Supporting Information

Tang-Schomer et al. 10.1073/pnas.1324214111

SI Materials and Methods

Silk Scaffold and ECM Gel Preparation. Silk solution was prepared from *Bombyx mori* cocoons as outlined previously (1). Porous scaffolds were made by a salt-leaching method as previously described (2, 3). To generate interlocking modular structures as the jigsaw puzzle process, a biopsy punch (McMaster-Carr) with concentrically arranged rings was used to punch out layered donut shapes. These structures were separated to prepare for cell seeding and reassembled into a composite structure with concentric rings. The scaffolds were autoclaved, coated with poly-L-lysine (10 μg/mL) for 1–2 h, washed two times with PBS, and dehydrated slightly before cell seeding. On adding concentrated dissociated cortical neuron suspension (20–30 million cells/mL), the scaffolds were saturated and immersed for 24 h. Scaffolds were washed extensively to remove unattached cells and prepared for cell culture.

Collagen gel was prepared from rat tail type I collagen I (3–4 mg/mL; BD Biosciences), 10× 199 media (Gibco), and 1 M NaOH by mixing at a ratio of 88:10:2. Fibrin gel was prepared by mixing fibrinogen (20 mg/mL; EMD Millipore) and thrombin (10 U/mL; Sigma) at a volume ratio of 2:5. Marigel was purchased from BD Biosciences.

To make a scaffold gel composite structure, the scaffolds were washed with media two times followed by washing with ECM gels in liquid form to replace the media within the scaffold. The composite structure was incubated at 37 °C for 30 min before media immersion for long-term culture.

Primary Cortical Neuronal Culture in 2D, Collagen Gels, and 3D Brain-Like Tissues. The brain tissue isolation protocol was approved by the Tufts University Institutional Animal Care and Use Committee and complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institutional Animal Care and Use Committee B2011-45). Cortices from embryonic day 18 (E18) Sprague–Dawley rats (Charles River) were isolated, dissociated with trypsin (0.3%; Sigma) and DNase (0.2%; Roche Applied Science) followed with trypsin inhibition with soybean proteins (1 mg/mL; Sigma), centrifuged, and plated at 1–4 million cells/scaffold in NeuroBasal media (Invitrogen) supplemented with B-27 neural supplement, penicillin/streptomycin (100 U/mL and 100 μg/mL), and GlutaMax (2 mM; Invitrogen).

For control cultures used for viability assays, cells were plated at 1,300, 1,900, 2,500, and 3,100 cells/mm^2 in 2D cultures (corresponding to 40,000, 60,000, 80,000, and 100,000 cells/well of a 96-well plate, respectively) and 400, 2,000, and 8,000 cells/mm^3 in collagen gels (corresponding to 20,000, 100,000, and 400,000 cells/50 μL gel, respectively).

For control cultures used for axon length measurement, cells were plated at 20, 2,000, and 20,000 cells/mm^3 in collagen gels (corresponding to 1,000, 10,000, and 1 million cells/50 μL gel, respectively). For collagen gel-based cultures for PCR and local field potential (LFP), cells were plated at 8,000 cells/mm^3 (corresponding to 2 million/250 μL or 4 million/500 μL gels).

For 3D scaffold-based systems, the scaffolds were immersed in high-density cortical cell suspensions (20–30 million cells/mL) for 24 h followed by extensive washes with media and subject to scaffold-only cultures or gel-infused composite structure-based cultures. Cell densities in 3D scaffold-based systems were determined by DNA content (Fig. S3). The media was changed one time per week for all culture systems.

Confined Compression Test. An Instron mechanical tester (Instron 3366) was used for confined compression testing. A customized chamber was machined using aluminum stock and stainless steel porous discs (40-μm pore size; McMaster-Carr). A stress–relaxation test was performed. Briefly, the sample was compressed stepwise at 5% of its height and relaxed for 500 s to allow for equilibrium to be established as secreted water flowed out through the porous disk. The compression–relaxation was repeated for 2 h, and the load-time diagram was recorded. The compressive modulus (or aggregate modulus) was calculated as the linear slope of the minima at equilibrium.

The aggregate modulus was used to approximate the Young modulus based on the formula $Ha = E(1-\nu)/[(1+\nu)(1-2\nu)]$, where Ha is the aggregate modulus, E is the Young modulus (or elastic modulus), and ν is the Poisson ratio, which is given as the ratio of the lateral deformation vs. the axial deformation: $\nu = \Delta L(lateral)/\Delta L(axial)$. In the confined compression test, the lateral deformation was small; therefore, ν was negligible, and the aggregate modulus was approximate to the Young modulus.

