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Statistical study of biomechanics of living brain cells during growth and maturation on artificial substrates

La Chen, Wenfang Li, Vanessa Maybeck, Andreas Offenhäusser, Hans-Joachim Krause*

Institute of Bioelectronics (ICS-8/PGI-8), Forschungszentrum Jülich GmbH, 52425 Jülich, Germany

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ABSTRACT

There is increasing evidence that mechanical issues play a vital role in neuron growth and brain development. The importance of this grows as novel devices, whose material properties differ from cells, are increasingly implanted in the body. In this work, we studied the mechanical properties of rat brain cells over time and on different materials by using a high throughput magnetic tweezers system. It was found that the elastic moduli of both neurite and soma in networked neurons increased with growth. However, neurites at DIV4 exhibited a relatively high stiffness, which could be ascribed to the high outgrowth tension. The power-law exponents (viscoelasticity) of both neurites and somas of neurons decreased with culture time. On the other hand, the stiffness of glial cells also increased with maturity. Furthermore, both neurites and glia become softer when cultured on compliant substrates. Especially, the glial cells cultured on a soft substrate obviously showed a less dense and more porous actin and GFAP mesh. In addition, the viscoelasticity of both neurites and glia did not show a significant dependence on the substrates' stiffness.

1. Introduction

The growth and development of the brain tissue, which includes neuronal and glial cells, is a highly complex process involving various biochemical and biophysical interactions [1–3]. Mechanical interaction with the environment plays a key role in various important processes such as neural progenitor cell differentiation, neuronal migration, axon growth, brain folding, and foreign body reaction [4–7]. As one of the softest tissues in our body, the structural and mechanical properties of brain cells are assumed to be generated by cytoskeletal components and the arrangement of organelles. They are influenced by the substrate and the coupling between cells and extracellular matrix [8]. For example, neurons usually show robust actin filaments and enhanced actin protrusions on soft substrates, while the astrocytes tend to spread out on stiffer substrates, with their actin cytoskeleton more organized compared to growth on compliant surfaces [9]. The nucleus was found to be mainly responsible for the solid-like behavior and the stress-stiffening response of the neuronal soma, whereas neurofilaments have a predominant contribution in the viscous behavior of the neurites [10]. It is assumed that nerve cells stiffen during the early development stage [4]. As in Ref. [10], the elastic modulus of a neurite is mainly dependent on the microtubules and neurofilaments. The expression of individual microtubule-associated proteins (MAP) and neurofilament proteins were found to change during the development period from embryo to adult brain [11,12]. In astrocytes, the glial fibrillary acidic protein (GFAP) expression also changes with growth, and with a half-life time of approximately 7.5 days when cultured in a serum supplemented medium [13]. Even for the adult brain, the tissue viscoelasticity also exhibits some age-related change [14]. However, so far, there is still no detailed report on the variation of the mechanical properties of brain cells during growth and maturation on foreign bodies. Although there are lots of reports on how the matrix modulus regulates the neuronal cell differentiation, growth, and morphology [3,9,15], the dependence of the mechanical properties of living brain cells on the stiffness of the matrix has been rarely investigated.

Up to now, numerous techniques have been developed to characterize the mechanical properties of cells [4]. Because of its high spatial resolution and its topographic characterization functionality, atomic force microscopy (AFM) is one of the most popular tools applied in the characterization of brain cell mechanics [8,16,17]. Magnetic tweezers, one powerful tool in biophysics, have

* Corresponding author.
E-mail address: h.-j.krause@fz-juelich.de (H.-J. Krause).

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also been widely used in cell mechanics studies. Due to ease-of-implementation and high force, single-pole magnetic tweezers have been used to study the neuronal mechanics and to simulate abrupt injury force [10,18]. In contrast to AFM, much less effort is required to handle living cells in a magnetic tweezers experiment [8]. However, possibly because of the fragility and the highly heterogeneous structure of neuronal cells, magnetic tweezers have not been commonly used to characterize the neuron's mechanical properties as have been done for other types of cells such as fibroblasts and endothelial cells [19]. In addition, it has been frequently observed that the cell mechanics show a large variation from cell to cell, usually exhibiting a log-normal distribution in stiffness [20–22]. Most AFM and single-pole magnetic tweezers setups have the drawback of low throughput. Only one cell at a time can be measured, making it difficult to obtain enough data to evaluate the stiffness distribution, especially for highly heterogeneous conditions like networked neurons. So these methods may then be used in systems that restrict the cell, such as patterning, to achieve a more homogeneous sample population [10]. Recently we have implemented a multi-pole magnetic tweezers device which combines the virtues of high force, high throughput, and good maneuverability [22,23].

In this work, we present the application of our high throughput magnetic tweezers to study living brain cells. To gain new insight into brain cell mechanics, the statistical mechanical properties of living neuronal and glial cells have been characterized in detail during *in vitro* growth and culturing on substrates with different stiffness. These changes over time and the response to the mechanical properties of foreign materials are particularly important for the testing of materials or devices that may be implanted in the brain. Furthermore, the cell to cell variability uncovered by this study highlights the need for high throughput methods to test environmental influences on cell mechanics.

