Supplemental Figures

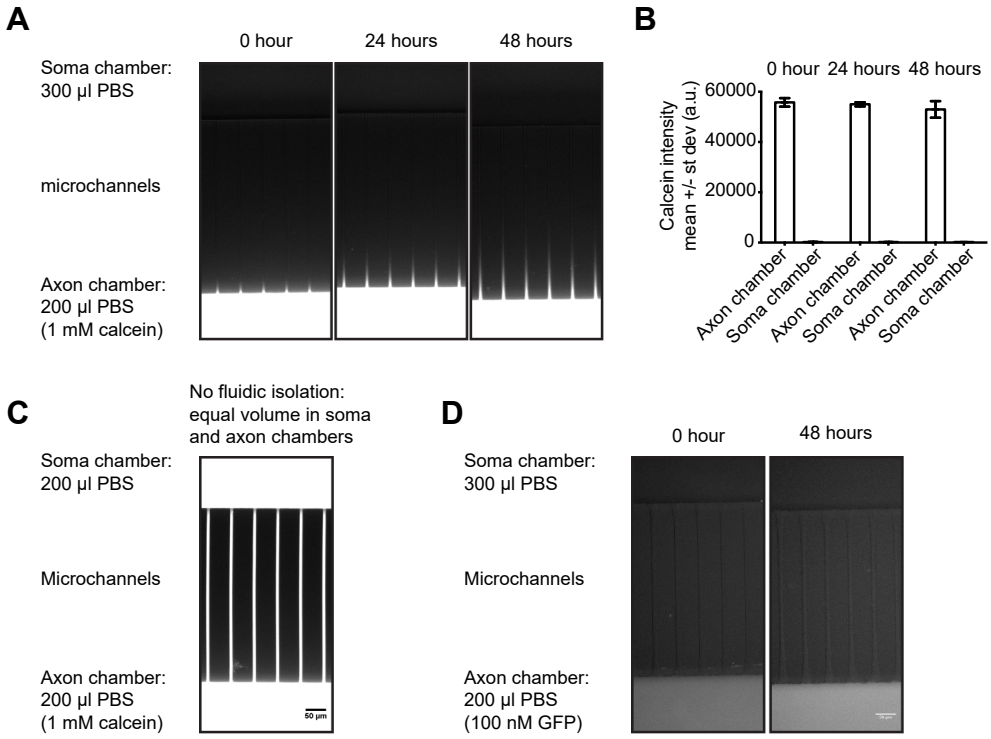

Figure S1. Experimental demonstration of fluidic isolation over 48 hours. Related to Figure 1.
(A) Representative example showing lack of calcein (623 Da) diffusion through microchannels over 48 hours. Fluorescent images of microchannels, including portions of the axon and soma chambers. Calcein (1 mM) was added to the axon side, and images were acquired at 0, 24, and 48 hours **(B)** Quantification of fluorescent intensities from ROIs in the axon and soma chamber at 0, 24, and 48 hours. **(C)** When equal volumes of media are used in the axon and soma sides of the microfluidic device, fluidic isolation does not occur, and calcein (1 mM) readily diffuses through the microchannels. The representative image was taken 5 min. after adding the dye to the axon side. **(D)** Representative images using GFP (27 kDa) as a tracer for fluidic diffusion. GFP (100 nM) was added to the axon side and images were acquired at 0 and 48 hours; diffusion of GFP into the microchannels does not occur. Values are averages ± standard deviations from 3 independent experiments with n >3 for each condition. Scale bar: 50 µm.

