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PRINCIPAL INVESTIGATOR: George A. Oyler, M.D., Ph.D.

CONTRACTING ORGANIZATION: Synaptic Research
Baltimore, MD 21227-3831

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George A. Oyler, Barry Gertz, Archana Kotiya

E-Mail: George@synapticresearch.com; Barry@synapticresearch.com; archana@synapticresearch.com

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Synaptic Research
1448 S Rolling Road
Baltimore, MD 21227-3831

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14. ABSTRACT
Botulinum neurotoxin has no cure once it has entered neurons. One approach to finding a treatment is to determine the molecules that regulate the half-life of the toxin in the cell. The serotype BoNT/A has a very long half-life (months) whereas the serotype BoNT/E has a short half-life (days). We have made a chimeric protein consisting of the light chains (LCs) of both toxins and found the half-life to be short, similar to light chain E (LcE). This finding suggests that the molecules controlling intracellular degradation of LcE are dominant over those that control LcA. We have also completed an assessment of neuronal proteins that bind each LC by Yeast 2 Hybrid (Y2H) analysis. We have assessed the contributions of these molecules to LC half-life by knock-down experiments. Furthermore, we have produced a designer ligase which is comprised of a VHH antibody binding region specific for LcA that is coupled to a ubiquitin ligase molecule. When delivered intracellularly as a plasmid by transfection, this molecule has been shown to shorten the half-life of LcA by ubiquitination and targeting to the proteasome. We have inserted the designer ligase into a protein delivery system and have found the protein to be unstable. We are working on producing a more stable chimeric protein and producing adequate amounts for in vitro testing.

15. SUBJECT TERMS- Botulinum toxin, ubiquitin, chimeric toxin light chains, LcA, LcE, Yeast 2 hybrid, intracellular therapy.

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INTRODUCTION

A feature of botulinum neurotoxin (BoNT) intoxication is the extraordinary persistence of light chain (LC) activity within neuronal cells. Serotypes A, B and C1 remain active for months whereas serotype E is active for only days. By comparing certain properties of long vs short persisting light chains, it should be possible to elucidate the mechanism(s) responsible for persistence. In this series of studies, we will assess the role that the ubiquitin system plays in the persistence of BoNT with the intent of developing therapeutics that exploit the system to inactivate the toxin. These include developing a LC-targeted ubiquitin ligase that selects LCs for degradation by proteasomes as well as inhibiting deubiquitinating enzymes (DUBs) that remove ubiquitins from LCs, protecting them from degradation.

BODY

Aim 1: Support for project entitled "Therapeutic Approach for Botulinum Intoxication Targeting Degradation of the Light Chain" (CBM.THRTOX.01.10.RC.020; PI:Michael Adler).

Synaptic Research will develop dichain hybrids consisting of Clostridium botulinum toxin light chains (LCs) from serotypes A (long-lived) and E (short-lived). These hybrids will be used to assess which amino acid residues are important in determining LC half-life by mutation and deletion experiments. The role of deubiquitinating enzymes in extending LC/A half-life will be analyzed using siRNA technology.

Project Milestones (1 Year):

Month	Activity	Task
1-2	Construct AE and EA dichains, test activity	1
3-4	AE or EA lentiviral production, transduction, selection and testing	1
5-6	Deletion and mutation of LC residues, persistence testing	2
7-8	Construct stable cell line with minimum domain	3
9-12	Targeted knockdown of deubiquitinating enzymes, persistence testing	4

Task 1: Determination of persistence of BoNT/AE dichain constructs.

A major goal of this proposal is to determine whether the persistence of BoNT/LcA is due to an active process such as deubiquitination or the short half-life of BoNT/LcE is due to an active process such as E3 ubiquitin ligase recognition of BoNT/E Lc leading to its ubiquitination and subsequent degradation by the ubiquitin proteasome pathway. To determine whether one process dominates over the other, chimeric A/E and E/A molecules (both chains full length) were produced and their half-lives were determined.

The following mammalian expression vector-based constructs were produced for this study:
1. SBP-CFP-BoNT/LcA1 (single chain)
2. SBP-CFP-BoNT/LcE (single chain)
3. SBP-CFP-BoNT/LcA1-E (chimeric molecule)
4. SBP-CFP-BoNT/LcE-A1 (chimeric molecule)
5. SBP-CFP-BoNT/LcA1-A1 (molecular weight control)
6. SBP-CFP-BoNT/LcE-E (molecular weight control)

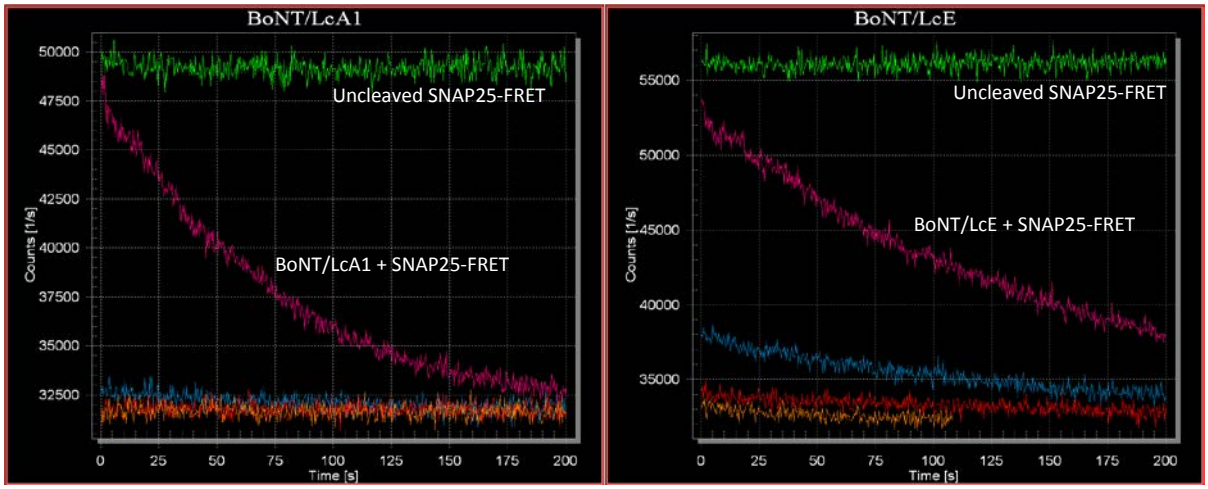
The nomenclature above uses SBP to indicate a 40 amino acid N-terminal streptavidin binding protein tag, followed by CFP or cyan fluorescent protein tag, and the BoNT/Lc coding sequence.

To study the parameters that confer stability to BoNT/LcA1 and the role of cellular mechanisms leading to rapid degradation of BoNT/LcE, genes encoding these two LCs were fused and the stability of the chimeric molecules was determined. Chimeras SBP-CFP-BoNT/LcA1-E and SBP-CFP-LcE-A1 were constructed by fusion of BoNT/LcE and BoNT/LcA1 with cyan fluorescent protein tag (CFP) fused to its N-terminus. These constructs were tested for activity *in vitro* using SNAP25-FRET reporter. Half-life of these chimeric LCs was determined in human embryonic kidney cells, HEK293, using cycloheximide protein inhibition experiments. Replicated SBP-CFP-LcA1-A1 and SBP-CFP-LcE-E were used as controls to determine whether the increased mass of the A1/E or E/A1 constructs results in altered clearance by the ubiquitin proteasome system. Samples were analyzed by Western blotting using polyclonal rabbit anti-GFP antibody that binds to the CFP tag.

Enzymatic activity determination of BoNT/LCs by FRET analysis

To determine the enzymatic activity of BoNT/LCs, the plasmids were transfected into HEK293 cells using Lipofectamine. Two days later, the cells were lysed and the lysates were mixed with the CFP-SNAP25-YFP fusion protein (SNAP25-FRET, expressed in bacteria). This fusion protein shows Forster Resonance Energy Transfer (FRET) phenomenon. Cleavage of SNAP25-FRET substrate by BoNT/LC leads to loss in FRET; i.e., loss of YFP and gain of CFP signal when excited at the same wavelength (438nm).

The enzymatic activity of all 6 LC constructs is shown in Figure 1, below. The results demonstrate cleavage of SNAP25-FRET substrate by all BoNT/LC constructs including the chimeric molecules LcE-A1 and LcA1-E proteins, indicating that the molecules are enzymatically active and the expressed proteins must be folded correctly.



