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Glutamate affects dendritic morphology of neurons grown on compliant substrates

Michelle L. Previtera^{1,2} and Bonnie L. Firestein^{2,3}

¹Graduate Program in Molecular Biosciences, Rutgers, the State University of New Jersey, 604 Allison Road, Piscataway NJ 08854-8082

²Department of Cell Biology and Neuroscience, Rutgers, the State University of New Jersey, 604 Allison Road, Piscataway NJ 08854-8082

³Department of Biomedical Engineering, Rutgers, the State University of New Jersey, 604 Allison Road, Piscataway NJ 08854-8082

Abstract

Brain stiffness changes in response to injury or disease. Similarly, as a secondary consequence, glutamate is released from neurons and astroglia. Two types of glutamate receptors, N-methyl-D-aspartate (NMDA) and α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, sense mechanotransduction, leading to downstream signaling in neurons. Recently, our group reported that these two receptors affect dendrite morphology in hippocampal neurons grown on compliant substrates. Blocking receptor activity has distinct effects on dendrites, depending on whether neurons are grown on soft or stiff gels. In the current study, we examine whether exposure to glutamate itself alters stiffness-mediated changes to dendrites in hippocampal neurons. We find that glutamate augments changes seen when neurons are grown on soft gels of 300 or 600 Pa, but in contrast, glutamate attenuates changes seen when neurons are grown on stiff gels of 3000 Pa. These results suggest that there is interplay between mechanosensing and glutamate receptor activation in determining dendrite morphology in neurons.

Keywords

Glutamate; substrate rigidity; dendrite branching; dendritic arborization; hippocampal neurons; hydrogels; polyacrylamide

Introduction

In disease states and during repair, the overall stiffness of the brain changes. For example, brain stiffness decreases in Alzheimer's Disease¹, during demyelination², and after stroke³. In contrast, neurogenesis is thought to increase brain stiffness⁴. These changes have been detected by magnetic resonance elastography or ultrasound-based elastography in humans or

Corresponding author: Bonnie L. Firestein, Ph.D., Department of Cell Biology and Neuroscience and Graduate Program in Biomedical Engineering, Rutgers, the State University of New Jersey, 604 Allison Road, Piscataway, NJ 08854-8082; Phone: 848-445-8045; firestein@biology.rutgers.edu.

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murine models. Alterations in stiffness are detected by tissues, which then respond by altering their morphology and/or behavior^{5–6}. In the case of neuronal behavior, increasing the stiffness of fibrin gels (concentrations of 9.5 to 141 mg/ml) on which dorsal root ganglion (DRG) neurons are grown results in decreased neurite outgrowth⁷. It was also reported that there are higher cell densities for DRG neurons grown on intermediate stiffness polydimethylsiloxane (PDMS) substrates than found on more or less stiff substrates within a very small range⁸. Substrates that mimic the stiffness of the intact brain favor cortical neuronal growth over astroglial growth⁹, and softer substrates favor polarization of neurites in hippocampal neurons¹⁰. Furthermore, we previously reported that in hippocampal culture, stiffer substrates promote increased dendrite branching in neurons¹¹ while in spinal cord culture, dendrite extension increases when cells are plated on stiffer gels^{12–13}. These effects also depend on plating density¹⁴ and stiffness-dependent astrocyte growth and morphology^{9, 15}.

We recently reported that substrate stiffness-dependent effects on dendrite morphology are mediated by N-methyl-D-aspartate (NMDA) and α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors¹⁴. We used antagonists to show that these receptors modulate responses by hippocampal neurons to substrate stiffness. What remains to be seen is whether application of glutamate itself has any effect on the morphology of neurons and response to stiffness. This is important since secondary insults occur after the initial injury¹⁶. In specific, glutamate has been identified as the main neurotransmitter responsible for neuronal death after injuries, such as TBI^{16–18}. Understanding how changes to stiffness in the brain coupled with glutamate exposure alter the dendritic arbor will aid in our understanding of how to aid those post-TBI. Furthermore, studying this phenomenon will elucidate drug targets for the treatment of patients who have experienced a TBI.

