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Piggybacking on the Cholera Toxin: Identification of a CTB-binding Protein as an Approach for Targeted Delivery of Proteins to Motor Neurons

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KEYWORDS • Affimer • Drug delivery • Motor neuron • Cholera toxin

ABSTRACT: A significant unmet need exists for the delivery of biologic drugs such as polypeptides or nucleic acids, to the central nervous system (CNS) for the treatment and understanding of neurodegenerative diseases. Naturally occurring bacterial toxins have been considered as tools to meet this need. However, due to the complexity of tethering macromolecular drugs to toxins, and the inherent dangers of working with large quantities of recombinant toxin, no such route has been successfully exploited. Developing a method where a bacterial toxin's non-toxic targeting subunit can be assembled with a drug immediately prior to *in vivo* administration has the potential to circumvent some of these issues. Using a phage-display screen, we identified two antibody mimetics, Anti-Cholera Toxin Affimer (ACTA)-A2 and ACTA-C6 that non-covalently associate with the non-binding face of the cholera toxin B-subunit (CTB). In a first step toward the development of a non-viral motor neuron drug-delivery vehicle, we show that Affimers can be selectively delivered to motor neurons *in vivo*.

INTRODUCTION

Research into treatments for degenerative diseases of motor neurons such as amyotrophic lateral sclerosis (ALS), is hampered in part due to the considerable challenges associated with delivery of molecular probes to the central nervous system (CNS).[1] To complicate matters, recent trends towards the use of biologics as drugs have resulted in a shift in focus from small molecules to macromolecules such as nucleic acids and antibodies.[2] Typical strategies for cellular penetration and macromolecule delivery have relied in the past on the use of virally derived cell-penetrating peptides (CPPs).[3-5] But whilst efficient, these vectors lack specificity in both cell targeting and mechanism of cell entry.[6] Viral delivery methods, such as those based on the Herpes Simplex virus, have been used successfully to transfer therapeutic genes into the CNS for treatment of conditions such as ALS and spinal cord injury.[7-9] Viral-mediated gene therapy has been limited however by concerns over vector safety and expense of production.[10] A significant need exists for the development of novel technologies to deliver biologics into motor neurons via peripheral administration routes.

AB_5 bacterial toxins comprise a toxic A-subunit associated with a non-toxic pentameric B-subunit that enables entry to certain cell types through interactions with highly specific surface markers to trigger receptor-mediated endocytosis.[11-13] Cholera toxin B-subunit (CTB), binds to ganglioside GM1, a membrane-tethered carbohydrate highly expressed at the neuromuscular junction.[14, 15] CTB therefore represents a potential candidate to target motor neurons *in vivo* for delivery of a cargo.

Indeed, the ability of CTB to undergo retrograde transport in neurons has led to extensive use of CTB-conjugates as neuronal tracers.[16-18] Tinker *et al.* previously demonstrated that by mimicking the full-length holotoxin, fluorescent CT chimeras could be produced by replacing the toxic A1 domain with a fluorescent protein.[19] Chen et al. expanded on this work by expressing fusions with heat-labile enterotoxin IIA, and showing that these could be successfully delivered to the cytosol of neurons in mammalian cell culture.[20] This approach, however, requires expression of A2 fusions with the desired cargo. Successful formation of AB_5 complexes relies on assembly of the B_5 pentamer around the A2 α-helix, making co-expression a necessity (Figure 1).[21] For development of a tool to deliver a payload of any kind, co-expression would ideally be avoided, as not all molecules of interest are amenable to forming fusions, e.g. nucleic acids and antibodies, and separate expression would prevent extended handling of potentially toxic complexes. Identifying a molecule capable of associating with preassembled CTB pentamer in a non-covalent manner could circumvent these problems, since the cargo of choice could be ligated to the CTB- binding component. Each component could then be produced independently and assembled immediately prior to administration.

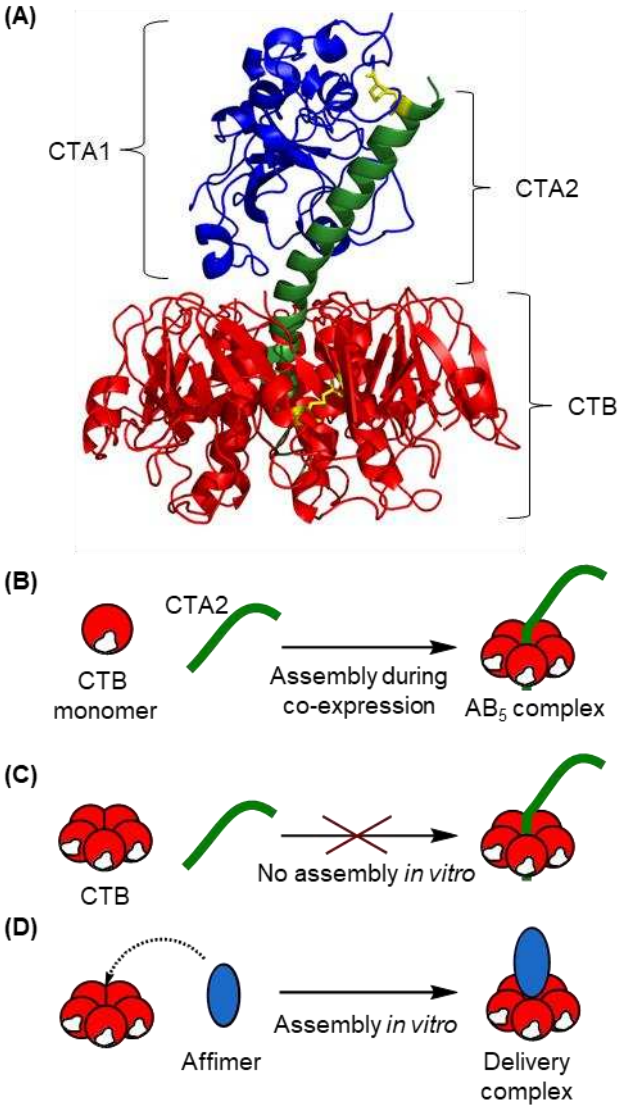

Figure 1. (A) Crystal structure of the AB_5 cholera toxin showing the CTA1 subunit (green) associated to CTA2 subunit (green) via a disulfide bond (yellow) which in turn is non-covalently associated with the B_5 subunit CTB (red). (B) Co-expression of A- and B-components leads to assembly of AB_5 complex *in vivo*, while (C) no spontaneous assembly of AB_5 complexes occurs upon mixing the B-pentamer with the A2 peptide. (D) The approach described in this paper enabling separate expression of components and simple assembly *in vitro* to form a delivery complex

Antibody mimetics represent a group of engineered protein scaffolds which reproduce the molecular recognition of antibodies while possessing enhanced properties. These improvements include: minimal size, stability, and ease of expression.[22, 23] Specificity for a particular target is obtained by screening diverse libraries using technologies such as phage, mRNA, or ribosome display that allow the binding capability of the isolated protein to be linked back to its coding sequence.[24-26] This strategy for high-throughput identification of binding proteins has led to the discovery of novel interactions with affinities comparable to those attained with antibodies.[27] One recent addition to the family of antibody mimetics is the Affimer, a thermostable scaffold based on a phytocystatin consensus sequence, with two variable nine-residue loops responsible for substrate binding.[28, 29] The availability of this

scaffold library led us to postulate that we could identify an Affimer to target the non-binding face of CTB to replicate the formation of an AB_5-type complex *in vitro*, and that this complex would be capable of internalisation into motor neurons and transportation to the cell body in a retrograde fashion to deliver a payload.

Herein we report the first step toward developing this novel technology, in which we use phage display to identify Affimers associating with the non-binding face of CTB. Two binders were identified from an initial screen, termed ACTA-A2 and ACTA-C6, and were shown to form stable complexes with CTB. The technology was then validated *in vivo* by tongue injection in mice followed by detection in neuronal cells of the brain.

RESULTS AND DISCUSSION

Identification of CTB-binding Affimers

CTB for use in phage selections and other downstream processes detailed in this paper was expressed in *Vibrio sp.* 60 and subsequently purified directly from the culture supernatant,[30] as the protein is released from the periplasm following accumulation. Approaches to identify target-specific binding proteins by phage display typically rely on modification of the target to permit immobilisation to a solid support.[31-33] Recombinant bacteriophage libraries can then be tested against the fixed target in multiple screens to obtain a series of binders. However, modification can be non-specific, resulting in the target being displayed in a variety of orientations. It was critical that Affimer-association did not interfere with the ability of CTB to bind to GM1 as this would impede cellular uptake; thus presentation of CTB in a range of orientations was undesirable. CTB-GM1 complex has a particularly high binding affinity for a protein-carbohydrate interaction (K_d = 43 nM for the monovalent protein-oligosaccharide interaction),[34, 35] therefore, it was possible to perform phage display screens against unmodified CTB complexed to ganglioside GM1 which was adhered to a microtitre plate surface. This approach ensured that the binding face of CTB was obscured during screening, and that the non-binding face was targeted exclusively. Four consecutive rounds of phage display were conducted in this manner.

Phage ELISA allowed identification of fifteen unique clones of Affimer-displaying phage particles that could bind to CTB (Figure S1). A plate-based Affimer-lectin binding assay (ALBA) was established to prioritize a subset of binders for full characterisation. Affimer coding sequences were subcloned from the phage vector into pET11, allowing addition of a His-tag for purification and a C-terminal cysteine residue for conjugation of the Affimer to a biotin-maleimide reagent. Biotinylated Affimer was then evaluated for its ability to bind to GM1-coated plates in the absence or presence of CTB. The amount of Affimer remaining on the plate surface following extensive wash steps was quantified using a streptavidin-HRP conjugate in the presence of the fluorogenic HRP-substrate Amplex® Red and hydrogen peroxide (Figure 2). Affimers A2, B11, C6, and D3 showed the highest binding relative to the control wells and were thus taken forward for further study.

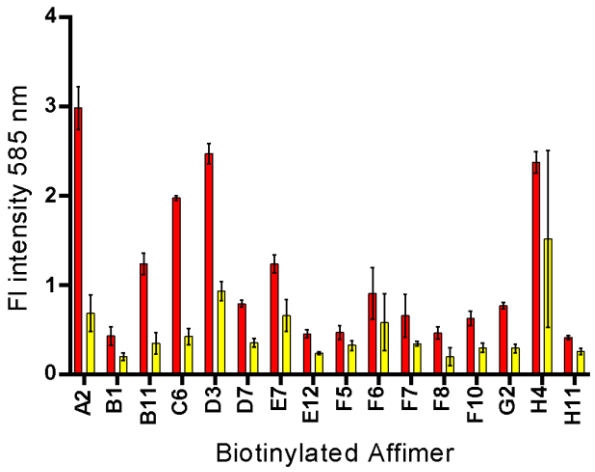

Figure 2. Analysis of CTB-binding Affimers using the Affimer-lectin binding assay (ALBA). Each data point represents the signal produced following incubation of a biotinylated Affimer with (red) or without (yellow) CTB, detected using a Streptavidin-HRP conjugate in the presence of Amplex Red and hydrogen peroxide. The fluorescence of the Amplex Red reaction product resorufin was detected at 585 nm and measured in technical triplicate. Error bars show the standard deviation.

CTB-binding Affimers Demonstrate Selectivity

The binding specificity of the Anti-Cholera Toxin Affimers (ACTAs) was investigated by comparison of their ability to bind to CTB and its *E. coli* homologue, heat-labile enterotoxin B-subunit (LTB). The LTB lectin is 80% identical to CTB, with several of the differentiating residues exposed on the surface of the protein.[36, 37] The five ACTA proteins that bound most strongly to CTB were re-evaluated against both CTB and LTB in an ALBA assay (Figure 3A). ACTA-B11, C6 and D3 appear not to discriminate between CTB and LTB. Interestingly, however, ACTA-A2 demonstrated strong selectivity, binding exclusively to CTB. Discrimination between these two highly similar proteins is significant since current commercial antibodies are unable to do so. This assay gave some insight into where ACTA-A2 may be binding, since of the 17 residues that differ between CTB and LTB, only five of these are surface exposed on the non-GM1-binding face of CTB. These residues were therefore considered to be the most likely to be forming interactions with the ACTA variable loops (Figure S2).

To tease out the nature of this interaction, five CTB-LTB hybrids were produced, each carrying a single amino acid mutation corresponding to the five residues postulated to mediate ACTA-A2-CTB selectivity (Figure S3). The hybrid proteins were expressed, and the protein-containing media tested directly in the ALBA with ACTA-A2 biotin (Figure 3B).

