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Localized bimodal response of neurite extensions and structural proteins in dorsal-root ganglion neurons with controlled polydimethylsiloxane substrate stiffness

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ARTICLE INFO

Article history:
Accepted 8 December 2010

Keywords:
Polydimethylsiloxane
Actin
Dorsal root ganglia
Elasticity
Cell adhesion

ABSTRACT

Neuron morphology, adhesion, axon or neurite outgrowth, and neuron–glial cell interactions are influenced by cues from growth factors as well as extracellular matrix molecules linked to the structural scaffolding. Both chemical and physical events affect neural response, as cells respond to soluble, and insoluble chemical cues and neuron–material interface cues including the mechanical properties of the substrate itself. Both types of cues directly influence neural behaviors and the array of structural elements including microtubules, actin filaments, neurofilaments, and filament-associated proteins. In this manuscript, we examine the relationship between the physical substrate environment and neuron morphology in primary dorsal-root ganglia (DRG) neuron co-cultures including glial cells and DRG neurons. By culturing DRGs on polydimethylsiloxane (PDMS) substrates of varying elasticity we found that structural protein responses, neurite extensions, and protein distributions varied between substrates, indicating a physical relationship between cultured surface rigidity and cellular morphology. In addition, we found higher cell densities for both DRG neurons and glial cells grown on semi-rigid polydimethylsiloxane substrates (PDMS ratio of base to curing agent of 35:1) than found on more rigid (15:1) or more flexible (50:1) substrates, indicating a localized bimodal response within a very small difference of elasticity on PDMS. These results imply that physiological relevancy may be best discovered by examining and replicating physical parameters such as tissue stiffness. This work is important in fields including biomaterials, neuron–material interactions, and neuroscience.

1. Introduction

Neuroscientists have probed a variety of complex neural responses by dictating input stimulation including the control of the chemical, electrical, and mechanical environments (Weiss, 1934; Lo et al., 2000). Environmental control and stimulation studies have been conducted for a wide range of physiologically important neural cells including, neurons, axons, and nerve fibers. Less studied are efforts to control the physical environment through manipulation of the material interface, though this has serious implications, as neurons can sense and respond to a variety of external stimuli including mechanical stretching, compression, touch, and pressure. The physical environment is a critical component of the nervous system's ability to convert mechanical inputs to electrical signals (Lin et al., 2009; Drew et al., 2004). Substrate interactions and stiffness in particular has recently gained much attention as a means of influencing cell responses in areas including mechanobiology, motility, and stem cell differentiation (Chou et al., 2009; Yeung et al., 2005; Engler et al., 2004; Leipzig and Shoichet, 2009). This link to substrate stiffness is very important for neurons, as the peripheral nervous system is supported by tissue that is much softer than conventional glass coverslips, the standard for cell culture and neural response studies. Furthermore, within neural cells, the physical environment includes a complex set of interactions between extracellular links and intracellular structures such as cytoskeleton. The cytoskeleton plays an obvious role in terms of structure, but it is also implicitly linked to intricate processes including neurite outgrowth and calcium signaling. To date, relatively few studies have examined neurite outgrowth and the relationship to cytoskeletally linked molecules in neurons with respect to the mechanics of substrate interactions. Using a more physiologically relevant system for examining cell behavior would provide significant advantages over many conventional approaches.

When examining cell–substrate questions, it is important to note that requisite advances in the fields of material science have been made that permit the successful interfacing of polymer substrates with

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0021-9290/$ - see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.jbiomech.2010.12.006

a variety of cell types from cardiac cells to neurons (Jacot et al., 2008; Teixeira et al., 2009). In particular, significant advancements in the development and use of elastomeric polymers have provided greater control over the cellular mechano-physical milieu. For example, culturing mammalian cells on hydrogels of adjustable stiffness has been shown to alter extent of spreading and rates of motility (Pelham and Wang, 1997; Wong et al., 2003; Kidoaki and Matsuda, 2008). In addition, protein-based extracellular matrix gels such as fibrin, collagen, or a mixture of collagen and laminin have been used to fabricate two- or three-dimensional cell culture substrates with adjustable stiffness (Byfield et al., 2009; Breuls et al., 2009). Furthermore, synthetic polymeric materials, such as polyacrylamide (PA), have been used to examine compliance dependence of cytoskeletally regulated activities in living cells (Peyton and Putnam, 2005). These diverse elastomeric materials have also been successfully employed in the area of neuroscience. Studies using PA gels revealed that lower substrate flexibility allowed cells to more adequately adhere to Glyoxyl agarose (GA) gels and induced favorable neurite branching (Cullen et al., 2007). Also, neurons grown on softer substrates showed a greater (3 ×) degree of branching compared to those grown on stiffer gels lacking extracellular matrix molecules (Flanagan et al., 2002). Further, Balgude et al. (2001) reported an inverse correlation between the rate of neurite extension and the stiffness in agarose gel substrates. These approaches have also provided experimentally measurable benefits *in vivo*. For example, when soft hydrogels were implanted into an injured feline central nervous system, neuronal growth and a reduction in the glial scar were observed (Woerly et al., 2004).

