The Influence of Glia on the Neuronal Response to Mechanical Loading *in vitro*

Ann Mae DiLeonardi, Erika A. Matheis, Karin A. Rafaels

I. INTRODUCTION

In vitro models of brain injury offer a high degree of experimental control compared to *in vivo* experiments. Many in vitro models include only neurons in culture, but neurons are surrounded by glial cells in the brain [1]. Including other cell types in co-culture can dramatically change the response of those cells [2], but the role glia play in mechanically induced trauma in 2D cell culture models has not been fully determined. Therefore, the aim of this study is to determine whether glial cells in co-culture with neurons influence the response of the neurons to mechanical loading.

II. METHODS

Preparation of Cell Cultures

Embryonic day 18 rat hippocampi (BrainBits, LLC., Springfield, IL) were incubated in feeding media (Neurobasal™ media, 2% B-27, 0.4mM GlutaMAX) containing 0.3 mg/ml trypsin and 0.2 mg/ml DNase I for 20 minutes at 37°C and then titrated in plating media (feeding media containing 5% heat inactivated fetal bovine serum) using Pasteur pipettes with progressively smaller tip diameters. Neurons were plated at approximately 100 k cells per ml on 35 mm diameter BioFlex plates (Flexplate Int. Corp.) treated with 0.01 mg/ml poly-l-lysine (PLL). The anti-mitotic agent Cytosine β-D-arabinofuranoside (Ara-C, 1µM) was added to some wells 24 hours post-plating to prevent growth of glia. Feeding media was replaced four days after the initial plating, then one to two times per week. Cells were maintained at 37°C, 5%CO^2 for 12 days *in vitro* (DIV).

Cell stretching

On DIV12, cultures were stretched by applying a pressure pulse that deforms the silastic membrane on which cells are grown using the Cell Injury Controller II (CIC, Custom Design and Fabrication). This input resulted in 37% strain at a strain rate of 7.4 s^{-1}. Cultures were imaged prior to stretch and immediately and 4 hours post-stretch using a zyla 4.2 SCMOS camera attached to an inverted Nikon Eclipse Ti-U microscope.

Fixation and immunocytochemistry

For fixation, cells were incubated in 4% paraformaldehyde for 15 minutes at room temperature, washed in cold 1X PBS and stored at 4°C until staining. Immunocytochemistry was used to differentiate cell type. Briefly, cells were permeabilized with 0.25% Triton X-100 for 10 minutes and blocked with 10% bovine serum albumin (BSA) for 30 minutes. Cultures were incubated with the 1° antibodies diluted in 1X PBS containing 1% BSA (GFAP & Tubulin) for 1 hour, followed by a 1 hour incubation with the 2° antibodies. Lastly, cultures were washed in 1x PBS and mounted to slides using Prolong Diamond.

III. INITIAL FINDINGS

Immunofluorescence of glia-free cultures revealed immunoreactivity for tubulin and DAPI indicative of the presence of neurites and cell bodies, respectively, but did not display immunoreactivity for glial fibrillary acidic protein (GFAP), suggesting there were no glial cells present in those cultures (Fig. 1A). In contrast, glia-containing cultures had immunoreactivity for tubulin, DAPI and GFAP, suggesting the presence of glial cells in those cultures (Fig. 1B). Phase contrast imaging of live cultures showed neurites from glia-free cultures did not appear as healthy as the cultures with glia; there was evidence of 'beading' along their lengths prior to stretch, neurites appeared thinner and there was evidence of seemingly free-floating debris (Fig. 1C) compared to their glia-containing counterpart, where neurons appeared to cluster around glia (Fig. 1D). Following stretch, neurites from glia-free cultures exhibited more beading and breakage immediately post-stretch (Fig. 1E), which only worsened at 4 hours

A. M. DiLeonardi and K. A. Rafaels (e-mail: karin.a.rafaels.civ@mail.mil; tel: 410-278-9459) are scientists in the Weapons and Materials Research Directorate of the U.S. Army Research Laboratory (ARL). E. A. Matheis is a contractor for Survice Engineering Company working at ARL.

post-stretch (Fig. 1G). In glia-containing cultures, neurons are distinguished by the halo appearance surrounding and neurites protruding from their cell bodies, while glia cells are distinguished by the dark dots observed within and mesh-like appearance surrounding their cell bodies (Fig. 1D). Interestingly, in the glia-containing cultures, while there is evidence of neurite loss at both the immediate and 4 hour post-stretch time points, there is also evidence of acute neurite beading (arrowheads, Fig. 1F), which resolved itself by 4 hours post-stretch (Fig. 1H).