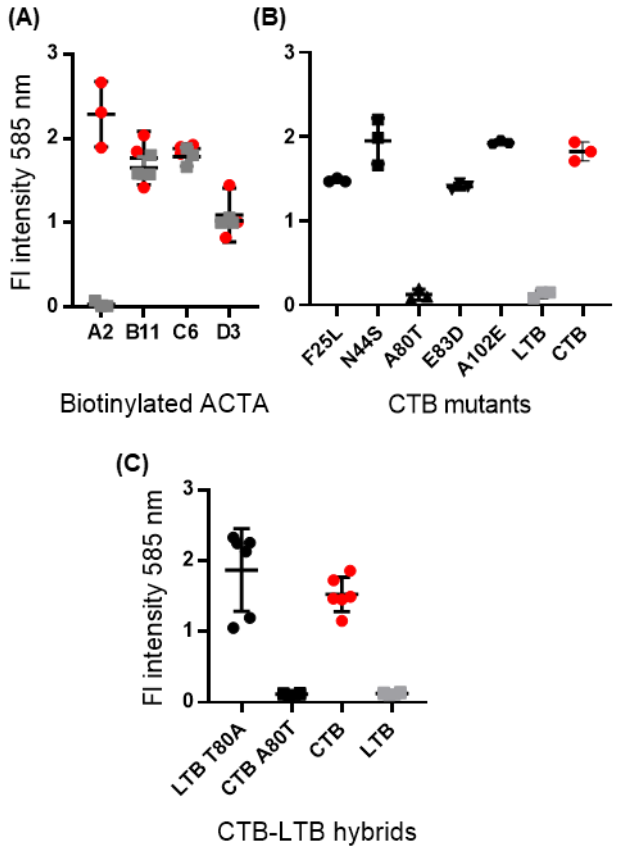

Figure 3. (A) CTB-binding Affimers demonstrate variable selectivity for CTB. Selectivity assays were carried out by incubating GM1 bound CTB (red circles) or GM1 bound LTB (grey squares) with biotinylated ACTA- A2, B11, C6, and D3 in an ALBA assay. (B) ACTA-A2 shows selectivity. Fluorescence detected following testing of ACTA-A2-biotin against a series of five CTB mutants (F25L, N44S, A80T, E83D, A102E, LTB and CTB). (C) A80T facilitates binding of ACTA-A2 to LTB. Fluorescence detected following testing of ACTA-A2-biotin against purified LTB A80T, LTB, or CTB. Signal detected plotted with error bars showing the standard deviation of three technical repeats (A and B) or six technical repeats (C).

CTB A80T was the only hybrid protein that no longer bound to ACTA-A2. We therefore generated the corresponding hybrid LTB T80A and tested its interaction with ACTA-A2 biotin in the ALBA (Figure 3C). The mutation resulted in a gain in function for LTB, demonstrating that residue A80 in CTB alone is responsible for the observed selectivity of ACTA-A2 for CTB over LTB. A80 lies in a cleft close to the central cavity at the interface of two CTB protomers (Figure 4) which likely provides a binding pocket for one of the variable regions of ACTA-A2. This site could potentially become unavailable when A80 is mutated to a larger amino acid, such as threonine as found in LTB. Since ACTA-B11, C6 and D3 do not discriminate between CTB and LTB, it is unlikely that they bind in the same way as ACTA-A2. Due to their different binding specificities for CTB and LTB, ACTA-A2 and C6 were selected for further biophysical characterisation.

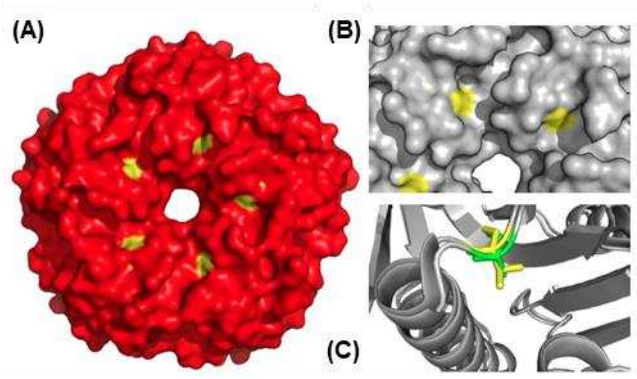

Figure 4. (A) Surface representation of the non-binding face of CTB, with residue A80 coloured in yellow. (B) A close-up image of the hypothesized ACTA-A2 binding groove, showing A80 in yellow. (C) A close-up of an overlay of CTB and LTB with A80 shown in green, and T80 shown in yellow.

Isothermal titration calorimetry (ITC) was applied to measure the binding interaction between each of ACTA-A2 and ACTA-C6 and CTB, with binding constants determined at 37 °C to simulate temperature *in vivo*. ITC data is summarized in Table 1. An exothermic heat change was observed for both Affimers, and K_d values of 1.1 µM and 97 nM were determined for ACTA-A2 and ACTA-C6, respectively. Importantly, this revealed that CTB binds to ACTA-C6 10-fold more tightly than to ACTA-A2. ITC also revealed that each ACTA bound to the CTB pentamer as a 1:1 complex. The 1:1 binding for ACTA-A2 to CTB is consistent with it binding to the central pore of the CTB pentamer with one of its loops projecting into the groove distinguished by A80. Interestingly, as ACTA-C6 also displays 1:1 binding, it suggests that this Affimer similarly binds in the central pore, even though it does not distinguish between A80 and T80 in CTB and LTB.

Table 1. Results of ITC experiments

Affimer	ACTA-A2-CTB	ACTA-C6-CTB
Temp (K)	308	308
ΔH° (kcal/mol)	-23.0 ± 0.87	-7.33 ± 0.14
ΔG° (kcal/mol)	-8.40 ± 1.65	-9.95 ± 0.19
Kd (nM)	1130 ± 210	97 ± 30
TΔS° (kcal/mol)	-14.64 ± 2.52	2.62 ± 0.33
n[a]	1.15 ± 0.03	1.05 ± 0.01
N[b]	3	2

[a] binding stoichiometry; [b] number of replicates

Having observed 1:1 binding stoichiometry, and that the A80T mutation was sufficient to prevent interaction between ACTA-A2 and CTB, we sought to identify which of the two variable loops (Figure S2) in the Affimer might interact with the central pore of the CTB pentamer, and which might interact with the groove containing the A80 residue. We generated peptides

corresponding to each of the variable loops in ACTA-A2 flanked by cysteine residues and cyclised these using dibromomaleimide[38] to constrain them in loop conformations, before purification by HPLC. We then used ITC to investigate their interaction with CTB (Figure S4). Neither peptide bound sufficiently strongly to determine binding parameters from a direct titration. ITC competition binding experiments, in which intact ACTA-A2 was titrated into a solution of CTB in the presence of the cyclic peptide, also indicated very weak binding of individual loops. While global fitting of the ITC data gave an estimate for the K_d of the loop 2 cyclic peptide of ~ 1 mM, it was not possible to measure binding of the loop 1 peptide.

Alanine-scanning mutagenesis of the ACTA-A2 loops proved to be a more insightful strategy to distinguish their binding sites. We qualitatively screened a series of ACTA-A2 mutants for binding to both CTB and LTB using the ALBA assay to identify residues which were essential for interaction (Figure S5). While various mutations led to a reduction in binding to CTB, the ACTA-A2 S55A mutant remained able to bind to CTB, but was also unique in gaining the ability to bind to LTB. ITC was used to confirm that the ACTA-A2 S55A mutant bound to CTB and LTB with similar affinity (1.7 μM and 8.4 μM, respectively). Our hypothesis is that this mutation in loop 1 of ACTA-A2 has a similar effect to the T80A mutation in LTB to remove a steric-block to interaction. We therefore suggest that loop 2 is responsible for interaction with the central pore of the CTB pentamer, and loop 1 with one of the five grooves containing residue A80 on the top face of the protein.

Protein delivery in cultured cells

We next assessed the ability of these novel complexes to be taken up into mammalian cells prior to validation *in vivo*. As a first proof of concept, ACTA proteins and CTB were labelled with Alexa Fluor 555 and 488, respectively. Each protein was labelled at a specific site to ensure that complex formation would not be impeded by chemical modification: Affimers harbouring a C-terminal cysteine were modified with maleimide fluorophores, while the N-terminal threonine residue of CTB was oxidized with sodium periodate and then modified with aminooxy fluorophores.[39] ACTA:CTB complexes were produced by combining CTB with the Affimers in a 1:1 ratio and subsequently purified by size-exclusion chromatography (Figure S6). A single peak containing ACTA-555 co-eluted with CTB-488 was collected, and the protein was administered to Vero cells, a cell-line derived from monkey epithelial cells expressing ganglioside GM1. The presence of labelled proteins internalized within the cells was readily detected at concentrations as low as 175 nM and 15 nM for ACTA-A2/CTB and ACTA-C6/CTB respectively (Figure S7 and Figure 5).

Analysis of the cell images reveals that CTB and ACTAs clearly co-localize and appear concentrated in discrete puncta in an area directly adjacent to the nucleus that is consistent with the morphology of the Golgi apparatus (Figure S8). CTB is known to enter recycling endosomes, and be transported to the trans-Golgi network from the cell surface, resulting in accumulation of CTB in the Golgi apparatus.[40] Preliminary analysis of complex localisation therefore appears to agree with previous observations and indicate that ACTA complexes do not alter the endocytic pathway of CTB.

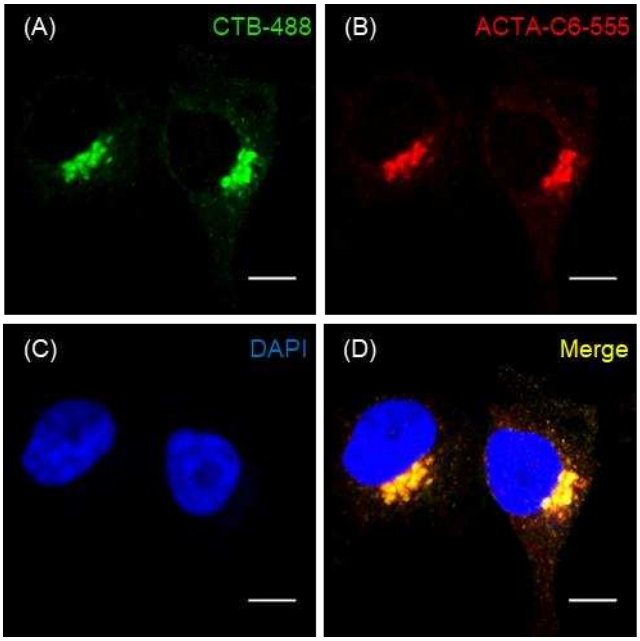

Figure 5. Vero cells treated with CTB-488/ACTA-C6-555 complex to a final concentration of 15 nM and stained with DAPI nuclear stain. Scale bars are 10 μm. (A) CTB-488 fluorescence, (B) ACTA-C6-555 fluorescence, (C) DAPI nuclear stain blue fluorescence, (D) merged image of 488, 555, 405 nm wavelengths.

Delivery of Affimers to motor neurons

Numerous studies report the ability of CTB to be endocytosed into neurons when delivered to the CNS.[41, 42] Neuronal tracing in this capacity has been achieved by intraperitoneal and intravenous injections for broad dispersal of CTB into the neurons of the spinal cord and brain.[43] A more targeted delivery approach that is useful for testing putative reagents, with labelling specifically of the neurons of the hypoglossal nucleus in the brainstem, is intramuscular administration of protein into the tongue,[44, 45] therefore, injections were carried out paralingually. C57BL/6 mice were treated with either 25 μg unlabelled CTB complexed with one of the two fluorescently-labelled Affimers (ACTA-A2-555 or ACTA-C6-555), or with one of the three individual components (5 μg ACTA or 20 μg CTB). After 24 hours, cross-sections of the brainstem were prepared and stained with a primary-secondary antibody pair to detect endocytosed CTB. Slides were then viewed by confocal microscopy (Figure 6).

CTB staining and ACTA fluorescence were both observed in the brainstems of mice treated with the complex, but not in mice treated with the fluorescent ACTA alone (Figure S9) demonstrating that ACTA internalisation was dependent on CTB. In a similar manner to earlier experiments carried out in Vero cells, localisation could broadly be assigned to a region peripheral to the motor neuron nucleus. Detection of ACTA-CTB around the nucleus indicates effective retrograde trafficking of the complex from the neuromuscular junction to the cell body, contained within the brainstem.

The two ACTA-CTB complexes showed different localisation patterns. After 24 hours, ACTA-A2 is observed in small punctate spots throughout the cell, while the fluorescence for ACTA-C6 is peripheral to the nucleus as observed in preliminary cell experiments (see Figure 5/Figure S9). These halos of ACTA-

C6-555 fluorescence largely overlay with the detected CTB, suggesting that the higher affinity ACTA-C6 is still mostly bound to its target after internalisation and trafficking to the cell body. Conversely ACTA-A2-555 fluorescence overlaid poorly with CTB, suggesting that the complex dissociates after internalisation and the stable Affimer is then being sequestered inside presumably endosomes, or lysosomes for degradation.

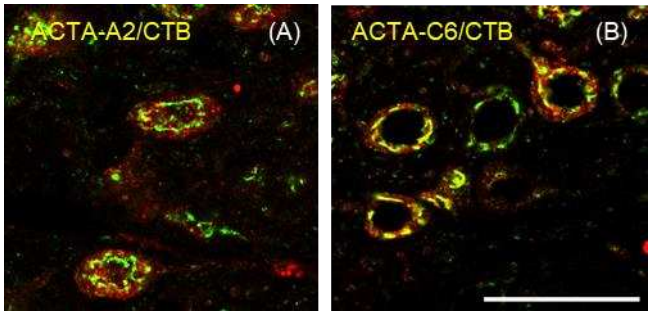

Figure 6. Cells of the hypoglossal nucleus in brainstem cross-sections for mice paralingually injected with ACTA-A2-555/CTB (left, image (A)) or ACTA-C6-555/CTB (right, image (B)), showing ACTA fluorescence (555 nm, red) overlaid with CTB immunolabelling (488 nm, green). Scale bar = 50 µm.

