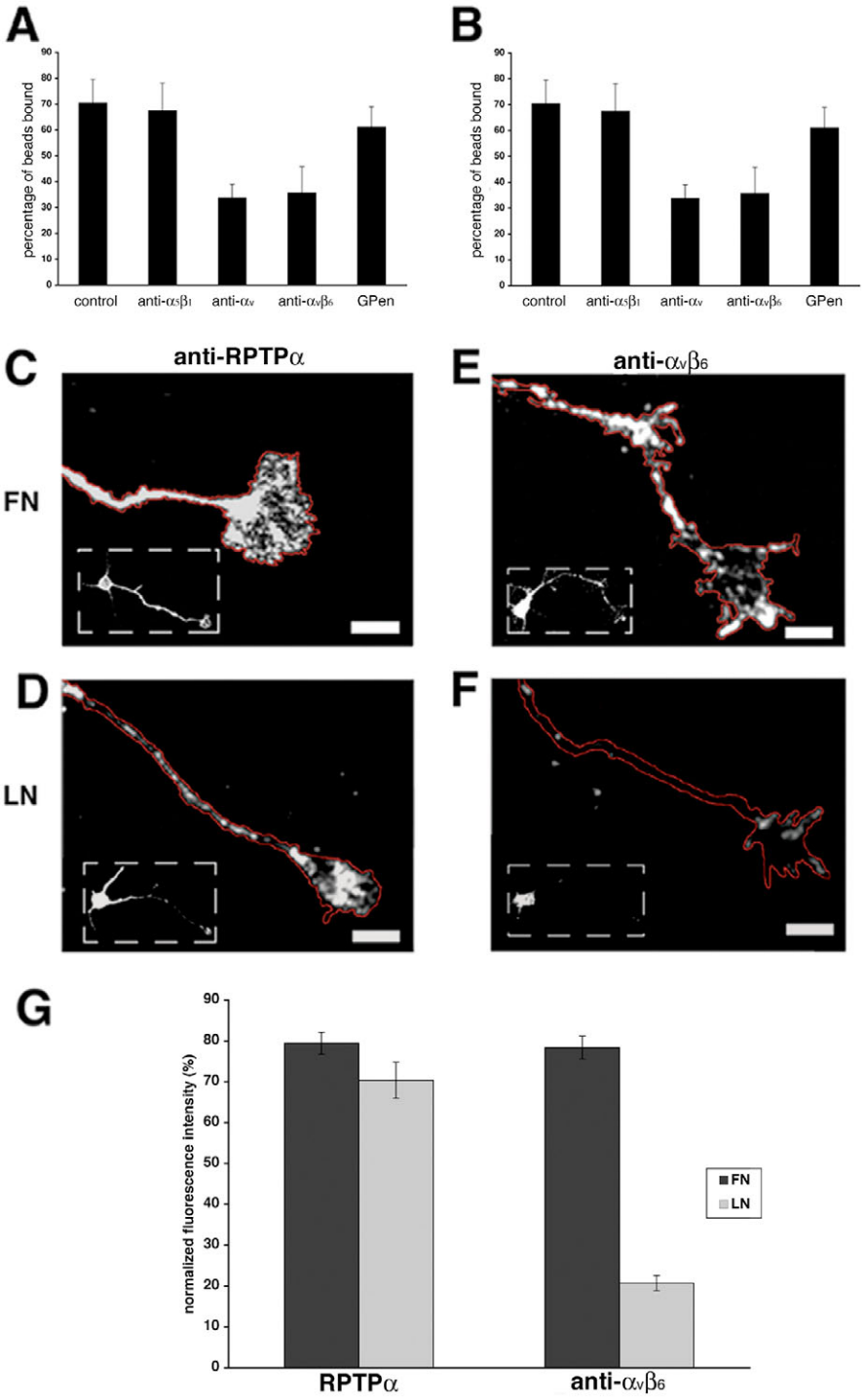

Fig. 2. $\alpha_v\beta_6$ integrins are required for the reinforcement of FN-cytoskeleton bonds at the leading edge of the growth cones. (A,B) Function-blocking antibodies against $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins had no effect on binding and the reinforcement of FN-coated beads, but blocking of the α_v and $\alpha_v\beta_6$ integrins reduced binding and reinforcement to the background levels (A,B). Localization of RPTPα and $\alpha_v\beta_6$ integrin in growth cones was ECM-specific. Both RPTPα and $\alpha_v\beta_6$ were localized to the leading edge of growth cones in neurons plated on FN-coated glass (C,D). On LN, RPTPα was localized to the growth cones, whereas $\alpha_v\beta_6$ integrin was expressed at low levels along the axons and in the growth cones (E,F). Insets provide image of the entire neuron. Bars 5 μm. (G) Fluorescence intensities of the RPTPα and $\alpha_v\beta_6$ signals were quantified, the data were normalized against nuclear fluorescence intensity and are presented as the mean ± s.e. for at least 20 representative neurons.

