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# Collagen-Dependent Neurite Outgrowth and Response to Dynamic Deformation in Three-Dimensional Neuronal Cultures

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**Abstract**—*In vitro* models of brain injury that use thick 3-D cultures and control extracellular matrix constituents allow evaluation of cell–matrix interactions in a more physiologically relevant configuration than traditional 2-D cultures. We have developed a 3-D cell culture system consisting of primary rat cortical neurons distributed throughout thick ($>500$ $\mu$m) gels consisting of type IV collagen (Col) conjugated to agarose. Neuronal viability and neurite outgrowth were examined for a range of agarose (AG) percentages (1.0–3.0%) and initial collagen concentrations ($[Col]_i$; 0–600 $\mu$g/mL). In unmodified AG, 1.5% gels supported viable cultures with significant neurite outgrowth, which was not found at lower ($\leq$1.0%) concentrations. Varying $[Col]_i$ in 1.25% AG revealed the formation of dense, 3-D neurite networks at $[Col]_i$ of 300 $\mu$g/mL, while neurons in unmodified AG and at higher $[Col]_i$ (600 $\mu$g/mL) exhibited significantly less neurite outgrowth; although, neuronal survival did not vary with $[Col]_i$. The effect of $[Col]_i$ on acute neuronal response following high magnitude, high rate shear deformation (0.50 strain, 30 $s^{-1}$ strain rate) was evaluated in 1.5% AG for $[Col]_i$ of 30, 150, and 300 $\mu$g/mL, which supported cultures with similar baseline viability and neurite outgrowth. Conjugation of Col to AG also increased the complex modulus of the hydrogel. Following high rate deformation, neuronal viability significantly decreased with increasing $[Col]_i$, implicating cell–matrix adhesions in acute mechanotransduction events associated with traumatic loading. These results suggest interrelated roles for matrix mechanical properties and receptor-mediated cell–matrix interactions in neuronal viability, neurite outgrowth, and transduction of high rate deformation. This model system may be further exploited for the elucidation of mechanotransduction mechanisms and cellular pathology following mechanical insult.

**Keywords**—Cell biomechanics, Neurite outgrowth, Hydrogel, *In vitro*, Mechanotransduction, TBI.

## INTRODUCTION

Traumatic brain injury (TBI) is caused by a physical insult to the head and may result in prolonged or permanent loss of sensory, motor, and/or cognitive functions,[1,48] representing a major health and socioeconomic problem.[38,55] A rapid insult to the brain produces diffuse strain patterns, which may lead to cell death if strain thresholds are surpassed. However, sublethal strain regimes may lead to persistent alterations in cellular signaling and function. The underlying mechanisms that translate bulk tissue deformation to cellular dysfunction remain poorly understood, but may be dictated by cell orientation, cell–cell connections, as well as cell–extracellular matrix (ECM) interactions.

Receptor-mediated cell–ECM interactions, in particular, are crucial in many homeostatic cellular processes including proliferation, migration, and differentiation,[13,15] and may be affected during various pathological states.[33,58] However, the role of cell–ECM adhesions in acute and chronic responses following traumatic neural loading is currently not known. In many non-neural systems, cells communicate with their immediate physical environment through such interactions and alter intracellular biochemical signaling in response to mechanical cues, a process referred to as mechanotransduction.[2,32,34] Transmembrane proteins such as integrins transduce mechanical stimuli from the ECM to the cytoskeleton and activate intracellular signaling molecules regulating the activity of enzymes (e.g. proteases, phosphatases, kinases) that can modify cytoskeletal organization and may result in changes in gene expression.[2,10,29,36,50,53] However, the mechanisms involved in transduction of non-physiological mechanical deformation are not fully understood. Non-homeostatic (i.e., pathological) mechanotransduction may play a role in cellular dysfunction in response to mechanical forces through integrin-mediated signaling events, increasing intracellular $Ca^{2+}$ concentration, causing membrane disruption via protein phosphory-

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lation, and/or activating cytoskeletal-cleaving enzymes; components of the secondary sequelae associated with TBI.[49] Central nervous system (CNS) neurons are normally protected from high rate strain and may not have inherent mechanisms to homeostatically transduce mechanical stimuli that other cells (e.g., vascular smooth muscle cells, osteoblasts) possess and may therefore have a high sensitivity to mechanical forces, causing abnormal biochemical responses leading to cell dysfunction and possibly death.

The study of mechanotransduction under both physiologic and pathologic states requires the use of experimental systems that mimic the cytoarchitecture and microenvironment found *in vivo*. Fundamental differences exist between cells cultured in planar (i.e., two-dimensional or 2-D) vs. three-dimensional (3-D) configurations, such as the distribution of cell–cell and cell–ECM interactions, which can alter cell morphology and subsequent signaling and function.[11,12,28,52,61] Cells cultured in 3-D have been found to exhibit more *in vivo*-like viability, growth, proliferation, response to biochemical stimuli, gene expression, and differentiation.[20,27,30,43] Models consisting of neurons distributed throughout a 3-D matrix material have previously been developed.[6,7,46,47,60] Dorsal root ganglia (DRG) extend neurites through hydrogel matrices in a manner dependent on the physical properties (e.g., agarose pore size[16] and stiffness[5]), ligand concentration (e.g., collagen[59] and RGD peptides in fibrin[51]), and substrate geometry.[62] Embryonic cortical neurons have been plated within 3-D matrices of collagen and various hydrogels (e.g., poly[*N*-(2-hydroxypropyl)-methacrylamide],[60] poly(acrylate),[46] and agarose[47]). Enhanced survival and neurite outgrowth was observed in collagen (0.4–0.5 mg/mL) as compared to hydrogels lacking ECM ligands, which produced varying degrees of cell viability and a paucity of neurite outgrowth, together indicating that growth and survival of primary cortical neurons are improved by specific cell–matrix interactions.[46,47,60]

Most *in vitro* models developed to study the response of neural cells to mechanical deformation utilize planar cell culture, and include mechanical stretch[19,23,54] and hydrodynamic shear stress[40,45] systems. Differences in cell morphology and the spatial distribution of cell–cell/cell–ECM interactions in planar vs. 3-D cultures may be important, especially in models of traumatic cellular injury as bulk deformation is translated to cells through physical coupling, which may be unrealistically represented in 2-D culture. Furthermore, models amenable to systematic control of cell culture parameters (e.g., cell composition, matrix constituents) provide a framework for the elucidation of the roles of specific factors in deformation transfer to cells and associated acute responses. Accordingly, we previously developed and characterized the 3-D Cell Shearing Device (CSD), a custom electro-mechanical device capable of reproducibly imparting variable rate and magnitude shear deformation to 3-D cell-containing matrices.[39]

The overall goal of this study was to investigate the roles of matrix mechanical properties and composition in neuronal survival, neurite outgrowth, and the response to traumatic loading. We therefore engineered a bioactive scaffold by controlling matrix mechanical properties and ligand density to find optimal ranges for neuronal survival and neurite outgrowth. This cell culture model was then used to study the response to high rate bulk shear deformation.

