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Neurite branching on deformable substrates

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Abstract

The mechanical properties of substrates underlying cells can have profound effects on cell structure and function. To examine the effect of substrate deformability on neuronal cell growth, protein-laminated polyacrylamide gels were prepared with differing amounts of bisacrylamide to generate substrates of varying deformability with elastic moduli ranging from 500 to 5500 dyne/cm^2. Mouse spinal cord primary neuronal cells were plated on the gels and allowed to grow and extend neurites for several weeks in culture. While neurons grew well on the gels, glia, which are normally co-cultured with the neurons, did not survive on these deformable substrates even though the chemical environment was permissive for their growth. Substrate flexibility also had a significant effect on neurite branching. Neurons grown on softer substrates formed more than three times as many branches as those grown on stiffer gels. These results show that mechanical properties of the substrate specifically direct the formation of neurite branches, which are critical for appropriate synaptic connections during development and regeneration.

Keywords

Axon; Dendrite; Development; Gel; Polyacrylamide; Motility; Neuron; Regeneration

INTRODUCTION

Cells respond to a myriad of signals in order to appropriately divide, differentiate, migrate, and establish connections with other cells. While it has long been recognized that cells receive chemical signals through the action of ligands binding to membrane and cytoplasmic receptors, it is becoming increasingly clear that other factors, such as the mechanical aspects of the environment mediated through transmembrane adhesion receptors, also have profound effects on cellular function. Pioneering studies by Hay and colleagues in the 1970s showed that cells cultured in 3D deformable collagen gels adopt a morphology distinct from that routinely observed on glass or plastic [1]. In studies that compared morphology and migration of fibroblasts on glass, in 3D collagen gels, and in intact corneal tissue, it was clear that the cellular behavior in deformable collagen gels more closely mimicked that of the cells in their normal host tissue [2].

Further studies have continued to document effects of substrate deformability on cellular behavior. Fibroblasts grown on plastic or in stressed collagen gels that were adhered to a surface generated prominent stress fibers, while cells grown in relaxed collagen gels, which were not

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attached to a surface and thus were more compliant, had no stress fibers [3]. Using a similar collagen gel system, Arora *et al.* have shown that substrate deformability can influence a cell's ability to respond to growth factors [4]. Fibroblasts grown on plastic up-regulate levels of alpha smooth muscle actin 9-fold in response to TGFβ1, while cells in stiff collagen gels increase levels 3-fold and cells in relaxed collagen do not appreciably change levels at all.

Recent work from Wang and colleagues has shed more light on how cells respond to mechanical cues through the use of polyacrylamide gels to generate substrates with precise mechanical properties over a wide range of substrate deformabilities. In this system, polyacrylamide is cross-linked with varying amounts of bisacrylamide to generate gels that do not differ in polymer mass but do differ in deformability due to the numbers of cross-links between linear acrylamide polymers. Initial studies using these deformable substrates showed that both cellular morphology and motility were affected by the deformability of the substrate: cells on softer polyacrylamide gels spread less and exhibited increased motility and lamellipodial activity [5]. Further studies showed that cell movement could be guided by the physical parameters of the substrate such that cells migrating on stiffer regions of gels (Young's modulus = 300 k dyne/cm^2) avoided moving onto softer regions (Young's modulus = 140 k dyne/cm^2), and if tension was generated in the substrate by a pulling force migrating cells would reorient toward the region of increased tension [6].

Most studies of the effects of substrate deformability on migrating cells have been conducted on non-neural cells, but tissue mechanics are also likely to affect neuronal development. Neuronal cells extend neurites over sizeable distances in the body to contact target innervation sites such as peripheral muscles. The proper extension and branching of these processes is critical to development of appropriate neuronal circuits and target innervation. The extension of neuronal processes (dendrites and axons, collectively termed neurites) occurs through the activity of a distal growth cone that extends lamellae and filopodia much like a locomoting fibroblast. Likewise, the cytoskeletal structure of the growth cone and the rearrangements that occur during advance, retraction, and pausing behaviors are similar to those used at the leading edge of non-neuronal cells [7–10]. Therefore, neuronal cells are likely to respond to mechanical cues of the environment in a manner similar to migrating cells. The current study investigates this hypothesis using mouse primary spinal cord neurons plated onto polyacrylamide gels of varying deformability.