substrates inhibited both neurite elongation and axon differentiation. There was no difference evident in the LN rigidity response with the loss of RPTPα. This is not surprising, because rigidity response appears to be ligand-specific in other cell types and RPTPα has been specifically implicated in FN rigidity response (Jiang et al., 2006). Therefore, we suggest that RPTPα mediates the FN-specific rigidity response, whereas rigidity of LN matrices affects (an) alternate pathway(s).

Src-family-kinase activation and p130Cas phosphorylation are regulated by RPTPα in the FN rigidity response at the leading edge of the growth cone

First, we tested the effect of a broad Src-family-kinase (SFK) inhibitor (10 μM SU6656) on the neurite extension and rigidity response to FN substrates. Similar to RPTPα$^{-/-}$ neurons, neurons cultured in the presence of the SFK inhibitor, showed no preference for the soft matrices, and there was no difference in neurite extension between substrates of different rigidities in the presence of inhibitor (Fig. 5A). However, the average neurite length was reduced in the presence of the inhibitor compared with control, probably due to inhibition of various rigidity-independent regulatory pathways involving SFKs. Although SFK inhibition had a negative effect on axon differentiation, the rigidity dependence of differentiation was also abolished, similar to RPTPα$^{-/-}$ neurons (Fig. 5B). Thus, it appeared that, in addition to being involved in pathways regulating neurite extension (Robles et al., 2005), SFK(s) are indispensable in the RPTPα-mediated FN-rigidity response. In addition, SFK inhibitor had also decreased the neurite elongation rates in neurons cultured on LN, but it had no effect on the LN rigidity response (data not shown). To test whether SFK inhibition had a direct effect on FN rigidity response, we used the SFK inhibitor in the laser-tweezer assay with FN-coated beads. As expected, the bead binding was not affected, but the reinforcement frequency was reduced (Fig. 5C).

Second, we determined the effect of FN rigidity on localization of Fyn and its direct substrate p130Cas. In RPTPα$^{+/+}$ neurons, endogenous Fyn was consistently enriched at the leading edge of the growth cones on rigid FN substrates but not on soft ones (Fig. 5C). By contrast, we observed low-level Fyn accumulation in RPTPα$^{-/-}$ neurons regardless of substrate rigidity (Fig. 5D). This result indicates that Fyn recruitment to the leading edge is stimulated by rigidity-dependent RPTPα activation.

Similarly, there was a decrease in the level of phosphorylated p130Cas, with a decrease in substrate rigidity (Fig. 5E), that was congruent with the stretch-dependence of p130Cas activation previously shown in fibroblasts (Sawada et al., 2006; Tamada et al., 2004). In RPTPα$^{-/-}$ neurons, we detected only low levels of phosphorylated p130Cas at the edges of the growth cones, regardless of substrate rigidity (Fig. 5F). Thus, we speculate that formation of a force-transducing integrin-RPTPα complex at the leading edge followed by Fyn-mediated, force-dependent p130Cas

