Botulinum toxin type A acts peripherally by inhibiting acetylcholine release from the presynaptic neuromuscular terminals, thus weakening muscle contraction, and its clinical benefit depends primarily on the toxin's peripheral action. However, a number of experimental studies in animals and in human beings have provided ample evidence supporting a central action of BT-A.

Botulinum toxin blocks the gamma motor endings of jaw muscles in the rat and produces parallel denervation of extrafusal and intrafusal fibers. Evidence of fusimotor denervation suggests that BT-A alters activity in muscle spindle afferents (Filippi et al., 1993, Rosales et al., 1996). One approach for studying muscle spindle la afferents in humans is to elicit the tonic vibration reflex (TVR). Trompetto et al., (unpublished observations) have tested the TVR before and after BT-A injection in patients with hand dystonia. The special sensitivity of the TVR to suppression by BT-A injection -found by the authors- could be mediated by the chemonervation of intrafusal muscle fibers, leading to a reduction in spindle inflow to the central nervous system during vibration. Studies of the reciprocal inhibition (Priori et al., 1995, Modugno et al., 1998) between agonist and antagonist muscles in patients with arm dystonia and in patients with essential tremor and studies of f wave in patients with focal dystonia (Wohlfarth et al., 2001) suggested that BT-A injected in the upper limb muscles can modify the excitability of the spinal cord. On the contrary in patients with cranial dystonia botulinum toxin treatment has little influence upon the excitability of brainstem interneurons (Valls-Solé et al., 1993, Giralda et al., 1996). Evidence of changes in cortical functional organisation after BT-A has been provided by studies with the long latency reflexes (Naumann et al., 1997), somatosensory evoked potentials (Kanovsky et al., 1998), magnetic stimulation of cortical motor areas (Byrnes et al., 1998, Gilio et al., 2000) and positron emission tomography (PET) (Ceballos-Baumann et al., 1997). These studies demonstrated that the injection of BT-A in the upper limbs transiently modify the topography and the excitability of the sensory-motor cortical areas.

In conclusion BT-A has complex mechanisms of action. In addition to acting directly at the neuromuscular junction, the toxin alters sensory inputs to the central nervous system, thus indirectly inducing secondary central changes (Currà et al., 2004). It is possible that some of the long-term clinical benefits of BT-A treatment may also reflect plastic changes in motor output after the reorganization of synaptic density. AB is a Consultant/Lecturer for Allergan Inc.

#S02 Characterization of the Protein Receptor Binding Site of Botulinum Neurotoxins B and G. T. BINZ1, A. RUMMEL2, T. EICHNER2, T. KARNATH1, S. MAHRHOLD1, A. GUTÇAITIS1, T. WEIL3, H. BIGALKE1. Depts. 1Biochemistry and 2Toxicology, Hannover Medical Univ., Hannover; 3Merz Pharmaceuticals GmbH, Frankfurt/M, Germany.

Synaptotagmins I and II are integral synaptic vesicle proteins that have been suggested to act as protein receptors for BoNT/B and BoNT/G. The luminal segment of both isoforms that becomes accessible for BoNTs in the synaptic cleft upon neurotransmitter release has been shown to interact with the ??-trefoil (HCC)-domain of the toxins. In order to identify the synaptotagmin binding site within the HCC-domain we conducted a computer based search. Potential interaction sites were subjected to site directed mutagenesis. Various mutated BoNTs were tested for their capability to interact with synaptotagmin by GST-pull-down assays. Effects on neurotoxicity were measured at mouse hemi-diaphragm preparations. The results obtained suggest that the protein receptor interaction site lies adjacent to the established ganglioside binding pocket thus promoting rapid access to the temporarily surface exposed protein receptor. Our molecular characterization of the toxin-receptor interaction may also serve as a basis for the design of efficient binding inhibitors that simultaneously block the access to glycolipid and protein receptor. Support: German Research Council (BI 660/2-1) and Human Frontier Science Program (RGY0027/2001B).

#S03 Endocytosis and Membrane Dynamics in Motor Neurons. KATRIN DEINHARDT, STEPHANIE BOHNERT, CAROLE VERASTEGUI, OTTO BERNINGHAUSEN1, GIAMPIETRO SCHIAVO. Molecular Neuropathobiology Laboratory, Cancer Research UK London Research Institute, UK. 1Department of Biological Sciences, Imperial College, London, UK.

Axonal retrograde transport is responsible for the central delivery of endogenous ligands and signalling molecules, and constitutes the gateway for the entry in the central nervous system for pathogens and virulence factors. Among these, tetanus neurotoxin (TeNT) has been recently used as a paradigm to clarify both the molecular pathogenesis of tetanus and the machinery controlling membrane dynamics and axonal transport in motor neurons (MN). TeNT binds specifically to MN presynaptic nerve terminals, where it is internalized and retrogradely transported along the axon to the cell body. Previous studies showed that the TeNT receptor complex, which is comprised of polysialogangliosides and GPI-anchored proteins, resides in lipid microdomains. We now provide experimental evidence that the internalization machinery for TeNT is clathrin-dependent. Our findings suggest that this novel internalization and sorting route is an example of lipid-raft as well as clathrin-dependent endocytosis, two pathways that have until very recently been viewed as mutually exclusive. The mixed population of TeNT transport carriers all display neutral pH, suggesting that TeNT does not enter a classical endosomal pathway that leads to degradation. However, a subpopulation of these carriers is positive for the small GTPase Rab7. Rab7 is a regulator of vesicular traffic, which is involved in controlling transport to and from late endosomes. These results suggest that for TeNT trafficking, components of the classical endocytic pathway are used in a novel context. They provide insight on the coordination of this highly specialized neuronal route with other regulated endocytic processes, such as synaptic vesicle recycling, and constitutive membrane turnover in primary MNs under normal and pathological conditions. This study is funded by Cancer Research UK.
Characterization of Clostridial botulinum Neurotoxin Channels in Neuroblastoma Cells. AUDREY FISCHER, MAURICIO MONTAL. Section of Neurobiology, Division of Biological Sciences, University of California San Diego, La Jolla, CA 92039-0366.