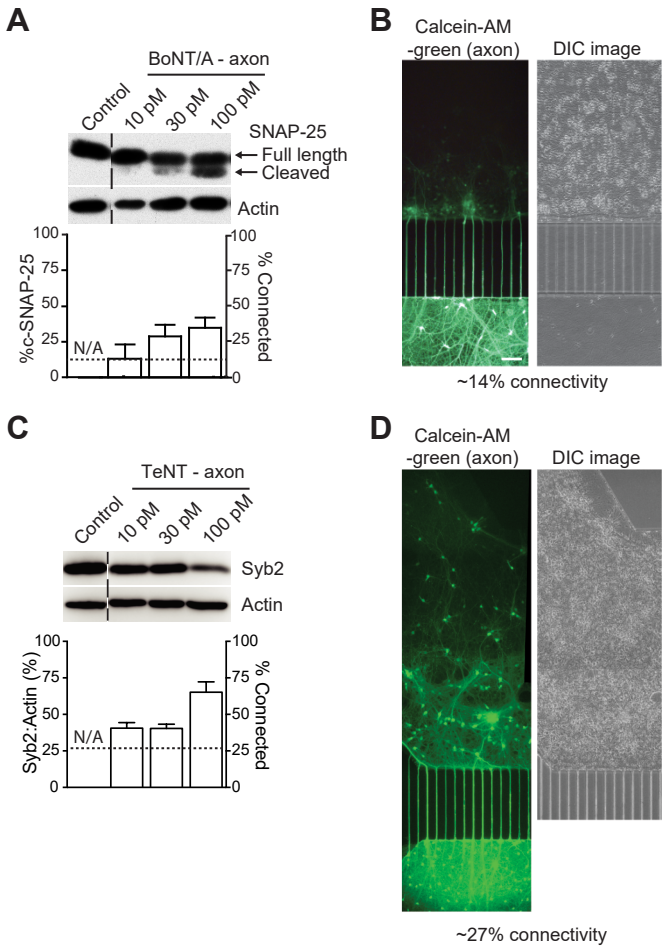

Figure S2. Distal effects of BoNT/A and TeNT at pM concentrations are observed following extended incubation. Related to Figure 1.

(A) BoNT/A dose-response in microfluidic devices. Immunoblotting of lysates from the soma chamber, 16 days after the indicated toxin treatment on the axon side; > 30% cleavage was observed using 100 pM toxin. **(B)** Representative image of Calcein-AM-green staining, when added to the axon chamber, showing the connectivity within the soma chamber. Connectivity is lower (14%) than in the experiments described in the main text (24%) due to lower initial cell density. A lower initial cell density was necessary for the cells to grow and remain viable during these longterm experiments (total of 30 days *in vitro*). **(C)** TeNT dose-response in microfluidic devices. Immunoblotting of lysates from the soma chamber, 6 days after the indicated toxin treatment on the axon side; > 65% cleavage was observed using 100 pM TeNT toxin. **(D)** Representative image of Calcein-AM-green staining, when added to the axon chamber, showing the connectivity within the soma chamber. Values are averages ± SEM from 3-4 independent experiments with n = 3-5 for each condition. Scale bar: 100 µm.