A further experiment to establish the longevity of the complex *in vivo* was carried out by administering the complex paralingually and sacrificing the mice after one week. Few ACTA-A2-555 positive cells were detected, with nearly all remaining fluorescence appearing clustered in punctate spots (Figure 7). ACTA-C6-555, however, was readily detected in the hypoglossal nucleus, with much of ACTA-C6-555 fluorescence overlaying with immunostained CTB, indicating a longer-lived half-life for the ACTA-C6/CTB complex. We propose that the lower affinity ACTA-A2 dissociates from CTB rapidly, resulting in a reduced half-life *in vivo* compared to ACTA-C6, which remains bound for longer, leading to an extended half-life. Of note is that Affimers are derived from phytocystatins, a group of lysosomal protease inhibitors found in plants. Lysosomal degradation of these proteins might therefore be surprising. Alternatively, it is possible that ACTA-A2 either persists in the motor neuron, but without the fluorescent tag, or it may have been exocytosed. Either way, ACTA-A2-555 is observable in the cells for a shorter time than ACTA-C6-555. It would be interesting to compare the degree of neuroprotection offered by these complexes with an appropriate payload. An amenable payload could be the calcium binding protein parvalbumin. In a mouse ALS model, transgenic overexpression of the calcium binding protein parvalbumin slowed progression of symptoms and increased survival when compared to the ALS mice with normal levels of expression.[46] In humans, such transgenesis is not feasible, particularly since genetic causes of ALS remain unknown and most cases are classified as sporadic.[47] Therefore, utilising parvalbumin (or similar proteins with neuroprotective potential) as a payload in the CTB-Affimer complex could enable its delivery to motor neurones and so reduce their vulnerability to degeneration in ALS.

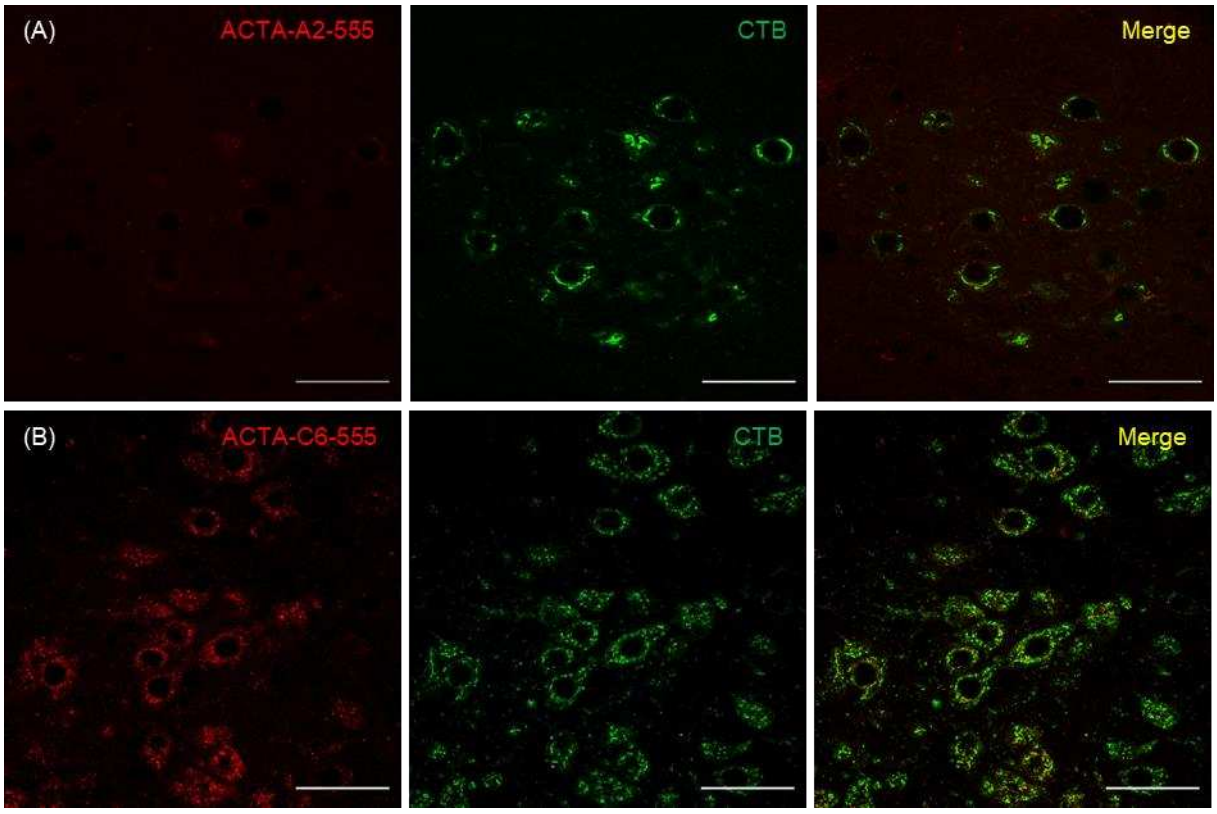

Figure 7. Hypoglossal nucleus in cross-section of brainstems of mice injected paralingually with (A) ACTA-A2-555 + CTB and (B) ACTA-C6-555 + CTB. Mice were sacrificed one week following injections, with CTB detected by immunolabelling. Scale bars = 50 µm.

CONCLUSIONS

CTB has been extensively studied as a tool for neuronal tracing and protein delivery. In this manuscript we expand on the capabilities of CTB by proposing a novel method for carrying macromolecular cargo into the brainstem of a mouse as a first step to developing a motor neuron-selective protein delivery system. By exploiting the GM1-binding properties of the cholera toxin B-subunit, two CTB-binding Affimers were identified through phage display that target the face distal to the GM1-binding sites of CTB. Using an ELISA-type assay, selectivity for CTB over its *E. coli* homologue LTB was demonstrated for ACTA-A2, but not for ACTA-C6. Fluorescently-labelled ACTA-CTB assemblies were injected intramuscularly into the tongues of mice and were readily detected in the motor neurons of the hypoglossal nucleus. Intracellular distribution *in vivo* was different between ACTA-A2/CTB and ACTA-C6/CTB treated mice, with ACTA-C6/CTB remaining largely associated, whilst ACTA-A2/CTB appeared to have fully disassembled. After 24 hours ACTA-A2 was detectable in discrete vesicles but had significantly reduced after one week. The weaker interaction of the ACTA-A2/CTB complex was hypothesized to be facilitating disassembly *in vivo*. A lower affinity may be preferable for release of cargo following internalisation and may be necessary to achieve dispersion throughout the neurons of the body. As a proof of concept, we have shown that CTB can be used as a vehicle to deliver a piggybacking cargo into motor neurons. Beyond the use of the CTB:Affimer approach described here, this approach could be applied to any antibody-mimetic and lectin pair to develop a modular protein assembly for intracellular delivery of macromolecular cargo. This strategy therefore has the potential to be adapted to delivery of molecular cargoes to a wide variety of cell types beyond those which interact with CTB.

ASSOCIATED CONTENT

The Supporting Information is available free of charge at http://pubs.acs.org."

Full experimental methods, protein sequences, additional phage ELISA results, additional fluorescence microscopy images, isothermal titration calorimetry data

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All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

ACTA, anti-cholera toxin Affimer; ALS, amyotrophic lateral sclerosis; CNS, central nervous system; CPP, cell-penetrating petides; CTB, cholera toxin B-subunit; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; ALBA, Affimer-lectin binding assay; LTB, heat-labile enterotoxin B-subunit; ITC, isothermal titration calorimetry.

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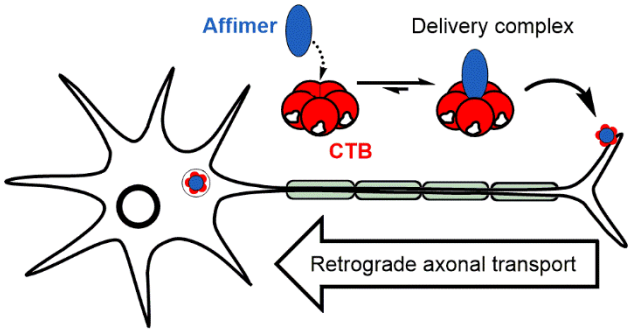


SUPPORTING INFORMATION FOR

Piggybacking on the Cholera Toxin: Identification of a CTB-binding Protein as an Approach for Targeted Delivery of Proteins to Motor Neurons

Matthew R. Balmforth, Jessica Haigh, Vajinder Kumar, Wenyue Dai, Christian Tiede, Darren C. Tomlinson, Jim Deuchars, Michael E. Webb, and W. Bruce Turnbull

Supplementary figures

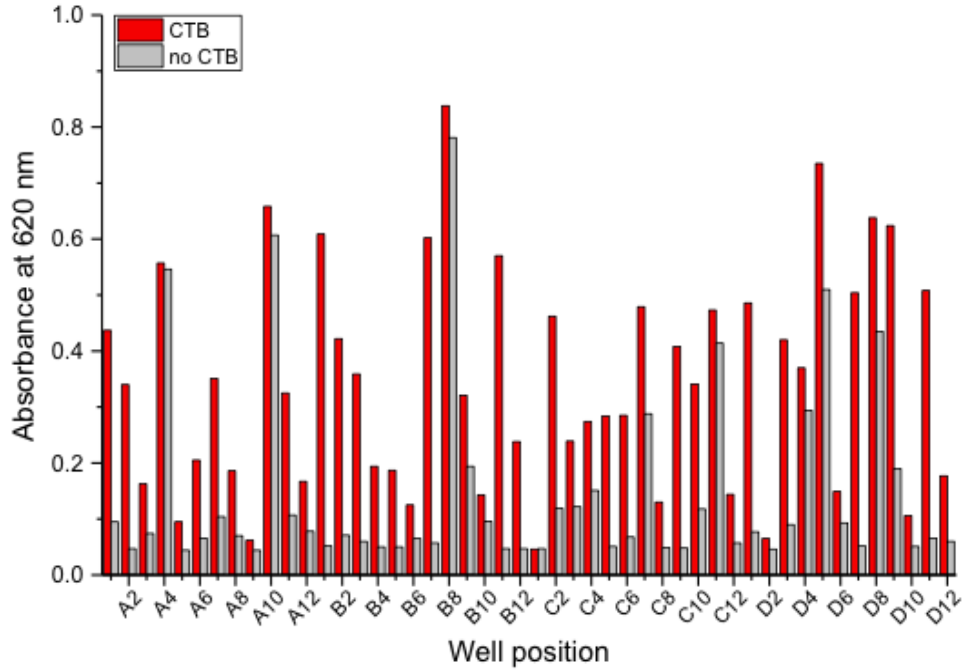


Figure S1. Analysis by phage ELISA of 48 Affimers isolated following panning against wild-type CTB. A single species of Affimer is tested against either CTB bound to GM1 (red bars), or GM1 alone (grey bars). Absorbance at 620 nm is plotted in a bar chart against the well position of the tested Affimer to facilitate identification.

	Variable loop 1								
A2	Q	H	E	R	S	H	W	V	D
B1	P	P	D	S	T	E	Q	Q	R
B3	E	F	S	S	S	R	R	V	K
C4	V	D	Q	K	P	P	A	R	M
C6	M	D	L	N	A	G	L	P	R

Variable loop 2								
H	N	Q	F	F	D	Y	F	I
K	W	P	G	K	F	N	K	Y
G	L	S	T	I	G	K	I	L
K	N	F	W	F	P	S	Q	N
Q	G	L	K	K	L	K	F	T

Figure S2. Primary sequences of the variable loops of ACTAs identified following selections against CTB. Colour scheme corresponds to RasMol colour grouping by traditional amino acid properties.

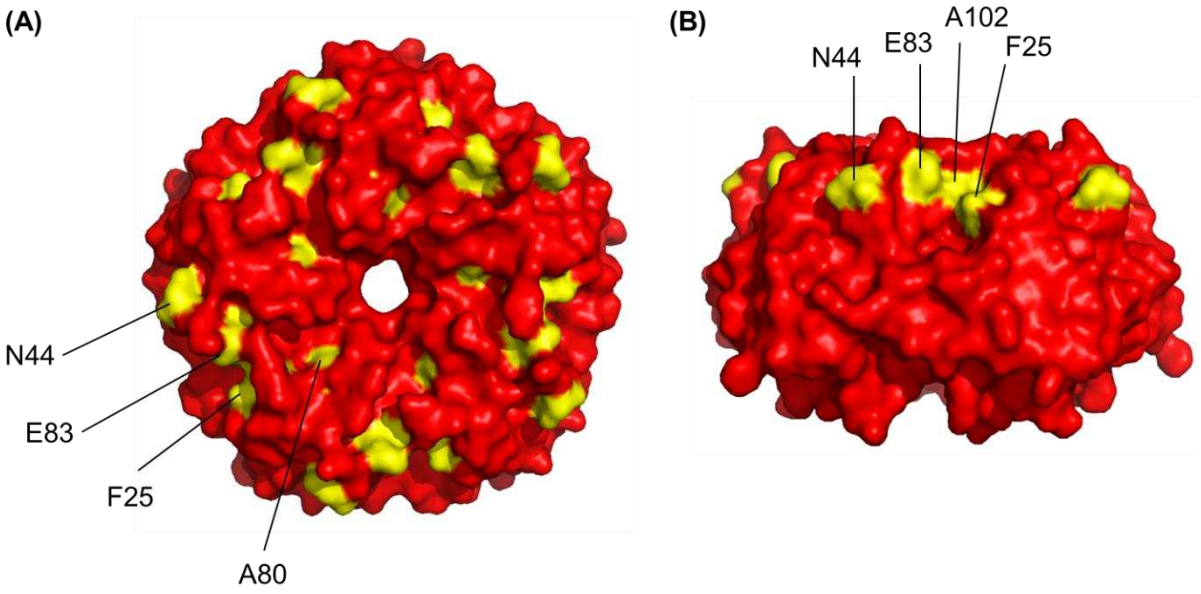


Figure S3. There are five surface exposed residues per protomer that differ between the top faces of CTB and LTB. Panel (A) is a surface representation of the top face of CTB, and shows the locations of four of these residues (F25, N44, A80, E83 highlighted in yellow); panel (B) is a side on view of CTB with a different subset of the surface exposed residues differing to LTB coloured in yellow (F25, N44, E83, A102).