PDMS is a widely used elastomeric material that has been implemented in a variety of cell-based applications. PDMS also provides an advantage in regards to controlling the physical environment as the elastic modulus can be modified in the kPa range by altering the weight ratio of base to curing agent during fabrication. This engineered system has been used to study many cell responses including examinations of cell spreading and cytoskeletal morphologies in fibroblasts (Chou et al., 2009), and investigations of stretch-activated action potential in DRG neurons under mechanical stimulation (Lin et al., 2009). Here, we used a ligand-coated PDMS system to more closely mimic a physiological environment and to probe *in vitro* structural morphologies in DRG neurons. We used this elastomeric approach to first understand the effect of substrate stiffness on cell survival including DRG neurons and glial cells. We then examined the distribution of structural proteins including microtubules, actin filaments, and microtubule-associated protein 2 (MAP2) with respect to controlled Young's modulus (e.g., high stiffness in glass versus lower stiffness in PDMS). We believe these results will provide a better understanding of DRG neuron responses in a more realistic physiological environment, as they relate to substrate elasticity, and could be important in a wide range of applications including cell–material interactions, neural tissue engineering, and neural regeneration.

2. Materials and methods

2.1. Polydimethylsiloxane

Polydimethylsiloxane (PDMS, Sylgard 184) was purchased from Dow Corning Corp (Midland, MI, USA), and the ratios of the elastomeric base to curing agent were altered to control the Young's modulus. For example, with a ratio of 35:1, the elastic modulus of PDMS is approximately 88 kPa (Lin et al., 2009). We used this capacity to produce PDMS substrates with elasticities that were more physiologically relevant than conventional glass, which has an elastic modulus of ~70 GPa, and prepared PDMS substrates with different base to curing agent ratios at room temperature.

2.2. Dorsal-root ganglion primary culture

Twenty-four CD1 mice of 8–12 weeks old were used to obtain the DRG-glial cell primary co-cultures. The usage of these animals was approved by the Institute of Animal Care and Use Committee of China Medical University, Taiwan, following the Guide for the use of Laboratory Animals (National Academy Press). Mice were euthanized using CO_2 to minimize suffering. DRGs and glial cells were acutely dissociated and processed as described previously (Lin et al., 2008). The DRG neurons were then seeded on either glass coverslips or PDMS coated with poly-L-lysine (0.1%; Sigma, St. Louis, MO, USA). Coverslips with DRG neurons were then placed in petri dishes with Dulbecco's modified Eagle's medium containing 1% penicillin/streptomycin and 10% fetal calf serum, and incubated at 5% CO_2 at 37 °C for 6, 24, and 48 h time spans for our experiments.

2.3. Immunofluorescent microscopy and image analysis

After culturing DRG neurons on each of the substrates, DRGs were washed with phosphate buffer saline (PBS) three times, fixed with 4% paraformaldehyde, and incubated with PBS containing 1% bovine serum albumin, 0.1% Triton X-100 for staining within the cell, and 0.02% sodium azide for blocking, followed by a primary antibody at 4 °C overnight. This approach is similar to immunostaining protocols that have been used previously with DRGs (Lin et al., 2009). The primary antibodies used were guinea pig-anti-PGP9.5 (Chemicon, Temecula, CA, USA), mouse anti-actin (Sigma, St. Louis, MO, USA), mouse anti-tubulin (Sigma, St. Louis, MO, USA), and mouse anti-MAP2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies were 6 μM Alexa Flour® 488 donkey anti-mouse (Molecular Probes, Carlsbad, CA, USA), goat anti-guinea pig (Molecular Probes, Carlsbad, CA, USA), and 594 IgG (Molecular Probes, Carlsbad, CA, USA). The stained DRG neurons were mounted with VECTASHIELD-DAPI (Vector, Burlingame, CA, USA), sealed under a coverslip, and then examined using an epi-fluorescent microscope (Olympus, BX-51, Japan) with a 100× high numerical aperture (NA = 1.4) oil immersion objective for imaging the distribution of the structural proteins in DRG neurons and glial cells. In addition, the images were analyzed using NIH ImageJ software (public software downloaded from http://rsb.info.nih.gov/ij/download.html). Six primary cultures in total were employed, and 20 images were captured for each group. All statistic data are presented as the mean ± standard error. Statistical significance between glass coverslips and PDMS group was tested using the ANOVA test, followed by a post-hoc Tukey's test ($p < 0.05$ was considered statistically significant).

3. Results

DRGs and glial cells are believed to have significant roles within the peripheral nervous system (PNS). Recent studies identified glial cells participating in the synaptic transmission and modulation of neural activity (Filosa et al., 2009; Auld and Robitaille, 2003), which indicates that glial cells are likely important for neuron survival and function. Furthermore, our previous study (Lin et al., 2009; Cheng et al., 2010) showed that soft PDMS systems could be used to examine mechanical responses in DRG neurons. As external mechanics imposed on cells is related to substrate elasticity, we were motivated, in this study, to examine such substrate effects on DRG-glial cell co-cultures. To investigate the effect of substrate stiffness on DRG neuron growth, we examined neuron density and neurite outgrowth after culturing a DRG neuron–glial cell co-culture on glass coverslips with poly-L-lysine. We used this DRG neuron–glial cell co-culture for all of our experiments. To determine cell type and number (DRG/glial) within our cultures, we stained them for the protein gene product 9.5 (PGP 9.5), a member of the ubiquitin hydrolase family of proteins used as general neuron markers (Calzada et al., 1994; Thompson et al., 1983); cells that were positive for PGP 9.5 were considered DRG rather than glial cells. The total number of cells including DRG neuron normalized by the culture area on glass was relatively low at 6 h after cell culture (Fig. 1A and B), yet after 48 h, the number of DRG neurons in the normalized area was found to be relatively higher (Fig. 1C and D). The results were quantified and plotted as a bar chart in Fig. 1E and F. An increase in the amount of time that the cells were cultured on the substrates from 6 to 48 h increased the density of DRG neurons cultured on glass coverslips. In addition, when primary DRG neurons were cultured on glass substrates, the neurites did not appear to develop significant extensions after 6 h, but such extensions were visible after 48 h.