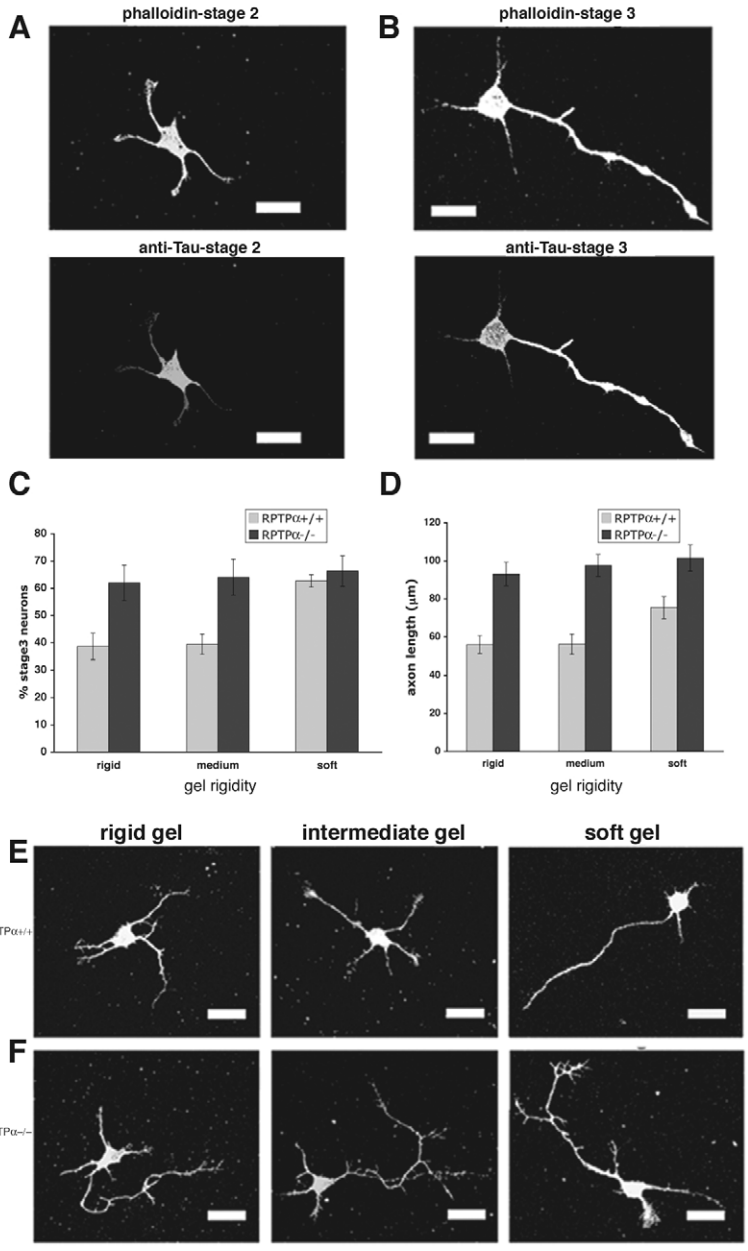

Fig. 3. Neurite extension on FN depends on RPTPα and matrix rigidity. (A,B) Primary neurons isolated from the hippocampi of RPTPα$^{+/+}$ and RPTPα$^{-/-}$ mice were plated on FN-coated polyacrylamide gels of decreasing rigidities, incubated for 48 hours, fixed and visualized using phalloidin. Anti-Tau immunofluorescence was used as axonal marker. Typical (A) stage 2 and (B) stage 3 neurons are shown. Bars, 10 μm. (C,D) Control neurons showed faster differentiation on soft than on rigid substrates; RPTPα$^{-/-}$ neurons, however, showed a high level of differentiation irrespective of matrix rigidity (C). Similarly, control neurons extended longer neurites on soft than on rigid surfaces, whereas RPTPα$^{-/-}$ neurons extended long neurites on both soft and rigid surfaces (D). Results shown in C and D (mean ± s.e.) were statistically significant (P<0.01). (E,F) Phalloidin staining of RPTPα$^{-/-}$ and RPTPα$^{+/+}$ neurons. Neurites of RPTPα$^{-/-}$ neurons appeared more meandering than the neurites of control neurons. Bars, 15 μm.

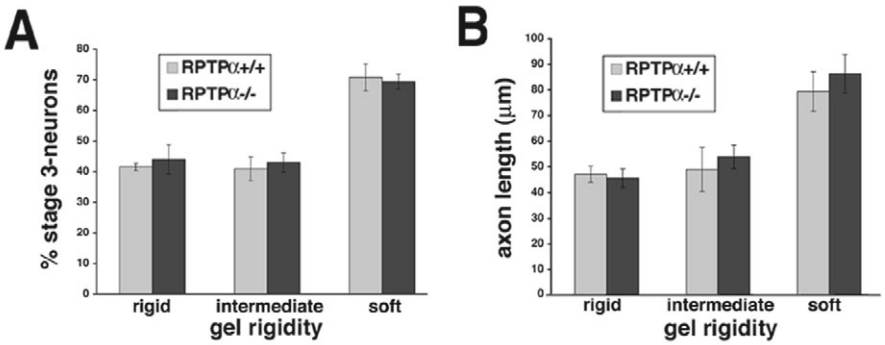

Fig. 4. LN rigidity response is RPTPα independent in neurons. (A) Axon differentiation is inhibited by increasing rigidities of LN-coated substrates in both control and knockout neurons. (B) Neurite extesion is stimulated by soft LN-coated substrates, and loss of RPTPα had no effect on this behavior. The results (mean ± s.e.) were statistically significant (P<0.01).

phosphorylation is required for the inhibition of extension in neurons on rigid substrates, whereas the same components are required for enhancement of extension in fibroblasts on rigid substrates.

