Entry of a Recombinant, Full-Length, Atoxic Tetanus Neurotoxin into Neuro-2a Cells

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Tetanus neurotoxin (TeNT) and botulinum neurotoxin (BoNT) are clostridial neurotoxins (CNTs) responsible for the paralytic diseases tetanus and botulism, respectively. CNTs are AB toxins with an N-terminal zinc-metalloprotease light chain that is linked by a disulfide bond to a C-terminal heavy chain that includes a translocation domain and a receptor-binding domain (HCR). Current models predict that the HCR defines how CNTs enter and traffic in neurons. Recent studies implicate that domains outside the HCR contribute to CNT trafficking in neurons. In the current study, a recombinant, full-length TeNT derivative, TeNT(RY), was engineered to analyze TeNT cell entry. TeNT(RY) was atoxic in a mouse challenge model. Using Neuro-2a cells, a mouse neuroblastoma cell line, TeNT HCR (HCR/T) and TeNT(RY) were found to bind gangliosides with similar affinities and specificities, consistent with the HCR domain containing receptor binding function. Temporal studies showed that HCR/T and TeNT(RY) entered Neuro-2a cells slower than the HCR of BoNT/A (HCR/A), transferrin, and cholera toxin B. Intracellular localization showed that neither HCR/T nor TeNT(RY) localized with HCR/A or synaptic vesicle protein 2, the protein receptor for HCR/A. HCR/T and TeNT(RY) exhibited only partial intracellular colocalization, indicating that regions outside the HCR contribute to the intracellular TeNT trafficking. TeNT may require this complex functional entry organization to target neurons in the central nervous system.
While the cellular basis for the trafficking patterns of this fusion protein needs further resolution, these observations question whether or not the HCR domain is necessary and sufficient to traffic the CNTs to their respective physiological substrates.

Since domain exchanges between CNTs can yield unexpected phenotypes (20), comparison of HCR trafficking relative to full-length CNT is an important question. We chose to characterize the entry of the holo-TeNT, since phenotypes of the HCs are complex, with HCA interacting with membranes independent of a pH gradient and pH dependency ascribed to the HCR domain within holo-CNTs (21). The ability to localize full-length CNTs has been limited by the ability to produce recombinant full-length CNTs with epitope tags to detect intracellular localization. The current study characterizes a recombinant full-length, atoxic TeNT which contains Arg372Ala and Tyr375Phe mutations within the catalytic site [TeNT(RY)] and possess epitopes to allow detection of the LC and HC domains. TeNT(RY) bound gangliosides with the same specificity as HCR/T, consistent with the localization of receptor binding function within the HCR domain. Intracellular localization measurements showed that TeNT(RY) and HCR/T trafficked at similar rates but only partially colocalized within intracellular vesicles. This indicates that regions outside the HCR domain contribute to the intracellular trafficking of TeNT.

MATERIALS AND METHODS

Production and purification of recombinant proteins. Hemagglutinin (HA)-tagged and FLAG-tagged heavy chain receptor (HCR) binding domains of tetanus (HCR/T; residues 865 to 1315) and botulinum neurotoxin serotype A2 (Clostridium botulinum strain A2 Kyoto F, HCR/A, residues 870 to 1296) were produced and purified as previously described (22, 23). Briefly, DNA encoding three HA repeats (YPYDVPDYA) was subcloned downstream of the His6 epitope and upstream of the HCR sequence in pET28a (Novagen), and this construct was then confirmed by DNA sequencing. Escherichia coli BL21 (DE3) was transformed with the expression plasmid. Transformants were plated overnight on LB agar containing 50 μg/ml of kanamycin (Km) and stored at −80°C in 12% (vol/vol) glycerol. For protein expression, E. coli was grown overnight on LB-Km plates that were inoculated into 400 ml of LB-Km. Cells were grown for 2 h at 30°C at 225 rpm (optical density at 600 nm [OD600] of ~0.6), and then 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, followed by overnight culture at 16°C (225 rpm). Cells were harvested and lysed with a French press. The lysates were centrifuged at 38,000 × g for 20 min to remove the insoluble fraction, and the supernatant was vested andlysed with a French press. The lysates was centrifuged at 38,000 rpm for 20 min to remove the insoluble fraction, and the supernatant was used. This indicates that regions outside the HCR domain contribute to the intracellular trafficking of TeNT.
of tetanus toxin into a neuronal cell line.

