Supporting Information

Epitope Targeting of Tertiary Protein Structure Enables Target-Guided Synthesis of a Potent In-Cell Inhibitor of Botulinum Neurotoxin**

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Standard Materials

All amino acids were purchased from Aapptec as the Fmoc carboxylic acid with standard TFA side-chain protecting groups for solid phase peptide synthesis. HATU (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and PEG₄ (Fmoc-NH-PEG₄-CH₂CH₂COOH, Fmoc-18-amino-4,7,10,13,16-pentaoxaoctadecanoic acid) were purchased from ChemPep. DIEA (diisopropylethylamine), triethylsilane (TES) and TFA (Trifluoroacetic acid) were purchased from Sigma Aldrich. TentaGel beads were purchased as 90μm S-NH₂ beads, 0.29mmol/g, 2.86x10⁶ beads/g from Rapp Polymere (Germany), Rink Amide resin was purchased from Anaspec and C-terminal biotinylated peptides were synthesized on Biotin NovaTag resin (EMD Millipore).

Cyclic Peptide Library Construction

Cyclic peptide libraries were synthesized on a Titan 357 split-and-mix automated peptide synthesizer (Aapptec) via standard FMOC SPPS coupling chemistry using 90μm TentaGel S-NH₂ beads. All cyclic libraries included a C-terminal Lys(N3) residue, 18 L-stereoisomers of the natural amino acids, minus Cysteine and Methionine, at each of five randomized positions followed by a Propargylglycine residue. Libraries were cyclized overnight at RT after synthesis with 1.5 eq. CuI and 5 eq. L-ascorbic acid in 20% piperidine:NMP. Resin was chelated extensively with 5% w/v sodium diethylthiocarbamate trihydrate and 5% v/v DIEA in NMP. For the in situ click screen, an azide or alkyne click handle was coupled to the N-terminus following cyclization. At least a five-fold excess of beads is used when synthesizing libraries to ensure oversampling of each sequence. Amino acid side-chains are protected by TFA labile protecting groups that are removed all at once following library synthesis using a 95:5:5 ratio of TFA: H₂O: TES.

Scaled Up Peptide Synthesis

Bulk synthesis of peptide sequences was performed using standard FMOC SPPS peptide chemistry on either the Titan 357 automated peptide synthesizer (AAPPTEC) or a Liberty 1 microwave peptide synthesizer (CEM Corporation). The typical scale was 300mg on Rink Amide Resin, unless otherwise noted. Click cyclized peptides were cyclized overnight at RT after synthesis with 1.5 eq. CuI and 5 eq. L-ascorbic acid in 20% piperidine:NMP. Resin was chelated extensively with 5% w/v sodium diethylthiocarbamate trihydrate and 5% v/v DIEA in NMP. Peptides were cleaved from the beads with deprotected side-chains using a 95:5:5 ratio of TFA: H₂O: TES. The peptides were purified on a prep-scale Dionex U3000 HPLC with a reverse-phase C18 column (Phenomenex). All peptides are checked for correct mass and impurities using MALDI-TOF MS and are lyophilized to a powder for long-term storage at room temperature. Concentrated peptide stocks for assays are made by dissolving powder in small amounts of DMSO and measuring the A280 (peptides) or A360 (dinitrophenol-containing peptides) absorbance via Nanodrop to determine the stock concentration. A280 extinction coefficients were calculated using the sequences and the “peptide properties calculator” provided by Northwestern University online (http://www.basic.northwestern.edu/biotools/proteincalc.html), the A360 extinction coefficient used for dinitrophenol-containing peptides was 1.74E+4 M⁻¹cm⁻¹.
Helix Click-stabilized Inhibitor Variant Synthesis

The peptidomimetic inhibitor Dab(Dinitrophenol)-RWT-Dab-ML as reported by Zuniga et al., (2011) was scaled up as outlined above with a C-terminal PEG₄ biotin and an N-terminal PEG₄ biotin. The N-terminally labeled variant showed no detectable binding in a point ELISA format or inhibition in an in vitro BoNT LC activity assay, this is consistent with the crystal structure indicating a partially buried N-terminus when bound. The C-terminally labeled variant was used as a control during the screening of various cyclic variants (Figure S7). Sixteen variants were synthesized. All were based on the inhibitor above, with an (i, i+3) substitution of propargylglycine and azidolysine. Both possible arrangements of these residues were tested for each substitution as well as an up to two residue glycine extension on the C-terminus. All compounds were cyclized as detailed above.