We next investigated the relationship between neuronal structure/morphology and substrate stiffness. We controlled the physical properties of the PDMS on the coverslips by altering the ratio between the

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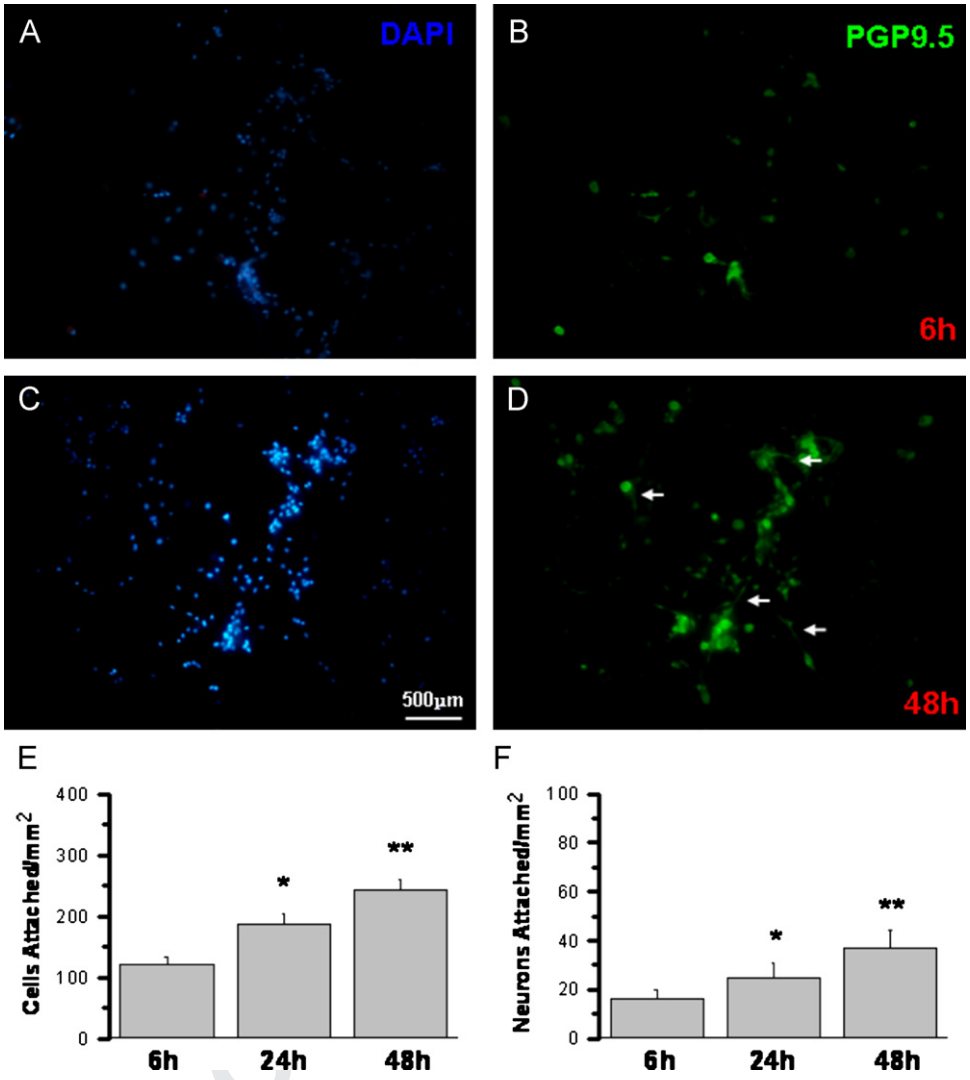

Fig. 1. Primary cultures of DRG–glial cell co-cultures on glass over time. DRG neurons and glial cells were found in low cell density 6 h after being seeded on glass coverslips coated with poly-L-lysine (A, B). DRG neurons and glial cells were found in higher cell densities 48 h after being seeded (C, D). DAPI (blue) and PGP 9.5 (green) were used to indicate that the cells examined were, indeed, DRG neurons (B and D). Quantification of the number of total cells (E), and neurons (F) normalized by the total surface area of the substrate for cells and neurons after 6, 24, and 48 h time periods. Cells that were positive for PGP 9.5 were considered DRG and not glial cells. We used this DRG neuron–glial cell co-culture for all of our experiments. Arrows indicate the neurite. (scale bar = 500 μm). ($^{*}p < 0.05$ compared to 6 h, $^{**}p < 0.01$ compared to 6 h. The error bars represent s.e.m. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

