

## **Botulinum neurotoxin type-A enters a non-recycling pool of synaptic vesicles**

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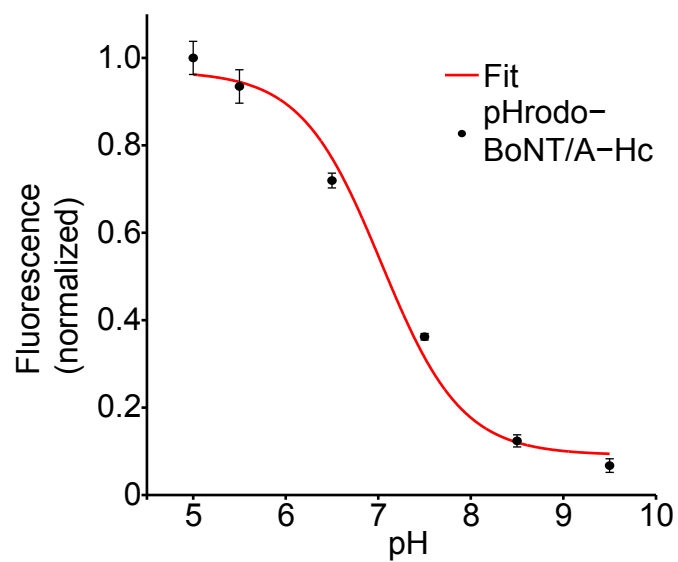
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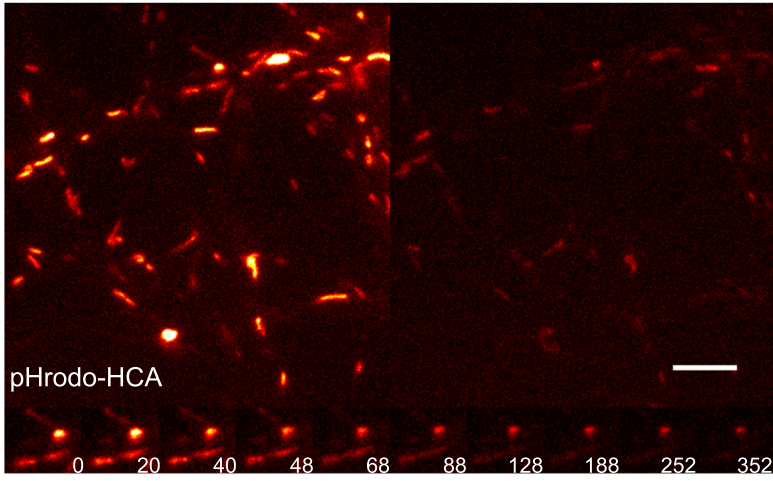
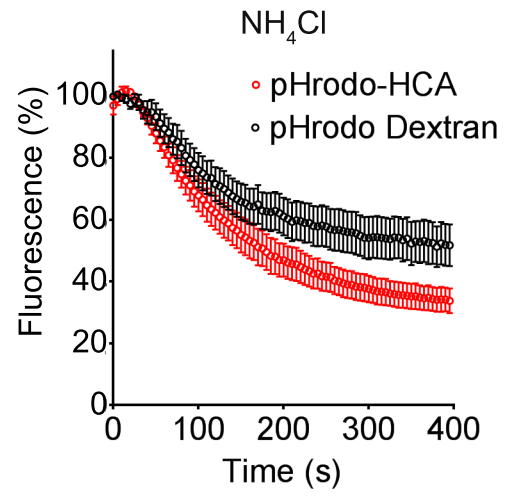
**Supplementary Figure S1: Normalized fluorescence intensity of pHrodo-BoNT/A-Hc in vitro at varying pHs.** The data were plotted as mean $\pm$ sem ( $n=4$ ) and fit to a Henderson-Hasselbach equation ( $r^2 = 0.99$ ), which gave a pKa value of 7.03 (95% confidence intervals = 0.401).

**Supplementary Figure S2: NH<sub>4</sub>Cl-evoked destaining of presynaptic nerve terminals labeled with pHrodo-BoNT/A-Hc or pHrodo-dextran reduces fluorescence to comparable levels.**

Hippocampal neurons (14-17 days *in vitro*) were loaded with either pHrodo-BoNT/A-Hc (300 nM) (A) or pHrodo-dextran (0.1 mg/ml) during a 2 min high K<sup>+</sup> stimulation. Neurons were left to recover for 12-15 min prior to the addition of NH<sub>4</sub>Cl (50 mM), which alkalizes the lumen of labeled SVs and thus quenches the pHrodo fluorescence. (A) Representative nerve terminals labeled with pHrodo-BoNT/A-Hc prior to destaining and 400 s following the addition of NH<sub>4</sub>Cl. Enlargements show the response of a representative nerve terminal to the destaining over time. Scale 5  $\mu$ m. (B) Normalized fluorescence of nerve terminals loaded with pHrodo-BoNT/A-Hc ( $n=6$ ) or pHrodo-dextran ( $n=5$ ) in response to NH<sub>4</sub>Cl. Data are plotted as mean $\pm$ sem. (C) The change in fluorescent intensity of pHrodo-BoNT/A-Hc was measured *in vitro* in response to pH change from either pH 4 to 8 or from pH 8 to 4 ( $n=3$  experiments).

**Supplementary Movie S1: BoNT/A-Hc-containing vesicles are distributed throughout hippocampal nerve terminals.** A three-dimensional reconstruction of a representative presynaptic region that has endocytosed HRP-BoNT/A-Hc (green vesicles and endosomes). Synaptic vesicles and endosomes that do not contain HRP-BoNT/A-Hc are shown in red. The presynaptic plasma membrane is displayed in yellow. Scale 200 nm.



**A****B****C**