The channel and chaperone activities of Clostridial botulinum neurotoxin (BoNT) A were investigated in neuroblastoma cells under conditions that closely emulate those prevalent at the endosome. Channel activity occurs in bursts interspersed between periods of little or no activity. The channels are voltage dependent, opening only at negative voltages to a main conductance of ~90 pS and display a conspicuous subconductance of ~10 pS. Within bursts, the channel resides preferentially in the open state. A salient feature of the BoNT channel is that it is closed at positive voltages under conditions in which the orientation and the magnitude of the pH gradient, as well as the polarity and magnitude of the membrane potential compare fairly well with those prevailing across the endosomal membrane: pH 5.3 and positive potential on the compartment containing the BoNT and pH 7.0 and negative potential on the opposite compartment. This suggests that the BoNT heavy chain channel would be closed in the endosome until it is gated by the BoNT light chain to initiate its translocation across the membrane into the cytosol. This work was supported by the U.S. Army Medical Research and Materiel Command under Contract/Grant/Intergovernmental Project Order DAMD17-02-C-0106.

Update on Botulinum Toxin Treatment of Focal Dystonias (Excluding Cervical Dystonia). MARIE-HELENE MARION. Department of Neurology, St George's Hospital and Medical school, London SW17 0QT, UK.

After 15 years of development of the use of Botulinum toxin A (BTX-A) treatment in various focal dystonias, recent studies have focused on: (1) systematic reviews to determine the efficacy and safety of the treatment (Costa et al., Cochrane-Database-Syst-Rev, 2005); (2) assessment of its long term effect; (3) impact on the quality of life, and (4) determining the equivalence of dosage between the 2 formulations of the BTX-A (Botox® and Dysport®).

Many neurologists are still reluctant to treat task-specific dystonias. Recent reviews on long term follow up of writer's cramps(Marion et al., Rev Neurol 159:10,923-927, 2003) and musician's cramps (Schuel et al., Neurology 64:341-343, 2005) treated with BTX should give more confidence to neurologists to use it more widely in these indications. It may be important, though, to accept that BTX treatment has its limits. The challenge in the future will be to recognise this, in order, for example, to select patients for neurosurgery such as deep brain stimulation.

An Update on Other Hyperkinasias. ANDRES CEBALLOS-BAUMANN.

Differential Movements of Synaptic Vesicles Belonging to Different Vesicle Pools. MICHAEL GAFFIELD, SILVIO RIZZOLI1, WILLIAM J. BETZ. Department of Physiology & Biophysics, University of Colorado Medical School, Denver, Colorado, USA. 1Current address: Max-Planck-Institut fur biophysikalische Chemie, Goettingen, Germany.

The defining feature of a chemical synapse is the collection of synaptic vesicles in the presynaptic terminal. These vesicles participate in a cycle that permits them to be used repeatedly during sustained activity. While vesicles appear to be homogeneous, both ultrastructurally and biochemically, functional studies suggest the existence of different vesicle 'pools.' For example, the 'recycling pool' is thought to comprise those vesicles that undergo exocytosis first during repetitive stimulation, before vesicles in the 'reserve pool' are mobilized. Are the pools segregated morphologically? It is natural to predict that vesicles in the recycling pool are located close to the presynaptic membrane, and reserve pool vesicles farther away. We tested this hypothesis and found that in frog motor nerve terminals recycling pool vesicles are not clustered near the sites of exocytosis, but instead are scattered, almost randomly, throughout the vesicle cluster.
Next, we measured the motions of vesicles in the two pools (using the technique Fluorescence Recovery After Photobleaching (FRAP) in nerve terminals stained with a fluorescent dye, FM1-43). We found that, in resting nerve terminals, vesicles in the recycling pool are mobile, while those in the reserve pool are not. Reserve pool vesicles can be mobilized by certain drugs, like forskolin, which activate the cyclic-AMP pathway. The movements of vesicles appear to be from diffusion, and are not significantly perturbed by agents that interfere with the cytoskeleton. Funding for this study was provided by the National Institutes of Health and the Muscular Dystrophy Association.

#S09 Clostridial Neurotoxins: Receptors, Modes of Entry, and Detection. EDWIN R. CHAPMAN. Dept. Physiology, University of Wisconsin, Madison, WI 53704.

Botulinum neurotoxins (BoNTs) cause botulism by entering neurons and cleaving proteins that mediate neurotransmitter release; disruption of exocytosis results in paralysis and death. The receptors for BoNTs are thought to be composed of both proteins and gangliosides. Our efforts are directed toward 1) working out the precise pathways of entry for each of the toxins 2) identifying the protein components of the toxin receptor complexes 3) devising new ways to assay for toxin activity and entry into cells and 4) reconstituting toxin translocation across lipid bilayers. We will present data which address the identity of toxin receptors and the modes of entry into cells; we will also describe a FRET-based assay that can be used to monitor toxin activity and which might prove useful for high throughput screening of toxin inhibitors. These studies are supported by grants from the NIH and AHA.

