β1-integrin Cytoskeletal Signaling Regulates Sensory Neuron Response to Matrix Dimensionality

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Abstract

Neuronal differentiation, pathfinding and morphology are directed by biochemical cues that *in vivo* are presented in a complex scaffold of extracellular matrix (ECM). This microenvironment is three-dimensional (3D) and heterogeneous. Therefore, it is not surprising that more physiologically-relevant cellular responses are found in 3D culture environments rather than on two-dimensional (2D) flat substrates. One key difference between 2D and 3D environments is the spatial arrangement of cell-matrix interactions. Integrins and other receptor proteins link the various molecules presented in the extracellular environment to intracellular signaling cascades and thus influence a number of neuronal responses including the availability and activation of integrins themselves. We have previously reported that a 3D substrate induces an important morphological transformation of embryonic mouse dorsal root ganglion (DRG) neurons. Here, we investigate the hypothesis that β1-integrin signaling via focal adhesion kinase (FAK) and the RhoGTPases Rac and Rho influences neuronal morphology in 2D vs 3D environments. We report that β1-integrin activity and FAK phosphorylation at tyrosine 397 (FAKpY397) are linked to neuronal polarization as well as neurite outgrowth and branching. Rac and Rho expression are decreased in 3D vs 2D culture but not correlated with β1-integrin function. These results suggest that proper β1-integrin activity is required for elaboration of physiologic DRG morphology and that 3D culture provides a more appropriate milieu to the mimic *in vivo* scenario. We propose that neuronal morphology may be directed during development and regeneration by factors that influence how β1-integrin, FAK and RhoGTPase molecules integrate substrate signals in the 3D microenvironment.

Keywords

Dorsal root ganglia; neurite outgrowth; culture dimensionality; β1-integrin; focal adhesion kinase; signaling

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INTRODUCTION

Extracellular cues are critical for cellular maturation and function, and *in vivo*, growth factors and extracellular matrix (ECM) molecules are presented in a three-dimensional (3D) context. Neuronal differentiation, growth cone motility and branching events are regulated by molecular cues provided by surrounding cells, and *in vivo* these are arranged in a complex 3D network of ECM that creates heterogeneous local microenvironments (Kollins and Davenport, 2005; Zhang et al., 2005). It is therefore not surprising that more physiologically-relevant responses are found by culturing cells in 3D environments (e.g., embedded inside a gel matrix) rather than on two-dimensional (2D) surfaces (e.g., glass coverslips or tissue culture plasticware) (Harris, 1985; Hoffman, 1993; Cullen et al., 2007). We have recently reported that sensory neurons respond to the arrangement of external signals presented in 2D vs 3D environments by modulating morphology, neurite extension and branching (Ribeiro et al., 2012). Most significantly, in 3D the neurons adapt *in vivo*-like features on a timescale that recapitulates the developmental program, establishing that a simple 3D environment alone can provide optimized conditions for normal development to occur.

One of the fundamental differences between 2D and 3D environments is the nature of cell-matrix interactions. β1-integrins are a major class of transmembrane proteins that transmit signals between the ECM and the cytoskeleton and have been linked to neuronal cell migration (Graus-Porta et al., 2001; Belvindrah et al., 2007a; Belvindrah et al., 2007b), neuritogenesis (Gupton and Gertler, 2010; Loubet et al., 2012), neurite pathfinding (Robles and Gomez, 2006), outgrowth during regeneration (Carlstrom et al., 2011; Tan et al., 2011) as well as synapse formation and plasticity (Huang et al., 2006; Warren et al., 2012). Pairing with one of twelve α subunits, β1-integrins form receptors for various ECM molecules including collagens, laminins and fibronectin (Pylayeva and Giancotti, 2006).

The types of ECM molecules and how they are presented in the neuronal environment influence β1-integrin availability, activation and signaling (Condic and Letourneau, 1997; Condic, 2001; Tan et al., 2011). β1-integrins exist within three activation states: inactive, active, and clustering (Clark and Brugge, 1995); clustering of β1-integrins leads to cell-matrix adhesions via recruitment of cytoskeletal proteins, RhoGTPases (e.g., Rac and Rho) and non-receptor tyrosine kinases, including focal adhesion kinase (FAK) and Src (Mitra et al., 2005). In growth cones, these cell-matrix adhesions occur as small clusters called point contacts, which are similar to focal adhesion complexes, the much larger formations observed in non-neuronal cells (Gomez et al., 1996; Renaudin et al., 1999).

Many studies of β1-integrin function in neurons have been carried out *in vitro* on 2D substrates and yielded some results that conflict with those found *in vivo*. A notable example involves FAK, which is a scaffolding protein that integrates signals from both matrix and soluble cues. It is generally understood for most cell types that upon binding to activated β1-integrin or certain growth factors, autophosphorylation of FAK at tyrosine (Y) 397 (FAKpY397) causes Src recruitment and activation, which then phosphorylates FAK at Y861 (Cary et al., 1996; Arold, 2011). FAK autophosphorylation occurs predominantly intramolecularly between adjacent FAK molecules; however, interactions between FAK

molecules are not stable and significant clustering is required for FAK autophosphorylation (Toutant et al., 2002; Arold, 2011). This clustering is more likely when cells are adhered to a 2D substrate where matrix molecules are planar and cell-matrix contacts are polarized to one surface of the cell (Kanchanawong et al., 2010). In environments where FAK is not able to form large clusters, which may occur in 3D culture where matrix molecules and cell-matrix contacts are spread throughout the cell surface, autophosphorylation of FAK at Y397 may be less probable (Arold, 2011). Consistent with this idea, several groups have reported decreased FAKpY397 in 3D culture as well as intact tissue, thus suggesting that signaling via FAK differs in 3D as compared to the relatively well-studied cascades that occur in 2D environments (Cukierman et al., 2001; Damianova et al., 2008; Min et al., 2012; Serrels et al., 2012). FAKpY397 also appears to be linked to mechanosensation of substrate stiffness, wherein stiffer 2D substrates were associated with increased FAKpY397 (Shi and Boettiger, 2003; Wozniak et al., 2003; Friedland et al., 2009). Interestingly, in several of these studies, FAKpY861 was also measured and did not vary with either culture dimensionality or substrate stiffness (Cukierman et al., 2001; Damianova et al., 2008; Friedland et al., 2009). Thus, variations in FAK concentration and phosphorylation that occur due to altered cell-matrix interactions may play a role in regulating how FAK integrates various signals in 2D vs 3D environments.

In neurons, it is clear that FAK influences neuronal function, but much of the details of how FAK regulates neurite outgrowth are not well understood and likely depend on context (type of cell, type of matrix molecule, activation mechanism) (Lemons and Condic, 2008; Myers and Gomez, 2011; Monje et al., 2012). FAK is highly expressed in growing axons and growth cones (Menegon et al., 1999) and is thought to physically integrate extracellular cues (Ren et al., 2004; Rico et al., 2004; Mitra et al., 2005; Tomar and Schlaepfer, 2009; Myers and Gomez, 2011; Myers et al., 2012). Removal of FAKpY397 signaling in 2D culture leads to decreased growth cone motility and neurite retraction as well as increased branching and synapse formation, with the net effect of these processes being increased neurite growth (Rico et al., 2004).