(a) **(b)**

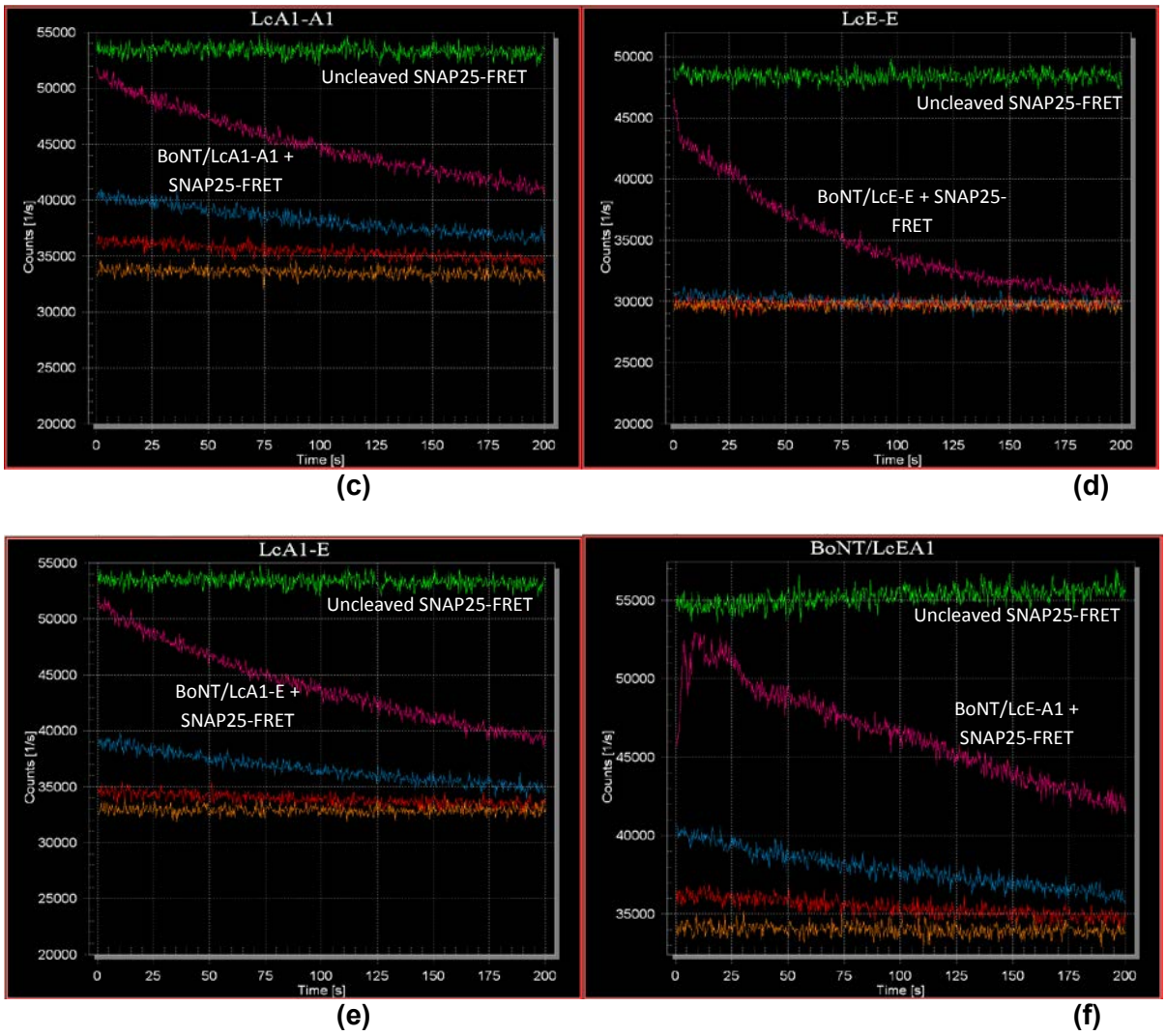


Figure 1: Förster Resonance Energy Transfer (FRET) based analysis of enzymatic activity of BoNT/LCs. Time-based cleavage of SNAP25-FRET substrate by (a) BoNT/LcA1 (b) BoNT/LcE (c) BoNT/LcA1-A1 (d) BoNT/LcE-E (e) BoNT/LcA1-E and (f) BoNT/LcE-A1. The magenta line shows the first 200 seconds of activity, blue line shows 200-400 seconds, red line 400-600 seconds and the orange line shows the final 600-800 seconds of activity.

Half-life determination of BoNT/LCs by cycloheximide chase – Single chains

The half-life of BoNT/LCs was determined in HEK293 cells using cycloheximide, a protein synthesis inhibitor. HEK293 cells in 6-well plates at 70% confluency were used for transfection using Lipofectamine reagent. For each well, 1.5 µg of plasmid DNA was used at a DNA:Lipofectamine ratio of 1:3. Forty-eight hours after transfection, the transfected cells were treated with cycloheximide at a concentration of 50 µg/ml. At various time points after cycloheximide addition, cells were washed twice with DPBS and lysed using lysis buffer (DPBS + EDTA-free protease inhibitor cocktail + 0.1% Triton X-100). Total amount of protein was estimated in these cells lysates using a BCA protein analysis kit and Western blot analysis was performed by loading equal amount of protein for different time points shown in Figures 2, 3 and 4. Blot bands were quantified using ImageJ software.

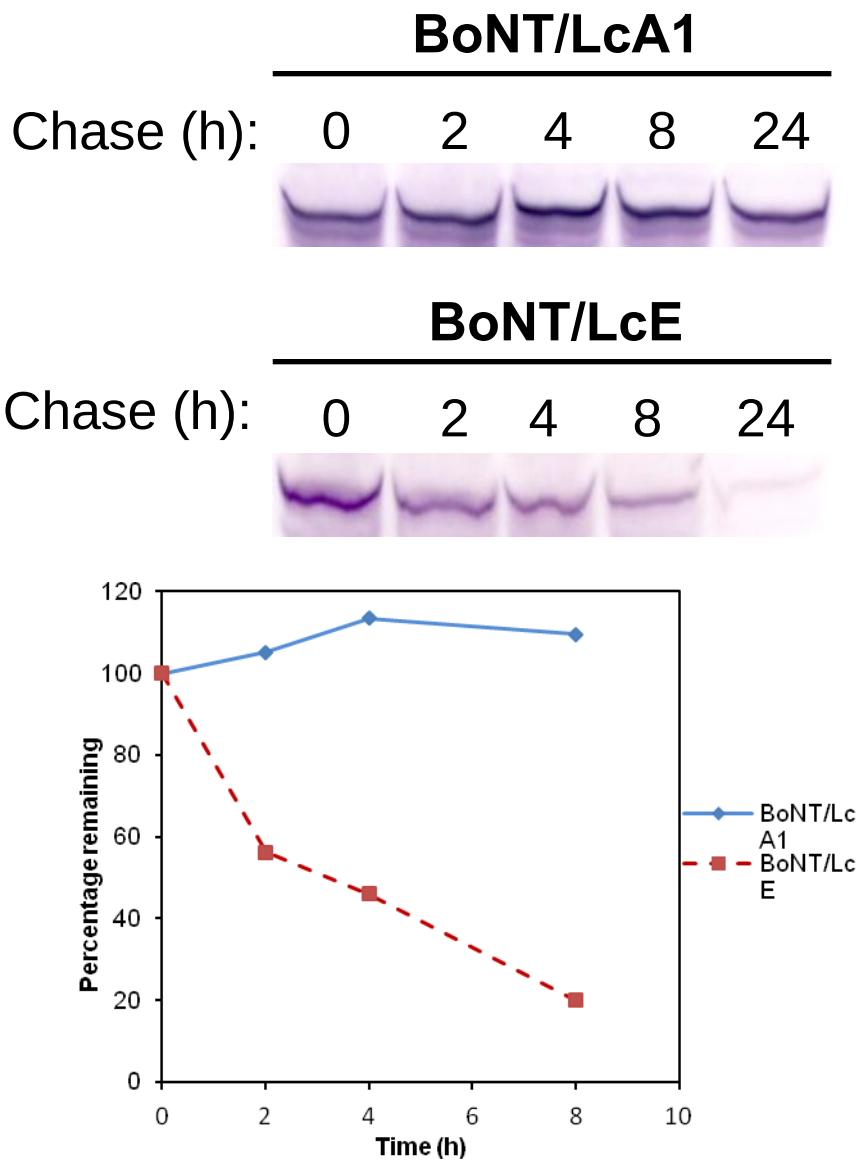


Figure 2: Degradation of BoNT/LcA1 and BoNT/LcE in HEK293 cells assessed by cycloheximide chase and SNAP-25 cleavage.

BoNT/LcA1 was found to be stable with approximately same levels of the light chain at 24h whereas the half-life of BoNT/LcE was found to be less than 4h indicating difference in involvement of cellular factors responsible for degradation of these two light chains.