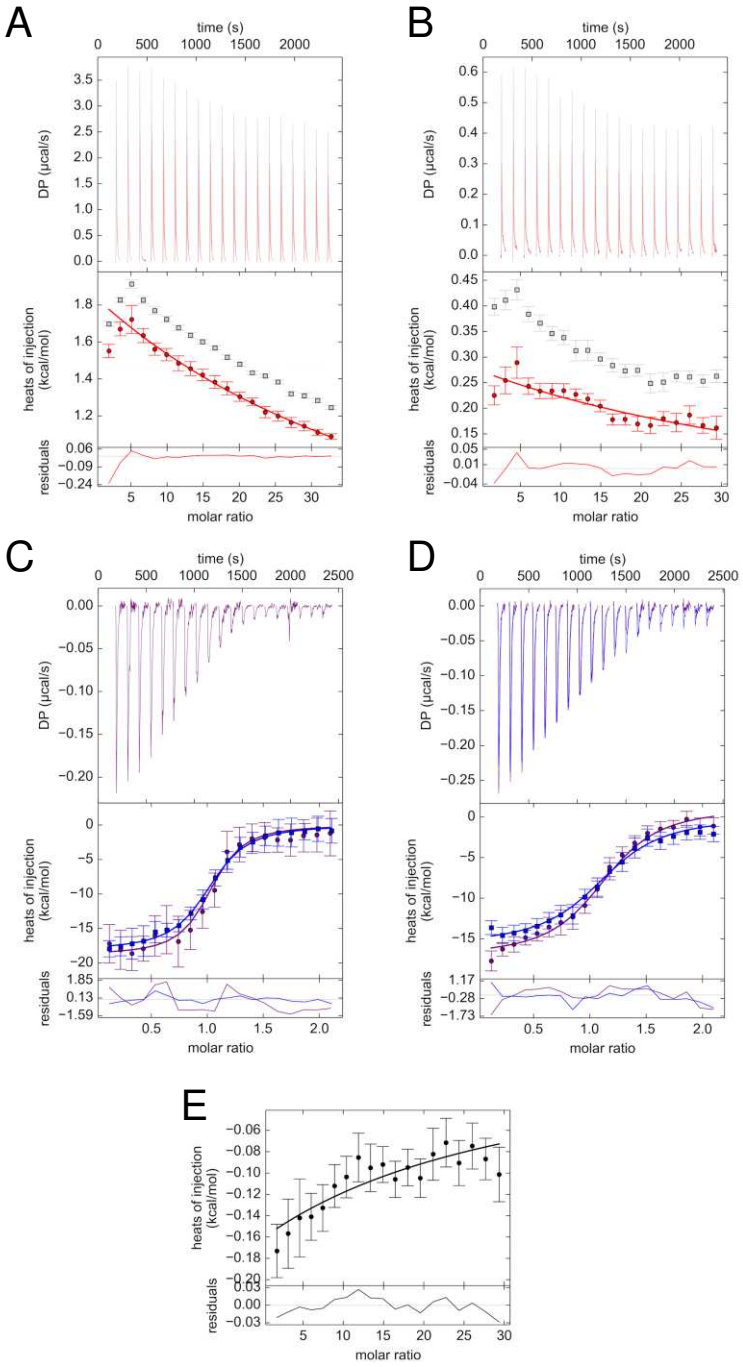


Figure S4. Assessment of interaction between cyclic peptides corresponding to loops of ACTA-A2 and CTB by ITC. (A) Titration of 7.8 mM cyclic peptide A (loop 1) into 50 µM CTB (red) and buffer (black). (B) Titration of 7 mM cyclic peptide B (loop 2) into 50 µM CTB (red) and buffer (black). (C) Titration of 90 µM ACTA-A2 into 9 µM CTB pentamer in the presence (purple) and absence (blue) of 3.5 mM cyclic peptide A. (D) Titration of 135 µM ACTA-A2 into 13 µM CTB in the presence (purple) and absence (blue) of 1.125 mM cyclic peptide B. (E) Difference between traces from panel (B).

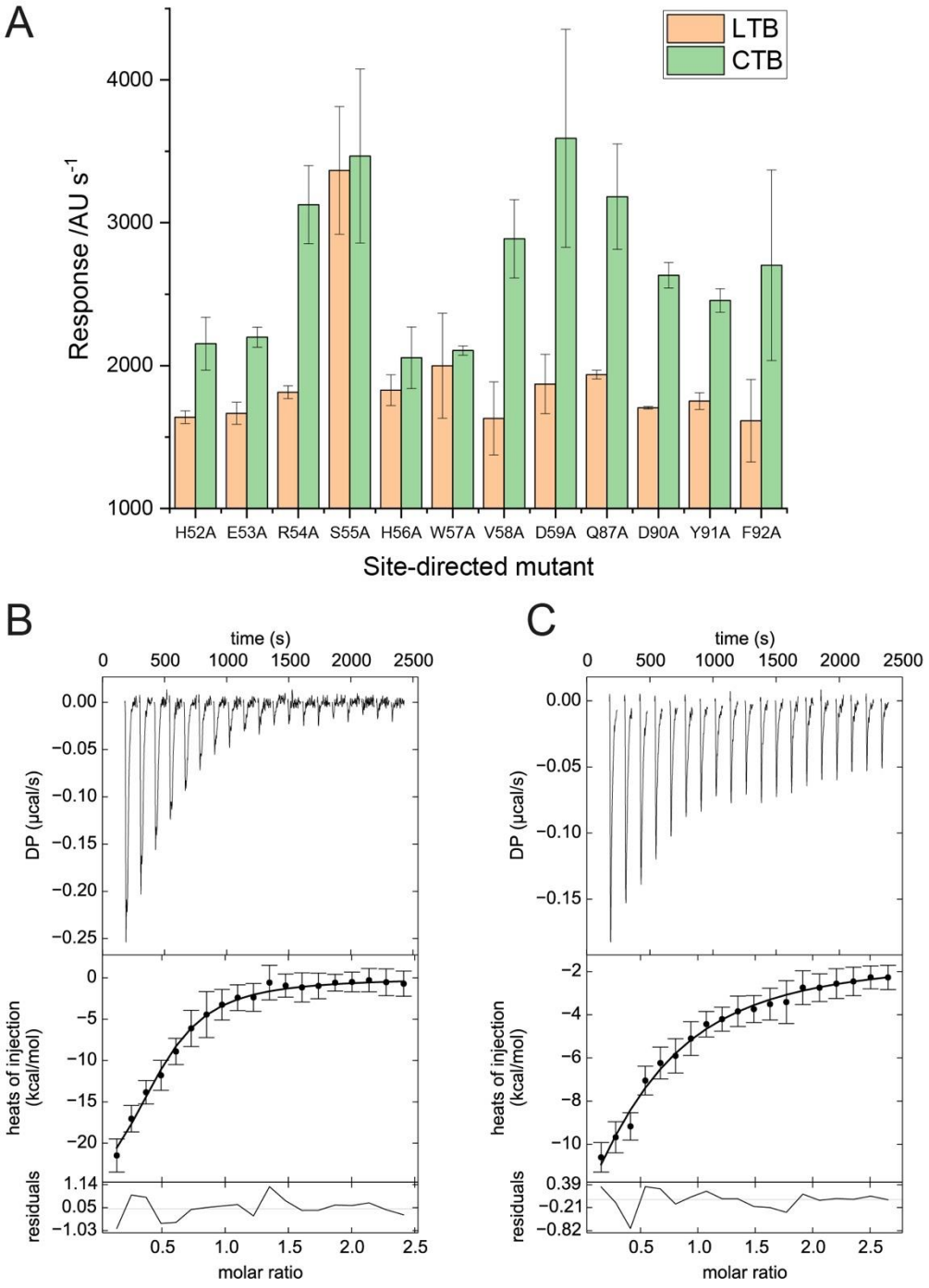


Figure S5. Identification of residues required for interaction of ACTA-A2 with LTB and CTB. (A) ALBA-based screening of binding of ACTA-A2 loop 1 (H52-D59) site-directed mutants and a subset of loop 2 (Q87-F92) site-directed mutants against LTB and CTB. (B) ITC analysis of ACTA-A2-S55A interaction with CTB. (C) ITC analysis of ACTA-A2-S55A interaction with LTB (in presence of 1.1 mM cyclic peptide B).

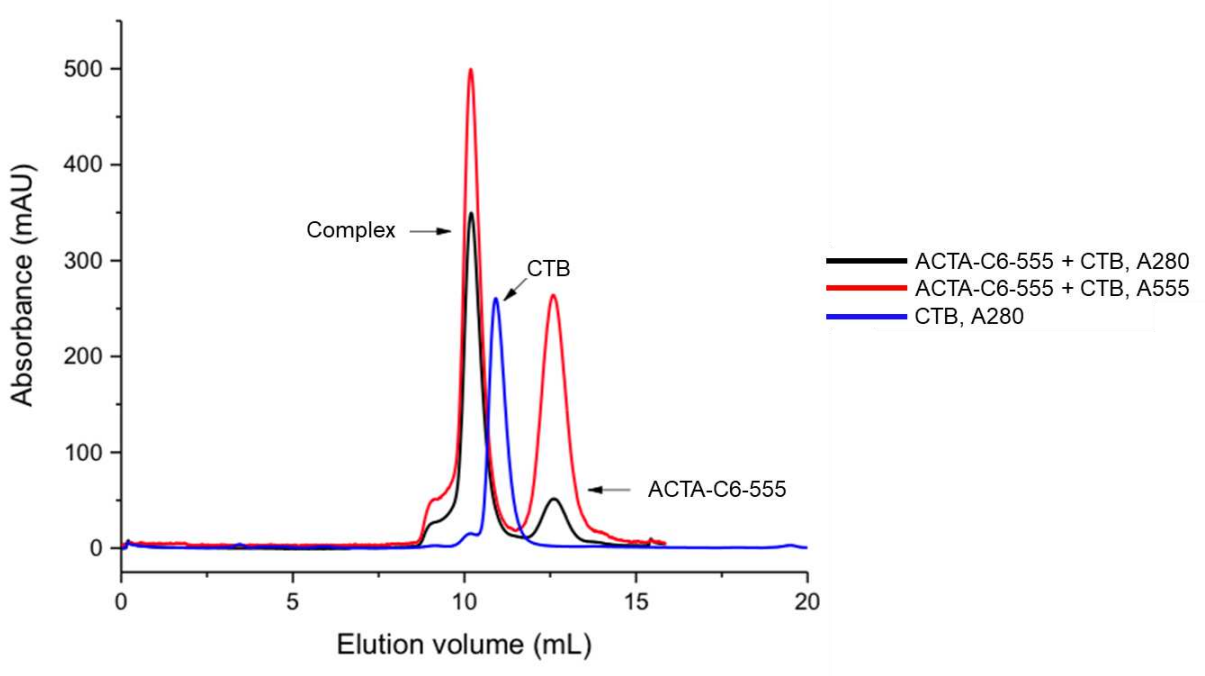


Figure S6. Size-exclusion chromatogram of the purification of C6-555:CTB complex using a Superdex 75 10/300 GL gel filtration column, with detection at 280 nm (black) and 555 nm (red) absorbance. Arrows denote the complex formed and free unbound C6-555 (Affimer C6 labelled with Alexa Fluor 555). Only fractions containing the complex were used in subsequent experiments. A chromatogram of CTB (blue) has been overlaid as a reference to show the shift in elution volume of the complex.

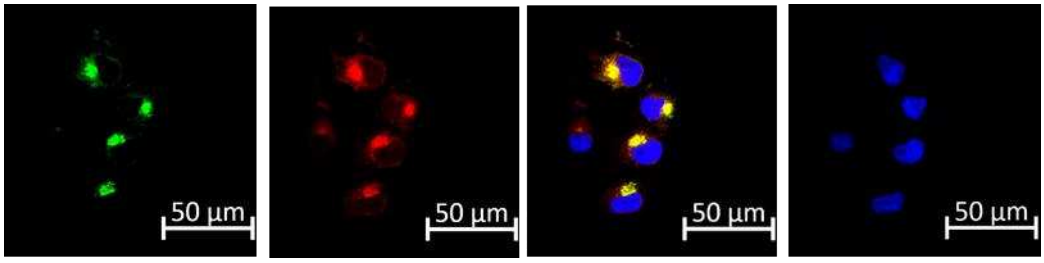


Figure S7. Vero cells treated with 175 nM CTB-488 + 3A2-555 complex.