## MATERIALS AND METHODS

### *Preparation of Protein-Conjugated Agarose Gels*

Agarose was chosen as the matrix backbone for this system because it lacks cell surface receptor binding domains, its physical properties may be tailored based on concentration,[5,63] and procedures have been established for the controlled coupling of bioactive ligands.[6,17] Collagen has previously been used for successful 3-D cortical neuronal culture,[46,47] integrin receptors that recognize collagen have been demonstrated for a range of neuronal sub-types,[3,4,8,9,41,56] and it is a major component in our positive control matrix, Matrigel.[35] Collagen Type IV (Col) (Sigma, St. Louis, MO) was suspended at 2 mg/mL in 0.25% acetic acid. A heterobifunctional amine reactive and photoreactive crosslinker, sulfosuccinimidyl (perfluoroazidobenzamido) ethyl-1,3-dithiopropionate (SFAD) (Pierce, Rockford, IL), was added (1:100 molar ratio) and the protein/crosslinker solution was incubated in the dark at room temperature for 4 h, followed by dilution in ultrapure water to obtain a range of protein concentrations. SeaPrep agarose (BioWhittaker Molecular Applications, Rockland, ME) was prepared at 4% (w/v) in ultrapure water. Equal volumes of protein-crosslinker solution and molten agarose solution were combined, which yielded a 2% stock agarose gel with appropriate collagen concentrations to yield the desired initial collagen concentrations ($[Col]_i$) of 30, 150, 300, and 600 $\mu$g/mL. Mixtures of agarose and protein-crosslinker were gelled at 4 °C for 20 min, after which they were exposed to UV light (306 nm, 25 W, 6300 mW/cm$^2$) for 20 min to mediate the photocrosslinking reaction and to sterilize the gels. Resulting gels were rinsed in sterile phosphate buffered saline (PBS, pH = 7.4) for 4 days to remove unreacted crosslinker and uncoupled protein. For control gels, Col was mixed with agarose according to the same procedures described, without the addition of SFAD.

Photoimmobilization of Col was verified via immunocytochemistry. Briefly, Col presence in Col-coupled agarose gels (referred to as "Col-AG"; $n = 7$) and agarose gels mixed with Col without crosslinker (referred to as "Col + AG"; $n = 6$) were compared to unmodified, agarose-only gels (referred to as "AG"; $n = 8$). Both Col + AG and Col-AG were at a concentration of 300 $\mu$g Col/mL, and where then exposed to the UV immobilization procedure and subsequently rinsed in PBS for four days. To assess the remaining Col in the agarose, samples were incubated with an antibody recognizing Col IV (AB748, 1:20, Chemicon, Temecula, CA) and then a fluorescently conjugated secondary antibody (Alexa 488, 1:500, Molecular Probes). Fluorescent intensity readings were collected using a microplate reader and were normalized to background readings attained from the AG samples. One-way ANOVA was performed followed by Tukey's pair-wise comparisons ($p < 0.05$ required for significance).

### *Rheological Characterization*

The complex moduli of AG, Col-AG, and Matrigel (referred to as "MG") were determined using a Bohlin CVO rheometer (East Brunswick, NJ). AG and Col-AG gels were cast and punched into discs (12 mm diameter × 1 mm thick) and loaded onto the rheometer. MG was added to the rheometer when fluid-like (~4 °C), and the base plate temperature was then increased to 37 °C to permit gelation. The viscous and elastic moduli were determined for AG (1.0–4.0%), Col-AG (1.5% AG at $[Col]_i = 30$, 150, and 300 $\mu$g/mL), and MG (7.5 mg/mL in Neurobasal medium) under low oscillatory shear strain (0.005) in a parallel plate configuration at 37 °C in an humidified environment. The stress response was measured, and the complex modulus was determined through a frequency sweep of 0.01–3.20 Hz (corresponding to the linear response range of the hydrogel). Matrix rheological properties were determined for AG (1.0%, $n = 4$; 1.5%, $n = 11$; 2.0%, $n = 4$; 2.5%, $n = 4$; 3.0%, $n = 3$; 4.0%, $n = 3$), Col-AG (1.5% AG at $[Col]_i = 30$, 150, and 300 $\mu$g/mL; $n = 4$, 7, 8, respectfully), and MG (7.5 mg/mL; $n = 4$). General linear model ANOVA was performed with agarose percentage, collagen concentration, and frequency as independent variables and complex modulus as the dependent variable, followed by Tukey's pair-wise comparisons ($p < 0.05$ required for significance).

### *Harvest and Dissociation of Primary Cortical Neurons*

All procedures involving animals conformed to guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Georgia Institute of Technology. All cell reagents were obtained from Invitrogen (Carlsbad, CA) or Sigma (St. Louis, MO) unless otherwise noted. Cortical neurons were harvested from Sasco Sprague-Dawley rat dams (Charles River, Wilmington, MA) at embryonic day 17. Following anesthesia with halothane (Fisher Scientific, Pittsburgh, PA) and decapitation, fetuses were removed by Caesarian section, and the cerebral cortices were isolated and rinsed in sterile $Ca^{2+}$- and $Mg^{2+}$-free Hanks Balanced Salt Solution (HBSS). Trypsin (0.25%, + 1 mM EDTA) was added for 10 min at 37 °C, then cortices were rinsed, and DNase (0.15 mg/mL with $MgSO_4$ (0.25 mg/mL) in HBSS) was added. The tissue was agitated using a vortex for 30 s, centrifuged at 1000 rpm for 3 min, and the pelleted cells were resuspended in culture medium (Neurobasal medium + 2% B-27 + 500 $\mu$M L-glutamine). Cell concentration and initial viability were determined using a hemocytometer and evaluating the exclusion of trypan blue.