Half-life determination of BoNT/LCs by cycloheximide chase – Replicated control chains

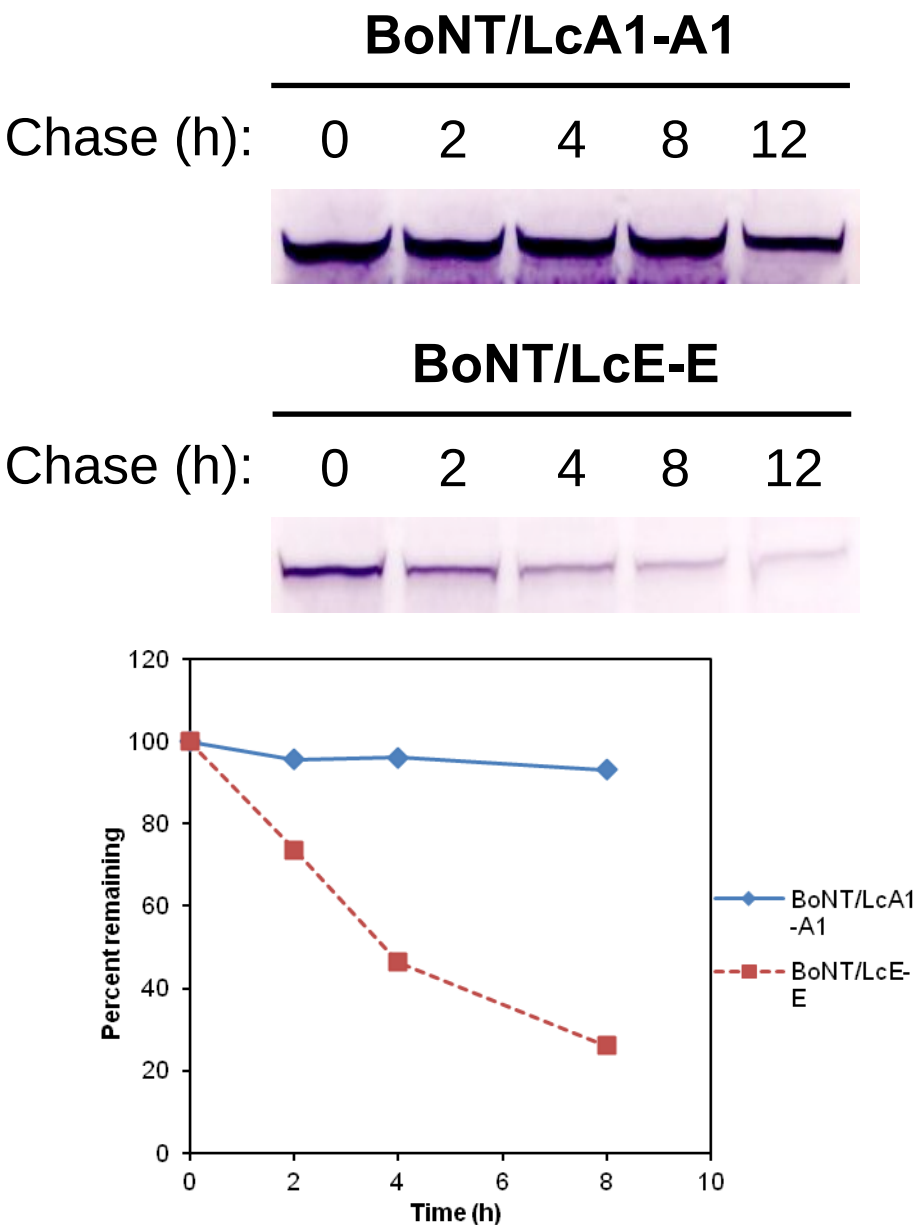


Figure 3: Degradation of BoNT/LcA1-A1 and BoNT/LcE-E in HEK293 cells assessed by cycloheximide chase and SNAP-25 cleavage.

For half-life experiments of the control constructs, BoNT/A1-A1 showed persistence similar to BoNT/LcA1 and BoNT/LcE-E followed the degradation pattern of BoNT/LcE indicating that the increased mass of the replicated LC does not show altered clearance by the ubiquitin proteasome pathway.

Half-life determination of BoNT/LCs by cycloheximide chase – Chimeric chains

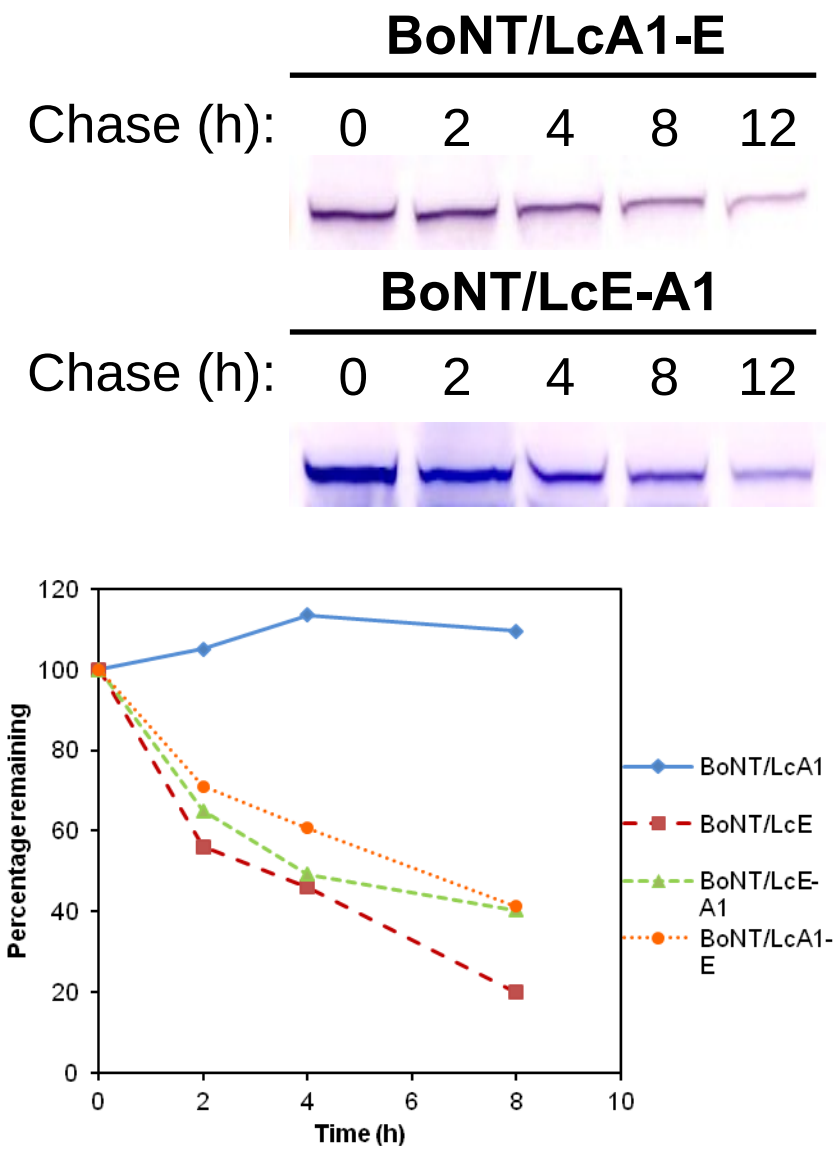


Figure 4: Degradation of BoNT/LcA1-E and BoNT/LcE-A1 in HEK293 cells assessed by cycloheximide chase and SNAP-25 cleavage.

As shown in Figure 4, BoNT/LcA1 fused to BoNT/LcE at either at N-terminal or C-terminal of LcE is short-lived with a half-life of approximately 4h. Cycloheximide chase studies for BoNT/LcE-A1 in HEK293 cells clearly demonstrate its half-life is more similar to BoNT/LcE than to BoNT/LcA1.

These results indicate that the cellular mechanisms responsible for rapid degradation of BoNT/LcE are dominant over the mechanisms responsible for persistence of BoNT/LcA. In the case of the fusion protein, persistence of BoNT/LcA can be abbreviated, possibly by recruiting ubiquitin activity. Thus, persistence of BoNT/LcA may be successfully overcome by developing ubiquitin designer ligase-based therapeutics that will target BoNT/LcA for proteasomal degradation.

Task 2: Mutation of dileucine residues and systematic deletion of residues from AE dichain to map stability-conferring domains of A.

The factors or parameters that confer stability to LCs of botulinum toxin can be assessed by mutation of dileucine residues and systematic deletion of residues from LcA-LcE chimeras to provide a map of stability-conferring domains of A.

Mutation of dileucine residues

The di-leucine signal sequence was mutated to di-alanine to determine the role of di-leucine in the persistence of BoNT/A. According to Wang et al (2011), di-leucine residues in LcA at positions 427-428 play an important role in the longevity of this molecule. The following construct was produced:

- SBP-CFP-BoNT/LcE-A1 427LL428 to 427AA428 (mutated molecule)

Assessment of the role of specific amino acid residues in the persistence of BoNT/LcA1 were assessed by focusing on the role of a di-leucine motiff in amino acids 427 and 428 of the BoNT/LcA1.

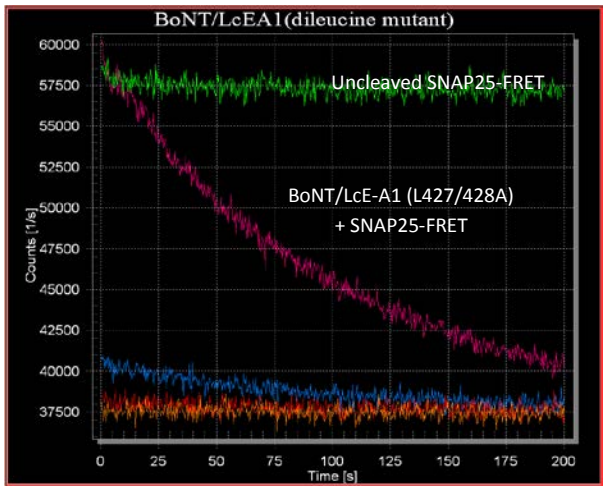


Figure 5: Förster Resonance Energy Transfer (FRET) based analysis of enzymatic activity of BoNT/LcA1(L427/428A).

