Supplementary Material

## Supplementary Figures

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**Figure S1.** MS confirmation of synthesized FRET peptide reporter of BoNTA.



**Figure S2.** Evaluation of the cytotoxicity and cell-penetrating activities of BoNTA proteins in N2a cells. **(A)** The effects of CPP fusion on the cytotoxicity of BoNTA. **(B-C)** Immunofluorescence (IF) analysis of the cell-penetrating activity of CPP-BoNTA with 4 h incubation. **(B)** Representative images. Scale bars, 20 m. **(C)** Quantification of the mean fluorescence intensity of BoNTA positive cells. **(D-E)** Histogram showing TissueFAXS cytometry analyses of co-localization of BoNTA proteins and SV2 receptors in mouse N2a cells (Related to Figure 2C). SV2 **(D)** and BoNTA **(E)** are analyzed in Texas Red and FITC channels respectively. Three biological replicates are performed and 1,000 cells are analyzed for each replicate. **(A and C)** The data are shown as mean ± SD (*n* = 3). Statistical analysis is performed using Student’s *t* test.

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**Figure S3.** Analyses of time-dependent muscle-weakening effects of **(A)** Botox **(B)** BoNTA **(C)** TAT-BoNTA **(D)** Pep1-BoNTA **(E)** ZFP3-BoNTA **(F)** BoNTA-ZFP3 **(G)** ZFP3-BoNTA-ZFP3 using DAS assay.

## Supplementary Table

**Supplementary Table 1.** Primer sequences for CPP-BoNTA cloning.

|  |  |
| --- | --- |
| Primer name | Sequence ( 5’ to 3’ ) |
| BoNTA-FWD | gcgcgtctagaatggattataaggatgacgacgacaagggttccggttcccatcatcaccaccatcacagttccggcgtggacctgggtttcgagaacctgtacttccagggtatgcctttcgttaataaa |
| BoNTA-REV | atataaagcttttacagagggcgttcgcccca |
| TAT-BoNTA-FWD | gcgcgtctagaatggattataaggatgacgacgacaagggttccggttcccatcatcaccaccatcacagttccggcgtggacctgggtttcgagaacctgtacttccagggtggtcgtaaaaaacgccgc |
| TAT-BoNTA-REV | atataaagcttttacagagggcgttcgcccca |
| Pep1-BoNTA-FWD | gcgcgtctagaatggattataaggatgacgacgacaagggttccggttcccatcatcaccaccatcacagttccggcgtggacctgggtttcgagaacctgtacttccagggtaaagagacctggtgggaa |
| Pep1-BoNTA-REV | atataaagcttttacagagggcgttcgcccca |
| ZFP3-BoNTA-FWD | gcgcgtctagaatggattataaggatgacgacgacaagggttccggttcccatcatcaccaccatcacagttccggcgtggacctgggtttcgagaacctgtacttccagggtgaaaaaccgtacaagtgc |
| ZFP3-BoNTA-REV | atataaagcttttacagagggcgttcgcccca |
| BoNTA- ZFP3-FWD | gcgcgtctagaatgccttttgtgaacaagca |
| BoNTA- ZFP3-REV | gcgcgaagcttcttgtcgtcgtcatccttataatccatggaaccggaaccgtgatggtggtgatgatggaaacccaggtccacgccggaactaccctggaagtacaggttctcgccggtatgggtacgttg |
| ZFP3-BoNTA-ZFP3-FWD | gcgcgtctagaatggattataaggatgacgacgacaagggttccggttcccatcatcaccaccatcacagttccggcgtggacctgggtttcgagaacctgtacttccagggtgagaagccgtataaatgcc |
| ZFP3-BoNTA- ZFP3-REV | agtacttctcgacaagcttagccggtatgggtacgttggtgtgcc |

