Exon-Skipping Antisense Oligonucleotides to Correct Missplicing in Neurogenetic Diseases

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Alternative splicing is an important regulator of the transcriptome. However, mutations may cause alteration of splicing patterns, which in turn leads to disease. During the past 10 years, exon skipping has been looked upon as a powerful tool for correction of missplicing in disease and progress has been made towards clinical trials. In this review, we discuss the use of antisense oligonucleotides to correct splicing defects through exon skipping, with a special focus on diseases affecting the nervous system, and the latest stage achieved in its progress.

Introduction

ALTERNATIVE SPLICING IS THE PROCESS by which exons of primary transcripts can be spliced into alternative arrangements to produce structurally and functionally different messenger RNA (mRNA) and protein variants. The splicing process can be regulated by different *cis*- and *trans*-acting factors that influence splice site selection (Johnson et al., 2009). Point mutations in a gene may weaken or strengthen splice sites; enhancer or silencer elements or lead to their destruction. This in turn causes alteration of splicing events and consequent disease.

Additionally, most eukaryotic genes contain several pseudoexons, sequences resembling perfect exons, which are nonetheless ignored by the splicing machinery (Dhir and Buratti, 2010). Aberrant pseudoexon inclusion due to deep intronic mutations has been uncovered in recent years as a frequent cause of human diseases. When a pseudoexon inclusion leads to premature insertion of a termination codon in the mRNA, the term “nonsense exon” is used, also indicating that the mRNA undergoes rapid degradation by nonsense-mediated decay (NMD) pathways (Dhir and Buratti, 2010).

It has been observed that 50% of human genetic diseases arise due to mutation that affect the splicing process, including numerous that are associated with neurogenetic diseases (Matlin et al., 2005; Mills et al., 2012). The brain, in fact, being the most complex organ, expresses a relatively higher number of alternatively spliced genes, some of which were found to be linked to neurological and neurodegenerative diseases. Because these diseases cannot presently be cured and are degenerative, they place a great burden on caregivers and represent some of the most costly diseases for society.

Antisense Oligonucleotide-Mediated Splicing Modulation

The most widely studied gene therapy approach for the correction of aberrant splicing is the use of antisense oligonucleotides (AONs) that target the pre-mRNA. AONs are short synthetic modified nucleic acids that bind RNA through base pairing and enable modulation of its expression (Southwell et al., 2012). (For a thorough overview on the applications of antisense-mediated exon skipping, see van Roon-Mom and Aartsma-Rus, 2012).

Design of AONs that can bind to splice sites or to exonic or intronic enhancing *cis*-regulatory sequences in the pre-mRNA [exonic splice enhancers (ESEs) or intronic splice enhancers (ISEs)] leads to masking of these regions and the inability of *trans*-splicing regulatory factors to bind effectively, thus inducing switching between alternative splice isoforms via exon skipping (Fig. 1). In the same way, by masking of cryptic splice sites, AONs have been used for the correction of cryptic splicing.

As point mutation or deletions can disrupt the open reading frame in a transcript, AONs have also been used to induce the skipping of one or more additional exons, to restore the transcript reading frame, allowing the production of an internally deleted, but sometimes (partially) functional protein (Turczynski et al., 2012). This strategy has its most advanced examples in the exon skipping approaches to the treatment of Duchenne muscular dystrophy (DMD, Aartsma-Rus, 2012).

Further, AONs-mediated exon skipping can also be used to induce reading frame disruption and protein knockdown (Disterer and Khoo, 2012). However, to achieve a robust therapeutic effect, it is essential that sufficient amounts of AONs are taken up specifically by the tissue affected by the

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FIG. 1. Methods of antisense oligonucleotide-mediated modification of splicing: (A) Alternative exon: antisense oligonucleotides (AONs) directed toward splice sites or exonic enhancers induce exon skipping. (B) Pseudoexon: AONs directed toward cryptic splice sites restore the normal transcript. (C) Nonsense exon: AONs targeting cryptic splice sites of nonsense exons restore normal transcripts. (D) Exonic cryptic splice site: AONs, targeting the exonic cryptic splice site, restore normal splicing.

disease, which is often not the case. Moreover, other techniques such as RNA interference have shown higher efficacy and feasibility. On the other hand, protein knockdown via exon skipping may exhibit advantages over other techniques that simply knock down gene expression: the alternative isoforms may have dominant negative behavior towards the normal isoform, present additional therapeutic properties, or may avoid side effects that are associated with near total downregulation of the normal isoform.

In principle, AONs appropriately targeted to block exonic or intronic splicing silencers (ESSs or ISSs) can also restore exon inclusion in the context of disease-associated exon skipping. The exon-inclusion strategy has proven effective *in vitro* and *in vivo* as a possible therapy for spinal muscular atrophy (SMA), as extensively described in recent reviews (Hua and Krainer, 2012; Douglas and Wood, 2013).

Notably, most advanced AON-mediated exon skipping approaches are clinical phase 3 trials for DMD (Arechavala-Gomez et al., 2012) and earlier clinical trials are in preparation for SMA (Porensky and Burges, 2013).

Different Oligonucleotide Chemistries are Available for the Correction of Splicing

The currently used AONs are rarely regular RNA or DNA oligonucleotide, as alternative AON chemistries have been developed to improve affinity, boost stability in the circulation and in target cells, and enhance cell penetration and nuclear accumulation. This issue will be here only briefly outlined and the reader is referred to (Saleh et al., 2012) for a more complete discussion of AON chemistry.

The non-bridging oxygen in the phosphate backbone has been replaced with a sulfur atom, generating phosphorothioate (PS) AONs (De Clercq et al., 1969). This modifi-

cation enhances cellular uptake and improves resistance to nucleases but reduces the affinity of the AON to the target RNA. Moreover, the PS modification does not abrogate the ability, proper of DNA oligos, to induce RNase H cleavage of the target RNA.

Addition of a methyl or a methoxyethyl group to the 2'-O atom of the ribose sugar (2'OMe and 2'OMOE, respectively) renders the AON-target RNA hybrid RNase H-resistant and increases the affinity of the AON for the target RNA (Sproat et al., 1989; Manoharan et al., 1999). Most AONs currently under study for splicing corrections have both the 2'O and the phosphorothioate (PS) modification (2'OMe-PS and 2'OMOE-PS), probably because they have a good safety profile and their synthesis is relatively inexpensive.

2'OMe-PS were the first AONs to be used for exon skipping (Sierakowska et al., 1996; Khang et al., 1998) and the first to be used for dystrophin exon skipping in cultured primary muscle cells from *mdx* dystrophic mice (Dunckley et al., 1998). Some years later, exon skipping and dystrophin protein recovery was demonstrated upon delivery of 2'OMe-PS into *mdx* mice, aided by the nonionic block copolymer pluronic F127, and injected either locally in skeletal muscles (Lu et al., 2003) or systemically via tail vein (Lu et al., 2005). 2'OMe-PS AONs targeting dystrophin exon 51 (GSK-2402968/PRO051/driscapersen) have proven successful in intramuscular clinical trials in DMD patients (van Deutekom et al., 2007) and have demonstrated significant dystrophin restoration, good safety and tolerance in systemic clinical trials (Goemans et al., 2011).