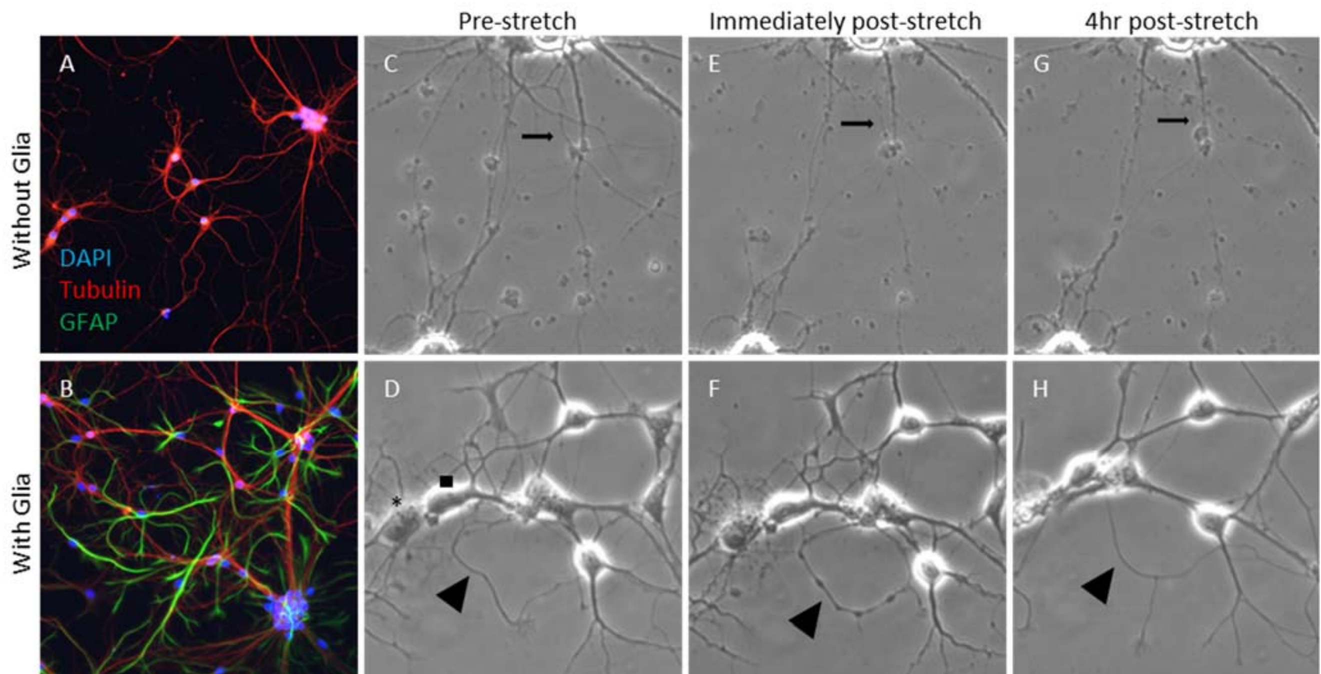

Fig. 1. Representative fluorescent images from cultures without glial cells (A) and with glial cells (B). DAPI stains the nucleus of all cells, Tubulin stains the processes of just neurons and GFAP stains only glial cells. Representative phase contrast images of glia-free cultures pre-stretch (C), immediately post-stretch (E) and 4 hours post-stretch (G). The arrow denotes a neurite that degenerates over the time course. Glia-containing cultures pre-stretch (D), immediately post-stretch (F) and 4 hours post-stretch (H). Asterisk is above a glia cell. Square is above a neuron. Arrow heads denote a neurite that displays beading and recovers over the time course.

IV. DISCUSSION

Our initial findings suggest that neurons are healthier in glia-containing cultures compared to their glia-free counterparts. In addition, we observed evidence of neurite recovery in glia-containing cultures following mechanical stretch *in vitro*. This finding is not surprising considering that glia offer support to neurons via release of neurotransmitters, secretion of trophic factors, synthesis and release of molecules to shape the extracellular matrix, among other functions [3]. Furthermore, glial cells play a role in neuronal-fate determination associated with disease or damage [4], as exhibited by reactive gliosis following mechanical trauma [5].

In culture, neurons prefer softer substrates, which glia can provide, as evidenced by other *in vitro* models growing neurons on top of a layer of astrocytes [6-7]. This model differs by allowing the neurons to adhere directly to the deformable membrane for better characterization of the mechanical loading on the neurons, while still obtaining some of the benefits of glia, i.e. reduced neurite breakage observed in our study. The soft, compliant environment of the glia before [7] and the increased compliance after [8] may help explain the positive influence of glia in culture.

Glia also serve as chemical ‘buffers’ for neighbouring neurons. Elevated intracellular calcium can precipitate cellular injury and death [9], but glia can buffer free calcium, reducing the load on the neurons [10], though this benefit may be reduced following injury [11]. We have yet to quantify damage to the glia at this strain level, but we did observe a change in the filamentous structure following mechanical stretch at later time points, as has been observed by others [8]. Future studies will quantify neurite beading and breakage in glia-free and glia-containing cultures, as well as alterations to glia as a function of mechanical injury.

V. REFERENCES

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