MATERIALS AND METHODS

Preparation of glass and polyacrylamide gel substrates

German glass coverslips (25 mm, Assistant/Carolina Biological Supply, Burlington, NC) were used for plating neurons directly onto glass. Preparation of coverslips and polyacrylamide gels was according to the protocol of Pelham and Wang [5,11], with the following modifications. Gels were prepared with 3% acrylamide and bisacrylamide (bis) ranging from 0.04 to 0.2%. Final gel thickness after polymerization was ~100 μm.

A bifunctional cross-linker (50 μM sulfo-SANPAH (Pierce, Rockford, IL) in 200 mM HEPES, pH 8.5) was used to attach poly-D-lysine to the polyacrylamide gels [5]. Poly-D-lysine (10 μg/ml in water, Boehringer Mannheim, Ingelheim, Germany) was applied to polyacrylamide gels or cleaned coverslips at 0.52 μg/cm^2 and left on gels for 1–2 h at 37°C for cross-linking and on coverslips for 5 min at room temperature. After removing poly-D-lysine, 167 μg/ml matrigel (Becton Dickinson, Franklin Lakes, NJ) in EMEM was applied at 8.67 μg/cm^2 to both coverslips and polyacrylamide gels and removed after 2 h at room temperature. Matrigel does not form a gel at the dilution used in this study, but coats the surface with a mixture of extracellular matrix proteins. Coverslips and gels were rinsed once with EMEM and kept

overnight in EMEM in a humidified 37°C incubator. Several hours before plating cells, EMEM was removed and replaced with neuronal culture medium (see below).

Cell culture

Primary spinal cord neuronal cultures were prepared as described [12] from E13.5. mouse embryos (C57BL/6J, Jackson Labs, Bar Harbor, ME). Neurons were grown in neuronal culture medium consisting of EMEM, 9 μg/ml insulin (Sigma, St. Louis, MO), 180 μg/ml transferrin (Sigma, St. Louis, MO), 9 μg/ml BSA (Gibco, Rockville, MD), 29 μg/ml putrescine (Sigma, St. Louis, MO), 23 ng/ml selenium (Sigma, St. Louis, MO), 18 ng/ml T3 (Calbiochem, San Diego, CA), 8.2 ng/ml hydrocortisone (Sigma, St. Louis, MO), 12 ng/ml progesterone (Sigma, St. Louis, MO), 10 ng/ml NGF (Becton Dickinson, Franklin Lakes, NJ), 2% horse serum (Gibco, Rockville, MD), 5% FCS (Gibco, Rockville, MD), and 1% penicillin/streptomycin (Gibco, Rockville, MD).

Rheology, imaging and data analysis

Gel viscoelasticity was measured with an RFS-II fluid rheometer (Rheometrics, Piscataway, NJ) after polymerizing 1 ml of gel solution in a sample holder with cone and plate geometry. Measurements were acquired at room temperature, with 5% strain at 10 rad/s frequency. Cells were imaged using a Nikon Diaphot 300 microscope and images captured with a silicon-intensified-target camera (DAGE-MTI 64L, Michigan, IL) and Scion capture card (Scion, Frederick, MD). Images were collected in NIH Image and compiled with Adobe Photoshop 5.0. Imaged cells were selected at random, and all visible processes of selected neurons were imaged.