Discussion

Recent studies showed that increasing matrix rigidity has different regulatory effects on differentiation and motility in different cells (Engler et al., 2004; Engler et al., 2006). There is a general question of whether the cells use different molecular machinery to sense the rigidity of the environment or rather process differently the output of a similar sensory machinery. Here, we propose a RPTPα-dependent mechanism of FN rigidity response employed by neuronal growth cones. The molecular components of this pathway appear to be similar to the one we observed to stimulate fibroblasts on rigid FN surfaces (Jiang et al., 2006; Kostic and Sheetz, 2006) but, in neurons, rigid surfaces inhibit extension and differentiation.

Although FN is not present at high levels in adult brain, it is known to support survival and migration of neural cells during development (Chun and Shatz, 1988; Pearlman and Sheppard, 1996; Sheppard et al., 1995) and in transplants (Tate et al., 2002). In addition, FN is upregulated after cerebral injury (Egan and Vijayan, 1991; Tate et al., 2007), in epileptic seizures (Hoffman et al., 1998), and it also ameliorates effects of ischemic stroke (Sakai et al., 2001). We used laser tweezers to test whether reinforcement of FN-coated bead links to the cytoskeleton in cultured hippocampal neurons and confirmed that here, similar to fibroblasts, reinforcement depends upon RPTPα in the growth cones. However, the binding of FN to the growth cones was predominantly through the $\alpha_v\beta_6$ integrin rather than the $\alpha_v\beta_3$ integrin in fibroblasts.

Whereas the FN-null mutation is early embryonic lethal, knockouts of FN-specific integrin subunits exhibit less-severe phenotypes (Yang et al., 1999). High expression levels of α_v and β_6 integrins have been detected in the hippocampus (Chan et al., 2003; Pinkstaff et al., 1999), whereas β_3 integrin is expressed at very low levels. Although 80% α_v-integrin-knockout embryos die in mid-gestation (Bader et al., 1998), conditional knockout studies revealed that neuron-targeted deletion of α_v integrin results in cerebral hemorrhage, axon degeneration and seizures (McCarty et al., 2005). However, no role of the β_6 integrin subunit in the CNS has been reported – not surprising given that β_6-integrin-knockout mice show defects in epithelial tissues and lung, but do not display any neurological abnormalities (Huang et al., 1996). Nevertheless, one can speculate that other FN integrin receptors can compensate for the absence of β_6 subunit. Here, we confirmed the expression of $\alpha_v\beta_6$ integrins in hippocampal neurons and demonstrated that it is required for reinforcement of FN-cytoskeleton links in the growth cones. This suggests that integrin subunits are not redundant in fine-tuning of cell adhesion and motility.

Further, the RPTPα ablation did not affect the reinforcement of VN-bead–cytoskeleton links as in fibroblasts, which is consistent with a low binding affinity of $\alpha_v\beta_6$ integrins for VN. We speculate that VN binds to integrin receptors that have no effect on RPTPα activation, perhaps $\alpha_8\beta_1$ (Bossy et al., 1991). Thus, the common integrin subunit involved in FN rigidity detection in neurons and fibroblasts is the α_v subunit. Interestingly, α_v is required in processes such as radial migration (Anton et al., 1999) and LTP (Kramar et al., 2003; Kramar et al., 2006), which are impaired in RPTPα$^{-/-}$ mice.

