Supporting Information

Insights into the mechanisms by which clostridial neurotoxins discriminate between gangliosides.

Joshua R. Burns [#†], *Gregory S. Lambert* [#] *and Michael R. Baldwin* [*]

Department of Molecular Microbiology and Immunology, University of Missouri School of Medicine, Columbia, Missouri, U.S.A.

Supporting Information

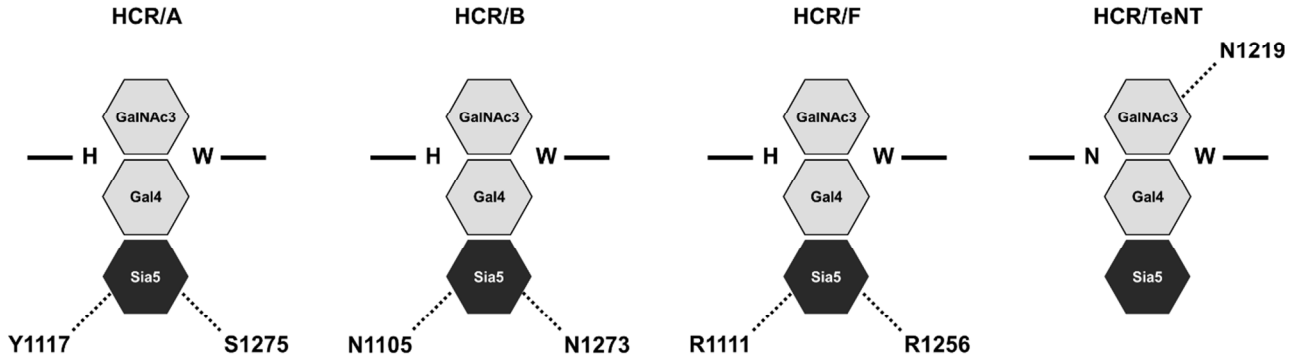

Figure S1. Schematic of HCR-mediated ganglioside binding. In each of the panels monosaccharides are represented by hexagons. GalNAc3 and Gal4 are colored in light grey while Sia5 is highlighted in dark grey. HCR amino acid residues likely involved in ganglioside binding are represented using their single letter codes. Hydrogen bonds are indicated by dotted lines between the amino acid in question and the indicated sugar.

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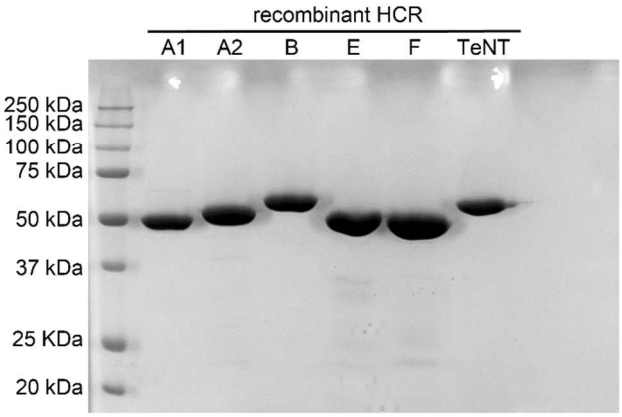

Figure S2. Representative gel showing purified HCR proteins. Representative wild-type and variant HCRs (7.5 μg) were resolved by SDS-PAGE and visualized by staining with Coomassie Blue. Unless otherwise stated all proteins employed in this study were stably expressed and estimated to be at least 90% pure as determined by SDS-PAGE followed by visualization with Coomassie Blue.

