

Mouse Hind Limb Assay

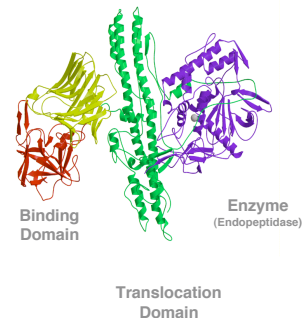
Session 4B

**ICCVAM/NICEATM/ECVAM Scientific Workshop on
Alternative Methods to Refine, Reduce, and Replace the
Mouse LD50 Assay For Botulinum Toxin Testing**

14 November 2006

The 3 R's

- *Reduce*
 - Number of animals used in assays
- ***Refine***
 - **Non-lethal in vivo potency assay assessments**
- *Replace*
 - In vitro assays



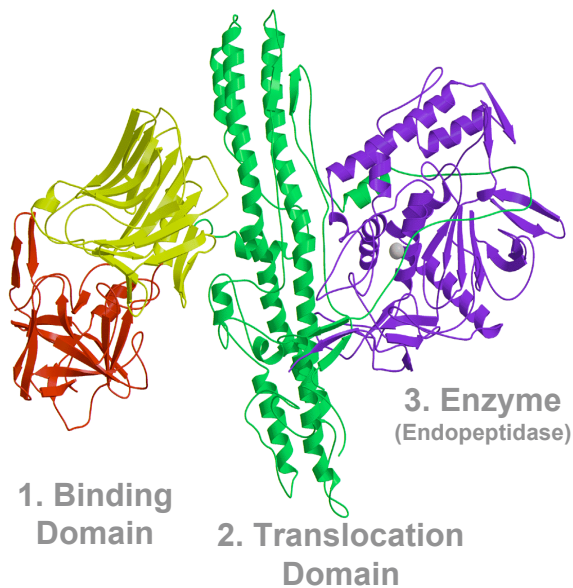
Challenges With Measurement of Formulated Product Potency

- **Quantity of botulinum toxin (ng level)**
 - Assay limit of detection
 - Losses due to surface adsorption
- **Formulation excipients**
 - Interference with analytical methods
- **Complex multi-step mechanism of action**

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Assessment of All Portions of BoNT Necessary



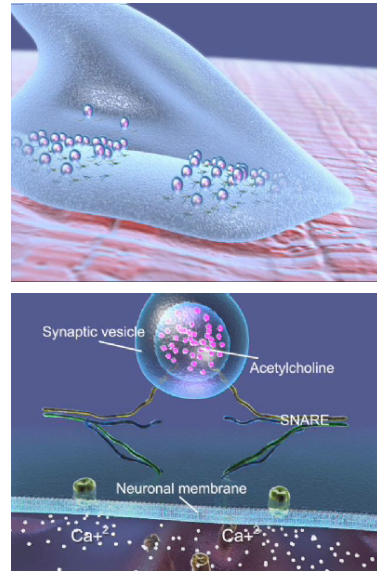
1. Binding domain allows protein to attach to nerve
 2. Translocation domain moves enzyme from one compartment to interior of nerve
 3. Enzyme continues to work inside nerve
- Potent and long duration of effect