The roles of Rac and Rho in regulation of neuronal morphology are similarly complex, and moreover, FAK and GTPases are known to regulate each other dynamically in the cyclic regulation of cell-matrix adhesion and cytoskeletal structures (Woo and Gomez, 2006; Thoumine, 2008; Myers et al., 2011; Loubet et al., 2012). To better elucidate the role of matrix presentation on β1-integrin signaling and neuronal morphology, we investigated the expression of β1-integrin, total FAK, FAKpY397, FAKpY861, Rac and Rho in embryonic mouse dorsal root ganglion (DRG) neurons and PC12 cells.

EXPERIMENTAL PROCEDURES

Unless otherwise noted, reagents were obtained from Fisher Scientific (Pittsburgh, PA, USA), Sigma Aldrich (St. Louis, MO, USA) or Invitrogen (currently Life Technologies (Carlsbad, CA, USA)).

Mice

C57Bl/6^J mice (Jackson Laboratory, Bar Harbor, ME, USA) were used. All animal studies were approved by the UMBC and University of Maryland School of Medicine Institutional Animal Care and Use Committees (IACUC) and performed in accordance to NIH guidelines.

Antibodies

Antibodies against total β1-integrin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for immunofluorescence and Cell Signaling Technologies (Danvers, MA, USA) for immunoblotting. Antibodies against FAK and FAKpY397 were obtained from BD Biosciences (San Jose, CA, USA) for immunofluorescence and Cell Signaling for immunoblotting. FAKpY861 was detected with anti-FAKpY861 from Invitrogen. Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), βIII tubulin and neurofilament 160 (NF160) were from Sigma Aldrich. The β1-integrin function-blocking antibody HMβ1-1 and isotype control (Armenian Hamster IgG2, λ1 isotype) as well as antibodies against Rac1 and Rho were from BD Biosciences. Fluorescent antibodies and horse radish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA).

DRG neuron culture

DRG were dissected from mouse embryos at E12.5–E14.5 following established methods (Banker and Goslin, 1998) and cultured according to Ribeiro *et al.* (Ribeiro et al., 2012). Tissue was dissociated with 0.025% trypsin (Invitrogen) for 20 min, triturated, and filtered through a 25-μm nylon mesh (Sefar, (Depew, NY, USA)) to remove tissue clumps and most non-neuronal cells. Using this method, cultures were >95% neurons as assessed by positive βIII tubulin and NF160 immunoreactivity.

Neurons were seeded at $1.5×10^4$ cells/cm^2 onto 2D collagen-coated coverslips (~7 μg/cm^2, pre-coated with 0.1 mg/ml with poly-L-lysine) and at $5×10^5$ cells/ml within 3D collagen gels (1 mg/ml, 20 μl) and cultured for 2 days in 24-well plates. In all cases, rat-tail type I collagen (BD Biosciences) was used. The cell densities have been previously determined to be sufficiently low to minimize cell-cell contact. For immunoblotting ~$1.0×10^5$ cells/cm^2 were cultured in collagen-coated 6- or 12-well tissue culture plates (2D), and ~$5.0×10^6$ cells/ml were cultured within 3D collagen gels (1 mg/ml, 30–40 μl). Cells were maintained in serum-free medium: Dulbecco's Modified Eagle Medium supplemented with 1X N2 supplement, 100 U/ml of penicillin/streptomycin, 20 mM of L-glutamine and 50 ng/ml of 7S Nerve Growth Factor (NGF, (EMD Millipore, Billerica, MA, USA)). For 1 mg/ml collagen gels, 10X Hank's Buffered Salt Solution, 1N NaOH, deionized water, collagen, 50 ng/ml NGF were combined with cells to generate 3D substrates. The collagen/cell solution was allowed to gel in a culture incubator for 30–45 min prior to addition of culture medium.

PC12 cell maintenance and culture

PC12 cells (CRL-1721.1, ATCC, (Manassas, VA, USA)) were cultured in complete medium: Dulbecco's Modified Eagle Medium enriched with 10% fetal bovine serum, 5%

horse serum and 100 μg/ml penicillin-streptomycin at 37°C and 5% CO_2 (Yu et al., 1999). Cells were removed from cell culture substrates using 0.025% trypsin and were cultured for 2 days in complete medium supplemented with 100 ng/ml NGF on 2D collagen-coated coverslips or within 3D collagen gels as per methods for DRG neurons. Cell densities depended on the following method of analysis: for immunocytochemistry and image analysis, 0.75×10^4 cells/cm^2 were seeded on coverslips (2D; ~7 μg/cm^2 collagen) and 2.5×10^5 cells/ml were encapsulated for 3D cultures (1 mg/ml, 20 μl). For immunoblotting, 1.5×10^4 cells/cm^2 were cultured on 10-mm collagen-coated tissue culture plates (2D) and 1.6×10^6 cells/ml were cultured within 3D collagen gels (1 mg/ml, 50 μl).

β1-integrin inhibition

A function-blocking blocking antibody to β1-integrin, HMβ1-1 (Yanagawa et al., 2001; Sarin et al., 2005), was selected for analysis of β1-integrin inhibition. To determine inhibitor concentration to be tested with DRG neurons, PC12 cells were cultured on collagen-coated coverslips for 2 days with β1-integrin inhibitor (0, 20 or 50 μg/ml). Samples were imaged under phase contrast microscopy (Olympus IX-81, Center Valley, PA, USA) and the images were used to determine cell density, total neurite length per cell, and the percentage of cells expressing neurites by ImageJ software (NIH). In DRG neuron studies, 20 μg/ml of inhibitor were added to the medium and collagen/cell solution. An isotype control (Armenian Hamster IgG2, λ1 isotype) was also tested at the same concentration as the inhibitor.

Immunocytochemistry and image analysis

Samples were fixed in a buffered 4% formalin solution for 20 min, washed 3 times with phosphate buffered saline (PBS) and then incubated with blocking solution (10% lamb serum in PBS) for 30 min (in 2D) or 2 h (in 3D). 2D cultures were incubated in primary antibodies to β1-integrin, FAK, FAKpY397 and FAKpY861 (each 1:100) for 30–60 min and then appropriate fluorescently-labeled secondary antibodies for 30–60 min. 4′,6-diamidino-2-phenylindole (DAPI, 300 nM, Sigma) was used to visualize cell nuclei. Procedures for 3D cultures were similar but had washing steps (>30 min each) and overnight antibody incubations. Samples were imaged with confocal laser scanning microscopy (Leica TCS SP5, (Buffalo Grove, IL, USA)) and analyzed with LAS AF software (Leica). Sets of neurons used for fluorescence quantification were imaged under the same confocal settings for each channel (objective, zoom, gain and offset). Analyses of fluorescence intensity were performed using Volocity imaging software (PerkinElmer).

