We describe five infants with severe hypotonia, feeding difficulties and varying degrees of respiratory distress. The diagnosis of PWS was initially not clinically apparent therefore they underwent neurophysiological assessment, including stimulated single fibre EMG (SSFEMG) of orbicularis oculi. SSFEMG was considered abnormal if the grand average of the mean consecutive difference (MCD), of the intervals between the stimulus and the potential (the jitter), was greater than 26 μs, or if more than 10% of the individual measurements were greater than 34 μs. All five infants showed significant abnormalities in SSFEMG, suggestive of a neuromuscular transmission disorder. Jitter measurements ranged from 34.6 to 68.7.

One infant was initially diagnosed with congenital myasthenic syndrome and responded well to treatment with pyridostigmine. Cytogenetic studies subsequently confirmed the diagnosis of PWS in all five cases. This is the first report of abnormal SSFEMG findings in infants with PWS. We suggest that these infants may have an abnormality in neuromuscular junction transmission contributing to their early hypotonia and inactivity, which improves with maturation. PWS should therefore be considered in the differential diagnosis of hypotonic infants with neuromuscular junction abnormalities diagnosed by SSFEMG.

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G.P.74
Clinical aspects of presynaptic neuromuscular transmission defect in anti-GQ1b IgG antibody-related disorders
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Guillain–Barre syndrome (GBS), Miller Fisher syndrome (MFS) and Bickerstaff’s brainstem encephalitis (BBE) are immune-mediated disorders with overlapping clinical features, whereby anti-GQ1b IgG antibody is detected in variable frequencies. In vitro studies of MFS have demonstrated anti-GQ1b IgG antibody-mediated presynaptic damage at the neuromuscular junction. Previous studies have provided electrophysiological evidence of presynaptic neuromuscular transmission defect in anti-GQ1b positive MFS patients, which persisted up to 3 months from initial presentation. In this study, we compared incremental responses antibody-positive MFS and BBE (Group 1) with antibody-negative GBS and MFS variants (Group 2) using repetitive nerve stimulation (RNS) as an electrophysiological measurement of presynaptic neuromuscular transmission defect. With 20 Hz RNS, Group 1 showed significantly greater incremental responses compared to Group 2 (t-test, p = 0.0006). With 50 Hz RNS, Group 1 also showed significantly greater incremental responses compared to Group 2 (t-test, p = 0.002). Repeat studies at 6 months demonstrated return of increments to within normal limits in all three patients in Group 1. There was significant correlation of the initial increments (mean of 20 Hz and 50 Hz) and anti-GQ1b IgG antibody titer (Pearson’s correlation coefficient r = 0.76, p = 0.011). In addition to MFS, we have also shown that presynaptic dysfunction can occur in BBE. To this end, incremental responses in upper limb muscles may serve as surrogate markers for NM transmission defect in these disorders. As immunotherapy is the mainstay of treatment in these conditions, our findings of presynaptic neuromuscular transmission defect may serve as a potential parameter in selecting patients for future therapeutic trials.

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ADVANCES IN THERAPY FOR NEUROMUSCULAR DISORDERS – POSTER PRESENTATIONS

T.P.16
Restoring the reading frame in large DMD duplication mutations results in dystrophin expression in vivo
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Antisense oligonucleotide (AON) therapy has been shown to be an effective treatment for restoring dystrophin expression in mdx mice and Duchenne muscular dystrophy (DMD) patients with out-of-frame mutations. However, to date, limited exon skipping studies have been conducted in DMD patients with large out-of-frame duplication mutations. Whilst exon skipping-one or two exons is a highly efficient process in cell culture, efficiency falls when targeting multiple exons, thus producing little of the desired end product. Therefore, we sought to understand the expression and function of large DMD duplication proteins when only two exons are removed in order to restore the reading frame. We constructed large “in-frame” DMD duplication mutation plasmids and evaluated their expression in vivo. Two out-of-frame DMD duplication mutations (dup ex22–29 and dup ex18–30) were modified to express in-frame human dystrophin transcripts (del ex21–22 dup ex23–29 and del ex17–18 dup ex19–30). In addition, an original “in-frame” DMD duplication mutation (dup ex3–25) was cloned to determine if over expression of the duplicated transcript would result in protein expression. Plasmids were electroporated into TA muscles of mdx mice, and human dystrophin expression was evaluated 7 days later. Human dystrophin expression was detected at the sarcolemma for all the constructs, confirming the duplicated proteins were stable. In addition, all three proteins recruited the nNOs protein. Whilst this study indicates DMD patients with out-of-frame duplication mutations may be suitable candidates for AON therapy, further work to analyse the protective potential of restored in-frame duplication mutations is necessary. We are currently investigating DMD duplication protein function through the exclusion of Evans blue dye post treadmill exercises. In addition, plasmids will be administrated into neonatal mdx mice to assess the ability of the constructs to prevent muscle fibre degeneration.