At a cellular level, the response of hippocampal neurons to rigid FN surfaces was essentially opposite to that of fibroblasts, i.e. extension of neurites was inhibited, whereas spreading of fibroblasts was stimulated by increased matrix rigidity (Jiang et al., 2006; Kostic and Sheetz, 2006). In addition, rigid matrices inhibited axon differentiation. Given that RPTPα$^{-/-}$ mice display hippocampal abnormalities (Petrone et al., 2003), and that RPTPα was implicated in force transduction and

Fig. 5. Rigidity response in the growth cones is SFK-dependent, and tyrosine phosphorylation of p130Cas requires RPTPα activity and rigid matrix. (A-G) RPTPα$^{+/+}$ neurons were plated on FN-coated substrates of varying rigidities and treated or not with the SFK inhibitor (10 μM SU6656) after cells had adhered to the substrate. (A,B) After a 48-hour incubation, there was no difference in treated RPTPα$^{+/+}$ neurons on rigid versus soft surfaces. However, axon elongation and differentiation were inhibited in neurons treated with SFK compared with untreated controls. (C) Growth cones of treated neurons displayed decreased reinforcement of FN-coated beads in the laser tweezers experiments. (D,E) Immunostaining of Fyn revealed lower levels of edge accumulation on soft than on rigid matrices in RPTPα$^{+/+}$ neurons. In growth cones of RPTPα$^{-/-}$ neurons there was a decreased edge accumulation regardless of rigidity. (F,G) Immunostaining of phosphorylated p130Cas (anti-phospho-Y615-Cas) showed high levels of phosphorylated p130Cas in the presence of RPTPα and rigid matrices. In RPTPα$^{+/+}$ neurons on soft matrix, and in RPTPα$^{-/-}$ neurons – regardless of the matrix rigidity – the observed levels of phosphorylated p130Cas were significantly lower. (H) Fluorescence intensities of the Fyn and phosphorylated p130Cas signals were quantified, normalized against nuclear fluorescence intensity and are presented as the mean ± s.e. for at least 20 representative neurons.