#S10 Penetration and Translocation of Fluorescent Botulinum Toxins in Cultured Hippocampal Neurons. CLAUDIA VERDERIO, GIAMBATTISTA BONANNO, CESARE MONTECUCCO, MICHELA MATTEOLI. Dept. of Medical Pharmacology and CNR Institute of Neuroscience, Center of Excellence for Neurodegenerative Diseases, University of Milano; Sper., Univ. of Padova; Dip. di Scienze Biomed. Sper., Dip. di Scienze Biomed. Sper., Univ. of Padova.

We have shown recently that synapses of hippocampal interneurons both in culture and in situ do not express detectable levels of the SNARE protein SNAP-25. The sensitivity of excitatory and inhibitory neurons to botulinum neurotoxins type A or type E (BoNT/A and BoNT/E), which proteolyze SNAP-25, was investigated in hippocampal neurons in culture. Synaptic vesicle recycling was specifically blocked at glutamatergic but not GABAergic synapses upon 2 hour intoxication with 10-375 nM BoNT/A, whilst selectivity for glutamatergic synapses was lost after prolonged (16 hours) exposure to 100nM BoNT/A. A preferential effect at glutamatergic synapses was also observed with BoNT/E but in a narrow concentration range. The different effectiveness of BoNT/A or /E at inhibitory terminals did not result from unequal penetration or different lifetime of the toxins in interneurons, which were assessed with fluorescent BoNT derivatives. Conversely, exogenous expression of SNAP-25 within inhibitory neurons provided sensitivity to BoNT/A. Taken together, these results indicate that BoNT/A or /E inhibit preferentially the release of excitatory versus inhibitory neurotransmitters and indicate that the level of SNAP-25 expression may account for the different effects of these neurotoxins at the two types of terminals. (Supported by Telethon Italia).

#S11 High-Throughput Yeast-Cell Assays for BoNT Proteases. NEIL GREEN, WENTIAN Luo, HONG FANG. Department of Microbiology and Immunology, Vanderbilt University Medical School, Nashville, TN, 37232.

The seven botulinum neurotoxin light chains (BoNT/LC) (serotypes A-G) are highly specific endopeptidases, which cleave SNARE proteins that are essential for neurotransmitter release from presynaptic membranes. High-throughput cell based assays could provide a system with which to rapidly identify intracellular inhibitors of the seven LC proteases. To this end, we have developed a variety of yeast-based assays for the LCs of serotypes B, D, and G. These assays permit a genetic selection for inhibition of protease activity, and we have developed assays to genetically select for restoration of protease activity. These assays are now being used to screen for LC protease inhibitors. We have also used our assays to probe protease/substrate specificity by genetic selection inside cells. Additional yeast-based assays are under development to monitor protease activities of the remaining BoNT/LC serotypes. This study is supported by NIH Grant 5R01AI58011-02 to N.G. and NIH Grant 1R21AI062812 to H.F.

#S12 Trojan horse or Proton Force: Finding the Right Partner(s) for Toxin Translocation. JOHN R. MURPHY. Section of Molecular Medicine, Department of Medicine, Boston University School of Medicine, Boston, MA 02118.

The botulinum neurotoxins, anthrax toxins, and diphtheria toxin are all known to require passage through an acidic compartment in order to deliver their respective catalytic (C) domains to the cytosol. While much is known about their mode of action and structure function relationships, little is known of the mechanism(s) by which their C-domains are translocated across a vesicular membrane and delivered to the cytosol. We have used in vitro translocation of the C3 domain of the fusion protein toxin DAB3891L-2 from purified early endosomes as a model system to examine this process. Using translocation of ADP-ribosyltransferase as an assay, a cytosolic translocation factor (CTF) complex has been purified 650-800-fold from human T-cell and yeast extracts. The heat shock protein (Hsp) 90, its yeast ortholog Hsp 82, thioredoxin reductase, and β-COP have been identified by mass spectrometry sequencing and shown to play an essential role in the delivery of the C-domain from the endosomal lumen to the external milieu. Since C-domain translocation and release from early endosomes requires a CTF-complex, we reasoned that a toxin specific motif involved in protein-protein interaction(s) was likely to be involved in the delivery process. A 10 amino acid motif in transmembrane helix 1 of diphtheria toxin that is conserved in anthrax edema and lethal factors, and botulinum neurotoxin serotypes A, C, and D was identified by mass spectrometry sequencing and shown to provide a system with which to rapidly identify intracellular inhibitors of the seven LC proteases. To this end, we have developed a variety of yeast-based assays for the LCs of serotypes B, D, and G. These assays permit a genetic selection for inhibition of protease activity, and we have developed assays to genetically select for restoration of protease activity. These assays are now being used to screen for LC protease inhibitors. We have also used our assays to probe protease/substrate specificity by genetic selection inside cells. Additional yeast-based assays are under development to monitor protease activities of the remaining BoNT/LC serotypes. This study is supported by NIH Grant 5R01AI58011-02 to N.G. and NIH Grant 1R21AI062812 to H.F.
S13 Spasticity in Children with Cerebral Palsy. H. KERR GRAHAM. Hugh Williamson Gait Laboratory and Orthopaedic Department, The Royal Children's Hospital, Melbourne, Australia.

Cerebral palsy encompasses a wide range of clinical phenotypes which are best classified according to the movement disorder, topographical distribution and gross motor function (GMFCS). Spasticity and spastic-dystonia are the most common movement disorders in children with cerebral palsy and contribute to a wide range of functional impairments and secondary musculoskeletal deformities. Maintenance of muscle length, avoidance of progression from dynamic to fixed deformity and maximising functional potential are recognized goals in the management of younger children with cerebral palsy. This can best be accomplished in the context of an integrated spasticity management program which includes the use of focal, regional and generalized interventions for spasticity and muscular hypertonia including such interventions as intramuscular injection of Botulinum neurotoxin A and phenol neurolysis, selective dorsal rhizotomy (SDR) and intrathecal Baclofen (ITB). At The Royal Children's Hospital, children with cerebral palsy are identified and entered in a statewide register and followed by a multidisciplinary team. All of the above spasticity management options are offered, integrated with a program of corrective orthopaedic surgery and strengthening. In this presentation, the Spasticity Compass will be described as a guide to appropriate spasticity management in children with cerebral palsy as well as the integrated management algorithm which illustrates the role of spasticity management, musculoskeletal surgery and strengthening. Funding for this study was provided by the National Research Council (NH & MRC) Clinical Centre of Research Excellence (CCRE) Grant. Dr. Graham has grant support from Allergan.