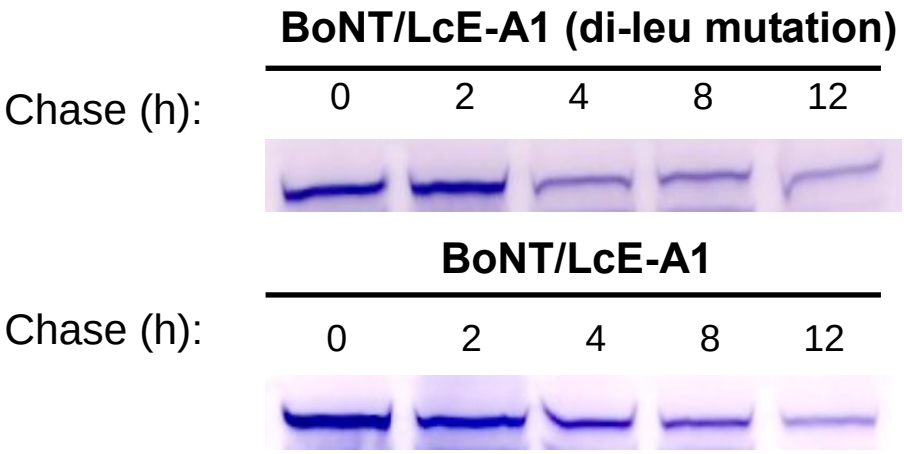


Figure 6: Degradation of BoNT/LcE-A1 (L427/428A) and BoNT/LcE-A1 in HEK293 cells assessed by cycloheximide chase.

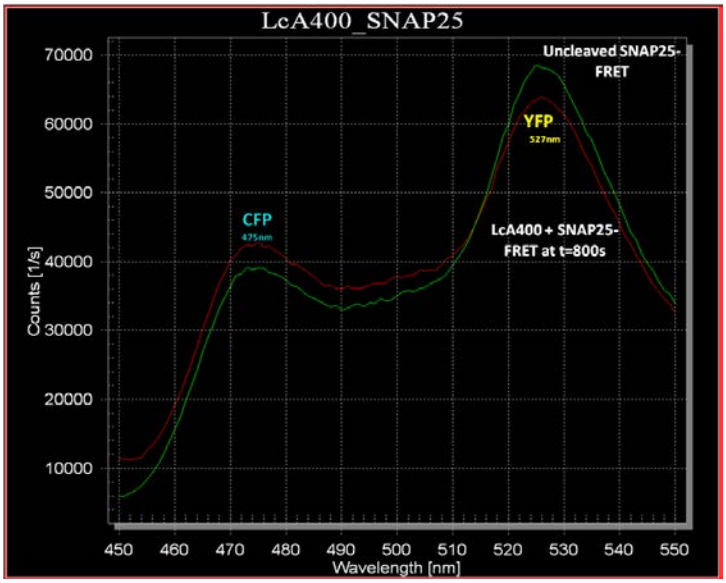
The results show that the mutated LC was active but no effect was noted on chimeric protein half-life. Note that both chimeric proteins have a shortened half-life due to the influence of LcE and no further shortening could be attributed to the mutated residues.

Deletion Studies

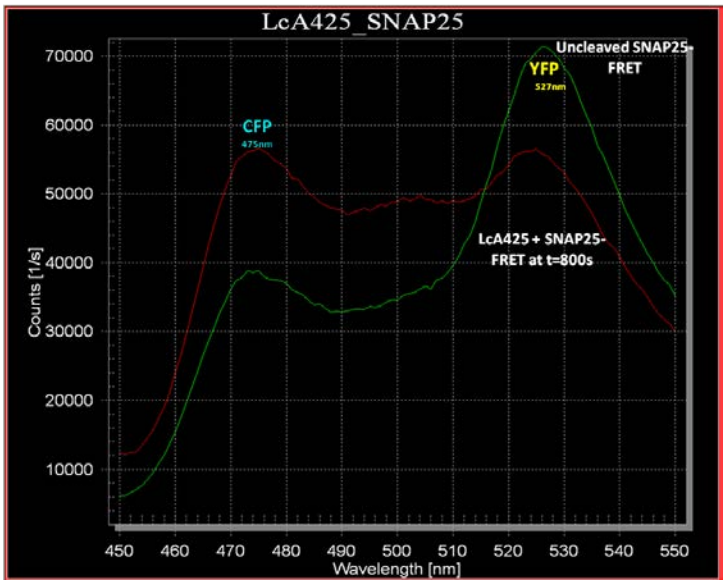
To determine the domain of LcA that conferred stability to the molecule, deletions were made at the C-terminus of LcA1 resulting in two truncated forms: LcA1 (1-425) and LcA1 (1-400). These constructs were tested for activity *in vitro* using SNAP25-FRET reporter. Half-life of these chimeric LCs was determined in human embryonic kidney cells, HEK293, using cycloheximide protein inhibition experiments.
The following mammalian expression vector-based constructs were produced for these studies:
1. SBP-CFP-BoNT/LcA1 (1-425) (Referred as LcA425)
2. SBP-CFP-BoNT/LcA1 (1-400) (Referred as LcA400)
The nomenclature above uses SBP to indicate a 40 amino acid N-terminal streptavidin binding protein tag, followed by CFP fluorescent protein tag, and ending with the truncated BoNT/LcA1 coding sequence.

Enzymatic activity determination of truncated BoNT/LcA by FRET analysis

To determine the enzymatic activity of truncated BoNT/LcA1 constructs, CFP-SNAP25-YFP fusion protein (SNAP25-FRET, expressed in bacteria) in which CFP acts as a donor and YFP as acceptor was used. Cleavage SNAP25-FRET substrate by BoNT/LC leads to loss in FRET; i.e., loss of YFP and gain of CFP signal when excited at the same wavelength (438nm). 10000 RFU (Relative Fluorescence Units) of each light chain was used for the experiment.



(a)



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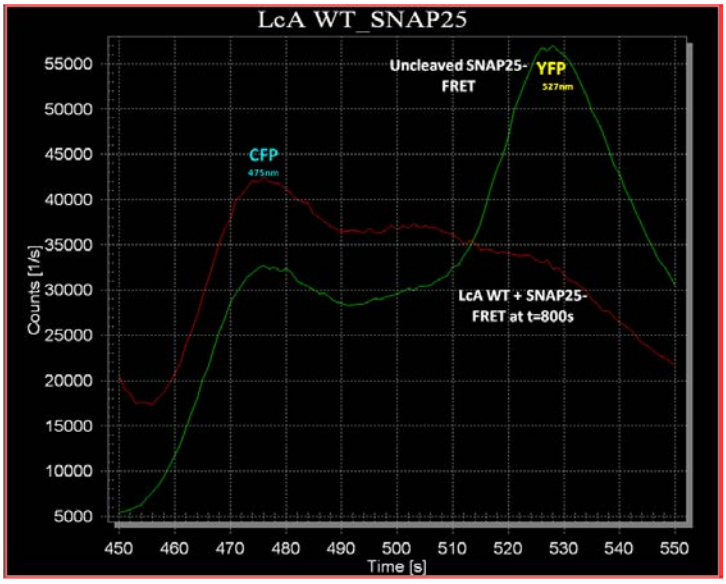


(c)

Figure 7: Förster Resonance Energy Transfer (FRET) based analysis of enzymatic activity of BoNT/LCs. Emission-scan of SNAP25-FRET substrate with and without (a) BoNT/LcA400, (b) BoNT/LcA425 and (c) Wild type BoNT/LcA.

As shown in Figure 7a, LcA400 lost its activity after deletion of 38 C-terminus amino acids demonstrated by its similarity to the curve of uncleaved substrate. LcA425 maintained activity (Fig. 7b) when 13 C-terminal amino acids were deleted but it was not as active as wild-type LcA (Fig. 7c). Since the deletions did not include the enzymatically active site of the LC, the substantial loss of activity of the truncated light chains could be due to protein misfolding.

Half-life determination of truncated BoNT/LcA1 by cycloheximide chase

The half-life of truncated BoNT/LCs was determined in HEK293 cells using cycloheximide. HEK293 cells in 6-well plates at 70% confluence were used for transfection using Lipofectamine reagent. For each well, 1.5 µg of plasmid DNA was used at a DNA:Lipofectamine ratio of 1:3. 48h after transfection, the transfected cells were treated with cycloheximide at a concentration

of 50 µg/ml. At various time points after cycloheximide addition, cells were washed twice with DPBS and lysed using lysis buffer (DPBS + EDTA-free protease inhibitor cocktail + 0.1% Triton X-100). Total amount of protein was estimated in these cells lysates using a BCA protein analysis kit and Western blot analysis was performed by loading equal amount of protein for different time points shown in Figure 8. Blot bands were quantified using ImageJ software.