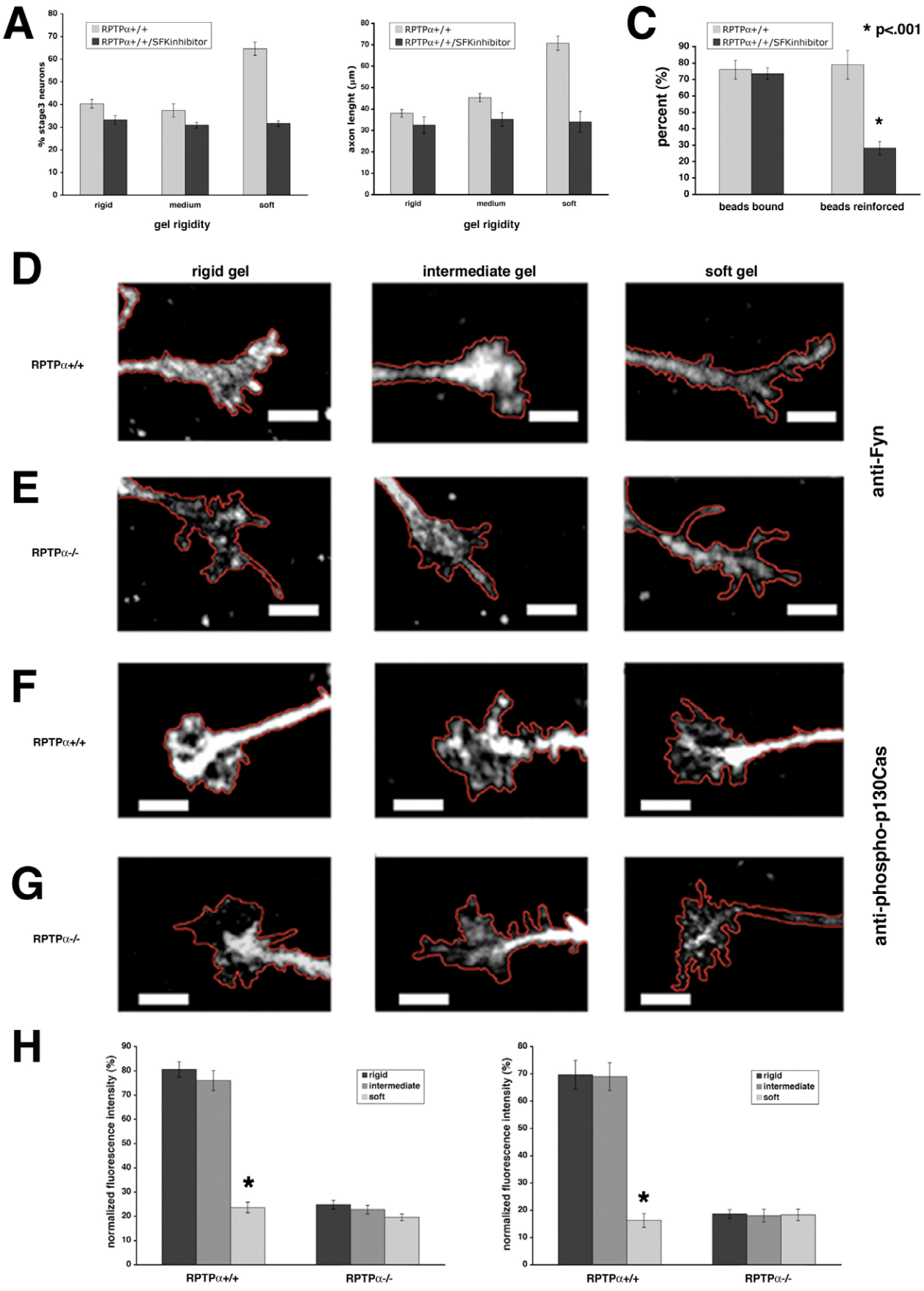

Fig. 5. See previous page for legend.

rigidity response in fibroblasts (Jiang et al., 2006; von Wichert et al., 2003), we speculated that RPTPα deletion would affect the rigidity response in neurons as well. Consistent with our hypothesis, RPTPα$^{-/-}$ neurons did not respond to the rigidity of FN-coated substrates, unlike controls. By contrast, the LN rigidity response was not affected by the absence of RPTPα, indicating FN specificity of those integrin(s) that activates RPTPα. Rigidity response on LN appears to be mediated independently of RPTPα.

These findings are consistent with previous observations (Engler et al., 2004; Flanagan et al., 2002), but beg the question of how rigidity was detected by the neurons. Two major models are suggested: first, the mechanism of rigidity sensing involves different molecular components, similar to the different response pathways for FN and collagen rigidity (Kostic and Sheetz, 2006; Wang et al., 2001); second, the sensing mechanism is the same in fibroblasts and neurons but the response to the sensory signal could be different. Whereas the difference in β subunit between the neurons and fibroblasts is consistent with either model, the common components at the leading edges of growth cones and lamellipodia indicate that the machinery involved in sensing rigidity is the same.

Similar to the our earlier results in fibroblasts, we observed rigidity-dependent RPTPα-mediated recruitment of Fyn and p130Cas phosphorylation at the leading edge of the growth cones. Ubiquitously expressed SFKs, Fyn and Src have been previously implicated in neurite extension (Liu et al., 2004), NMDA-receptor phosphorylation (Cheung and Gurd, 2001; Le et al., 2006), and neuronal migration (Kuo et al., 2005). However, Fyn – unlike Src – seems to be indispensable in hippocampal development, long-term potentiation (LTP) and spatial memory as shown by knockout studies (Grant et al., 1995; Grant et al., 1992; Kojima et al., 1997). Src and Fyn also appear to have different roles in other cell types (Kostic and Sheetz, 2006; von Wichert et al., 2003). When we used a general SFK inhibitor, the rigidity response in hippocampal neurons was abolished, but the neurite extension was also inhibited. Thus, SFKs appear to be required for both rigidity-dependent and rigidity-independent neurite extension, whereas the effect of the phosphatase appears to be exclusively rigidity-dependent. Further, we observed the rigidity-dependent Fyn recruitment at the leading edge; hence we speculate that neuronal rigidity response requires Fyn activity. However, neurite extension is also regulated though multiple pathways involving RPTPα and/or Fyn that seem rigidity-independent (Bodrikov et al., 2005; Robles et al., 2005). Future studies will hopefully provide more insight into the distinct function of these pathways.

Further, we found a correlation between rigidity sensing and p130Cas tyrosine phosphorylation – which is involved in many motility pathways (Kostic and Sheetz, 2006; Tamada et al., 2004; Vuori and Ruoslahti, 1995) – to rigidity-dependent Fyn recruitment. Since p130Cas$^{-/-}$ mice die in utero before the brain has developed it is difficult to determine the effect of p130Cas ablation on CNS development and function (Honda et al., 1998). Nevertheless, in vitro studies showed that p130Cas is required for neurite extension in cerebellar neurons (Huang et al., 2006). Further, ethanol-stimulated Fyn-mediated p130Cas phosphorylation has been reported (Nishio and Suzuki, 2002). Once phosphorylated, p130Cas could signal to many different pathways that normally promote growth and not differentiation. Thus, our model could explain how increased matrix rigidity promotes growth and motility of fibroblasts, but inhibits differentiation of neurons. Although the model is plausible, much more is needed to prove the exact roles of the components.