S14 The Skills to Apply Botulinumtoxin - Palpation - EMG - Muscle Stimulation - Sonography. F. HEINEN, S. BERWECK, A.S.SCHROEDER, S.H. LEE. Department of Pediatric Neurology and Developmental Medicine, Children's University Hospital Munich, Germany.

Targeting muscles is relevant in the treatment of children with CP (Berweck et al., Lancet Vol 363, No.9494:249-250) The variety of technical support will be demonstrated. In CP a multilevel approach is needed. This can be achieved with sonography guidance and the 'BOTOX-12Plus-Concept' (more than 12 U/kg b.w. BOTOX) without affecting safety or incidence of secondary non-response due to neutralising antibodies. Patients and Methods: Analysis of 141 patients who received 349 treatments with 'new' BOTOX (83 treatments with <12 U/kg b.w. vs. 266 treatments with >12 U/kg b.w. Results: Mean number of treated muscles per treatment session: 12Plus:>6 muscles. No severe side effects were observed. During a three-years-period 3 patients tested positive for secondary non-responders due to neutralising antibodies(1.3%). Conclusion: Multi-level treatment involving more muscles can be realised with sonography and the "BOTOX-12Plus-Concept". Sonography is shown as a tool for easy, quick, painless and anatomically precise, visually controlled injection of botulinum toxin. Support: Educational grants from Allergan, Elan, Ipsen, and Merz. FH is a Consultant/Lecturer for Allergan, Ipsen, Solstice and Merz.

S15 EMG Guidance and Electrical Stimulation for Injection of Botulinum Toxin. NATHANIEL H. MAYER1, ALBERTO ESQUENAZI2. Department of Physical Medicine and Rehabilitation, Temple University1 and MossRehab1,2, Philadelphia, PA 19026.

When treating muscle overactivity in the upper motoneuron syndrome (UMNS), a number of techniques for identifying injection sites for botulinum toxin have been reported. Among these techniques, electromyographic (EMG) guidance and electrical stimulation (e-stim) guidance are probably the commonest. Children indicated that the importance of EMG or electrical stimulation guidance to treat dystonia or spastic muscles was based more on theoretical or preclinical data than on controlled clinical trials (Childers, Phys Med Rehabil Clin N Am 14:781, 2003). Some authors have opined that needle EMG guidance is rarely required when injecting botulinum toxin (Jankovic, Muscle Nerve. 24(11):1568, 2001). Others have found relatively low rates of accuracy without EMG guidance in focal hand dystonia (Molloy et al., Neurology. 58(5): 805, 2002) or e-stim in juvenile cerebral palsy (Chin et al., J Pediatr Orthop. 25(3):286, 2005). This paper will review current evidence for methodological considerations regarding types of guidance in the clinical delivery of botulinum toxin with special emphasis on muscle identification in UMNS. Video demonstration of EMG and e-stim techniques will be incorporated into this paper for purposes of discussion. Preliminary results of a clinical study examining motor point localization versus distributed technique will also be reviewed. This study was supported by a NIDRR Model TBI Systems of Care Grant to the Moss Rehabilitation Research Inst.

S16 Spasticity Associated with Multiple Sclerosis. MIKE BARNES. Hunters Moor Regional Neurological Rehabilitation Centre, Newcastle upon Tyne, UK.

In many people spasticity is the major disabling symptom in multiple sclerosis. There are very few epidemiological studies on the prevalence of troublesome spasticity in MS, but a review of the available literature indicates that, at some point in the course of the disease, around 60% of people with MS will need treatment for their spasticity. Other work has demonstrated that there is a high unmet need in this population and a considerable amount of inappropriate prescribing. Adductor spasticity is a particularly disabling problem. Muscle spasms are also a major problem for this population, often painful and often disturbing sleep. The management of spasticity remains multidisciplinary and will usually involve a range of different treatments and management strategies. Botulinum toxin has a key role to play in the overall spasticity management programme. I will summarise the available data that confirms that botulinum toxin is a useful adjunctive therapy for the management of spasticity in MS. The evidence does demonstrate that botulinum toxin has a major role to play in the management of a difficult symptom in a disabling condition.

S17 Patient Selection and Functional Measures in Post-Stroke Spasticity. ALLISON BRASHEAR. Department of Neurology, Indiana University School of Medicine, Indianapolis, IN USA.