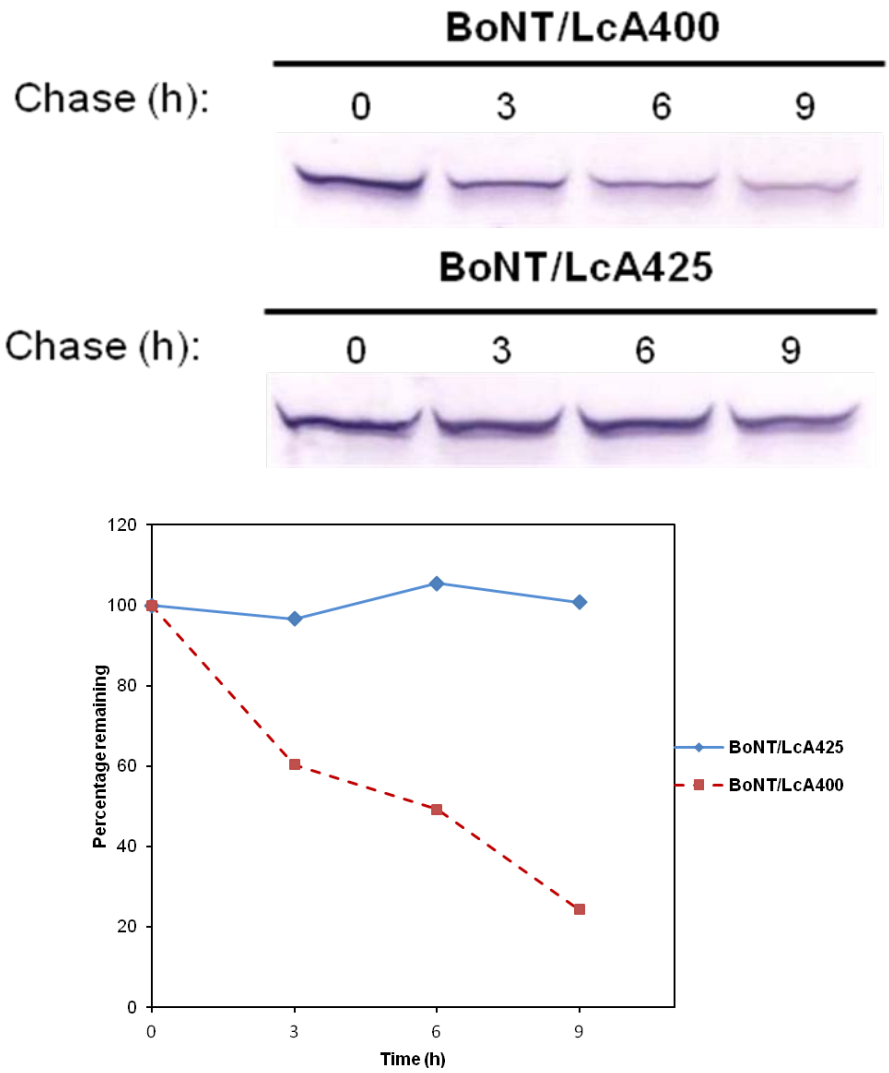


Figure 8: Degradation of BoNT/LcA400 and BoNT/LcA425 in HEK293 cells assessed by cycloheximide chase.

BoNT/LcA425 was found to be stable with approximately same levels of the light chain at 9h whereas the half-life of BoNT/LcA400 was found to be approximately 5h. We cannot conclude that the domain lying within 400-425 confers stability to the LcA molecule since BoNT/LcA400 is enzymatically inactive. Improper folding of this molecule alone could contribute to its shortened half-life.

Enzymatic activity determination and half-life studies with B8-BoNT/LcE

In a second approach, it was decided to study whether the cellular mechanisms leading to rapid degradation of BoNT/LcE could be employed to target the BoNT/LcA using a camelid heavy-chain-only antibody (VHH), B8, which binds to BoNT/LcA within cells. This approach was not part of the original application but was approved by Dr. Patrick McNutt.

The following construct was made to carry out this study:

- SBP-YFP-B8-BoNT/E

The enzymatic activity of B8-BoNT/LcE fusion protein was confirmed using CFP-SNAP25-YFP fusion protein (SNAP25-FRET, expressed in bacteria) as described previously.

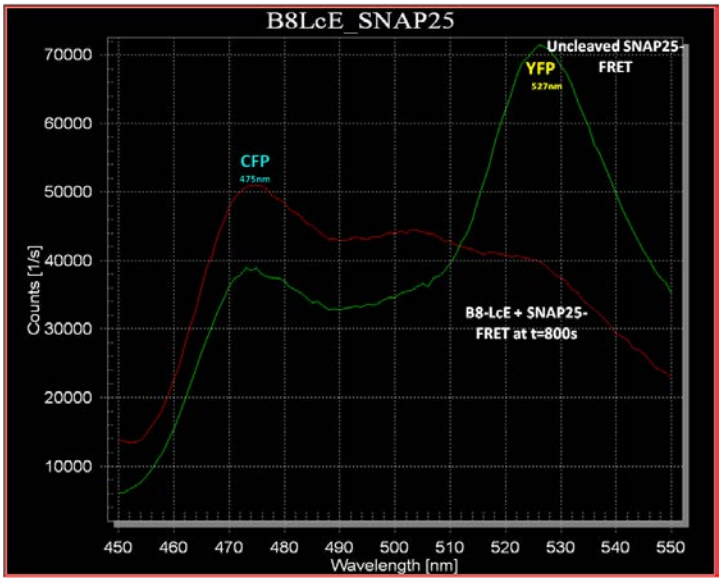
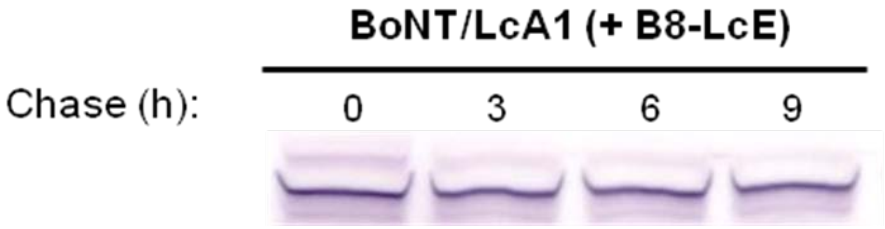


Figure 9: Förster Resonance Energy Transfer (FRET) based analysis of enzymatic activity of B8-BoNT/LcE. Emission-scan of SNAP25-FRET substrate with and without B8-BoNT/LcE.

To determine the effect of B8-BoNT/LcE on the half-life of BoNT/LcA1, HEK293 cells were co-transfected with 1.5 µg each of BoNT/LcA1 and B8-BoNT/LcE plasmid DNA using Lipofectamine reagent. For cycloheximide chase, same protocol was used as previously discussed.



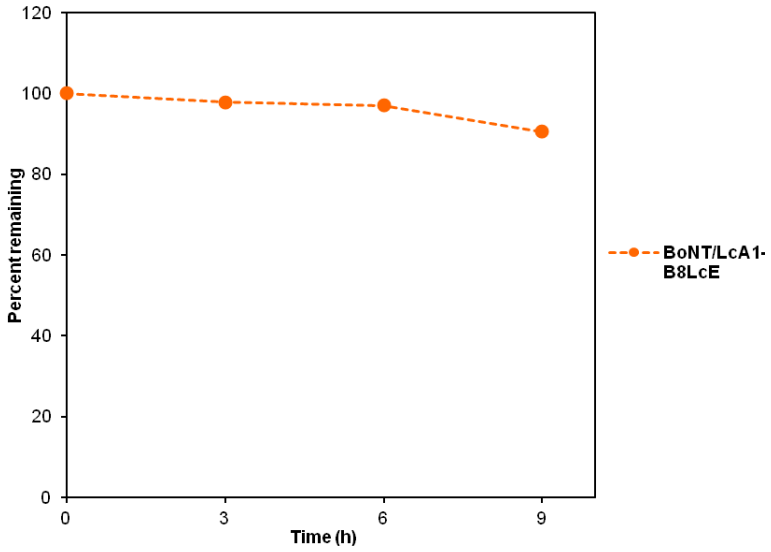


Figure 10: Degradation of BoNT/LcA1 in the presence of B8-BoNT/LcE in HEK293 cells assessed by cycloheximide chase.

The results demonstrate that the enzymatic activity of LcE was normal after ligation to the B8 VHH camelid antibody (Fig. 9). Results in Fig. 10 show that binding of LcE to LcA via the VHH bridge did not alter the degradation kinetics of LcA. However, the possibility existed that the VHH was not functional in the B8-LcE complex.

Thus, we pursued the investigation of the functionality of the B8-LcE molecule. To determine if the VHH B8 antibody was active when combined with LcE, we attempted to pull-down B8-LcE with its target, LcA. HEK-293 cells were co-transfected with B8-LcE and LcA2(444). Fluorescent tags were genetically appended to B8-LcE (YFP) and LcA2 (CFP) allowing co-localization analysis by FRET. The cells were lysed and LcA2, which contains a 6xHis tag, was pulled down using nickel coated beads. Protein was eluted from the beads using immidizole and assessed by Western blot for B8-LcE using anti-GFP antibody and by FRET analysis. Neither assay showed the presence of B8-LcE indicating that the B8 was not functional.

These experiments were then repeated with B8-YFP as a control. LcA pulled down B8-YFP but not B8-LcE confirming that the B8 on LcE was not functional. At this point, and after discussions with Dr. McNutt, we decided to end this path of investigation.

