

Spatial Confinement Instigates Environmental Determination of Neuronal Polarity

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MATERIALS AND METHODS

Gold-coated coverslips, master fabrication, microcontact printing with hexadecanethiol, and backfilling with amide-linked glycol-terminated alkanethiol were performed as previously described.^{1,2}

CD-1 timed pregnancy mice were sacrificed and E18 hippocampus pairs were dissected (Charles River Laboratories, Wilmington, MA). E18 mouse hippocampi were stored in Hibernate E (BrainBits, LLC, Springfield, IL) and later dissociated using a GentleMACS system in conjunction with the Neural Tissue Dissociation Kit (P) and Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA). Mouse Laminin I (Trevigen, Gaithersburg, MD) was deposited onto patterned substrates at 24 µg/mL for 1 h prior to plating. Neurons were seeded onto patterned substrates in Neurobasal medium supplemented with 2% B27 supplement, 1% Glutamax, 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA). Cells were stored at 37 °C, 5% CO₂. After four days *in vitro*, cultures were subjected to 0.02% saponin extraction as previously described followed by fixation in a 3.7% paraformaldehyde/PHEM buffer and permeabilization with 0.1% Triton X-100.³ Substrates were blocked in 10% normal goat serum (Invitrogen, Carlsbad, CA) and neurons were stained using anti-tau, clone 5E2 antibody (Millipore, Temecula, CA), AlexaFluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA) AlexaFluor 594 phalloidin (Invitrogen, Carlsbad, CA), and anti-laminin antibody (Millipore, Temecula, CA) which was conjugated to AlexaFluor 350 carboxylic acid, succinimidyl ester (Invitrogen, Carlsbad, CA). Coverslips were subsequently mounted using ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, CA). Fluorescent images were obtained using a Nikon TE2000-PFS microscope running NIS-Elements imaging software and equipped with an EXFO X-Cite UV illuminator and Photometrics CoolSNAP camera.

Images were analyzed and individual neurons were reported as containing a single axon, multiple axons, or no axon. Single axon, multiple axons and no axon classifications were similar for both patterned and non-patterned hippocampal neurons at day 4 (Table 1).

	Non-Patterned Neurons	Patterned Neurons
One Axon	71.79%	85.24%
Multiple Axons	17.95%	10.00%
No Axon	10.26%	4.76%
Sample Size	39	210

Table 1. Neurons were characterized according to the number of neurites staining positive for tau. Both patterned and non-patterned neurons were saponin extracted, fixed and stained at day 4.

Those with a single axon were classified as growing on either the long path or short path. Data was collected and analyzed for each starburst pattern. Graphs were plotted and R^2 and p-values were calculated using KaleidaGraph 3.6 (Synergy Software) and Prism 5 (GraphPad Software, Inc.).

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