base and the curing agent from 15:1 to 35:1 to 50:1 and compared our results to those found when using more conventional glass substrates. The relative stiffness of each substrate, 10:1 PDMS, 15:1 PDMS, 35:1 PDMS, 50:1 PDMS, and glass, are 1882, 173, 88, 18 kPa and 70 GPa, respectively (Lin et al., 2009; Cheng and LeDuc, 2008; Cheng et al., 2009). The PDMS and glass were both first coated with poly-L-lysine, based on conventional approaches in neurobiology (Lin et al., 2008). This is known to increase neuron attachment while accelerating the growth of the cells (James et al., 2000). Fig. 2A and B reveals that neurite extensions were pronounced for cells grown on glass after 48 h. When culturing the neurons on 15:1 ratio of PDMS, the cells appeared to have a smaller number of extensions after 48 h as well as a lower density of cells when normalized by the culture area (Fig. 2C and D). However, when the PDMS ratio between base and curing agent was increased from 15:1 to 35:1, the cultured DRG neurons produced more extensions and were found in a higher number per area (Fig. 2E and F). This apparent trend did not continue, as an increase in the PDMS ratio from 35:1 to 50:1 produced results similar to those found for DRG neurons cultured on 15:1 PDMS, suggesting a bimodal response (Fig. 2G and H). We further investigated the relation between neurite extension and substrate stiffness. Fig. 3A–D depicts the neurite extensions of DRG neurons on glass and PDMS substrates with different stiffnesses after 6 h culture. Neurons extended their neurites on glass (Fig. 3A; $N=20$, 31.3 ± 1.9 μm), but not on 10:1 and 15:1 PDMS (Fig. 3B and C). On 35:1 PDMS, neurons can initiate neurite extensions as on glass (Fig. 3D; $N=20$, 23.2 ± 1.6 μm); neurite extensions were not observed on 50:1 PDMS (Fig. 3E). When culturing DRG neurons on glass and PDMS substrates of different stiffnesses for 48 h, DRG neurons extended their neurites to approximately 282.4 ± 17.5 μm on glass (Fig. 3F). This neuronal behavior was not observed when culturing DRG neurons on PDMS substrate with the ratio of 10:1 and 15:1 (Fig. 3G and H). However, when the ratio between base and curing agent of PDMS substrate was increased from 15:1 to 35:1, the cultured DRG neurons exhibited significant neurite extensions (Fig. 3I). Consistent with previous cell density results there was no trend for an increase in extensions with a greater (50:1) base to curing agent ratio, again suggesting a bimodal response within these PDMS elasticity range studies (Fig. 3J). To quantify these responses, the numbers of cells were counted and normalized over their culture area (Fig. 4A and B). Our results show that the number of cells per area was 204.2 ± 10.7,

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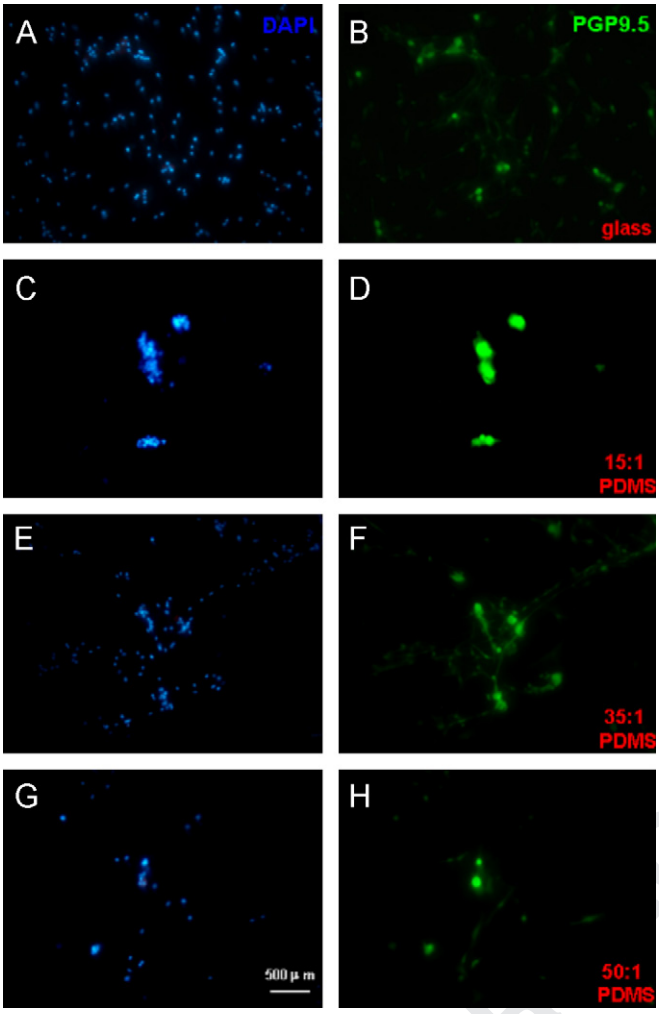

Fig. 2. Primary cultures of DRG neurons on substrates with differing elasticities cultured for 48 h. DAPI (blue), and PGP 9.5 (green) imaging of DRG neurons and glial cells after 48 h for (A) cells stained with DAPI on glass substrates, (B) neurons stained with PGP 9.5 on glass substrates, (C) cells stained with DAPI on 15:1 base to curing agent ratio of PDMS, (D) neurons stained with PGP 9.5 on 15:1 base to curing agent ratio of PDMS, (E) cells stained with DAPI on 35:1 base to curing agent ratio of PDMS, (F) neurons stained with PGP 9.5 on 35:1 base to curing agent ratio of PDMS, (G) cells stained with DAPI on 50:1 base to curing agent ratio of PDMS, and (H) neurons stained with PGP 9.5 on 50:1 base to curing agent ratio of PDMS. DAPI was used through immunostaining to image the nucleus and PGP 9.5 was used to determine the respective numbers of DRG neurons and glial cells (scale bar=500 μm). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

