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Microtopographical features generated by photopolymerization recruit RhoA/ROCK through TRPV1 to direct cell and neurite growth

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Abstract

Cell processes, including growth cones, respond to biophysical cues in their microenvironment to establish functional tissue architecture and intercellular networks. The mechanisms by which cells sense and translate biophysical cues into directed growth are unknown. We used photopolymerization to fabricate methacrylate platforms with patterned microtopographical features that precisely guide neurite growth and Schwann cell alignment. Pharmacologic inhibition of the transient receptor potential cation channel subfamily V member 1 (TRPV1) or reduced expression of TRPV1 by RNAi significantly disrupts neurite guidance by these microtopographical features. Exogenous expression of TRPV1 induces alignment of NIH3T3 fibroblasts that fail to align in the absence of TRPV1, further implicating TRPV1 channels as critical mediators of cellular responses to biophysical cues. Microtopographic features increase RhoA activity in growth cones and in TRPV1-expressing NIH3T3 cells. Further, Rho-associated kinase (ROCK) phosphorylation is elevated in growth cones and neurites on micropatterned surfaces. Inhibition of RhoA/ROCK by pharmacological compounds or reduced expression of either ROCKI or ROCKII isoforms by RNAi abolishes neurite and cell alignment, confirming that RhoA/ROCK signaling mediates neurite and cell alignment to microtopographic features. These studies demonstrate that microtopographical cues recruit TRPV1 channels and downstream signaling pathways, including RhoA and ROCK, to direct neurite and cell growth.

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Introduction

Cells respond to biochemical and biophysical cues in their microcellular niche to establish and maintain functional tissue architecture and intercellular networks. For example, the development of functional neural circuits requires precise targeting of axons and dendrites determined by growth cone responses to diverse biochemical and biophysical gradients and borders. Likewise, precise guidance of *de novo* neurite growth towards the stimulating elements of a neural prosthesis will likely be crucial to overcome spatial signaling resolution limitations of current prosthetics such as the cochlear implant or the developing retinal implant [1]. Accordingly, tissue engineers have developed a variety of biochemically and/or physically patterned substrates to precisely guide neurite growth. Developing a thorough understanding of growth cone responses to user-defined biochemical and biophysical cues represents a primary objective in neural-biomaterial interaction studies. Extracellular matrix, cell surface, and diffusible biochemical guidance cues direct growth cone pathfinding by recruiting well-defined attractive and repulsive intracellular signaling pathways [2, 3]. In addition to these diverse biochemical cues, neurites also respond and orient to micro- and nano-scale biophysical features in their environment [4–8]. However, the mechanisms by which growth cones translate these biophysical cues into directed neurite growth have not been elucidated.

Topography is believed to induce tension in the cytoskeleton and the cell membrane that is subsequently transduced into biochemical signals to modulate cellular responses [9]. Recently, the transient receptor potential (TRP) channels have been shown to play important roles in both biochemical neurite guidance [10–13] and mechanical sensing in sensory neurons [14, 15]. These clues hint to a possible role of TRP channels in neurite guidance by topographic features.

Several intracellular signaling pathways recruited by transmembrane receptors to mediate growth cone responses and axon guidance in response to specific chemotactic guidance molecules have been identified. In particular, Rho-family GTPases, including Rac, Cdc42, and RhoA, function as critical mediators of growth cone responses to biochemical guidance cues. Generally, RhoA promotes repulsion while Rac and Cdc42 promote attraction, though they function in complex and interactive ways [2, 16, 17]. In contrast to the signals recruited to mediate neurite pathfinding in response to chemotactic molecules, the mechanisms by which cells in general, and growth cones in particular, sense and respond to environmental biophysical features remain relatively unknown. We postulate similar signaling pathways might be involved in biophysical neurite guidance.

Spiral ganglion neurons (SGNs) are the target neurons stimulated by cochlear implants (CIs), the most successful neural prosthesis in clinical use. SGNs typically have 1–2 primary neurites in a dissociated culture system. The small number of neurites coupled with the motivation to improve CI performance by precisely guiding neurite growth to specific prosthesis electrodes makes dissociated SGN cultures an excellent model for exploring neurite alignment in response to physical cues.

Here we employed the spatial and temporal control of photopolymerization, i.e. the use of light for polymer formation, to fabricate micropatterned methacrylate platforms that direct neurite growth and Schwann cell alignment. We implicate TRP vanilloid subtype 1 (TRPV1) channels as critical mediators of cell and neurite responses to surface topographical cues. We further identify a signaling node activated by these biophysical cues that involves the Rho GTPase, RhoA, and its downstream effector kinase, Rho associated kinase (ROCK). Our findings provide insights into how cells and growth cones sense and respond to topographical cues that direct cell and neurite growth. These studies also inform efforts to design biomaterials suitable for next generation prosthetics that offer enhanced integration with target tissues.