**Binding of TeNT(RY) to Neuro-2a cells.** TeNT utilizes two gangliosides as functional plasma membrane receptors (7, 23, 25, 26). Dual ganglioside binding is necessary and sufficient for HCR/T entry into neurons and nonneuronal cells, such as HeLa cells (7). TeNT prefers to bind b-series gangliosides (GD1b, GT1b, and GQ1b) (27), and in solid-phase assays, HCR/T binds most avidly to GT1b, GD1b, and GD1a (23). Knockout mice that do not produce b-series gangliosides or complex gangliosides are resistant to TeNT (28, 29). To test the ganglioside binding potential of TeNT(RY) relative to HCR/T, Neuro-2a cells (ATCC CCL-131) were loaded with exogenous gangliosides. Neuro-2a cells are derived from a mouse neuroblastoma and contain minimal amounts of endogenous complex gangliosides. After incorporation of exogenous gangliosides (GT1b, GD1b, GD1a, or GM1a), the binding of HCR/T and TeNT(RY) was determined. As expected from previous experiments (7), at 4°C HCR/T bound, in a dose-dependent manner, to GT1b- and GD1b-enriched cells (Fig. 2). HCR/T bound minimally to GD1a and GM1a, except at the highest concentration (40 nM) (Fig. 2A). The amount of TeNT(RY) bound

**RESULTS**

**Properties of TeNT(RY).** Recombinant, atoxic full-length tetanus toxin [TeNT(RY)] (1,315 amino acids) was engineered with two point mutations within the light chain (R372A and Y375F). The N terminus contained His6 and 3× FLAG epitopes, while the C terminus contained HA and Strep epitopes (Fig. 1A). *E. coli* produced TeNT(RY) as a soluble ~150-kDa protein as determined by SDS-PAGE (Fig. 1B). Production of TeNT(RY) in *E. coli* was optimized, with purified yields ranging from 2 to 8 mg/liter of batch culture. TeNT is an AB toxin which is cleaved by trypsin into a single-chain or dichain (trypsin-nicked) TeNT(RY) (5 to 7), when administered at 500-fold-higher concentrations than the amount of wild-type TeNT needed to cleave VAMP2 (data not shown). TeNT(RY) was next used to probe the entry properties of full-length tetanus toxin into a neuronal cell line.

5D blind method, wide-field modality, and 20 iterations. The Hoechst channel was excluded from the deconvolution.

**Mouse challenge model.** Female ICR mice (18 to 22 g) were injected intraperitoneally with 5 µg/mouse of either the single-chain or dichain (trypsin-treated) forms of the recombinant TeNT(RY) (equivalent to ~125,000 50% lethal doses [LD50] of wild-type TeNT) (1) and then monitored for 48 h, when survival was scored. All mice survived challenge with 5 µg of the single-chain or dichain forms of recombinant TeNT(RY) and showed no symptoms of tetanus intoxication. All animal experiments were approved by and conducted according to guidelines from the University of Wisconsin Animal Care and Use Committee.

**Data analysis.** Image analyses of intensity were performed using ImageJ 1.46r. The colocalization of deconvolved cells was analyzed using Pearson’s colocalization coefficients in Nikon Elements AR. Graphs were created using GraphPad Prism 5, and figures were compiled using Adobe Photoshop CS3. Statistics were performed by analysis of variance (ANOVA) (see Fig. 4 to 7), using GraphPad Prism 5, or by Student’s t test (see Fig. S1 in the supplemental material), using Excel.

**A** Schematics of recombinant TeNT(RY) and HCR/T. Native TeNT is composed of LC, HCT, and HCR domains; the recombinant protein contains mutations within the active site at R372 and Y375. In addition, N-terminal His6 and FLAG epitopes and C-terminal HA and Strep epitopes are present. Recombinant HCR/T contains N-terminal His6 and FLAG or HA epitopes. (B) TeNT(RY) was incubated alone (−) or with trypsin (+) at room temperature for the indicated time points. Samples were subjected to SDS-PAGE, alone (−) or with β-mer (+), and stained with Coomassie blue (top and middle) or by immunoblotting (bottom), using anti-FLAG–HRP-conjugated antibody (1:20,000).
Therefore, toxin B (CTxB), reached 50% internalization by 1 min, respectively (Fig. 3A). In comparison, TeNT(RY) was internalized by 50% of the cells at ~7 min (Fig. 3A). HCR/T was internalized at a similar rate as TeNT(RY), with 50% internalization at ~5 min. The HCR domain of BoNT/A (HCR/A), which utilizes both a ganglioside and a defined protein, synaptic vesicle protein 2 (SV2), as receptors, exhibited 50% internalization of cells at ~1.5 min (Fig. 3B). Entry of HCR/A was faster than entry of HCR/T or TeNT(RY), suggesting that different mechanisms are utilized by BoNT/A and TeNT to enter Neuro-2a cells.