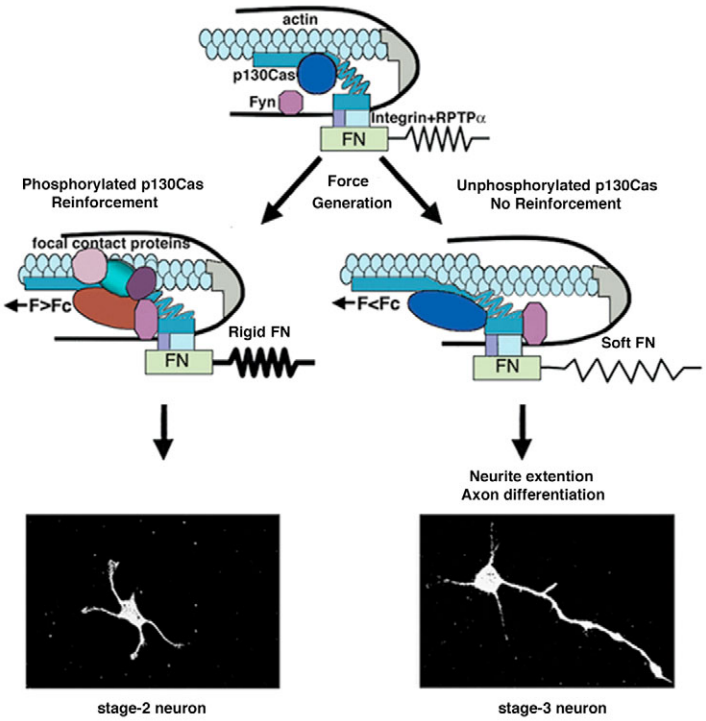

Fig. 6. Proposed molecular mechanism of FN-specific reinforcement and rigidity response in hippocampal neurons. In RPTPα$^{+/+}$ neurons, matrix rigidity triggers force-dependent activation of the RPTPα, followed by activation of Fyn, which consequently phosphorylates stretch-sensitive p130Cas. This results in the recruitment of the focal-contact proteins causing the reinforcement of the growth-cone–substrate links. This reinforcement has a negative effect on the neurite extension. On the soft matrices, the force exerted by the actin-myosin network in response to matrix rigidity, does not reach a threshold critical for the reinforcement of the FN-cytoskeleton bonds and subsequent focal-contact formation. Thus, the neurite extension is stimulated on soft matrices.

It is perhaps surprising that the same molecular components might be implicated in the opposite rigidity responses of such different cells as fibroblasts and neurons. One could correlate differences in rigidity responses to different motility types in neurons and fibroblasts. The growth cones pull neurites forwards and are smaller than fibroblast lamellae. They distinguish FN from LN by forming contacts on FN similar to focal contacts in fibroblasts (Gomez et al., 1996). We speculate that stiff substrates support formation of focal contacts, which in turn stabilize interactions between growth cones and the matrix. If those contacts are not disassembled rapidly as in fibroblasts, then growth cones might have a reduced velocity of progression resulting in shorter neurites (Fig. 6). Therefore, similar rigidity responses could lead to different cellular responses in different cells.

We conclude that the major components of the rigidity-sensing apparatus are present in the growth cone like they are in the fibroblast and show similar rigidity-dependent changes that affect motility and differentiation. We suggest that the rigidity-sensing mechanism involves an α_v integrin-dependent RPTPα activation that recruits and activates Fyn at the leading edge where it phosphorylates p130Cas in a rigidity-dependent manner. This pathway appears to be crucial for matrix-rigidity-dependent regulation of neurite extension and axon differentiation. FN plays an important regulatory role in both normal development and a variety of pathological processes in the brain. Thus, FN assembly on fibers or other rigid elements could control motility.

Materials and Methods

Primary hippocampal neuron culture

Hippocampi were dissected from RPTPα$^{+/+}$ and RPTPα$^{-/-}$ P0 mice and incubated for 30 minutes in 2.5% trypsin solution. Neurons were dissociated through a series of decreasing-diameter pipettes and resuspended in Neurobasal-A medium supplemented with 2% B-27 supplement, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all materials from Invitrogen). Neurons were plated on coverglass or gels, incubated with 20 μg/ml polylysine and subsequently coated with 50 μg/ml laminin (LN; BD Biosciences) or 50 μg/ml fibronectin (FN; Roche).