Sandwich ELISA Assays for Initial Evaluation of BoNT LC Binding

These assays were conducted to test the binding of the various ligands to the BoNT LC protein. For this assay, all samples were taken in triplicate for statistical purposes. The target protein used was BoNT LC – GST conjugate (List Labs). Peptide ligands were first immobilized onto Neutravidin ELISA plates (Pierce) for one hour at 1μM concentration. A PEG₄-biotin, was used as the no-ligand blank, as the GST proteins has significant background binding to a blank Neutravidin plate. The plates were then blocked with 5% BSA overnight. Protein was incubated at a concentration of 100nM (or as indicated) for sample wells and the blank wells. GST protein alone (Abcam) was also incubated with the ligands and blanks at the same concentration as a control. The proteins were incubated for three hours at room temperature, then washed three times with 1xTBS + 0.05% Tween-20. The protein was then detected with 1:10,000 anti-GST mouse mAb (Fisher) for one hour, washed three times with 1xTBS + 0.05% Tween-20 and developed with a 1:1 mixture of TMB substrate for ten minutes. The samples were plotted by subtracting the GST blanks and averaging the sample wells, when included error bars indicate one standard deviation above and below.

Competitive sandwich ELISAs were performed as above, except as indicated the competitor peptide was preincubated for one hour with the BoNT LC protein at the indicated concentration. Capture, detection and development proceeded as above. Competition for the same binding site is observed as a drop-off in signal as the preincubated concentration of competitor peptide increases.

FRET-based in vitro BoNT-LC Activity Assay

FRET-based BoNT-LC activity assays were carried out as suggested by the manufacturer of the FRET pair labeled BoNT substrate (List Labs, SNAPtide #523). All time-resolved fluorescence assays were performed in a Flexstation3 fluorescent plate reader at 37 degrees. Briefly, 8 μM of FRET-labeled substrate was prepared in Assay Buffer (50 mM HEPES, pH 7.4, containing 0.3 mM ZnCl₂, 1.25 mM DTT and 0.05% Tween-20) in a black fluorescence 96-well plate. BoNT LC-GST conjugate was diluted to the indicated concentration (generally 1 nM unless noted otherwise) and preincubated either with the indicated concentration of inhibitory peptide ligand, or a 0.05% DMSO
control. At time = 0, the plate reader adds the BoNT-LC to the substrate, and begins monitoring the fluorescence. The excitation wavelength was set to 490 nm, and the emission to 523 nm with a cutoff at 495 nm. Wells were monitored for approximately one hour. Initial velocities of cleavage were plotted against inhibitor concentration.

**Fluorescence Polarization Assays**

Inh-1 and L2 were synthesized with fluorescein for fluorescence polarization (FP) assays. The BoNT LC binding assays were completed by making a 1μM starting protein concentration and diluting 3x in Assay Buffer down a series of 10 wells in a black 96 well polystyrene plate. Ligand was added to the wells for a final concentration of 20nM. The plate was incubated at room temperature for one hour with shaking, and read on a Flexstation3 plate reader.

The data were fitted by subtracting the average of the low concentration baseline to zero in order to use the Hill fit. Using GraphPad Prism, the curves were fitted to a Hill function using a common saturation point.

**Secondary Binder Epitope-Targeted Screen**

Screens were performed using a library with the form Pra-Pra-XXXXX-Lys(N3)-TG, with the azido and propargyl amino acids closest to the bead clicked together to form a closed cycle. The peptide library was a comprehensive 5-mer containing 18 L-amino acids, excluding Met and Cys due to stability reasons. The N-terminus contained an alkyne amino acid for in situ click with the Lys(N3) on the target 13-mer-epitope fragment. Screens were performed using 400mg of dried library beads swelled at least six hours in 1x TBS (25mM Tris-Cl, 150mM NaCl, 10mM MgCl2, pH = 7.5) buffer and blocked in Blocking Buffer (1x TBS + 1% BSA + 0.05% Tween-20) overnight. Screening against the BoNT LC 166-179 epitope was carried out in two main steps: a scrambled epitope pre-clear and a target epitope product screen.