Rat and mouse cortical tissues were dissected from adult animals (Institutional Animal Care and Use Committee B2011-45), stored in chilled PBS, and tested within 1 h of animal euthanasia. All tissue samples were trimmed into 5-mm-diameter, 2-mm-height discs for mechanical testing.

Immunocytochemistry. Cells were fixed with 4% (vol/vol) paraformaldehyde (Fisher Scientific) for 20 min, washed, and permeabilized with 0.1% Triton X-100 (Fisher Scientific) including 4% (vol/vol) goat serum (Sigma) for 20 min followed with incubation of primary antibodies overnight at 4 °C. After three 10-min washes, cells were incubated with secondary antibodies for 1 h at room temperature followed by extensive washes. Antibodies included anti–β3-tubulin (rabbit, 1:500; Sigma), anti-GFAP (mouse, 1:1,000; rabbit, 1:500; Sigma), antimicrotubule-associated protein-2 (rabbit, 1;500; Sigma), and goat anti-mouse or -rabbit Alexa 488 and 568 (1:250; Invitrogen) secondary antibodies. Fluorescence images were acquired on a Leica DM IL fluorescence microscope using excitation/emission (Ex/Em) of 470/525 nm for Alexa 488 and Ex/Em of 560/645 nm for Alexa 568.

Three-Dimensional Confocal Imaging and Image Analysis. A Leica TCS SP2 (Leica Microsystems) confocal microscope was used to capture the source image stacks. The Fiji ImageJ Simple Neurite Tracer plugin was used for 3D axon tracing and measurements (4). Briefly, a start point and an endpoint of an axon in the 3D stack were picked by the user. The tracer program began searching for a path between these two points by creating a series of expanding spheres along each pixel with a light intensity greater than zero. A line was formed when the edges of two spheres intersected, and the automatic tracing continued until a complete axon path was constructed of all of the individual intersection center points.

LFP Measurements. LFPs were recorded with a custom-built recording probe consisting of two parallel tungsten electrodes (50-μm-diameter tip and 250-μm separation) with the signal electrode positioned to record local activity in the 3D culture systems. Recordings were conducted in Hepes-buffered artificial cerebrospinal fluid (CSF) consisting of 125 mM NaCl, 5 mM KCl, 5 mM Glu, 10 mM Hepes, 3.1 mM $CaCl_2$, and 1.3 mM $MgCl_2$ titrated to pH 7.4 using 1 M NaOH and supplemented with 10 mM fresh glucose before use. Signals were amplified with an amplifier (AM-Systems), filtered at 0.1 Hz to 10 kHz, and digitized at 50 kHz. Acquisition was made using Clampex, version 9.2 (Molecular Devices). For static cultures, baseline signals were obtained for 10 min before

drug application. A 10-μL droplet of 1 mM tetradotoxin (~20 μM final concentration) was applied near the electrodes. After the signal stabilized in ~1 min, recording was resumed for another 10 min. For the impact experiments, the electrode was removed during and repositioned after the weight drop to prevent damage from the impact. The postinjury recording contained an ~2-min delay to exclude artifacts associated with reinsertion.

The Clampfit (Molecular Devices) program was used for analysis. Power spectra were obtained by fast Fourier transformation average of a rectangular window over a time period of either 10 min (for cumulative effects) or 27 s (for time-dependent evolution analysis). To plot time-evolved changes, a 10-min trace was divided into 22 segments ($t0$–$t21$) with a window size of 27 s. The total power (millivolts^2) at each time point was calculated as the sum of the 0- to 50-Hz power spectrum (millivolts^2 per hertz). The average of these spectral segments represented the average total power of a 10-min trace.

Impact Injury of 3D Cultures. A custom-made weight-drop setup (a 10.9-g screw as the weight and a hollow tube with an ~15-mm i.d. for the height) was used for impact injury [modified from the model by Marmarou et al. (5)]. The test sample was replaced into artificial CSF solution (1 mL) before injury and situated beneath the tube. On weight drop, the sample was immediately removed. Solution samples were collected (100 μL) before and after injury for liquid analysis. Calculation of compressive distance (x) was based on Newton's gravitational formula $mgh = 1/2kx^2$, where m is the weight (0.0109 kg), g is the gravitational constant (9.91 m/s^2), and h is the height (0.095, 0.19, and 0.38 m); k is the spring constant given as the Young modulus (E) multiplied by the original area (A_0) divided by the original length (L_0) of the test sample. For this study, the aggregate modulus was given to proximate the Young modulus (E) as described above.