Immunoblotting

Cells in 2D and 3D cultures were lysed directly in RIPA (radioimmuno precipitation assay) lysate buffer containing protease and phosphatase inhibitor cocktails (Pierce, Rockford, IL, USA). The 3D gels were ground using a micropestle and homogenized with QIAshredder homogenizer (Qiagen (Valencia, CA, USA)) for 2 min at $15,000 \times g$ in a microcentrifuge. We used centrifugation at $10,000 \times g$ for 25 min at 4°C to separate the protein extracts from the cells. The total protein concentration of each lysate was determined with a micro BCA protein assay (ThermoScientific, Rockford, IL, USA). Cell lysates (20 μg) were separated on SDS-PAGE gels and immunoblotted. Incubation with primary antibodies to β1-integrin

(1:1000), FAK (1:1000), FAKpY397 (1:500), FAKpY861 (1:500), Rac (1:750), Rho (1:750) and GAPDH (1:10000) was followed by HRP-conjugated secondary antibodies and ECL substrate (Pierce). Band intensities were quantified (ImageJ) and normalized for loading control to GAPDH, a housekeeping gene that is stably and constitutively expressed at high levels in most tissues and cells (Thellin et al., 1999; Paszek et al., 2005).

Individual 2D and 3D cultures of PC12 cells yielded sufficient protein for immunoblotting and thus three cultures of PC12 cells were analyzed in triplicate for each experimental condition. 3D cultures of DRG neurons, however, yielded very low amounts of total protein. Therefore, extracts from 8 cultures in 2D and 3D were pooled, concentrated and analyzed in triplicate.

Quantitative assessment of neurite outgrowth

Volocity and Neurolucida (MBF Bioscience (Williston, VT, USA)) imaging software packages were used to reconstruct and measure process extension. Sholl analysis was used to determine branching features of DRG neurons (Benson and Cohen, 1996; Watson et al., 2006; Ribeiro et al., 2012). The quantified outgrowth features included the number of primary neurites, the total number of branch points per cell, the total neurite length per cell, and the length of the longest neurite for each cell. Primary neurites emerged directly from the soma. Total neurite length was defined as the sum of all neurites plus all the branches deriving from a single cell. Neurites <15 μm in length were excluded. Neurons were excluded if the processes interacted with other cell soma or neurite processes. Length distributions of 15 neurites were analyzed from 3 separate experiments (3 samples each, n 60).

Statistical analysis

Statistical analysis was performed using Analyse-it Statistical software (Leeds, UK) via parametric or non-parametric analyses, as appropriate, with a minimum significance level set at $p<0.05$ (>95 % confidence level). All data was tested for normality using normal probability plots. Statistical significance of differences between two Gaussian populations was determined using two-tailed, unpaired t-test. Statistically significant differences between more than two experimental groups were determined by analysis of variance (ANOVA). Statistical significance between the non-Gaussian populations was determined using a non-parametric test (Mann-Whitney or Kruskal-Wallis) followed by post-hoc analysis. Bonferroni correction was used for multiple comparisons.

RESULTS

As in our previous work (Ribeiro et al., 2012), we focus on the influence of culture dimensionality on neuronal morphology. Herein we use two cell models, embryonic mouse DRG neurons and PC12 cells. Experiments focus on DRG neurons as they better represent the *in vivo* scenario, but PC12 cells were also used for immunoblotting experiments where sufficient quantities of protein could not be obtained in individual DRG cultures. Data yielded from the analyses of immunofluorescence and immunoblotting were similar for the two cell types.

β1-integrin signaling is altered in 2D vs 3D culture

To begin to test how cell-matrix signaling alterations in 2D vs 3D culture yield strikingly different neuronal phenotypes (Ribeiro et al., 2012), we hypothesized that β1-integrin is a key mediator in cell-matrix interactions and that β1-integrin activation results in adhesion formation and subsequent activation of FAK. To visualize β1-integrin adhesion complexes, DRG neurons were cultured on 2D collagen-coated coverslips or within 3D collagen gels for 2 days, and then fixed and processed for immunofluorescence imaging. Generally, in 2D culture, areas of positive reactivity for β1-integrin, FAK and the phosphorylated forms of FAK at Y397 and Y861 are present throughout the cell as punctate regions (Figure 1A–D) where FAK, FAKpY397 and FAKpY861 each colocalized with β1-integrin (Figure 2A–C). In 3D, areas of β1-integrin, FAK and FAKpY861 reactivity are diffuse whereas FAKpY397 reactivity is weak or absent (Figure 1E–H); FAK and FAKpY861 were colocalized with β1-integrin (Figure 2D–F). Quantitative measures of whole cell fluorescence confirmed reduction of FAKpY397 reactivity in 3D culture as compared to 2D culture with no significant differences in FAK or FAKpY861 reactivity (Figure 2G). Similar findings were obtained for PC12 cells (Figure 3).

Proteins were extracted and analyzed by immunoblot. β1-integrin and FAK were detected at similar levels for DRG neurons in both 2D and 3D cultures, indicating that overall expression levels of these molecules was not altered by culture dimensionality (Figure 4A). Relative expression levels of all molecules were determined for PC12 cell cultures (Figure 4B). In contrast to DRG cultures, expression of β1-integrin and FAK were decreased in 3D as compared to 2D. Whereas expression levels of the phosphorylated forms of FAK were also reduced in 3D vs 2D, the possible amount of phosphorylation depends on the total FAK expression. When normalized by total FAK in each condition, only FAKpY397 was significantly reduced (25%) in 3D vs 2D culture (Figure 4C).

Inhibition of β1-integrin significantly affects neuronal morphology

To further test the hypothesis that β1-integrin is a key mediator of cell morphology in 2D and 3D culture, we inhibited β1-integrin function with HMβ1-1, a function-blocking antibody. We cultured PC12 cells on collagen-coated coverslips (2D) in the presence of 0, 20 and 50 μg/ml inhibitor, in the previously reported effective range (Yanagawa et al., 2001; Sarin et al., 2005). In the presence of β1-integrin inhibitor, fewer cells adhered, yet a greater percentage of those adherent cells expressed neurites. Cells in the presence of 20 μg/ml HMβ1-1 had the greatest total neurite length per cell (114.5 ± 32.5 μm) compared to 0 and 50 μg/ml HMβ1-1 (76 ± 32.5 μm and 84 ± 31 μm, respectively) (Figure 5). Balancing the effects of cell adhesion and neurite growth, we selected 20 μg/ml for experiments with DRG neurons. Initial imaging studies confirmed that this concentration of inhibitor allowed DRG neuron adhesion to collagen-coated coverslips to a similar degree as PC12 cells and resulted in similar punctate areas of positive β1-integrin reactivity in all cases (Figure 6). From these results, we inferred that inhibiting β1-integrin under these conditions resulted in β1-integrin function that was decreased vs controls but still present.