Botulinum toxin treatment is a unique treatment for focal post-stroke spasticity which allows focal and incremental doses to the spastic limb. Patient selection and reproducible outcome measures are essential to demonstrating the clinical benefit of treatment from injections with botulinum toxin in clinical trials and in managing patients in the clinical setting. Patient selection should include those with increased tone that interferes with activities of daily living important to the patient and/or caregiver. Tone alone cannot be a determinant of the need for botulinum toxin treatment. In some circumstances, increased tone may allow improved function of the spastic limb. In addition, those who expect improvement in the pre-stroke state will be disappointed. Determining which patients to treat, assessing and measuring functional outcomes will be needed to document benefit of treatment. Managing patient and caregiver expectations will also improve the benefits perceived with botulinum toxin treatments. Functional measures developed for stroke outcome studies are not sensitive or specific enough to assess change after a focal treatment like botulinum toxin. Attention to hand hygiene, limb position, use of the limb in activi-
ties of daily living, such as dressing and eating, and assessment of pain are targets for functional improvement in patients with post-stroke spasticity of the upper limb treated with botulinum toxin. The Disability Assessment Scale (DAS) is the first measurement tool to be developed specifically for assessing change after botulinum toxin treatment in the upper limb. To date no such scale exists for the lower extremity. While the DAS is a self-report tool, it is a first step in quantifying change in function after treatment. Measurement of change in function when tone is decreased with botulinum toxin treatments will provide the best indicator of success of treatment with botulinum toxin. How to quantify improvement in a reproducible fashion in hygiene, pain, dressing and limb position and other important activities of daily living remains to be determined. AB is a Consultant/Lecturer for Allergan, Solstice Neurosciences, and Merz; and has grant support from Allergan, Solstice Neurosciences and Ipsen.

S18 Sprouting at the Neuromuscular Junction and Factors Influencing the Duration of Botulinum Toxin Action. JORDI MOLGÓ. Laboratoire de Neurobiologie Cellulaire et Moléculaire, Centre National de la Recherche Scientifique, Gif sur Yvette 91198 cedex, France.

The local injection of botulinul neurotoxins (BoNTs) into adult skeletal muscles of animals and humans blocks quantal acetylcholine release, resulting in muscle paralysis. Although a marked atrophy of skeletal muscle occurs during paralysis, there is no evident damage of motor nerve terminals. The duration of neuromuscular blockade depends upon: i) BoNT serotype; ii) dose used; and iii) animal species. Higher doses are needed to paralyze amphibian, versus mammalian, skeletal muscles. According to their duration of action, BoNTs can be classified into three groups: long-lasting (BoNT/A and BoNT/C), intermediate (BoNT/B and BoNT/F), and short-lasting (BoNT/E). In mammals, neuromuscular blockade is not permanent, as functional recovery returns within a few weeks to months. It is still unclear why the recovery-time in rodents is shorter than in humans after BoNTs exposure. In mammals muscle inactivity following a single injection of BoNT/A, /C, /D or /F elicits, within 3-5 days, a nerve outgrowth or sprouting along intramuscular axons at the nodes of Ranvier (nodal sprouting), and at motor nerve terminals (terminal sprouting) of the neuromuscular junction. The Progression of events leading to the first nerve-muscle contacts, and differentiation of new endplates after BoNTs exposure is a remarkable demonstration of synaptic plasticity. Throughout this period, important communications occur between the synaptic partners involving diverse signaling molecules, and synaptic proteins. Supported in part by grants (#026065093, #0334046004) from the Direction des Systèmes de Forces et de la Prospective.

S20 Is Light Chain Subcellular Localization a Factor Influencing Botulinum Toxin Duration of Action? ESTER FERNÁNDEZ-SALAS, LANCE E. STEWARD, PATTON E. GARAY, SHIAZAH MALIK, RAMILLA O. LEWIS, JOANNE WANG, HELEN HO, SARAH W. SUN, MARCELLA A. GILMORE, JOSEPH V. ORDAS, JOSEPH FRANCIS, AND K. ROGER AOKI. Biological Sciences Dept., Allergan, Irvine, CA 92612.

Botulinum neurotoxin type A (BoNT/A) is used in the treatment of neuromuscular and autonomic disorders and pain, with a therapeutic effect lasting from 3 to 12 months. Studies in animal and cell culture models suggest that this long inhibition of exocytosis is due to the persistence of the light chain (LC/A). We have reported that LC/A localizes to the plasma membrane while LC/E, with the shortest duration of effect, resides in the cytoplasm. At the plasma membrane LC/A co-localizes and interacts with SNAP25, and resides in close proximity to the SNARE complex. Cell fractionation has confirmed the presence of LC/A in the membrane fraction and the presence of LC/E in the cytoplasmic fraction. Deletions and mutations of sequences at the N- and C-terminus of LC/A produced changes in localization and activity. In conclusion, we have identified localization signals in LC/A, and are conducting studies to establish a link between LC subcellular localization and duration of effect in cells.

EF-S is an employee of Allergan.

S19 Do Newly Formed Synaptic Contacts Account for Functional Recovery from Botulinum Toxin Type A? CLARKE R. SLATER, ALEXANDER A. ROGOZHIN, K.K. PANG. School of Neurology, Neurobiology & Psychiatry, University of Newcastle, Newcastle upon Tyne, NE2 4HH, UK.

During recovery from botulinum toxin type A (BoTxA), motor axon sprouts grow out from the neuromuscular junction (NMJ) and make new synaptic contacts with the muscle. While it is generally considered that evoked quantal release at these new sites accounts for the initial functional recovery from BoTxA, this has never been directly tested. We used focal extracellular recording of nerve-evoked endplate currents (EPCs) from mouse epitrochleoonconeous muscles to assess quantal release, from both new contacts and the original neuromuscular junction, after a single injection of BoTxA. Quantal release first appeared about 2 weeks after BoTxA at both new contacts and original NMJs and was always greater at the original NMJs. The total area of new synaptic contacts, determined on single muscle fibers from alpha-bungarotoxin labelling of AChRs, was less than 25% of that of the original NMJs. We conclude that in our material, the sprouts are not the major source of evoked quantal release during the initial recovery from BoTxA. Funding by The Wellcome Trust. BoTxA (Botox) was kindly donated by Allergan.
S21 BoNT for Gastrointestinal Disorders: Therapy and Mechanisms. P. JAY PASRICHA. University of Texas Medical Branch, Galveston, TX.