Internalization of TeNT(RY) into Neuro-2a cells. Pearson’s coefficients were determined to measure protein-protein colocalization. Experimental colocalization ranges were established for this system. As a measure of maximum colocalization, the colocalizations of the HA (LC) and FLAG (HC) epitopes on TeNT(RY) bound to Neuro-2a cells at 4°C were determined to have a Pearson’s coefficient of 0.80 (Fig. 4, 5, 6, and 7; see also Fig. S1 in the supplemental material). The minimum colocalization, determined by measuring colocalization of CTxB bound to Neuro-2a cells at 4°C with SV2c, an intracellular protein enriched in synaptic vesicles, was determined to have a Pearson’s coefficient of 0.03 (Fig. 4 to 7; see also Fig. S1). After a 20-min incubation, the Pearson’s coefficient of LC and HC on TeNT(RY) was 0.77, which indicated that the LC and HC domains had not dissociated during this time course. In contrast, colocalization of TeNT(RY) with SV2c was low, at ~0.2.

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FIG 2 Ganglioside binding profiles of HCR/T and TeNT(RY). Neuro-2a cells were loaded with exogenous ganglioside GT1b, GD1b, GD1a, or GM1a. After loading, cells were washed and cooled to 4°C in DPBS. Cells were then incubated with 2.5, 10, or 40 nM HCR/T (A) or TeNT(RY) (B) in cold buffer for 30 min. Cells were fixed and processed for immunofluorescence. The epifluorescence images shown are of GT1b-treated Neuro-2a cells with 40 nM HCR/T and TeNT(RY) (HA), and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Intensity was quantified by averaging the intensity of HA staining in a field, subtracting background fluorescence, and normalizing to the intensity of intracellular SV2c (HA/SV2c). Intensity was then normalized to set the signal from 40 nM HCR/T bound to GT1b to 1 in both sets of data.

FIG 3 TeNT(RY) and HCR/T are endocytosed at a similar rate into Neuro-2a cells. (A) Neuro-2a cells were treated with exogenous GT1b [for TeNT(RY) and HCR/T uptake] or GM1a [for CTxB uptake]. Cells were treated with 60 nM transferrin (Tfn) or 40 nM TeNT(RY), HCR/T, or CTxB for 2, 5, 10, or 20 min at 37°C. In a separate experiment, cells were incubated with Tfn or CTxB for 0.5, 1, or 2 min at 37°C. Cells were processed for immunofluorescence analysis. TeNT(RY) and HCR/T were detected using anti-HA antibody, and CTxB and Tfn were obtained as Alexa-labeled conjugates. Individual cells were analyzed, and an internalized cell was defined as containing intracellular signal in two or more vesicles. At least three independent replicates were performed with a minimum of 200 cells analyzed per time point for each replicate. The time at which 50% of the cells contain intracellular signal (t1/2) was determined for each protein. (B) Neuro-2a cells were treated with exogenous GT1b and treated with 40 nM HCR/A and HCR/T for 0.5, 1, 2, and 5 min at 37°C. Cells were processed and analyzed as described in panel A, except the minimum number of cells analyzed was 180 per time point for each replicate.
Partial colocalization of intracellular TeNT(RY) and HCR/T in Neuro-2a cells. TeNT(RY) was engineered with an HA epitope on the C terminus of the HC domain and incubated with FLAG epitope-tagged HCR/T on ganglioside-enriched Neuro-2A cells. At 20 min, the Pearson’s coefficient for HCR/T and TeNT(RY) was 0.50, indicating a partial colocalization of the two proteins (Fig. 5). The Pearson’s coefficient of differentially epitope labeled HCR/T was 0.69 (Fig. 6), statistically different from the colocalization between HCR/T and TeNT(RY). The different coefficients indicate that the intracellular localization of TeNT(RY) and HCR/T represents partial colocalization. Inspection of the merged images in Fig. 6 showed intracellular vesicles containing both TeNT(RY) and HCR/T and also vesicles which contained either TeNT(RY) or HCR/T. In both experiments, the colocalization of TeNT(RY) and HCR/T with SV2 was low, ~0.2. This indicates that regions outside the HCR contribute to TeNT intracellular trafficking.