Here, we performed a focused study to investigate how neurons respond to glutamate when grown on compliant substrates. Compliant substrates can be deformed by the cells on which they are grown versus non-compliant substrates, such as glass coverslips, which cannot be deformed by the cells. We first grew hippocampal neurons on substrates of different stiffnesses, and after growth of the dendritic arbor, we applied a sublethal concentration of glutamate to simulate secondary injury in the penumbra of the injury site. We found that the expression of total NMDA and AMPA receptors did not change in response to substrate stiffness; however, glutamate inhibits stiffness-promoted dendrite number on stiff but not soft gels. In contrast, glutamate increases dendrites when neurons are grown on soft gels.

Materials and Methods

Polyacrylamide (PA) gel preparation

Polyacrylamide (PA) gels were made as we previously described^{11, 14}. Briefly, gels were made with 7.5% acrylamide and 0.02, 0.03, or 0.1% N,N8-methylene-bis-acrylamide(bis) (both degassed) in 50 mM HEPES, pH 8.2. Gels were polymerized on Rain-X(Houston,TX)-treated glass coverslips and functionalized twice. The gels were coated with poly-D-lysine (Sigma).

Hippocampal neuron culture

Experiments were performed as previously described^{11, 14}. Briefly, neurons were plated onto 300, 600 and 3000 Pa gels. On day *in vitro* (DIV) 7 or 8, neurons were transfected with pEGFP for dendrite visualization using the calcium phosphate method¹⁹. On DIV 10, cultures were treated with vehicle control or with 10 μ M L-glutamate. Neurons were fixed on DIV 12, imaged at 200X using an Olympus Optical IX50 microscope (Tokyo, Japan) with a Cooke Sensicam CCD cooled camera, fluorescence.

Sholl analysis

Images were analyzed using the Bonfire dendrite counting program^{20–21}. The experimenter was blinded to the condition when performing dendrite analysis. Data shown are a combination of three different cultures (n=3).

Results

Glutamate has different effects on the dendritic arbor for neurons grown on soft versus stiff compliant substrates

Since we previously showed that glutamate receptors are involved in mediating morphological responses of neurons to substrate stiffness¹⁴ but did not show that glutamate itself can have an effect on the dendritic arbor of these neurons, we plated neurons on gels and treated them with vehicle or glutamate during the time of active dendritogenesis, DIV 10–12^{11, 14}. In line with our previously published reports^{11, 14}, we found that as substrate stiffness increases, the number of primary and secondary dendrites increases as well as the number of terminal and branch points (Figure 1A,B); these increases are most significant when neurons are plated on 3000 Pa gels. Interestingly, when neurons are grown on compliant substrates and treated with glutamate, the effects of stiffness on the number of primary and secondary dendrites are abolished (Figure 1A,B). In contrast, the effects of stiffness on the number of dendrite terminal and branch points are not abolished when neurons are grown on 3000 Pa gels and treated with glutamate. However, we observed effects on neurons treated with glutamate when the neurons are grown on soft substrates (300 and 600 Pa; Figure 1C). Furthermore, the number of dendrite branch points significantly increases for neurons grown on 600 Pa gels when they are treated with glutamate (Figure 1C).

Glutamate treatment eliminates differences in dendritic intersections when neurons are grown on 600 and 3000 Pa gels

Sholl analysis was performed to assess dendrite patterning of the overall arbor. Dendritic intersections are counted at concentric circles that are at a fixed distance from the cell body using semi-automated software developed in our laboratory^{20–21}. The number of intersecting dendrites is plotted versus distance from the cell body. Thus, we used Sholl analysis to assess the effects of glutamate on the entire dendritic arbor. As shown in Figure 1D, the number of dendritic intersections significantly increases when neurons are grown on stiffer surfaces. Furthermore, this increase occurs throughout the arbor, up to 200 μ m from the cell body. In contrast, when neurons are treated with glutamate, neurons grown on 3000 Pa gels

do not exhibit the same increase when compared with neurons grown on 600 Pa gels; however, neurons on both the 600 and 3000 Pa gels still demonstrate increased dendritic intersections when compared to neurons grown on 300 Pa gels (Figure 1E).