**Step 1: Scrambled Epitope Preclear**

Swelled library beads were blocked overnight in blocking buffer then washed with 1x TBS three times. The scrambled epitope (Lys(N3)-NGTLFNLSEVRH-PEG4-Biotin) Figure S11) was added at a concentration of 5uM in Blocking Buffer and incubated shaking at room temperature for four hours. The beads were washed three times with Blocking Buffer. 1:10 000 Mouse mAb anti-Biotin alkaline phosphatase conjugate (Abcam) was added in Blocking Buffer for one hour, followed by three washes in Blocking Buffer, three washes 1x TBS + 0.05% Tween-20, and three 15 minute washes with a high-salt TBS buffer (1x TBS with 750mM NaCl), then let shake in high salt buffer for one hour. The beads were then washed three times with BCIP buffer (100mM Tris-Cl, 150mM NaCl, 1mM MgCl2, pH = 9.0) and developed by adding 15mL BCIP buffer plus 13μL BCIP and 26μL NBT (two part system, Promega). Development proceeded for 30 minutes. After development the beads were quenched with concentrated HCl to 0.1N and washed with MilliQ water ten times to remove substrate. Beads were placed in a plate in 0.05N HCl. The purple beads (typically approximately 1-5%) were removed by micropipette and discarded. The remaining beads were incubated in NMP 4 hours to remove trace purple precipitate from the BCIP/NBT reaction, then were washed five times with methanol, five times with water, five times with 1xTBS and reblocked overnight in Blocking Buffer.
**Step 2: Target Epitope Product Screen**

Beads remaining from the preclear were washed three times with Blocking Buffer, then incubated with 5uM of the target epitope (Lys(N3)-SFGHEVLNLTRN, Figure S10) in Blocking Buffer for 12 hours to allow for an in situ click reaction to occur. The beads were then washed three times with 1x TBS and incubated for 1 hr with a 7.5M Guanadine-HCl buffer (pH = 2.0) to remove all target epitope not attached covalently to the beads. These beads were then washed ten times with 1x TBS, reblocked overnight in Blocking Buffer, then incubated for one hour with a 1:10 000 Mouse mAb anti-Biotin alkaline phosphatase conjugate (Abcam) in Blocking buffer for 1 hr to detect for the presence of the biotinylated target epitope clicked to the hit beads. Antibody incubation was followed by three washes in Blocking Buffer, three washes 1x TBS + 0.05% Tween-20, and three 15 minute washes with a high-salt TBS buffer (1x TBS with 750mM NaCl), then let shake in high salt buffer for one hour. Afterwards, the beads were again washed three times in BCIP buffer and developed as per the preclear. Purple beads are removed from the screen via pipette as hit beads. These hits were incubated in the guanidine-HCl buffer to remove attached streptavidin, washed ten times with water and sequenced via edman degradation on a Procise CLC system from Applied Biosystems. See Table SX for sequences from product screen.

**Kinetic in situ click target-guided synthesis of divalent inhibitor**

This screen was performed using a library with the form Pra-XXXXX-Pra-NYRWL-Lys(N3)-TG, with the azido and propargyl amino acids closest to the bead clicked together to form a closed cycle. The peptide library was comprehensive from length zero to length five containing only four possible residues (Glycine, D-Leucine, Aminoisobutyrate and D-Proline, see Scheme 2 in main text). The N-terminus contained an alkyne amino acid for in situ click with the Inh-1 –Biotin – Lys(N3) anchor inhibitor (Figure S14). Screens were performed using 50 mg of dried library beads swelled at least six hours in 1x TBS (25mM Tris-Cl, 150mM NaCl, 10mM MgCl2, pH = 7.5) buffer and blocked in Blocking Buffer (1x TBS + 1% BSA + 0.05% Tween-20) overnight. Screening was performed with the Inh-1 –Biotin – Lys(N3) anchor inhibitor compound and the BoNT LC protein.