Botulinum toxin (BTX) is one of the most potent inhibitors of acetylcholine from nerve endings and this accounts for its toxic properties as well as its therapeutic application in a variety of neuromuscular syndromes. This talk focuses on the growing use of BTX in the so-called "spastic" disorders of the gastrointestinal tract. These include achalasia, where the short-term efficacy of intrasphincteric injection of BTX has been well established now. However, because of the chronicity of this condition, repeated injections of the toxin may be required at regular intervals. By contrast, the relatively short duration of action may be an advantage in disorders such as chronic anal fissure, where the benefit of this therapy has now been demonstrated in hundreds of patients. There are many other sphincteric and non-sphincteric syndromes in the gut where the efficacy of this agent is being actively tested. These include non-cardiac chest pain, gastroparesis and sphincter of Oddi dysfunction. Skeletal muscle sphincters such as the upper esophageal sphincter or the external anal sphincter/suburethral muscle may also be targeted with good effect. In some of these conditions, local injection of BTX may serve as a useful therapeutic trial, facilitating the decision to institute more invasive forms of therapy. The cumulative short-term experience with BTX in the gut to date suggests that it is a relatively simple and safe therapy. The use of BTX represents a novel approach for gastrointestinal motility disorders and the rapidly expanding list of successful applications holds promise for more widespread use of similar agents in the future. Additional studies on long term outcome and safety are eagerly awaited.

In addition to its therapeutic importance, the study of BoNT in the GI tract has interesting and important implications for understanding the full spectrum of the biological effects of this toxin. Thus, recent work from our lab demonstrates novel sites and/or mechanisms of action that have previously not been described in the skeletal muscle system. BoNT A injections result in blockade of excitatory neuromuscular transmission in the pyloric sphincter as well as a reduced response to exogenous Ach, SubP and KCl suggesting that it might directly affect smooth muscle. Further, "gene chip" studies demonstrate a striking local plasticity in the expression of several important genes that may be of importance in the regenerative response. Funded by grants from the U.S. Food and Drug Administration and Allergan.

S22 Use of BoNT for Hyperhidrosis and Gustatory Sweating. DEE ANNA GLASER. Department of Dermatology, Saint Louis University School of Medicine, St. Louis, MO 63104

Hyperhidrosis manifests as excessive sweating, beyond the physiologic needs to maintain normal thermoregulation. Generalized sweating is usually secondary in nature whereas localized forms may be idiopathic or secondary. The differential diagnosis is extensive and requires appropriate evaluation. Approximately 2.8% of the population has hyperhidrosis (Strutton DR et al., J Am Acad Dermatol2(2)274-86,2004) with the most common sites being the axilla, palms, soles, face and groin. Numerous studies have demonstrated the effectiveness and safety of BoNT for the treatment of hyperhidrosis. A recent North American multi-center double-blind placebo-controlled study of 2 doses of BoNT-A in 322 patients with primary axillary hyperhidrosis confirmed the efficacy of BoNT-A with 75% of treated patients achieving a ≥2 point reduction in the hyperhidrosis disease severity scale. A greater than 75% reduction in sweat production was achieved in >80% of treated subjects and there was significant improvement in quality of life measurements.

There were no significant differences between the 2 doses studied and the mean duration of benefit was 7 months.(in press). Gustatory sweating (GS) is a common complication of surgery or injury in the region of the parotid gland, stemming from aberrant regeneration of secretomotor parasympathetic neurons. GS responds very well to low doses of BoNT-A with treatment responses lasting as long as 3 years (Laccourreye et al., Ann Otol Rhinol Laryngol;107:52-5,1998). BoNT-B can be used to treat hyperhidrosis but has been associated with a high incidence of side effects including dry mouth and indigestion (Nelson L. et al., Br. J. Plast. Surg. 58:228-32,2005; Baumann L. et al., Dermatol. Surg. 31:263-70, 2005). This study was funded by Allergan, Inc.

S23 Management of Bladder, Prostatic and Pelvic Floor Disorders with Botulinum Neurotoxin. A. ALBANESE1, G. BRISINDA2, 1National Neurological Institute, Catholic University of Milan, Milan; 2Department of Surgery, Catholic University of Rome, Italy.

Since its introduction in the late 1970s for the treatment of strabismus and blepharospasm, botulinum toxin (BoNT) has been increasingly used in the interventional treatment of several other disorders characterized by excessive or inappropriate muscle contractions. The use of this pluripotential agent has extended to a plethora of conditions including: focal dystonia; spasticity; inappropriate contraction in most sphincters of the body such as those associated with spasmodic dysphonia, esophageal achalasia, chronic anal fissure, and vaginismus; eye movement disorders; other hyperkinetic disorders including tics and tremors; autonomic disorders such as hyperhidrosis; genitourinary disorders such as overactive and neurogenic bladder, nonbacterial prostatitis and benign prostatic hyperplasia; and aesthetically undesirable hyperfunctional facial lines. In addition, BoNT is being investigated for the control of pain, and for the management of tension or migraine headaches and myofacial pain syndrome.

BoNT injections have several advantages over drugs and surgical therapies in the management of intractable or chronic disease. Systemic pharmacologic effects are rare; permanent destruction of tissue does not occur. Graded degrees of relaxation may be achieved by varying the dose injected; most adverse effects are transient. Finally, patient acceptance is high. In this paper, clinical experience over the last years with BoNT in urologically impaired patients will be illustrated. Moreover, this paper presents current data on the use of BoNT to treat pelvic floor disorders.

S24 Re-Engineering The Target Specificity Of Clostridial Neurotoxins: a Route To Novel Therapeutics.