### *3-D Primary Cortical Neuronal Cultures*

Neurons were cultured in 3-D within either unmodified agarose (AG), collagen-conjugated agarose (Col-AG), or Matrigel (MG) in custom cell culture chambers consisting of a glass coverslip base with lateral PDMS containment (Dow Corning, Midland, MI). Agarose gels (AG or Col-AG) were heated to 50 °C for 30 min to melt the agarose. Col heated to this temperature has previously been shown to return to physiological conformation upon returning to 37 °C.[18] The gels were then cooled to 37 °C and the appropriate volume of cell suspension was added to yield 5000 cells/mm$^3$. After gentle mixing, the cell and agarose mixture was transferred to each well and evenly spread out to create 500–750 $\mu$m thick cultures. Cultures were placed at −20 °C for 60 s and then at 4 °C for 15 min to ensure rapid and complete agarose gelation and 3-D cell entrapment, after which 0.5 mL of pre-warmed medium was added and each culture was placed in a tissue culture incubator (37 °C, 5% $CO_2$, 95% humidified air). MG cultures were plated by combining MG solution and cell suspension to yield 5000 cells/mm$^3$ in 7.5 mg/mL MG. After gentle mixing, the cell and MG mixture was transferred to each well and evenly spread out to create 500–750 $\mu$m thick cultures, and then placed in tissue culture incubator (37 °C, 5% $CO_2$, 95% humidified air) for 30 min to allow gelation of the MG, after which pre-warmed medium was added (0.5 mL/culture) and the chambers were returned to a tissue culture incubator. For AG,

Col-AG, and MG cultures, half of the medium was replaced every 2–3 days *in vitro* (DIV).

Culture viability was assessed using fluorescent probes for distinguishing live and dead cells (LIVE/DEAD Viability/Cytotoxicity Kit; Molecular Probes, Eugene, OR). Cell cultures were incubated with 2 $\mu$M calcein AM and 4 $\mu$M ethidium homodimer-1 at 37 °C for 30 min and rinsed in PBS. After viability/cytotoxicity staining, cells were viewed using a Laser Scanning Confocal Microscope (Zeiss 510; Zeiss, Oberkochen, Germany). For each culture, three to five *z*-stacks (460 $\mu$m × 460 $\mu$m × ≥100 $\mu$m thick) were acquired from randomly selected regions. Confocal images were viewed using LSM 5 Image Browser (Zeiss). Culture viability was assessed by quantifying the number of live and dead cells. Neurite outgrowth was calculated by counting the number of neurites (≥10 $\mu$m in length) departing somata from 12 to 24 randomly selected live neurons per culture.

Neuronal cultures were plated in unmodified AG and viability was assessed at 1 DIV (1.0%, $n = 4$; 1.5%, $n = 4$; 2.0%, $n = 4$; 3.0%, $n = 3$) and 8 DIV (1.0%, $n = 4$; 1.5%, $n = 4$). Using separate cultures, viability and neurite outgrowth were assessed at 2 DIV in neuronal cultures in AG (1.25%, $n = 3$), Col-AG (1.25%; $[\text{Col}]_i = 300$ $\mu$g/mL or 600 $\mu$g/mL, $n = 3$ each), and MG (7.5 mg/mL, $n = 3$). Culture thickness was measured; viability was quantified as a function of depth into the matrix (based on 100 $\mu$m thick sections from the top, middle, and bottom). The number of neurites per neuron and the percentage of live neurons extending neurites were counted. In other cultures, viability and neurite outgrowth were assessed at 7–8 DIV in AG (1.25%, $n = 4$), Col-AG (1.25%; $[\text{Col}]_i = 300$ $\mu$g/mL or 600 $\mu$g/mL, $n = 3$ and 4 respectively), and MG (7.5 mg/mL, $n = 6$). General linear model ANOVA was performed followed by Tukey's pair-wise comparisons when appropriate ($p < 0.05$ required for significance).

### *3-D In Vitro Model of TBI*

Neuronal cultures in Col-AG or MG were mechanically loaded using the 3-D CSD, a custom electro-mechanical device capable of reproducibly subjecting 3-D cell-containing matrices to high rate, simple shear deformation.[39] Cultures were plated in custom deformable cell culture chambers designed to interface with the 3-D CSD, and were deformed by the linear displacement of the chamber top plate with respect to the fixed cell reservoir base (Fig. 1a). Bulk shear strains up to 0.50 (45° shear angle) at strain rates up to 30 $s^{-1}$ simulate the spatial and temporal strain patterns associated with inertial TBI,[42] and result in heterogeneous 3-D strain fields throughout the cell-containing matrices dependent upon the initial cellular orientation within the matrix.[39]

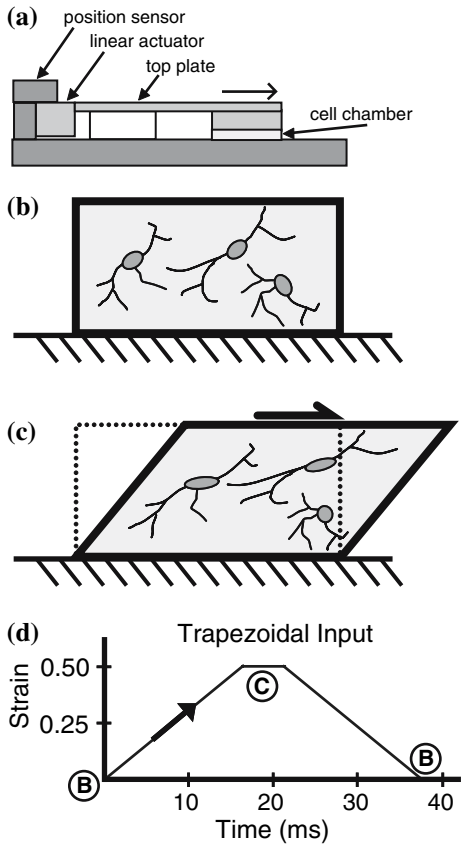


**FIGURE 1. (a) Schematic of our custom-built electro-mechanical 3-D Cell Shearing Device (CSD) interfacing with neuronal cultures. (b) Side view showing an undeformed 3-D neuronal cell culture in the cell chamber. (c) High rate deformation was imparted to the 3-D cell-containing matrices through the horizontal motion of the cell culture top plate while the culture base was held fixed. (d) Mechanical loading of the 3-D cell-containing matrices followed a symmetrical trapezoidal input to 0.50 shear strain at a 30 $s^{-1}$ strain rate, resulting in a 16.7 ms rise time and 5 ms hold time for the deformation. The points of no deformation (b) and peak deformation (c) are indicated on the loading profile.**

Neurons were plated in Col-AG (1.5%; $[\text{Col}]_i = 30$, 150, or 300 $\mu$g/mL) or MG (7.5 mg/mL). At 7 DIV, cultures were subjected to high rate loading (Col-AG: $n = 5$, 6, 7, respectively; MG: $n = 8$) or static control conditions (Col-AG: $n = 4$ each; MG: $n = 11$). At the time of loading, cell culture medium was removed from the culture, the top plate was gently mounted above the cell-containing matrices, and the chambers were loaded into the device. Neuronal cultures were coupled to the 3-D CSD and subjected to high rate deformation using symmetric trapezoidal input (0.50 strain, 30 $s^{-1}$ strain rate, 16.7 ms rise time, 5 ms hold time) (Fig. 1c, d) or left as static controls (0.00 strain, 0 $s^{-1}$ strain rate; Fig. 1b). After the deformation (or control conditions) was applied, the top plate was removed, fresh

medium was added (0.5 mL/well), and the cultures returned to the tissue culture incubator. Culture viability was assessed at 24 h post-insult. Using two-way ANOVA, matrix and loading condition were independent variables and viability was the dependent variable, followed by Tukey's pair-wise comparisons ($p < 0.05$ for significance). Furthermore, regression analyses were performed evaluating relationships between complex modulus, $[Col]_i$, and post-insult viability ($p < 0.05$ for significance).