2'OMOE-PS AONs have been used in cell lines to redirect splicing of murine interleukin-5 receptor alpha chain (il5r α , Karras et al., 2000) and of myD88 (Vickers et al., 2006) and to correct aberrantly spliced reporter enhanced green fluorescent protein (EGFP; Sazani et al., 2001). Importantly,

2'OMOE-PS have been recently used successfully for exon inclusion of the *SMN2* gene as a potential approach for the treatment of SMA in cultured cells (Hua et al., 2007) and *in vivo* by intracerebroventricular (ICV) infusion or injection in a mouse model of SMA (Hua et al., 2010). In this latter work, a side-by-side comparison was also made between an 18-mer 2'OMOE-PS and an overlapping 20-mer 2'OMe-PS AON. The 2'OMOE-PS was found to be more effective after ICV infusion into adult mice central nervous system (CNS) and to elicit less unwanted proinflammatory effects (Hua et al., 2010).

In a different available oligonucleotide chemistry, a methylene bridge connects the 2'-O and the 4'-C of the ribose, forcing the nucleotide in the "endo" conformation, in what has been dubbed "locked nucleic acid" (LNA; Obika et al., 1998). This modification leads to a very high affinity for the target nucleic acid.

Aartsma-Rus and colleagues (2004) reported that an AON completely made of LNA was very effective for exon skipping in cells derived from an exon 45-deleted DMD patient. However, this AON also showed reduced specificity, probably due to the very high affinity of the 14-mer LNA with the target (Aartsma-Rus et al., 2004). In the applications that use oligonucleotides as steric inhibitors, the specificity issues associated with LNA have been solved by using a mixmer of LNA and DNA backbone sequence (Elayadi et al., 2002) or LNA and 2'OMe backbone (Arzumanov et al., 2001; Fabani and Gait, 2008). Roberts and colleagues (2006) have shown that upon intraperitoneal (IP) injection in the EGFP splice-switching mouse model, a 16-mer LNA/DNA mixmer containing 8 LNA units alternating with the DNA units, and with an all-PS backbone, showed much higher potency in the liver, colon, and small intestine than an overlapping 2'OMe 18-mer AON (Roberts et al., 2006).

In addition to the described negatively charged oligonucleotides (2'OMe-PS, 2'OMOE-PS, and LNA), two more oligonucleotide chemistries have been used in attempts to modulate splicing: peptide nucleic acids (PNAs) and phosphorodiamidate morpholino oligomers (PMOs). Both these types of charge-neutral oligonucleotides are resistant to exo- and endonucleases and RNase H cleavage.

PNAs have a 2-aminoethyl glycine backbone linked to nucleobases (Larsen et al., 1999) and show high affinity to both RNA and DNA targets and good sequence specificity (Egholm et al., 1993). It is common to incorporate additional cationic lysine residues in the PNA oligonucleotide, to get good water solubility, and to improve cell entry by facilitating the binding to the negatively charged surface of cells. Karras and colleagues have shown that a 15-mer PNA containing a single C-terminal Lys residue had similar efficiency as a 2'OMOE-PS AON in modulating the splicing of the *IL5R- α* pre-mRNA when electroporated into BCL1 lymphoma cells (Karras et al., 2001). In the absence of a transfection agent, an 18-mer PNA containing four Lys residues (PNA-4Lys) was shown to be taken up by HeLa cells better than a PNA of identical sequence with one Lys residue only and to modulate reporter EGFP splicing (Sazani et al., 2001). In the same conditions, 2'OMe-PS and 2'OMOE-PS AONs of identical sequence were not able to induce splicing correction (Sazani et al., 2001). The same PNA-4Lys 18-mer, when IP-injected in the splice-switching EGFP mouse, was very effective in inducing EGFP production in a number of organs,

such as kidney, liver, heart, and lung, whereas the PNA with only a single Lys residue was inactive (Sazani et al., 2002).

However, a PNA-4Lys 14-mer was not effective in inducing dystrophin exon skipping in exon-45-deleted DMD patient myoblasts (Aartsma-Rus et al., 2004), probably due to its short length.

A 20-mer PNA without Lys addition was shown to be moderately effective in dystrophin exon skipping in the *mdx* mouse model upon local muscle injection (Yin et al., 2008a). The same authors showed recently that, upon tail vein injection into *mdx* mice, a 25-mer PNA without Lys is more effective than the 20-mer PNA in dystrophin exon skipping and doses up to 100 mg/kg were well tolerated (Yin et al., 2010).

PMOs consist of morpholine rings that are connected through phosphorodiamidate groups (Summerton and Weller, 1997). Generally, PMOs used are longer than 2'OMe-PS AONs due to their slightly lower affinity. Schmajuk and colleagues (1999) have shown that a PMO 18-mer was 20-fold more effective than a corresponding 2'OMe-PS 18-mer in modulating splicing in free uptake in cell culture (Schmajuk et al., 1999).

As PMOs are charge neutral, they are difficult to transfect. Gebiski and colleagues (2003) successfully transfected PMOs in *mdx* mouse muscle cells in combination with a sense oligo (leash) and Lipofectin, to allow formation of a cationic lipoplex. In these cells they showed efficient dystrophin exon skipping with a 25-mer PMO, and exon skipping and dystrophin production was also observed by intramuscular injection of the cationic lipoplex in the *mdx* mouse (Gebiski et al., 2003). The same PMO 25-mer was also effective in generating dystrophin in a number of muscle types when injected intravenously and uncomplexed in *mdx* mice (Alter et al., 2006). Direct comparisons in the *mdx* mouse of the PMO 25-mer to a 2'OMe-PS of identical sequence and length, suggested that PMO generates higher exon skipping and dystrophin production upon both local and systemic delivery than 2'OMe-PS (Fletcher et al., 2006). However, leash transfection of a 22-mer PMO into exon 45-deleted DMD patient's muscle cells did not result in as good exon skipping as for a 20-mer 2'OMe-PS ON (Aartsma-Rus et al., 2004). The reason for these discrepancies might be different effectiveness of 2'OMe-PS and PMO AONs depending on the target exon sequence as well as on AON length, as also suggested by exon skipping studies in a range of human exons in humanized DMD mice (Heemskerk et al., 2009).

A PMO 30-mer targeting dystrophin exon 51 (AVI-4658/eteplirsén) has proven successful in intramuscular clinical trials (Kinali et al., 2009). In recent systemic studies, eteplirsén has demonstrated significant dystrophin restoration, good safety, and tolerance (Cirak et al., 2011).

To facilitate delivery into cells *ex vivo* and *in vivo*, different cell-penetrating peptides (CPPs) have been conjugated to PMOs, yielding pPMOs (peptide-conjugated PMOs). While toxicity has been associated with moderate doses of the peptide conjugates (Abes et al., 2006; Burrer et al., 2007), pPMOs have been used *in vivo* for effective oligo delivery at doses below those causing observed toxicity (Amantana et al., 2007). The systemic injection of a pPMO conjugated to different Arg-rich peptides and targeting exon 23 led to dramatic enhancement of dystrophin production and exon skipping in the skeletal muscles and heart of *mdx* mouse

(Jearawiriyapaisarn et al., 2008; Wu et al., 2008; Yin et al., 2008b, 2009, 2011; Wu et al., 2012).