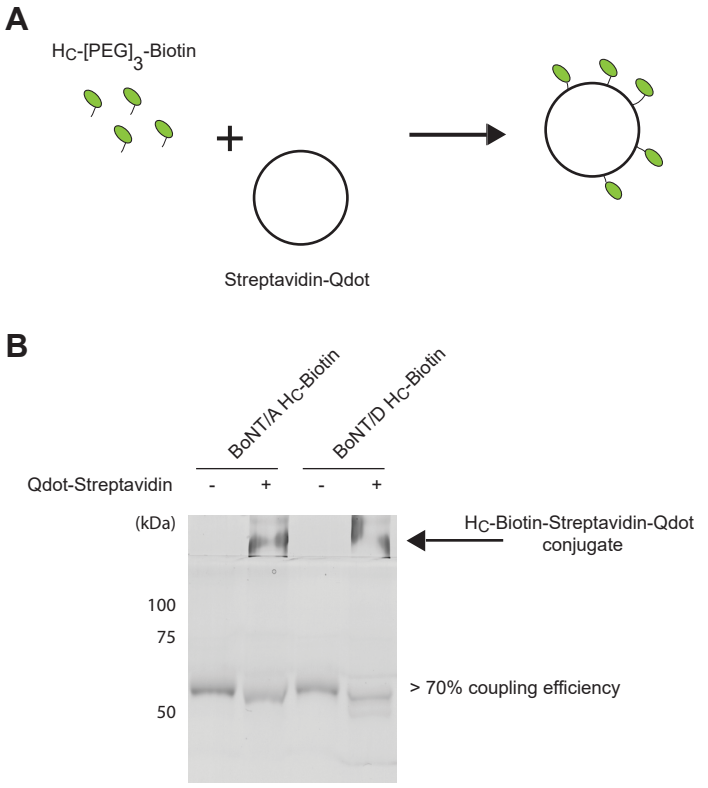

Figure S3. Overview of Qdot – H_C coupling procedure. Related to Figure 5.
(A) Biotinylated toxin H_C fragments were incubated with streptavidin coated Qdots overnight at 4°C to form H_C-Qdot conjugates. **(B)** H_C-Qdot conjugates were subjected to SDS-PAGE, in the absence of β-mercaptoethanol, to measure coupling efficiency, which ranged from 30% to 80%. Qdot labeled H_C domains are trapped in the stacker gel; only free H_C domains enter the separating gel.

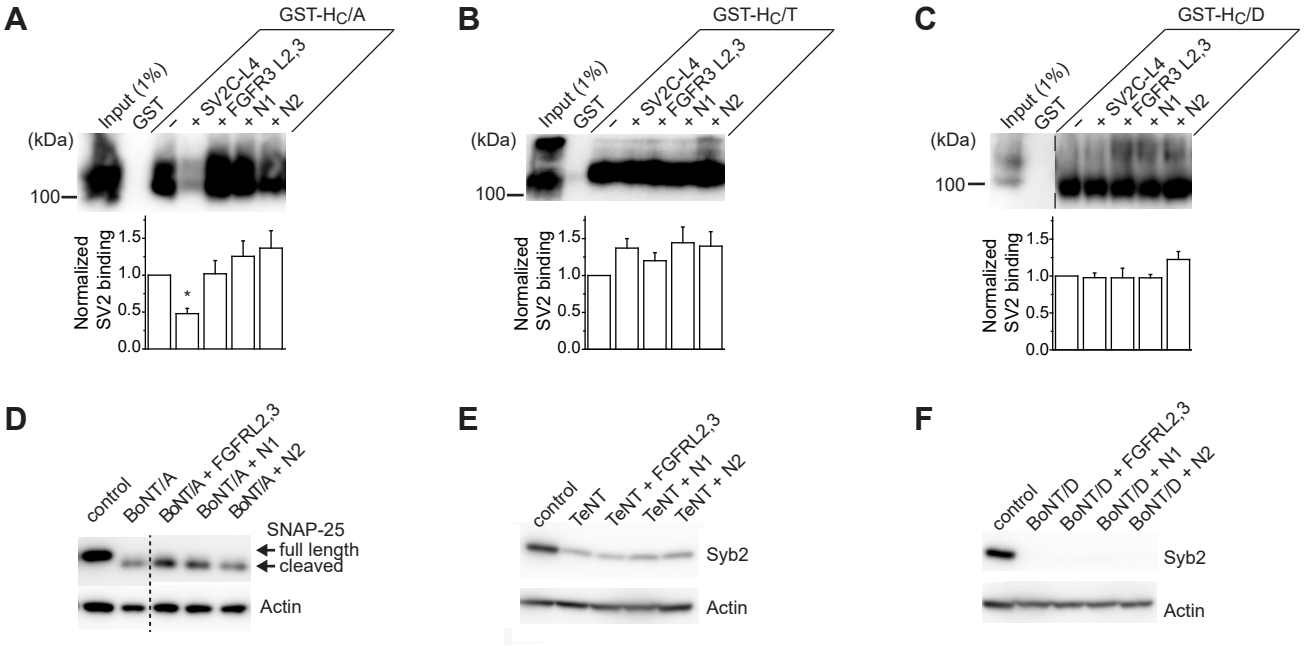

Figure S4. FGFR3L2, 3, N1 and N2 do not interfere with H_C-SV2 interactions, or cleavage of SNAREs in mass neuronal cultures. Related to Figure 6.

(A-C) Binding of native SV2 to immobilized H_C fragments was not affected by the alternative receptor fragments, FGFRL2,3, N1 or N2. Immunoblots are representative of 3-5 independent experiments. **(D-F)** Pre-incubation of toxins with receptor fragments had no effect on toxin-mediated substrate cleavage when applied directly to neurons grown in a dish. Representative blots are shown; experiments were carried out with 3-5 independent rat litters.