## RESULTS

### *Col-AG Matrix Characterization*

The ability to chemically conjugate Col to an agarose backbone was assessed by evaluating the presence of Col in SFAD-conjugated agarose (Col-AG) in comparison to the presence of Col mixed with agarose without crosslinker (Col + AG). Immunostaining following the rinsing period revealed a statistically significant increase in the amount of Col present in the Col-AG vs. the Col + AG samples ($p < 0.05$) (Fig. 2). This demonstrated the effectiveness of the Col crosslinking reaction in coupling the Col to the agarose backbone. Additionally, the complex modulus was determined for AG (as a function of AG percentage), Col-AG (as a function of $[Col]_i$), and MG (7.5 mg/mL) under oscillatory shear strain for a range of frequencies where the material properties were linear. In agarose-only gels, the complex modulus increased proportionally with the AG percentage (data not shown), with values similar to those previously reported.[5,63] For a fixed AG percentage (1.5%), the complex modulus was determined based on $[Col]_i$ (ranging from 30 to 300 $\mu$g/mL), revealing that complex modulus increased as a function of $[Col]_i$, with significant differences between all groups with the exception of the Col(30 $\mu$g/mL)-AG vs. AG, which were statistically equivalent. Thus, over the frequency range evaluated, the order of complex moduli was Col(300 $\mu$g/mL)-AG > Col(150 $\mu$g/mL)-AG > Col(30 $\mu$g/mL)-AG = AG, thus revealing a dependence between $[Col]_i$ and complex modulus within the range of [Col] evaluated. Furthermore, the complex moduli were greater in all agarose hydrogels (AG and Col-AG) compared to Matrigel ($p < 0.001$) (Fig. 3).

### *Cell Culture Characterization*

Baseline characterization of cortical neuronal culture development in unmodified agarose was performed to gain insight into the requisite physical properties permitting survival and neurite outgrowth.

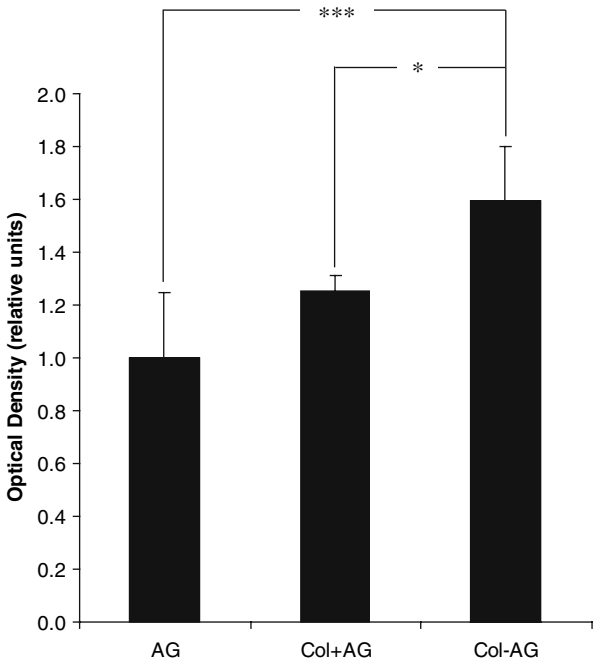


**FIGURE 2. Efficacy of the collagen-agarose crosslinking reaction was confirmed by comparing the presence of fluorescently-labeled collagen in SFAD-conjugated agarose (Col-AG) to the presence of fluorescently-labeled collagen mixed with agarose without crosslinker (Col + AG). There was a statistically significant increase in the fluorescence intensity in the Col-AG group vs. the Col + AG group (*, $p < 0.05$) and the AG group (***, $p < 0.001$). Data were normalized to background reading attained from unmodified AG (no Col) gels; data presented as mean ± standard deviation.**

This analysis was performed at 1 and 8 DIV in order to assess the immediate and longer-term effects of AG concentration, and hence physical properties, on cortical neuronal survival. Over the range of AG concentrations evaluated (1.0–3.0%), neuronal viability at 1 DIV depended significantly on agarose percentage (viability was 37 ± 4.5% for 1.0% AG; 60 ± 23% for 1.5% AG; 68 ± 14% for 2.0% AG; 82 ± 8% for 3.0% AG). Higher concentration, and hence stiffer, gels resulted in increased survival (1.0% AG vs. 3.0% AG, $p < 0.01$; 1.0% AG vs. 2.0% AG, $p < 0.05$). Additionally, a subset of these parameters were evaluated at 8 DIV, revealing that agarose concentrations ≤1.0% continued to yield minimal neurite outgrowth and poor survival (< 50%), whereas agarose concentrations of 1.5% resulted in viable cultures (~80%) with significant neurite outgrowth by this time point (Fig. 4). While early viability was maximal with the highest AG%, subsequent studies revealed that higher percentage agarose gels (> 2.0%) were not suitable for 3-D neurite outgrowth as the cortical neurons maintained a spherical morphology over longer time-points (data not shown). Thus, there was a range of matrix mechanical properties, based on a convergence of