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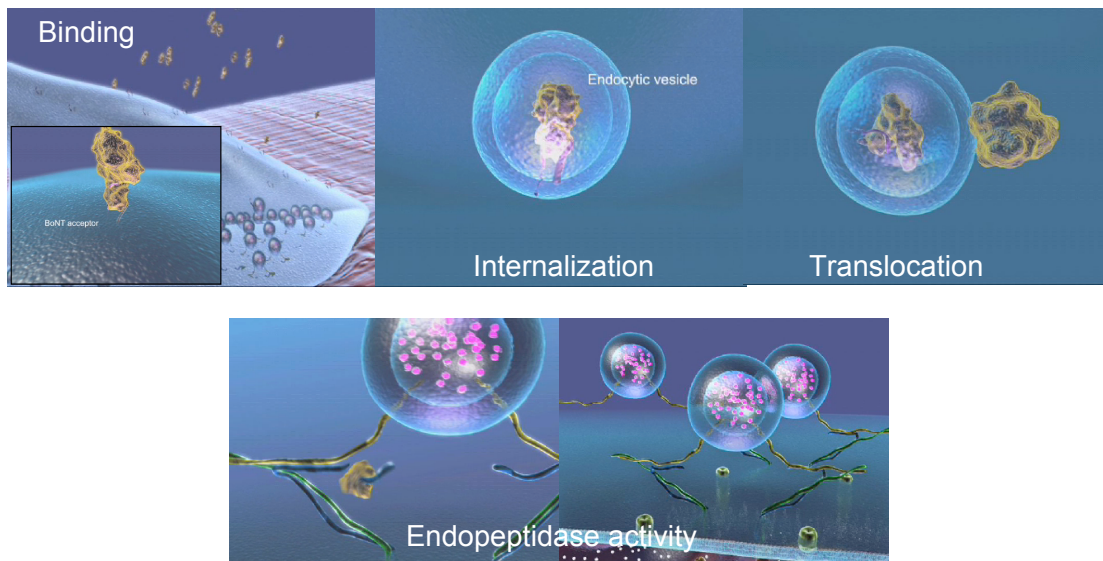
Motor Nerve Neurotransmitter Release

- **Calcium-dependent response to action potential**
- **Vesicle docking, fusion and release**
- **Mediated by specialized proteins**



Adapted from animations provided by www.neurotoxininstitute.org

Botulinum Toxin Type A Action In Motor Neuron



Adapted from animations provided by www.neurotoxininstitute.org

History of Non-lethal Animal Assays For Botulinum Toxin (BoNT)

- ***Regional limb paralysis***
 - Hind limb paralysis (Duchen, 1968; Sugiyama, 1975)
 - Median paralysis units (Pearce et al, 1995)
 - Tail Suspension Test & Activity Meter (Aoki et al, 1995)
 - Murine digit abduction score (Aoki, 2001; Munier et al, 2003)
 - Rat digit abduction span (Jurasinski et al, 2001; Rosales et al, 2006)
 - Voluntary Running (Keller, 2006)
- ***NIBSC abdominal ptosis (Sesardic et al, 1996)***

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IM Efficacy Evaluations

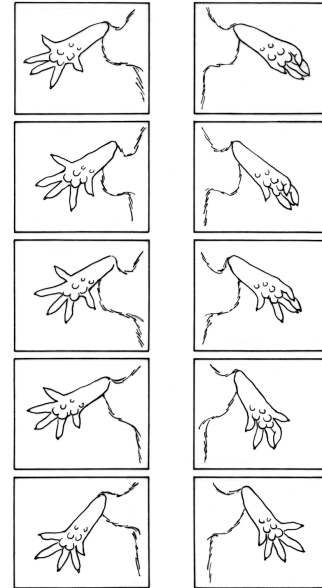
- **Digit Abduction Score (DAS) assay**

- IM local efficacy (ED50)