Neurite length was determined from images of neurons using the line selection tool and measure function in NIH Image software (National Institutes of Health, Bethesda, MD). The number of branches was found by tallying up the number of places where one neurite appeared to split into two. Since neurites would occasionally fasciculate together, branches were defined on the basis of neurite diameter and the angle at which the processes extended from the shaft. Branches tend to be of similar diameter as the shaft and extend at larger angles while defasciculating processes tend to be about half the diameter of the shaft and extend at smaller angles. Therefore, not all the regions where processes diverged were counted as branches. All data were analyzed using Kaleidagraph software (Synergy Software, Reading, PA).

Protein analysis

For matrigel quantification gels were prepared of smaller diameter (5 mm) than those used for plating cells. These 5 mm gels and control glass coverslips (also 5 mm diameter) were treated with poly-D-lysine and matrigel as described above. In order to remove bound proteins from the gels and coverslips, they were placed with the protein-linked surface downward on boiling SDS-PAGE sample buffer. Since the matrigel protein components are not covalently bound to the gel (the poly-D-lysine is cross-linked onto the gels and matrigel layered on top) or the glass they were removed by the SDS and βME in the sample buffer. The protein samples from either three gels or three coverslips were pooled then loaded into wells of a 10% SDS-PAGE gel. The amount of matrigel in each sample was detected by Western blot using an antibody against laminin (rabbit polyclonal, diluted 1:1000, Sigma, St. Louis, MO), which is a major component of matrigel.

RESULTS

Preparation and characterization of matrigel-coated poly-acrylamide gels

Polyacrylamide gels were prepared as substrates for neuronal cells by adapting the method of Pelham and Wang [5] (see Materials and Methods). A rheometer was used to assess the deformability of the gels by imposing a small deformation on the substrate and measuring the resulting force. Variation of the bisacrylamide concentration in 3% polyacrylamide gels produced materials with a range of shear moduli from 500 to 5500 dyne/cm^2 (Fig. 1a). Subsequent experiments used gels prepared with 0.04–0.2% bisacrylamide since within this range a small change in cross-linker concentration produced a large change in shear modulus. Polyacrylamide gels cross-linked with different amounts of bisacrylamide do not differ in amount of polymer, but only in the density of covalent cross-links. The pore size of these gels is on the order of 100 nm [13–15], which is sufficiently small that cells(~15 μm) and cellular processes (such as growth cones at the ends of neurites of neuronal cells, ~5 μm) are too large to enter the gel matrix (Fig. 1b).

Polyacrylamide gels were covered with poly-D-lysine and matrigel to allow cell attachment. The matrigel was layered on as a dilute solution so that the matrix proteins coat the surface but do not make a gel. The amount of matrigel on the polyacrylamide gels and glass coverslips prepared for culturing neurons was quantified to ensure that gels varied in deformability but not in amount of matrix coating. Western blot analysis of samples using an antibody to laminin, one of the major components of matrigel, confirms that the amount of matrigel on the gels remains consistent regardless of gel deformability (Fig. 1c). The amount of matrigel on glass was also roughly equivalent to that on the gels (data not shown).

Neurons thrive on polyacrylamide gels and are affected by substrate deformability

Neuronal cultures prepared from embryonic mouse spinal cord contain a mixture of glia and multiple types of neurons, predominantly sensory neurons from dorsal root ganglia, motor neurons, and interneurons. These cells grow as a fairly dense culture on glass (Fig. 1d) and can thrive for several weeks. The same culture conditions were used to plate cells on polyacrylamide gels of varying deformability.

Plating of spinal cord cells on matrigel-laminated poly-acrylamide gels yielded cultures that are sparser than comparable cultures plated at the same density on glass. No cells adhered to polyacrylamide gels that were not coated with matrigel (data not shown). No glia were observed on any of the gels even after several weeks in culture, a time point at which comparable cultures on glass are primarily made up of glial cells since glia continue to divide while neuronal cells are post-mitotic. These data suggest that these mesh-like deformable substrates are not conducive to glial growth (Figs. 2 and 3).