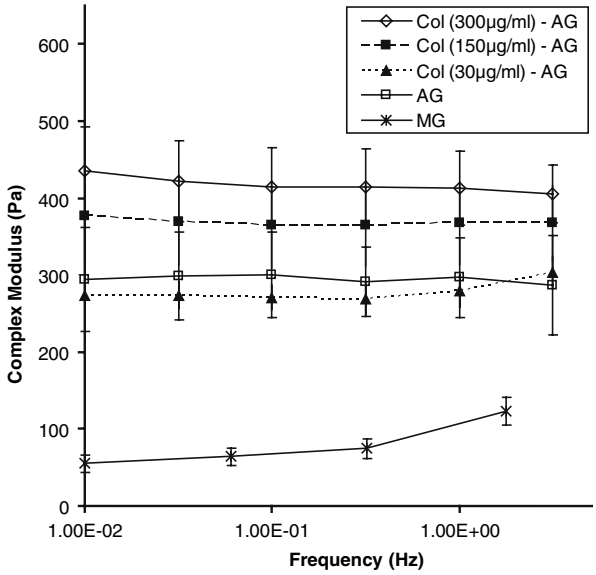


**FIGURE 3. The complex modulus was determined for Matrigel (MG), unmodified agarose (AG), and collagen-conjugated agarose (Col-AG) hydrogels. There were statistically significant differences between all groups with the exception of the Col(30 $\mu$g/mL)-AG and AG. The complex moduli were greater in all agarose hydrogels (AG and Col-AG) compared to MG ($p < 0.001$). In Col-AG samples, there was a correlation between increasing $[Col]_i$ and increasing complex modulus within the range of $[Col]_i$ evaluated. Data presented as mean ± standard deviation.**

hydrogel pore size (constrictivity) and sufficient mechanical integrity (increasing complex modulus), where cortical neurons were found to thrive in unmodified agarose. Based on these findings, AG percentages in the range of 1.25–1.50% were chosen for studies to optimize neuronal survival and neurite outgrowth as a function of $[Col]_i$.

Accordingly, neuronal viability and neurite outgrowth were evaluated in unmodified AG and Col-AG ($[Col]_i$ = 300 and 600 $\mu$g/mL) gels in comparison to a positive control MG matrix at 2 DIV (Fig. 5a–d). Viability did not vary based on $z$-position or based on collagen presence or concentration (Fig. 6); however, there were statistically significant increases in the percentage of neurons extending neurites as well as the mean number of neurites per neuron in MG and Col(300 $\mu$g/mL)-AG vs. AG ($p < 0.05$), indicating that neurite outgrowth in 3-D cultures is dependent on $[Col]_i$ at certain agarose concentrations (Fig. 7a, b). Furthermore, neurite outgrowth and neuronal viability were also evaluated at 7–8 DIV (Fig. 5e–h). At this time point, neurite outgrowth in Col(300 $\mu$g/mL)-AG was statistically equivalent to that of MG in terms of the percentage of neurons extending neurites and the mean number of neurites per neuron ($p > 0.50$) (Fig. 7a, b). However, neurite outgrowth was significantly reduced in AG ($p < 0.001$) and in Col(600 $\mu$g/mL)-AG ($p < 0.05$) compared to MG. It was also observed that the majority of somata in AG or Col-AG maintained a near spherical morphology, regardless of whether or not they extended neurites. Conversely, neurons cultured in MG developed a range of complex (non-spherical) morphologies with virtually all live neurons extending neurites. Neuronal survival, however, did not vary between Col(300 $\mu$g/mL)-AG, Col(600 $\mu$g/mL)-AG, and AG, and the viability in all these groups was statistically lower than that in MG ($p < 0.001$) (Fig. 8). Viability and neurite outgrowth studies revealed that 300 $\mu$g/mL was the threshold collagen concentration above which there was no added benefit to having more collagen present; in fact, neurite outgrowth decreased with more collagen. Thus we used a range of collagen concentrations less than 300 $\mu$g/mL for the shear deformation experiments, where the cells plated in this range had similar baseline viability and neurite outgrowth, thus isolating collagen concentration as an independent variable.

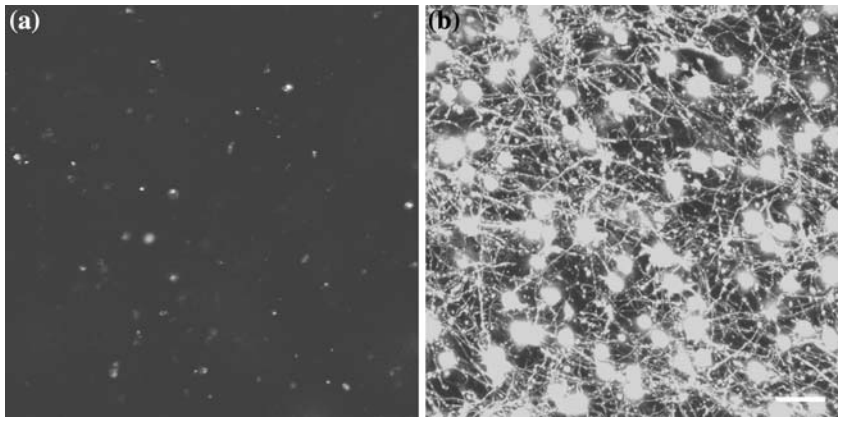


**FIGURE 4. Photomicrographs of calcein-AM stained neurons plated in (a) 1.0% AG and (b) 1.5% AG at 7–8 DIV. Neurons in 1.0% AG exhibited fewer neurite extensions and decreased viability compared to neurons plated in 1.5% AG. (scale bar = 50 $\mu$m).**

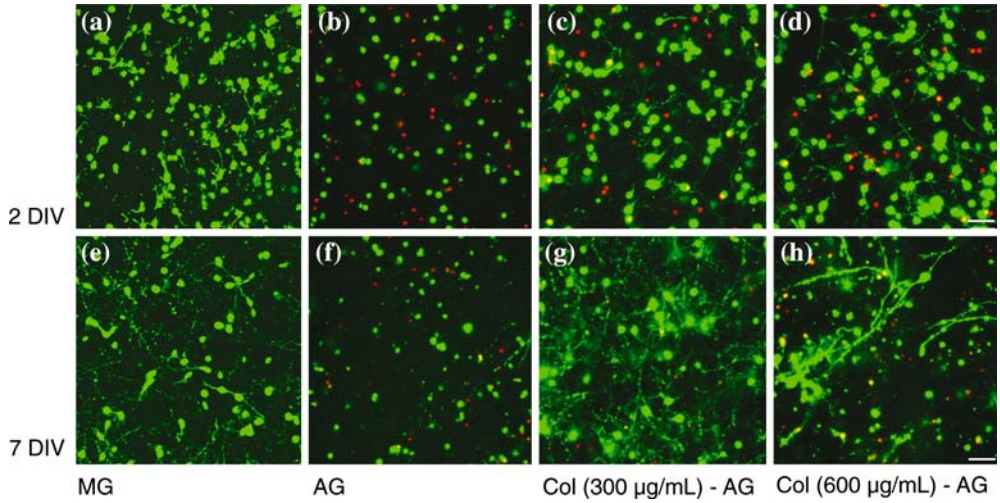


**FIGURE 5. Neuronal viability was evaluated neuronal cultures in (a, e) MG, (b, f) AG, (c, g) Col(300 $\mu$g/mL)-AG, and (d, h) Col(600 $\mu$g/mL)-AG at 2 DIV (a-d) and 7 DIV (e-h) by staining with calcein-AM (live cells stained green) and ethidium homodimer (dead cells stained red). (scale bar = 50 $\mu$m).**

