1 Supplementary Information

2 Retargeting the *Clostridium botulinum* C2 toxin to the neuronal cytosol

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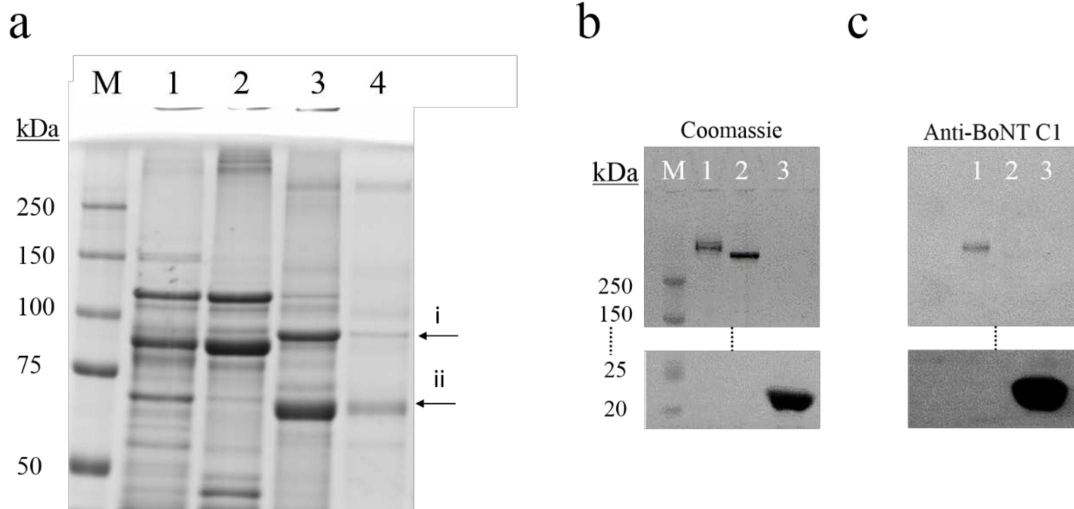
5 **Figure S1. Characterization of recombinant proteins by SDS-PAGE and western blot. (a)**
6 SDS-PAGE of GST-purification of protein C2II-C1. Expected masses: GST-C2II-C1 (~117
7 kDa), C2II-C1 (~91 kDa), trypsin activated C2II-C1 monomer (~71 kDa), trypsin activated C2II-
8 C1 oligomer (>>250 kDa). SDS-PAGE lanes: molecular ruler (M), 1: ultracentrifuged
9 supernatant, 2: resin loaded before thrombin, 3: resin loaded after thrombin, 4: supernatant
10 separated from resin after thrombin digestion. Arrows indicate the presence of a protein band
11 doublet during purification in lane 4: i) full length C2II-C1, ii) truncated C2II-C1. **(b/c)**
12 Oligomerization of C2IIΔD4 and C2II-C1 and anti-BoNT C1 reactivity. Samples of supernatant
13 from GST-purification of C2II-C1 (**S1a**, lane 4) were activated by trypsin, separated by SDS-
14 PAGE and visualized by Coomassie Blue G-250 **(b)** and western blot analysis with anti-BoNT
15 C1 **(c)**. Purified BoNT C1 H_{CC} (~23 kDa) was used as a positive control for antigenicity. Lanes:
16 M: molecular ruler, 1: activated C2II-C1, 2: activated C2IIΔD4, 3: BoNT C1 H_{CC}.