Task 3: Construct stable cell line with minimal domain of BoNT/A LC that maintains stability of original dichain.

After discussions with Drs. Adler and McNutt, it was decided not to pursue this task since we were unable to determine a minimal domain of BoNT/A that maintained its stability.

Task 4: Perform targeted siRNA knockdown of candidate deubiquitinating enzymes (DUBs) to determine their role in persistence mediated by interaction with BoNT/A LC using the cell lines constructed in Task 3.

We studied the effects of VCIP135 knockdown on transiently expressed BoNT/LcA1 in HEK293 cells. We were able to successfully knockdown VCIP135 using siRNA (Fig. 11). We have shown that the half-life of BoNT/LcA1 is significantly shortened by siRNA knock-down of VCIP135 in the HEK293 cells expressing BoNT/LcA1 (Fig. 12). VCIP135 is the deubiquinating enzyme recruited by p47, which is one of the host cellular factors that specifically interact with BoNT/LcA1 and not BoNT/LcE. We will attempt to pulldown VCIP135 from cell lysate either by

bacterial expressed His-BoNT/LcA1 bound to a Ni column or capture of transient expressed His-BoNT/LcA1 in mammalian cells using a Ni column to confirm this interaction. However, preliminary evidence would seem to indicate that the association of VCIP135 with BoNT/LcA1 and/or complexes associated with BoNT/LcA1 is transient in nature. As such, we would not be able to precipitate a sufficient amount of VCIP135 for detection.

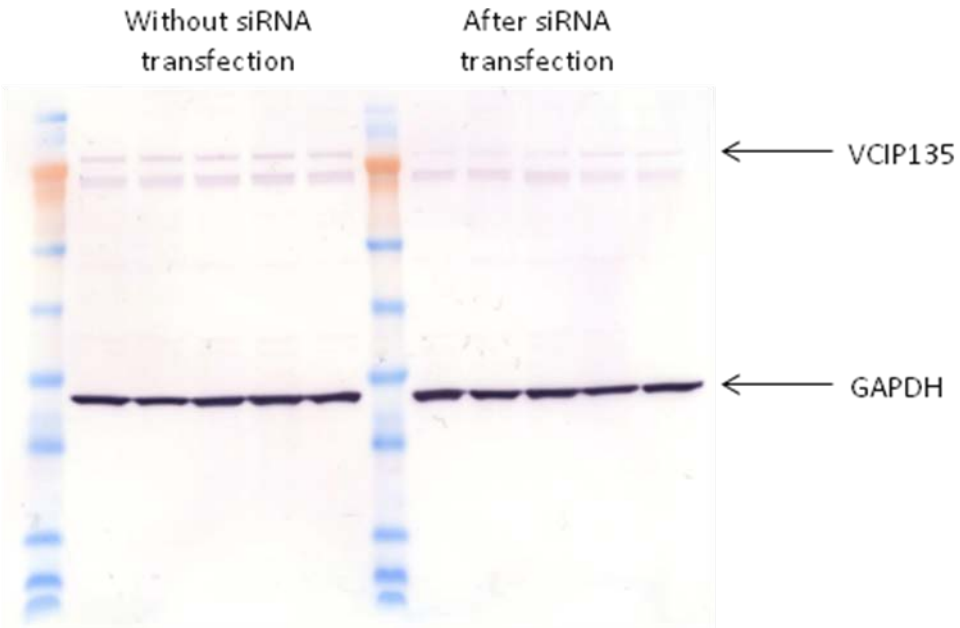


Figure 11. VCIP135 knock-down in HEK293 cells by transient transfection of siRNA. The expression levels of VCIP135 and GAPDH were monitored and detected on Western blot using anti-VCIP135 and anti-GAPDH antibody.

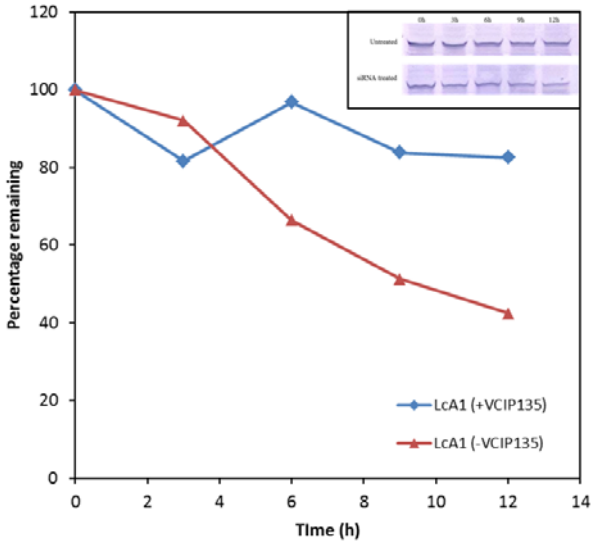


Figure 12. Degradation of BoNT/LcA1 in HEK293 cells, with and without VCIP135, assessed by cycloheximide chase. Quantitative analysis of BoNT/LcA1 levels on the blots (example shown in insert) was done using Image J software. The amount of BoNT/LcA1 at various times post addition of cycloheximide was calculated and plotted.

Summary of results obtained for Aim 1:

- Chimeric molecules, made by fusion of genes encoding BoNT/LcE and BoNT/LcA1, were successfully expressed in HEK293 cells. The expressed chimeric molecules were found to actively cleave SNAP25-FRET substrate.
- Cycloheximide protein synthesis inhibitor studies to determine the stability of the light chain constructs in HEK 293 cells indicated the following half-lives:
  BoNT/LcA1:100% of initial light chain level at 24h
  BoNT/LcE: $t_{1/2}$ ~ 4h
  BoNT/LcA1-A1: Over 95% of initial light chain level at 12h
  BoNT/LcE-E: $t_{1/2}$ ~ 4h
  BoNT/LcA1-E: $t_{1/2}$ ~ 4h
  BoNT/LcE-A1: $t_{1/2}$ ~ 4h
- Cycloheximide studies with BoNT/LcA1-E and BoNT/E-A1 chimeric constructs demonstrated a half-life of 4h similar to that of BoNT/LcE which suggest that the mechanisms leading to the rapid degradation of BoNT/LcE dominate over the cellular mechanism leading to BoNT/LcA1 persistence.
- Short half-life of BoNT/LcE-A1 and BoNT/LcA1-E chimeras suggest that BoNT/A1 half-life can be shortened by recruiting the ubiquitination activity of BoNT/E by fusion and BoNT/LcA1 designer ligase can be successfully employed to overcome the persistence of BoNT/LcA1.
- Assessment of the role of a di-leucine motiff at amino acids 427 and 428 of the BoNT/LcA1 showed no effect of the mutation on the half-life of LcA in the A/E chimeric protein. This result was not unexpected in view of previous results showing that the A/E chimeric protein exhibited a shortened half-life due to LcE.
- Deletion experiments were inconclusive as they showed that deletion of the 38 C terminal amino acids (LcA400) resulted in a significant decrease of half-life of LcA but also diminished enzymatic activity suggesting that the truncated protein was folded improperly. Thus, it is not clear as to whether the shortened half-life was due to elimination of critical sequences for protein longevity or improperly folded protein.
- The half-life of BoNT/LcA1 is significantly shortened by siRNA knock-down of VCIP135 in the HEK293 cells expressing BoNT/LcA1. VCIP135 is the deubiquinating enzyme recruited by p47, which is one of the host cellular factors that specifically interact with BoNT/LcA1 and not BoNT/LcE.

Aim 2: Support for project entitled "High-throughput characterization of the BoNT interactome and intoxication-induced transcriptional changes" (CBM.THRTOX.05.11. RC.014; PI Patrick McNutt).

Synaptic Research will use a Yeast 2 Hybrid (Y2H) system to screen a neuronal cell library using LcA and LcE as bait. Following identification and validation of cellular proteins that interact with LCs and are important to intoxication, the Y2H system will be used to rapidly conduct initial screening for small molecules that disrupt the target protein-LC interaction.

Project Milestones (2 Years):

Month/Year	Activity	Task
1-12/1 1-6/2	**Characterize the BoNT interactome using yeast two-hybrid (Y2H) analysis**	**1**
1-4/2	**Assess the importance of at least three validated interactions to toxin activity – impact of mutation**	**2a**
5-8/2	**Assess the importance of at least three validated interactions to toxin activity – affinity pulldowns**	**2b**
9-12/2	**Assess the importance of at least three validated interactions to**	**2c**

	toxin activity – FRET analysis	

Task 1: Characterize the BoNT interactome using yeast two-hybrid (Y2H) analysis (Years 1 and 2).

The Y2H screen of proteins from adult human brain that bind to LcA1 and LcE has been completed. The screen was satisfactory from the technical point of view. Altogether, over 220 million interactions (22-fold the library complexity) were tested. In the initial tests, the bait fusion was neither toxic nor auto activating the yeast two-hybrid system. As a result, there was little or no background noise. Several preys were found with a good confidence scores. This suggests that the bait was well folded in yeast. In addition, we have found SNAP25 as prey in this screening which is consistent with the correct folding of the bait fusion.

The following table summarizes the gene hits with good to very high confidence scores.