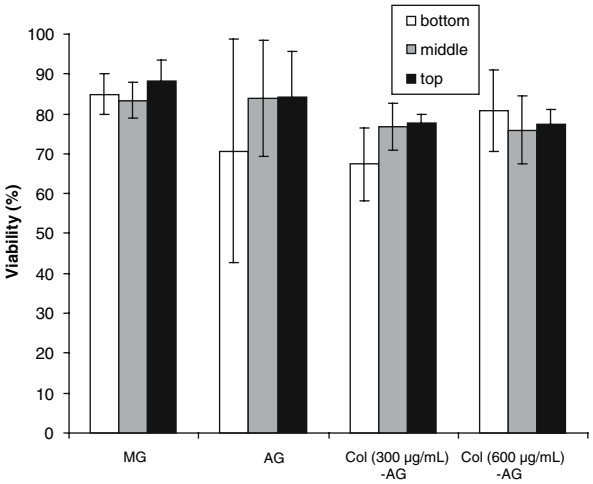


**FIGURE 6. Viability of neuronal cultures in MG, AG, Col(300 $\mu$g/mL)-AG, and Col(600 $\mu$g/mL)-AG did not vary at 2 DIV based on *z*-position or based on collagen presence or concentration. ($p > 0.05$).**

### *Response to Mechanical Loading*

To assess the roles of matrix mechanical properties and ligand density on the neuronal response to high rate deformation, 3-D neuronal cultures were plated in Col-AG ($[Col]_i$ = 30, 150, or 300 $\mu$g/mL) or MG and, at 7 DIV, mechanically loaded using the 3-D CSD (0.50 strain, 30 $s^{-1}$ rate) or subjected to static control conditions. Culture viability was used as the primary indicator of injury severity and was assessed at 24 h post-insult (Fig. 9). Neurite outgrowth and cell viability were similar in neuronal cultures over the range of $[Col]_i$ used; however, the viability in static control cultures was statistically lower in the Col-AG vs. MG cultures ($p < 0.001$). Post-insult neuronal viability depended on the matrix type ($p < 0.05$), mechanical loading ($p < 0.001$), and interactions between these factors ($p < 0.001$). Following loading in the MG cultures, there was a significant decrease in neuronal viability vs. MG static controls ($p < 0.001$). However, the response in the Col-AG cultures was dependent upon $[Col]_i$, as the viability in mechanically loaded cultures decreased as $[Col]_i$ increased. Specifically, the neuronal viability did not decrease relative to the respective static control at the lowest $[Col]_i$ evaluated (30 $\mu$g/mL); however, the viability was reduced compared to respective static controls for $[Col]_i$ of 150 $\mu$g/mL ($p < 0.05$) and 300 $\mu$g/mL ($p < 0.01$). The post-loading neuronal viability was similar in the Col(300 $\mu$g/mL)-AG and the MG cultures, potentially indicating a limit in injury-induced neuronal death based on the bulk loading parameters and time post-insult. Also, in order to account for differences in the baseline viability of the different static control groups, this analysis was repeated with the post-loading viability normalized to viability of their respective static control. The normalized post-insult viabilities of MG, Col(150 $\mu$g/mL)-AG, and Col(300 $\mu$g/mL)-AG groups did not statistically vary ($p > 0.2$); however, the normalized viability of Col(30 $\mu$g/mL)-AG was statistically higher than the other groups ($p < 0.05$). Thus, the post-insult reduction in culture viability was equivalent in MG compared to agarose cultures with higher $[Col]_i$, but not low $[Col]_i$.

Regression analyses were performed to evaluate relationships between the matrix mechanical properties and ligand density on post-loading neuronal viability. Post-insult viability significantly varied with $[Col]_i$ in

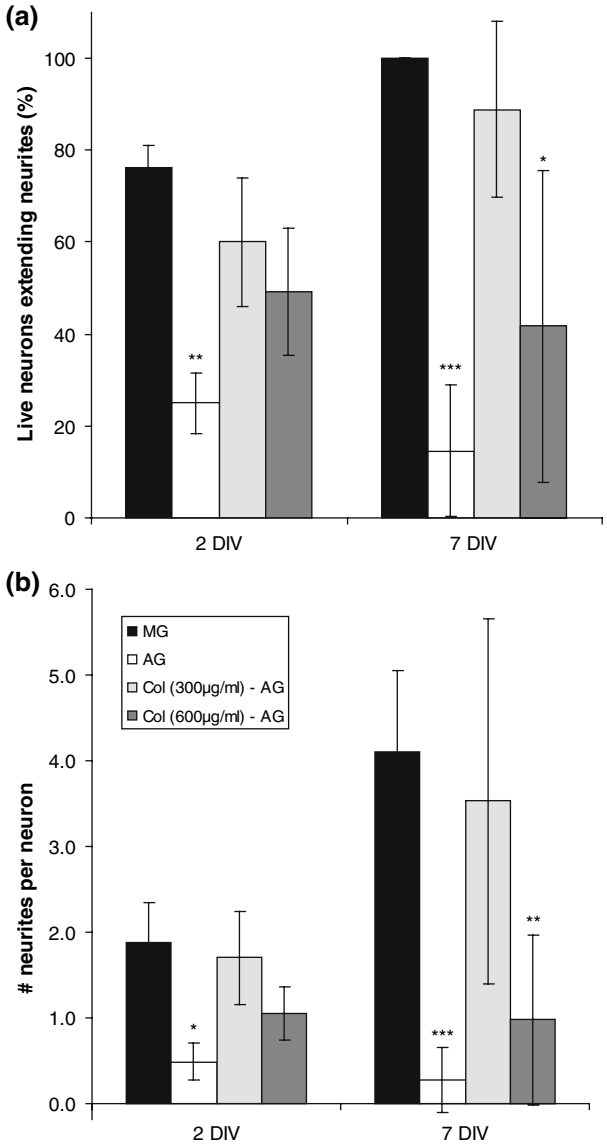


**FIGURE 7. Neurite outgrowth in neuronal cultures in MG, AG, Col(300 $\mu$g/mL)-AG, and Col(600 $\mu$g/mL)-AG varies with Col content. At 2 DIV there were statistically significant decreases in the (a) percentage of live neurons extending neurites and (b) the number of neurites per neuron in AG and Col (300 ug/mL) compared to MG (**, $p < 0.01$; *, $p < 0.05$); statistically there was no difference between MG, Col(300 $\mu$g/mL)-AG, and Col(600 $\mu$g/mL)-AG. At 7 DIV the (a) percentage of neurons extending neurites and (b) the number of neurites per neuron were statistically less than MG for cultures in AG (***, $p < 0.001$) and Col(600 $\mu$g/mL)-AG (*, $p < 0.05$; **, $p < 0.01$); statistically there was no difference in neurite outgrowth between MG and Col(300 $\mu$g/mL)-AG ($p > 0.05$). Data presented as mean ± standard deviation.**