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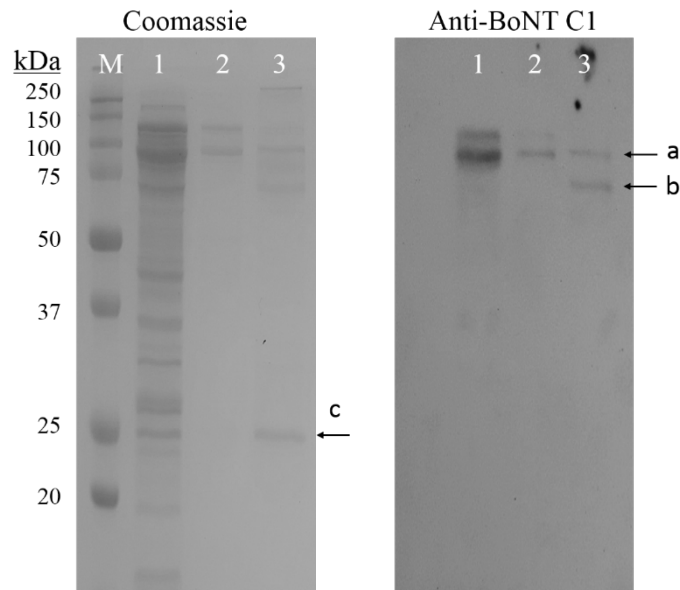
18 **Figure S2. SDS-PAGE and anti-BoNT C1 western blot of C2II-C1 purification.** This set of
19 data is an extension of the purification gel from **Fig. S1a** to demonstrate the antigenicity of the
20 doublet bands present during purification. Expected masses: GST-C2II-C1 (~117 kDa), C2II-C1
21 (~91 kDa), trypsin activated C2II-C1 monomer (~71 kDa), trypsin activated C2II-C1 oligomer
22 (>>250 kDa). SDS-PAGE lanes: molecular ruler (M), 1: ultracentrifuged supernatant, 2: resin
23 loaded before thrombin, 3, resin loaded after thrombin. Arrows indicate bands in the final elution
24 fraction after thrombin treatment: a) full length C2II-C1, b) truncated C2II-C1, c) GST tag



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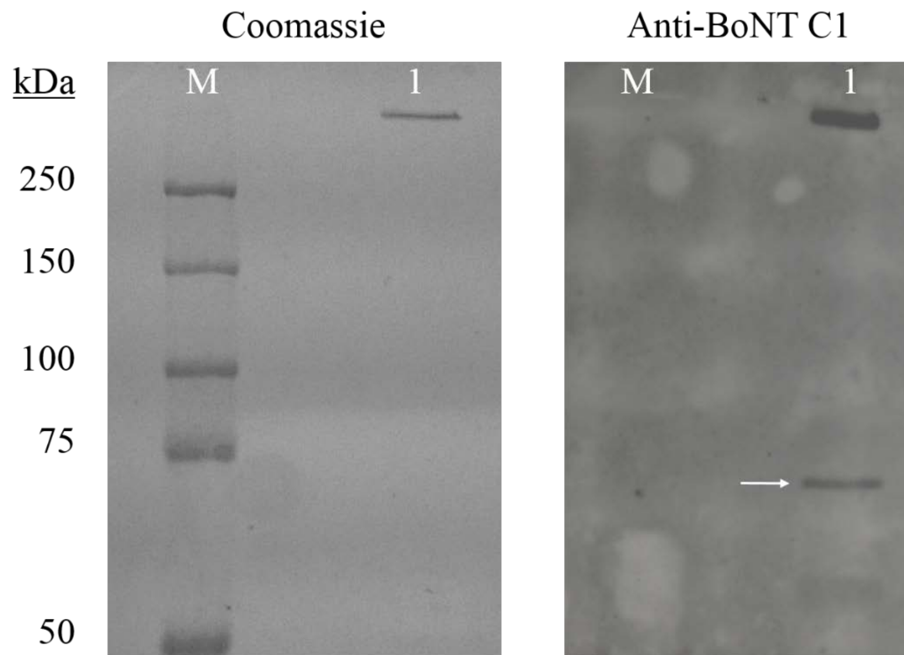
27 **Figure S3. SDS- PAGE and anti-BoNT C1 western blot of extensively heated C2II-C1**
28 **oligomer.** Expected masses: trypsin activated C2II-C1 monomer (~71 kDa), trypsin activated
29 C2II-C1 oligomer (>>250 kDa). SDS-PAGE lanes: molecular ruler (M), 1: trypsin activated
30 C2II-C1. A white arrow indicates the full length C2II-C1 monomer.