Supplemental Movies

Movie S1. BoNT/D- H_C undergoes fast axonal retrograde transport in a non-acidified organelle. Related to Figure 5. Axons of rat hippocampal neurons grown in microfluidic chambers were incubated with Alexa-labelled BoNT/D- H_C (green) and subsequently Lysotracker (red). Movies were acquired at 1 or 3 Hz for 2-5 minutes. In the video, axon side is to the left, soma side is to the right. This movie is a representative example of experiments performed on cells from 4 separate rat litters.

Movie S2. BoNT/D- H_C undergoes fast axonal retrograde transport in neurons lacking SV2 protein receptor. Related to Figure 5. SV2A(-/-)B(-/-) mouse hippocampal neurons were grown in microfluidics chambers for 14 days prior to experiments. BoNT/D- H_C-Qdot was loaded into the axon side of the microfluidic and incubated for 3-5 hours prior to imaging. Sale bar is 10 μm, movies were acquired at 1 Hz for 1 minute. In the video, distal end of a microchannel is visible with soma side at the bottom of the movie. This movie is a representative example of experiments performed on cells from 2 separate mouse litters.

Supplemental Experimental Procedures

Materials and mouse lines

Monoclonal antibodies directed against syb2 (1:1000, Cl. 69.1,), SV2 (1:1000, pan-SV2), synaptophysin (1:1000, Cl. 7.2), and SNAP-25 (1:1000, Cl. 71.1) were provided by R. Jahn (Max-Planck-Institute for Biophysical Chemistry, Gottingen, Germany). An antibody specific for the BoNT/A cleaved form of SNAP-25 was obtained from Research and Diagnostics (1:200, MC-6053, Las Vegas, NV). Guinea pig anti-vesicular glutamate transporter 1 (vGlut1, 1:2000, AB5905) and chicken MAP2 (1:1000, AB5543) antibodies were obtained from Millipore (Billerica, MA). Mouse anti-actin (1:1000, AB8226) and VCP (1:2000, AB11433) antibodies were obtained from Abcam (Cambridge, MA). Anti-BoNT/A (CR2) and anti-BoNT/D (8DC1.2) antibodies were provided by J. Marks (University of California, San Francisco, CA). Anti-TeNT antibody was from US Biological (T2962, Salem, MA). Calcein-AM (C3100, C3099), LysoTracker (L7526), Alexa dyes (A10254, A20341), Qdot 625 Streptavidin conjugate (A10196), EZ-Link Amine-PEG3-Biotin (21347B), EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (22980), and Zeba^{TM} spin columns (89882), were from Life Technologies (Grand Island, NY). TeNT holotoxin was obtained from List Biological Laboratories (190, Campbell, CA). BoNT/A Holotoxin, from strain Hall A hyper, was purified as previously described (Malizio et al., 2000). BoNT/D was isolated from strain D 1873 as described (Pellett et al., 2015). The specific activity in mice was determined to be 1.25×10^8 mouse LD_{50} Units (U)/mg for BoNT/A and 1.15×10^8 mouse LD_{50} Units (U)/mg for BoNT/D (Pellett et al., 2015). SV2A, SV2B, and SV2A/B knockout mouse lines were previously described (Janz et al. 1999). Purified recombinant FGFR3b L2,3, and FGFR3b L2,3 protein expression vector were provided by K. Roger Aoki (Allergan, Irvine, CA)(Jacky et al., 2013). Nidogen peptides N1 (THIYQWRQT) and N2 (NQTWSYHID) were synthesized by Biomatik (Wilmington, DE).

Immunocytochemistry

Cells in microfluidic chambers were fixed (4% paraformaldehyde in PBS), permeabilized (0.3% Triton X-100, 1% BSA, in PBS), blocked (10% BSA in PBS), and stained with anti-MAP2, -vGlut1, and -c-SNAP-25 (for BoNT/A experiments) or -syb-2 (for TeNT and BoNT/D experiments) antibodies. Microfluidic devices were carefully removed at the end of the staining procedures and coverslips were mounted (Fluoromount-G, #0100-01, SouthernBiotech Birmingham, AL) for imaging. Neurons were imaged using a 60X SC objective lens on an Olympus FV1000 confocal microscope (Center Valley, PA). A minimum of three images, from each region of a microfluidic device, were collected, with a minimum of three microfluidics per condition. Raw images were analyzed using Imaris 8 (Oxford Instruments, Concord, MA) and ImageJ; data were plotted using Prism 6 software (GraphPad Software Inc., LaJolla, CA).