In contrast, neuronal cells grow well on the gels, and 1 day after plating these cells began to extend neurites and adopt recognizable neuronal morphologies. Although the neurons are sparse they survive well and extend neurites for long distances. Figure 2 shows neurons cultured for 7 days on a soft gel (0.08% bisacrylamide, ~2300 dyne/cm^2). Neurites extend quite far on the gels, on average 0.8 mm from the cell body, and form multiple branches along their length. Neurons also grow well and extend processes on stiffer gels (0.2% bisacrylamide, ~5500 dyne/cm^2; Fig. 3a), although the neuronal processes have fewer branches than comparable processes on softer gels. In order to compare branching on gels, both soft and stiff, to that on glass, it was necessary to prepare sparse neuronal cultures because the usual procedure for plating these cells on glass yields dense cultures that make it impossible to trace a single neurite and observe branch points. Neurons in sparse cultures on glass also grow long neurites, but generate relatively few branch points (Fig. 3b). Quantification of the number of branches along neurites

of neurons cultured on glass and on a range of deformable gels shows that cells grown on softer substrates (~500–3000 dyne/cm^2) have significantly more branches than those on stiffer substrates (~3000–5500 dyne/cm^2) or glass (Fig. 4). The branching frequency on the stiffest gel (5500 dyne/cm^2) was similar to the branching frequency of neurons on glass. Increased branch formation on softer substrates appears to be due primarily to the mechanical differences of the substrates since the amount of matrigel bound to the gels was approximately constant, and the slight variations did not scale with neurite branching.

DISCUSSION

Neurons appear to be exquisite biosensors of mechanical rigidity, and are able to generate differing branching densities over a fairly narrow range of substrate deformability (see Fig. 4, branching density on ~2500 dyne/cm^2/0.08% bis gels vs. ~3500 dyne/cm^2/0.1% bis gels). The highest branching density was observed on softer substrates whose mechanical properties correlate well with those of sections of bovine spinal cord (shear modulus ~500 dyne/cm^2, L.A. Flanagan and P.A. Janmey, unpublished observation) and of porcine and human gray matter (average ~2000 dyne/cm^2) [16]. The results described here also fit well with previous studies that show that neurite outgrowth is induced by externally generated tension [17–19]. In addition, cultures of neurons in agarose gels have shown that increasing gel stiffness, with an accompanying increase in polymer density, decreases the rate of neurite extension from chick dorsal root ganglia explants [20]. The data presented here extend these results by investigating a broad range of substrate deformability under conditions where the network mesh size and therefore surface topography is constant, and documents a specific effect of mechanics on neurite branching.

The increased branching of neuronal processes on soft materials contrasts with studies of other cell types, notably fibroblasts, in which cells prefer to extend on the stiffest materials [6]. In addition to this qualitative difference, there is also a large difference in the magnitudes of shear moduli over which these two cell types respond. Fibroblasts show little dependence on stiffness over the range investigated here (500–5500 dyne/cm^2), and only display morphologic and motility differences at much higher stiffness (140 000–300 000 dyne/cm^2, [6]), which the neuron would react to as infinitely stiff or equivalent to glass. These data show that distinct cell types can respond radically differently to external mechanical stimuli. Exploiting these mechanosensing differences may be useful for the design of materials that optimize growth or function of selected cell types.

Developmental biology studies have highlighted the importance of branch formation during establishment of connections in the nervous system [21–24]. Further research on axonal extension has shown that the primary growth cone is responsible for delineating future branch points along the axon [25]. The neuronal growth cone is also able to reorient growth in response to mechanical stimuli [26]. The effect of substrate stiffness on neurite branch formation may fit with a model in which neuronal growth cones on softer substrates are able to engage in more pausing behavior and dynamic reorganization that lead to branch formation, which would be similar to fibroblasts that were more dynamic and also had fewer focal adhesions on softer substrates [5]. In any case, it is most likely in neurite outgrowth that the growth cone is the primary responder to external tension.