We then carried out detailed analyses of the effect of β1-inhibitor treatment on DRG neuron morphology (Figure 7). We found that β1-integrin inhibition resulted in decreased neurite

growth in both 2D and 3D culture, but the extent of this effect was ~2x greater in 3D, resulting in an average neurite length per cell that was 82% shorter in the presence of HMβ1-1. Effects of β1-integrin inhibition on polarity and branching were similarly exacerbated in 3D culture. In 2D, ~70% of cells were multipolar (2 or more primary neurites) and ~70–80% of the cells had 0 or 1 branch points regardless of β1-integrin inhibition. In 3D, β1-integrin inhibition resulted in neurons that were largely multipolar (72% with 2 or more neurites) vs unipolar (74% with 1 neurite) with no inhibitor; neurites were unbranched (77% with 1 or fewer branches) with HMβ1-1 vs branched (64% with 2 or more branches) in control conditions. In sum, the morphology of neurons in 3D with β1-integrin inhibition is strikingly similar to that of neurons in 2D culture (with or without inhibition). Treatment with isotype control antibody yielded no differences in cell morphology vs untreated controls.

β1-integrin inhibition and 3D culture synergistically influence FAK phosphorylation at Y397

To test whether the influence of β1-integrin inhibition on cell morphology in 3D is related to FAK phosphorylation, we examined levels of β1-integrin, FAK, FAKpY397 and FAKpY861 in PC12 cells cultured in 2D and 3D with 0 and 20 μg/ml β1-integrin inhibitor (Figure 8). If these processes are related, we expected that when comparing cultures with and without β1-integrin inhibition, expression levels of these molecules be significantly altered in 3D, but relatively unchanged in 2D culture. We found that β1-integrin, FAK and FAKpY861 expression were reduced by ~20–30% in 2D culture in the presence of inhibitor, while in 3D the expression levels of β1-integrin as well as FAKpY397 were decreased more drastically (37% and 59%, respectively). As reported above, with no inhibition, FAKpY397 normalized by FAK was 25% lower in 3D vs 2D. With inhibition, the effect of culture dimensionality was more pronounced: FAKpY397 was 74% lower in 3D vs 2D. FAKpY397 expression in 3D cultures was also reduced by 62% with β1-integrin inhibition vs no treatment. There were no significant differences between cultures with 20 μg/ml isotype control antibody vs no treatment (Figure 8D). We note that sample sizes in the studies with inhibitor were larger (n=5–7 cultures) than for those of the isotype control (n=4–5 cultures); thus mean values resulting from the isotype control studies have a greater standard error of the mean than for inhibitor studies.

Rac and Rho expression levels are reduced in 3D vs 2D cultures regardless of β1-integrin inhibition

We also examined levels of Rac and Rho in PC12 cells cultured in 2D and 3D with 0 and 20 μg/ml β1-integrin inhibitor (Figure 9). Treatment with the β1-integrin inhibitor had no significant effect on levels of Rac and Rho expression. However, expression of these molecules was reduced in 3D cultures as compared to 2D. The effect on Rac was more pronounced than for Rho, with respective ~80–90% and ~40–50% reductions in expression in 3D vs 2D.

DISCUSSION

We have previously reported that a 3D substrate induces an important morphological transformation of embryonic mouse DRG neurons. Here, we investigate the hypothesis that β1-integrin signaling via FAK is linked to differences in neuronal morphology in 2D vs 3D environments. We demonstrated that β1-integrin, FAK, FAKpY397 and FAKpY861 are present throughout DRG neurons and PC12 cells in 2D; these cell-matrix adhesions were punctate and reminiscent of point contacts reported by others (de Curtis and Malanchini, 1997; Leventhal et al., 1997; Robles and Gomez, 2006; Carlstrom et al., 2011; Eva et al., 2012; Myers et al., 2012). In 3D, however, areas of β1-integrin, FAK and FAKpY861 reactivity were diffuse and FAKpY397 reactivity was significantly reduced vs 2D. These findings agree with previous studies of cells in 3D matrices showing altered cell-matrix adhesion morphology and FAKpY397 expression (Cukierman et al., 2001; Damianova et al., 2008) and support the idea that the 3D presentation of matrix around the cell results in locally decreased intracellular concentrations of β1-integrin and FAK and result in altered FAK phosphorylation.

We further probed this signaling response by disrupting β1-integrin signaling with HMβ1-1, a function-blocking β1-integrin inhibitor. The inhibitor did not alter the formation of punctate β1-integrin adhesions in 2D culture, but in agreement with others, β1-integrin inhibition had a significant effect on neurite growth (Tucker et al., 2005; Plantman et al., 2008; Carlstrom et al., 2011). β1-integrin inhibition resulted in decreased neurite lengths in 2D and 3D culture and distinct effects on neuronal morphology with culture dimensionality. In 2D, neurons were largely multipolar with unbranched neurites regardless of β1-integrin inhibition. In 3D with no treatment, neurons were unipolar with branched neurites, a morphology which is reminiscent of the *in vivo.* In 3D with β1-integrin inhibition, however, neurons appeared more like those in 2D culture and were multipolar with unbranched neurites. These results suggest that proper β1-integrin activity is required for elaboration of physiologic DRG morphology and that 3D culture provides a more appropriate milieu to the mimic *in vivo* scenario.

Inhibition of β1-integrin function also had distinct effects on the expression levels of signaling molecules in 2D vs 3D culture. Whereas the treatment of HMβ1-1 inhibitor in 2D culture resulted in small reductions in the expression of β1-integrin and FAK, the inhibitor had no effect on FAK phosphorylation at Y397. In 3D culture, inhibition also resulted in decreased β1-integrin expression (vs no treatment), but the effect on FAKpY397 was more striking, with a net 74% reduction compared to 2D and 62% compared to 3D with no treatment.

Our studies thus suggest that FAKpY397 is a major link that influences β1-integrin function in 2D vs 3D culture: the multipolar/unbranched morphology in 2D had the highest FAKpY397 expression level, the multipolar/unbranched morphology in 3D with β1-inhibition had the lowest FAKpY397 level and the unipolar/branched morphology in 3D with no treatment had intermediate FAKpY397 level. To begin to explain these results, we considered the suggested role of FAK in regulating RhoGTPase activity and thus adhesion formation and stability (Rico et al., 2004; Myers et al., 2012). We found up to 2-fold lower

Rho expression in 3D vs 2D cultures. This finding is similar to studies of breast epithelial cells which report decreased Rho expression in soft 3D gels as compared to stiffer 3D gels and 2D substrates (Wozniak et al., 2003). The effect of culture dimensionality on Rac expression was more profound, with up to 7x lower expression levels in 3D vs 2D. Interestingly, Rac and Rho levels did not change with β1-integrin inhibition, indicating that neuronal signaling via these GTPases is not strongly dependent on β1-integrin function under these conditions.