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33 **Figure S4. SDS-PAGE of activated C2II-C1 and C2ΔD4.** These are full length versions of
34 gels appearing in **Figure S1b,c.** Samples of trypsin-activated GST-purified C2II-C1 were
35 separated by SDS-PAGE and visualized by **(a)** Coomassie Blue G-250 and **(b)** western blot
36 analysis with anti-BoNT C1. Purified BoNT C1 H_{CC} (~23 kDa) was used as a positive control for
37 antigenicity. Lanes: M: molecular ruler, 1: activated C2II-C1, 2: activated C2IIΔD4, 3: BoNT C1
38 H_{CC}.

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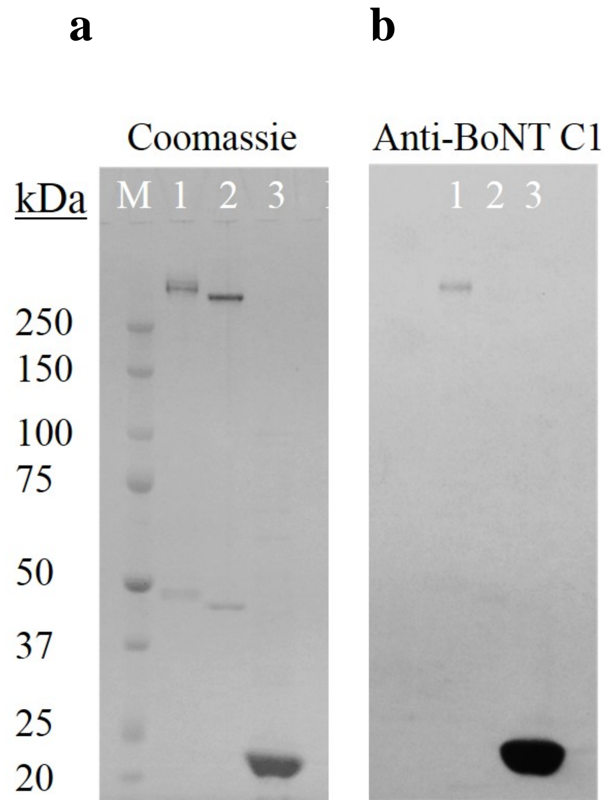

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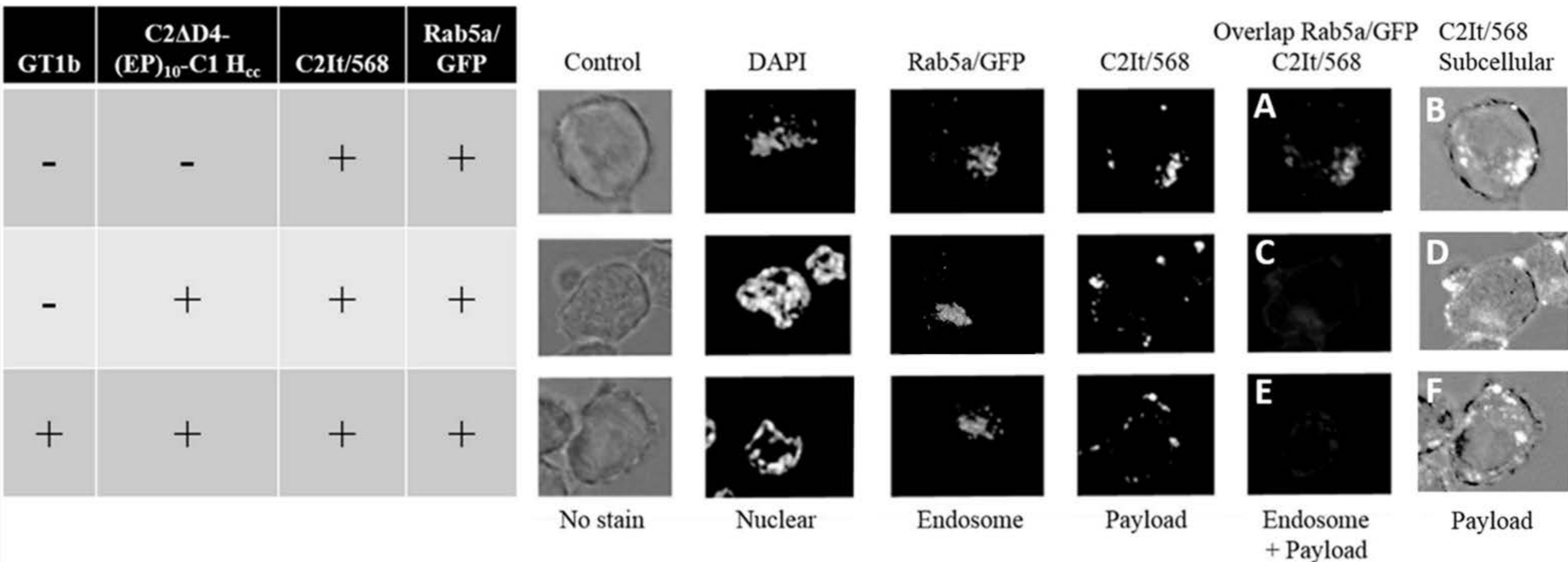