In a different approach, a non-peptidic cell-penetrating moiety, consisting in an octaguanidinium dendrimer, has been attached to the 3' end of a PMO (Li and Morcos, 2008). Such delivery-enabled PMOs, called "vivo-morpholinos," are efficiently uptaken in cells (Li and Morcos, 2008) and have been successfully injected systemically in *mdx* mice showing good activity in exon skipping and dystrophin production and no signs of toxicity at the injected doses (Wu et al., 2009).

Success in the preclinical studies involving exon-skipping treatment of DMD has prompted studies aimed at extending the range of application of these antisense oligonucleotides from neuromuscular to many other genetic diseases. In the following pages, we will review AON-mediated exon skipping treatments that have been studied in relation to the central nervous system (CNS) and for neurogenetic diseases.

Exon Skipping as a Therapeutic Approach for Neurogenetic Diseases

Ataxia telangiectasia

Ataxia telangiectasia (AT) is a progressive autosomal recessive neurodegenerative disorder resulting from mutations in the ataxia telangiectasia mutated (*ATM*) gene (Perlman et al., 2003). To date, close to 600 *ATM* mutations have been identified (www.hgmd.cf.ac.uk/ac/gene.php?gene=ATM). The phenotypic changes include progressive cerebellar ataxia, increased cancer incidence, and chromosome instability (Teraoka et al., 1999). Worldwide incidence for this disease is estimated at 1 in 40,000 to 1 in 100,000 individuals. Disease onset is in early childhood, usually before age 5.

The *ATM* gene (located on chromosome 11q22.3) includes 66 exons and encodes a 13-kb mature transcript with an open reading frame of 9,168 nt. The first two exons, 1a and 1b, are spliced differentially in alternative transcripts. The ATM protein is a serine/threonine kinase with 30 phosphorylation targets (Bakkenist et al., 2003) constitutively expressed in all tissues, primarily in the nucleus. It has a role in the control of cell cycle checkpoints, repair of double-stranded DNA breaks, responses to oxidative stress and apoptosis, and is a potent tumor suppressor.

Approximately half of the mutations in AT have been reported as splicing mutations (Teraoka et al., 1999; Nakamura et al., 2012; Cavalieri et al., 2013) that either create new cryptic splice sites or interfere with splice regulatory elements. Some of these mutations elicit the activation of 5' or 3' cryptic splice sites within exonic regions, leading to the deletion of a part of the exon. Du and colleagues designed a customized mutation-based approach for AT by transfecting antisense PMOs in immortalized lymphoblastoid cell lines from three different AT patients (Du et al., 2007). Cell line TAT[C] is homozygous for the mutation 7865C>T (A2622V) that creates a new 5' splice site in exon 55. This, in turn, results in the deletion of 64 nt in the spliced mRNA, frameshifting, and the appearance of a premature stop codon. The ATM protein produced is therefore shorter and possibly the mutated mRNA would undergo NMD. Cell line IRAT9 is homozygous for the mutation 513C>T (Y171Y), which activates a stronger 3' cryptic splice site within exon 8, resulting in a deletion of the first 22 nt of exon 8, frameshifting and the

appearance of a premature stop codon. This in turn causes the ATM protein to be truncated. Cell line AT203LA carries in heterozygosis the mutation IVS28-159A>G, that results in the insertion of a 112-nt segment of intron 28 in the mRNA, frameshifting, and premature translation termination (Fig. 2).

For TAT[C] and IRAT9 cells, PMOs were designed to mask the aberrantly activated cryptic splice sites (Fig. 2A, B), and correction was observed at transcriptional level with a 40% of pre-mRNA being spliced in the normal manner. PMOs could redirect aberrant splicing as early as 8 hours after treatment and this effect lasted at least 84 hours. The maximum effect, however, occurred at 48 hours. At the protein level, less than 10% ATM protein was induced at 24 hours of treatment, as compared with the ATM protein level in controls and as measured by western blots on nuclear extracts. However, the ATM protein level continued to increase until 84 hours, and the restored ATM protein was functional for kinase activity, as evaluated by ATM autophosphorylation and downstream transactivation of ATM substrates p53 and SMC1.

For the AT203LA cell line, two different PMOs were designed, one targeting the 3' splice site and one targeting the 5' splice site of the pseudoexon (Fig. 2C). Only the latter PMO showed effective blocking of pseudoexon inclusion and almost completely restored normal splicing, as evaluated both at the mRNA and protein levels.

The most debilitating feature of AT is the progressive loss of Purkinje cells in the cerebellum and the accompanying progressive ataxia. Therefore, for any compound to be effective in treating AT patients, it will have to cross the blood-brain barrier (BBB) and target brain cells, particularly Purkinje cells. Following their study in cells, Du and colleagues further extended the exon skipping strategy into preclinical studies, by investigating whether the coupling of an arginine-rich CPP to PMOs could enable their delivery to brain and cerebellum (Du et al., 2011). They showed that the targeting efficiency of these pPMOs is greatly improved in *ex-vivo* experiments, compared with PMOs of identical sequence: when the pPMO were tested on previously established cell lines (TAT[C], AT203LA) a complete abolishment of mutant transcripts along with 80% correction of ATM protein level was observed. This work was extended to the *in vivo* systemic administration of fluorescent pPMOs in wild type mice, via tail vein injection, which showed efficient uptake in the brain, and in Purkinje cells in particular (Du et al., 2011). However, no report is presently available on the *in vivo* exon-skipping efficacy of these pPMOs.

Nakamura and colleagues studied *ATM* mutations in eight families of Japanese AT patients (JPAT) where 16 different mutations were found. Six of these mutations were involved in splicing, four of which were novel (Nakamura et al., 2012). Their exon-skipping analysis was performed on lymphoblastoid cell lines of patients JPAT 11/12 bearing a splicing mutation (c.2639-384A>G) in heterozygosity with a large deletion (c.6807+272_7516-275del5350). Mutation c.2639-384A>G creates a cryptic 5' splice site resulting in the inclusion of a 58-nt pseudoexon (Fig. 2D). This leads to a frameshift and a predicted secondary premature stop codon. They used a PMO (AMO-J11) to target the cryptic 5' splice site, and delivered it to patient's lymphoblastoid cell line via the Endo-Porter system (Summerton, 2005). A complete ablation of mutant transcript was observed by reverse

FIG. 2. Schematic representation of five splicing mutations identified in the ataxia telangiectasia mutated (*ATM*) gene, and locations of blocking antisense oligonucleotides that restore normal splicing (Du et al., 2007, 2011; Nakamura et al., 2012; Cavalieri et al., 2013).

transcription–polymerase chain reaction (RT-PCR), in a PMO dose-dependent manner, and the full-length *ATM* protein was recovered. They also used a *vivo*-morpholino of identical sequence, which was able to induce a significant amount of functional protein at lower concentrations than the PMO, as validated by the western blot (Nakamura et al., 2012). However, authors report possible cytotoxic effects of the *vivo*-morpholino at the used concentration, on the lymphoblastoid cell line.