We note that the dynamics of cell-matrix adhesions and cytoskeletal structures underlie cell morphology: the cyclical process of stabilizing and destabilizing adhesions and actin/ microtubule fibers affects spreading, migration and morphogenesis in non-neuronal cells (Wozniak et al., 2003; Mitra et al., 2005). The balance of these dynamics also influences neuronal growth and branching (Woo and Gomez, 2006; Thoumine, 2008; Myers et al., 2011; Loubet et al., 2012) (Figure 10). As integrators of signals from the extracellular environment, both FAK and RhoGTPases are influenced in part by β1-integrin activity and clustering ("outside-in" signaling) and the integration of FAK and RhoGTPase signaling in turn alters adhesion formation and stability ("inside-out" signaling) (Loubet et al., 2012). Despite known effects of single molecules on resulting neuronal morphologies, the details of the dynamic interactions between these molecules are not yet clear (Bilimoria and Bonni, 2013) and likely vary with specifics of the cell type and age as well as the substrate type, density, adhesivity and presentation (Wozniak et al., 2003). Given the significant differences in cell morphology and β1-integrin cytoskeletal signaling with matrix dimensionality noted herein, future studies of adhesion stability and turnover as affected by β1-integrin, FAK and GTPase signaling dynamics will lend further insight into the mechanisms underlying neuronal morphology.

Acknowledgments

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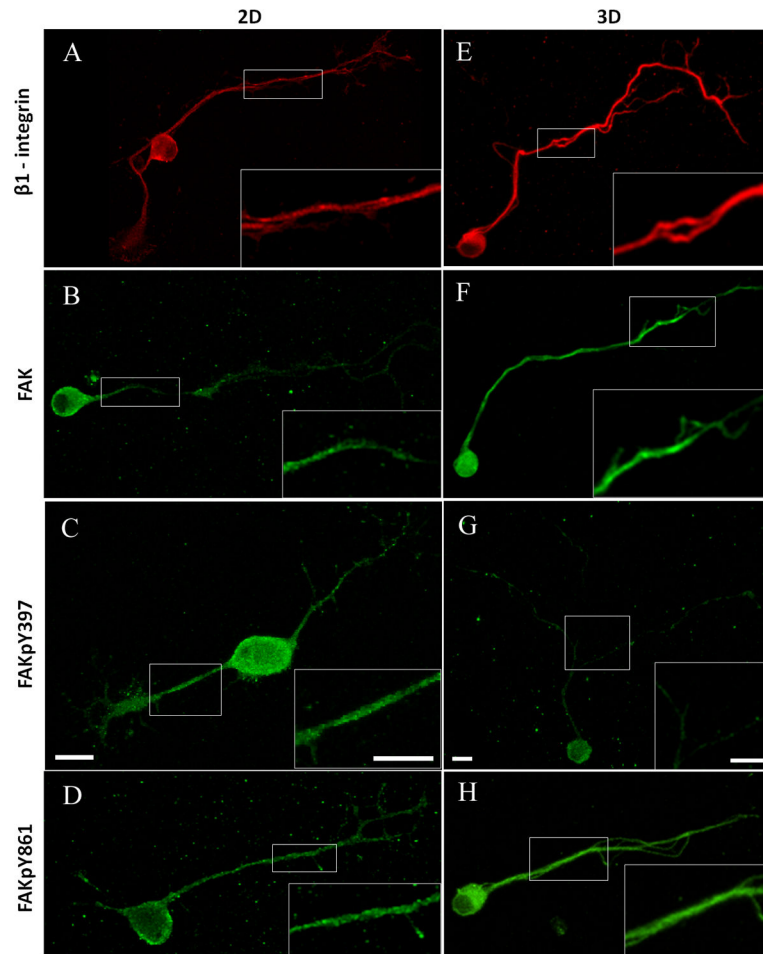
Figure 1. Morphology of β1-integrin, FAK and pFAK reactivity is altered in 2D vs 3D culture
In 2D culture of DRG neurons, areas of reactivity for β1-integrin (A), FAK (B), FAKpY397 (C), and FAKpY861 (D) are punctate throughout the cell, whereas in 3D culture, reactivity of β1-integrin (E), FAK (F) and FAKpY861 (H) are diffuse and FAKpY397 reactivity (G) is weak or absent. Insets show magnified views of the areas of positive reactivity. Scale bars, 10 μm.
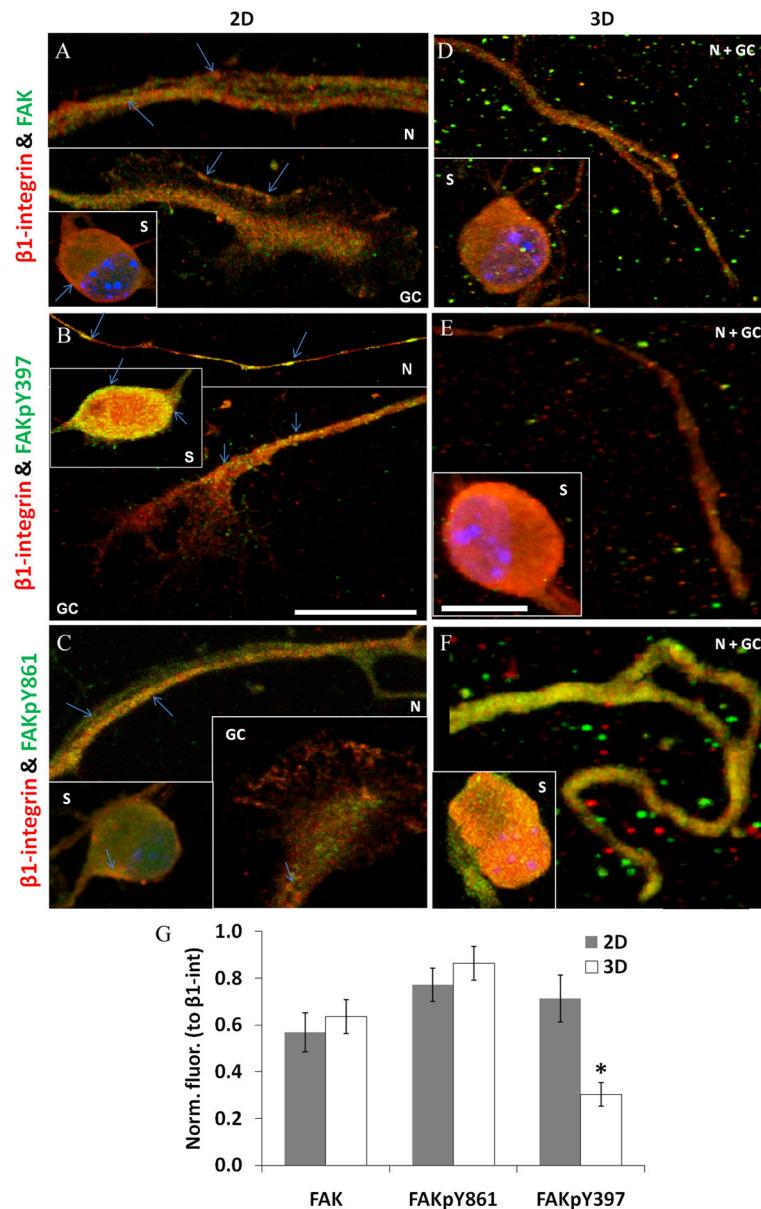
Figure 2. Phosphorylation of FAK at Y397 is altered in 3D culture compared to 2D culture
In 2D culture of DRG neurons, β1-integrin reactivity is colocalized with FAK (A), FAKpY397 (B) and FAKpY861 (C) in puncta (arrows) located throughout the cell soma (s), neurite (n) and growth cones (gc). In 3D culture, colocalization patterns of β1-integrin with FAK (D) and FAKpY861 (F) reactivity are diffuse, whereas colocalization between β1-integrin and FAKpY397 (E) is not apparent due to relatively low expression of FAKpY397 in 3D culture. Whole cell fluorescence intensity was measured for FAK, FAKpY397 and FAKpY861 and normalized to that of β1-integrin and confirms a reduction of FAKpY397 expression in 3D in comparison to 2D (G). Bars represent mean ± SEM and asterisks denote significant difference from 2D ($p < 0.05$) for $n > 9$ cells in each condition.
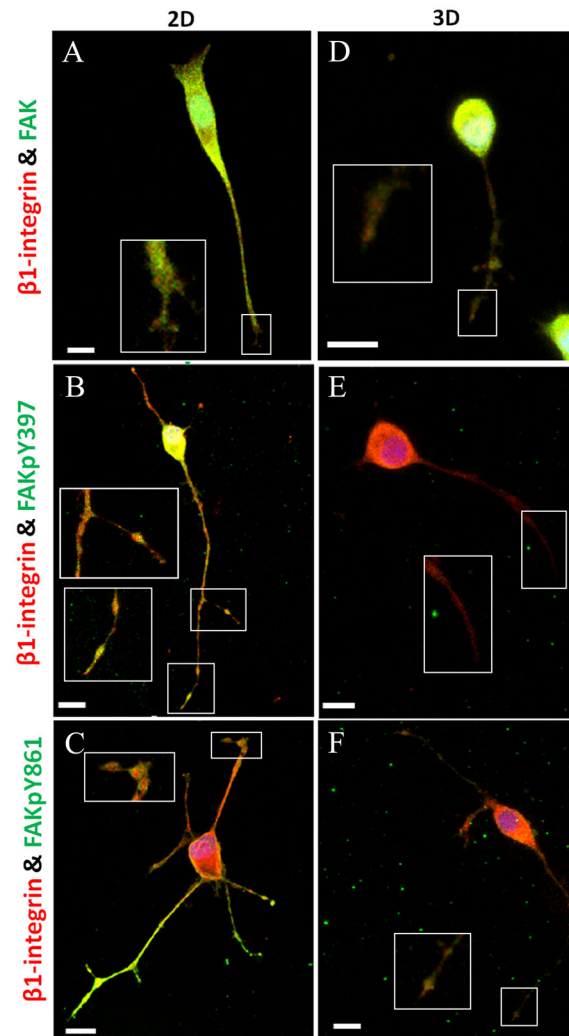
Figure 3. Morphological patterns of β1-integrin, FAK and pFAK in PC12 cells are similar to those in DRG neurons
In 2D culture, reactivity for β1-integrin is colocalized with FAK (A), FAKpY397 (B) and FAKpY861 (C) in puncta located throughout the cell, whereas in 3D culture, colocalization patterns of β1-integrin with FAK (D) and FAKpY861 (F) are diffuse and FAKpY397 reactivity is weak or absent (E).
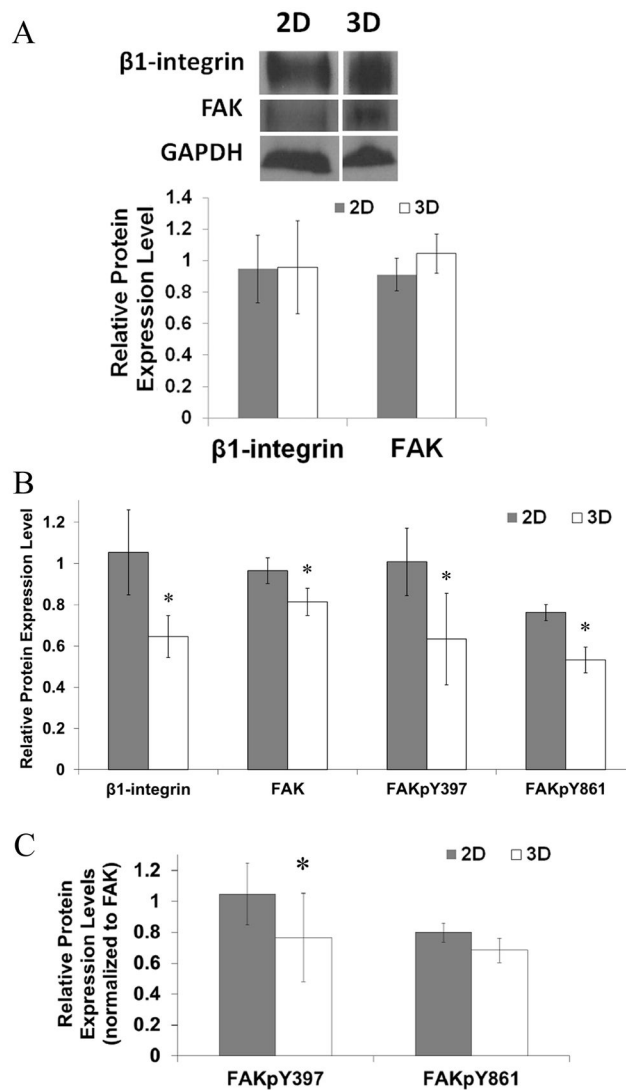
Figure 4. Expression of FAKpY397 is decreased in 3D culture