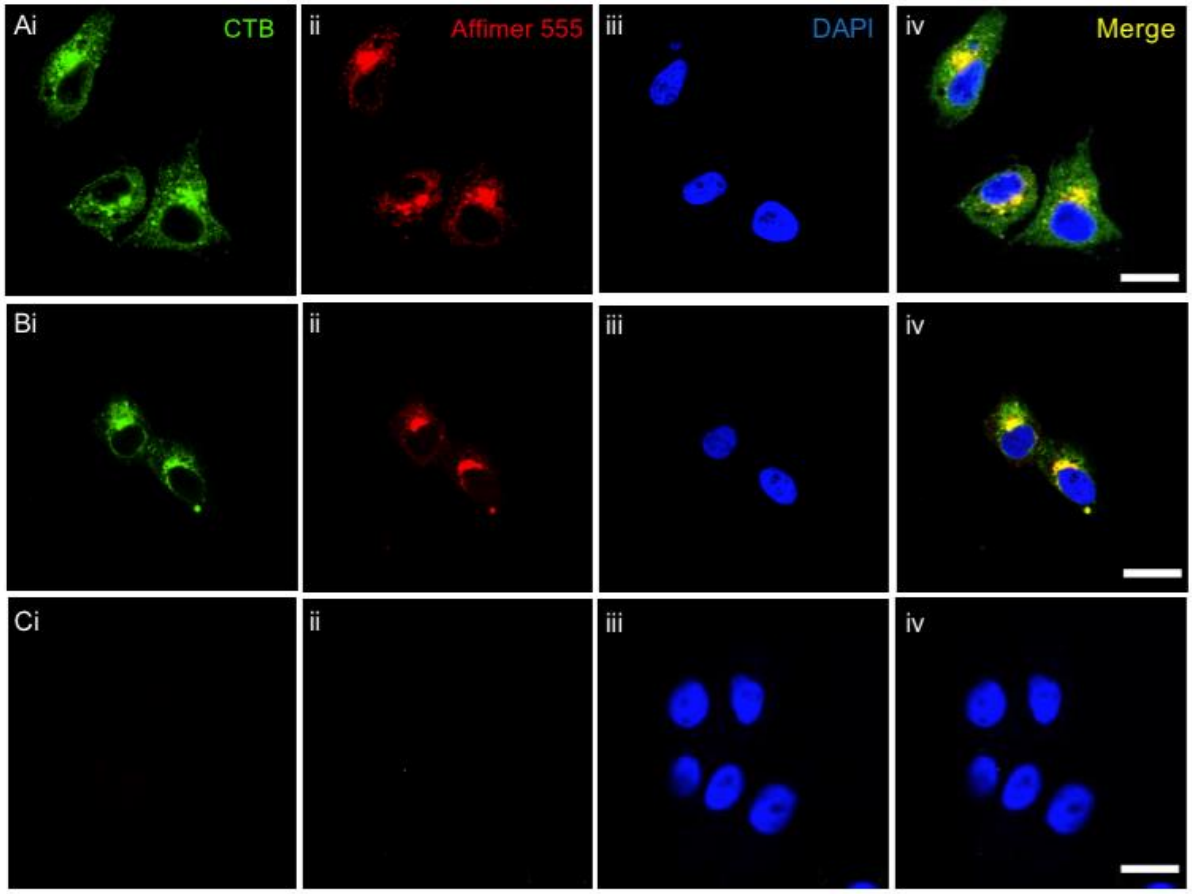


Figure S8. Vero cells incubated with (A) ACTA-C6-555 + CTB, (B) ACTA-A2-555 + CTB, (C) ACTA-C6-555 only, at 750 nM, fixed after six hours. CTB was detected using immunofluorescent labelling (panel i) and nuclei were stained with DAPI (panel iii). Scale bars are 20 µm.

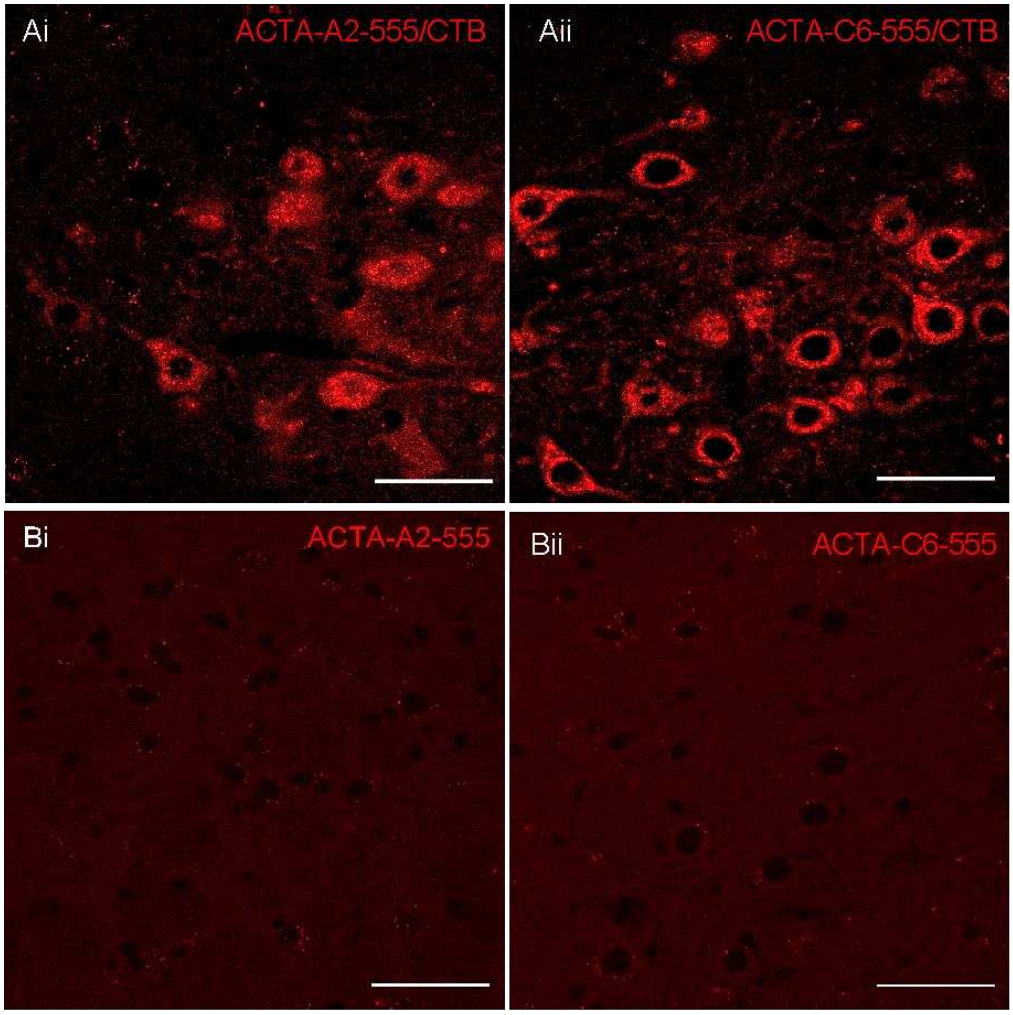


Figure S9. Magnified cross-sections of the brainstem focusing on the hypoglossal nucleus showing (A) mice injected paralingually with ACTA-555/CTB, ACTA-A2 (i), ACTA-C6 (ii) (B) mice injected paralingually with ACTA-555 alone, ACTA-A2 (i), ACTA-C6 (ii). Scale bars are 50 μm.

1 Materials and Methods

1.1 Standard Buffer Solutions

All common buffers were adjusted to pH 7.4, unless otherwise stated, and made up with 15 MΩ water to the required volume.

Phosphate Buffer: 50 mM NaH_2PO_4, 150 mM NaCl

Phosphate Buffered Saline (PBS): 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl

PBS-T: 50 mM NaH_2PO_4, 150 mM NaCl, 0.05% TWEEN20

Tris Buffered Saline 1X (TBS): 50 mM Tris-HCl, 150 mM NaCl

Lysis Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 1 μg mL^{-1} DNAse I, 1x Vol Halt Protease Inhibitor

Lactose Elution Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 300 mM Lactose

Ni-NTA Wash Buffer: 20 mM Imidazole, made up to desired volume with lysis buffer

Ni-NTA Elution Buffer: 500 mM Imidazole, made up to desired volume with lysis buffer

Blocking Buffer: 20% v/v 10× Casein Blocking Buffer in *PBS-T*

Cell Cleansing Buffer: 0.2 M acetic acid, 0.5 M NaCl, pH 2.8

Solubilisation Buffer: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.1% Triton-X 100

1.2 Media

Miller's Lysogeny Broth (LB) Medium: 1.0% w/v Tryptone 0.5% w/v Yeast Extract 1.0% w/v NaCl

LB Agar Antibiotic Plates: 1.5% w/v Agarose, 2.5% w/v LB Medium. The agar was supplemented with a final concentration of 100 μg/ml ampicillin or 50 μg/mL kanamycin to generate the selective medium.

2x Tryptone/Yeast Media (2TY): 1.6% w/v Tryptone, 1.0% w/v Yeast Extract, 0.5% w/v NaCl

High Salt LB Growth Media: 1.0% w/v Tryptone, 0.5% w/v Yeast Extract, 2.0% w/v NaCl

Cell Culture Media: DMEM/F-12 GlutaMAX, 10% v/v foetal bovine serum, 5% v/v Penicillin-Streptomycin (Sigma)

1.2.1 Polymerase Chain Reaction

Standard polymerase chain reaction (PCR) was used to amplify DNA for gel extraction, or for product analysis, to ensure that all primers resulted in a single, specific DNA fragment being produced. In all cases, New England Biolabs (NEB) enzymes and buffers were used. Standard PCR was set up in 50 μl reactions on ice, and contained the following:

1× Phusion High Fidelity Polymerase buffer (NEB)
500 nM Forward primer
500 nM Reverse primer
400 μM dNTP
1-25 ng DNA template
1 unit Phusion High Fidelity Polymerase (NEB)

This solution was made up to 50 µl with sterile H_2O, and the following cycling conditions were used:

95 °C	5 minutes	
95 °C	20 seconds	30 Cycles
T_m	20 seconds	
72 °C	45 seconds	
72 °C	5 minutes	
4 °C	Hold	

An extension time of 45 seconds was suitable for amplification of DNA segments less than 1 kb in size. A further 20 seconds was added for every additional kb of DNA to be amplified.

PCR products were purified using Qiagen QIAquick PCR Purification Kits, used as per the manufacturer's instructions.

1.2.2 Site Directed Mutagenesis

Quikchange site-directed mutagenesis was carried out to change nucleotides within a plasmid in a site-specific manner. All primers were designed using *Agilent Technologies QuikChange Primer Design* program.[1] Polymerase chain reactions were set up as follows:

1x KOD Hot Start Polymerase buffer (VWR)
140 nM Forward primer
140 nM Reverse primer
1.5 mM $MgSO_4$
200 µM dNTP
50 ng DNA template
1 unit KOD Hot Start Polymerase (VWR)
ddH_2O to 50 µl

Cycling conditions:

95 °C	2 minutes	
95 °C	20 seconds	
55 °C	20 seconds	20 cycles
70 °C	2 min 30 sec	
72 °C	10 minutes	
4 °C	Hold	

The PCR product was combined with 1 µl DpnI, and incubated at 37 °C for two hours, before transformation into *E. coli* XL10 cells.

1.2.3 Heat-shock Transformation of XL10/BL21 Gold *Escherichia coli*

Chemically competent *E. coli* cells were transformed via heat-shock. *E. coli* XL10-Gold cells were used for plasmid production, whilst BL21 Gold cells were employed for protein expression.

[1] https://www.genomics.agilent.com/primerDesignProgram.jsp

10 µl of cells were combined with 1 µl of plasmid DNA in sterile H_2O, and kept on ice for 30 minutes. Following this, the cells were placed in a 42 °C water-bath for 90 seconds, and then immediately supplemented with 500 µl room-temperature LB medium and incubated at 37 °C for 1 hour. The cells were then isolated by centrifugation for 4 minutes at 13000 RPM. 400 µl supernatant was then removed, and the cells were resuspended in the remaining LB medium. 100 µl of the transformed *E. coli* cells were then plated out onto LB agar ampicillin or kanamycin plates, and incubated overnight at 37 °C.

1.2.4 Small Scale Plasmid DNA Purification

XL10 colonies were picked under sterile conditions from LB-agar plates, and added to 5 ml 2TY medium, supplemented with ampicillin or kanamycin, to ensure selective growth of the picked colonies. This pre-culture was then shaken at 160 rpm for 16 hours at 37 °C. The plasmid was then isolated via alkaline lysis using the QIAprep® Spin Miniprep Kit High-Yield Protocol.

1.2.5 Affimer Expression

E. coli BL21 (DE3) cells, harbouring the pET11-Affimer-HIS6 plasmid, were collected under sterile conditions from a glycerol stock or from a LB-agar plate, and added to 5 ml LB medium together with ampicillin or kanamycin, to ensure selective growth. This pre-culture was shaken at 200 rpm for 18 hours at 37 °C, before being used to inoculate 400-1000 mL LB-media containing the appropriate antibiotic. Following inoculation, cultures were induced with 0.5 mM IPTG at OD 0.6, and incubated at 30 °C for at least six hours.

1.2.6 CTB Expression from *Vibrio sp. 60*

For the production of wild-type CTB, a preculture of the *Vibrio sp.60* stock was initially set up in 100 mL high salt LB growth media, with 100 µg mL^{-1} of ampicillin. This was incubated at 30 °C for 18 hours, before being used to inoculate 5 x 1 L high salt LB growth media. Once the OD_{600} reached 0.6-0.8, 240 mg/L IPTG was added to the flasks to induce protein expression. Following IPTG induction, the cells were incubated at 30 °C for another 24 hours, before cell pellet isolation by centrifugation at 17000 x*g* for 25 minutes. The supernatant was retained, as this contained the protein of interest, and was thus separated from the cell pellet, and purified by ammonium sulfate precipitation.

Solid ammonium sulfate was added and dissolved to a final concentration of 57% w/v. The saturated solution was then stirred for two hours using a magnetic stirrer at room temperature. The solution was then centrifuged at 18,000 x*g* for 30 minutes, and the supernatant was discarded, whilst the pellet was resuspended in 20 mL PBS. This in turn was centrifuged at 18,000 x*g* for 10 minutes to remove insoluble material, and to allow the suspension to be filtered through a 0.8 µm Sartorius Minisart filter. Finally, the filtered suspension was separated on a lactose affinity column at 4°C, and eluted with lactose elution buffer. The eluate was dialysed 4 times against high salt PBS.

