Effects of Environment Dimensionality in DRG Neurons Process Outgrowth: 3D Better Mimics *In Vivo* Features

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Statement of Purpose: Cells cultured in engineered 3D microenvironments better represent *in vivo* cellular behavior than cells cultured in 2D configurations; e.g., cells cultured in 3D scaffolds have been found to exhibit physiological growth, proliferation, gene expression and differentiation[1]. One of the fundamental differences between 2D and 3D culture is the distribution of cell-cell and cell-ECM interactions, which can alter signaling mechanisms regulating neuronal viability and neurite outgrowth. Previous studies with neurons cultured in 3D matrices have shown that neurite extension is dependent on the substrate physical properties, ligand concentration and geometry, indicating that neuronal growth and survival are improved by specific cell-matrix interactions that may be different in 2D vs 3D[2]. The purpose of this study is to determine the effect of environment dimensionality on neurite outgrowth. Our work has determined that process extension and branching of sensory neurons are altered in 3D culture in response to the recognition and adhesion of the neurons to their surrounding matrix, adapting morphological features that more closely mimics those occurring *in vivo*[3]. The results from this study will provide a foundation to design optimal biomaterials for the development of therapeutics for nerve repair and neurodegenerative disorders.

Methods: DRG neurons were isolated from E13.5-E14.5 mice according to established methods[3]. Dissociated neurons were seeded at $1.5x10^4$ cells/cm^2 onto 2D collagen coated coverslips (~7 µg/cm^2), on top of 2D collagen gels (1 mg/ml, thickness ~ 1.5 mm), and within 3D collagen (1 mg/ml) gels (20 µl) at $5x10^5$ cells/ml and cultured for 2 days. Cells were maintained in serum free medium (DMEM supplemented with 1X N2, 100 U/ml of Penicillin/Streptomycin, 20 mM of L-Glutamine and 50 ng/ml of NGF; Invitrogen). After fixation in 4% formalin, neurons were processed using immunohistochemistry for neuronal phenotypic markers, βIII-tubulin and NF160 (Sigma). For neurite outgrowth studies subsequent confocal imaging (Leica SP5) slices were reconstructed and analyzed using the Neurolucida system; Kruskal-Wallis statistical test was used to determine significance. Cell viability was analyzed using a Click-iT® TUNEL Imaging Assay (Invitrogen); significance was determined using a student t-test.

Results: DRG neurons clearly adapt their morphology to the characteristics of their environment. In 2D, the growth cones were large and rich in lamellipodia, and adhered to the substrate and flattened out, like in many other neuronal types for several 2D systems[5]. In 3D gels, the growth cones more closely resembled those *in vivo*: they were smaller, with a tipped shape, and had fewer filopodia and lamelipodia (Fig. 1).

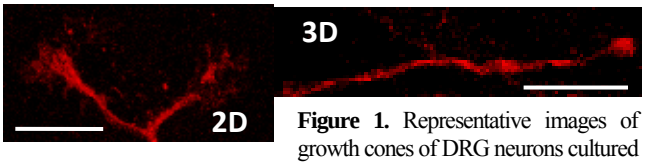

Figure 1. Representative images of growth cones of DRG neurons cultured in 2D and 3D collagen. Neurons are positively labeled for βIII-tubulin. Scale bars, 10 µm.

The branched structure analysis of the DRG neurons showed that neurite outgrowth and branching is altered between neurons cultured in 2D collagen surfaces or 3D collagen gels. Neurons cultured in 3D usually have a single primary neurite, and a shorter distance to the first branch, in comparison to the two neurites elongating from the neuron cell body in neurons cultured in 2D. These results indicate that over the same culture period, neurons cultured in 2D resemble primitive bipolar cells whereas in 3D they adapt a pseudounipolar morphology which is characteristic of later stages of *in vivo* embryonic development. Neurons cultured in 3D also have a longer average neurite length and show a more bifurcated structure than those cultured in 2D (Fig 2). No differences are observed in total neurite length. To test if these observations could be related to neuronal mechanosensing of the decreased stiffness of the gels, we cultured the neurons on top of soft 2D collagen gels. The number of cells extending neurites and the length of the neuronal projections were dramatically decreased in this setting. The cells typically adapt a bipolar morphology, as on collagen-coated glass (2D) and have even fewer branches (Fig 2).

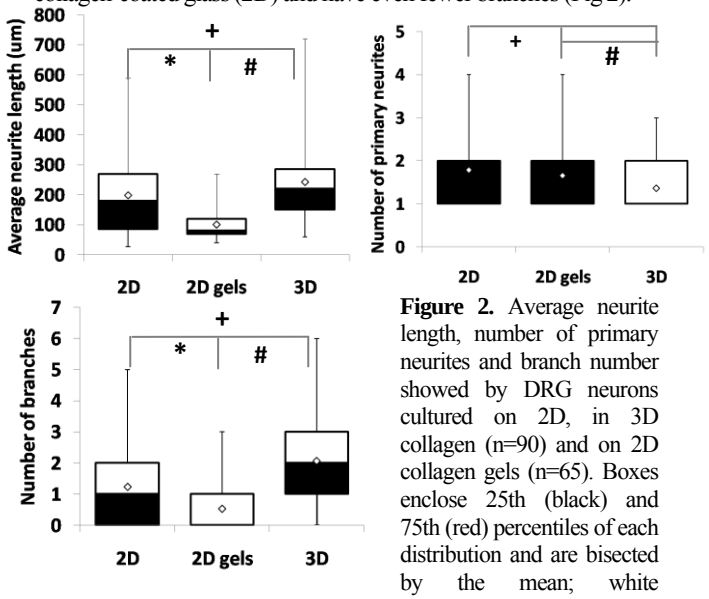

Figure 2. Average neurite length, number of primary neurites and branch number showed by DRG neurons cultured on 2D, in 3D collagen (n=90) and on 2D collagen gels (n=65). Boxes enclose 25th (black) and 75th (red) percentiles of each distribution and are bisected by the mean; white diamonds represent the mean; whiskers indicate the maximum and minimum. Symbols denote significant differences ($p<0.05$) between: *2D and 2D gels; #2D gels and 3D; + 2D and 3D.

Conclusions: We conclude that dimensionality plays a major role in DRG neuronal branching, growth cone morphology and outgrowth mechanisms. Neurons sense the three-dimensionality of their environment and respond by extending longer neurites, branching more and adapting a different morphology that when cultured in 2D; in sum, the morphology adapted in 3D more closely mimics neurons *in vivo*. The results from this study challenge the use of traditional 2D tissue culture for understanding the *in vivo* structure of neurons and the mechanisms involved in neuronal behavior in 3D environments.

References: 1. Irons, HR., et al., J Neural Eng. 2008, 5:333-341. **2.** Cullen, DK., et al., Annals of Biomed. Eng. 2007, 35:835-846. **3.** Ribeiro, A. et al, J. Neuroscience, 2010 (in prep). **4.** Banker, G, K Goslin. *Culturing Nerve Cells*. 1998, 2nd ed. MIT Press. **5.** Harris, WA et al., J. Neurosci. Research, 1985, 13:101-122.

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