# 3 Supplementary Data

**Protein sequence**

**BoNTA full-length sequence (BoNTAFL)**

MPFVNKQFNYKDPVNGVDIAYIKIPNVGQMQPVKAFKIHNKIWVIPERDTFTNPEEGDLNPPPEAKQVPVSYYDSTYLSTDNEKDNYLKGVTKLFERIYSTDLGRMLLTSIVRGIPFWGGSTIDTELKVIDTNCINVIQPDGSYRSEELNLVIIGPSADIIQFECKSFGHEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPLLGAGKFATDPAVTLAHELIHAGHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDSLQENEFRLYYYNKFKDIASTLNKAKSIVGTTASLQYMKNVFKEKYLLSEDTSGKFSVDKLKFDKLYKMLTEIYTEDNFVKFFKVLNRKTYLNFDKAVFKINIVPKVNYTIYDGFNLRNTNLAANFNGQNTEINNMNFTKLKNFTGLFEFYKLLCVRGIITSKTKSLDKGYNKALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITSDTNIEAAEENISLDLIQQYYLTFNFDNEPENISIENLSSDIIGQLELMPNIERFPNGKKYELDKYTMFHYLRAQEFEHGKSRIALTNSVNEALLNPSRVYTFFSSDYVKKVNKATEAAMFLGWVEQLVYDFTDETSEVSTTDKIADITIIIPYIGPALNIGNMLYKDDFVGALIFSGAVILLEFIPEIAIPVLGTFALVSYIANKVLTVQTIDNALSKRNEKWDEVYKYIVTNWLAKVNTQIDLIRKKMKEALENQAEATKAIINYQYNQYTEEEKNNINFNIDDLSSKLNESINKAMININKFLNQCSVSYLMNSMIPYGVKRLEDFDASLKDALLKYIYDNRGTLIGQVDRLKDKVNNTLSTDIPFQLSKYVDNQRLLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSKVNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIINCMENNSGWKVSLNYGEIIWTLQDTQEIKQRVVFKYSQMINISDYINRWIFVTITNNRLNNSKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDLYDNQSNSGILKDFWGDYLQYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFHQFNNIAKLVASNWYNRQIERSSRTLGCSWEFIPVDDGWGERPL

**WT-BoNTA (His6-BoNTAFL)**

MDYKDDDDKGSGS**HHHHHH**SSGVDLGFENLYFQG-BoNTAFL

**TAT-BoNTA (His6-TAT-BoNTAFL)**

MDYKDDDDKGSGS**HHHHHH**SSGVDLGFENLYFQG**GRKKRRQRRRPQ**GGSGGSGGS-BoNTAFL

**Pep1-BoNTA (His6-Pep1-BoNTAFL)**

MDYKDDDDKGSGS**HHHHHH**SSGVDLGFENLYFQG**KETWWETWWTEWSQPKKKRKV**GGSGGSGGS-BoNTAFL

**ZFP3-BoNTA (His6-ZFP3-BoNTAFL)**

MDYKDDDDKGSGS**HHHHHH**SSGVDLGFENLYFQG**EKPYKCPECGKSFSASAALVAHQRTHTGEKPYKCPECGKSFSASAALVAHQRTHTGEKPYKCPECGKSFSASAALVAHQRTHTG**GGSGGSGGS-BoNTAFL

**BoNTA-ZFP3 (BoNTAFL-ZFP3-His6)**

BoNTAFL-GGSGGSGGS**EKPYKCPECGKSFSASAALVAHQRTHTGEKPYKCPECGKSFSASAALVAHQRTHTGEKPYKCPECGKSFSASAALVAHQRTHTG**ENLYFQGSSGVDLGF**HHHHHH**GSGSMDYKDDDDK

**ZFP3-BoNTA-ZFP3 (His6-ZFP3-BoNTAFL-ZFP3)**

MDYKDDDDKGSGS**HHHHHH**SSGVDLGFENLYFQG**EKPYKCPECGKSFSASAALVAHQRTHTGEKPYKCPECGKSFSASAALVAHQRTHTGEKPYKCPECGKSFSASAALVAHQRTHTG**GGSGGSGGS-BoNTAFL-GGSGGSGGS**EKPYKCPECGKSFSASAALVAHQRTHTGEKP YKCPECGKSFSASAALVAHQRTHTGEKPYKCPECGKSFSASAALVAHQRTHTG**