**Step 1: Inh-1 Interaction Preclear**

Swelled library beads were blocked overnight in blocking buffer then washed with 1x TBS three times. The anchor inhibitor (Inh-1 –Biotin – Lys(N3)) Figure S14) was added at a concentration of 10uM in Blocking Buffer and incubated shaking at room temperature for four hours. The beads were washed three times with Blocking Buffer. 1:10 000 Mouse mAb anti-Biotin alkaline phosphatase conjugate (Abcam) was added in Blocking Buffer for one hour, followed by three washes in Blocking Buffer, three washes 1x TBS + 0.05% Tween-20, and three 15 minute washes with a high-salt TBS buffer (1x TBS with 750mM NaCl), then let shake in high salt buffer for one hour. The beads were then washed three times with BCIP buffer (100mM Tris-Cl, 150mM NaCl, 1mM MgCl2, pH = 9.0) and developed by adding 15mL BCIP buffer plus 13μL BCIP and 26μL NBT (two part system, Promega). Development proceeded for 30 minutes. After development the beads were quenched with concentrated HCl to 0.1N and washed with MilliQ water ten times to remove substrate. Beads were placed in a plate in 0.05N HCl.
The purple beads (approximately 1%) were removed by micropipette and discarded. The remaining beads were incubated in NMP 4 hours to remove trace purple precipitate from the BCIP/NBT reaction, then were washed five times with methanol, five times with water, five times with 1xTBS and reblocked overnight in Blocking Buffer.

**Step 2: Target-guided Synthesis Product Screen**

Beads remaining from the preclear were washed three times with Blocking Buffer, then incubated with 10uM of the anchor inhibitor (Inh-1 –Biotin – Lys(N3)) Figure S14) and 100nM BoNT LC in Blocking Buffer for either 2 or 12 hours to allow for an in situ click reaction to occur. The beads were then washed three times with 1X TBS and incubated for 1 hr with a 7.5M Guanidine-HCl buffer (pH = 2.0) to remove all Inh-1 not attached covalently to the beads. These beads were then washed ten times with 1x TBS, reblocked overnight in Blocking Buffer, then incubated for one hour with a 1:10 000 Mouse mAb anti-Biotin alkaline phosphatase conjugate (Abcam) in Blocking buffer for 1 hr to detect for the presence of the biotinylated Inh-1 clicked to the hit beads. Antibody incubation was followed by three washes in Blocking Buffer, three washes 1x TBS + 0.05% Tween-20, and three 15 minute washes with a high-salt TBS buffer (1x TBS with 750mM NaCl), then let shake in high salt buffer for one hour. Afterwards, the beads were again washed three times in BCIP buffer and developed as per the preclear. Purple beads are removed from the screen via pipette as hit beads. These hits were incubated in the guanidine-HCl buffer to remove attached streptavidin, washed ten times with water and sequenced via edman degradation on a Procise CLC system from Applied Biosystems. See Table SX for sequences from product screen.

**iCell Neuron Culture**

The cryopreserved hiPSC-derived neurons (iCell Neurons) were supplied by Cellular Dynamics International. The neurons were an ~98% pure (Tuj1+/Nestin-) pan-neuronal population of GABAergic, glutamatergic, and a very small percentage (< 1%) of dopaminergic neurons. Neurons were thawed according to the provided instructions, and live cells were counted by the Trypan Blue exclusion method. Cells were seeded at a density of 50,000 cells per well into Poly-D-Lysine Coated 96 Well plates (Corning) and 8.3 ug/cm² human Laminin (Sigma) unless otherwise indicated and incubated in neuronal medium with supplement as provided by Cellular Dynamics International. 50% of the medium was replaced every 3 days. Neural outgrowth staining (Life Technologies) showed substantial neurite growth and maturation 3 days after plating (Figure S18). Active synaptic vesicle recycling upon K⁺/Ca²⁺ depolarization was monitored by FM1-43 (Life Technologies) staining and nearly all neurons showed repeatable staining and destaining by day 15 after plating (Figure S19).

**Neuronal BoNT Toxicity Assays**

For all neuronal toxicity assays, 3 day post plating hiPSC-derived neurons were exposed to the indicated concentrations of BoNT/A holotoxin (List Labs) with indicated inhibiting compounds in 50uL of neuronal medium allowed to pre-incubate for 1 hr at 37 degrees. A negative control without toxin was always included. After 24 hours of exposure time, the toxin solution was removed and cells were lysed in 50 ul of 1x lithium dodecyl sulfate
(LDS) loading buffer (Invitrogen). The cell lysates were analyzed by western blot for SNAP-25 cleavage and beta-tubulin as a loading control. SNAP-25 was probed using Ms anti-SNAP-25 SMI-81 (Abcam) as a primary and goat anti-Ms H&L Alexafluor 790 (Life Technologies) as a secondary and imaged using an Odyssey instrument at the 800 nm wavelength. beta Tubulin was probed using Rb anti-beta Tubulin (Life Technologies) as a primary and goat anti-Rb H&L Alexafluor 680 (Life Technologies) as a secondary and imaged using an Odyssey instrument at the 700 nm wavelength. Cleaved SNAP-25 bands appeared below the intact SNAP-25 band, and each were quantified by densitometry in ImageJ and the relative fraction of intact SNAP-25 indicated in Figures 2 and 3.