HCR/T and HCR/A entry into Neuro-2a cells. HCR/A and HCR/T had different rates of internalization (Fig. 3). To determine if HCR/A and HCR/T trafficked to the same vesicles, Neuro-2a cells were incubated with HA-HCR/A and FLAG-
Figure 7 shows that HCR/A trafficked to an SV2-positive compartment in a compacted perinuclear region. The Pearson’s coefficient for HCR/A and SV2 was 0.82, a coefficient expected of proteins that were completely experimentally colocalized. In contrast, the Pearson’s coefficient for the colocalization between HCR/A and HCR/T was low, at ~0.20. This Pearson’s value was lower than anticipated based on a visual inspection of the colocalization in Figure 7, which had overlap of HCR/T with HCR/A and SV2c in the perinuclear region. However, inspection of the individual images in the z-stack of the merged image in Figure S2 in the supplemental material showed that the majority of HCR/T localized to vesicles that were peripheral to the compact SV2 vesicles. Together, the data showed that the majority of HCR/T traffic was independent of HCR/A and SV2, with only a fraction of HCR/T within SV2-positive vesicles. HCR/T-associated vesicles did not contain the endocytic markers clathrin, Rab5, EEA1, and LAMP1, while the HCR/A-associated vesicles possessed the synaptic vesicle marker proteins SV2c, synaptosomal-associated protein 25 (SNAP25), vesicle-associated membrane protein 2 (VAMP2), and acetylcholine transferase (AChT) (data not shown). Colocalization of HCR/A or HCR/T with SV2c at earlier time points showed Pearson’s coefficients similar to those of the 20-min incubations (data not shown). This indicates that the HCR/A-SV2 association is an early trafficking event.
This study describes the properties of full-length tetanus neurotoxin. The toxicity of TeNT(RY) was tested in mice, in which both trypsin-treated and untreated TeNT(RY) were atoxic. Thus, TeNT(RY) appears to be a useful reagent to study neurotoxin entry, since tetanus toxin is not a select agent, and in the United States, humans are immunized with tetanus toxoid during the DT and DTaP vaccinations (31). HCR/T and TeNT(RY) displayed similar properties when assayed for binding gangliosides (Fig. 2) and for the rate of internalization (Fig. 3) but showed only partial colocalization within Neuro-2a cells (Fig. 5). This is consistent with the HCR domain defining the interaction with host cell receptor, while regions outside the HCR contribute to the fate of intracellular trafficking. The observed complexity of TeNT trafficking relative to BoNT/A, which colocalized within SV2-enriched vesicles in this study, is consistent with the necessity of TeNT to traffic through multiple vesicles to reach the CNS, while BoNT is delivered from the SV in the periphery of the neuron. The complex trafficking has been observed in cortical neurons, where HCR/T trafficked through both activity-dependent and -independent trafficking pathways, whereas HCR/A trafficked through solely activity-dependent pathways (11). One difference with the fraction of HCR/T that colocalized with HCR/A in Neuro-2a cells relative to colocalization observed in primary cortical neurons is that in Neuro-2a cells, the colocalization appears to occur from within the intracellular vesicle population, while in cortical neurons, the colocalization occurs at the plasma membrane during SV endocytosis. The significance of this differential trafficking is under investigation.

The receptor binding activity of TeNT has been defined within the HCR (32). The ganglioside preference of TeNT(RY) and HCR/T were the same (Fig. 2), with the highest binding to the b-series gangliosides GT1b and GD1b, suggesting that while there is a preferred ganglioside for each of the ganglioside binding pockets, the overall affinity for b-series gangliosides is greater than that of a-series gangliosides. This is supported by crystallographic and biochemical characterization, which showed that the two ganglioside binding sites are within the HCR (7, 23, 25, 26). TeNT(RY) binding was higher than HCR/T to untreated Neuro-2a cells, which may reflect a nonganglioside interaction located outside the HCR. The translocation domain of CNTs is sufficient for insertion into a lipid bilayer (16), indicating that the HCT is capable of interacting with lipids independent of the HCR domain. We hypothesize that the increased association of TeNT(RY) to untreated Neuro-2a cells is due to interactions between the cell membrane and the holotoxin, possibly the translocation domain. Experiments are under way to determine if a membrane-interacting site can be ascribed to regions outside the HCR domain. Recent work with chimeric botulinum and tetanus neurotoxins adds to the complexity of the trafficking of CNTs. Wang et al. found that efficient retrograde trafficking of TeNT was dependent on the presence of the entire toxin (19) and that replacing any domain of TeNT with the homologous domain of BoNT/A resulted in a toxin with induced flaccid paralysis in mice. This also suggests that the HCR domain is not the sole determinant in the entry and trafficking of CNTs. We found that TeNT(RY) and HCR/T partially co-localized within Neuro-2a cells, statistically less than HCR/T co-localized with HCR/T, supporting a role for domains outside the HCR in the trafficking of TeNT into Neuro-2a cells. Continued assessment of HCR/T and TeNT trafficking in Neuro-2a cells may resolve the components within TeNT that are responsible for the unique trafficking pattern.