CONCLUSION

While it is well established that neurons can respond to multiple molecular cues during neurite outgrowth and branching [27,28], only a few studies have investigated effects of the physical environment that neurons encounter [20,29]. The results of this study suggest that mechanical parameters have profound effects on neuronal morphology, specifically branching, and may

be one of the cues that neurons integrate when making critical pathfinding decisions. Furthermore, the fact that neurons can respond to substrate deformability by altering branch formation may have important implications for design of appropriate matrices for neuronal regeneration [30,31] and for formulating models that describe how branching occurs during development [16,21].

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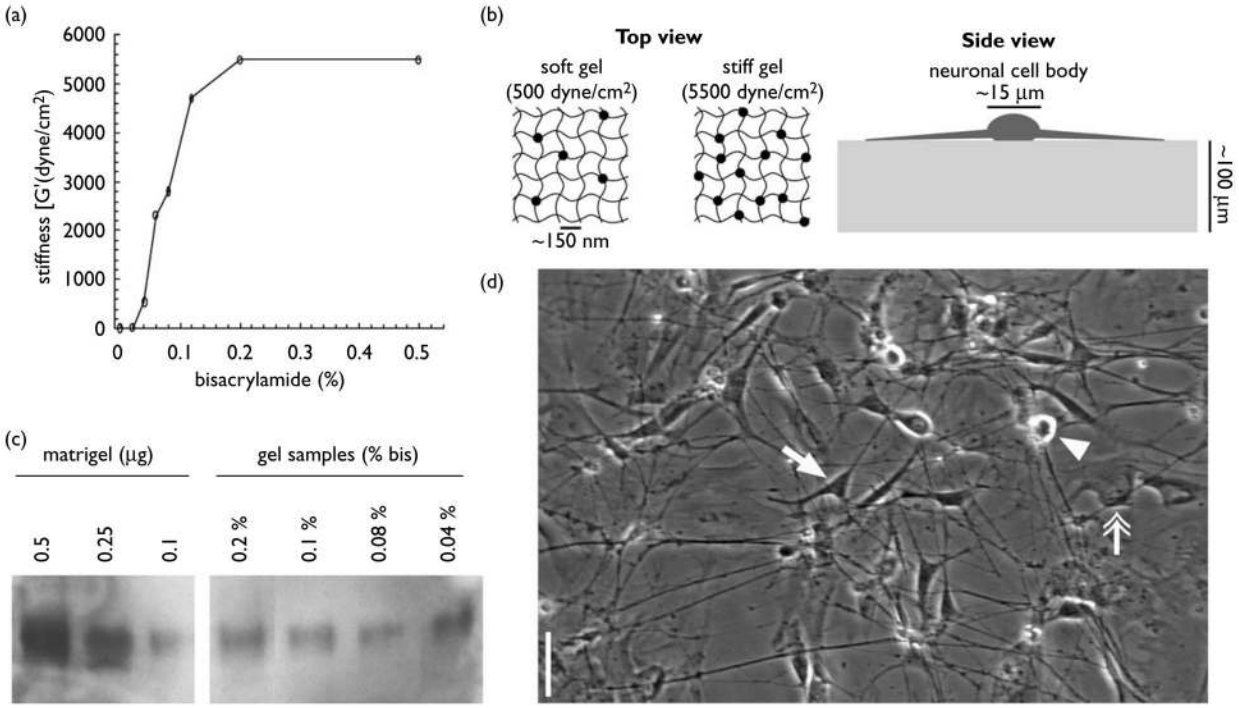

Fig. 1.