1.2.7 Expression and Purification of CTB variants from *E. coli*

1 L LB media was inoculated with an overnight culture of BL21 cells transformed with the relevant pSAB-CTB plasmid and protein expression induced with 0.5 mM IPTG. The induced culture was incubated for ~20 hours, and then centrifuged at 10,000 x *g* for 10 min at 4 °C to pellet the cells. The supernatant was retained and combined with ammonium sulfate to precipitate the protein as outlined above. The presence of the surface exposed H13 in CTB confers affinity for Ni-NTA resin, offering an alternate purification route. This purification strategy was often employed when expressing multiple CTB variants, as purification by Ni-NTA chromatography could be set up in a higher throughput manner than lactose affinity chromatography. A less pure eluate was obtained via this route, necessitating purification by gel filtration.

1.2.8 Cell Disruption for Protein Purification

Cells were pelleted by centrifugation for 10 minutes at 10,000 x*g*. The supernatant was discarded, and the remaining cell pellet was resuspended in a lytic cocktail containing 8 mL lysis buffer, 0.8 mL BugBuster (Novagen). This suspension was incubated at room temperature on a rocker for 40 minutes for standard proteins, or 20 minutes, followed by an additional 20 minute incubation at 50 °C, to be carried out when purifying Affimers. The lysate was then clarified by centrifugation at 10,000 x*g* for 30 minutes, followed by filtration through a 0.45 µm Sartorius filter.

1.2.9 Purification of His-tagged Proteins

2 mL Ni-NTA slurry was transferred to a Bio-Rad Econo-Pac® Chromatography Column, and washed with 25 mL lysis buffer to dilute and remove the resin storage solution. The liquid was drained, and the clarified cell lysate was transferred to the column containing the resin, which was capped at both ends, and placed on a rocker for 30 minutes to facilitate binding of the His-tagged protein to the Ni-NTA resin. The lysate was drained from the column, and the settled resin was washed with 50 mL Ni-NTA Wash Buffer. The bound material was then eluted with 10 mL Ni-NTA Elution Buffer and collected in 2 mL fractions.

1.2.10 Purification by Gel Filtration Chromatography

Following elution from affinity columns, protein solutions were further purified by gel filtration chromatography. All columns were stored in 20% ethanol, and all solutions to be applied directly to size exclusion columns were filtered through a 0.2 µm Sartorius Minisart filter. Columns were equilibrated initially with water followed by 1.5 column volumes of an appropriate protein buffer. When displacing the ethanol, flow rates were set at half the standard flow rate to prevent exceeding the pressure limit of the column.

For large-scale purification of proteins, a HiLoad™ 16/60 Superdex™ 75 prep-grade column (GE Healthcare) was attached to an Äkta Purifier FPLC system, and flowed at a rate of 1 mL min^{-1}. Protein samples ranging from 500 µl to 5 mL in volume, and between 10 kDa and 80 kDa in mass, were loaded onto this prep-grade column.

For analytical scale purification of proteins, a Superdex™ 75 Increase 10/300 column was attached to the FPLC system, and flowed at a rate of 0.5 mL min^{-1}. Volumes ranging from 50 µl to 1 mL were loaded onto the analytical column, with a mass range of 10 – 80 kDa.

Proteins samples for purification or analysis were injected manually into the injection loop of the FPLC system. The loop was then flushed with buffer (1.5 x the loop volume) in an automated process to apply the loop contents to the resin. Fractions were collected once the void volume of the column had been reached, and were analysed by a detector measuring absorbance at 280 nm, or 488/555 nm if purifying fluorescently-labelled proteins.

1.2.11 Concentration of Protein Samples

Following size exclusion chromatography, protein samples were concentrated using Amicon Ultra-15 Centrifugal Filter Units with cut-off range of 3 - 30 kDa. 5 mL fractions were transferred to the filter units, and centrifuged at 3,500 x *g* until the protein was concentrated to an appropriate volume. As the protein collects on the filter, resuspension of the protein every 15 minutes using a pipette was necessary.

1.2.12 Protein labelling

1.2.12.1 Labelling Affimers with Alexa Fluor 555 C2 maleimide

Affimers carrying a C-terminal cysteine residue were concentrated to 150 µM in TCEP phosphate buffer (50 mM phosphate, 150 mM NaCl, 0.5 mM TCEP, pH 7.2). 400 µl Affimer was combined with 0.94 mg Alexa Fluor 555 C2 maleimide (aliquoted and lyophilised) (equal to five molar equivalents). Samples were incubated at room temperature for four hours in foil, before purification using a PD-10 desalting column, concentration to 250 µl, and a final purification by gel filtration using an analytical Superdex 75 increase column. Concentration of labelled Affimer was calculated using an equation provided by Thermo Fisher Scientific:

$$[ACTA]M = \frac{(A_{280} - (A_{555} * 0.08)) * dilution\ factor}{E\ cm^{-1}\ M^{-1}}$$

1.2.12.2 Labelling CTB with aminooxy Alexa Fluor 488

CTB was labelled with aminooxy Alexa Fluor 488 by oxidising the N-terminal threonine, and combining the subsequent oxidised CTB with two molar equivalents of the lyophilised fluorophore. CTB (500 µM [protamer concentration], in phosphate buffer only), L-methionine (10 equivalents), and $NaIO_4$ (5 equivalents) in phosphate buffer, were incubated together at room temperature out of light for 45 minutes, before buffer exchanging into phosphate buffer (**pH 6.8**) using a PD10 desalting column. The 1 mL eluate was then concentrated to 0.5 mL (~500 µM), and added to 0.4 mg lyophilised fluorophore (2 molar equivalents) and 4.5 µl aniline (neat). To reduce the number of fluorophores per pentamer, the amount of fluorophore can be reduced to 0.4 molar equivalents. After a 20 hour incubation, the protein was purified using a G25 desalting column, concentrated to 0.5 mL, and purified by gel filtration.

1.3 Phage Display

1.3.1 Preparation of ligand-coated plates

1.3.1.1 Streptavidin plate preparation

Streptavidin-coated plates were prepared by coating Nunc MaxiSorp flat-bottom 96 well plates with 100 µl 100 nM streptavidin diluted in *PBS*. The plate was sealed, and incubated at 37°C overnight to adsorb the protein to the plate surface. The following day, the 96-well plate was washed with *PBS-T*, and then blocked with *blocking buffer* overnight at 37°C. The commercial blocking buffer is casein-based, and blocks sites in the wells not occupied by streptavidin to prevent non-specific binding. After the incubation, the plate was washed once more with *PBS-T* prior to use.

1.3.1.2 GM1 plate preparation

GM1-coated plates were prepared by coating Nunc MaxiSorp flat-bottom 96 well plates with 100 µl 1.3 µM solution of GM1 ganglioside in methanol. The methanol was allowed to evaporate, thereby adsorbing the ganglioside to the plates. The GM1 plates were blocked overnight by incubating them at 37°C with a casein-based blocking buffer. The following day, the plate was washed once with 300 µl PBST on a plate washer immediately before use.

1.3.2 Panning the phage library using streptavidin-coated strips

In the first step, streptavidin-coated plastic wells were prepared for pre-panning the phage-Affimer library. 300 µl *blocking buffer* was added to four wells of a Pierce streptavidin-coated 8-well strip (Thermo Scientific). The strip was sealed, and incubated overnight at 37°C. Three of the wells were to be used for pre-panning the library, with the final well required for panning the phage against the biotinylated target protein. A colony of ER2738 cells was transferred into 5 mL 2TY supplemented with 12 µg mL^{-1} tetracycline (2TY tet) for overnight growth.

The wells were washed three times with *PBS-T* using a plate washer. 100 µl *blocking buffer* and 20 µl of CTB-biotin (1.5 µM/100 µg mL^{-1}) were added to the fourth well, for capture of the target to the streptavidin, whilst 100 µl *blocking buffer* only was added to the first three pre-panning wells. The strips were sealed once more, and incubated for two hours at room temperature on a vibrating platform shaker. Buffer from the first pre-pan well was removed, and was replaced with 100 µl *blocking buffer* and 5 µl of the phage library. The phage was then mixed and incubated in the well on a vibrating platform for 40 minutes. The buffer from the second pre-pan well was removed, and the solution from the first pre-pan well was transferred. The transferred phage was incubated for 40 minutes once more, and the same process was repeated for the third pre-pan well. This process was employed to remove phage-Affimer particles that bind to a component of the well, such as the bound streptavidin, or to other adsorbed proteins such as casein. The wells containing the target protein were washed six times, before adding the phage from the pre-pan wells. A two-hour incubation at room temperature followed on the vibrating platform shaker to allow the transferred phage to bind to the immobilised target.

1.3.3 Phage elution and propagation

A fresh culture of ER2738 *E. coli* cells was grown to an OD_{600} of approximately 0.6 by diluting the overnight culture 1/15 into 8 mL 2TY, and incubating the solution at 37°C for one hour.

Whilst the culture was growing, the panning well was washed 27 times with *PBS-T*. The phage was then eluted by adding 100 µl of 0.2 M glycine, pH 2.2, and incubating the solution for ten minutes at room temperature before neutralisation with 15 µl of 1M Tris-HCl, pH 9.1. The resulting solution was transferred immediately to the fresh 8 ml culture of the ER2738 *E. coli* cells. To ensure that all bound material had been successfully eluted, 100 µl of triethylamine (1.4% in *PBS*) was added to the well, and left for six minutes. 50 µl 1M Tris-HCl, pH 7.0 was added to neutralise the triethylamine, and the solution was transferred to the 8 mL culture. The culture was placed in a 37 °C incubator for one hour, and shaken once, by hand, after 30 minutes.

1 µl of the culture was combined with 100 µl 2TY, and plated out onto a LB-agar carbenicillin plate. The remainder of the culture was centrifuged to pellet the cells, and resuspended into 100 µl media. The entire suspension was transferred and spread across a second LB-agar ampicillin plate. The plates were incubated at 37 °C overnight. The following day, the colonies on the plate containing 1 µl of cells were counted, and multiplied by 8,000 to determine the total number per 8 ml of cells. Successful panning rounds typically generated between 0.5 – 2 x 10^6 cells.

5 mL 2TY supplemented with 100 µg/ml carbenicillin (2TY carb) was added to the plate containing the full culture suspension, and a plastic spreader was used to scrape the cells into the solution. The 5 mL was transferred to a falcon tube, and a further 2 mL 2TY was used to scrape any remaining cells from the plate surface. The OD_{600} was measured of a 1:10 dilution, and the

suspension was diluted into 2TY to make an 8 mL culture of OD_{600} = 0.2. The dilution was incubated at 37 °C, 230 rpm, for one hour.

0.32 µl M13K07 helper phage (titre ca. 10^{14}/ml) was added to the culture after one hour, and left to incubate at 37 °C, 90 rpm, for 30 mins. The helper phage also infect the bacteria, providing the phage-Affimer with additional components to form infectious particles. The helper phage also confers resistance to kanamycin. To select only the bacteria harbouring phage-Affimer particles and the helper phage, 16 µl of kanamycin (25 mg ml^{-1}) was added, and the culture was incubated overnight at 25 °C, 170 rpm.

The phage-infected cultures were centrifuged at 3,500 *xg* for 10 mins to pellet the infected cells, leaving the newly formed phage-Affimer particles in solution. The supernatant was retained and transferred to fresh tubes. 125 µl phage-containing supernatant was retained for the next panning round.

1.3.4 Phage purification and storage

To purify the phage, 2 ml of PEG-NaCl precipitation solution (20% (w/v) PEG 8000, 2.5M NaCl) was added to the phage-containing supernatant, mixed, and incubated overnight at 4 °C. The following day, the precipitated phage were pelleted at 4,816 *xg* for 30 mins, and the supernatant was discarded. The pellet was resuspended in 320 µl TE buffer, transferred to a 1.5 mL Eppendorf tube, and centrifuged at 16,000 *xg* for 10 mins. The supernatant contained purified phage, which could be used directly in a subsequent panning round, or could be combined with 50% glycerol and stored at -80 °C for long-term storage.

1.3.5 Panning the phage library using streptavidin-coated magnetic beads

After propagation of the phage from the first panning round, a second panning round was carried out to expose the phage to the same target, but presented in a different manner. Rather than exposing the phage to CTB-biotin captured onto streptavidin-coated plastic wells, the biotinylated target would be presented on streptavidin-coated magnetic beads. This difference in presentation should minimise the number of background binders carried through the screen by presenting a very different binding surface.

As in the first round, a colony of ER2738 *E. coli* cells was cultured overnight in 5 mL 2TY tet. 20 µl streptavidin beads (Dynabeads MyOne Streptavidin T1) were combined with 100 µl *blocking buffer* and incubated overnight at room temperature on a Stuart rotator.

The next day, the beads were centrifuged at 800 *xg* for one minute, placed onto a magnetic Eppendorf rack, and resuspended in 100 µl fresh *blocking buffer*. The magnetic rack draws the beads to the side of the tube, allowing buffer to be changed without losing the streptavidin beads. 125 µl of the phage retained from the initial propagation step, or 5 µl of the purified phage, was combined with blocking buffer to a total volume of 250 µl in Eppendorf LoBind Tubes. 25 µl of the pre-blocked streptavidin beads was added to the phage, and incubated on a Stuart rotator for one hour. Beads were separated from the phage by centrifugation and placement on the magnetic rack. The supernatant was transferred to a tube containing another 25 µl of pre-blocked streptavidin beads, and the pre-panning process was repeated.

15 µl CTB-biotin (1.5 µM) was combined with 200 µl blocking buffer, and 50 µl streptavidin beads, before being placed on a Stuart rotator for 30 minutes to bind. The beads were washed three times with 500 µl blocking buffer by removing the buffer, resuspending the beads, and then immobilising the beads to the magnetic rack, removing the buffer, and iterating the process. The supernatant containing the pre-panned phage was then transferred to the beads containing the captured CTB-biotin, and placed on a Stuart rotator for 45 minutes. After the incubation, the beads were washed manually 27 times with PBS-T.