Glutamate treatment increases dendritic intersections in neurons grown on soft gels and decreases intersection in neurons grown on stiff gels

We next asked whether glutamate has different effects on the dendritic arbor, depending on the substrate stiffness on which neurons are grown (Figure 2). Quite interestingly, we found that glutamate increases dendritic intersections in neurons grown on the softest gels studied (300 Pa) and that glutamate decreases dendritic intersections in neurons grown on the stiffest gels studied (3000 Pa). In contrast, glutamate has no effect on dendritic intersections when neurons are grown on gels of intermediate stiffness (600 Pa). Thus, the overall arbor, as determined by Sholl analysis, is unaltered in neurons grown on 600 Pa gels although dendrite terminal and branch points increase, suggesting a local effect of glutamate on the arbor.

Discussion and Conclusion

Our previous work suggested that NMDA and AMPA receptors affect dendrite branching patterns in neurons grown on gels of differing stiffnesses¹⁴. Specifically, blocking NMDA receptors increases dendrites grown on soft gels, with moderate effects on distal arborization in neurons grown on stiffer gels¹⁴. Blocking AMPA receptors decreased distal dendrites in neurons grown on stiff gels but had little or no effects on neurons grown on soft gels¹⁴. In this study, we directly ask whether glutamate itself plays a role in modulating response to stiffness or whether additional glutamate confers additional effects. As shown in Table 1, the effects of glutamate differ greatly, depending on whether neurons are grown on soft versus stiff substrates. Our data suggest that glutamate augments stiffness-mediated changes in dendrites in neurons grown on soft (300 and 600 Pa) gels and attenuates stiffness-mediated effects in neurons grown on stiff (3000 Pa) gels.

How can glutamate mediate mechanotransduction effects? In the hippocampus, it is believed that mechanical injury, such as that which occurs in traumatic brain injury, disrupts the ratio of excitatory and inhibitory neurotransmission²², with glutamate being the primary excitatory neurotransmitter. Glutamate can then bind to the NMDA receptor, which can then mediate mechanotransduction²³, dependent on the receptor subunit composition²³. Furthermore, this mechanosensitivity is governed by serine 1323 in the carboxyl terminus of the NR2B subunit²⁴. AMPA receptors on cortical neurons also respond to stretch injury²⁵, and NMDA receptor and CaMKII activation are involved in this response²⁶. Increases in calcium-permeable AMPA receptors occur due to alterations in extrasynaptic, NR2B-containing NMDA receptors²⁷. Thus, there is precedence for glutamate acting as a modulator of mechanotransduction; however, glutamate has not yet been studied for its effects on neuronal morphology in concert with mechanotransduction.

Global concentrations in the brain may affect dendrites. Although debated, the concentration of glutamate in the brain is believed to be in the micromolar range^{28–33}. Synaptic cleft concentrations are thought to be on the order of 150–200 μM ³⁴. Our studies included

treatment of neurons with concentrations of 10 μ M glutamate, within the ambient concentration in the brain and at a much lower level than what we have determined to be neurotoxic in our cultures³⁵. Thus, even if glutamate levels are not released at toxic amounts after injury, ambient glutamate itself may modulate effects of the injury.