Figure S5. Channel separated localization of C2It-568 and early endosomes in N2A cell culture. A/B – Intracellular C2It/568 without GT1b or the C2II-C1 delivery unit co-localizes with the cellular periphery and/or early endosomes. C/D – Lack of co-localization of C2II-C1-delivered C2It/568 with early endosomes is consistent with a low intracellular signal when N2A cells are not treated with GT1b. The major abundance of C2It/568 in this treatment is confined to the cellular periphery. E/F – Intracellular presence of C2It/568 mediated by C2II-C1 in the presence of GT1b. Signals are not co-localized with early endosomes, indicating endosomal escape dependent upon delivery facilitated by C2II-C1.

42 **Table S6**. Primers for Cloning of C2IIΔD4-$(EP)_{10}$-C1 H_{CC} (C2II-C1), C1 H_{CC}, C2IIΔD4, and
43 C2I. C2It was directly subcloned from synthetic DNA. C2It was extended by overlapping PCR
44 to generate C2I. This extension of C2It was amplified from synthetic DNA prior to
45 incorporation.

C2IIΔD4F	CGCGGATCCATGCTGGTCTCC
C2IIΔD4-GS(EP)R	CCGGCTCTGGTTCCGGTTCAGAACCGGTGATCACTTTGACCA GAATATTCATG
(EP)GS C1 H_{CC}F	CCAGAACCAGAGCCAGAACCAGGTTCTACCAACGTTGTCAAA GACT ATTGGGG
C1 H_{CC}R	CGGGAATTCTTATTCTGAAACCGGGAC
GS$(EP)_{10}$GSF	AACCGGAACCAGAGCCGGAACCGGAACCGGAACCGGAGCCA GAACCAGAGCCAGAACC
C1 H_{CC}F	CGCGGATCCATGGGCACCAACGTTGTCAAAGACTATTGG
C2IIΔD4R	CGGGAATTCTTA GGTGATCACTTTGACCAG
C2IF	CGCGGATCCATGCCGATTATTAAAGAACCGATTGACTTCATC AACAAACCGG
C2IR	CCGGAATTCTTAGATTTCTTTGTTTTGGATACCTTCAGCATCA AT
C2IOF	GCAAGAACTGGACTTTTACAACAAAGGCTCGGAAGCCTGGGG TGCGGAAAACTATG
C2IOR	CATAGTTTTCCGCACCCCAGGCTTCCGAGCCTTTGTTGTAAAA GTCCAGTTCTTGC

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