Tandem Liquid Chromatography/MS Analysis. MS analysis of neurotransmitters was performed on a triple quadruple mass spectrometer (3200Q TRAP LC/MS/MS system; MDS Inc.) using an aminopropyl column (Luna NH_2; Phenomenex) (6). The gradient method used Solvent A, a 95:5 water/acetonitrile solution containing 20 mM ammonium acetate and 20 mM ammonium hydroxide (pH 9.5), and Solvent B (acetonitrile). Solution samples were mixed with 1,000 ng ^{15}N-labeled Glu (Sigma) and run with a flow rate of 150 μL/min with gradient t = 0 min, 85% (vol/vol) B; t = 15 min, 0% B; t = 28 min, 0% B; t = 30 min, 85% (vol/vol) B; and t = 40 min, 85% (vol/vol) B. Positive ionization mode was used to detect Glu ($C_5H_{10}NO_4^+$; Q1 mass = 148.14; Q3 mass = 84). Glu standards were used to obtain their characteristic retention time. For quantification, signals were integrated from peaks centered at the retention time. The ratio of the peak of the signal of interest over that of the internal control was calculated and converted to concentration.

Gene Expression Analysis. RNA was extracted from 2D cultures (1 million cells/well of a six-well plate at ~1,900 cell/mm^2), collagen gel-based cultures (8,000 cells/mm^3 and 2 million cells/250 μL gel), and 3D scaffold gel composite cultures (5-mm diameter × 2-mm height) using a QIAshredder and the RNeasy Mini Kit (Qiagen Inc.). The RNAs were washed and eluted according to the manufacturer's protocol, quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific), and further purified by DNase treatment (Sigma). cDNAs were synthesized using an iScript Reverse Transcription Supermix Kit (Bio-Rad).

Primers specific for rat neural cell adhesion molecule L1, growth-associated protein 43, synaptosomal-associated protein 25, and GAPDH were designed using the Primer BLAST tool (National Center for Biotechnology Information) and synthesized at a concentration of 25 nM (Invitrogen) (Fig. S4*A* shows primer sequences). Primer efficiency was validated to be between 95% and 105% using serial dilutions of commercial rat brain RNA (Clontech) (Fig. S4*B*).

PCRs were carried out using an Mx 3000 qPCR System (Stratagene) with SYBR Green PCR Master Mix (Invitrogen). Each reaction was run in duplicate, with C_t values automatically determined using the Stratagene software and averaged to reduce technical error. Expression levels for each gene of interest (GOI) were normalized to GAPDH expression as determined from reactions run on the same plate. For each group, fold change relative to the 24-h time point was calculated using the $\Delta\Delta C_t$ method. Asymmetric error bars show the maximum and minimum fold change as calculated based on the SD of the $\Delta\Delta C_t$ values. Statistically significant differences were determined using two-way ANOVA with Bonferroni posttests to compare $\Delta\Delta C_t$ values at each time point. For the 2D and collagen groups, n = 3 replicates per time point per GOI. For the 3D scaffold group, n = 3, except for the 24-h and 1-wk time points, where n = 2 for all GOI, the 2-wk time point, where n = 2 for synaptosomal-associated protein 25, and the 3-wk time point, where n = 2 for neural cell adhesion molecule L1 and growth-associated protein 43 and n = 1 for synaptosomal-associated protein 25. Reduced replicate numbers were a result of low RNA yields and/or sample contamination as determined from control reactions.

DNA was extracted from 2D cultures (0.2, 0.4, 0.6, 0.8, and 1 million cells per well of a six-well plate) and 3D scaffold-only and 3D scaffold–collagen gel composite cultures using an All-Prep DNA/RNA/Protein Mini Kit. DNA quantity was measured using a PicoGreen dsDNA Assay Kit (Invitrogen).

Cell Viability Assay. AlamarBlue assay was used to assess cell viability of 2D plate cultures, collagen gel-based, and 3D brain-like tissue cultures, according to the manufacturer's protocol (Invitrogen).