Confidence	LcA1	LcE
A – Very High	EZR, MSN, SEPT4, SEPT7, SNAP25	AHSA1, MRFAP1L1, NEDD5, SYT1
B - High	APEH, ARHGEF11, KALRN, KIFAP3, NF2, PPP2R3A, RDX, STUB1, TTC3	DLST, ERAP1, GNAS, KIFAP3, PCDHGB2, SREK1
C - Good	HECTD1	LRP11, SET, SEZ6L2, FARP2

For LcA1, several of the preys with high confidence scores appear to be highly specific as they haven't appeared in previous screens of human libraries. This is the case for Septins 4 and 7 which have been shown previously to regulate bacterial entry into host cells (Mostowy et al, 2009). Several E3 ligases (STUB1/CHIP, TTC3 which is E3 ligase of Akt) also appeared in the screen as prey.

Two screenings were performed for LcE as the first screening obtained only 7 positive clones out of 112 million interactions tested. Two preys (FARP2 and NEDD5) were found in both LcE screenings (with both LexA and Gal4 fusion) which makes these interactions even more reliable. Of interest, three of the hits have coiled-coil domains which is the motif present in SNARE's (e.g. SNAP25) for BoNT/E binding to substrate. However, the SID (Selected Interacting Domain, which is in fact the minimal region common to the experimental fragments) for FARP2 and SMYD2 do not contain the coiled-coil domain. FARP2 is involved in neuronal guidance and also is called Ezrin like protein – Ezrin was also a previous protein of interest due to both pull downs and Y2H with BoNT/A (but not /E before).

The fact that Septins and Erzin/Ezrin domain proteins (FARP2 for BoNT/E) came up for both LcA1 and LcE is intriguing and suggests that further studies should be performed on these proteins. Also the Septins that interact with BoNT/A and BoNT/E are different members suggesting it is more than just sticky proteins (in which case you would expect that same Septins coming up on both BoNT/A and /E).

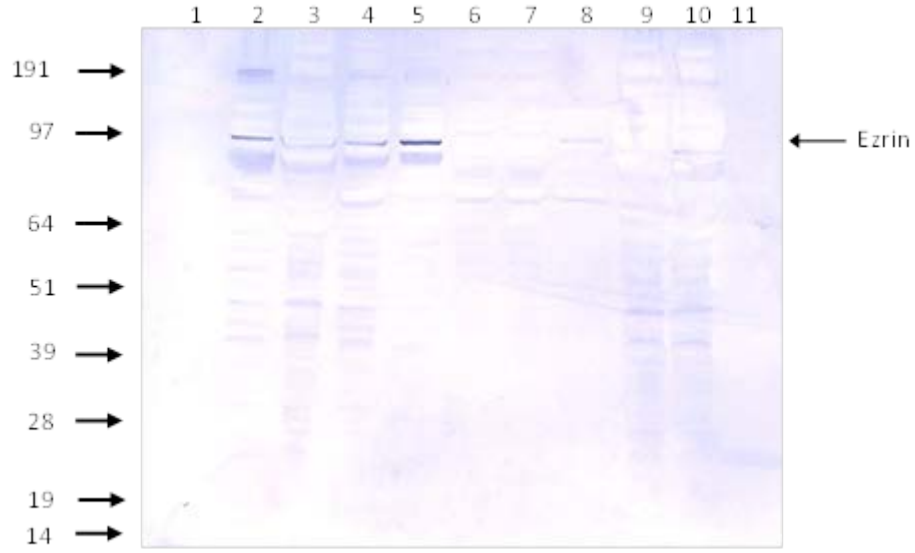
Dr. McNutt's group has identified mRNA levels in their ESNs which identify Ubiquitins, DUBs and other potential interacting proteins that should serve as a point of discussion in determining which proteins to prioritize for future studies.

Task 2: Assess the importance of at least three validated interactions to toxin activity (Year 2).

One of the validated interactions we plan to pursue in this Task is affinity pull-down of proteins from neuronal cell lysates that bind to LcA or LcE and characterize the proteins by Western blot.

While we have yet to prioritize the proteins, we have started to develop the pull-down assay. In a pilot experiment, we conjugated bacterial LcA and LcE expressing streptavidin binding protein to streptavidin (SA)-beads and incubated them with N2A cell lysates. After incubation, the beads were washed and the proteins eluted by boiling in SDS. The eluted proteins were run on Western blot using antibodies to Septin4 and Ezrin. Unfortunately, no bands were observed at the appropriate molecular weights for Ezrin or Septin4.

The experiment was repeated using a higher concentration of N2A and HEK293 cell lysate and a comparison of protein elution by SA and 6xHis-Ni affinity methods was made. This time, there was a faint bad that was detectable on Western blot using Ezrin antibody (but not Septin4 antibody):



SDS Gel parameters
Gel: NuPAGE® Novex 12% Bis-Tris Gel 1.0 mm, 12wells
Buffer: NuPAGE® MOPS SDS Running Buffer
Ladder: SeeBlue® Plus2 (Invitrogen)
Run: 150V, 1.5h
Transfer parameters
Voltage: 30V
Time: 1.5 h (in transfer buffer for two blots)
Blot analysis: WesternBreeze® Chromogenic Immunodetection Kit (Invitrogen)
1° antibody: Ezrin (C-15) antibody, 1:10000 (sc-6409)
2° antibody: Alkaline phosphatase-conjugated Anti-goat IgG
Detection: BCIP/NBT substrate for alkaline phosphatase

Lane	Sample
1	Ladder
2	N2a cell lysate
3	HEK293-LcA444 cell lysate
4	Ni-His-LcA444 flow through
5	Ni-His-444-N2a flow through
6	250mM imidazole Elution 1 (ppt)
7	500mM imidazole Elution 2 (ppt)
8	500 mM Elution 3 (sup)
9	Strep-SBP-LcA444 flow through
10	Strep-SBP-LcA444-N2a flow through
11	SDS loading dye Elution

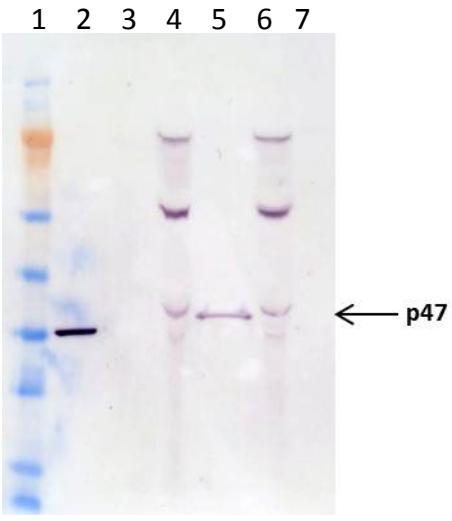
Figure 13. Western blot analysis of pulldown fractions from N2a cell lysate using Ezrin antibody

The results show that Ezrin can be detected in N2A cell lysate (Lane 2), in HEK293 cell lysate (Lane 3) and in both flow throughs (Lanes 4-5). Most important, a faint bad was observed for imidazole elution of the His-Ni beads (Lane 8) indicating that Ezrin bound to LcA444. The eluate precipitated so it was spun down and the supernatant (sup) and precipitated (ppt) fractions were run separately. Only the supernatant showed a band at the appropriate size. The SA elution and anti-Septin4 were unsuccessful. The latter is interesting in view of recent data from the McNutt lab showing that Septin4 mRNA is found in very low levels in ESNs. In the following experiments, we focused on His-Ni affinity for purification to evaluate other proteins and optimize the Western blot for more sensitive detection using chemiluminescence.

Lysate	293	M17	-	293	M17	-	293	M17
Column	-	-	+	+	+	+	+	+
LcA	-	-	-	-	-	+	+	+

Figure 14 LcA-His Pulldown Assay. Eluate from LcA-His purification was diluted 1:10 in IMAC loading buffer (final 25 mM imidazole) and reloaded onto Ni resin. Cell lysate (grown in 10 cm plate and lysed with Peirce IP buffer) was run over column, washed, and complex eluted with 500 mM imidazole. Eluates were run on a 4-12% NuPage gel with MOPS buffer (denatured and reduced), blotted onto PVDF, and probed using anti-Ezrin primary using Invitrogen Alk-Phos WesternBreeze kit.

We have successfully validated association of p47 by pulldown assays. His-tagged BoNT/LcA1 and BoNT/LcE were transiently overexpressed in HEK293 cells and pulled down using a Ni column. Co-precipitate was analyzed by Western blot and p47 was detected in lysate containing BoNT/LcA1 only (Fig. 15).



Lane	Sample
1	Ladder
2	Positive control
3	Negative control
4	BoNT/LcA1 cell lysate
5	BoNT/LcA1 pulldown elution
6	BoNT/LcE cell lysate
7	BoNT/lcE pulldown elution

Figure 15. Interaction of p47 with BoNT/LcA1 in HEK293 cells. Streptavidin-based pull-down of SBP-tagged BoNT/LcA1 and BoNT/LcE show interaction of p47 specifically with BoNT/LcA1 and not BoNT/LcE.

Aim 3: Support for project entitled "Assessment of the ubiquitin proteasome system as a therapeutic target for persistence of BoNT intoxication" (CBM.THRTOX.05.11.RC.023; PI Patrick McNutt).