Characterization of polyacrylamide gel substrates and neuronal cultures. (**a**) Shear moduli (G ′) of polyacrylamide gels are plotted as a function of increasing bisacrylamide cross-linker concentrations. (**b**) Schematic diagram depicting polyacrylamide gel substrates. The top view highlights that gels of differing deformability do not differ in polymer mass but only in number of cross-links. The relative differences between the size of a neuron and its processes and the pore size of the gels and thickness of the gel substrates are also shown (not drawn to scale). (**c**) Samples of matrigel (three different concentrations) or gels coated with matrigel (four different bisacrylamide cross-linker concentrations) were analyzed by Western blot with an antibody to laminin, a major component of matrigel. Gels of varying deformability do not differ in amount of matrigel coating. (**d**) Mouse primary spinal cord cultures on glass grow as dense cultures with a variety of neuronal subtypes (arrow and arrowhead) and underlying, very flat glial cells (double arrowhead). Scale bar = 50 µm.

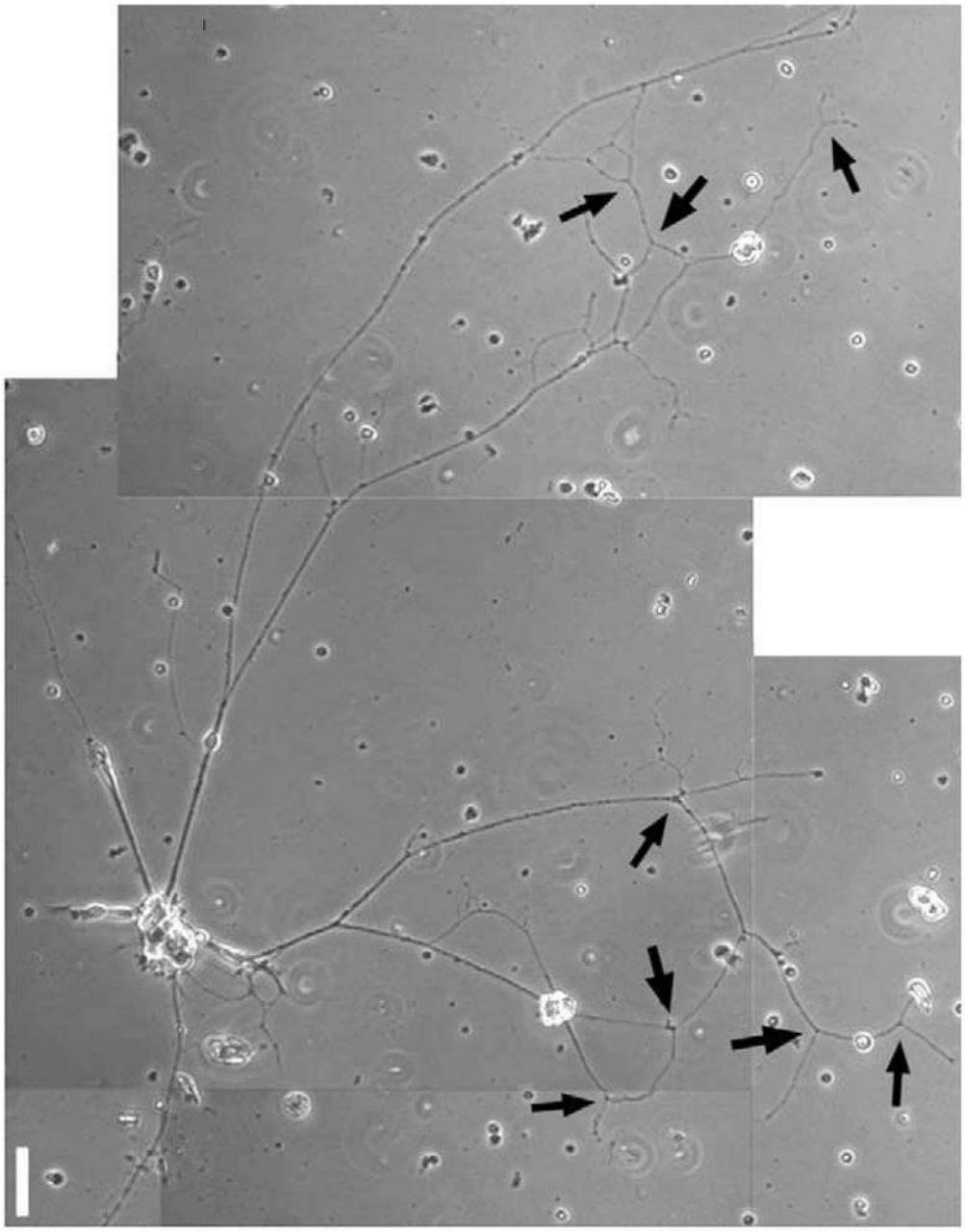

Fig. 2.

Spinal cord neurons on soft substrates. Neurons growing on a soft (0.08% bisacrylamide, 2300 dyne/cm^2) gel extend neurites with multiple branch points (representative branches marked by arrows). Scale bar = 50 μm.

