SUPPORTING INFORMATION

SUPPORTING METHODS

Ethics statement

All animal care and experimental protocols in this study were conducted under the guidelines set by the NIH Guide for the Care and Use of Laboratory Animals handbook. The protocols were reviewed and approved by the Animal Care and Use Committee (ACUC) at the University of Wisconsin - Madison (assurance number: A3368-01).

Antibodies and materials

Monoclonal antibodies directed against syb II (Cl. 69.1), syp (Cl. 7.2), and SNAP-25 (Cl. 71.1) were generously provided by R. Jahn (Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany). Rabbit polyclonal antibodies against BoNT/A and BoNT/B were described previously(1). Rabbit anti-ceb, mouse anti-β-actin, and biotin labeled anti-LDLR antibodies were purchased from Abcam (Cambridge, MA). FITC-CD11b antibody was purchased from BD Biosciences (Franklin Lakes, NJ). Vybrant DiO was purchased from Invitrogen (Carlsbad, California).

TeNT was purchased from List Biological Laboratories (Campbell, CA). BoNT/A, BoNT/B, BoNT/E, and BoNT/F were purified as described previously(1, 2). Chicken avidin, α-chymotrypsin VII, sodium thioglycollate broth, and human biotin-labeled Tr were purchased from Sigma-Aldrich (St. Louis, MO). The chicken avidin cDNA was generously provided by M.S. Kulomaa (University of Tampere, Finland).

Human recombinant MΦ colony-stimulating factor (M-CSF) and IFNγ were purchased from PeproTech (Rock Hill, NJ). LPS (Escherichia coli, serotype 0111:B4) was obtained from Sigma Chemical Co. (St. Louis, MO). MMP-9, MCP-1, and IL-6 recombinant proteins standards and ELISA antibodies were obtained from R&D (Minneapolis, MN) while TNFα standard and antibodies were from BD Biosciences (Franklin Lakes, NJ), respectively. PE conjugated CD115 antibodies were purchased from eBioscience (San Diego, CA).

Preparation of BoNT/BΔHC and biotinylation of toxins

Biotinylation of toxin was performed as previously described(1). Digestion of BoNT/B holotoxin to create BoNT/BΔHC was performed in a manner similar to previous protocols(3). 50~100 µg of BoNT/B was incubated with chymotrypsin at a toxin to enzyme ratio of 50:1. The mixture was incubated at 37°C and monitored every 3 d on an SDS-PAGE gel. This procedure was repeated until holotoxin was no longer detected by SDS-PAGE. PMSF (1 mM) was added to inhibit chymotrypsin activity.

Cell culture and CNT treatment procedures

Transient transfection of HEK-293 cells performed with Lipofectamine 2000 according to manufacturer’s protocol (Invitrogen, Carlsbad, California). For the AvLDLR experiments, HEK-293 cells were transfectioned with an empty pEGFP-N1 vector or one containing the AvLDLR gene. Additionally, either SNAP-25b or syb II, in pIRE2-EGFP, was also introduced via transfection at a 1:2 DNA ratio compared to the pEGFP-N1 vector. One day post-transfection, cells were incubated with 5 nM biotin-CNT in serum free media for 8 h before serum was added to a final concentration of 10%. Cells were harvested 3 d post-transfection and lysates were subjected to immunoblot analysis. For the Tr experiments, biotin-CNTs were incubated with avidin and Tr at
a 1:1.2:1.4 molar ratio, respectively. In the α-LDLR antibody experiments, 5 µl of antibody was used in place of Tr. Incubation procedures and analysis were performed as described for the AvLDLR experiments.

The murine RAW264.7 cell line was obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 with 5% cosmic calf serum (HyClone Logan, UT), 2 mM sodium pyruvate, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin. Cells were grown in 24-well plates with 0.5 ml medium and treated with 50 nM BoNT/B with or without antibodies (at a 1:1 molar ratio) for 6 h. Cells were subsequently stimulated with 500 pg/ml IFNγ and 100 ng/ml LPS. After 48 h, cell lysates were collected and ceb cleavage was assessed via immunoblot analysis. Adhesion assays were performed similar to above, after incubation with the BoNT/B the cells were resuspended at a density of 5x10⁶/mL and incubated with 5 µl of Vybrant DiO. Cells were seeded into a 48 well plate and fluorescence was analyzed using a Bio-Tek Synergy HT (Winooski, VT ) microplate reader.