61.2 ± 4.3, 76.3 ± 7.1, 108.3 ± 8.3, and 48.8 ± 5.7 for glass, 10:1 PDMS, 15:1 PDMS, 35:1 PDMS, and 50:1 PDMS, respectively, after 48 h. Furthermore, the neurite lengths measurements were averaged at 6, 24, and 48 h for both glass and 35:1 PDMS substrates. The results indicate that the DRG neurite length increased with longer culture times. The neurite lengths on glass were 31.3 ± 1.9 μm after 6 h in culture: these are longer than those grown on 10:1 (4.6 ± 0.4 μm), 15:1 (5.3 ± 0.2 μm), and 50:1 (6.1 ± 1.1 μm) PDMS substrates, but not on 35:1 PDMS substrates (23.2 ± 1.6 μm), as shown in Fig. 4C. The neurite lengths on glass were 282.4 ± 17.5 μm after seeding for 48 h; these are longer than those grown on 10:1 (39.3 ± 5.2 μm), 15:1 (45.3 ± 4.1 μm), 35:1 (161.2 ± 9.0 μm), and 50:1 (48.3 ± 3.6 μm) PDMS substrates, as shown in Fig. 4D. This bimodal PDMS response suggests that PDMS with a ratio of 35:1, while less ideal than conventional glass substrates, provides an advantageous environment for promoting neurite extensions and growth. It is also noted that

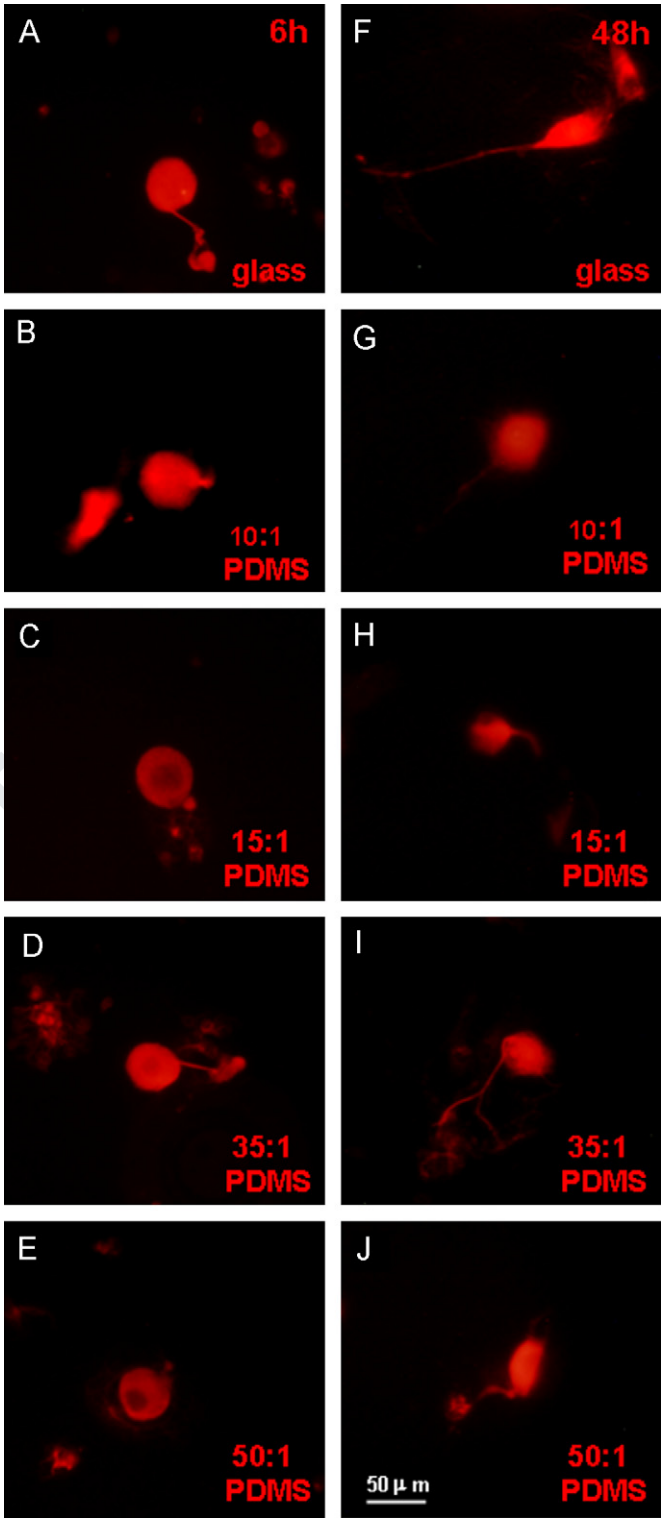

Fig. 3. Primary cultured DRG neurons on substrates with differing elasticities after 6 and 48 h. Fluorescent images of microtubules in DRG neurons cultured for 6 h (A–E) and 48 h (F–J). (A and F) Cells stained with microtubules on glass substrates, (B and G) neurons stained with microtubules on 10:1 base to curing agent ratio of PDMS, (C and H) cells stained with microtubules on 15:1 base to curing agent ratio of PDMS, (D and I) neurons stained with microtubules on 35:1 base to curing agent ratio of PDMS, and (E and J) neurons stained with microtubules on 50:1 base to curing agent ratio of PDMS (scale bar=50 μm).

controlling neuron density precisely is a challenging issue with primary DRG neuron culture, although there is far less than an order of magnitude difference.