A fresh 8 mL culture of ER2738 *E. coli* cells was set up, and the bound phage were eluted and used to infect the culture as described above. Plates were prepared and the phage propagated and stored as described previously. 200 µl phage supernatant was retained for the following panning round.

1.3.6 Panning the phage library against GM1-bound CTB

CTB is a lectin that binds with high affinity and specificity to GM1 ganglioside. The phage display protocol could therefore be modified to screen the phage library against GM1-bound unmodified CTB. GM1 plates were prepared, blocked, and washed as described in *GM1 plate preparation.* An overnight culture of ER2738 *E. coli* cells was prepared.

10 µl purified phage from the second panning round (CTB-biotin bound to streptavidin-coated magnetic beads) was added to 190 µl blocking buffer, and was pre-panned against four GM1-coated wells. 50 µl 1 µM solution of CTB was captured onto the GM1-coated surface of the fifth well, whilst the sixth well was retained as a negative control. Meanwhile, two 5 mL ER2738 *E.*

coli cell cultures were prepared by diluting the overnight culture 1:15, and incubating the solution at 37 °C for one hour, 230 rpm. The panning wells were washed six times with PBS-T, before addition of the phage from the third pre-panning well, which was split between the two panning wells. After a one hour incubation, phage were eluted, and used to infect the two 5 mL ER2738 *E. coli* cell cultures. The Plates containing single colonies were taken forward for analysis by phage ELISA.

1.3.7 Phage ELISA

An enzyme-linked immunosorbent assay (ELISA) was carried out to validate the binding of a phage-Affimer subset to a screened target.

200 µl aliquots of 2TY carb were transferred to a 96-well V-bottom deep well plate using a multichannel pipette. From the final panning round, 24 - 48 individual colonies were picked and used to inoculate the wells. The plate was then incubated overnight at 37°C, 1050 rpm. 200 µl 2TY was then transferred to the wells of a fresh deep well plate, which were inoculated with 25 µl of the overnight culture, before being incubated in turn for one hour at 37°C, 1050 rpm.

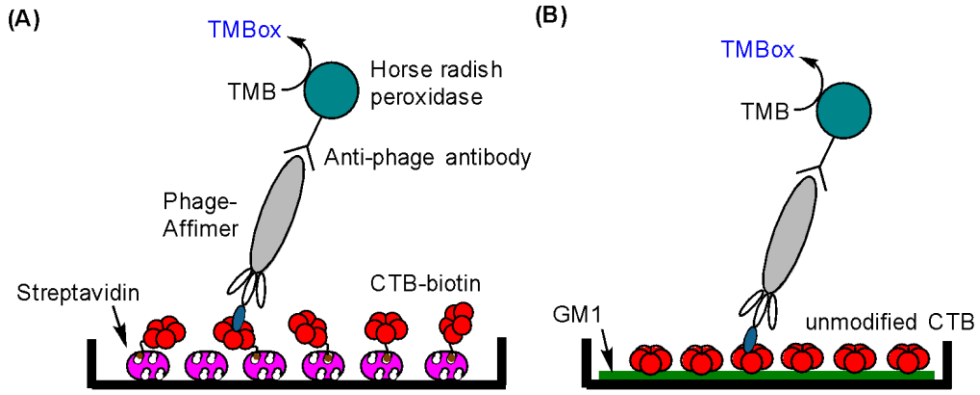


Figure S10 Phage ELISA schematic. (A) CTB-biotin is immobilised to streptavidin-coated plates, and presented to a phage-Affimer species. The bound phage is detected by anti-phage antibody, conjugated to horse radish peroxidase (HRP). Binding is detected by the addition of TMB and hydrogen peroxide, which is converted to a blue product by the peroxidase. The blue product absorbs at 620 nm and can be detected by plate reader. (B) Unmodified CTB is immobilised to GM1 ganglioside-coated plates, and presented to a phage-Affimer species. Detection of bound phage is facilitated through use of a peroxidase-conjugated antibody, and the peroxidase substrates TMB and hydrogen peroxide.

After one hour, 5 µl M13K07 helper phage (titre ca. 10^{14}/ml) was diluted into 5 ml 2TY carb, and 10 µl of the diluted stock was transferred into each of the wells of the one-hour culture. This culture was incubated for 30 minutes at room temperature, 450 rpm. The rpm was reduced to enable efficient infection of the cells with the helper phage, which was required in turn to supplement the phage-Affimer present in the infected cells with various complement proteins required for infectious phage-Affimer particles to escape into the surrounding media.

Finally, kanamycin was added to the cultures by diluting the stock 20 fold into 2TY, and transferring 10 µl of the diluted antibiotic into each of the wells. As the helper phage confers kanamycin resistance to infected bacteria, this would effectively kill any *E. coli* cells that had not taken up the helper phage. This was incubated overnight at room temperature, 750 rpm. The phage-infected culture was centrifuged at 3500 *xg* for ten minutes to pellet the bacteria, leaving the Affimer-expressing phage in the supernatant. The supernatant could then be used directly in the ELISA.

Plates were prepared with either streptavidin or GM1 ganglioside. To half of the plate, CTB-biotin (1 µM), or unmodified CTB (1 µM), was added and allowed to bind for one hour, before being washed three times with PBS-T to remove unbound material.

40 µl of phage-containing supernatant was then transferred to the first and seventh well of the plate such that each phage was tested against the target, as well as the negative control well. 10 µl undiluted 10x Casein Blocking Buffer was added to supplement the phage. Following a one hour incubation, the plate was washed 27 times with PBS-T.

Commercially acquired Anti-Fd-Bacteriophage HRP was diluted 1000 fold in blocking buffer, and 50 µl was added to each well. After another one hour incubation, the plate was washed 12 times with PBS-T. Binding was assayed by adding 50 µl SeramunBlau solution, which contained the TMB and hydrogen peroxide. The plate was allowed to develop for approximately four minutes, before the absorbance at 620 nm was measured using a plate reader.

1.4 Peptide synthesis

1.4.1 Materials and methods

All amino acids and resins were purchased from either Novabiochem, Merck or Sigma-Aldrich. All amino acids were N-Fmoc protected and side chains protected with Boc (Lys); OtBu (Asp, Ser, Thr); Trt (Asn, Gln); Pbf (Arg). Synthesis of all peptides was performed using a microwave assisted automated peptide synthesiser (CEM, Liberty or Liberty Blue). DMF used in peptide synthesis was of ACS grade and from Sigma Aldrich. Peptides were synthesised on an 0.25 mmol scale. Lyophilisation was performed using a BenchTop Pro with OmnitronicsTM from VirTis SP Scientific. Preparative HPLC was performed on an Agilent Technologies 1260 infinity controller in conjunction with an diode array detector. Mass spectrometry data were obtained on a Bruker Daltonics micrOTOF using electrospray ionisation (ES) MS instruments as appropriate.

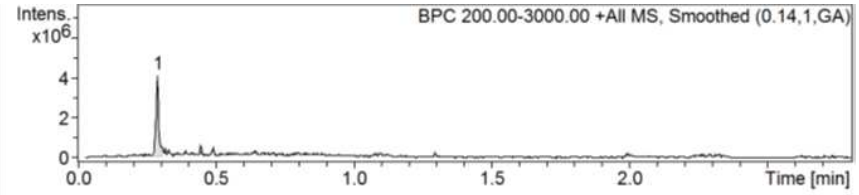
Cycles for Automated Peptide Synthesis - Peptides were prepared on a microwave assisted Liberty Blue CEM peptide synthesizer following a standard Fmoc solid phase peptide synthesis protocol with 2-chlorotrityl resin and diisopropylcarbodiimide and Oxyma Pure as coupling reagents. Arginine was subjected to double coupling as standard. After the final residue, the resin was removed from the reaction vessel and any further changes were performed manually.

Global deprotecton of tBu Group:- Following removal from the automated synthesiser, the resin was placed in a fritted empty SPE tube and washed with DMF (3 × 2 min spins), DCM (3 × 2 min spins) and methanol (3 × 2 min spins). The resin was isolated by filtration before being dried under high vacuum overnight. For cleavage, a cocktail consisting of H_2O: TIS: TFA (2.5: 2.5: 95) was added to the resin and the mixture was left to spin for 1 h at rt. The resin was filtered, and the filtrate added to cold diethyl ether to precipitate the peptide. This process was repeated. The peptide was analysed by LCMS

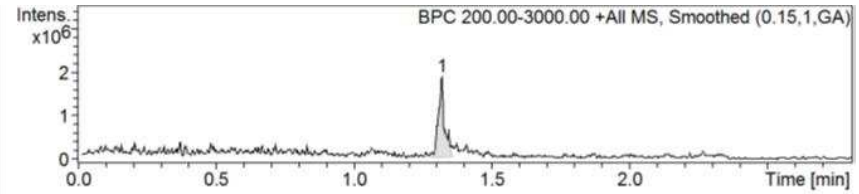
Crude peptides were dissolved in H_2O or DMF for purification by preparative UV-HPLC using Kinetex EVO C18 preparative column (reversed phase) on an increasing gradient of acetonitrile in water + 0.1% formic acid (v/v) at a flow rate of 20 mL min-1 . The eluent was monitored at 210, 254 and 280 nm with a diode array detector. Fractions containing purified peptide were combined, concentrated in vacuo and lyophilised.

1.4.2 Peptide cyclisation

Peptides were dissolved in acetonitrile:water (1:4) mixture and TCEP.HCl (1.2 equivalents) was added to ensure reduction of the cysteine residues. After stirring for 30-40 min at room temperature, 2,3-dibromomaleimide (1.2 equivalents) was added and the reaction mixture was stirred for an additional 4 h. After 4h, LCMS showed completion of reaction. The cyclic peptides were purified by preparative UV-HPLC. The masses of cyclic peptides were consistent with dithiosuccinimide products.[2]



LCMS total ion count chromatogram for cyclized loop 1.
HR-ESMS for cyclized loop 1: observed $[M+2H]^{2+}$ = 783.3036; $C_{64}H_{90}N_{22}O_{21}S_2$ requires 783.3041 (m/z for 2+ ion)



LCMS total ion count chromatogram for cyclized loop 2.
HR-ESMS for cyclized loop 2: observed $[M+H]^+$ = 1602.6156; $C_{74}H_{92}N_{17}O_{20}S_2$ requires 1602.6140 (m/z for 1+ ion)

[2] Tedaldi, L. M., PhD Thesis, University College London, 2010 pp129. https://discovery.ucl.ac.uk/id/eprint/1310478/

1.5 Biophysical Techniques

1.5.1 Isothermal Titration Calorimetry

Extensive dialysis of both proteins was required to match buffers before running experiments. All ITC experiments were performed using a MicroCal ITC_{200}. Water in the reference cell was replaced at the beginning of a set of experiments, using a 1 mL Hamilton syringe. The sample cell was then washed extensively with PBS-T to ensure that no protein remained from any previous experiments. The cell was additionally washed with water, followed by the experiment buffer, to remove any bubbles remaining from the detergent used in the previous wash step, and to equilibrate the sample cell. 300 µl protein 'receptor' was gently drawn up into a 1 mL Hamilton syringe, and purged from any bubbles. The sample cell was then gently filled. The tip of the syringe was then drawn up to the ledge formed at the interface between the sample cell, and the overflow reservoir. Any excess liquid was removed up to this point, thereby ensuring that the volume was standardised across all experiments.

The titration syringe was then loaded with ligand from a microcentrifuge tube, and the fill port was closed. The titration syringe was then placed back into the tube, and the titration syringe was purged, refilled, and then transferred into the sample cell. The calorimeter was heated to 25 °C or 35 °C before carrying out titrations.

An initial sacrificial titration of 0.5 µl was required, before, typically 19 x 2 µl titrations were carried out, each spaced out over 4 seconds, with a 120 second delay in between each injection. Experiments were initially carried out at 100 µM CTB, titrated into 10 µM Affimer. These concentrations were then varied, if necessary, to obtain a sigmoidal shape of curve. For each set of concentrations, a CTB titration into buffer was carried out to obtain a measure of the heat of dilution of CTB into buffer. These data could then be subtracted from the data obtained from ligand into receptor titrations to bring the baseline closer to zero, thereby facilitating curve fitting. Data were analysed and fitted using either a 'one site bind' model in the Origin Microcal software, or using the Nitpic and SedPhat software.

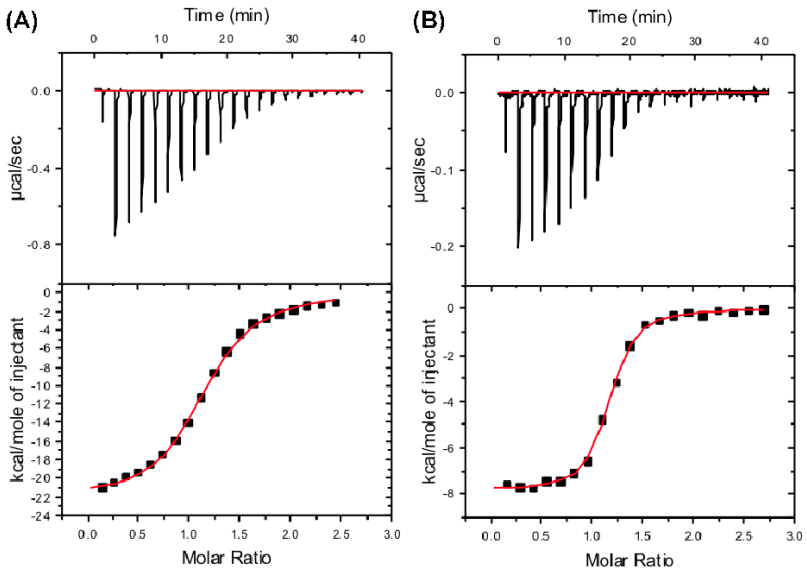


Figure S11. Results of ITC experiments (A) Thermogram and binding isotherm generated following the titration of 100 µM CTB into 10 µM C6 at 35°C by ITC. The data are fitted with a one-site binding model using Microcal Origin software following baseline subtraction using CTB into buffer titrations. (B) Thermogram and resultant Wiseman binding isotherm following high concentration titrations of 200 µM CTB into 17 µM A2 at 35°C by ITC. The data are fitted with a one-site binding model using Microcal Origin software following baseline subtraction using CTB into buffer titrations.