Materials and methods

Photopolymerization

Micropatterns on methacrylate polymer surfaces were generated using photopolymerization. Specifically, photomasks with alternating transparent or reflective bands were used to selectively block light that initiates the polymerization process, which, in turn, locally alters curing speed directly beneath the mask. Repeating raised and depressed features with microscale amplitudes and periodicities populate the entirety of the substrate surface upon removal of the mask from the final polymer film [4, 5]. In these studies, monomer mixtures of 40 wt% hexyl methacrylate (HMA, Sigma, St. Louis, MO) and 60% wt% 1,6-hexanediol dimethacrylate (HDDMA) were used with 1 wt% of 2,20-dimethoxy-2-phenylacetophenone (DMPA, Ciba, Tarrytown, NY) as the photoinitiator. Channel amplitudes were measured by a Wyko NT 1100 optical profiling system (Veeco, Plainview, NY) as described elsewhere [5]. Polymer substrates were coated with laminin [4]. The neurites of SGNs (Fig. 1D, E), dorsal root ganglion neurons (DRGNs), trigeminal ganglion neurons, and cerebellar granular neurons strongly align to these micropatterned substrates [4, 5].

Assessment of neurite and cell alignment

The entire area of each culture was imaged with the micropatterns positioned parallel to horizontal. Neurite lengths (T_L) and the alignment length (A_L) of the pattern were measured with ImageJ as previously described [4]. The ratio of T_L/A_L was used to assess the extent of neurite alignment to micropatterns (Fig. 1A). A value close to unity implies that the neurite strongly aligns to the pattern [4]. SGSC and NIH3T3 cell alignment was determined as previously described [4] by measuring the angle (θ) made between the pattern direction and the major axis of an ellipse drawn around the cell body (Fig. 1B). An angle close to zero implies strong alignment to the pattern [4].

Primary cultures

Spiral and dorsal root ganglia dissociated cultures were prepared from P1–5 rat pups and maintained as previously described [4, 18]. The cell suspension was plated in glass cylinders on polymer films. Experimental manipulation began 3–6 h later to allow for cell adhesion. H1152 (EMD Millipore, Billerica, MA), Y27632 (EMD Millipore), Rho Activator II (Cytoskeleton, Denver, CO, CN03), C3 transferase (Cytoskeleton, CT04), Ruthenium red (Tocris Bioscience, Bristol, UK), gentamicin (Sigma), SKF96365 (EMD Millipore), cpt-

cAMP (Sigma), 8-Br-cGMP (Sigma), GsTMx-4 (Peptides International, Louisville, KY) or the appropriate control carrier were added to the indicated cultures. Dissociated spiral ganglion Schwann cells (SGSCs) cultures were prepared as previously described [19, 20]. Briefly, dissociated SG cultures were plated on laminin-coated polymer films and maintained Dulbecco's Modified Eagle Medium (Life Technologies) supplemented with N2 (Life Technologies) and insulin (10 µg/ml, Life Technologies) in the absence of neurotrophic factors or serum for at least 96 h prior to experimental manipulation. In the absence of neurotrophic support, nearly all of the SGNs in the culture die within 48 h. By 96 h, >95% of cells were S100-positive SGSCs [19, 20].

Transfection of NIH3T3 cells

NIH3T3 cells were cultured on patterned polymers (2 µm A/50 µm P) and transiently transfected with a TRPV1 expression plasmid kindly provided by Dr. Yuriy Usachev (Iowa City, IA) or an empty vector using Lipofectamine 2000 (Life Sciences). A plasmid expressing green fluorescent protein (GFP) was co-transfected (1:3) to identify transfected cells. TRPV1 expression in GFP-positive cells was verified by immunostaining. For generation of a stable transfected cell line, NIH3T3 cells transfected with TRPV1 were treated with 500 µg/ml G418. After 3 weeks, individual colonies were selected, replated to generate single cell clones, and expanded in G418. TRPV1 expression was assessed by western blot and a clone with robust TRPV1 expression was used for further experiments.

Immunofluorescence

Immunolabeling was performed as previously described [20]. The following primary antibodies were used in various combinations: anti-NF200 (1:400 Sigma), anti-S100 (1:400 Sigma), anti-phosphorylated ROCK2 (pROCK, phospho T249, 1:500, Abcam, Cambridge, MA), and anti-TRPV1 (1:100, Alomone Labs, Israel). Secondary antibodies were conjugated with Alexa Fluor-488, Alexa Fluor-546, Alexa Fluor-568, or Alexa Fluor-647 (Life Sciences).

Measurement of ROCK phosphorylation

Dissociated SGN cultures plated on 7–8 µm amplitude/50 µm periodicity patterned polymers or unpatterned polymers were fixed, immunolabeled with anti-pROCK and anti-NF200 antibodies, and imaged with a Leica TCS SP5 confocal microscope. The laser power and photomultiplier tube gain were set to be in a linear range and the settings were identical for all samples imaged. The growth cone and distal neurite were outlined based on NF200 labeling and the mean pROCK pixel intensity within this area was measured in ImageJ. Background fluorescence within a similarly sized region just outside of the growth cone was subtracted from the value obtained for pROCK fluorescence. The scale used was arbitrary but linear and consistent among all experiments.