The fact that glutamate has different effects on neurons depending on substrate compliance is an important finding since brain stiffness is altered with injury^{1–3} and glutamate that is released after injury could be beneficial or detrimental, depending on the change in stiffness. Reactive gliosis often occurs after injury, resulting in softening of astrocytes and favoring of dendrite outgrowth after injury³⁶. Glutamate could aid in the regrowth of dendrites in this scenario. In contrast, although the brain softens in patients with Alzheimer's Disease¹, plaques and fibrils found in these brains are stiffer than neurons and glia^{37–38}, which may overcome the beneficial effects of soft substrates on dendrites. In general, white matter is stiffer than gray matter as measured *in vivo*³⁹; however, the corpus callosum and corona radiata are stiffer than other areas of white matter⁴⁰, which would translate to inhibition of dendrite outgrowth. Taken together, these studies suggest that local stiffness *in vivo* influences dendrite outgrowth whether in disease or during normal brain function. Thus, alterations of neuronal responses to glutamate depending on brain region and injury status in relation to brain stiffness should be taken into account when designing implants.

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Figure 1. Dendritic arbors of neurons grown on elastic substrates with vehicle or glutamate treatment

A. Representative images of neurons quantitated in Figures 1 and 2. Neurons are expressing green fluorescent protein, and images were converted to black and white and inverted. Scale bar = 100 μ m. **B.** The number of primary and secondary dendrites increases with substrate stiffness when neurons are grown on PA gels and treated with vehicle (control). Stiffness effects on neurons grown on 3000 Pa gels are abolished when the neurons are treated with glutamate. ***p<0.001 determined by one-way ANOVA followed by Dunn's Test for Multiple comparisons versus neurons grown on 300 Pa gels. Xp<0.05 determined by Mann-

Whitney Test when comparing treated to control neurons grown on the same PA gels. **C.** The number of dendrite branch and terminal points increases in neurons grown on stiff gels whether the neurons are treated with vehicle or glutamate. $**p<0.01$ and $***p<0.001$ determined by oneway ANOVA followed by Dunn's Test for Multiple comparisons versus neurons grown on 300 Pa gel. The number of dendrite branch and terminal points increases for neurons grown on 600 Pa gels when the neurons are treated with glutamate. $^Xp<0.05$ determined by Mann-Whitney Test when comparing treated to control neurons grown on 600 Pa gels. The number of dendrite branch and terminal points increases for neurons grown on 600 Pa gels when the neurons are treated with glutamate. $^Xp<0.05$ determined by Student's t-Test when comparing treated to control neurons grown on 300 Pa gels. **D.** Sholl analysis for neurons grown on various gels and treated with vehicle. **E.** Sholl analysis for neurons grown on various gels and treated with glutamate. Bars represent $p<0.05$ by Mann-Whitney Test for 600 (grey) and 3000 Pa (black) vs. 300 Pa. n values (number of neurons) for vehicle control: 300 Pa = 34; 600 Pa = 44; 3000 Pa = 49. n values for glutamate treatment: 300 Pa = 27; 600 Pa = 43; 3000 Pa = 44. Data are represented by mean $\pm$ SEM.

Figure 2.

Sholl analysis of neurons treated with vehicle (control) compared to neurons treated with glutamate for each stiffness. Graphs of Sholl analysis for neurons grown on PA gels. Dendrites proximal to the cell body show increased dendritic arborization when neurons are treated with glutamate and grown on 300 Pa gels; however, dendrites proximal to the cell body show decreased dendritic arborization when neurons are treated with glutamate and grown on 3000 Pa gels. Dendritic arborization remains unchanged on 600 Pa gels when neurons are treated with glutamate. Bars represent $p < 0.05$ by Mann-Whitney Test. n values (number of neurons) for vehicle control: 300 Pa = 34; 600 Pa = 44; 3000 Pa = 49. n values for glutamate treatment: 300 Pa = 27; 600 Pa = 43; 3000 Pa = 44. Data are represented by mean $\pm$ SEM.

Table 1

Effects of glutamate treatment on stiffness-mediated changes to dendrites in neurons grown on gels of indicated compliance.

	Primary Dendrites	Secondary Dendrites	Dendrite Branch Points	Dendrite Terminal Points	Sholl Analysis
300 Pa	No effect	No effect	No effect	Augments	Augments
600 Pa	No effect	No effect	Augments	Augments	No effect
3000 Pa	Attenuates	Attenuates	No effect	No effect	Attenuates