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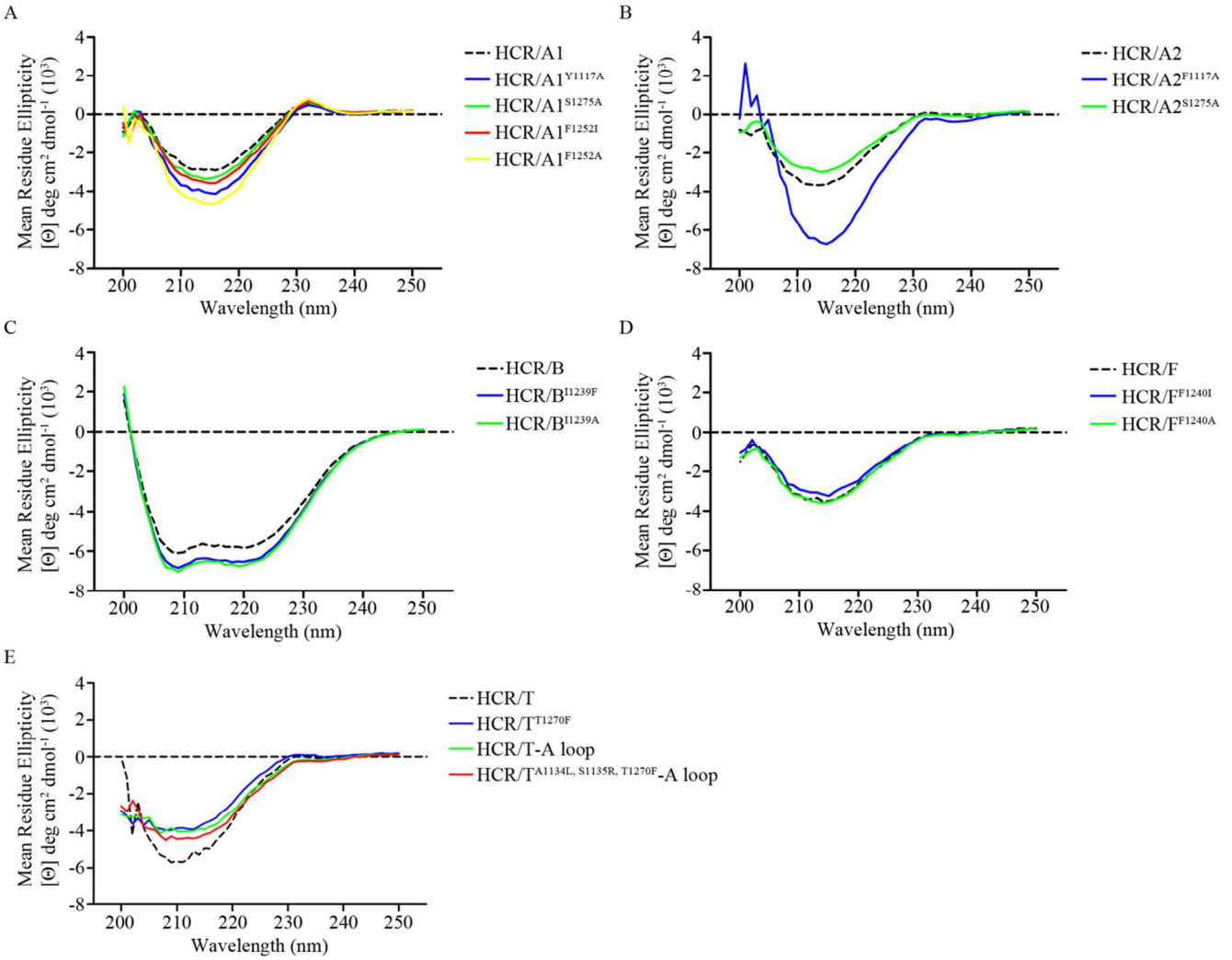

Figure S3. CD spectra of wild-type and mutated HCR domains. The far-UV CD spectra (198-240 nm) of HCR derivatives (0.5 mg/ml in water) was recorded using an AVIV model 202 spectrometer. (**A**) HCR/A1 and mutated derivatives, (**B**) HCR/A2 and mutated derivatives, (**C**) HCR/B and mutated derivatives, (**D**) HCR/F and mutated derivatives, and (**E**) HCR/T and mutated derivatives.