Protein extracts from 2D and 3D DRG neuron cultures (8 each) were pooled, concentrated and analyzed in triplicate by immunoblot (A). Protein extracts from 2D and 3D PC12 cell cultures (>3 each) were each analyzed in triplicate. Grayscale intensity values were normalized to GAPDH to compare relative expression levels (A, B) and PC12 cell samples were normalized again to total FAK to determine relative phosphorylation levels at Y397 and Y861 (C). Bars represent mean ± SEM and asterisks denote significant difference (p<0.05) from 2D culture.
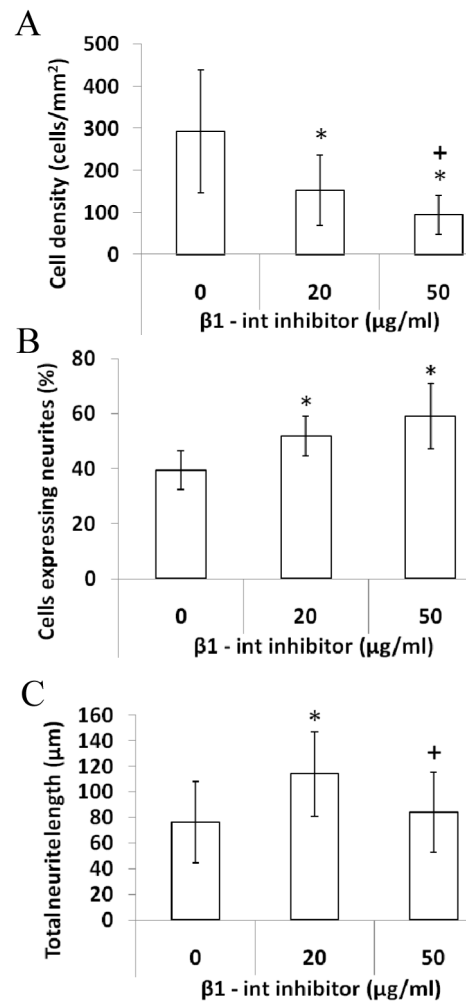
Figure 5. Density of adherent cells and neurite outgrowth are influenced by inhibition of β1-integrin function