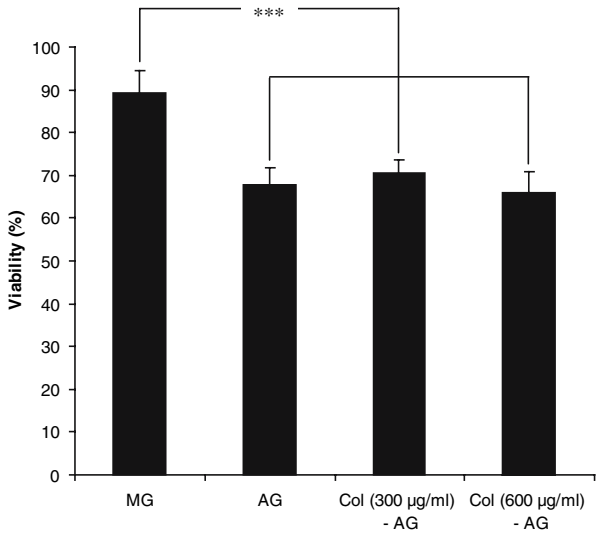


**FIGURE 8. The viability of 3-D neuronal cultures in MG, AG, Col(300 $\mu$g/mL)-AG, and Col(600 $\mu$g/mL)-AG was quantified at 7 DIV. There were no statistical differences in neuronal survival in between AG, Col(300 $\mu$g/mL)-AG, and Col(600 $\mu$g/mL)-AG cultures. However, the AG and Col-AG groups had significantly lower neuronal viability than MG cultures (***, $p < 0.001$). Data presented as mean ± standard deviation.**

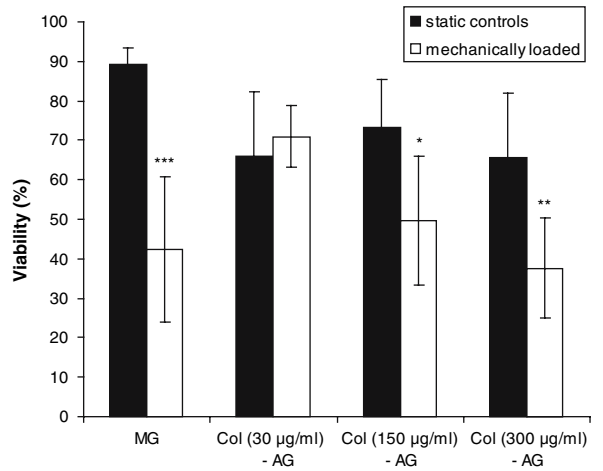


**FIGURE 9. The response to high rate deformation of 3-D neuronal cultures in Col-AG ($[Col]_i$ = 30, 150, and 300 $\mu$g/mL) was compared to the response in MG cultures. At 7 DIV, 3-D neuronal cultures were either subjected to static control conditions (0.00 strain, 0 $s^{-1}$ strain rate) or mechanically loaded (0.50 strain, 30 $s^{-1}$ strain rate), and culture viability was determined 24 h post-loading. The viability of static controls in Col-AG was statistically lower than in MG static controls ($p < 0.001$). Following high rate deformation, there was a significant reduction in culture viability in MG (***, $p < 0.001$) as well as in the Col(150 $\mu$g/mL)-AG (*, $p < 0.05$) and the Col(300 $\mu$g/mL)-AG (**, $p < 0.01$) cultures. However, in the lowest $[Col]_i$, Col(30 $\mu$g/mL)-AG, there was not a statistically significant decrease in viability following high rate deformation. Data presented as mean ± standard deviation.**

Col-AG cultures ($p < 0.001$), with a modest correlation coefficient ($R^2 = 0.53$) (Fig. 10a). Post-insult viability in Col-AG cultures also significantly varied with complex modulus ($p < 0.001$), and with a similar correlation coefficient ($R^2 = 0.56$) (Fig. 10b). These results indicate that just over half of the variability can be explained by either complex modulus or ligand density. Remaining variability may include that of the collagen concentration on a per culture basis, which was not factored into this analysis. Furthermore, these

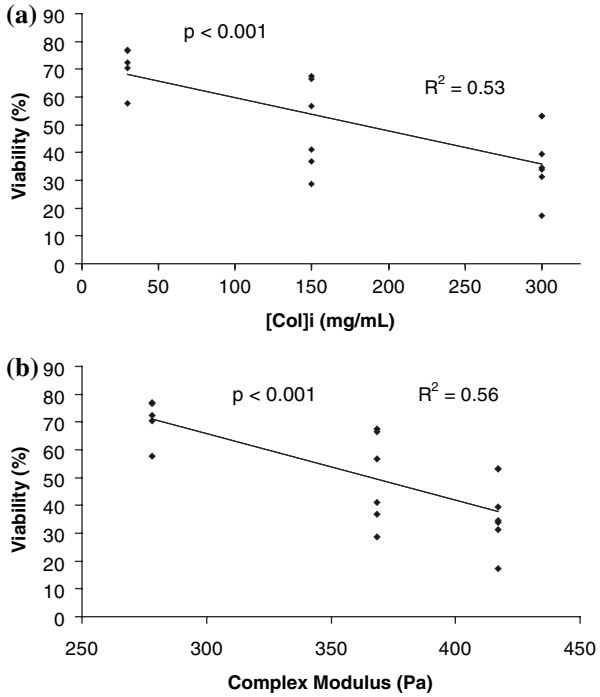


**FIGURE 10. Regression analyses were performed to assess the influence of matrix complex modulus and ligand density on the response to high rate deformation. In Col-AG cultures, decreasing post-loading viability scaled nearly linearly with (a) increasing ligand density as well as with (b) increasing complex modulus.**

individual trends became highly non-linear when the post-loading viability in MG cultures was added to the analysis based on the complex modulus of MG and the Col presence in MG,[35] indicating that complex relationships must be considered to describe post-insult viability under these conditions.