In Situ Rho GTPase Activity Assay

The method of labeling active, GTP-bound Rho GTPases *in situ* was performed as described elsewhere [21, 22]. Briefly, dissociated DRGNs were cultured on unpatterned polymers and patterned polymers with 7–8 µm amplitude and 50 µm periodicity for 24 h. PAK-GST

peptide (Human p21 activated kinase PBD, Cytoskeleton) and Rhotekin-RBD-GST peptide (Cytoskeleton) were used to detect Rac/Cdc42 and RhoA activity, respectively. Cultures were incubated with 5 µg/ml PAK-GST or Rhotekin-RBD in 3% BSA in DPBS at 4°C overnight and then fixed. Samples were then incubated with anti-GST (1:1000, Calbiochem) and anti-NF200 antibodies followed by Alexa Fluor 488 and Alexa Fluor 647 conjugated secondary antibodies and with Alexa Fluor 568 phalloidin (1:40, Life Sciences). Growth cones were identified by anti-NF200 and phalloidin labeling. Images of growth cones were captured with a Leica SP5 confocal microscope as above. The mean pixel intensity for PAK-GST and Rhotekin-RBD-GST labeling was determined as above.

Fluorescence resonance energy transfer

The fluorescence resonance energy transfer (FRET)-based probe, Raichu-RhoA, was a gift from Professor Michiyuki Matsuda and is described elsewhere [23, 24]. DRGNs were transfected with Raichu-RhoA plasmid DNA by Lipofectamine 2000 and imaged 48 h later using a Leica DMIRE2 microscope equipped with ET-C/YFP FRET (EX 436nm±10nm, EM 535nm±15nm, Chroma Technology) and ET-CFP (EX 436nm±10nm, EM 480nm±20nm, Chroma Technology) filters using MetaMorph software. The mean value of region adjacent to the growth cone was measured and set as background value. After background subtraction, the YFP/CFP ratio images were generated by MetaMorph software. The mean grey values in growth cones were measured to assess the FRET efficiency.

RhoA activity assay

RhoA activity in protein lysates from NIH3T3 or NIH3T3-TRPV1 cells, plated on patterned or unpatterned substrates, was determined using the G-LISA RhoA Activation Assay Biochem Kit (Cytoskeleton) according to the manufacturer's protocol using a Bio-RAD microplate reader (Model 680XR) plate reader. After subtraction of baseline levels, RhoA activity was standardized to the level in NIH3T3 cells lacking TRPV1 expression plated on unpatterned substrates for each repetition. The assay was performed in duplicate and repeated three times.

RNA interference

Dissociated SGNs were transfected with 100 nM DsiRNAs for ROCK1 (Integrated DNA Technologies, IDT, Coralville, IA, RNC.RNAI.N031098.12.1, CDS/18), ROCK2 (IDT, RNC.RNAI.N013022.12.4, CDS/27–28), 100 nM DsiRNAs for TRPV1 (IDT, RNC.RNAI.N031982.12.1, CDS/13 and RNC.RNAI.N031982.12.2, CDS-3'UTR/16), or DS Scrambled Neg (IDT) as negative control by Lipofectamine RNAiMAX (Life Sciences) 5–6 h after plating. Cultures were fixed 60 h after transfection.

Relative ROCK1/2 and TRPV1 expression levels were assessed by real-time reverse transcriptase polymerase chain reaction (RT-PCR) using the Applied Biosystems 7500 Real-Time PCR System with TaqMan® Gene Expression Cells-to-CT™ Kit (Life Technologies). Relative standard curve method was used to detect relative mRNA expression levels with TaqMan® Gene Expression Assay for ROCK1 (Life Technologies, Rn00579490_m1), ROCK2 (Life Technologies, Rn00564633_m1), TRPV1 (Life Technologies, Rn00583117_m1), or 18s as endogenous control (Life Technologies, Rn03928990_g1).

Samples transfected with DS Scrambled Neg was set as a calibrator. Expression experiments were performed in duplicate and repeated at least four times.

Results

TRPV1 channels contribute to neurite and cell guidance by topographical features

To explore the molecular events that underlie neurite responses to microtopographical features, we used the temporal and spatial control afforded by photopolymerization to generate methacrylate polymer films with micropatterns consisting of parallel lines of ridges and grooves (Fig. 2A–C). For these studies ridge amplitude ranges from 1–8 μm and is controlled by manipulating the light exposure time, as previously described [4, 5]. Periodicity of the pattern is fixed at 50 μm determined by spacing of the photomask. As demonstrated by angled cross-section SEM images, the photopolymerization process produces micropatterns with gradual sloping transitions between ridges and grooves (Fig. 2C). These gradual transitions are critical for the mechanistic studies described here as they do not physically constrain the neurite processes as often seen in patterns prepared by photolithography. Without such constraints, the neurite response and alignment can change significantly based on different topographical cues and following manipulation of receptor expression or intracellular signaling events. As previously described, SGN neurites strongly align to these micropatterns with higher channel depth and lower periodicity [4, 5].

We hypothesized that TRP channels, which are sensitive to biophysical stimuli and have been shown to regulate neurite pathfinding, contribute to the ability of growth cones to respond to microtopographical features. Thus, the effect of several TRP channel blockers on SGN neurite alignment to micropatterns was explored. SKF96365 (15 μM), a general inhibitor of TRP channels [10] significantly decreases SGN neurite alignment to physical microfeatures (Fig. 3A, C, F). Moreover, ruthenium Red (2 μM) and gentamicin (200 μM), which also block TRPV channels [25] also significantly decrease neurite alignment (Fig. 3A, C–E). However, application of GsMTx-4 (15 μM), a peptide inhibitor of mechanosensitive ion channels [26] has no significant effect on neurite alignment (Fig. 3B).