In a recent work, Cavalieri and colleagues identified a new *ATM* mutation c.1236-405C>T located in intron 11, which affects splicing by creating a novel cryptic 5' splice site, thus including a pseudoexon (Cavalieri et al., 2013). Patients bear the mutation in heterozygosis with the known missense mutation c.6326G>A. Cavalieri and colleagues (2013) used a PMO to mask the 5' cryptic splice site created by the mutation (Fig. 2E) in patient's lymphoblasts and reported a 26% increase of the correctly spliced mRNA as observed by RT-PCR, when cells were treated with 50 μ M PMO. Moreover, they used a *vivo*-morpholino of identical sequence and showed in patient's lymphoblasts an exon skipping efficiency of 50% to 95%, depending on AON concentrations used (1 μ M or 2 μ M). At these concentrations, the authors also report a cytotoxic effect of *vivo*-morpholinos, after 84 hours

of treatment. The functional protein activity determined through kinase activity was 50% of wild-type levels. Since AT patients with 5% to 20% of functional *ATM* have a mild AT phenotype, and *ATM* heterozygotes having 40% to 50% of normal *ATM* protein levels do not show any sign of disease, Cavalieri and colleagues have hypothesized that even a minimal rescue of functional *ATM* protein levels could provide therapeutic benefit to these patients.

Congenital disorder of glycosylation

Congenital disorder of glycosylation (CDG) is an autosomal recessive disorder that affects glycan synthesis. The most prevalent form of CDG, type 1a, has an incidence of 1 in 50,000 to 1 in 100,000 individuals, and is caused by mutations in *PMM2* gene, which is located on chromosome 16p13 and gene encodes phosphomannomutase 2 (*PMM2*), a key enzyme that controls the synthesis of GDP-mannose and is essential for the generation of N-glycans. Mutations in the *PMM2* gene lead to the hypoglycosylation of different proteins in different tissues (Dupré et al., 2000). CDG involves multi organ failure, but also leads to neurologic deficits including cognitive impairment, ataxia, pigmentary retinal degeneration, and neuropathy (Pearl et al., 2001). Clinical

presentation and course are highly variable, ranging from infants who die in the first year of life to mildly involved adults. N-glycosylation defect of cerebrospinal fluid β -trace protein, (a glycoprotein synthesized by glia-rich fractions of the brain) were observed in CDG type 1a patients (Pohl et al., 1997; Grünewald et al., 1999).

Since the total lack of the *PMM2* gene product is lethal, no patient with two copies of any inactivating mutation has ever been recorded. Patients are usually compound heterozygous. Though 95% of the mutations are missense in nature, there are yet a few mutations that cause pre-termination codons and splicing mutations (Vuillaumier-Barrot et al., 2006; Schollen et al., 2007; Vega et al., 2009). One such mutation has been targeted by Vega and colleagues, who reported the use of PMOs to revert a deep intronic mutation (c.640-15479C>T) that leads to the activation of a cryptic 5' splice site in intron 7 (Vega et al., 2009).

Two different 25-nt-long PMOs were designed, complementary to the 5' or 3' cryptic splice sites of the pseudoexon in intron 7 (Fig. 3) and both these PMOs were transfected at the same time into patient's fibroblasts carrying the c.640-15479C>T mutation in heterozygosity with missense mutation c.691G>A, via the Endo-Porter reagent (Summerton, 2005). There was a 100% restoration of the correctly spliced mRNA. The levels of PMM2 protein after transfection increased from 9% to 23% of the quantity detected for the control cell line. PMM2 enzymatic activity was rescued almost to 50% that of the control fibroblasts.

Fronto-temporal dementia and Parkinsonism linked to chromosome 17

Fronto-Temporal Dementia and Parkinsonism linked to chromosome 17 (FTDP-17) is an autosomal dominant neurodegenerative disorder showing cognitive impairment, behavioral and personality changes and motor symptoms. The incidence of FTDP-17 is unknown; however, it is an extremely rare condition. It is caused by mutations in the *MAPT* gene on chromosome 17q21, which encodes microtubule-associated protein tau. Over 100 families with 44 different mutations in the *MAPT* gene have been identified worldwide. The symptoms and onset age of FTDP-17 vary not only between families carrying different mutations but also between and within families carrying the same mutations. Pathological hallmarks of the disease are aggregation of tau into

neurofibrillary tangles (NFT) in neurons and/or glial cells. The pathogenetic mechanisms underlying the disorder are related to the altered proportion of tau isoforms or to the ability of tau to bind microtubules and to promote microtubule assembly. There is currently no cure for FTDP-17 and treatment is only symptomatic and supportive. The prognosis and rate of the diseases progression vary considerably among individual patients, ranging from life expectancies of several months to several years, and, in exceptional cases, as long as two decades after diagnosis (Tsuboi et al., 2009).

Tau is constitutively and abundantly expressed in both the central (CNS) and the peripheral nervous system (PNS). In the CNS, tau is enriched in the axons of mature and growing neurons, though low levels of tau are also present in oligodendrocytes and astrocytes.

Tau functions in microtubule (MT) assembly, influences MT stability, and is important for neurogenesis, axonal maintenance, and axonal transport. The human *MAPT* gene consists of 16 exons and its expression is regulated by complex alternative splicing (Andreadis, 2012). Exons 9–12 each encode a 31- to 32-amino-acid imperfect repeat that comprises the MT-binding domain of tau. In adult human brain, exons 2, 3, and 10 are alternatively spliced to produce six different tau isoforms. Exon 10 (E10) inclusion generates isoforms with four microtubule repeats called 4R tau. When E10 is skipped, the result is 3R tau isoforms. In fetal brain, only the shortest 3R tau isoform is expressed because of constitutive exclusion of exons 2, 3, and 10.

While several missense mutations in *MAPT* are known, approximately one-half of the mutations observed in FTDP-17 alter the relative levels of 4R versus 3R tau by affecting alternative splicing of E10 (Andreadis, 2012). These mutations include missense (N279K, N296H, G303V, and S305N), silent (L284L, N296N), and deletion mutations (Delta280K, Delta296N) in E10, and intronic mutations (E10–10, E10+3, E10+11, E10+12, E10+13, E10+14, E10+16, and E10+19) in introns 9 and 10. Most of these mutations increase E10 inclusion. The result is that the 4R/3R ratio, which is normally 1, is increased to 2–3. These mutations demonstrate that the 4R/3R ratio is crucial to the correct functioning of tau, although the mechanism by which this alteration leads to neurodegeneration and FTDP-17 is presently unclear.