Immunoblotting

The material in the soma chamber of microfluidic devices was solubilized with 100 µl of lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.05% SDS, 1 mM EDTA, 2 mM PMSF, and 1 µg/ml leupeptin/aprotinin/pepstatin) for 5 min on ice. Lysates were briefly vortexed, allowed to further solubilize on ice for 10 minutes, and insoluble material was removed by centrifugation (15,000 x g for 5 min). All samples were boiled in SDS loading buffer, separated by SDS-PAGE, and subjected to immunoblot analysis; immunoreactive bands were visualized using enhanced chemiluminescence and an Amersham Imager 600 (GE Healthcare, Buckinghamshire, UK). Immunoblot data were quantified by densitometry using Fiji software (Schindelin et al., 2012).

Live-cell fluorescence microscopy

Fluorescence images were acquired on an Olympus IX83 inverted microscope equipped with a cellTIRF 4Line excitation system with either an Olympus 60x/1.49 Apo N or 100x/1.49 UApo N objective (Center Valley, PA) and an Orca Flash4.0 C-MOS camera (Hamamatsu Photonics, Skokie, IL) running Metamorph software that was modified to run concurrently with Olympus 7.8.6.0 acquisition software from Molecular devices (Sunnyvale, CA).

Purification of H_C domains and FGFRL2,3

The H_C domains of TeNT, BoNT/D, and BoNT/A were purified as either GST or 6xHis fusion proteins in *E. coli* as previously described (Peng et al., 2012). A bacterial expression vector encoding BoNT/D H_C, fused to GST, was provided by

Dr. M. Dong (Harvard University, Southborough, MA) and was further mutated to include a tetra-cysteine sequence to enhance cysteine labeling. H_C fragments were cleaved from the GST tag using thrombin. Proteins containing a 6xHis sequence were purified using Ni^{2+} beads (GE HealthCare) and eluted using imidazole. All H_C protein fragments were stored at -20 ºC in 50 mM HEPES pH 7.4, 200 mM NaCl with 40% glycerol. Recombinant FGFRL2,3 was purified as previously described (Jacky et al., 2013) and stored at -80 ºC in 150 mM NaCl, 20 mM HEPES pH 7.4 with 10% glycerol.

Labeling H_C fragments with Qdots: 500 pmoles of purified H_C (TeNT, BoNT/A, BoNT/D) was incubated with 50 nmole of EZ-Link amine-PEG3-Biotin and 10 nmole of EDC (1:100:20 molar ratio) with shaking at RT for 2 hours. Biotinylated H_C domains were purified by buffer exchange through ZebaTM spin columns, followed by incubation with streptavidin-Qdot 625 conjugate at a 1.3:1, Qdot to H_C molar ratio, overnight at 4°C. Labeling efficiency was determined by quantifying free H_C relative to Qdot labeled H_C via SDS-PAGE and silver staining.

Labeling H_C fragments with organic dyes: Alexa-C5-maleimide 488 or 568 (2 mM final) was added drop-wise to 10 µM H_C and incubated, on a rotator, for 2 hours at room temperature. Excess dye was removed by buffer exchange through a Zeba^{TM} spin column. The labeling stoichiometry was 0.6-2.0 dye to protein.