Synaptic Research will develop a mouse spinal cord neuron model to complement the recESN platform developed by Dr McNutt. We will identify ubiquitin and DUB proteins derived from spinal cord neurons that bind to LC/A and LC/E consistent with longer term goals of the McNutt lab to assess the impact of candidate DUBs on LC/A persistence and evaluate the ability of pharmacologic entities to rescue intoxicated neurons. Furthermore, we will evaluate the ability of designer E3 ubiquitin ligases that ubiquitinate LC/A (recE3/A) to expedite recovery from intoxication.

Project Milestones (2 Years):

Month/Year	Activity	Task
1-3/1	**Treat purified bacterial LC/A and LC/E with lysates derived from spinal cord neurons**	**1**
3-6/1	**Evaluate differential association of LC/A and LC/E with ubiquitinases and DUBs from spinal cord lysates**	**1**
1-12/1	**Transduce spinal cord neurons with E3 ubiquitin ligase lentivirus**	**2**
1-12/2	**Characterize the activity of designer E3 ubiquitin ligases that target LC/A to resist or shorten BoNT intoxication**	**2**

Task 1: Evaluate differential association of LC/A and LC/E with ubiquitinases and DUBs in mouse spinal cord neurons using a pull-down assay (Year 1).

We are ready to assist the McNutt lab in their efforts:

- Synaptic Research has prepared adult rat brain primary neurons from which to provide lysates for the pull-down assay.
- We have entered into discussions with LifeSensors, a company that makes kits to detect ubiquitins and deubiquitins. They can provide the DUB inhibitor PR619, VU-1 antibody to ubiquitin and Ubi-Rhodamine and DiUbiquitin IQF substrates to measure the enzymatic activity of these enzymes.
- We have performed pilot experiments evaluating Ezrin and Septin4 binding from N2A and HEK293 cell lysates to LcA coated beads by western blotting (see Aim 2, Task 2). The results from these experiments will be useful in choosing parameters for pull down experiments using lysates from primary neurons and ESNs.

Task 2: Characterize the ability of designer E3 ubiquitin ligases that ubiquitinate LC/A to resist or rescue BoNT intoxication (Years 1 and 2).

We are optimizing the expression of C2I toxin expressing D5/B8 designer ligase and the C2II accessory protein as well as the purification of these proteins. We have determined that bacterial expression of C2I-D5/B8 will not produce enough functional protein for cell culture experiments. The vast majority of the protein is in inclusion bodies. We have attempted to

solubilize the protein and re-fold it using various media designed for this purpose with some success. However, the refolded proteins show no activity upon testing *in vitro.*

Thus, we have initiated studies with the baculovirus/insect cell expression system. We have synthesized the gene for C2IN-D5/B8 codon optimized for insect cell expression and have cloned this gene into the viral plasmid (baculomid) for infection of Sf9 insect cells. Insect cells went through 2 rounds of infection to produce high titer P2 virus. This high titer viral prep was used to infect SF9 cells for protein production. Multiple attempts were made using a range of MOI and time points with limited success. The protein was never efficiently expressed by the cells, while the positive controls showed nice expression. We also attempted to produce the protein using a mammalian expression system (HEK293 cells). Again, the cells did not efficiently produce the protein, and what little was produced was quickly degraded by the cell. Our results would indicate that the C2IN-D5/B8 is a not well suited for protein production in any number of protein expression systems. We believe this is due to the inherent misfolding/instability of the D5 portion. AS such we have begun efforts to redesign this portion of the chimera using other potentially more stable proteins.

KEY RESEARCH ACCOMPLISHMENTS

Aim 1

- Chimeric molecules, made by fusion of genes encoding BoNT/LcE and BoNT/LcA1, were successfully expressed in HEK293 cells. The expressed chimeric molecules were found to actively cleave SNAP25-FRET substrate.
- Cycloheximide studies with BoNT/LcA1-E and BoNT/E-A1 chimeric constructs demonstrated a half-life of 4h similar to that of BoNT/LcE which suggests that the mechanisms leading to the rapid degradation of BoNT/LcE dominate over the cellular mechanism leading to BoNT/LcA1 persistence.
- Short half-life of BoNT/LcE-A1 and BoNT/LcA1-E chimeras suggest that BoNT/A1 half-life can be shortened by recruiting the ubiquitination activity of BoNT/E by fusion and BoNT/LcA1 designer ligase can be successfully employed to overcome the persistence of BoNT/LcA1.
- Assessment of the role of a di-leucine motiff at amino acids 427 and 428 of the BoNT/LcA1 showed no effect of the mutation on the half-life of the A/E chimeric protein.
- Truncation of the C terminus of LcA to amino acid 400 and 425 was performed to determine the minimal domain associated with the long half-life of this protein. LcA400 was not enzymatically active. Although LcA425 was active, it's half-life was the same as wild type LcA. Thus, we were unable to determine the minimal domain.
- The half-life of BoNT/LcA1 is significantly shortened by siRNA knock-down of VCIP135 in the HEK293 cells expressing BoNT/LcA1. VCIP135 is the deubiquinating enzyme recruited by p47, which is one of the host cellular factors that specifically interact with BoNT/LcA1 and not BoNT/LcE.

Aim 2

- The Yeast 2 Hybrid (Y2H) screen of proteins from adult human brain that bind to LcA1 and LcE has been completed.
- The fact that Septins and Erzin/Ezrin domain proteins (FARP2 for BoNT/E) came up for both LcA1 and LcE is intriguing and suggests that further studies should be performed on these proteins.
- Ezrin in N2A cell lysates was shown to bind to LcA immobilized on streptavidin beads by pull-down and Western blot.

Aim 3

- C2I-D5/B8 has been expressed as an insoluble protein in bacteria. The protein has been solubilized and refolded, but has been shown to be inactive. Insect and mammalian

expression systems have also been unable to produce soluble C2IN-D5/B8. We are in the process of redesigning the D5 portion of the protein. It will be replaced with a more stable protein.

- C2II has been expressed as a partially soluble protein in bacteria. There is enough protein available to perform small scale cell culture studies to determine whether the protein can shuttle C2I into the cell. Current results indicate that the C2II protein heptamerizes after trypsinization suggesting that the protein is functional.

REPORTABLE OUTCOMES

Therapeutic Approach Targeting Degradation of BoNT/A Light Chain. A. A. Kotiya, Y. C. Tsai, Y. N. Chang, P. McNutt, A. A. Syed, K. McIntosh, B. Molles, M. Adler, G. A. Oyler, IBRCC 2012.

Cellular Protein Interactions with Botulinum Neurotoxin Light Chain. B. Gertz, J. K. Krady, K. McIntosh, A. A. Kotiya, G. A. Oyler, Y. C. Tsai, A. M. Weissman, C. B. Shoemaker, P. McNutt, IBRCC 2013.

CONCLUSION

Our hypothesis is that LcA has a longer half-life than LcE due to the presence of cell factors (deubiquitins or DUBs) that override the normal protein processing and degradation mechanisms (ubiquitination) that remove proteins from the cell cytoplasm. Our results using chimeric molecules of LcA and LcE demonstrated an overall half-life corresponding to LcE suggesting that short half-life dominates over long half-life and by extension, ubiquitination dominates over deubiquitination. These results support the strategy proposed in this project that delivery of ubiquitinating enzymes to BoNT intoxicated neurons should result in shortened half-life of the toxin. Knockdown studies of VCIP135, a DUB recruited by p47, supports this, as a significantly shorter half-life of LcA was observed in intoxicated cells.

The identification of molecules that bind to LcA and LcE by Y2H analysis will allow us to determine their role in LC survival and potentially target them therapeutically. We have analyzed three potential targets, Ezrin, Septin, and VCIP135. We were able to show involvement of these proteins in half-life of the LCs by pulldown, knowckdown, and/or FRET analysis. These results can be used in therapeutic targeting to decrease the half-life of LcA.

Our studies further support the use of designer ligase as a therapeutic approach for treatment of LcA intoxication. We will continue studies to express and purify the C2I-B8 based designer ligase. Once we have sufficient quantities of a soluble and active ligase in hand, we will perform cell culture studies on BoNT/A intoxicated primary neurons to determine whether the ligase can rescue the cells.

So What? Currently, there is no cure for botulinum poisoning once the toxin has entered a neuron. Moreover, the half-life of BoNT/A is very long, on the order of months, which means that a patient would need to be kept on life support (ventilator) for that period of time in order to survive. In the event of a large scale poisoning, many people would die due to lack of ventilators. The ability to identify and target cell factors that affect the half-life of toxins should allow therapeutics to be designed that would shorten the intracellular life of the toxin effecting a cure. One such product is the designer ligase, which has a VHH antibody region directed to the toxin light chain appended to an ubiquitin ligase which causes degradation of the toxin. Efforts continue to produce the designer ligase and test on neuronal populations of cells in vitro to determine whether they can protect the cells from intoxication.

REFERENCES

Wang, J., T.H. Zurawski et al. (2011). “A dileucine in the protease of botulinum toxin A underlies its long-lived neuroparalysis. Transfer of longevity to a novel potential therapeutic.” J Biol Chem **286**: 6375-6385.

APPENDICES
None.

SUPPORTING DATA
Included in text.