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T.P.17
Alternate translational initiation and amelioration of phenotype in the DMD gene
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Nonsense mutations within exon 1 of DMD do not result in severe DMD but instead lead to very mild BMD, due to an alternative initiation of translation at AUG codons within exon 6. This leads to translation of a nearly full-length but N-terminal truncated dystrophin lacking the first half of the canonical actin binding domain 1 (ABD1). We have identified the motif encoded in exon 5 that recruits ribosomes for alternate initiation of translation at AUG codons within exon 6, and using a dual luciferase reporter system we have determined that this motif is selectively activated in muscle cell lines but not fibroblasts or HEK cells. Our data suggest that this motif is an IRES (internal ribosome entry site) as complementary experiments have ruled out any promoter activity or aberrant splicing. The exceedingly

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mild clinical features of patients with an exon 1 DMD founder allele (p.Trp3X) suggest that the product of this IRES initiation is a highly functional protein. We are therefore exploring different strategies to induce IRES utilisation for therapeutic purposes in patients with mutations in exons 1 through 4. One of them is based on forced synthesis of this IRES protein isoform that can be induced by exon 2 skipping. This leads to a frameshift and premature stop codon in exon 3, which thereby force the use of the IRES. We have developed four different antisense sequences for efficient skipping of exon 2 that are incorporated into a short U7 RNA derivative that was previously used in other studies to induce efficient exon skipping in DMD. We are currently testing these antisense constructs in patient cells carrying mutations in the first exons to evaluate the potential positive benefit of this out of frame skipping strategy.

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T.P.18
A recipe for the rational design of efficient antisense oligonucleotides for DMD gene exon skipping
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Antisense oligonucleotides-mediated exon skipping to restore dystrophin expression for DMD therapy is promising in human clinical trials. Current AON design methods are semi-empirical involving either trial-and-error and/or preliminary experimentation. To increase the productivity of developing efficient AONs, a rational AON design approach is desirable. While pioneering retrospective studies have extracted many AON design variables, they were not tested prospectively to design AONs for skipping DMD exons. Here, we proposed a recipe for the design of efficient AONs that is based on the biophysical and biochemical mechanisms involved in the exon splicing process. The recipe was applied to design 23 novel AONs, each to skip one of nine DMD exons, and their exon skipping efficiencies were subsequently validated in wet experiments. 19 AONs were found to be efficacious in inducing specific exon skipping (83% of total), of which 14 were considered efficient (61% of total), i.e. induced exon skipping in >25% of total transcripts. The validated design variables facilitate the rational design of AONs that could expedite development of optimal AONs for therapy.

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T.P.19
Enhanced exon skipping in the 44 dystrophic mouse model of muscular dystrophy through refined oligomer design
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Antisense oligomer (AO) induced dual dystrophin exon skipping was studied in the B6Ros.Cg-Dmdm4d1S1/J (44+) mouse, an established mouse model of muscular dystrophy (DMD) by correcting the reading frame of the pre-mRNA with AO-mediated exon skipping. An antisense 18-mer 2‘-O-methyl RNA/ethyl-ene-bridged nucleic acid chimera AO targeting exon 45 of the dystrophin gene, AO85, can induce exon 45 skipping efficiently in cultured cells. AO85 is expected to facilitate dystrophin expression in 8-9% of all DMD patients. Here, we examined the kinetics of AO85-mediated exon 45 skipping in a cell-free splicing system. In vitro transcribed pre-mRNAs containing dystrophin exon 45 and part of its flanking introns within a hybrid minigene were incubated with HeLa cell nuclear extract, and the resultant mRNAs were amplified by semiquantitative reverse transcriptase-polymerase chain reaction. Time-course analysis revealed that the splicing process fitted well to first order kinetics. Addition of AO85 produced an extra spliced product, deleting exon 45 (Δexon 45), indicating AO85-mediated exon 45 skipping. Production of Δexon 45 increased linearly with increasing concentrations of AO85, reaching a maximum of nearly 80% of the transcripts. The half-maximal effective concentration (EC50) of AO85 was 58.0 nM. The percentage of Δexon 45 among the transcripts decreased inversely with the pre-mRNA concentration; Lineweaver–Burk plotting revealed a competitive fashion of AO85 action. The low EC50 indicates high potential of AO85 for clinical application.

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T.P.20
Antisense oligonucleotide induced dystrophin exon 45 skipping at a low EC50 in a cell-free splicing system
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Antisense oligonucleotides (AOS) can facilitate the expression of internally deleted dystrophin in dystrophin-deficient Duchenne muscular dystrophy (DMD) by correcting the reading frame of the pre-mRNA with AO-mediated exon skipping. An antisense 18-mer 2‘-O-methyl RNA/ethyl-ene-bridged nucleic acid chimera AO targeting exon 45 of the dystrophin gene, AO85, can induce exon 45 skipping efficiently in cultured cells. AO85 is expected to facilitate dystrophin expression in 8-9% of all DMD patients. Here, we examined the kinetics of AO85-mediated exon 45 skipping in a cell-free splicing system. In vitro transcribed pre-mRNAs containing dystrophin exon 45 and part of its flanking introns within a hybrid minigene were incubated with HeLa cell nuclear extract, and the resultant mRNAs were amplified by semiquantitative reverse transcriptase-polymerase chain reaction. Time-course analysis revealed that the splicing process fitted well to first order kinetics. Addition of AO85 produced an extra spliced product, deleting exon 45 (Δexon 45), indicating AO85-mediated exon 45 skipping. Production of Δexon 45 increased linearly with increasing concentrations of AO85, reaching a maximum of nearly 80% of the transcripts. The half-maximal effective concentration (EC50) of AO85 was 58.0 nM. The percentage of Δexon 45 among the transcripts decreased inversely with the pre-mRNA concentration; Lineweaver–Burk plotting revealed a competitive fashion of AO85 action. The low EC50 indicates high potential of AO85 for clinical application.

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T.P.21
Exon skipping for dysferlinopathies
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