Imaging and tracking analysis of labeled H_C fragments

H_C-Qdot conjugates were loaded into the axon side of microfluidic devices harboring 12-14 DIV hippocampal mouse neurons, at a concentration of 800 pM, and returned to the incubator for 6 hours. For time-lapse imaging, microfluidics were placed on a GM-8000 TOKAI HIT heated stage (Gendoji-cho, Fujinomiya-shi Shizuoka-ken, Japan) and were kept at 37°C throughout the experiment. Images were acquired within the microchannels, near the soma side. Raw image data were imported, as TIFFs, into Imaris 8 (Oxford Instruments, Concord, MA) and analyzed for instantaneous speed of processive H_C-Qdot tracks. Tracks were identified as processive if they moved more than 5 microns. All instantaneous speeds were grouped based on 0.2 micron/sec binning; data were plotted using Prism 6 software (GraphPad Software Inc., LaJolla, CA). Alexa labeled H_C fragments were loaded into the axon side of 12-14 DIV hippocampal rat neurons, at a concentration of 10-30 nM, and returned to the incubator for 4-24 hours. For LysoTracker experiments, the microfluidic device was separated from the coverslip, following H_C incubation, and treated with 50 nM LysoTracker red for 5 minutes in bath solution; coverslips were washed, and transferred to the microscope. Simultaneous visualization of green and red channels was achieved using a DV2-multichannel imaging system (Photometrics,Tucson, AZ). Time-lapse images were collected at either 1 or 3 Hz for 2-5 minutes. Raw images were saved as TIFF files and analyzed using Fiji imaging software. The red and green channels of the TIFF files were overlaid and retrograde transport was visualized by generating kymographs. A particle was defined as positive for both red and green channels if the signal was 5-10 arbitrary fluorescent units above the local background for more than three consecutive frames.

Validation of fluidic isolation in microfluidic devices

Microfluidic devices were assembled according to the manufacturers instructions. Empty microfluidics were loaded with 300 µl of PBS in the “soma” side and 200 µl of PBS plus a fluorescent marker in the “axon” side; the fluorescent markers were calcein (623 Da) and recombinant GFP (27 kDa). Fluidics were imaged at 0, 24, and 48 hours using 488 nm/525 nm excitation/emission filters. Fluorescence intensity was quantified using ImageJ.

Long-term toxin treatments in microfluidic devices

Rat hippocampal neurons grown in microfluidics for 11-13 days were treated with BoNT/A or TeNT in the axon side at the indicated concentrations. Following 48 hours of toxin exposure the axon chamber was washed to remove free toxin and allowed to incubate for an additional 14 days for BoNT/A and an additional 6 days for TeNT. The soma chamber was then lysed and the lysates probed for SNAP-25 cleavage for BoNT/A, and syb2 for TeNT, via immunoblot analysis.

In-dish inhibition experiments

Rat hippocampal neurons, grown in culture dishes for 12-14 days, were used for toxin inhibition experiments. Holotoxins were pre-incubated with FGFRL2,3, N1 or N2 for 60 minutes at 37°C. Mixtures were then added to neurons and incubated

for 2 hours; neurons were then washed and incubated overnight before the soma side was probed for SNARE cleavage by immunoblot analysis.

H_C-Qdot coupling

H_C coupling was measured by subjecting H_C-Qdot conjugates to SDS-PAGE on 13% gels, in the absence of reducing agents. Proteins were visualized via silver stain; briefly, gels were fixed with 10% glacial acetic acid/30% ethanol for 1 hour and washed with 20% ethanol for 30 minutes. Fixed gels were reduced using 1.3 mM sodium thiosulfate for 1 minute, and washed three times with deionized water. Gels were then incubated in 11.8 mM silver nitrate/0.0074% formaldehyde for 45 minutes before washing again, three times, with deionized water. Bands were resolved in developer solution containing 285 mM sodium carbonate, 32.5 µM sodium thiosulfate, and 0.0185% formaldehyde. Gels were scanned and analyzed via densitometry.

GST-H_C pull down assays to measure binding of native SV2

GST-H_C pull down assays were carried out using brain detergent extracts from adult rats as a source of native SV2. Brains were solubilized in extraction buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and protease inhibitors) and centrifuged to remove insoluble material. GST- H_C fusion proteins (300 nM BoNT/A, 2 µM TeNT and BoNT/D), immobilized on glutathione-Sepharose beads, were incubated with peptides comprising SV2C loop 4 (SV2CL4; 2-4 µM), FGFRL2,3 (2-4 µM), N1 or N2 (20 µM) for 10 minutes on ice. Rat brain detergent extract (1.5 mg) was added and samples were rotated at 4˚C for 1 hour. The immobilized beads were washed three times with 0.5 % Triton X-100 extraction buffer, boiled in SDS loading buffer, separated by SDS-PAGE, and subjected to immunoblot analysis.