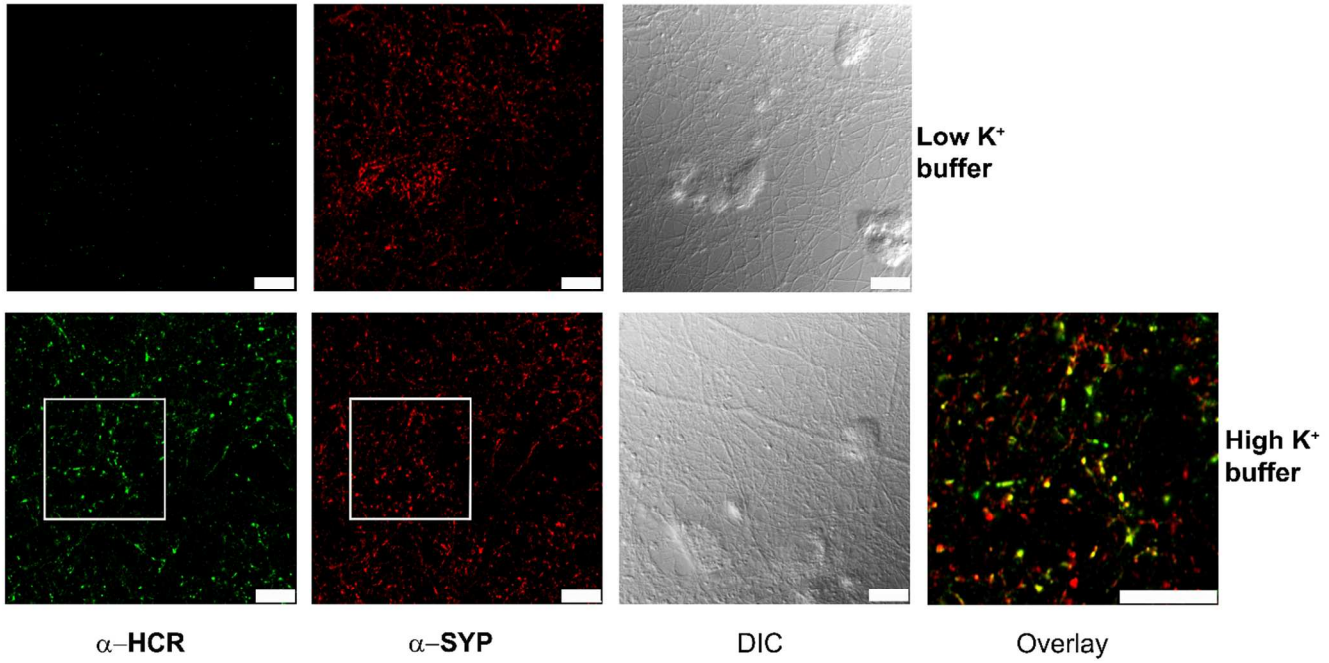

Figure S4. HCR/A2 enters neurons by way of recycling secretory vesicles. Rat cortical neurons were incubated with 50 nM HCR/A2 for 5 min at 37°C in either low K^+ buffer (*upper panels*) or high K^+ buffer (*lower panels*) to stimulate synaptic vesicle recycling. Cells were processed for immunocytochemistry and HCR/A2 was detected using a mouse anti-FLAG tag monoclonal antibody (green). Cells were co-stained with Synaptophysin (SYP) antibodies to identify neuronal synapses (red). An enlarged optical section of the region highlighted by the white box showing co-localization between the two proteins. Scale bar = 10 μm in all fields.

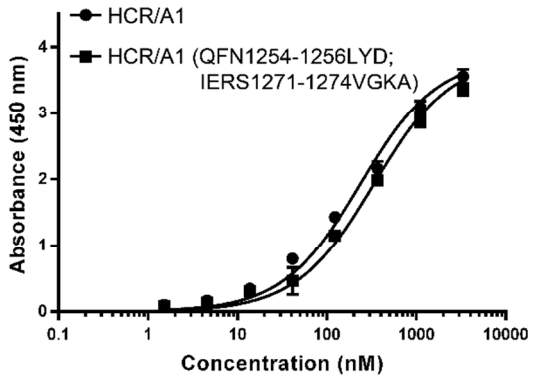

Figure S5. HCR/A1 and chimeric HCR/A1-A2 binding kinetics. Various concentrations of either wild-type HCR/A1 or HCR/A1 containing the indicated residues from HCR/A2 were examined for their ability to bind ganglioside GT1b. All values represent the arithmetic mean and standard deviation of at least four independent experiments performed in triplicate.