Kalbfuss and his colleagues have shown that AONs directed against *MAPT* exon 10 splice sites could suppress

FIG. 3. Congenital disorder of glycosylation type 1A. Diagram of splicing mutations and antisense oligonucleotides locations that rescue the aberrant transcript in congenital disorder of glycosylation type 1a causal gene *PMM2* (for phosphomannomutase 2) (Vega et al., 2009).

inclusion of E10 (Kalbfuss et al., 2001). The experiment was performed on COS-1 green monkey kidney cells by co-transfecting reporter minigenes (with a single point mutations either at position -1 , $+3$, or $+14$ relative to E10) with 17- to 21-nt-long 2'OMe PS AONs targeting either the 5' or the 3' splice site of E10 (Fig. 4). Both these AONs were successful in skipping E10 in the three minigenes, as assessed by RT-PCR. They further proved these results in rat pheochromocytoma PC12 cells, in which both AONs effectively excluded exon 10 from the endogenous wild type *MAPT* mRNA, as observed through RT-PCR. Western blots confirmed the reduction of tau 4R isoform. Furthermore, they observed that exon skipping-inducing AONs had a significant and specific physiological effect by destabilizing the microtubule cytoskeleton and altering cell morphology, when transfected in rat AR42J pancreatic acinar cells.

Donahue and colleagues have validated the use of the 5' splice site-targeting 2'OMe-PS AON described by Kalbfuss, in HeLa cells cotransfected with luciferase reporter minigenes, in the wild-type and mutant E10+14 versions (Donahue et al., 2006). Transfection of the AONs resulted in a decrease in luciferase expression of about 50%, relative to cells transfected with a control oligoribonucleotide. Real-time RT-PCR confirmed an increase of approximately 15 times of the 3R/4R mRNA ratio, relative to control treatment.

The work of Kalbfuss and colleagues (Kalbfuss et al., 2001) has been further extended by Peacey and colleagues by targeting E10 5' splice site with a bipartite 2'OMe-PS AON and comparing its efficiency in inducing E10 skipping to that of Kalbfuss' "continuous" 5' splice site-targeting 2'OMe-PS oligonucleotide (Peacey et al., 2012). The bipartite 2'OMe-PS AON was designed to interact with the 5' and 3' regions immediately flanking an hairpin located at the junction between exon 10 and intron 10, and consists of 10 bases complementary to the 3' flank, a linker of one adenosine, and 9 bases complementary to the 5' flank.

Cotransfection of the bipartite 2'OMe-PS or the "continuous" 2'OMe-PS along with wild-type minigene in SK-N-SH neuroblastoma cells induced exon skipping with a reduction in 4R of 68% and 66%, respectively. In addition, bipartite 2'OMe-PS and "continuous" 2'OMe-PS significantly increased expression of 3R tau by 37 and 65%, respectively. When tested on a E10+14 mutant form of the minigene, the bipartite 2'OMe-PS and the "continuous" 2'OMe-PS significantly reduced expression of 4R tau by 57

and 61%, respectively with concomitant increase in 3R tau expression. The effect of bipartite 2'OMe-PS AON on endogenous tau mRNA was verified in HEK 293 cells that showed 42% reduction of E10 inclusion.

Niemann-Pick disease type C

Niemann-Pick disease Type C (NPC) is an autosomal recessive disorder due to mutations in genes *NPC1* or *NPC2*, with an incidence of 1 in 150,000 individuals. NPC is involved in a significant reduction of life expectancy (Miao et al., 2012) and is mainly associated with progressive neurodegeneration and neurological, psychiatric, and hepatic manifestations. Approximately 50% of cases present before 10 years of age, but manifestations may first be recognized as late as the sixth decade. Neurological symptoms vary with age accompanied by hypotonia, delay in developmental motor milestones, learning difficulties, ataxia with cognitive deficits, and psychosis (Kheder et al., 2013).

In the NPC neurons, there is a gross alteration in the overall amounts of cholesterol and an accumulation of unesterified cholesterol and glycosphingolipids in the lysosome-like storage organelles in the cell bodies (Mukherjee et al., 2004).

Though most of the mutations in *NPC1* are missense, some splicing mutations have been found (Rodríguez-Pascau et al., 2009; Macías-Vidal et al., 2011). Work by Rodríguez-Pascau and colleagues focused on mutation c.1554-1009G>A, located in intron 9 of *NPC1* gene, leading to the insertion of a 194-nt pseudoexon, both in HeLa cells transfected with a mutant minigene and in patient fibroblasts, which carry the mutation in heterozygosis with in-frame deletion-insertion p.N961_F966delinsS (Rodríguez-Pascau et al., 2009). A PMO complementary to the cryptic 5' splice site was used to block the access of the splicing machinery to the pre-mRNA thus avoiding the formation of the aberrantly spliced transcript (Fig. 5). The PMO was able to prevent the inclusion of the pseudoexon and restored normal splicing upon delivery to patients' fibroblasts via the Endo-Porter system (Summerton, 2005). RT-PCR analysis shows the complete disappearance of the amplicon corresponding to the aberrant splicing, upon 48 hours of treatment with 10 μ M PMO (Rodríguez-Pascau et al., 2009).

Neurofibromatosis type 1

Neurofibromatosis type 1 (NFT 1) is an autosomal dominant disorder characterized by café-au-lait spots, cutaneous

FIG. 4. Frontotemporal dementia and Parkinson's disease. Schematic representation of Exon 10 splicing mutations in *MAPT* gene, which encodes microtubule-associated protein tau, and of the locations of antisense oligonucleotides that induce skipping of exon 10 (Kalbfuss et al., 2001; Peacey et al., 2012).

FIG. 5. Niemann Pick disease type C (NPC1). Scheme of splicing mutations and location of antisense oligonucleotides that restore the aberrant transcript in *NPC1* (Rodríguez-Pascau et al., 2009).

neurofibromas, Lisch nodules, inguinal and axillary freckling, and a high predisposition to develop certain types of tumors. It affects 1 in 3,000 individuals, especially targeting young children (Friedman et al., 1999). Affected individuals develop glial neoplasms (optic gliomas, malignant astrocytomas) and neuronal dysfunction leading to learning disabilities and attention deficits (Gutmann et al., 2012).

NFT1 is caused by mutations in the *NF1* gene, which is located on chromosome 17q11.2, contains 57 constitutive exons and encodes for an 11- to 13-kb mRNA transcript. The gene product neurofibromin negatively regulates Ras and acts as a tumor suppressor. Neurofibromin also plays important roles in cell growth, neuronal activity and bone metabolism (Hseuh et al., 2012).

About 44% of the mutations found in *NF1* are splicing mutations (Pros et al., 2008). A small subset of these mutations (20%) are deep intronic single nucleotide changes that, creating either a novel 5' or 3' site, increase pseudoexon inclusion into mature mRNA.