2. Materials and methods

2.1. Cell sample preparation

2.1.1. Cell culture and bead coating

Primary cortical-striatal co-cultures or mixed glia cultures were prepared from E18 Wistar rats. Cortices with striatum were isolated in ice cold Hanks Balanced Salt Solution without Mg^{2+} and Ca^{2+} (HBSS-, Gibco). Tissue was mechanically triturated approximately eight times in 2 mL HBSS- with a fire polished, silanized glass pipette until the cell suspension was homogeneously turbid. 4 mL HBSS+ (HBSS with Mg^{2+} and Ca^{2+}, Gibco) were added into the cell suspension. After incubation on ice for 3 min, the top 3 mL of the suspension (neuron cell suspension) were transferred to a new tube and subsequently the bottom 2 mL of the suspension (mixed glia suspension) were transferred to a separate tube. After a 200 g centrifugation (1100 rpm) for 2 min at 25 °C, neurons were resuspended in supplemented NB medium (10 mL NeuroBasal (Gibco), 100 μL B27 (Gibco), 0.25 mM Glutamax (Gibco), 500 μg gentamicin). Glial cells were resuspended in supplemented Dulbecco's Modified Eagle Medium (10 mL DMEM + F12, Glutamax (Gibco), 1 mL fetal bovine serum (Gibco), 100 μL non-essential amino acid (×100, Biochrom), 1 mM sodium pyruvate (Invitrogen), 500 μg gentamicin). Trypan blue staining was used to estimate viable cell density. 50 k –100 k cells were seeded on flame sterilized, poly-D-lysine coated (0.01 mg/mL PDL (Sigma) in Gey's balanced salt solution (Sigma), 2 h at 37 °C) cover slips in 24-well cell culture plate. Cultures were incubated at 37 °C, 5% CO_2. Half of the culture medium was changed every 3–4 days. Dynabeads® M-450 Epoxy superparamagnetic beads (Invitrogen) were coated with 25 μg/mL fibronectin according to the procedure recommended by the manufacturer. Before measurement, the fibronection-coated beads were diluted in DMEM and then added to each well (~1×10^5 beads per well). The plate was incubated for 30 min at 37 °C. The samples were rinsed twice with fresh warm DMEM to eliminate the unattached beads.

2.1.2. Preparation of soft substrates

Silicone base and curing agent (Sylgard 184, DOW Corning Corp.) were mixed thoroughly in ratio (m/m) of 10:1, 40:1, and 60:1. Clean glass cover slips were spin coated with these polydimethylsiloxane (PDMS) mixtures at a speed of 3000 rpm for 1 min, which resulted in a thickness of around 35 μm. After 1 h of settling to make the thin PDMS film on the cover slip flat, the curing procedure was carried out for 4 h at 80 °C followed by a slow cool down to room temperature, as given in Ref. [24]. The elastic modulus for samples PDMS10:1, PDMS40:1, and PDMS60:1 were 1697.9 kPa, 30.3 kPa, 1.7 kPa, respectively. To promote cell adhesion to the PDMS substrates, the samples were treated in oxygen plasma for 3 min. Afterwards, the samples were sterilized by UV light for half an hour. Then the protein coating and cell culture procedures were performed the same as mentioned for the common cover slip samples.

2.1.3. Immunofluorescent staining

The cell samples were washed three times with isotonic warm phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After washing the cells with PBS three times, the cell membranes were permeabilized with 0.3% Triton X100 (Sigma) in blocking buffer (BB, including: 50 mL PBS, 0.5 g bovine serum albumin, and 1 mL heat inactivated goat serum) for 10 min at room temperature. After washing with PBS three times again, BB was added and the samples were incubated overnight at 4 °C. The substrates were rinsed three times with PBS. The cell samples were incubated with primary antibody in BB in a dark chamber for 1.5 h. Then the substrates with cells were washed three times with PBS. The secondary fluorescent antibodies and DAPI were used together in BB. After 1.5 h incubation in the dark chamber, the substrates were subsequently rinsed with PBS and bidest water. For the detailed information of primary and secondary antibodies see Table S1 in the Supplementary Material. Finally, the cover slips were mounted on the object slides with mounting media and stored in the dark at 4 °C. The immunostained samples were examined using an Axio Imager 2 microscope which was equipped with 40×(EC Plan-Neofluar Oil DIC, N.A. 1.3) and 63×(Plan-Apochromat Oil DIC, N.A. 1.4) objectives (all of these were from Carl Zeiss AG).

2.2. Magnetic tweezers setup

The magnetic tweezers setup used in this work has been described in Refs. [22,23,25]. This magnetic actuator consists of a main hexapole yoke and a specific fluidic cell with magnetic tips. A magnetic field with high field gradient is generated in the workspace that is enclosed symmetrically by 3 sharp magnetic tips. These tips are made of FeCo–V alloy (VACOFLUX® 50, Vacuumschmelze GmbH & Co. KG) which owns a high saturation magnetization. With an effective workspace of 120 × 120 μm^2, forces up to 1 nN can be applied to the 4.5 μm Dynabeads® M-450 beads (see Fig. S1 in the Supplementary Material). By adjusting the coils' currents, both the force strength and direction can be controlled. In experiments, the cover slip with cells and magnetic beads was flipped upside down on the magnetic tips. Bright-field images of the cells and beads were taken by a scientific CMOS camera (Zyla 5.5, Andor Technology Ltd.) at a rate of around 30 frames/s. A water immersion objective (W Plan-Apochromat 40×/1.0 VIS-IR, Carl