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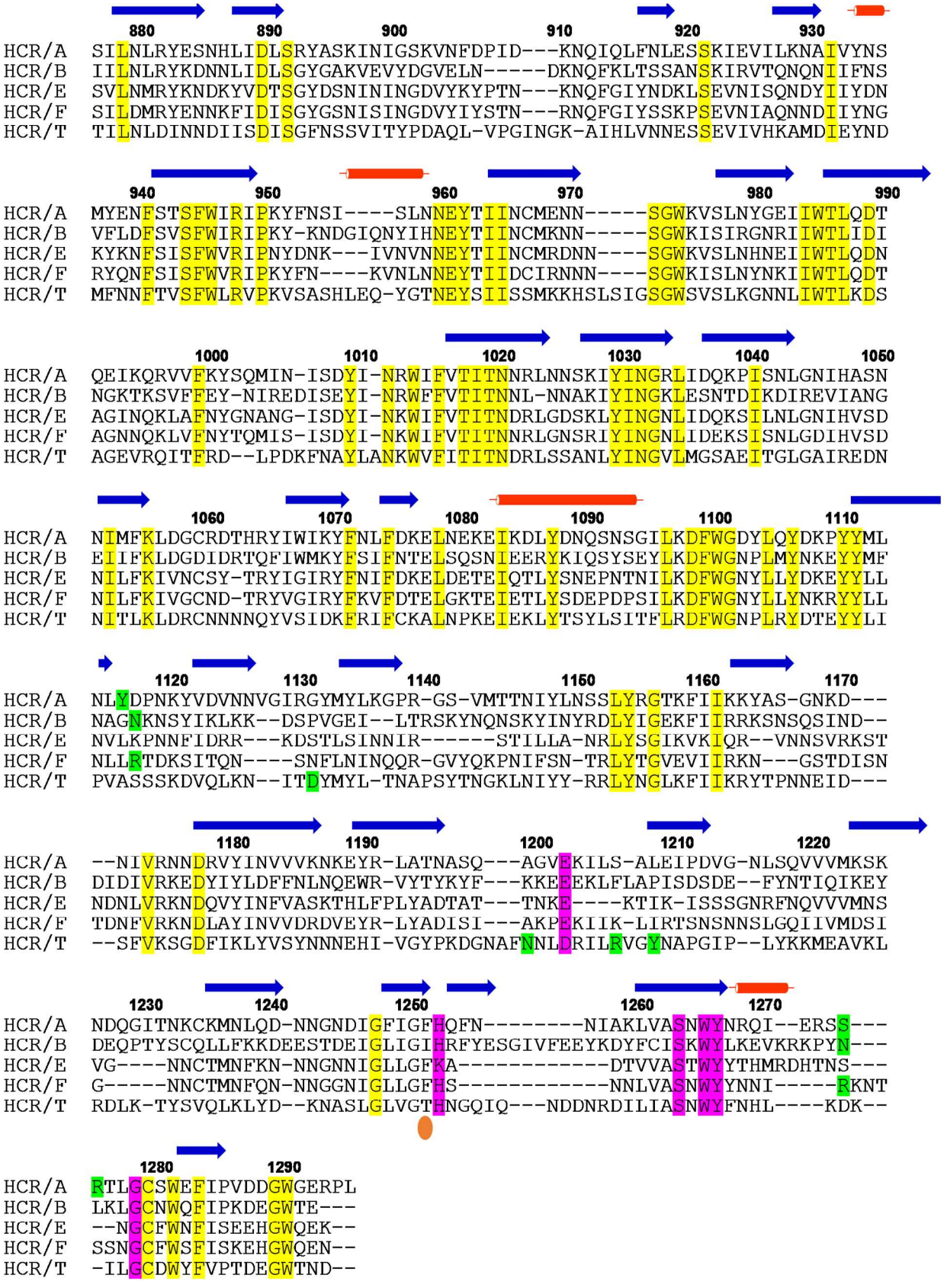

Figure S6. Structure based sequence alignment of BoNT (serotypes A, B, E and F) and TeNT HCR domains. HCR residues forming the conserved ganglioside binding motif (GBM) are highlighted in pink, while residues involved in binding specificity are colored in green. The semi-conserved hydrophobic residue unique to the BoNTs is indicated with an orange oval. Conserved residues are colored in yellow.