Based on the pharmacological inhibitor results, we focused on the TRPV1 channel as a potential mediator of neurite responses to biophysical microfeatures. Immunofluorescence confirmed TRPV1 expression in SGN growth cones (Fig. 4A–C). We used RNA interference (RNAi) to reduce the expression of TRPV1 in SGNs. Transfection of spiral ganglion cultures with dicer-substrate RNA oligonucleotides (DsiRNA) targeting TRPV1 significantly reduces mRNA expression as determined by real-time RT-PCR compared to cultures transfected with a scrambled, non-targeted oligonucleotide (Fig. 4D). Knockdown of TRPV1 significantly decreases SGN neurite alignment to micropatterns (Fig. 4E–G) implicating that TRPV1 channels contribute to neurite guidance by topographical features.

To further establish the role of TRPV1 in topographic guidance, we asked if induced expression of TRPV1 would sensitize cells to micropatterned substrates. We have previously shown that fibroblasts fail to align to the photopolymerized micropatterns used here [4]. NIH3T3 cells represent a fibroblast derived cell line that doesn't normally express TRPV1 [27]. We transiently co-transfected NIH3T3 cells with an expression plasmid

encoding the TRPV1 gene or an empty plasmid along with an expression plasmid for green fluorescent protein (GFP). The alignment of TRPV1-transfected NIH3T3 cells to the micropattern is significantly higher than NIH3T3 cells transfected with empty plasmid (Fig. 4H–J). Thus, expression of TRPV1 allows cells to become responsive to topographic cues. Taken together, these data demonstrate that TRPV1 channels contribute to neurite and cell alignment to topographical features.

Topographical features activate RhoA to mediate neurite and cell alignment

Next, we explored the possibility that Rho-GTPase family members contribute to neurite pathfinding by topographical surface features. Because DRGN growth cones respond well and align to these micropatterned surfaces [4, 5] and are more readily visualized than SGN growth cones in dissociated cultures, we used DRGN cultures to image activity of Rho family GTPases in response to topographic cues. We measured Rho and Cdc42/Rac activity in growth cones of DRGNs on micropatterned substrates by *in situ* Rho GTPase activity assays [21, 22]. Cultures were labeled with Rho-binding domain of Rhotekin (RBD-) or Rac/Cdc42-binding domain of Pak (PBD) - glutathione S-transferase (GST) tagged peptides followed by detection with anti-GST antibodies and fluorescent secondary antibodies. We compared fluorescence intensity in growth cones that were engaged with the sloping transition (edge) of the micropattern with growth cones that remained in the relatively flat region of the pattern. The average RBD-GST labeling is significantly higher in growth cones interacting with the edge of the pattern compared with growth cones in the flat regions (Fig. 5A). The extent of RBD-GST labeling of growth cones interacting with the edge is comparable to growth cones in flat regions in cultures treated with a RhoA activator (RAII, 1 $\mu\text{g/ml}$). Conversely, PBD-GST labeling is significantly higher in growth cones remaining in the flat regions of the pattern compared to those at the pattern edge (Fig. 5B). These results suggest that interaction with pattern edges increases RhoA activity and decreases Cdc42/Rac activity. We also used a FRET based biosensor [23, 24] transfected into DRGNs to compare RhoA activity in growth cones on micropatterned and unpatterned substrates. Similar to the results of *in situ* Rho GTPase activity assays, RhoA activity is significantly higher in growth cones on patterned substrates compared to unpatterned substrates (Fig. 5C–E).

To investigate the extent to which RhoA activity influences neurite guidance by micropatterns, we treated SGN cultures on micropatterned substrates with a RhoA activator or inhibitor. Neurite alignment on micropatterns depends on channel amplitude for a given feature spacing; channels with shallow amplitudes are less effective at inducing neurite alignment [4, 5]. Treatment of cultures with the RhoA activator, RAI (1, 2 $\mu\text{g/ml}$), increases neurite alignment on patterns of 1 μm channel depth (Fig. 6A–C). Conversely, treatment of cultures with RhoA inhibitor, C3 transferase (C3T, 1, 2 $\mu\text{g/ml}$), significantly reduces neurite alignment to micropatterns with 3 μm channel amplitude (Fig. 6D–F). These results demonstrate that RhoA activity substantially contributes to growth cone path-finding in response to biophysical cues of substrate micropatterns. Taken together these results suggest that interaction with pattern edges alters the activity of Rho-family GTPases in the growth cone to direct neurite growth.