- Local muscle weakness effect
 - Observations over time

- Repeated studies

- N = 5 - 6 experiments
 - 5 - 7 doses
 - 10 mice/dose



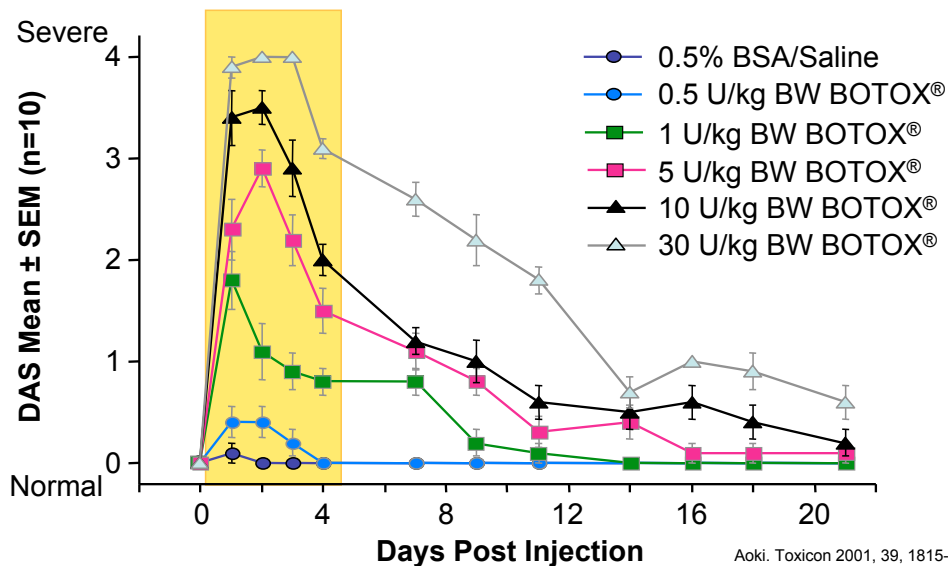
Non-injected Injected

Aoki. Toxicon 2001, 39, 1815-1820

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DAS Model of Muscle Weakening Efficacy in Mice

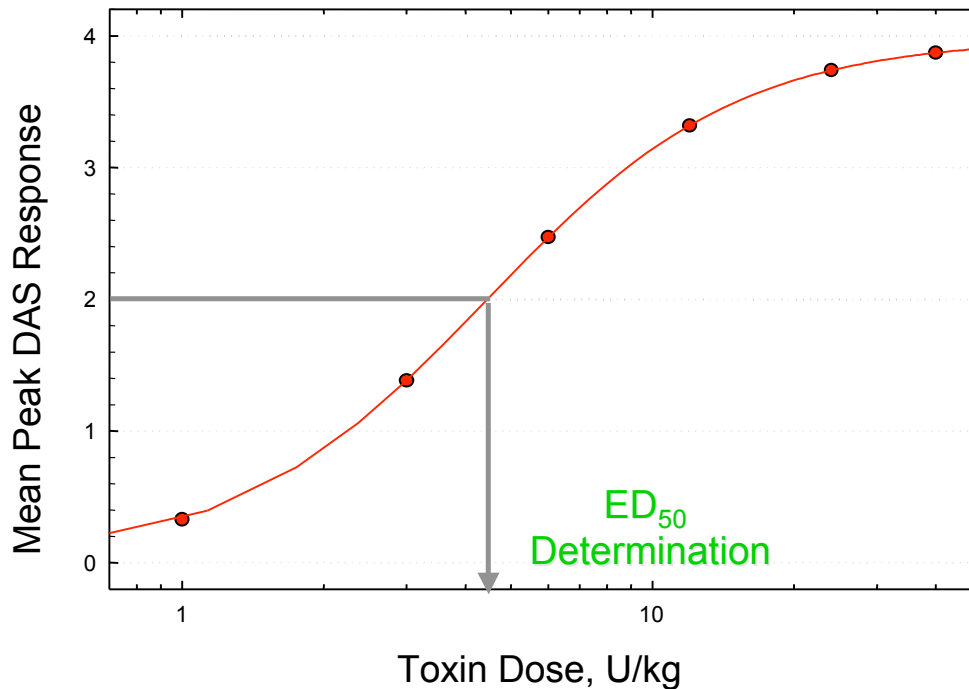


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DAS Dose Response Relationship Can Be Used For Relative Potency Comparisons