PC12 cells in 2D culture were treated with HMβ1-1 function-blocking antibody (0, 20, 50 μg/ml) for 2 days. In the presence of β1-integrin inhibitor concentration, cell density was lower (A), but a higher percentage of the adherent neurons expressed neurites (B). Cells had longer neurites in the presence of 20 μg/ml β1-integrin inhibitor vs cultures with 0 or 50 μg/ml inhibitor (C). Symbols denote significant differences ($p < 0.05$): * from 0 μg/ml β1-integrin inhibitor; + from 20 μg/ml β1-integrin inhibitor.
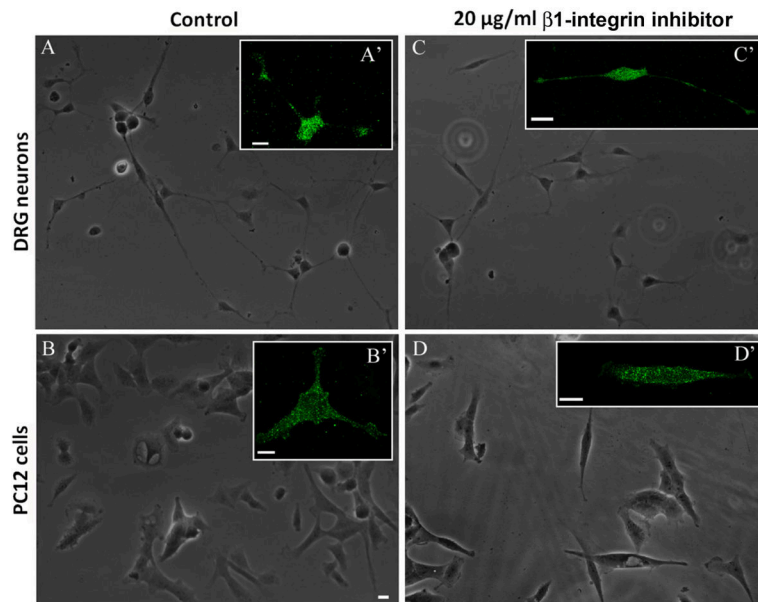
Figure 6. Morphology of β1-integrin reactivity is not altered by inhibition of β1-integrin
DRG neurons and PC12 cells were cultured on collagen-coated coverslips with 0 or 20 μg/ml β1-integrin inhibitor for 2 days and then fixed and processed for imaging under phase contrast for whole cell morphology (A–D) and fluorescence microscopy for β1-integrin reactivity (A′–D′). Scale bar, 10 μm.
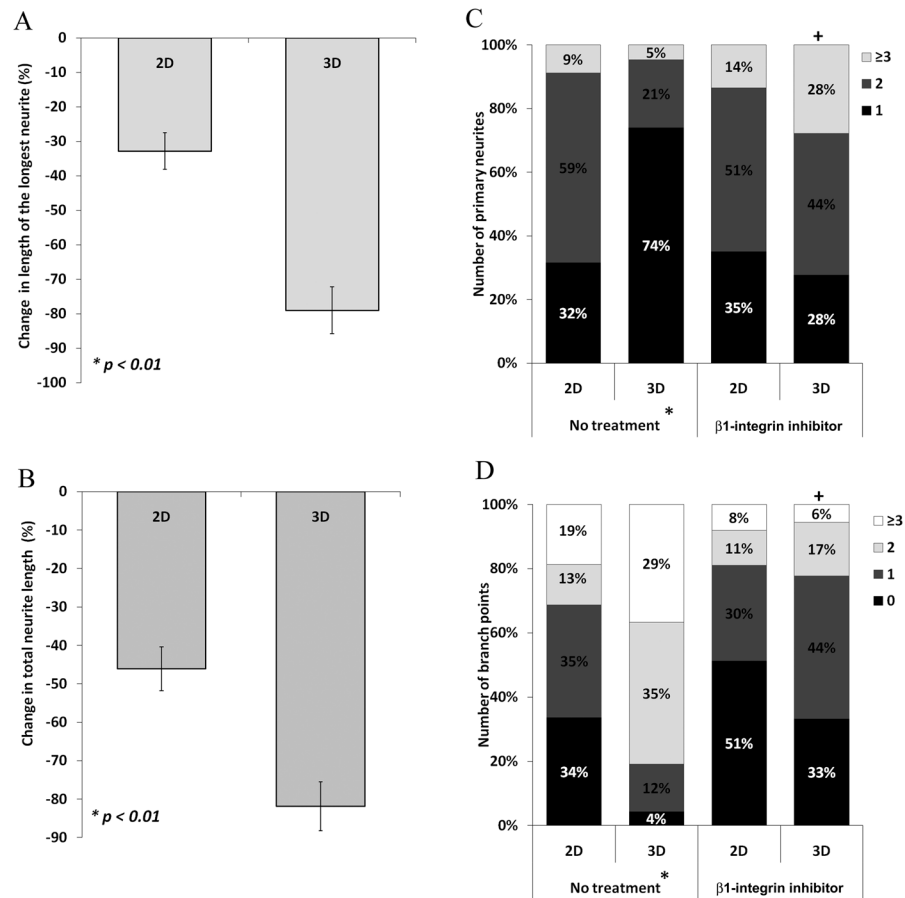
Figure 7. DRG neurons have an *in vivo*-like highly branched, unipolar morphology in 3D culture, but in the presence of β1-integrin inhibitor or 2D culture, are unbranched and multipolar