For protection assays, neurons were cultured and exposed to toxin as indicated above. At the indicated times, 10 uL of neuronal medium spiked with inhibitory compounds were added at an appropriate concentration to bring the total concentration in the well to 1 uM in a total volume of 60 uL. As above, 24 hours post exposure all cells were lysed with 1x LDS and analyzed by western blot.

**Synaptic Vesicle Recycling Assays**

hiPSC-derived neurons were cultured as indicated above and allowed to mature, with 50% replacement of neuronal medium every three days. After 21 days, the neurons showed synaptic vesicle staining and destaining using FM1-43 dye followed by K⁺/Ca²⁺ depolarization. These matured neuron cultures were exposed to the indicated BoNT/A holotoxin pre-incubated with inhibitor as indicated, and were then assayed with FM1-43.

For FM1-43 loading, control and toxin/inhibitor-exposed neurons were washed in Hank’s Balanced Salt Solution (HBSS) and depolarized for 5 min at 37 degrees with 56 mM KCl in isosmotic HBSS containing 2 mM CaCl2 and 2 mM FM1-43 (Life Technologies). Cultures were washed twice for 10 minutes in HBSS without Ca²⁺ and containing 0.5 mM EGTA, to wash surface membranes of the dye while preventing neuronal activation and loss of FM1-43 from within already labeled terminals. Labeled neurons were imaged in this rinse solution; some wells underwent a second round of depolarization (in the absence of FM1-43) to destain synaptic terminals and evaluate functional intoxication. Before imaging, all conditions were given a last wash with the suggested dilution of NucRed Live 647 live cell nuclear stain (Life Technologies). FM1-43 labeled cultures were photographed using an EVOS FL microscope. FM1-43 could be monitored on the RFP/GFP channels and the NucRed stained was monitored on the Cy5 channel.

Images were analyzed by ImageJ. We used a watershed analysis to find the edges of neuron cell bodies in the transmission channel and allocate a given intercellular area to each cell. These areas were then mapped onto the FM1-43 layer and puncta were counted in each area using the PunctaAnalyzer package while ignoring the previously identified cell bodies to find only synaptic vesicle puncta along neurites. The puncta count for each cell was averaged over two wells with two fields of view per well. A typical analysis is shown in Figure S20.

**Cell Penetration of STP-DBCO-Cy5**
The spontaneously translocated peptide (pIyIrIrGqf) previously reported was synthesized from D-stereoisomer amino acids with an N-terminal Lys(N3). DBCO-Cy5 was then coupled to the azide with 1.1 eq. using strain-promoted click chemistry in DMF overnight. To verify that this peptide could deliver a sizeable polar cargo to the neuronal cytosol, STP-DBCO-Cy5 was diluted to 1 μM concentration in neuronal media and added to the neurons for 30 minutes. After incubation the cells were washed once with neuronal medium and 2x with HBSS. One final wash was given with the suggested dilution of NucBlue Live Readyprobe (Life Technologies) in order to visualize the cell nuclei. Neurons were imaged live with an EVOS FL microscope system. NucBlue could be monitored on the DAPI channel, STP-DBCO-Cy5 could be monitored on the Cy5 channel. A representative view of cell showing Cy5 in the cytosol is shown in Figure S21.