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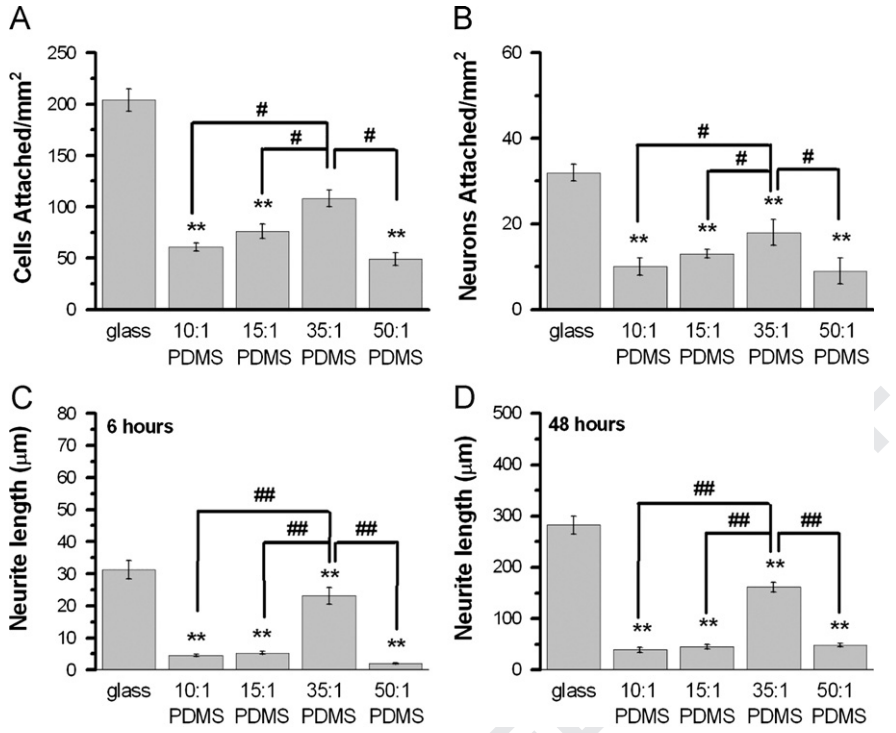

Fig. 4. Quantification of the number of total cells, DRG neurons normalized by the culture area, and neurite length on substrates with differing elasticities after 48 h of culturing. (A) Total cells, (B) DRG neurons numbers were determined for cultures on glass substrates, 10:1 base to curing agent ratio of PDMS, 15:1 base to curing agent ratio of PDMS, 35:1 base to curing agent ratio of PDMS, and 50:1 base to curing agent ratio of PDMS. DRG neurons appear to have a relatively low density for 10:1 PDMS substrates with increasing density for the 35:1 PDMS substrates. At 50:1 PDMS, the DRG neurons reversed the trend back to a lower density closer to that of the 10:1 PDMS. (C) The neurite lengths of single DRG neurons were determined while culturing DRG neurons on glass substrates, 10:1, 15:1, 35:1, and 50:1 base to curing agent ratio of PDMS for 6 h. (D) DRG neurite length versus glass substrate, 10:1, 15:1, 35:1, and 50:1 base to curing agent ratio of PDMS after 48 h culture ($^{**}p < 0.01$ compared to a glass substrate, $^{\#}p < 0.05$ compared to 35:1 PDMS, $^{\#\#}p < 0.01$ compared with 35:1 PDMS). The error bars represent s.e.m.

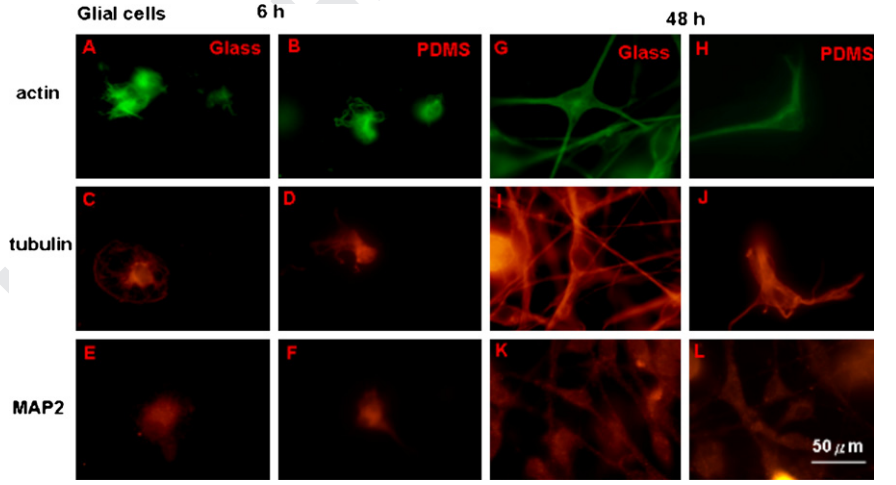

Fig. 5. Actin, microtubule, and MAP2 immunostaining of glial cells seeded on glass and 35:1 PDMS 6 and 48 h after plating. Actin immunostaining for glial cells on glass (A) and (B) 35:1 PDMS. Microtubule immunostaining through mouse-anti-microtubule antibody for glial cells on glass (C) and (D) 35:1 PDMS. MAP2 immunostaining through mouse-anti-MAP2 antibody for glial cells on glass (E) and (F) 35:1 PDMS. Actin immunostaining for glial cells on glass with areas of $1463.4 \pm 112.5\ \mu m^2$ (G) and (H) 35:1 PDMS ($1053.4 \pm 134.7\ \mu m^2$). Microtubule immunostaining through mouse-anti-microtubule antibody for glial cells on glass (I) and (J) 35:1 PDMS. MAP2 immunostaining through mouse-anti-MAP2 antibody for glial cells on glass (K) and (L) 35:1 PDMS.