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The ability to chemically couple proteins to LHN-fragments of clostridial neurotoxins in order to target cells other than the natural target of the neurotoxin, and thereby inhibit exocytosis, has been reported (Chaddock et al., Infect & Immun 68: 2587, 2000; Chaddock et al., Growth Factors 18: 147, 2000). This has the potential to be therapeutically beneficial where secretion plays a caus-
ative role in a disease or medical condition. Chemical coupling is, however, not a suitable basis for producing pharmaceutical agents. The production of recombinant fusion proteins containing the LHN-domain of clostridial neurotoxins and a targeting domain, and the ability of such recombinant fusion proteins to inhibit secretion from specific target cells, will be described. In particular, a novel protein consisting of the LHN-domain of botulinum neurotoxin type C and Epidermal Growth Factor (EGF) able to inhibit secretion of mucus from epithelial cells. The potential of such a molecule to prevent mucus hypersecretion in asthma and chronic obstructive pulmonary disease will be discussed. Supported in part by funding from Allergan and in part by the Health Protection Agency. KF receives grant support from Allergan and Ipsen.

S25 Trafficking and Post-Translational Modifications of BoNT Light Chains Within Cells. GEORGE A. OYLER1, YIEN CHE TSAI2, PAUL S. FISHMAN3, MICHAEL ADLER4, RANDALL KINCAID1. 1Veritas Labs, Rockville MD; 2NCI, Fredrick MD; 3Department of Neurology, University of Maryland and VAMC Baltimore; 4Neurotoxicology Branch, Pharmacology Division, USAMRICD, Aberdeen Proving Ground, MD.

We have investigated the cellular trafficking and post-translational modifications of botulinum neurotoxin (BoNT) light chains (LC) for serotypes A and E. While both BoNT/A and /E LCs are endoproteases directed towards SNAP-25, they differ greatly in presynaptic terminal persistence. BoNT/A LC is extremely stable in the presynaptic terminal while BoNT/E is short lived. To investigate trafficking of BoNT/A and /E as a mechanism of persistence, fusions to YFP have been made. Confocal microscopy reveals that both LC A and E are membrane associated and targeted to lipid raft microdomains. Targeting to lipid rafts requires palmitoylation of cysteine residues in A and E LC. Cysteine mutagenesis results in failure of lipid raft targeting but retained membrane association. Since the trafficking of LC A and E is similar, other mechanisms must account for differences in persistence. Studies of ubiquitin-proteasome degradation of LC A and E show that LC E is much more extensively ubiquitinated than LC A. These differences in ubiquitin-proteasome degradation potentially account for the shorter half-life of LC E. Since the extreme persistence of LC A is a major complication of intoxication, we have developed a BoNT LC A and E directed "designer" E3 ubiquitin ligases to facilitate the degradation of LC A and shorten its persistence. We demonstrate that such designer E3 ligases are able to specifically ubiquitinate LC A and E and significantly shorten the half-life of the LC in cells. These investigations demonstrate proof of concept for novel molecular therapies of BoNT intoxication.

S26 Structural Aspects of SNARE - Clostridial Neurotoxin Interactions. AXEL T. BRUNGER, MARK A. BREIDENBACH. Howard Hughes Medical Institute and Department of Molecular and Cellular Physiology, Neurology and Neurological Sciences, and Stanford Synchrotron Radiation Laboratory, Stanford, CA 94305.

Clostridial neurotoxins (CNTs) impair neuronal exocytosis through specific proteolysis of essential proteins called SNAREs. SNARE assembly into a low-energy ternary complex is believed to catalyse membrane fusion, precipitating neurotransmitter release; this process is attenuated in response to SNARE proteolysis. Site-specific SNARE hydrolysis is catalysed by the CNT light chains, a unique group of zinc-dependent endoproteases. The means by which a CNT primarily identifies and cleaves its target SNARE has been a subject of much speculation; it is thought to use one or more regions of enzyme-substrate interaction remote from the active site (exosites). Recently we solved the first structure of a CNT endopeptidase in complex with its target SNARE at a resolution of 2.1 Å: botulinum neurotoxin serotype A (BoNT/A) protease bound to human SNAP-25. The structure, together with enzyme kinetic data, reveals an array of exosites that determine substrate specificity. Substrate orientation is similar to that of the general zinc-dependent metalloprotease thermolysin. We observe significant structural changes near the toxin’s catalytic pocket upon substrate binding, probably serving to render the protease competent for catalysis. We have also solved the 2.2 Å x-ray crystal structure of tetanus light chain protease (TeNT-LC). As expected, the overall structure of TeNT-LC is similar to the other known CNT light chain structures. Differences between TeNT-LC and the other CNT light chains are mainly limited to surface features such as unique electrostatic potential profiles. An analysis of surface residue conservation reveals a pattern of relatively high variability matching the path of substrate binding around BoNT/A, possibly serving to accommodate the variations in different SNARE targets of the CNT group. This work was funded by National Institute of General Medical Sciences grant 1-ROI-MH63105-01 to ATB.

S27 Risk Factors for Botulinum Toxin Immunoresistance and Molecular Recognition of Toxin-A by Antibodies of Immunoresistant Patients. M. ZOUHAIR ATASSI1, BEHZOD Z. DOLIMBEK1, JOSEPH J. JANKOVIC2, LANCE E STEWART3, K. ROGER AOKI4. 1Department of Biochemistry, 2Parkinson’s Disease Center and Movement Disorders Clinic, Department of Neurology, Baylor College of Medicine, Houston, Texas 77030; and 3Allergan, Inc., Irvine, CA 92612.