Comparison of BoNT Products

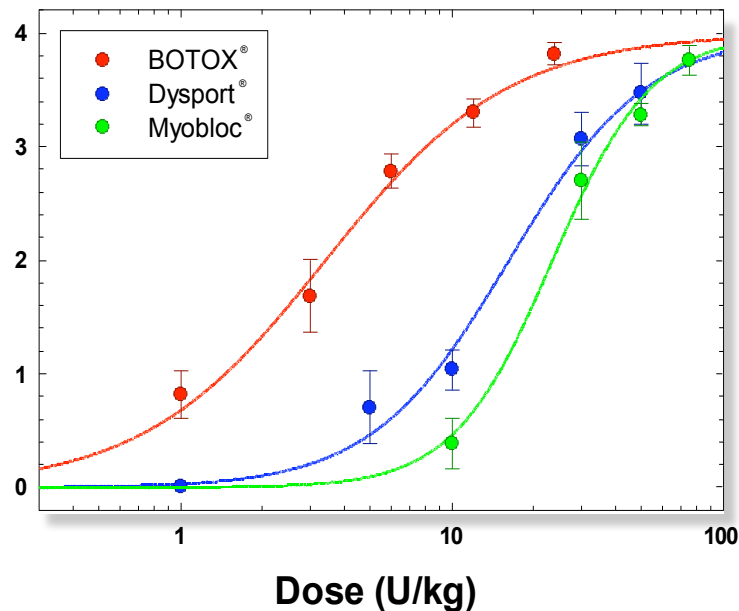
- ***Dose response determination***
 - BOTOX®, DYSPORT®, MYOBLOC®
- ***Effective local dose (ED50)***
- ***Systemic (IM-LD50) = Safety Margin***
- ***Ipsilateral atrophy (local migration)***

Comparison of BoNT Products

	BOTOX® 1989, US	Dysport® 1991, UK	Myobloc® 2000, US
Serotype	A	A	B
Complex Mr (kDa)	900	> 500	700 *
Package (units)	100	500	2,500 5,000 10,000
Neurotoxin Protein (ng/vial)	~5	12.5	25 50 100
Form	Vacuum-dried	Lyophilized	Solution
pH	~7	~7	5.6

* Elan data. Literature reports largest complex for B is 500 kDa and largest complex for A is 900 kDa

DAS Rank Order of Potency



Rank order of DAS potency: BOTOX® > Dysport® > Myobloc® (p < 0.05, one-way ANOVA and post hoc Tukey t-test).

Summary of Pharmacological Parameters for BOTOX[®], Dysport[®] and Myobloc[®]

Preparation	DAS ED ₅₀ U/kg (n)	im-LD ₅₀ U/kg (n)
BOTOX [®]	3.4 ± 0.3 (6)	69.2 ± 2.89 (4)
Dysport [®]	16.2 ± 1.1 (3)	168 ± 6.29 (7)
Myobloc [®]	23.4 ± 1.4 (5)	103 ± 2.55 (5)

Francis et al, EFNS, 2006

Strengths and Limitations of DAS assay

- **Strengths**
 - Clinical relevant measure of activity
 - local weakness
 - non-lethal endpoint
 - Applicable to other products
 - Dose response
 - Repeated measures
 - Local vs distal effects
 - Potential potency assay
 - Relative to a reference standard
- **Limitations**
 - Animals used (no reduction)
 - More training than LD50 assay required
 - Subjective score
 - Research assay
 - Requires optimization and validation for use as a potency release assay

**Inter-rater Reliability Comparison:
Abdominal Ptosis (NIBSC) vs Digit Abduction Score (Allergan)**

	<i>NIBSC (APt) – SC¹</i>	<i>AGN (DAS) – IM²</i>
Score	0, 1, 2, 3	0, 1, 2, 3, 4
Dose range (injection vol, ml)	0.05, 0.075?, 0.1, 0.15, 0.2 MLd50 (0.1 ml)	0, 0.4, 1, 5, 19, 30 U/kg (0.005 ml)
N per dose	8	10
Number of ratings (period)	1164 (@ 48 hours)	944 (over 4 days)
R	0.88 (1 vs 2) 0.91 (1 vs 3)	0.96
Identical scores	58%	83%
1 score diff		17%
2 score diff	< 4%	0.3%
Fold sensitivity vs LD50	~10 (vs ip-LD50)	14 (vs im-LD50)

1. Sesardic, et al Pharmacology & Toxicology 1996, 78:283-288
 2. Aoki. Toxicol 2001, 39, 1815-1820

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Hind Limb Assay (DAS) Summary

- ***Sensitive, reproducible, subjective score, non-lethal potency assay***
 - Requires more training than existing product potency assay
- ***Distinguishes between BoNT products***
 - Local vs systemic
- ***Requires validation and correlation with product release assay***