We next investigated the response of glial cells to varying substrate stiffness over time. At 6 h after seeding on glass, glial cells displayed favorable attachment and spreading (Fig. 5A; $N=20$, $1091.26 \pm 121.81\ \mu m^2$). PDMS with a 35:1 ratio of base to curing agent was used from the previous findings, since it allowed for more favorable cell densities, yet the spread morphology that was observed on glass was similar to glial cells seeded on the PDMS (Fig. 5B; $N=20$, $1078.81 \pm 124.63\ \mu m^2$).

Following this, we examined structural proteins such as actin and microtubules, as these are key elements for neurite outgrowth and mechanotransduction (Cheng et al., 2010; Bounoutas et al., 2009). Microtubules had a significant presence within the neuron soma, implying that this structure aided in the adherence and spreading of the cells for the glass coverslips group. The immunofluorescent staining for actin and microtubules for the PDMS was more localized, which may be attributable to the fact that the cell

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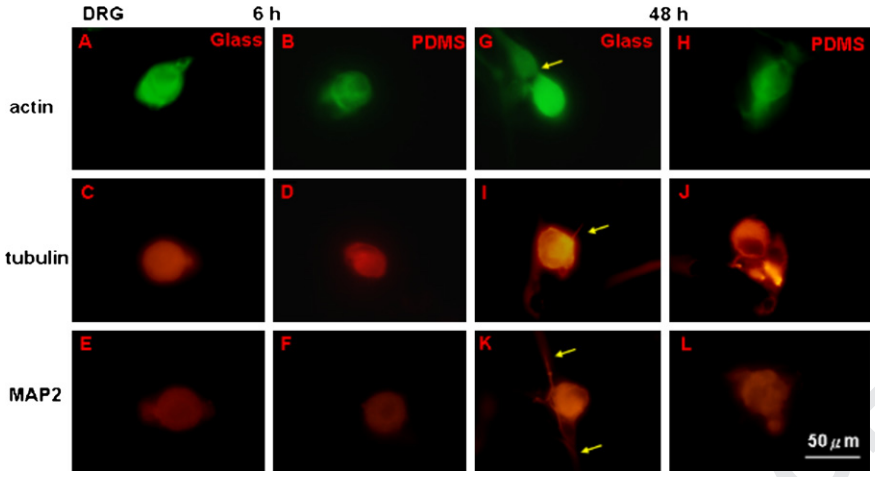

Fig. 6. Actin, microtubule, and MAP2 immunostaining of primary DRG neurons seeded on glass and 35:1 PDMS 6 and 48 h after plating. Actin immunostaining for glial cells on glass (A) and (B) 35:1 PDMS. Microtubule immunostaining through mouse-anti-microtubule antibody for glial cells on glass (C) and (D) 35:1 PDMS. MAP2 immunostaining through mouse-anti-MAP2 antibody for glial cells on glass (E) and (F) 35:1 PDMS. Actin immunostaining for glial cells on glass with areas of $1183.1 \pm 128.3\ \mu m^2$ (G) and (H) 35:1 PDMS ($1211.6 \pm 153.2\ \mu m^2$) Microtubule immunostaining through mouse-anti-microtubule antibody for glial cells on glass (I) and (J) 35:1 PDMS. MAP2 immunostaining through mouse-anti-MAP2 antibody for glial cells on glass (K) and (L) 35:1 PDMS. Arrows indicate the neurite.

was not spread over as large an area (Fig. 5A–D; $N=20$). We also investigated microtubule-associated proteins, which form cross-bridges between microtubules and other cytoskeletal components (Mukhopadhyay et al., 2004; Harada et al., 2002), although the specific characteristics of MAP2 are still unknown. After 6 h in culture, MAP2 was consistently distributed throughout the cells grown on both glass and PDMS substrate (Fig. 5E and F; $N=20$).

Next, we explored the changes that occurred over longer time periods with glass and PDMS substrates. After 48 h, glial cells cultured on glass showed a much greater degree of spreading with areas of $1463.4 \pm 112.5\ \mu m^2$ (Fig. 5G; $N=20$, $p<0.05$). The morphology of the glial cells after 48 h on 35:1 PDMS showed an enhanced spreading when compared to 6 h for either PDMS or glass, but did not have the same amount of spreading as cultures grown on glass after 48 h (Fig. 5H; $N=20$, $1236.2 \pm 81.6\ \mu m^2$). Furthermore, while the glial cells cultured on PDMS substrates for 48 h showed spreading at the 6 h time point, there was more spreading of glial cells cultured on glass after the same 48 h timespan. In addition, the presence and distribution of actin and microtubules was more distinct at 48 h for cells cultured on glass compared to PDMS (Fig. 5G–J). Similar distribution patterns were also observed for MAP2 as shown in Fig. 4K and L. These results demonstrate that longer culture times and the use of glass substrates may be more favorable for glial cell area and spreading. This response is complicated yet based on findings that substrate elasticity and three-dimensional surfaces can affect cell behavior. Controlling substrate elasticity and three-dimensional scaffolds has been shown, in various examples, to be advantageous to cell function. However, this does not necessarily translate into increased ability to visualize cell spreading or protein distribution. With fibroblasts, for example, morphology in a three-dimensional matrix has a more non-planar distribution (Beningo et al., 2004), which is more challenging to visualize through conventional epi-fluorescence microcopy. In addition, the mechanical environment, via substrate topography, affects cell orientation and polarization in neurites. Gomez et al. (2007) reported that physical and biochemical stimuli increase the rate of polarization and establishment of a single neuron when hippocampal neurons were cultured in a PDMS matrix. This effect was attenuated through controlling surface topography, which indicates a significant physical signal for controlling cell–substrate interactions for neurons and polarization.