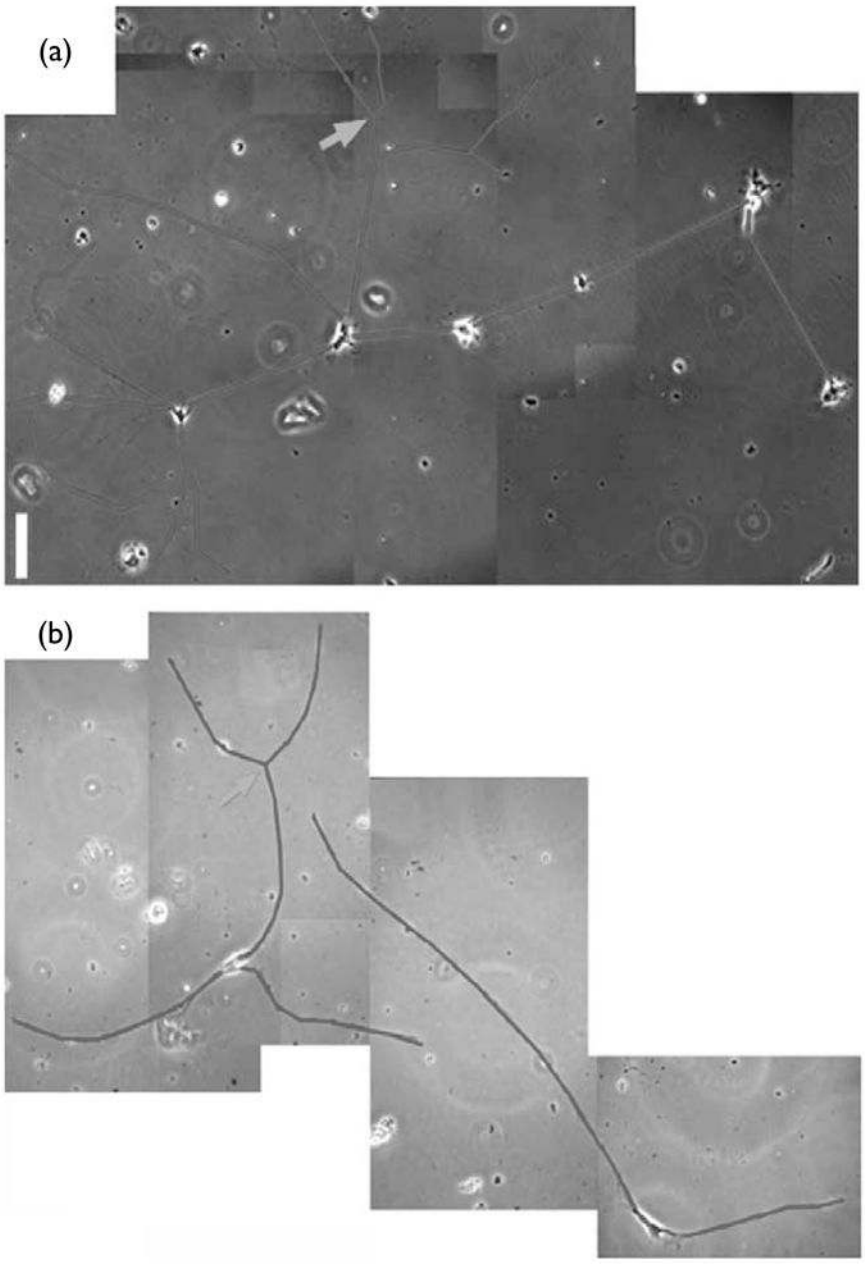

Fig. 3.

Spinal cord neurons on stiff substrates and glass. Neurons growing on (**a**) a stiff (0.2% bisacrylamide, 5500 dyne/cm^2) gel and (**b**) glass as sparse cultures. Neurites are highlighted in black and representative branch points are marked by arrows. Scale bar = 50 μm.

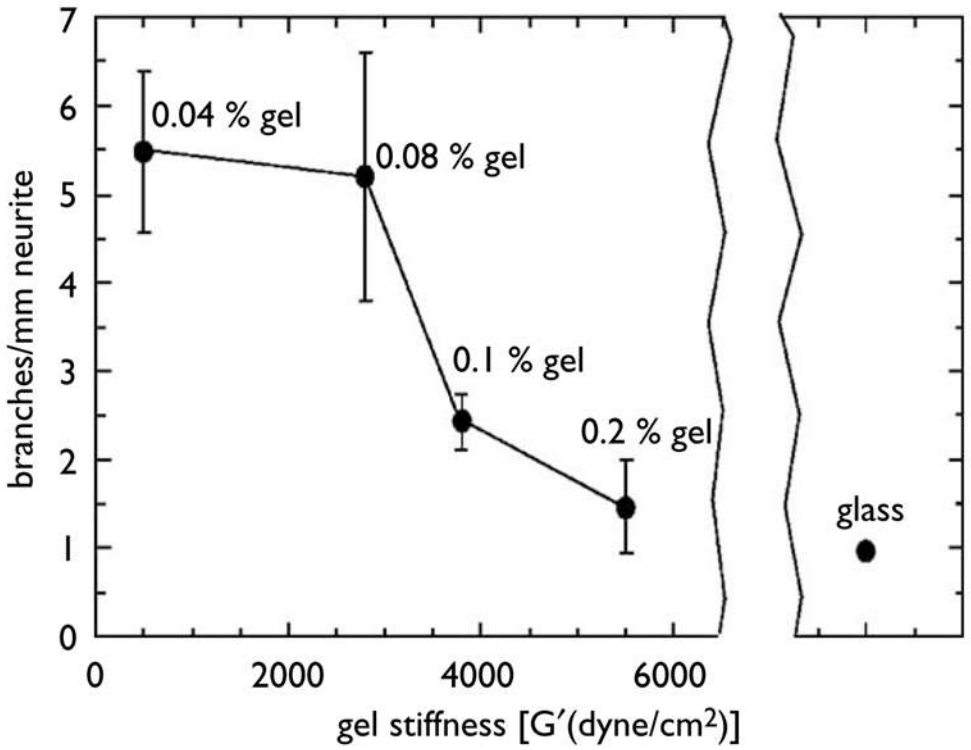

Fig. 4.

Quantification of neurite branching on deformable substrates and glass. Neurite branches per mm of neurite are plotted as a function of increasing substrate stiffness. Glass is considered to have an infinite stiffness compared to the gels. Numbers next to points indicate the percentage of bisacrylamide used for gels of that stiffness. Numbers of branches were counted for all the neurons on gels from several different experiments (0.04% n = 3, 0.08% n = 4, 0.1% n = 3, 0.2% n = 3). Error bars are s.e.m.