Activation of Rho-associated kinases (ROCK) by topographical features is required for neurite guidance

RhoA functions, at least in part, by activating Rho-associated kinases (ROCK). Accordingly, ROCK activity in SGN growth cones and distal neurites maintained on unpatterned or micropatterned substrates was assessed by quantifying immunofluorescence intensity in cultures immunostained with an antibody that detects phosphorylated ROCK (pROCK). pROCK immunofluorescence intensity is significantly higher in SGN distal neurites and growth cones on micropatterned substrates compared to those on unpatterned surfaces (Fig. 7A–C) suggesting activation of ROCK on patterned substrates. To explore the contribution of ROCK activation in topographic neurite guidance, cultures plated on micropatterned substrates were treated with H1152, a membrane-permeable ROCK inhibitor. H1152 (0.1, 1 μ M) significantly reduces neurite alignment (Fig. 7D, F–H). Likewise, Y27632 (10, 100 μ M), another small molecule ROCK inhibitor, significantly reduces SGN neurite alignment (Fig. 7E, F–G, I). H1152 (1 μ M) similarly reduces spiral ganglion Schwann cell (SGSC) alignment to micropatterns (Fig. 8A–C). To confirm that ROCK activity is required for neurite alignment to topographical cues, RNA interference was used to reduce the expression of ROCK1 or ROCK2 isoforms in SGNs. Transfection of spiral ganglion cultures with dicer-substrate RNA oligonucleotides (DsiRNA) targeting ROCK1 or ROCK2 significantly reduces mRNA expression as determined by real-time RT-PCR compared to cultures transfected with a scrambled, non-targeted oligonucleotide (Fig. 7J). Transfection with either ROCK1 or ROCK2 targeted oligonucleotides significantly reduces neurite alignment (Fig. 7K–N), further confirming that RhoA/ROCK signaling mediates neurite alignment to topographic cues. ROCK1 and ROCK2 each appear to be necessary for this behavior as knock-down of either isoform alone substantially reduces neurite alignment.

Activity of RhoA and ROCK is required in TRPV1-dependent cellular alignment to microtopographical features

To further investigate the relationship between TRPV1 and RhoA/ROCK signaling in the process of cellular alignment to topographical features, we created a NIH3T3 cell line stably transfected with TRPV1 (Fig. 9A). RhoA activity was compared in TRPV1-positive and TRPV1-negative NIH3T3 cells plated on smooth or micropatterned surfaces. RhoA activity is significantly higher in TRPV1-expressing NIH3T3 cells plated on micropatterned polymer films compared to cells plated on unpatterned surfaces (Fig. 9B). Thus, TRPV1 expression facilitates RhoA activation by micropatterned surfaces. As was the case with NIH3T3 cells transiently transfected with the TRPV1 expression plasmid, alignment of TRPV1 stably transfected NIH3T3 cells to the micropattern is significantly higher compared to NIH3T3 cells lacking TRPV1 expression (Fig. 9C–E). The ROCK inhibitor, H1152 (0.1 μ M), disrupts the alignment TRPV1-expressing NIH3T3 cells to micropatterned surfaces (Fig. 9E), confirming the requirement of RhoA/ROCK activity in mediating TRPV1-dependent cellular alignment to microtopographical features.

Discussion

During development and regeneration, cells, including neurons, respond to biophysical cues. Such biophysical forces include topographical features, stress and strain, vibration, and

mechanical loading. These forces regulate a variety of cellular processes including differentiation, alignment, extracellular matrix deposition, and gene expression to determine tissue architecture, remodeling, and intercellular communication [28]. Likewise, tissue engineers look to modification of physical micro- and nano-features in artificial platforms to achieve desired cellular responses and enhance integration with native tissue [28]. Here we utilized micropatterned substrates created by photopolymerization to identify channels and intracellular signals responsible for neurite and cellular alignment to microtopographical features.

CIs restore auditory perception, yet most recipients perform poorly with complex auditory tasks such as hearing in noise or music appreciation. Current CIs are placed 250 – 1000 μm away from the SGNs [29]. This distance between the electrodes and SGNs results in non-specific stimulation of SGNs over a wide frequency range which limits the number of functionally independent channels. CI users are, therefore, provided with poor tonal information due to limitations in spatial signaling specificity at the prosthesis-user interface. All neural prostheses face similar signal resolution challenges due to limited effective channels. To this end, neural tissue engineers have sought methods to enhance the prosthesis-tissue interface [30, 31].

Modification of prosthesis surfaces allows for modulation of biological responses without altering material bulk properties [32]. In particular nano- or micro-scale modification of surface topographical features modulates cell adhesion and proliferation and directs cell growth. For example, microstructures in platinum or silicone, commonly used materials for CI electrode arrays, enhance anti-adhesive and anti-proliferative properties of the surface against fibroblasts and direct neurite outgrowth [30, 31, 33]. Similarly, micro- and nanochannels created by photopolymerization strongly induce alignment of SGN neurites and SGSCs [4, 5]. Interestingly, fibroblasts fail to respond to these topographical cues, which may allow for reduced electrode encapsulation and lower electrode resistance [5, 33, 34]. SGN neurite and SGSC responses to these micro- and nano-channels depend on feature amplitude, periodicity, slope, and complexity; all of which can be finely tuned to modulate contact guidance [5, 35, 36]. Despite abundant evidence that cells respond to surface topographical features, the mechanisms by which cells sense and respond to these biophysical cues have not been elucidated. The micropatterns in this study were generated by photopolymerization and resulted in gradual, sloping transitions between the ridges and grooves (Figure 1C). These features differ from the sharp contrast features produced by lithographic methods [37] and are critical to our studies. Cells and growth cones are responsive to, yet not physically constrained by, these gradual transitions allowing for mechanistic experiments to enhance or inhibit the effects of the topographical features. Furthermore, photopolymerization allows for rapid, single-step creation of topographic features and enables precise tuning of pattern feature dimensions. In this work, feature amplitude was varied to increase or decrease the influence of the pattern on cell alignment. Modulating feature depth in this manner allowed us to explore the use of biochemical signals predicted to either disrupt or enhance alignment without masking their influence on neurite and cell contact guidance due to dominance by stark biophysical cues.

