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Hippocampal networks on reliable patterned substrates

Michael D. Boehler[b,1], Stathis S. Leondopulos[b,1], Bruce C. Wheeler[c], Gregory J. Brewer[a,b,*]

[a] Department of Neurology, Southern Illinois University School of Medicine, Springfield, IL 62794-9626, USA
[b] Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School of Medicine, Springfield, IL 62794-9626, USA
[c] J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, Gainesville, FL 32611-6131, USA

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ABSTRACT

Toward the goal of reproducible live neuronal networks, we investigated the influence of substrate patterns on neuron compliance and network activity. We optimized process parameters of micro-contact printing for reproducible geometric patterns of 10 μm wide lines of polylysine with 4, 6, or 8 connections at a constant square array of nodes overlying the recording electrodes of a multielectrode array (MEA). We hypothesized that an increase in node connections would give the network more inputs resulting in higher neuronal outputs as network spike rates. We also chronically stimulated these networks during development and added astroglia to enhance network activity. Our results show that despite frequent localization of neuron somata over the electrodes, the number of spontaneously active electrodes was reduced 3-fold compared to random networks, independent of pattern complexity. Of the electrodes active, the overall spike rate was independent of pattern complexity, consistent with homeostasis of activity. Lower mean burst rates were seen with higher levels of pattern complexity; however, burst durations increased 1.6-fold with pattern complexity ($n = 6027$ bursts, $p < 0.001$). Inter-burst interval and percentage of active electrodes displaying bursts also increased with pattern complexity. The extra-burst (non-burst or isolated) spike rate increased 4-fold with pattern complexity, but this relationship was reversed with either chronic stimulation or astroglia addition. These studies suggest for the first time that patterns which limit the distribution of branches and inputs are deleterious to activity in a hippocampal network, but that higher levels of pattern complexity promote non-burst activity and favor longer lasting, but fewer bursts.

1. Introduction

The study of coding in neural networks depends on the availability of reproducible and reliable live neuronal networks. Toward the goal of creating these networks, we and others have confined the growth of neurons to reproducible geometric patterns (Branch et al., 1998; Chang et al., 2003; Singhvi et al., 1994; St. John et al., 1997; Vogt et al., 2005). This reductionist approach is predicated on simplifying the complexity of the intact brain and even the hippocampal slice. The problem is exemplified by the enormous success of studies of synaptic function and plasticity both in the slice and in cultured neurons, with relatively scant information on how these neurons compute in the network in which they are embedded. Since each rat hippocampal neuron averages 10,000 connections (Braitenberg and Schuz, 2004), we wanted to determine how important this number was to overall viability and function. Our reductionist approach rigorously constrained connections between separated neurons to 4, 6 or 8 other neurons, compared to the random or uncontrolled connections in a typical 2-dimensional culture.

Our long-term goal has been to create patterned substrates at (1) cellular resolution (≤10 μm) with (2) viability sufficient to proceed through development of synapses with robust network activity (3 weeks), and (3) localize neuron somata over substrate electrodes to be able to monitor the network. The first goal to manufacture patterned substrates at cellular resolution was achieved in previous research by either ultraviolet photoresist chemistries (Kleinfeld et al., 1988; Corey et al., 1996), laser ablation of uniform substrates (Dulcey et al., 1991; Corey et al., 1991; Ravenscroft et al., 1998) or PDMS rubber stamps (Singhvi et al., 1994; Branch et al., 1998; Wheeler et al., 1999). PDMS stamps that we and others have employed to create geometric patterns of neuron substrate adhesion material are easily mass produced and have the ability to transfer adhesion promoters to any surface, including electrodes.

Our second goal of viability beyond one or two weeks was achieved with the development of covalent bonding of adhesion

* Corresponding author at: Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School of Medicine, Springfield, IL 62794-9626, USA. Tel.: +1 217 545 5230; fax: +1 217 545 3227.
E-mail addresses: mboehler2@siumed.edu (M.D. Boehler), stathisleon@gmail.com (S.S. Leondopulos), bwheeler@ufl.edu (B.C. Wheeler), gbrewer@siumed.edu (G.J. Brewer).
[1] These authors contributed equally to this work.

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promoters to glassy substrates by silane chemistries. An essential attachment of functional amino groups such as poly-lysine or trimethoxy-silyl-propyl-diethylene-triamine (DETA) selectively promotes neuron viability on patterned substrates (Kleinfeld et al., 1988; Hickman et al., 1994). Additionally, background materials of neuron-repulsive silanes with terminal fluorines (Ravenscroft et al., 1998) or poly-ethylene glycol (Branch et al., 2001) improve long-term compliance at cellular resolution of <20 μm wide patterns. These techniques permit high compliance patterned growth of neurons beyond the 3 week minimum for which network activity is robust in random cultures over electrode arrays. Despite these advances, robust network activity has not been reported at pattern resolutions below 20 μm.

The third goal of robust activity of patterned networks requires positioning the higher current-source density of the neuron somata in close proximity to the electrode. We achieved this localization by creating cell-sized areas of larger adhesion (20–30 μm diameter). As a result, the somata migrated to these areas from narrower paths occupied by axons and dendrites (Corey et al., 1991). Our unpublished observations of little or no activity for patterned networks prompted us to determine whether silane adhesion or neuron proximity to the electrode was at fault. With silanized substrates and 40 μm wide patterns, activity was observed on only 25% of the electrodes with somata on or within 20 μm (Nam et al., 2004). Better electrode coupling of the patterned amine to the electrode with an epoxy-silane (Nam et al., 2004) enabled high levels of activity to be recorded on 40 μm wide patterns, but again failed to enable sparser networks on 10 μm wide patterns (unpublished observations).