Understanding and accounting for the response of DRGs to substrates of varying elasticities is essential due to the intertwined nature of DRGs and glial cells. We seeded a DRG neuron–glial cell co-culture on either glass coverslips or soft (35:1) PDMS coverslips coated with poly-L-lysine based on our previous bimodal distribution findings in Figs. 1–3. Since the somas and neurites in DRG neurons usually do not exist at the same focal plane, we focused on structural proteins in DRG neurons in this study. After 6 h, the DRGs did not appear to spread differently on either substrate nor did they exhibit significant differences in their extensions (Fig. 6). Furthermore, the response of structural proteins such as actin filaments (Fig. 6A and B), microtubules (Fig. 6C and D), or MAP-2 (Fig. 6E and F) after 6 h in culture showed little discernable difference. In addition, we examined DRG neuron morphologies after culturing the cells on either glass coverslips or soft PDMS for 48 h. After 48 h there appeared to be increased spreading ($1183.1 \pm 128.3\ \mu m^2$) of cultures on both the glass (Fig. 6G; $N=20$) and PDMS substrates with areas of $1211.6 \pm 153.2\ \mu m^2$ (Fig. 6H; $N=20$). Actin filaments (Fig. 6G and H) and microtubules (Fig. 6I and J) were similarly distributed, although in comparison to 6 h of culture, the cells were more spread with the location of these structural proteins being distributed throughout the spread cell. At this time, the outgrowth in these single neurites was longer (Fig. 6, as indicated with arrows). The expression of MAP2 in DRG neurons at 48 h was similar to that observed after 6 h (Fig. 6K and L) for PDMS.

4. Discussion

Neurite extensions are important in early neuronal differentiation as they eventually form axons and dendrites. Thus, characterizing them with respect to substrate elasticity provided new understanding of neural behavior. Our findings are important as they elucidate the response of DRG neuron cell structure to soft matrices, which influences morphology, and the growth of neurite extensions. It has been suggested that neurite outgrowth begins immediately after neuronal attachments have formed (Dadsetan et al., 2009; Hodgkinson et al., 2007); the process is then modulated by the activation of membrane receptors and the extracellular matrix (Plantman et al., 2008; Hattori et al., 2007). The membrane receptors then activate intracellular signaling cascades to influence the actin and microtubule cytoskeleton—elements that are directly related to cell structure, mechanics, and spreading. In addition, activation of these receptors affects gene transcription to stabilize the neurite from a structural perspective (Fernyhough et al., 1989). This feedback system is important to balance extracellular and

Please cite this article as: Cheng, C.-M., et al., Localized bimodal response of neurite extensions and structural proteins in dorsal-root ganglion neurons with controlled polydimethylsiloxane.... Journal of Biomechanics (2010), doi:10.1016/j.jbiomech.2010.12.006

intracellular interactions that ultimately can affect both physical and chemical behaviors in neurons.

This study demonstrated the effects of substrate stiffness and its relationship to cell structure and morphology. To more fully understand this under-examined relationship, we cultured cells on PDMS of varying stiffness. We found that there was a significant increase in spreading and the number of neurite extensions after 48 h for cultures grown on both glass and PDMS. The numbers of cells normalized by culture area were found to change as a reflection of PDMS base to curing agent. Interestingly, a bimodal response was observed, as there were higher cell densities for both DRGs and glial cells grown on PDMS substrates (ratio of 35:1) than found on relatively soft or rigid substrates with PDMS (50:1 or 15:1 ratios). These ratios are directly correlated to elastic moduli of 173, 88, and 18 kPa for PDMS ratios of 15:1, 35:1, and 50:1, respectively. Spreading of DRG neurons also increased over time for cultures grown on both glass and PDMS, but cultures on glass showed a larger spreading area after 48 h. Major structural proteins in these cells, including actin filaments, microtubules, and MAP2, were also investigated. The correlation between increased spreading and the distribution of these proteins indicated the formation of structural proteins through spread neurons. We believe that these results will be of interest to a variety of areas including biomaterials, cell mechanics, cell–material interactions, and neural regeneration.

Conflict of interest statement

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgments

This work was supported by China Medical University CMU98-N2-10. This work was also supported in part by the National Science Foundation and the Office of Naval Research.

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Please cite this article as: Cheng, C.-M., et al., Localized bimodal response of neurite extensions and structural proteins in dorsal-root ganglion neurons with controlled polydimethylsiloxane.... Journal of Biomechanics (2010), doi:10.1016/j.jbiomech.2010.12.006