Human blood monocytes were purified from heparinized blood drawn from adult donors, as described previously(4). Briefly, blood was separated using a Percoll gradient and the monocyte layer recovered. Further enrichment of monocytes was performed through negative selection using a RosetteSep monocyte enrichment cocktail (Stemcell Technologies, BC) and lymphocyte separation media (Mediatech, VA). This procedure is in compliance with an approved human subject protocol through the University of Wisconsin Health Sciences Human Subjects Committee.

Purified monocytes were cultured in RPMI 1640 supplemented with 10% FBS (Hyclone Logan, UT), 2 mM sodium pyruvate, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin. Monocytes were differentiated with 20 ng/ml M-CSF for 7 d. BoNT/B, with or without antibody, was added 6 h prior to stimulation with 10 ng/ml IFNγ and 100 ng/ml LPS. After 48 h, the supernatant was collected from each well and stored at -70°C for subsequent cytokine analysis by ELISA. Cell lysates were collected and protein concentration was measured using a microBCA assay.

Rat hippocampal neurons were cultured as described previously(1). For BoNT/B and BoNT/BΔHC titration experiments, the indicated concentration of toxin was incubated with neurons for 48 h. Neuronal lysates were harvested as described previously and cleavage of syb II was assessed via immunoblot analysis(1).

**Mouse peritoneal injections and FACS analysis**

Adult C57/BL6 mice (18-22 g) were injected with a 3% sodium thioglycollate broth solution (400 µl) to induce MΦ enrichment in the peritoneal cavity. After four d, the mice were injected with 25 ng of BoNT/BΔHC, 42 ng of αB antibody, or a combination of both in 500 µl of sterile PBS. Control mice were injected with PBS alone. Mice were euthanized four hours later and cells within the intraperitoneal cavity were harvested. None of the mice exhibited any symptoms typical of botulinum poisoning and were healthy during the course of the experiment. Cells (1 X 10⁵) were incubated with 0.2 µg PE-CD115 or an isotype control antibody in FACS buffer (PBS with 0.25% BSA and 1 mM EDTA) for 30 min at 4°C. 10,000 events were collected using a BD Biosciences FACSCalibur flow cytometer (Flow Cytometry Facility, University of Wisconsin, Madison, WI) and CellQuest software (BD Biosciences). To determine the percentage of CD-115 positive MΦs, dead cells were gated out (marked by propidium iodide) before creating density plots of the remaining cells. A tight region was set to encompass PE-CD115 positive cells, but not cells incubated with the control antibody. Total MΦs were determined by the total
peritoneal cells x percentage of CD115 positive cells. FITC-CD11b experiments were performed similarly.

**ELISA and MTS assays**

A sandwich ELISA was performed to analyze TNFα and MMP9 secretion. The assay was developed using streptavidin conjugated to an HRP polymer, POLY-HRP-40 (Fitzgerald Industries, MA) and a one-component substrate, 3,3',5,5'-tetramethylbenzidine (BioFx Laboratories, MD). Color development was stopped by the addition of 0.18 M sulfuric acid. The absorbance at 450 nm was measured using an ELx800 universal microplate reader and data were analyzed with Ascent Software. The protein concentration was determined according to a standard curve using recombinant proteins.

Human BMDMs were stimulated as indicated and supernatants were collected for ELISA. Cell viability was assessed using the nonradioactive cell proliferation assay (3-4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt [MTS]-based assay; Promega, Madison, WI), according to the manufacturer’s protocol.