One possibility for poor long-term performance of networks at cellular resolution evaluated here was the frequent observation of uneven densities of pattern material. Commonly, the stamp is immersed in a neuron-adhesive ink such as a solution of the common polypeptide, poly-D-lysine, followed by removal of excess ink without drying. Potential processing problems that we addressed were: (1) the uniformity of inking, both as clustering of ink pools and amount of ink carried by the stamp, (2) the uniformity and concentration of ink transfer after stamping, (3) retention of the ink on the substrate and (4) ink density that best supported neuron viability and growth. By optimizing these parameters, we reliably created geometric patterns with square nodes connected by 4, 6 or 8 lines of 10 μm width for correlating pattern complexity with network spiking characteristics relative to the more common random cultures without stamped patterns.

A further goal of this line of investigation was to plate neurons at a density low enough to enable the association of neuron function with microscopically visible pathways. At densities below 500 cells/mm^2, currents from most neuronal somata would not be close enough to our electrodes (30 μm electrodes at 200 μm spacing) to detect activity above noise. However, we expected to mitigate this problem by virtue of the tendency of somata to migrate to the nodes of square patterns (Corey et al., 1991), where the substrate area was larger than available on a 5–10 μm wide line. This approach also requires alignment of pattern nodes to substrate electrodes.

Because low density cultures were expected to produce low levels of spiking activity, we used three approaches that increase spontaneous and/or evoked activity in random cultures with densities of 500 cells/mm^2. First, we used NbActiv4 culture medium containing creatine, estrogen and cholesterol to enhance synaptogenesis (Brewer et al., 2008). In random, non-patterned cultures, these ingredients increase synapse density 2-fold and result in 4-fold higher spike rates. Second, cultures were stimulated daily during development as we had shown previously that chronic stimulation improved activity (Brewer et al., 2009; Ide et al., 2010). Stimulation of 1–3 h/day for 2 weeks increases spontaneous spike rates 2-fold after 3 weeks in culture and recruits up to 50% more active electrodes as well as doubling the spike frequency within bursts. Evoked responses also increase 50%/stimulus. Third, we added astroglia which we had shown was particularly beneficial to the non-patterned culture (Boehler et al., 2007). Separate cultures of astroglia, harvested and applied in numbers equal to the neurons not only increased neuron survival in non-patterned cultures, but doubled spontaneous spike rates, with even higher instantaneous rates evoked in response to glutamate. Therefore, we evaluated the ability of each of these enhancements of unpatterned networks to increase the activity of patterned networks.

Our results show important process improvements for increased reliability and functionality of geometric patterns from micro-stamping. These methods enabled neurons to maintain viability for the weeks needed to develop synapses and spiking activity. The work presented here is to our knowledge the first to systematically evaluate the effect of the number of connections within a patterned substrate on network activity. Characterization of the nature of the activity with pattern complexity indicated longer burst durations but no change in average channel spike rate. Without stimulation, non-burst spike rates increased with pattern complexity.

2. Materials and methods

2.1. Mold and stamp fabrication

Silicon wafers coated with a 10 μm layer of SU-8 2010 photoresist (Microchem, Inc., Newton, MA) were micro-fabricated into molds to match the structural patterns of arrays (Chang et al., 2003). Multiple replicates of each pattern were created on one mold. Square geometric patterns of 10 μm depth were created on 200 μm centers as 20 μm diameter nodes connected with 10 μm lines making either 4, 6 or 8 connections. Wafers were cleaned with an oxygen plasma for 20 s at 55 W, followed by baking for 1 min at 110 °C. A spin-coated 10 μm layer of SU-8 2010 was deposited by rotation at 500 rpm for 5 s followed by 3500 rpm for 40 s. The SU-8 was baked on a 65 °C hotplate for 1 min followed by 3 min at 95 °C and 1 min at 65 °C. The mold was exposed to UV light for 26 s at 5.8 mW/cm^2 and baked as before. The pattern was developed in SU-8 developer for 2 min with mild agitation every 10–15 s. The pattern was cleared with a rinse of fresh SU-8 developer followed by a thorough rinse with 2-propanol and blown dry with nitrogen. To promote release of the stamps, the mold was silanized with 100 μl tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorsilane (United Chemical Technologies, Bristol, PA) under vacuum. Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) stamps were created by mixing the elastomer with curing agent at a 10:1 ratio, degassed and poured onto the mold, bounded by 1.2 mm diameter glass tubes to set the thickness, then cured overnight at an optimal temperature of 37 °C. In previous protocols elastomer mix was cured overnight at 60 °C which caused shrinkage of patterns. Cured PDMS slabs were removed from the mold and cut into 1 cm × 1 cm. A blank 12 mm × 12 mm coverslip (Fisher) was cleaned for 3 min with an O_2 plasma. The patterned stamp was attached to the cleaned slip as a backing substrate within 10 min of curing. Unused stamps were stored at room temperature.

2.2. Stamp surface modification and alignment

Fresh cut stamps were used for each experiment. All drying steps began with a stream of filtered nitrogen. All water was deionized to 18 MΩ. The stamp was first soaked stamp side up in 10% sodium dodecyl sulfate (SDS, Fisher) solution for 20 min (Chang et al., 2003) to bind hydrophobic sites on the PDMS and create a thin anionic film to electrostatically bind the cationic polylysine. Stamps were