1.5.2 Affimer-lectin binding assay

An Affimer-lectin binding assay (ALBA) was developed to validate the binding of Affimers to CTB

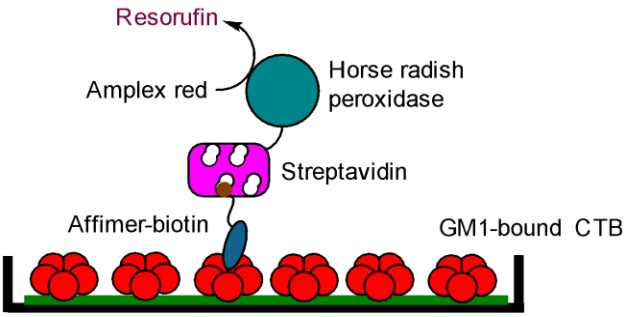


Figure S12. Schematic for an Affimer-lectin binding assay. A GM1-binding lectin, in this case CTB, is captured onto a GM1-coated surface. A biotinylated Affimer binding CTB is recognised by a streptavidin-peroxidase conjugate, which is quantified by addition of Amplex Red and hydrogen peroxide to form the fluorescent resorufin.

Affimers carrying a C-terminal cysteine were expressed, and biotinylated using biotin-PEG2-maleimide. Affimers were concentrated to 100 µM, and combined with a 20-fold molar excess of biotin-PEG2-maleimide in *phosphate buffer* supplemented with 1 mM TCEP. After an overnight incubation at room temperature, the Affimer was purified by using a PD-10 desalting column, or by dialysis into *phosphate buffer*.

50 µl GM1 ganglioside in methanol (1.3 µM) was added to wells (as required) of a Greiner Bio-one high binding black 96 well plate. The plate was then left in a lamina-flow hood until the methanol had completely evaporated. To block the plate and occupy any remaining adsorption sites, *blocking buffer* was added at a volume of 300 µl per well. The plate was covered, and incubated overnight at 37°C. Following incubation, the blocking solution was removed, and the plate was washed three times with *PBS-T*. A Combi Reagent Dispenser and standard tube dispensing cassette were used to facilitate accurate and rapid liquid transfer into the wells of the plate.

After the wash steps, 50 µl lectin (1 µM) was transferred to the appropriate wells, and left to bind for one hour at room temperature. Half the wells were deliberately left free of CTB to act as a control. The wash step was then repeated to remove any unbound CTB, and subsequently 100 µl biotinylated Affimer (250 nM) was added to each well. The Affimer was in turn allowed to bind for one hour before repeating the wash step a third time. In order to detect the presence of bound Affimer, 50 µl of a streptavidin-HRP complex (Ultra Streptavidin-HRP, Life Technologies), diluted at 1:1000 in *phosphate buffer*, was added to the wells of the plate. The one hour incubation and wash step was repeated, with an additional four washes, before one final rinse with *PBS* or *phosphate buffer*. The substrate used for peroxidase detection was a solution of Amplex Red and hydrogen peroxide in *PBS*, both at a final concentration of 5 µM. 50 µl of this substrate was added per well, and the plate was loaded into the plate reader for fluorescence detection at 585 nm.

1.6 Tissue Preparation and Analysis

1.6.1 Mammalian Cell Culture

1.6.1.1 Passage of cells

Vero cells were cultured in *cell culture media*, a supplemented Dulbecco's Modified Eagle's Medium (DMEM) containing foetal bovine serum, added due to the presence of essential growth factors for the cell proliferation, and a combination of antibiotics penicillin and streptomycin, to prevent the bacterial and fungal infections. Cultures were grown in T75 cell culture flasks at 37 °C with 5% CO_2 until 90% confluency, approximately after three days of culturing. At this stage, cells were washed once with 10 mL sterile *PBS*, and combined with 3 mL TrypLE Select (Life Technologies), a trypsin-based protease solution to detach cells from the flask surface. After 5 minutes at 37 °C, flasks were placed under a light microscope to verify complete detachment. If large numbers were seen to still be adhering to the surface, the flask was firmly tapped, and placed in the 37°C incubator for a further 3 minutes. 7 mL Cell Culture Media was added, neutralizing the trypsin, and the detached cells were transferred to a 15 mL falcon tube. The flask was washed twice with 10 mL PBS, and 20 mL Cell Culture Media was added, together with 1 mL of the resuspended cells. Cells were then returned to the incubator for culturing at 37 °C with 5% CO_2.

1.6.1.2 Cell seeding

For cell-based experiments, cells were 'seeded' onto 18 mm glass coverslips in 12-well plates. After passaging cells, the falcon tube containing the cells was retained, and used for cell seeding. A Neubauer Improved haemocytometer was used for cell counting by making a 1:10 dilution of the cells, and transferring 5 µl of the diluted sample onto the haemocytometer. A coverslip was then used to draw the cells onto the central grid by capillary action. A 5x5 grid at the centre of the haemocytometer was used for cell counting, and cells from five of the boxes were counted. This number was divided by two, and multiplied by 10^6 to calculate the number of cells per mL. For a 12-well plate, 1.2×10^5 cells per well were plated out. Cells were therefore diluted to 1.2×10^5 cells mL^{-1}, and 1 mL was transferred into the wells of a sterile 12-well plate, containing an 18 mm coverslip in each well. The plate was then incubated overnight at 37 °C with 5% CO_2 for use the following day.

1.6.1.3 Cell treatment with protein

Seeded cells in 12-well plates to be treated with protein were washed, by replacing the Cell Culture Media with 1 mL fresh media. Protein solution was then added directly to the media, and the cells were incubated for 4-6 hours at 37 °C, 5% CO_2 prior to cell fixation.

1.6.1.4 Cell fixation

Following treatment with protein, cells were exposed to a strong acid, to remove any surface-bound CTB, and then 'fixed', by adding a harsh cross-linking agent, effectively killing the cells and freezing them in a particular state.

Growth medium was removed, and the cells were washed three times with PBS. 0.5 mL Cell Cleansing Buffer was added, and incubated at 4 °C for 6 minutes. The cleansed cells were then washed an additional three times, before addition of 4% v/v paraformaldehyde (PFA) in PBS for 30 minutes at room temperature in a fume hood. The PFA was removed, and the cells were washed three times with PBS. Cells were then permeabilised with 0.1% Triton X-100 in PBS for one hour at room temperature in preparation for immunohistochemistry.

1.6.2 *In Vivo* Experimentation

All handling of live animals was carried out either by Dr Jessica Haigh, or Professor Jim Deuchars.

1.6.2.1 Animals

All animal experimentation was carried out under a Home Office License, and in accordance with the regulations of the UK Animals, Scientific Procedures, Act 1986. Experiments were performed on young male and female C75BL/6 adult mice, bred in house.

1.6.2.2 Intramuscular administration of protein

The tongue was targeted for intramuscular injections of protein and protein complexes, since it provided a route to deliver material into the motor neurones of the brainstem. Protein was prepared at a concentration of 50 µM in phosphate buffer, before lyophilisation for reconstitution prior to injection at a concentration of 200 µM.

Paralingual injections were carried out by heavily sedating mice with isoflurane, protruding the tongue from the mouth using forceps, and injecting 2 µl of protein material using a glass micropipette mounted onto a 10 µl Hamilton syringe. Mice were perfused after 24 – 72 hours.

1.6.2.3 Transcardial perfusion for tissue fixation

Mice were deeply anaesthetised by injection of sodium pentobarbitone (60 mg kg^{-1}) into the abdominal cavity. To ensure that the mice were fully sedated, pedal reflexes were tested. The abdomen of the animal was then dissected transversely, and a thoracotomy was carried out to reach the heart. The left ventricle of the heart was pierced, and a blunt needle was inserted and retained in position with a clip. The right atrium was then cut, and a phosphate solution (0.1 M NaH_2PO_4, pH 7.4) was applied through the needle to flush the circulatory system. Perfusion was then carried out by replacing the phosphate solution with approximately 150 mL 4% PFA.

1.6.2.4 Dissection and Tissue Preparation

The brainstem was removed by dissection of the spinal cord, opening of the skull, and separation of the meninges from the brain. The brain was then removed from the skull, ensuring not to disturb the brainstem and upper half of the spinal cord. The brain and spinal cord was then transferred into a 50 mL falcon tube with 4% PFA, and kept for 24 hours at 4°C. The PFA was

then removed and replaced with PBS, and the brainstem was separated from the hind brain and spinal cord for sectioning. The brainstem could then be sectioned. All sections were taken transversely on a VT1000S vibrating microtome at 50 µm thickness. The sectioned slices were collected and transferred into PBS in a 24-well plate.

1.6.3 Immunohistochemistry

Tissue sections, or cultured cells, were permeabilised in 1 mL *solubilisation buffer* for one hour at room temperature. The primary antibody (e.g. rabbit anti-CTB, chicken anti-GFP) was diluted 1:1000 in *solubilisation buffer*, and 0.5 mL was applied to the tissue/cells. After an overnight incubation at 4 °C, the wells were washed three times with PBS, and a 1:1000 dilution of a fluorescent secondary antibody (Alexa Flour 488 or 555) in PBS was made and 0.5 mL added to the sample. A one hour incubation at room temperature ensued, followed by three more washes with PBS. The sample was then air-dried onto a glass microscope slide, before addition of 10 µl of Vectashield Mounting Medium, and protection of the sample with a cover slip ready for visualisation. To stain the nuclei in a sample, Vectashield Mounting Medium with DAPI was used. This medium contains DAPI stain, a fluorescent stain that tightly associates to AT-rich regions of DNA in the nuclei of cells, thus facilitating visualisation of the nuclei. Microscope slides were covered with foil, and kept overnight at room temperature to dry, before being sealed with a generic nail varnish.

1.6.4 Confocal Microscopy

For confocal analysis of samples, an Axio Imager Z2 LSM880 upright confocal microscope (Zeiss) equipped with a 405 nm diode laser, Argon 458/488/514 nm, DPSS 561 nm and HeNe 633 nm lasers and a GaAsP detector. Images were captured using the Zeiss LSM Image browser software, and processed using Zen lite 2.3 software.

For wide-field analysis of samples, a Nikon Eclipse E600 microscope equipped with epifluoresence and Q-Imaging Micropublishing 5.0 camera was used. Images were captured using AcQuis image capture software.

1.7 CTB/LTB protein sequences

Protein sequences for LTBh, LTBh T80A, El Tor CTB, and CTB A80T. Highlighted in yellow is residue A80.

LTBh (H74-114)	APQSITELCS	EYHNTQIYTI	NDKILSYTES	MAGKREMVII
LTBh T80A	APQSITELCS	EYHNTQIYTI	NDKILSYTES	MAGKREMVII
CTB (El Tor)	TPQNITDLCA	EYHNTQIYTL	NDKIFSYTES	LAGKREMAII
CTB A80T	TPQNITDLCA	EYHNTQIYTL	NDKIFSYTES	LAGKREMAII

LTBh (H74-114)	TFKSGATFQV	EVPGSQHIDS	QKKAIERMKD	TLRITYLTET
LTBh T80A	TFKSGATFQV	EVPGSQHIDS	QKKAIERMKD	TLRITYLTEA
CTB (El Tor)	TFKNGAIFQV	EVPGSQHIDS	QKKAIERMKD	TLRIAYLTEA
CTB A80T	TFKNGAIFQV	EVPGSQHIDS	QKKAIERMKD	TLRIAYLTET

LTBh (H74-114)	KIDKLCVWNN	KTPNSIAAIS	MEN
LTBh T80A	KIDKLCVWNN	KTPNSIAAIS	MEN
CTB (El Tor)	KVEKLCVWNN	KTPHAIAAIS	MAN
CTB A80T	KVEKLCVWNN	KTPHAIAAIS	MAN

1.7.1.1 ACTA protein sequences

Binding loop sequences are shown in red.

ACTA-A2

GVGASAATGV RAVPGNENSL EIEELARFAV DEHNKKENAL LEFVRVVKAK EQQHERSHWV
DTMYYLTLEA KDGGKKKLYE AKVWVKHNQF FDYFINFKEL QEFKPVGDAA AAHHHHHHHH

ACTA-A2-cys

GVGASAATGV RAVPGNENSL EIEELARFAV DEHNKKENAL LEFVRVVKAK EQQHERSHWV
DTMYYLTLEA KDGGKKKLYE AKVWVKHNQF FDYFINFKEL QEFKPVGDAC AAAHHHHHHH
H

ACTA-C6

ASAATGVRAV PGNENSLEIE ELARFAVDEH NKKENALLEF VRVVKAKEQM DLNAGLPRTM
YYLTLEAKDG GKKKLYEAKV WVKQGLKKLK FTNFKELQEF KPVGDAAAAH HHHHHHH

ACTA-C6-cys

ASAATGVRAV PGNENSLEIE ELARFAVDEH NKKENALLEF VRVVKAKEQM DLNAGLPRTM
YYLTLEAKDG GKKKLYEAKV WVKQGLKKLK FTNFKELQEF KPVGDACAAA HHHHHHHH