We have synthesized, purified, and characterized sixty 19-residue peptides that overlapped consecutively by 5 residues and spanned the entire 848-residue heavy (H) chain of botulinum neurotoxin A (BoNT/A). We employed these synthetic peptides to map the entire H chain of BoNT/A for the regions that bind antibodies (Abs) in anti-BoNT/A antisera of humans and of different animal species and the regions recognized by T-lymphocytes in high responder mouse strains. We have also mapped the Ab-recognition profiles in sera of 28 Cd patients that have become immunoresistant to BOTOX® (BoNT/A)-treatment. The pattern of recognition varied from patient to patient, but a relatively small set of peptides were recognized by most of the patients. These were peptide N25 (H chain residues 785-803), C10 (981-999), C15 (1051-1069), C20 (1121-1139) and C31 (1275-1296). We will discuss the risk factors in cervical dystonia (CD) patients that cause unresponsiveness to BOTOX® treatment as a result of immunoresistance. The results have shown that the immune response to BoNT is influenced by dose, duration of treatment, frequency of immunization, quality of the toxin, a prior immune response to an immunologically cross-reacting toxin (e.g., tetanus neurotoxin), and the MHC of the host. Once a patient becomes immunoresistant to one toxin then switching to another toxin will often be of limited and short-lived benefit, because the patient may become immunoresistant to the second toxin. This work was supported by a grant from Allergan and by the Welch Foundation due to the award to M. Z. Atassi of the Robert A. Welch Chair of Chemistry. MZA is a Consultant/Lecturer for and receives grant support from Allergan.
S28 Clinical Application of Stabilized Low Molecular Weight (150kDa) Botulinum type A Neurotoxin Preparation for Treating Muscle Hyperactivities. TAKASHI SAKAMOTO1, RYUJI KAJI1, SHUNJI KOZAKI2, KEIJI OGUMA3, TETSUHIRO HARAKAWA4. 1Tokushima University, 2Osaka Prefectural University, 3Okayama University, 4Chemo-Sero-Therapeutic Research Institute (Kaketsuken, Inc.), Japan.

Clinical symptoms of acute botulism by contaminated food are characterized by the early onset and the lack of immunity toward botulinum toxin even at life-threatening paralysis after exposure to a huge amount of the toxin. The clinical action of therapeutic botulinum toxin preparations is on the other hand longer than the acute botulism and may become apparent after a few weeks. The risk of developing antibodies is low, but not negligible. This discrepancy may be explained by the large molecular weight of the therapeutic preparations added by non-toxin components, which could retard the release of the 150 kDa neurotoxin or act as an adjuvant to enhance immunogenicity. The lack of non-toxin components has been believed to increase instability of the preparation.

We have developed a stabilized neurotoxin with low molecular weight (150 kD) type A botulinum toxin (NTX), which can be stored at room temperature. We compared the actions of NTX and the conventional type A preparation of 900 kD (BTX) with regard to the decreasing compound muscle action potentials (CMAPs) after tibial nerve stimulation in the rat and the immunogenicity after challenging with the toxoids made from either toxin preparation of the same clinical efficacy of reducing CMAPs. We found significantly earlier onsets and increased durations of the clinical action and reduced rate of antibody development after challenging equivalent doses. After having the approval of the entire protocol by the institutional review board of Tokushima University, we then tried to use NTX for those patients with spasticity and severe dystonia refractory to BTX treatments. We observed marked clinical improvements in the walking speed, pain, and the range of motion in these patients. We believe that NTX is promising for the clinical use in large muscles with repeated doses, perhaps in spasticity.

This work was supported by a Research Grant on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation. RK is on the Allergan Speakers Bureau and has been involved in collaborative research with Kaketsuken.

S29 Recombinant Engineered Antibody Potently Neutralizes Known and Novel A Type Botulinum Neurotoxins. I. GEREN1, J. LOU1, C. GARCIA1, A. RAZAI1, C. FORSYTH1, T. SMITH2, J. BROWN2, C. PEREZ2, W.H. TEPP3, E.A. JOHNSON3, L.A. SMITH4, J.D. MARKS1. 1Dept. of Anesthesia, University of California, San Francisco, CA; 2Toxinology Division, USAMRIID, Ft. Detrick, MD; 3Dept. of Food Microbiology and Toxicology, UW Madison, WI.

To define the extent of BoNT/A diversity, more than 100 BoNT/A producing Clostridial strains were characterized by restriction mapping and DNA sequencing. Besides the known BoNT/A1 and A2 subtypes, two additional subtypes were identified, BoNT/A3 and BoNT/A4. BoNT/A2, A3, and A4 toxins differed by 10%, 15%, and 13% at the amino acid level compared to BoNT/A1. To determine the impact of subtype variability on immune recognition, we studied the binding and neutralization capacity of 8 monoclonal antibodies (mAbs) to BoNT/A1, BoNT/A2, and BoNT/A3. All 8 mAbs bind BoNT/A1 with high affinity, but 5 of the 8 mAbs showed a marked reduction in binding to BoNT/A2 and/or BoNT/A3. Binding to BoNT/A4 could not be determined due to the low amounts of toxin produced. Binding results predicted in vivo toxin neutralization; mAbs that bound A2 or A3 toxins with low affinity had minimal neutralizing capacity. To develop a recombinant BoNT/A antitoxin, molecular evolution was used to broaden mAb specificity and increase affinity, yielding a combination of three mAbs able to bind and potently neutralize BoNT/A1, A2, A3, and A4. We conclude that successful development of recombinant antitoxin requires defining subtype variability and the screening and molecular evolution of a large panel of mAbs. Supported by: DAMD17-03-C-0076, NIAID R21 AI53389-01 and U01 AI056493.