Work by Pros and colleagues aimed at restoring normal splicing using PMOs in primary fibroblast and lymphocyte cell lines derived from six NFT1 patients bearing three different deep intronic mutations (c.288+2025T>G, c.5749+332A>G and c.7908-321C>G) (Pros et al., 2009). PMOs were designed against the mutant cryptic 5' splice sites, and delivered via Endo-porter reagent (Summerton, 2005) (Fig. 6). PMO treatment effectively restored normal *NF1* splicing at the mRNA level for the three mutations

FIG. 6. Neurofibromatosis type 1 (NF1). Schematic representation of four *NF1* deep intronic mutations and of the location of specific phosphorodiamidate morpholino oligomers (PMOs) used to restore the correct splicing (Pros et al., 2009; Fernández-Rodríguez et al., 2011).

studied in the different cell lines analyzed. The efficiency of *NF1* splicing correction in fibroblasts after 24 hours of treatment ranged from 87 to 100% in the different mutations. These authors also found that PMOs had a rapid effect in fibroblasts that lasted for several days, acting in a sequence-specific manner and interfering with the splicing mechanism. A lower degree of aberrant splicing correction (30%–70%) was observed in lymphocyte cell lines, and a higher concentration of PMO, together with a longer time of exposure, was needed to produce similar effects to those seen in fibroblasts. These differences are probably due to the greater difficulty of lymphocyte transfection. To test whether the correction of aberrant *NF1* splicing also restored neurofibromin function to wild-type levels, the authors measured the amount of Ras-GTP after PMO treatment in primary fibroblasts, as an indirect indication of the GTPase activity of neurofibromin. The results clearly show a PMO-dependent decrease in Ras-GTP levels, which is consistent with the restoration of neurofibromin function.

Fernández-Rodríguez and colleagues analysed the genetic basis of a benign phenotype observed in a NFT1 patient carrying the mutation c.3198-314G>A in intron 19a of the *NF1* gene (Fernández-Rodríguez et al., 2011). This deep intronic mutation creates a new cryptic 3' splice site that uses two different 5' splice sites present in the wild type sequence, generating two aberrantly spliced transcripts leading to the inclusion of two different pseudoexons, both of which would generate the same putative truncated protein. A minigene construct containing mutation c.3198-314G>A indicated the production of both mutated and wild type transcripts, with a low proportion of the mutated form due to leaking in the splicing mechanism, contributing to the benign nature of *NF1* mutation and consequent mild phenotypic effect observed in the patient.

As a personalized therapeutic approach for *NF1* mutations, PMOs were delivered in patient's fibroblasts. Three different PMOs were designed, which the authors named antisense morpholino oligomers (AMOs): AMO-A, blocking the 3' splice site; AMO-D1, blocking the first 5' (donor 1) splice site; and AMO-D2, blocking the second 5' (donor 2) splice site (Fig. 6). Reduction of the mutant transcripts was validated by RT-PCR. However, a complete correction was observed only when a combination of the three PMOs was used. In order to provide insights into the functional validation, and in agreement with the results obtained by Pros and colleagues (Pros et al., 2009), a significant reduction of Ras-GTP was observed in fibroblasts treated with specific PMOs.

Neurofibromatosis type 2

Neurofibromatosis type 2 (NFT 2) is an autosomal dominant disorder that affects about 1 in 33,000 newborns, characterized by tumors of the nervous system and ocular abnormalities. The presence of bilateral vestibular nerve schwannomas is the most distinctive feature of NFT 2, but patients can develop other clinical manifestations such as schwannomas in other cranial, spinal and peripheral nerves, and also other types of tumors, like meningiomas (both intracranial and intraspinal) and ependymomas (low-grade CNS malignancies). This cancer syndrome is caused by mutations in the tumor suppressor *NF2* gene that is located on chromosome 22q12 and that codes for the tumor suppressor protein merlin.

Human merlin mainly located in adherens junctions and is predominantly found in nervous tissue, but also in several other fetal tissues. Merlin links receptors at the plasma membrane to their cytoplasmic kinases to promote contact-mediated growth inhibition. It is also involved in interaction with other cytoplasmic and nuclear proteins that affect cell cycle progression (Beltrami et al., 2013). Around 85% of the germline *NF2* mutations are point mutations, among which approximately 25% seem to affect splicing. In the recent work by Castellanos and colleagues, a patient with a deep intronic mutation (g.74409T>A, NG_009057.1) was identified (Castellanos et al., 2013). This mutation leads to the inclusion of a nonsense pseudoexon of 167 nt in the mature mRNA, between exon 13 and exon 14, thus resulting in a truncated Merlin protein. Castellanos and colleagues have been successful in devising a therapeutic strategy for the first time for an NFT 2 case, using a PMO targeting the deep intronic mutation (Fig. 7).

Patient-derived fibroblasts were treated with three different PMO concentrations (5, 10, and 20 μ M) for 24 hours, and a complete inhibition of pseudoexon inclusion was already observed at the lowest concentration tested. PMOs were delivered via the Endo-porter system (Summerton, 2005). Merlin protein levels, although starting to recover at 24 hours, were not significantly increased until 48 hours after PMO delivery. Merlin transcript levels were not affected by control PMO treatment in patient-derived fibroblasts or in fibroblast derived from a non-*NF2* individual treated with PMO, further supporting the sequence-specific effect of PMO treatment. Based on the role that Merlin has in contact-dependent inhibition of proliferation, the functionality of

FIG. 7. Neurofibromatosis type 2. Schematic representation of the identified *NF2* deep intronic mutation and PMO location (Castellanos et al., 2013).

recovered Merlin protein was tested by BrdU incorporation assay. Cell proliferation was reduced by 41% at 24 hours and by 67% at 48 hours when compared with control PMO-treated cells. Functional restoration of Merlin was also proven, by showing that F-actin polymerization abnormalities were eliminated in PMO-treated cells (Castellanos et al., 2013).

Megalencephalic leukoencephalopathy with subcortical cysts type 1

Megalencephalic leukoencephalopathy with subcortical cysts type 1 (MLC1) is a rare type of leukodystrophy (incidence ~1 in 500,000 individuals) associated with mutations in *MLC1* gene. The essential features of this disease include macrocephaly (large head) with onset in infancy, motor delay followed by motor disability in the form of progressive spasticity and ataxia, seizures and cognitive decline. MLC1 is an oligomeric plasma membrane (PM) protein, mainly expressed in glial cells and neurons. Most disease-causing missense mutations dramatically reduce the total and PM MLC1 expression levels in mammalian cells (Duarri et al., 2008).

In a recent work, Mancini and colleagues have described a homozygous *MLC1* deep intronic mutation (c.895-226T>G), which leads to the reduction of transcripts due to the inclusion of a 264 nt nonsense exon between exons 10 and 11 (Mancini et al., 2012). Studies performed with the mutated minigene transfected in HeLa cells showed only a partial inclusion of the pseudoexon, suggesting a “leaky” mechanism of the mutation. A PMO was used to mask the cryptic 5′ splice site activated by this mutation in a patient’s lymphoblastoid cell line (Fig. 8). This led to complete abrogation of the 264-nt pseudoexon, as analyzed by RT-PCR.

Pelizaeus-Merzbacher disease

Pelizaeus-Merzbacher disease (PMD) is a recessive X-linked dysmyelinating disorder of the CNS. PMD affects 1 in 400,000 individuals and its onset is usually in early infancy. The most characteristic early signs are nystagmus (rapid, involuntary, rhythmic motion of the eyes) and hypotonia (low muscle tone). Motor abilities are delayed or never acquired, mostly depending upon the severity of the mutation.