Briefly, alamarBlue reagent was mixed in culture media (1:10; vol/vol) at different time points (day in vitro 24 h, 3, 5, and one time every week up to 9 wk) and incubated for 3 h at 37 °C. The mixture was replaced into a new 96-well plate, and the fluorescence was read on a microplate spectrophotometer at Ex/Em of 570/585 nm (SpectraMax M2; Molecular Devices Corp.). Triplicate samples were used for each measurement (per time point per group). Three to four replicate cultures per group per time point (2D, collagen gel-based, 3D scaffold-only, and scaffold–gel composite cultures) in a 96-well format were used for this assay. Identical-sized silk scaffolds without cells were used as blank controls for 3D scaffold-based cultures. For quantification, readings from blank 2D plates or 3D blank controls were subtracted from all measurements.

Statistical Analysis. Data are means ± SEMs, except where otherwise noted. Analysis used Student t test, except where otherwise noted. For all tests, $P < 0.05$ was considered significant.

1. Rockwood DN, et al. (2011) Materials fabrication from Bombyx mori silk fibroin. *Nat Protoc* 6(10):1612–1631.
2. Nazarov R, Jin HJ, Kaplan DL (2004) Porous 3-D scaffolds from regenerated silk fibroin. *Biomacromolecules* 5(3):718–726.
3. Meinel L, et al. (2004) Engineering bone-like tissue in vitro using human bone marrow stem cells and silk scaffolds. *J Biomed Mater Res A* 71(1):25–34.
4. Longair MH, Baker DA, Armstrong JD (2011) Simple Neurite Tracer: Open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics* 27(17): 2453–2454.
5. Marmarou A, et al. (1994) A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics. *J Neurosurg* 80(2):291–300.
6. Bajad SU, et al. (2006) Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography-tandem mass spectrometry. *J Chromatogr A* 1125(1):76–88.

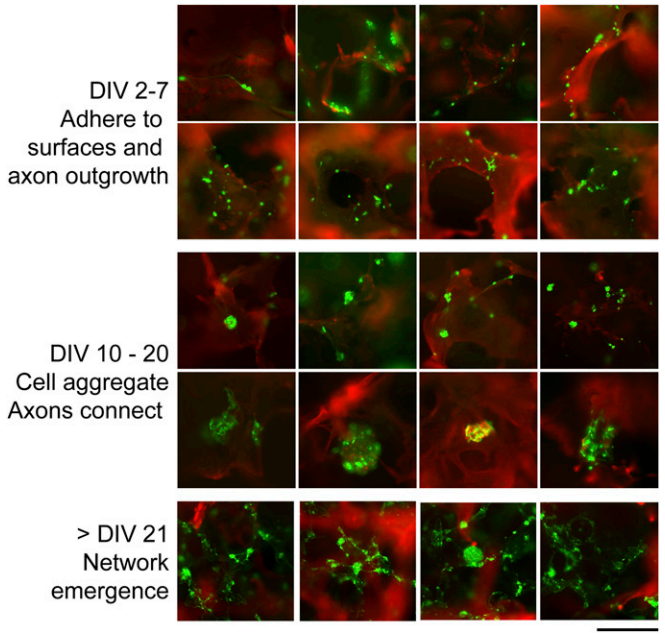

Fig. S1. Neuronal growth on convoluted 3D surfaces of silk scaffolds. Neurons were live-stained with calcein acetoxymethyl (green), and silk structure autofluoresces in red. DIV, day in vitro. (Scale bar: 500 μm.)

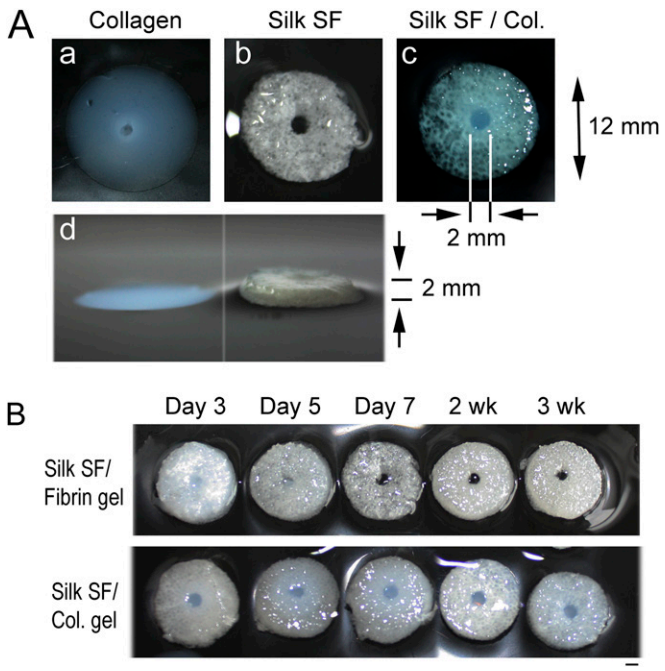

Fig. S2. ECM gels in the 3D brain-like tissues. (*A*) Dimensions of (*c*) silk scaffold (SF)–collagen gel composite structures compared with (*a*) similar-sized collagen gels and (*b*) silk SFs. (*d*) After 1 wk, the collagen gel shrank in height. (*B*) Stable collagen gel vs. unstable fibrin gel in the composite structures. Matrigel showed similar degradation as fibrin gel by 1 wk. SF, scaffold. (Scale bar: 2 mm.)