Synthesis of Inh-2 and Inh-2-STP Peptide Bicycles

Compound Inh-2 was synthesized on Rink Amide resin beginning with the synthesis of the L-2 cyclic peptide NH2-Pra-NYRWL-Lys(N3) on resin. This peptide was cyclized and chelated as detailed previously. The linker region was then coupled resulting in the compound NH2-G-Aib-I-[Pra-NYRWL-Lys(N3)] where square brackets indicate a closed cycle. The custom “Tz4” residue Fmoc-Lys(N3-Boc-Pra-OH)-Otbu was then coupled on resin. After Fmoc deprotection, either Fmoc-Lys(Mtt)-OH or Fmoc-Lys(Biotin)-OH (both Chempep) were then coupled depending on whether an orthogonally addressable amine or a biotin handle is needed (for Inh-2 and Inh-2-STP respectively). After Fmoc deprotection, the Inh-1 peptide is coupled as detailed previously resulting in the compound Fmoc-NH-Dab(DNP)-R-Lys(N3)-T-Dab(Boc)-Pra-L-Lys(MTT or Biotin) - Tz4-G-Aib-I-[Pra-NYRWL-Lys(N3)] where square brackets indicate a closed cycle. For Inh-2-STP synthesis, the MTT protecting group is removed from the Lys(MTT) by 10 washes with 1% TFA in DCM. The protection group removal was monitored by the yellow color of the eluent. DBCO-acid (Sigma) was coupled to the free amine, and STP (see synthesis above) was coupled with 1.1 eq. using strain-promoted click chemistry in DMF overnight. This compound is then cyclized and chelated again as detailed above to create the bicyclic compound. This peptide is cleaved as normal with 95:5:5 TFA:H2O:TES and purified by reverse phase HPLC. The structure and MALDI-TOF spectrum of these compounds can be found in Figures S5, S15 and S17.
Figure S1: Click cyclized BoNT LC inhibitor (Inh-1) (Biotinylated)
Sequence Dab(DNP)-R-[Lys(N3)-T-Dab-Pra]-L-PEG₄-Biotin where square brackets indicate a closed cycle. Expected m/z 1518.77, observed m/z 1518.61.
Figure S2: Click cyclized BoNT LC inhibitor (Inh-1) (Fluorescein)
Sequence Dab(DNP)-R-[Lys(N3)-T-Dab-Pra]-L-Lys(PEG₅-Biotin) where square brackets indicate a closed cycle. Expected m/z 1779.82, observed m/z 1780.11.

Figure S3: L-2 cyclic secondary binder (Biotinylated)
Sequence Biotin-PEG₅-[Pra-NYRWL-Lys(N3)] where square brackets indicate a closed cycle. Expected m/z 1516.75, observed m/z 1516.35.
Figure S4: L-2 cyclic secondary binder (Fluorescein)
Sequence Fluorescein-PEG₅-Pra-NYRWL-Lys(N3) where square brackets indicate a closed cycle. Expected m/z 1648.72, observed m/z 1648.89.

Figure S5: Divalent Inhibitor with in situ selected G-Aib-l linker (Inh-2)
Sequence NH₂-Dab(DNP)-R-[Lys(N₃)-T-Dab-Pra]-L-Lys(Biotin) - Tz4-G-Aib-l-[Pra-NYRWL-Lys(N3)] where square brackets indicate a closed cycle. Expected m/z 2862.44, observed m/z 2861.34.

Figure S6: Literature 310 helical BoNT LC inhibitor (biotinylated)
Sequence Dab(DNP)-RWT-Dab-ML-PEG₄-Biotin. Expected m/z 1586.81, observed m/z 1586.16.
**Figure S7: Preliminary ELISA curves of inhibitory ligands.**
Candidate substrate mimic ligand BoNT LC binding was evaluated by sandwich ELISA and compared to the literature inhibitor (blue).

**Figure S8: Representative in vitro BoNT LC activity assays.**
Typical time-fluorescence traces showing cleavage of SNAP-25 FRET substrate with various concentrations of BoNT LC, with the initial 2000 seconds zoomed in and fit to a Vmax initial slope to gather kinetics data. Vmax observed as a function of BoNT LC concentration shown.
Figure S9: Sandwich ELISA binding curve of Inh-1 compound.
Colourimetric sandwich ELISA curve showing binding of Inh-1 to BoNT LC-GST conjugate. Sandwich ELISA was performed as described above with immobilized biotinylated Inh-1, and GST-tagged BoNT LC and probed with anti-GST mAb-HRP. Curve was fit to one site specific Hill curve with h = 1.36, EC50 = 68 +/- 4 nM.

Figure S10: MALDI-TOF spectrum of biotinylated BoNT LC fragment.
Amino acids 166-179 of BoNT LC. Sequence Lys(N3)-SFGHEVNLTRN-PEG₄-Biotin. Expected m/z 2099.09, observed m/z 2099.45.
Figure S11: MALDI-TOF spectrum of biotinylated scrambled fragment.
Sequence Lys(N3)-NGTLFNSELRH-PEG₄-Biotin. Expected m/z 2099.09, observed m/z 2098.06.