In cultured cells, Tfn is endocytosed via a well-defined pathway into early endosomes and recycling endosomes prior to recycling back to the plasma membrane. The entry of Tfn observed here is in line with the rates of entry in other cell types (33). CTxB utilizes GM1a as a receptor and enters cells through many mechanisms, all of which converge into early endosomes, prior to retrograde trafficking to the trans-Golgi membrane and subsequently to the endoplasmic reticulum (ER). The faster entry of CTxB than HCR/T and TeNT(RY) was unexpected, given the use of gangliosides as receptors for each protein, but may be due to the active entry of CTxB via clustering of GM1a molecules (34), as compared to passive entry of HCR/T and TeNT(RY). Alternatively, this may be due to slower entry of HCR/T and TeNT(RY) due to the utilization of an accessory factor, possibly a host protein, in addition to gangliosides (35).

Since HA-tagged HCR/T and FLAG-tagged HCR/T possessed similar rates of entry that were different from that of HA-tagged HCR/A, the epitope could influence the absolute rate of entry, but the relative differences in the rates of entry of HCR/A and HCR/T were not due to the epitope. The epitope tags on HCR/T and HCR/A were located on the N terminus, spatially distant from the known receptor binding regions. Two epitope tags on TeNT(RY) were present on the C terminus and could have interfered with binding and internalization. However, as the intensities of bound HCR/T and TeNT(RY) were not statistically significantly different (Fig. 2), the C-terminal epitopes on TeNT(RY) did not appear to affect binding. In CNTs, the N terminus of the HCR is a jelly roll domain, and the C terminus is a beta-trefoil domain containing the known ganglioside binding sites. The extreme C terminus of the HCR folds back toward the N terminus, suggesting that a C-terminal tag would be spatially distant from the known receptor binding sites.

While Neuro-2a cells appear to be useful to measure the entry of tetanus toxin, other cell lines are also capable of internalizing tetanus toxin. Adrenal chromaffin cells, after the addition of exogenous ganglioside, are intoxicated by TeNT, which blocks evoked release of catecholamine (36). Differentiated PC12 cells, a rat pheochromocytoma cell line, internalize HCR/T and TeNT (7, 37) mediated by the C-terminal subdomain of the HCR (38). The intoxication of PC12 cells by TeNT is dependent on the presence of cholesterol (10), but a further mechanism for TeNT entry has not been defined. The observation that HCR/T is functional for entry into ganglioside-loaded HeLa cells confirms that TeNT is capable of being internalized via an SV-independent mechanism (7), likely in a manner similar to the entry of TeNT into motor neurons (12). While our earlier studies (11) observed that HCR/T entry into cortical neurons was primarily membrane depolarization independent (independent of SVs), intracellular localization of HCR/T was difficult to resolve due to the limited ability to resolve intracellular trafficking patterns within overlapping axons in culture. The advantage of the Neuro-2a cells used in this study was the ability to resolve the intracellular movement of BoNT and TeNT derivatives from the plasma membrane to intracellular vesicles. Fischer and Montal (39) also utilized Neuro-2a cells for patch clamp experiments. Neuro-2a cells are derived from a neuroblastoma, making these cells more similar to the sympathetic nervous system instead of the central nervous system.
cells release catecholamines when stimulated (as opposed to ace-tylcholine from motor neurons or glycine from inhibitory neu-rons) and have been shown to incorporate gangliosides to induce surface activity (40).

The two-receptor model proposes that BoNTs bind dual recep-tors: a protein and ganglioside (41). Protein receptors for the ma-jority of botulinum neurotoxin serotypes have been defined, but the identity of a protein receptor for tetanus has remained elusive (18). In addition, TeNT entry is complicated by the requirement for two entry mechanisms, one into motor neurons and subse-quently into inhibitory interneurons (42). TeNT entry into motor neurons has been characterized using the HCR domain. HCR/T does not colocalize with VAMP2 and is able to enter BoNT/A- and BoNT/D-treated neurons (12). The retrograde movement of HCR/T is dependent on Rab5 and Rab7 and occurs in Rab7 vesi-cles which are also positive for low-affinity neurotrophin receptor, TrkB, and brain-derived neurotrophic factor (BDNF) (14). These vesicles are of neutral pH and vATPase excluded (13). Little is known concerning the mechanism by which TeNT is transcyto-sed, but TeNT intoxication of inhibitory interneurons requires entry into a vesicle, which will undergo acidification, making en-dosomes an attractive mechanism of entry. The complexity of TeNT trafficking may be required to enter multiple neuronal cell types; this is facilitated by functions within multiple domains of the toxin.

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