**Statistical methods**

Statistical significance was evaluated by two-tailed unpaired Student’s t-test: *p < 0.05, **p < 0.01, ***p < 0.001.
**Figure S1: Model of entry via the AvLDL chimeric receptor.** (a) Schematic of the AvLDL chimeric receptor. A GFP was fused to the C-terminal intracellular end of the LDLR TMD (aa 788-861) and monomeric avidin was fused to the extracellular N-terminus. (b) Model of the experimental protocol. HEK-293 cells were transfected with the AvLDLR chimera, the appropriate SNARE, and then incubated with biotin-CNTs. Avidin binds the biotin-CNTs and the complex is internalized into endosomes, where the acidic environment induces translocation of the L chain into the cytosol where it cleaves SNAREs.
Figure S2: Functional entry of biotin-CNTs into fibroblasts that express a chimeric receptor. (a) HEK-293 cells expressing the chimeric receptor and the appropriate SNARE target for each CNT were lysed and subjected to immunoblot analysis after treatment with the toxins. Biotin-BoNT/A and biotin-BoNT/E were able to enter HEK-293 cells expressing AvLDLR and cleave 81% and 54% of SNAP-25, respectively. The arrows denote full-length (f) and the cleaved (c) form of SNAP-25. Cell lysates were probed for β-actin as a loading control. (b) Biotin-TeNT, biotin-BoNT/F, and biotin-BoNT/B behaved similarly and were able to enter and cleave 84%, 54% and 61% of syb II in cells expressing AvLDL, respectively. HEK-293 cells that expressed a control vector were resistant to the action of the CNTs.
Figure S3: CNTs, linked to an antibody or Tr, enter nonneuronal cells without expression of chimeric receptors. (a) Model of the experimental protocol. The biotin-toxin is attached to avidin and in turn attached to a biotin-antibody or biotin-Tr. Tr, or the antibody, mediate binding to native cell surface proteins and internalization via endocytosis. Functional entry of the toxin into the cytosol was monitored by cleavage of exogenously introduced SNAREs. (b) Immunoblots showing that both biotin-BoNT/A and biotin-BoNT/E, when linked to an antibody directed against the extracellular domain of the LDLR, cleaved 45% of SNAP-25 in HEK-293 cells. The arrows denote full-length (f) and the cleaved (c) form of SNAP-25. Cell lysates were probed for β-actin as a loading control. (c) When linked to biotin-Tr with avidin, biotin-BoNT/A and biotin-BoNT/E cleaved 46% and 33% of SNAP-25, respectively. (d) Similarly, biotin-BoNT/B, biotin-TeNT, and biotin-BoNT/F, when attached to Tr through avidin, cleaved 28%, 74%, and 89% of syb II, respectively.
Figure S4: Treatments on human BMDMs do not cause decreases in metabolic activity or reductions in MMP-9 release. (a) MTS assay indicating no significant decrease in metabolic activity in response to the indicated treatments as compared to control (n=5). (b) ELISA of matrix metalloprotease-9 (MMP-9) release; secretion was not inhibited by retargeted BoNT/B. Error bars represent SEM, n≥3. (c) MTS assay indicating no decrease in metabolic activity upon titration of BoNT/B. Error bars represent SEM, n≥3.
Figure S5: Retargeted BoNT/B selectively reduces the release of TNFα. The release of TNFα, matrix metalloproteinase-9 (MMP-9), monocyte chemotactic protein-1 (MCP-1), and interleukin-6 (IL-6) from BMDMs were assayed by ELISA. Only TNFα release was reduced by the retargeted toxin. Error bars represent SEM, n≥2.
Figure S6: Retargeting of BoNT/BΔHC to MΦs; injection into mice reduces MΦ recruitment in vivo. (a) Schematic of the structure of BoNT/B with the indicated chymotrypsin hyper-sensitive site between the HC and HN domains. (b) SDS-PAGE gel of untreated and chymotrypsin-treated BoNT/B stained with Coomassie blue. Full-length holotoxin migrates at 150 kDa while the chymotrypsin fragment (BoNT/BΔHC) migrates at 113 kDa. (c) The smaller chymotrypsin fragment was not detected, probably due to further proteolytic degradation. The disulfide bond in the holotoxin was reduced by β-mercaptopethanol (BME) into 100 kDa and 50 kDa fragments, corresponding to the heavy chain (H) and L chain, respectively. The chymotrypsin fragment was reduced to ~54 and ~59 kDa fragments that correspond to the L chain and the HN domain, respectively. (d) Treatment of hippocampal neurons with BoNT/BΔHC, with and without αB, did not result in cleavage of syn II as compared to BoNT/B holotoxin. Cell lysates were probed for synaptophysin (physin) as a loading control.
Figure S7: Cell adhesion and surface expression of CD11b on RAW 264.7 cells is not affected by retargeted BoNT/B. (a) RAW 264.7 cells, treated with indicated conditions, were incubated with DiO and quantified with a fluorescent plate reader. Error bars represent SEM, n=8. (b) Density plots of RAW 264.7 cells incubated with an FITC-CD11b antibody analyzed with flow cytometry. Surface expression of CD11b was similar across all tested conditions.
SUPPORTING REFERENCES

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