Laser trap bead assays

Silica beads (0.64 μm in diameter) were activated with avidin as described previously (Jiang et al., 2003) and coated with 10 μg/ml biotinylated full-length FN (Roche) or 10 μg/ml biotinylated full-length vibronectin (VN; BD Biosciences). BSA-coated beads (Sigma) were used as a control. Optical gradient laser trap set at 100 mW (40 pN/μm) (Axiovert TV 100; Carl Zeiss MicroImaging, Inc) was equipped with a 100× objective and calibrated as described (Choquet et al., 1997). The beads were held at the smooth edges of the growth cones for 3-5 seconds and then the trap was turned off to check for binding. If the bead was bound, the trap was turned back on and the rearward movement of the bead was recorded using a cooled CCD camera. The fraction of the beads bound and moving rearwards was calculated as the mean ± standard error (s.e.) for at least three independent experiments and statistical significance of the results confirmed by *t*-test ($P<0.01$). The total number of beads included in the analysis was at least 35 beads for each condition. The rearward movements of the beads were further analyzed using Nanotracker, with a tracking accuracy of 3-5 nm for 0.64-μm beads. The MSD values were calculated using an algorithm modified from Qian et al. (Qian et al., 1991).

Neurite extension on polyacrylamide substrates

The FN-coated or LN-coated polyacrylamide substrates were prepared as described previously (Pelham, Jr and Wang, 1997). The substrate flexibility was manipulated by maintaining the total acrylamide concentration at 5%,while varying the bis-acrylamide component between 0.25% (rigid), 0.1% (intermediate) and 0.025% (soft) (E=7.5 kPa, E=4 kPa and E=500 Pa, respectively) (Engler et al., 2004). The uniformity of coating was examined by coating with Cy5-conjugated (Amersham Biosciences) proteins, visualized by confocal microscopy. Experiments were performed 48 hours after plating. Neurite extension was quantified for at least 50 neurites for each condition and statistical significance of the results confirmed by *t*-test ($P<0.01$). Data are presented as the mean± s.e. of at least three independent experiments. In experiments with SFK inhibitor, 10 μM SU6656 (Calbiochem) was added after neurons had adhered to the substrate; cells were then further incubated for total time of 48 hours.

Antibodies

The following antibodies were used: mouse monoclonal anti-RPTPα (BD Transduction Laboratories), mouse monoclonal anti-$\alpha_v\beta_6$ (USBiological), mouse monoclonal anti-Tau (Biosource), affinity-purified rabbit polyclonal anti-phosphorylated Y165Cas (Cell Signaling Technology), mouse monoclonal anti-Fyn (Chemicon), goat anti-mouse Ig conjugated with Alexa-Fluor-477 (Molecular Probes), goat anti-rabbit Ig conjugated with Alexa-Fluor-477 (Molecular Probes), goat anti-mouse Ig conjugated with Alexa-Fluor-568 (Molecular Probes) and phalloidin conjugated with Alexa-Fluor-477 (Molecular Probes). To block binding of FN-coated beads, anti-$\alpha_5\beta_1$ (Chemicon), anti-α_v (BD Pharmingen), and anti-$\alpha_v\beta_6$ (USBiological) antibodies were added at 10 μg/ml final concentration 30 minutes prior to the experiments. GPen (0.5 mM) was used for blocking $\alpha_v\beta_3$ integrins as described previously (von Wichert et al., 2003).

Immunocytochemistry

Hippocampal neurons were plated onto FN-LN-coated coverglass or FN-LN-coated polyacrylamide gels. After 48 hours incubation for the described time, cells were fixed in 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. Fixed cells were incubated with primary antibodies for 1 hour followed by washing and incubation with appropriate fluorescent secondary antibodies. Fluorescent signals were visualized by confocal microscopy.

Microscopy and analysis

Images of immunofluorescently stained samples were acquired using a Fluoview confocal microscope (Olympus, Melville, NY) equipped with 40×, 60×, and 100× objectives. Analysis of acquired images was performed with the image analysis program, ImageJ (by W. Rasband (NIH, Bethesda, MD http://rsb.info.nih.gov/ImageJ).

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