Causes of PMD include *PLP1* deletions, duplications, and point mutations. While deletions are reported only rarely, duplications account for 60%–70% of cases, and point mutations have been identified in 20% of cases. Mutations in the *PLP1* gene can also give rise to spastic paraplegia type 2

(SPG2), which is an allelic form of the disease. A spectrum of CNS disorders from mild SPG2 to severe congenital PMD is associated with the mutations in this gene.

Various types of *PLP1* mutations result in missplicing: Bonnet-Dupeyron et al. (2008) reported that of the 33 mutations they detected, 7 were splicing mutations, including one considered as a missense in exon 2 and a nucleotide substitution in intron 3 outside the classical donor and acceptor splicing sites; Hobson et al. (2002) reported a 19-bp deletion in intron 3 causing missplicing of PLP1; Hübner et al. (2005) described a nucleotide exchange (c.762G>T) at the 3′ border of exon 6 that resulted in partial skipping of exon 6 in the PLP1 mRNA; Lassuthová et al. (2013), finally, recently described three new PLP1 splicing mutations.

PLP1 encodes proteolipid protein (PLP), a major four-pass transmembrane protein in CNS myelin, and is abundantly expressed in oligodendrocytes (Woodward et al., 2008).

The *PLP1* gene undergoes alternative splicing and produces the PLP protein and a shorter protein isoform DM20, which lacks 35 amino acids from the intracellular domain. Two competing 5′ splice sites residing within the 3′ portion of exon 3 are responsible for the alternative splicing of the gene transcript (Nave et al., 1987; Gabern et al., 2007).

Recent work by Regis and colleagues has focused on an exonic missense mutation c.436C>G in the *PLP1* gene of a patient affected by Pelizaeus-Merzbacher disease (Regis et al., 2013). This mutation led to introduction of regulatory motifs that perturb splicing, leading to a loss of the major PLP transcript. An antisense-based approach was used for *in vitro* correction and restoration of PLP transcript in the murine oligodendrocyte precursor cell line Oli-neu transfected with mutated reporter minigenes (Regis et al., 2013) (Fig. 9). A PMO was designed to target the mutated region in a co-transfection experiment of the mutant minigene along with the PMO in Oli-neu cells. Using real-time PCR the authors estimated the increase of the PLP/(DM20+PLP) mRNA ratio as 58% of the wild-type ratio. It remains to be estimated the PMO effect at the protein level, and whether the recovered PLP protein, which bears a mutated amino acid, is functional.

Concluding Remarks

As AONs-induced exon skipping is proving a powerful tool for the correction of DMD and progress has been made towards clinical trials, the scientific community is learning the principles of successful oligonucleotide design and

FIG. 8. Megalencephalic leukoencephalopathy with subcortical cysts type 1 (MLC1). Schematic representation of the identified *MLC1* deep intronic mutation and specific PMO location (Mancini et al., 2012).

FIG. 9. Pelizaeus-Merzbacher disease. Schematic representation of the identified *PLP1* gene mutation and specific PMO location (Regis et al., 2013).

delivery to skeletal and cardiac muscles. Parallel to the development of exon skipping strategies for DMD, the ability to investigate and identify the genetic causes of other genetic diseases has been improving, thanks to new sequencing methodologies. The number of identified splicing mutations and deep intronic mutations inducing inclusion of pseudoexons is accordingly increasing.

The time appears therefore ripe for extending exon-skipping strategies to many other diseases. In this review, we cover, to the best of our knowledge, the use of antisense oligonucleotides to correct splicing defects through exon skipping, with a special focus on diseases affecting the nervous system. The papers presented here for nine different neurogenetic diseases provide proofs of principle that exon skipping can become a therapy for many neurogenetic diseases. However, all the reviewed papers show efficacy either on reporter minigenes in transfected cells or in patient-derived cells in culture (Table 1). To move this therapeutic approach towards clinical application, *in vivo* preclinical studies will be needed, which will be made possible by the availability of animal models of these diseases, bearing splicing mutations. For FTDP-17, for example, two such mouse models are available: the T279 mouse (Dawson et al., 2007) that expresses human N279K mutated tau protein from a minigene, which allows alternative splicing of exon 10 and is under the regulation of the human TAU promoter; and the E10+16C>T mouse, which bears a transgenic human tau minigene with the intronic mutation E10+16C>T (Umeda et al., 2013). Both models show progressive neurodegeneration and tau accumulation.

Since the treatment of neurogenetic disorders with AONs may require life-long repeated dosing, it would be preferable to be able to administer such agents systemically. AONs, indeed, have a relatively long half-life and do not elicit immunogenic responses.

The principal obstacle that hampers the systemic delivery of therapeutic AONs to the CNS is the blood-brain barrier (BBB) (Chen et al., 2012; Wong et al., 2012). Molecules can only enter the brain interstitial fluid by transport through the brain capillary endothelial cells. However, modified AONs are taken up extremely fast in neurons and glial cells with a long residence, once inside the blood brain barrier (BBB).

It has been demonstrated that AONs with a PS backbone have a saturable BBB transport system, which allows them to easily access the CNS when systemically administered *in vivo*. A PS 42-mer injected in the tail vein of mice over-expressing amyloid precursor protein entered the cerebro-

spinal fluid (CSF) and the parenchymal space of the brain; transport was especially high in the hippocampus (Banks et al., 2001; Erickson et al., 2012). However, as many studies indicated lately that BBB is altered in a wide spectrum of brain diseases, care should be taken when extending studies on wild-type animals or specific disease models, to other disease models.

In *mdx* mice, nanoparticles have been used to deliver 2'OMe-PS AONs to muscles by intraperitoneal injections (Rimessi et al., 2009; Ferlini et al., 2010). Notably, the AON doses needed for exon skipping and dystrophin restoration were much lower than with 2'OMe-PS alone. Several studies have been recently focused at the development of nanoparticles for brain drug delivery (reviewed in Masserini, 2013) in general and for the delivery of nucleic acids in particular (reviewed in Pérez-Martínez et al., 2011). It is likely, therefore, that future attempts to systemic delivery of exon-skipping AONs to the brain will take advantage of the knowledge on nanoparticles that is currently accumulating.

Extracellular vesicles in the form of exosomes, the focus of much ongoing research, might also prove capable of delivering AONs across the BBB (Lee et al., 2012). Research in drug delivery to the brain is also attempting transient BBB opening, via bacterial proteins, ultrasounds, or small molecules (such as mannitol or bradykinin).

Delivering AONs via the nasal passages could provide a noninvasive way to bypass the BBB and avoid toxicity due to systemic administration (Dhuria et al., 2010). Small interfering RNAs, for example, have been delivered intranasally to the olfactory bulbs of mice via the olfactory nerve pathway, either naked (Renner et al., 2012) or in cell-penetrating peptide-modified nanomicelles (Kanazawa et al., 2013). Partially destroying the BBB.