Our data demonstrate that TRPV1 channels mediate neurite and cell growth in response to microtopographical cues by contributing to the activation of RhoA and ROCK. TRP inhibitors and DsiRNA knock-down of TRPV1 reduced SGN neurite alignment to the micropatterns while exogenous expression of TRPV1 expression in NIH3T3 cells induced alignment to the micropatterns. TRPV1 is a transmembrane nonselective cation channel that detects various physical and chemical stimuli, such as thermal, osmotic, and/or acidic stimuli, as well as endogenous and exogenous agonists including endocannabinoid lipids and capsaicin [25, 38, 39]. TRPV1 is required for mechanically evoked purinergic signaling in bladder urothelial cells [40] suggesting that it is also responsive to mechanical stimuli. Recently, TRPV1 channels were found to respond to plasma membrane mechanical deformation in Merkel cells, which act as mechanoelectric transducers [41]. These recent data, in line with our study, suggest that TRPV1 channels are responsive to mechanical stimuli.

The biophysical events leading to activation of mechanosensitive channels have not been firmly established. In the tether model, ankyrin repeat domains connect the cytoskeleton, membrane channels and extracellular matrix, and function as gating springs [42, 43]. The N-terminal region of TRPV1 contains six ankyrin repeats [44]. This connection between TRPV1 and cytoskeleton might facilitate TRPV1 to sense the membrane and cytoskeletal deformation of cytoskeleton and act as a mechano-gated channel. However, the exact molecular mechanisms linking TRP channel gating to biophysical cues remains to be determined.

Localization of TRPV1 expression in the growth cone in DRGNs and, as demonstrated here, SGNs, suggests a role in neurite guidance [45]. TRPV1 binds dynamically with intact microtubules to modulate their assembly/disassembly and cytoskeletal remodeling [46]. In DRGNs and F11 cells, TRPV1 activation by application of resiniferatoxin (RTX) or capsaicin results in rapid disassembly of dynamic microtubules, causing growth cone retraction and collapse and formation of varicosities along neurites [45, 47]. Interestingly, TRPC channels have also been implicated in neurite guidance [10, 11]. Further, TRPV1 is present in both pre- and post-synaptic spines of cortical neurons and regulates the morphology and the function of these structures [48]. Apparently, TRPV1 contributes to neuronal pathfinding and network formation in response to both biochemical and, as demonstrated here, biophysical cues, in addition to its well-described role in detection of noxious stimuli and pain signaling.

Our results demonstrate that RhoA and ROCK are activated by surface microtopographical features and that they mediate neurite and cell growth in response to surface microtopographical cues. Rho-family GTPases, including Rac, Cdc42, and RhoA, are critical mediators of growth cone responses to a variety of biochemical guidance cues. Generally, RhoA is activated by chemorepulsive molecules including netrins, semaphorins, ephrins, Slits and central myelin components and is known to mediate growth cone collapse and repulsion turning [2, 49, 50]. Conversely, activation of Rac and Cdc42 generally promotes neurite extension and attractive turning [50]. Our data demonstrate that interaction with pattern edges alters the activity of Rho-family GTPases in the growth cone to direct neurite growth. *In situ* Rho GTPase assays, FRET probes, and RhoA assays each indicate

that RhoA is activated in growth cones and TRPV1-expressing NIH3T3 cells on micropatterned surfaces. Interestingly, mechanical stretch in mesangial cells leads to RhoA activation, which is essential for stretch-induced production of the matrix protein fibronectin; events that are prevented by cGMP [51]. Meanwhile it appears that Rac/Cdc42 are active in regions of the growth cone that remain in the grooves away from the pattern edges. Inhibition of RhoA disrupts neurite alignment to micropatterned surfaces whereas RhoA activators enhance alignment of neurites maintained on micropatterns with low feature amplitudes. Thus microtopographical features, similar to chemorepulsive cues, activate RhoA to direct neurite pathfinding.

ROCK is a principal effector of RhoA that mediates growth cone turning or collapse in response to chemorepulsive cues. Here we find that ROCK is phosphorylated in growth cones and neurites maintained on micropatterned surfaces, consistent with increased RhoA activity on micropatterned surfaces. Further, inhibition of ROCK disrupts neurite, SGSC, and TRPV1-positive NIH3T3 cell alignment to micropatterns. Interestingly our data suggest that ROCK1 and ROCK2 isoforms independently contribute to neurite responses to microtopographical features as siRNA-mediated knock-down of either isoform disrupts alignment to the micropattern. These observations are consistent with a model of topographical cues activating RhoA/ROCK to elicit a repulsive turning of the growth cone away from the pattern edge. The effects of activated RhoA and ROCK on growth cone collapse and neurite retraction are linked to reorganization of both the actin cytoskeleton and microtubules and intermediate filaments including vimentin [2]. For example, ROCK phosphorylates and activates myosin light chain (MLC) and LIM kinases to modulate actin reorganization and neurite extension [2]. Recent data suggest that ROCK functions to enhance focal adhesion maturation, actin polymerization and focal adhesion kinase phosphorylation thereby influencing cellular response to surface topographical features [52].