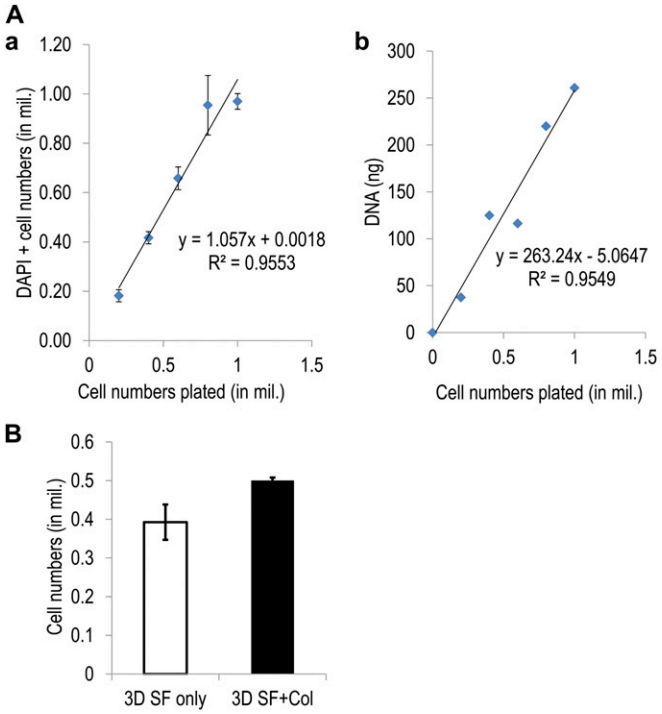

Fig. S3. Determining cell numbers in 3D cultures. (*A*) Linear correlations between cell numbers and DNA quantity for cortical cultures. (*a*) DAPI-positive cells correlate with initial cell plating numbers in 2D cultures. (*b*) DNA content measured with Picogreen assay (Invitrogen) to correlate with initial cell plating numbers in 2D cultures. (*B*) Cell numbers determined from DNA quantity extracted from 3D brain-like tissues and calculated according to the standard curve in *A*, *b*. The 3D brain-like tissues were 5 mm in diameter and 2 mm high based on a 96-well format and used for tissue viability and gene expression experiments compared with 50-μL collagen gel-based 3D cultures (*Materials and Methods*). SF, scaffold.

A

Gene	Forward primer	Reverse primer
NCAM-L1	CACCAGTGAGAGGGTGAGTG	CTCCAGTACATGGTGTCCTTT
GAP-43	AACGGAGACTGCAGAAAGCA	GCCTCGGGGTCTTCTTTACC
SNP-25	TGGATGAGCAAGGCGAACAA	TCCTGATTATTGCCCCAGGC
GAPDH	GATGGTGAAGGTCGGTGTGA	GGGATCTCGCTCCTGGAAG

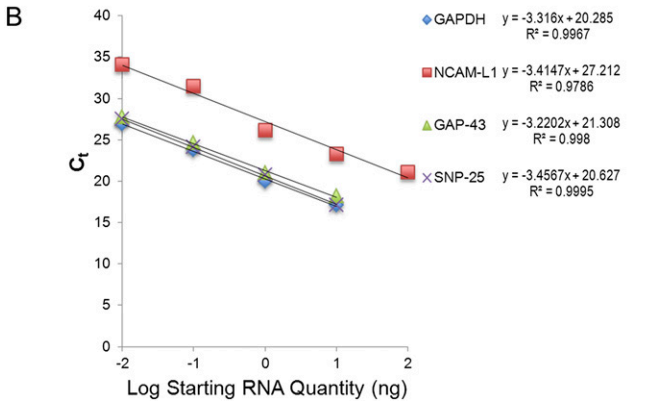

Fig. S4. Primer designs (*A*) and efficiency tests (*B*) for PCR. GAP-43, growth-associated protein 43; NCAM-L1, neural cell adhesion molecule L1; SNP-25, synaptosomal-associated protein 25.