## DISCUSSION

We developed a 3-D culture system that can be exploited to analyze the effects of controlled matrix composition and bulk matrix mechanical properties on neuronal viability, neurite outgrowth, and the neuronal response to high rate deformation. This neurobiological system demonstrates the dependence of culture environment and architecture on neuronal function and has implications for the development of tissue equivalents for both *in vitro* and *in vivo* applications. Neuronal viability and neurite outgrowth were evaluated over a range of agarose and collagen concentrations in order to optimize culture parameters in this 3-D engineered system. In the acute period following cell seeding, AG percentage, and hence matrix complex modulus, dictated neuronal viability with softer AG gels resulting in reduced survival. The collagen content of Col-AG matrices significantly influenced the matrix complex modulus, and in these matrices at 2 DIV for a fixed AG percentage, neuronal survival did not vary based on $[Col]_i$, but neurite outgrowth was found to be enhanced at the mid-range $[Col]_i$ assayed. This trend continued at 7 DIV, with neurite outgrowth demonstrating a non-linear relationship with respect to $[Col]_i$, but survival not being influenced by $[Col]_i$. These results suggest that neuronal viability and neurite outgrowth in 3-D depend on interrelated biochemical and physical properties, with initial neuronal survival dependent on matrix complex modulus and neurite outgrowth varying with ligand density. Overall, optimized agarose percentages (1.25–1.50%) with a photoimmobolized ECM ($[Col]_i$ = 30–300 $\mu$g/mL) permitted development of thick, 3-D neuronal cultures with a network of interconnecting neurites throughout all spatial dimensions, with neurite outgrowth, but not survival, statistically equivalent to a positive control matrix.

It is desirable for 3-D culture development and tissue engineering applications to have a fundamental understanding of interactions between physical and biochemical matrix properties in the ability to support the viability and growth of cells. Parameters of neurite outgrowth such as growth rate and neurite branching depend on matrix mechanical properties (in 3-D and 2-D)[5,22] as well as ligand presence/density.[6,7,37,51,59] Neuronal survival and neurite outgrowth in 3-D matrices may be influenced by intrinsic (e.g., neuronal maturation, receptor expression) as well as extrinsic (e.g., matrix mechanical properties, ligand concentration) signals. For example, the effects of agarose concentration on DRG neurite outgrowth revealed matrix stiffness and pore size differentially influence the rate and degree of neurite extension, with maximal neurite outgrowth occurring in low concentration (< 1.00%) gels.[5,7,16] However, low concentration hydrogels have been shown to be unsuitable for the survival and neurite outgrowth of cortical neurons in the present study as well as previous work,[47] underscoring that different intrinsic mechanisms exist between different neuronal sub-types, and thus engineered systems must be optimized for a particular neuronal population. DRG neurite outgrowth was studied in collagen matrices of varying concentrations, and hence stiffness, finding that neurite extension was maximized in lower (0.6 mg/mL) rather than higher (2 mg/mL) concentration gels.[59] Taken together, these studies suggest that matrix stiffness may be a good predictor of neurite outgrowth in the absence of ligand presence.[5] However, the addition of significant ligand binding revealed a non-linear relationship between ligand concentration and neurite outgrowth in the present study, as well as previous work demonstrating intermediate ligand densities resulted in maximal DRG neurite extension

with higher densities inhibiting outgrowth.[51] Thus, optimized neurite outgrowth and survival for a particular neuronal subtype may require a specific range of ligand density, matrix complex modulus, and pore size, with optimized ranges for one factor potentially related to the state of other factors. Our model system is advantageous in that it is relatively simple and increasing levels of complexity (including other ECM ligands and additional cell types) can systematically be added to further manipulate culture properties.

Neuronal cultures in optimized Col-AG ranges were utilized to investigate the effects of matrix mechanical properties and ligand density on acute mechanotransduction events associated with high rate shear deformation (0.50 strain, 30 $s^{-1}$ strain rate). This 3-D model of traumatic neural loading may be suitable for the investigation of defined strain transfer to individual neurons and analysis of the roles of specific parameters in the subsequent pathology with a temporal and spatial resolution not currently possible *in vivo*. Specifically, given morphological and force transduction alterations between cells cultured in 2-D and 3-D, our system may better recreate complex, 3-D cellular biomechanics associated with traumatic loading *in vivo*, and may be more appropriate to model the effects of specific receptor-mediated interactions in acute mechanotransduction events and subsequent intracellular signaling following a defined mechanical input. The bulk mechanical properties of brain tissue vary based on the type of sample and the testing parameters, but it should be noted that the stiffness of the hydrogels presented in this study are comparable to that of native brains.[25]

In the current study, the post-loading viability in Col-AG neuronal cultures decreased with increasing $[Col]_i$ and with increasing complex modulus, indicating that both factors may work in concert to translate bulk shear deformation to cells. The physical properties of the matrix material, including complex modulus and pore characteristics (e.g., the mean pore size for 1.5% AG is 150 nm [7]) influence the degree to which cells were contained within the matrix material, and as such they may dictate whether forces deforming the gel (i.e. bulk shear deformation) were translated to and thus deform the cells within the gel. Additionally, receptor-mediated cell–matrix interactions may also influence the translation of forces from bulk matrix deformation to cellular deformation. Cell–ECM adhesions may increase the fidelity of deformation transfer from matrix to cellular deformation, and serve as areas where forces are acutely translated to create local stress concentrations. This stress distribution may influence the probability of structural failure, and resulting biophysical disruptions of the plasma membrane, for example, may alter ionic homeostasis,[24,40] initiating abnormal neuronal signaling and leading to cell death.[49] Additionally, cell surface receptor-mediated mechanotransduction may contribute to biochemical alterations following high rate deformation, possibly initiating neuronal dysfunction and/or death. While mechanotransduction is generally considered in a homeostatic context (e.g., cells relying on mechanical stimulation to sense environmental cues), it may also contribute to neuronal dysfunction by altering the balance of phosphatase/kinase activity[14,26,44] and activating $Ca^{2+}$-dependent proteases that degrade cytoskeletal elements.[21,31,57] Future studies will aim to determine the contribution of specific receptor-mediated cell–ECM interactions to altered, and potentially detrimental, intracellular signaling events following high rate loading.

This is the first report of a ligand concentration-dependent response to high rate mechanical loading in a 3-D configuration, demonstrating that both the biochemical and physical properties of the culture environment are important considerations. This 3-D cellular model of neural trauma is capable of systematically evaluating the role of cell–ECM interactions in the response to a well-defined deformation. An increased understanding of deleterious biochemical pathways initiated by acute mechanotransduction events may uncover potential therapeutic targets for prevention of widespread cellular dysfunction, death, and neurodegeneration following neural trauma. Furthermore, this system has several applications in neurobiology and tissue engineering, both of which require control over the extracellular culture configuration and related interactions in order to better mimic tissue composition and properties.

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