The mechanisms by which TRPV1 activates RhoA/ROCK signaling to modulate neurite guidance by microtopographical cues has not been established. TRPV1 activation leads to calcium influx and cytoplasmic calcium mediates growth cone turning and axon guidance, at least in part, by modulating Rho family GTPase activity [53]. Further, RhoA disrupts actin cytoskeleton and microtubule assembly in the growth cone to mediate growth cone retraction [54]. TRPV1 has likewise been shown to disrupt microtubule assembly in the growth cone leading to collapse [45,47]. We therefore speculate that activation of TRPV1 results in calcium transients that modulate Rho-family GTPase activity and thereby alter actin and microtubule dynamics in the growth cone to direct neurite growth. Future experiments will help clarify whether calcium signaling provides such a mechanistic link between TRPV1 activity and RhoA signaling.

Conclusions

Our data point to a model in which mechanical deformation of the cell membrane by interaction with micropattern edges activates TRPV1 channels and initiates a signaling cascade involving RhoA/ROCK to induce neurite and cell alignment. These responses to biophysical cues involve similar intracellular signaling mechanisms as those recruited by

chemorepulsive molecules indicating that biophysical and biochemical cues can recruit overlapping intracellular signaling cascades to mediate cell alignment and growth.

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Figure 1.

Method of measuring neurite and cell alignment to micropatterned surfaces. **A.** The overall length of the longest neurite extending from the cell body and the end-to-end distance of the micropattern are measured in using the segmented line tool in ImageJ. Neurite alignment is defined as the ratio of total neurite length (T_L) to aligned length (A_L) i.e. the end-to-end distance. Values close to unity imply strong alignment to the pattern. **B.** Cell alignment is determined by determining the angle (θ) of an ellipse drawn around the long axis of the cell to the horizontal using ImageJ.

Figure 2.

Photopolymerized micropatterns direct neurite growth. **A.** Schematic demonstrating micropattern fabrication via photopolymerization. Pre-polymer mixture of HMA, HDDMA and photoinitiator is UV cured beneath 50- μ m periodicity glass-chrome Ronchi rule photomasks for patterned samples (upper row), or with glass microscope slides for unpatterned samples (lower row). Pattern feature height is tuned by modulating total exposure time. **B-C.** Representative SEM micrographs of top-down (**B**) and angled cross-section (**C**) views of micropatterned substrates. **D-E.** SGN neurites grow in random

directions on unpatterned substrates (D) but orient to the direction of the pattern (horizontal) on micropatterned surfaces (E).

Figure 3. Inhibition of TRP channels decreases neurite alignment to micropatterned surfaces. **A, C-F.** SKF96365 (15 μM , $n = 75$), Ruthenium Red (2 μM , $n = 105$) and gentamicin (200 μM , $n = 110$) each significantly decrease SGN neurite alignment on micropatterned substrates compared to control cultures ($n = 99$). **B.** GsMTx-4 (15 μM , $n = 57$), a peptide inhibitor of mechanosensitive ion channels, has no significant effect on neurite alignment ($n = 64$ in control group). SGN neurite alignment to micropatterns is similar for cultures maintained with or without GsMTx-4. * $p < 0.05$, Kruskal-Wallis one way ANOVA on ranks followed

by Dunn's method (A). n value in this figures are the number of neurites scored in each condition. Data represent means $\pm$ SEM.

Figure 4.

TRPV1 mediates neurite and NIH3T3 cell alignment to micropatterned surfaces. **A-C.** Expression of TRPV1 in SGN growth cones immunostained with anti-NF200 (A, green) and anti-TRPV1 (B, red) antibodies with combined labeling (C). **D.** Relative TRPV1 gene expression determined by real-time RT-PCR in cultures transfected with scrambled or TRPV1-targeted DsiRNA oligonucleotides. **E.** Transfection of cultures with TRPV1-targeted DsiRNA oligonucleotides significantly decreases SGN neurite alignment on micropatterned substrates compared to transfection of a scrambled oligonucleotide ($n = 97$

in each group). **F, G.** Images of SGN neurite alignment in cultures treated with scrambled (F) or TRPV1-targeted DsiRNA oligonucleotides (G). **H.** Transfection of NIH3T3 cells with a TRPV1 expression vector ($n = 120$) significantly increases 3T3 alignment to micropatterns compared to cells transfected with an empty plasmid ($n = 155$). **I, J.** Images of NIH3T3 cells cultured on micropatterns and co-transfected with empty and GFP expression plasmids (I) or TRPV1 and GFP expression plasmids (J). * $p < 0.05$, Student t-test (D), or Mann-Whitney test (E, H). Data represent means $\pm$ SEM.

Figure 5.