A, B: In the presence of 20 μg/ml β1-integrin inhibitor, neurite lengths are decreased; this effect is ~2x greater in 3D than 2D culture. C, D: In 2D culture, most neurons are multipolar and have 0–1 branches regardless of β1-integrin inhibition. In 3D culture with no treatment, most neurons are unipolar and have 2+ branches, but when β1-integrin is inhibited, neurons are multipolar and have 0–1 branches. Symbols denote significant differences (p<0.05): * between 2D and 3D culture within the same culture conditions, + from 0 μg/ml β1-integrin inhibitor. n 60 neurites per condition.
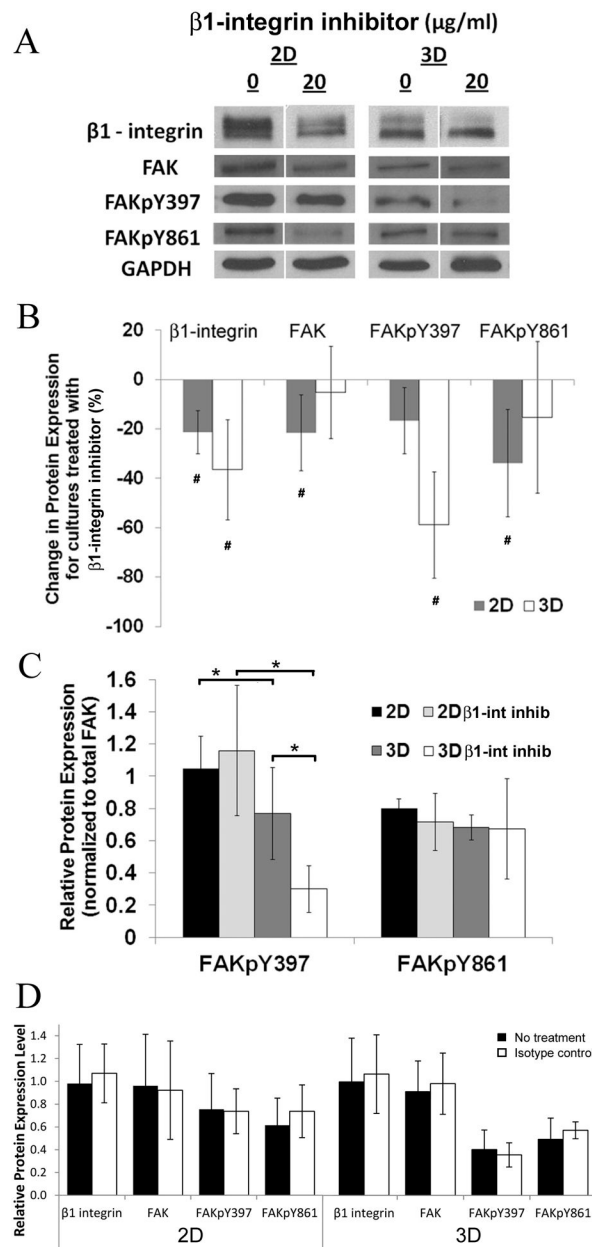
Figure 8. Reduction of phosphorylation of FAK at Y397 in 3D culture is compounded with β1-integrin inhibition

Protein extracts from PC12 cell cultures were analyzed in triplicate by immunoblot (A). Grayscale intensity values were normalized to GAPDH and the change in expression levels in cultures with β1-integrin inhibition by HMβ1-1 (20 μg/ml) were compared to cultures with no treatment (0 μg/ml inhibitor) (B). 2D cultures with β1-integrin inhibition had reduced expression of β1-integrin, FAK and FAKpY861 but no significant change in FAKpY397 (B); when normalized by total FAK expression, phosphorylation at Y397 and Y861 were similar in 2D regardless of β1-integrin inhibition (C). 3D cultures with β1-integrin inhibition had significantly decreased expression of β1-integrin and FAKpY397. There were no significant differences between 0 μg/ml β1-integrin inhibitor and treatment

with an isotype control antibody (D). Bars represent mean ± SEM. Per condition, n=5–7 in A–C, n= 4–5 in D. Symbols denote significant differences (p<0.05): * between culture conditions, # from 0 μg/ml β1-integrin inhibitor.
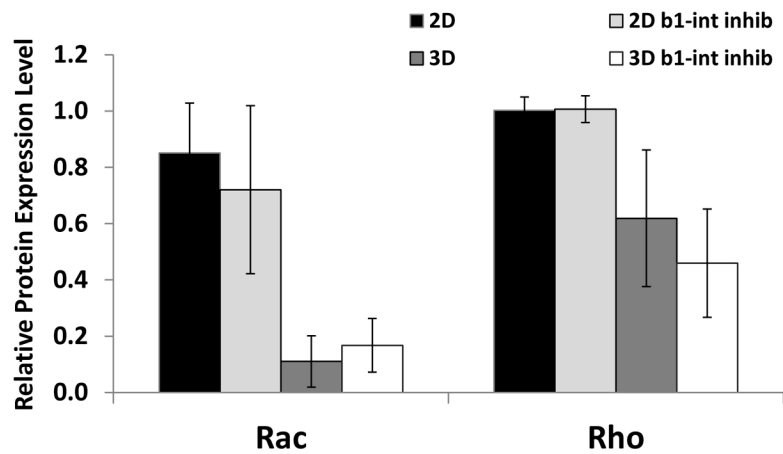
Figure 9. Expression levels of Rac and Rho are reduced in 3D vs 2D cultures regardless of β1-integrin inhibition

Protein extracts from PC12 cell cultures were analyzed in triplicate by immunoblot; expression levels of Rac and Rho were normalized to GAPDH and the change in expression levels in cultures with β1-integrin inhibition by HMβ1-1 (20 μg/ml) were compared to cultures with no treatment (0 μg/ml inhibitor). In 3D cultures, levels of Rac and Rho were reduced by ~80–90% and ~40–50%, respectively, vs 2D cultures regardless of treatment with HMβ1-1. Bars represent mean ± SEM; n=9 per condition.
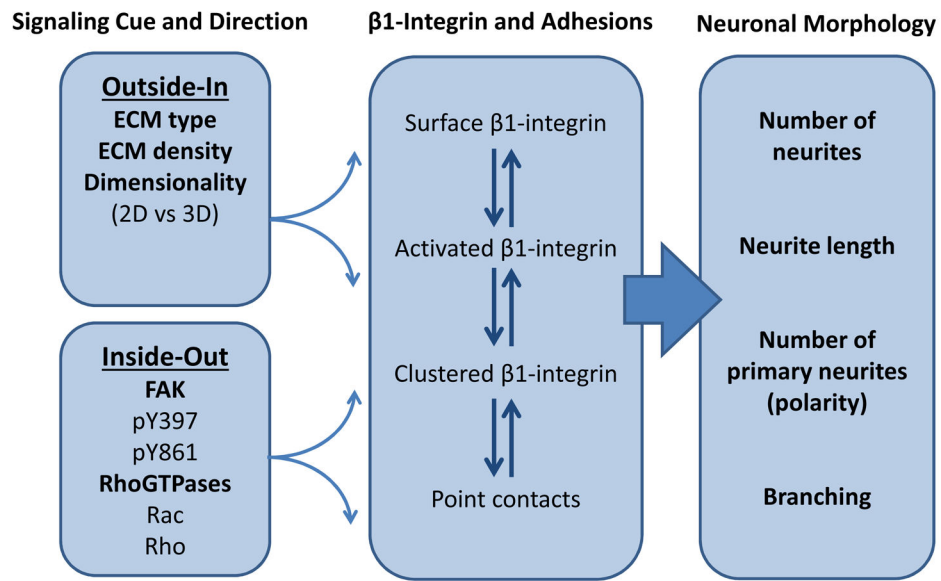
Figure 10. β1-integrin-FAK-GTPase interactions in neuronal morphology
Cues from outside the cell, such as extracellular matrix (type, concentration) are known factors that influence cell response. These outside-in signals via the ECM alter integrin activation, binding, clustering and point contact formation. On the inside of the cell, FAK and RhoGTPase molecules alter cytoskeletal dynamics, including adhesion formation and stabilization. Herein, we propose that β1-integrin-cytoskeletal signaling is also strongly influenced by the presentation of ECM as a 2D substrate vs an encompassing 3D environment.