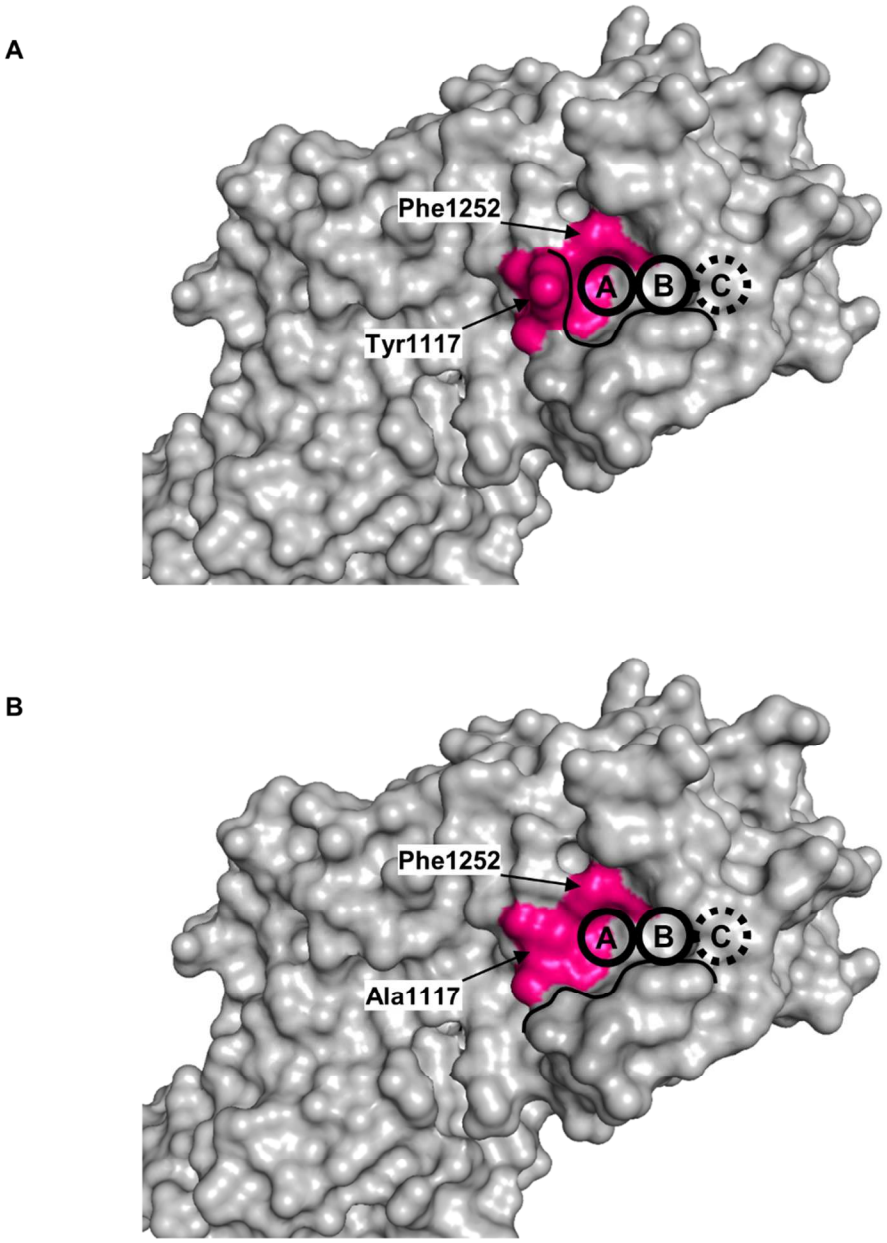

Figure S7. Ganglioside binding pocket of HCR/A1. Surface representation (grey color) of HCR/A1 (**A**) and HCR/A1^{Y1117A} (**B**). The ganglioside binding pocket with defined subsites (A-C) is highlighted using dark circles. The positions of Tyr1117/Ala1117 and the semi-conserved hydrophobic residue (Phe1252 for HCR/A1) that largely define the A subsite are colored pink.

"Adapted with permission from Hamark, C.; Berntsson, R. P.; Masuyer, G.; Henriksson, L. M.; Gustafsson, R.; Stenmark, P.; Widmalm, G., Glycans Confer Specificity to the Recognition of Ganglioside Receptors by Botulinum Neurotoxin A. *Journal of the American Chemical Society* **2017,** *139* (1), 218-230. Copyright (2017) American Chemical Society."