Micropatterned surfaces activate RhoA to mediate neurite alignment. **A-B.** Interaction of DRGN growth cones with pattern edges increases RhoA activity and decreases Cdc42/Rac activity detected by *in situ* Rho GTPase activity assay. The average RBD-GST labeling (**A**), indicating RhoA activity, is significantly higher in growth cones interacting with sloping transitions (edge) of the pattern ($n = 25$) compared with growth cones in relatively flat regions ($n = 23$). The extent of RBD-GST labeling of growth cones interacting with the edge ($n = 25$) is comparable to growth cones in flat regions that are in cultures treated with a RhoA activator, RAI ($1 \mu\text{g/ml}$, $n = 12$). Conversely, PBD-GST labeling (**B**), indicating Cdc42/Rac activity, is higher in growth cones remaining in the flat regions of the pattern ($n = 36$) compared to those at the pattern edge ($n = 49$). **C-E.** RhoA activity detected by a

FRET based biosensor is significantly higher in growth cones on patterned substrates (**D**, $n = 26$) compared to unpatterned controls (**C**, $n = 25$). $*p < 0.05$, one way ANOVA with post hoc Tukey (A), or Student t-test (B,E). Data represent means $\pm$ SEM.

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Figure 6.

RhoA activity facilitates neurite alignment to micropatterned surfaces. **A.** Treatment of cultures with RAII (1, 2 µg/ml) significantly increases neurite alignment on patterns of 1 µm channel depth compared to cultures without RAII ($n = 100$ in each group). **B-C.**

Representative images of cultures in the absence (**B**) or presence (**C**) of RAII and immunostained with anti-neurofilament 200 (NF200). **D.** Treatment of cultures with Rho inhibitor C3 Transferase (C3T, 1, 2 µg/ml) significantly reduces neurite alignment to micropatterns of 2–3 µm channel depth compared to cultures without C3T ($n = 100$ in each group). **E-F.** Images of cultures in the absence (**E**) or presence of C3T (**F**). * $p < 0.05$, Kruskal-Wallis one way ANOVA on ranks followed by Dunn's method (A, D). Data represent means $\pm$ SEM.

Figure 7.

ROCK activity facilitates neurite alignment to micropatterned surfaces. **A-C.** pROCK immunofluorescence intensity is significantly higher in SGN distal neurites and growth cones on micropatterned substrates (C) compared to those on unpatterned surfaces (B) ($n = 42$ in each group). **D-I.** SGN neurite alignment on micropatterned substrates is significantly reduced in cultures treated with ROCK inhibitors H1152 (0.1, $n = 250$; 1 μM , $n = 178$) or Y27632 (10, $n = 149$; 100 μM , $n = 129$) compared to control ($n = 120$ and 132, respectively). **J.** Transfection of spiral ganglion cultures with DsiRNA oligonucleotides targeting ROCK1 or ROCK2 significantly reduces mRNA expression as determined by real-time RT-PCR compared to cultures transfected with a scrambled, non-targeted oligonucleotide. **K-N.** Neurite alignment in cultures transfected with ROCK1 DsiRNA ($n = 81$) or ROCK2 DsiRNA ($n = 83$) is significantly decreased compared to alignment when treated with a scrambled oligonucleotide ($n = 80$) on micropatterned substrates. * $p < 0.05$, Student t-test (A, J) or Kruskal-Wallis one way ANOVA on ranks followed by Dunn's method (D, E, K). Data represent means $\pm$ SEM.

Figure 8.

Inhibition of ROCK disrupts spiral ganglion Schwann cells (SGSCs) alignment to micropatterned surfaces. **A.** Treatment of SGSC cultures with ROCK inhibitor H1152 significantly reduces SGSC alignment to micropatterns compared to SGSCs maintained in the absence of H1152 ($n = 50$ in each group). SGSC alignment in cultures treated with H1152 and grown on micropatterned surfaces is nearly random, comparable to SGSC grown on unpatterned surfaces. **B-C.** Images of SGSC alignment in cultures plated on micropatterned surfaces and maintained in the absence (B) or presence (C) of H1152. $*p < 0.05$, Kruskal-Wallis one way ANOVA on ranks followed by Dunn's method. Data represent means $\pm$ SEM.

Figure 9.

TRPV1 transfection of 3T3 cells significantly increases 3T3 alignment to micropatterns by facilitating RhoA/ROCK activity. **A**. Western blot of protein lysates from NIH3T3 and NIH3T3-TRPV1 cells probed with anti-TRPV1 antibody. The blots were stripped and reprobed with anti- β -actin antibody to verify equal protein loading. **B**. RhoA activity in NIH3T3 cells on patterned (Pat) substrates or NIH3T3-TRPV1 cells on unpatterned (Unpat) or patterned substrates normalized to 3T3 cells on unpatterned substrates. * $p < 0.05$, one way ANOVA followed by Tukey post hoc test. **C,D**. NIH3T3 or NIH3T3-TRPV1 cells on patterned substrates and labeled with Alexa Fluor 568 phalloidin. **E**. Alignment of NIH3T3

or NIH3T3-TRPV1 cells on unpatterned or patterned substrates in the presence or absence of H1152 (0.1 μ M) ($n = 100$ in each group). ** $p < 0.05$, Kruskal-Wallis one way ANOVA on ranks followed by Dunn's method compared to all other conditions. Data represent means $\pm$ SEM.

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