Figure S12: List of hit sequences of epitope targeted screen.
Beads were sequenced by Edman degredation and colors indicate similar sidechain residues to better illustrate homology, dashes indicate amino acids involved in the click cycle.
Figure S13: Inh-1 and L2 Binding Site Verification Competitive ELISAs
Competitive sandwich ELISAs were performed as above, except as indicated the competitor peptide was preincubated for one hour with the BoNT LC protein at the indicated concentration. Capture, detection and development proceeded as above. Competition for the same binding site is observed as a drop-off in signal as the preincubated concentration of competitor peptide increases. Lit. Inhibitor is the structure shown in Figure S6, while Epitope and Scrambled refer to Figures S10 and S11, respectively. SNAP 25 peptide is commercially available from List Labs.

Figure S14: Click cyclized BoNT LC inhibitor (Inh-1) for target-guided screen.
Sequence Dab(DNP)-R-[Lys(N3)-T-Dab-Pra]-L-Lys(Biotin)-Lys(N3).
Expected m/z 1510.77, observed m/z 1510.90.
Figure S15: Divalent Inhibitor with PEG4 linker. Sequence NH$_2$-Dab(DNP)-R-[Lys(N$_3$)-T-Dab-Pra]-L-Lys(Biotin) - Tz4-PEG$_4$-[Pra-NYRWL-Lys(N3)]. Expected m/z 2797.38, observed m/z 2799.45.

Figure S16: Single point sandwich ELISA comparing BoNT ligand variants.
Peptides were immobilized on neutravidin resin at 1 uM concentration and blocked in 3% BSA for 3 hours. Bound ligands were incubated with 50 nM BoNT LC-GST conjugate or buffer blank for three hours. ELISAs were probed with anti-GST-HRP mAb as detailed in protocols and developed.
Figure S17: Divalent Inhibitor with g-Aib-l linker and STP (Inh-2-STP).
Sequence NH₂-Dab(DNP)-R-[Lys(N₃)-T-Dab-Pra]-L-Lys(DBCO-STP) - Tz4-G-Aib-l-[Pra-NYRWL-Lys(N₃)], where STP is D-peptide Lys(N₃)-piylirlrGqf. Expected m/z 4547.25, observed m/z 4545.67.
Figure S18: Neurite outgrowth in iCell Neurons.
Neurons 4 days after plating were stained according to the manufacturer’s instructions with Neurite Outgrowth and Viability stains (Life Technologies), consisting of a Calcein viability stain (green) and a Dil C18 membrane stain (blue). All cells show a morphology consistent with neuronal outgrowth. Scale bars are 300 um.
Figure S19: Synaptic vesicle recycling in iCell Neurons.
FM1-43 stain (yellow) and nuclear stains (red) on live neurons after 21 days post-plating. 
A) FM1-43 labeled control cells, no depolarization B) FM1-43 labeled BoNT intoxicated cells, after depolarization, 
C) FM1-43 labeled control cells, after depolarization, D) Unlabeled BoNT intoxicated cells. All labeling and depolarization is described in the SI procedures. Scale bars are 300 um.
Figure S20: Image analysis of synaptic vesicle intensity.
Images were analyzed by ImageJ. We used a watershed analysis to find the edges of neuron cell bodies in the transmission channel and allocate a given intercellular area to each cell. These areas were then mapped onto the FM1-43 layer and puncta were counted in each area using the PunctaAnalyzer package while ignoring the previously identified cell bodies to find only synaptic vesicle puncta along neurites. The puncta count for each cell was averaged over two wells with two fields of view per well.
Figure S21: Evaluation of cytosolic delivery of STP with polar cargo.
Sequence of peptide is Cy5-DBCO-Lys(N3)-pliyRlrGqf. Cells were treated at 1 uM concentration for 30 minutes. After incubation the cells were washed once with neuronal medium and 2x with HBSS. One final wash was given with the suggested dilution of NucBlue Live Readyprobe (Life Technologies) in order to visualize the cell nuclei.

Figure S22: Effect of STP on Inh-2 rescue in neurons.
Rescue assays were performed as described above and in the main text. Fraction of intact SNAP-25 was calculated as noted above. Inh-2 without the STP showed no rescue effect when given to neurons 1 hour after exposure to a 55 U bolus dose of BoNT/A holotoxin, while Inh-2 showed significant protection.