Even if systemic delivery would represent a less invasive therapeutic protocol, it still has several disadvantages: the majority of AONs end up in the liver and kidney for each of the different AON chemistries (Sazani et al., 2002; Fluiter et al., 2003; Swayze et al., 2007; Aartsma-Rus and van Ommen, 2009). Moreover, although some AONs have been shown to cross the BBB, approximately 100-fold higher doses must be delivered systemically to achieve AONs concentrations similar to those achieved by intra-CNS delivery (Banks et al., 2001; Erickson et al., 2012) increasing the risk of toxicity.

Many animal studies have therefore employed alternative routes of administration (intraparenchymal injections in specific brain areas, intracerebroventricular injections, intrathecal injections), sometimes administering the AONs

TABLE 1. CURRENT USE OF ANTISENSE OLIGONUCLEOTIDES FOR MISSPLICING CORRECTION IN NEUROGENETIC DISEASES

<i>Disorder</i>	<i>Prevalence</i>	<i>Target gene</i>	<i>Target site</i>	<i>System in which efficacy has been tested</i>	<i>Oligo type</i>	<i>References</i>
Ataxia telangiectasia	1/40,000 to 100,000	<i>ATM</i>	Intronic and exonic cryptic splice sites	Patient-derived lymphoblastoid cell lines homozygous for mutation c.7865C>T or c513C>T or heterozygous for mutation IVS28-159A>G Patient-derived lymphoblastoid cell lines with mutation c.2639-384A>G (in heterozygosis with large deletion c.6807+272_7516-275del5350) Patient-derived lymphoblastoid cell lines with mutation c.1236-405C>T (in heterozygosis with missense mutation c.6326G>A)	PMO pPMO PMO vivo-morpholino PMO vivo-morpholino	Du et al., 2007 Du et al., 2011 Nakamura et al., 2012 Cavalieri et al., 2013
Congenital disorder of glycosylation	1/50,000 to 100,000	<i>PMM2</i>	Intronic 5' and 3' cryptic splice sites	Patient fibroblasts with intronic mutation c.640-15479C>T (in heterozygosis with missense mutation c.691G>A)	PMO	Vega et al., 2009
Frontotemporal dementia and Parkinsonism linked to chromosome 17	Incidence rate unknown	<i>MAPT</i>	Exon 10 5' and 3' splice sites	Green monkey kidney COS-1, SK-N-SH, or HeLa cell lines transfected with minigenes containing mutations S305S; E10+3; E10+14. Rat PC12 cell line	2'OMe-PS	Kalbfuss et al., 2001; Donahue et al., 2006; Peacey et al., 2012
Niemann Pick disease type C	1/150,000	<i>NPCI</i>	Intronic cryptic 5' splice site	Patient fibroblasts with intronic mutation c.1554-1009G>A (in heterozygosis with in-frame deletion insertion p.N961_F966delinsS)	PMO	Rodriguez-Pascual et al., 2009
Neurofibromatosis type 1	1/3000	<i>NFI</i>	Intronic cryptic 5' splice site	Patient fibroblasts and lymphocytes with intronic mutations c.288+2025T>G; c.5749+332A>G; c.7908-321C>G Patient fibroblasts with intronic mutation c.3198-314G>A	PMO PMO	Pros et al., 2009 Fernández-Rodríguez et al., 2011
Neurofibromatosis type 2	1/33,000	<i>NF2</i>	Intronic cryptic 5' splice site	Patient fibroblasts with mutation g.74409T>A	PMO	Castellanos et al., 2013
Megalencephalic leukoencephalopathy with subcortical cysts type 1	1/500,000	<i>MLC1</i>	Intronic cryptic 5' splice site	Patient-derived lymphoblastoid cell lines homozygous for mutation c.895-226T>G	PMO	Mancini et al., 2012
Pelizaeus-Merzbacher disease	1/400,000	<i>PLP1</i>	Exonic cryptic 5' splice site	Murine oligodendroglial Oli-neu cell lines transfected with minigene containing mutation c.436C>G	PMO	Regis et al., 2013

AONs, antisense oligonucleotides; PMO, phosphorodiamidate morpholino oligomer; 2'OMe-PS, 2' O-methyl phosphorothioate antisense oligonucleotides; pPMO, peptide conjugated PMO.

through the use of micro-osmotic pumps (Smith et al., 2006; Hua et al., 2010).

In recent years, oligonucleotides have been successfully employed in preclinical studies on neurodegenerative diseases and for some diseases are being tested in clinical trials. For the therapy of Huntington's disease (HD), AONs that catalyze RNase H-mediated degradation of huntingtin mRNA were proven effective by intraparenchymal bolus delivery directly to the striatum of mice (Carroll et al., 2011) and by transient infusion into the CSF of symptomatic HD mouse models and nonhuman primates (Kordasiewicz et al., 2012). For Alzheimer's disease, AONs directed against the A β region of amyloid precursor protein (APP) were injected intracerebroventricularly (Kumar et al., 2000; Banks et al., 2001; Chauhan et al., 2002; Chauhan et al., 2007; Fiorini et al., 2013), or systemically in the tail vein (Banks et al., 2001). In amyotrophic lateral sclerosis (ALS) AONs continuously infused intraventricularly have been demonstrated to distribute widely throughout the CNS of rodents and primates (Smith et al., 2006; Winer et al., 2013). Results of a phase 1 clinical trial with an AON against superoxide dismutase 1 (SOD1), delivered intrathecally in patients with SOD1 familial ALS have been recently reported (Miller et al., 2013). For SMA, AONs have been ICV infused in the mouse model by means of a micro-osmotic pump and have been observed to be uptaken from CSF by motoneurons of both upper and lower spinal regions (Hua et al., 2011; Passini et al., 2011). A single ICV injection was sufficient to rescue SMA in embryonic or neonate mice (Porensky et al., 2012). On these premises, a phase 1 clinical trial for SMA has been started, which consists in a single AON intrathecal injection (ClinicalTrials.gov identifier NCT01494701).

In proceeding toward oligonucleotide-based exon-skipping therapies for neurogenetic diseases, several issues must be considered. Not only effective AON chemistry and design should be found, but the chemistry should be such that the AON can be synthesized easily and cost effectively on at least gram scale or higher and under good manufacturing practice. Moreover, the best delivery route should be found for the specific disease, aiming at the affected part of the CNS or PNS and keeping in mind that the disease itself could alter the normal physiology, including the BBB.

Finally, as we are dealing often with rare diseases and with patient-specific mutations, we are confronted with issues affecting the development and implementation of personalized medicine and placing new demands upon the regulatory frameworks that control the licensing of medicinal products. For example, at present, each AON for each specific mutation needs its own approval procedure from the European Medicines Agency or from the U.S. Food and Drug Administration.

The lessons learned in the delivery of oligonucleotides to the brain for the cure of the above-mentioned neuromuscular and neurodegenerative disorders, together with the experience gained in the successful design of exon-skipping AONs for the cure of DMD will hopefully make personalized exon-skipping therapies for several neurogenetic disorders a reality in the next few years.

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