Retarget Botulinum Neurotoxin's Enzymatic Domain to Specific Neurons Using Diverse Neuropeptides as Targeting Domains

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Introduction

Many disease states are caused by miss-regulated neurotransmission. A small fraction of these diseases can currently be treated with botulinum neurotoxin type A (BoNT/A). BoNT/A is composed of three functional domains – the light chain (Lc) is a zinc metalloprotease that cleaves intracellular SNAP25 which inhibits exocytosis, the translocation domain (Td) that enables the export of the light chain from the endosome to the cytosol, and the receptor binding domain (Rbd) that binds to extracellular gangliosides and synaptic vesicle glycoproteins while awaiting internalisation [1]. Current endeavours are directed towards retargeting Bont/A as well as finding safer methods of preparation and administration. Recently, our laboratory has developed a SNARE based linking strategy to recombine non-toxic BoNT/A fragments into a functional protein by simple mixing [2]. This SNARE based linking strategy permits the step-wise assembly of highly stable macromolecular complexes [2,3]. Onto these three SNARE peptides, diverse functional groups can be attached to the N- or C- terminus by direct synthesis and/or by genetic design. To enhance the therapeutic potential of BoNT/A, this method enables the rapid assembly of a large array of neuropeptide-SNAREs to their cognate LcTd-SNARE. A substitution of the Rbd with various neuropeptide sequences permits a large throughput combinatorial assay of LcTd to target new cell types. In this study, we have fused LcTd to 3 different Synaptobrevin sequences; we also use a small protein staple, and 26 different Syntaxin-neuropeptide fusions (permitting the assay of 78 new chimeric LcTd proteins with modified targeting domains). These neuropeptides such as, but not exclusively, somatostatin (SS), vasoactive intestinal peptide, substance P, opioid peptide analogues, Gonadotropin releasing hormone, and Arginine Vasopressin, which natively function through G protein coupled receptors (GPCR) can undergo agonist induced internalisation upon activation. The ability of our new constructs, once endocytosed, to inhibit neurotransmitter release was tested on different neuronal cell lines with immunoblotting of endogenous SNAP25. This cleavage by Lc reflects the ultimate readout of t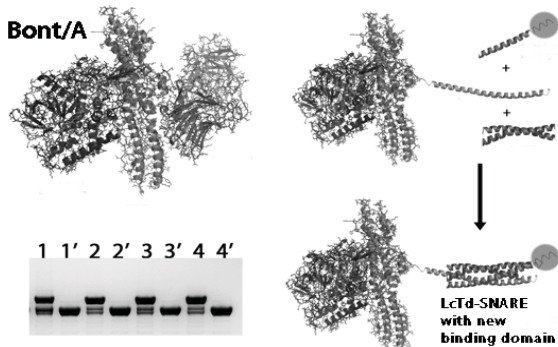he enzyme's efficacy, which incorporates the cell surface binding, internalisation kinetics, translocation of the Lc to the cytosol, and finally the enzymatic cleavage of SNAP25. Internalisation of the toxins can also be monitored with confocal microscopy and FACS by the substitution of the staple peptide for a fluorescent homologue. Figure 1 shows that whole boNT/A (upper left) can have its Rbd replaced with SNARE peptides, which will fuse together to form highly stable chimeric proteins with an altered targeting domain (right). Figure 1 also shows 4 different neuropeptide synthaxins in complex, resolved on SDS-PAGE gel (bottom left lanes 1-4, boiled 1'-4').

Fig. 1. SNARE-linked botulinum neurotoxins used for the retargeting of Bont/A.

Results and Discussion

The reassembled toxins were tested on mouse neuroblastoma 2A (N2A), rat pheocytochroma (PC-12), human neuroblastoma (SH-5YSY) cells as well as rat hippocampus, cortex and dorsal root ganglion neurons at nM concentrations from 18 to 42h. The resulting cleavage bands, as revealed by western immunoblotting, were quantified a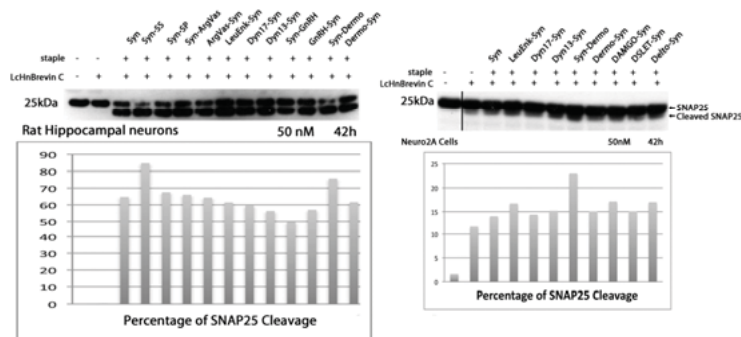s previously described [4]. Figure 2 shows a typical example of a SNAP25 cleavage assay. The same concentration of LcHn-BrevinC can have variable efficacy once pre-complexed with various neuropeptide targeting domains. As a noted example, we can see that the LcHn/Dermorphin complex has higher efficacy on N2A cells as well as on hippocampus neurons compared to the use of other Syntaxins, while LcHn-SS has a higher

Fig. 2. SNAP25 cleavage assay on rat hippocampal neurons (left) and N2A cells (right) with corresponding densitometric analysis.

efficacy on Hippocampus neurons. These results could reflect the cell surface expression of opioid and somatostatin receptors respectively, as well as the permissive structure activity of each neuropeptide in complex.

To this end, further structural refinement of the peptides implicated in these complexes could capitulate a higher potency and more selective medicinal enzyme. SNARE peptide stapling facilitates these structural refinements. Modifications in the SNARE motifs could enhance the versatility of the staple peptide, while modifications of the new targeting domains and its cross-linkers could enhance the structure activity (i.e. binding affinities, internalization activity). The SNARE based protein stapling is thus a versatile avenue for the efficient and high throughput subunit rearrangement of diverse protein that can exploit the advantages of both direct synthesis and recombinant expression to yield interesting *de novo* protein assemblies.

Assembled LcTd-SNARE complexes have also demonstrated to be important cell penetrating moieties (as well as BoNT/A fragments individually; data not shown), this ability can be advantageous to enhance the cell penetration of chimeric proteins of diverse interest that have been assembled using SNARE protein stapling. Our results have also shown that the internalized BoNT/A-SNARE complexes (with fluorescent staple) co-localize with acidic organelles inside cells (data not shown); a necessary step in the intracellular activation of the light chain. Current endeavors are also directed towards minimizing the cell penetrating properties of SNARE peptides to lead to exclusive endocytosis via GPCR internalization and minimizing any potential off target side effects.

Acknowledgments

References

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