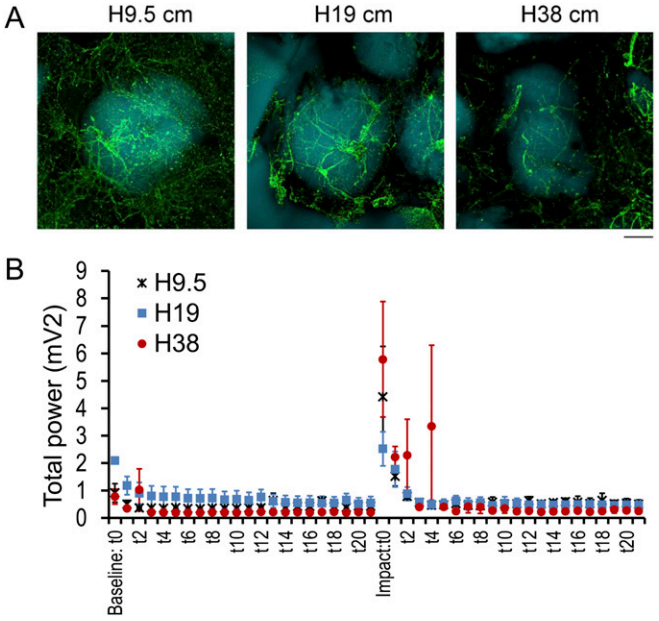

Fig. S5. Cellular damage and electrophysiological responses after impact injury. (*A*) Fluorescence images of 3D brain-like tissues fixed immediately after impact. Images are 2D sums of confocal image stacks of neurons immunostained with β3-tubulin in green superposed with bright-field images of silk structure in dark gray. (Scale bar: 100 μm.) H, height. (*B*) Time-evolved changes of total power (millivolts^2) of LFP signals obtained over a 20-min duration (10 min of baseline and 10 min postinjury). Each segment (*t*0–*t*20) represents a 27-s window.

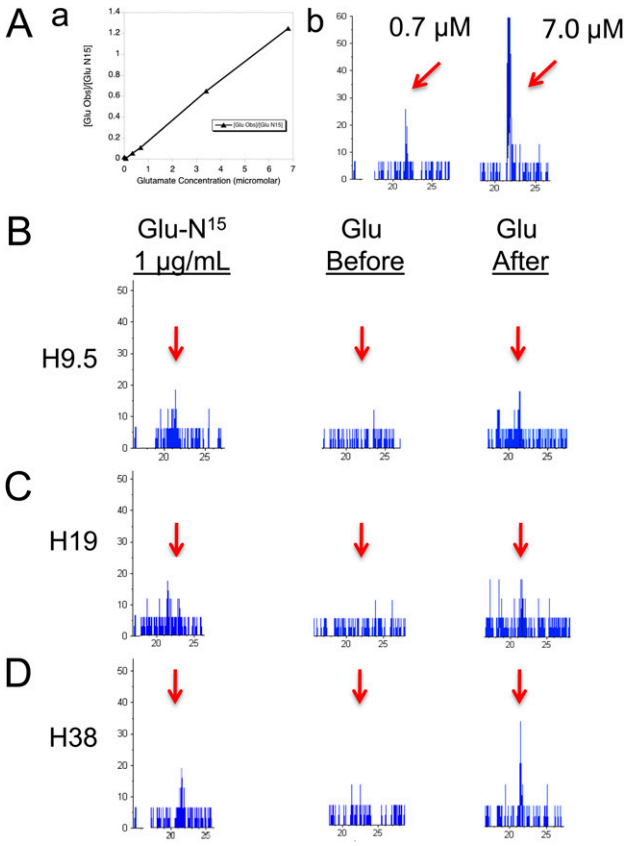

Fig. S6. Glu measurements with tandem liquid chromatography/MS. (*A*) Glu calibration curve (determined from a Glu internal standard Glu-N^{15}). (*a*) Standard curve. (*b*) Glu peaks of 0.7 and 7 μM at retention time of ∼21 min. (*B*–*D*) Glu detection before and after impact injury of differing severity at (*B*) H9.5, (*C*) H19, and (*D*) H38. Column 1 shows internal standard Glu-N^{15} peaks. Columns 2 and 3 are Glu peaks before and after injury. The observed Glu signal was integrated from peaks centered at ∼21 min retention time (marked by red arrows). H, height.