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## Developing an antisense oligonucleotide treatment for Spinocerebellar Ataxia Type 3

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# 2

## **Antisense oligonucleotides in therapy for neurodegenerative disorders**

Melvin M. Evers, Lodewijk J. A. Toonen, Willeke M. C. van Roon-Mom (2015).  
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## ABSTRACT

Antisense oligonucleotides are synthetic single stranded strings of nucleic acids that bind to RNA and thereby alter or reduce expression of the target RNA. They can reduce expression of mutant proteins by breakdown of the targeted transcript, but they can also restore protein expression or modify proteins through interference with pre-mRNA splicing. There has been a recent revival of interest in the use of antisense oligonucleotides to treat several neurodegenerative disorders using different approaches to prevent disease onset or halt disease progression and the first clinical trials for spinal muscular atrophy and amyotrophic lateral sclerosis showing promising results. For these trials, intrathecal delivery is being used but direct infusion into the brain ventricles and several methods of passing the blood brain barrier after peripheral administration are also under investigation.

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## INTRODUCTION

There has been a recent revival of interest in the use of antisense oligonucleotides to treat neurodegenerative disorders. Antisense oligonucleotides are synthetic single stranded strings of nucleic acids, between 8-50 nucleotides in length, that bind to RNA through standard Watson-Crick base pairing. Antisense oligonucleotides interfere with gene expression by altering RNA function. Depending on sequence and modifications, antisense oligonucleotides can alter RNA function through several distinct mechanisms, making them a diverse tool. They can be used to restore protein expression, reduce expression of a toxic protein, or modify mutant proteins to reduce their toxicity. Antisense-mediated gene inhibition was first introduced by Stephenson and Zamecnik in 1978 <sup>1</sup>. Using a DNA molecule of 13 nucleotides in length with modifications at the 3' and 5' OH moieties, they showed inhibition of replication and cell transformation of the Rous sarcoma virus. Since then, modifications to the backbone and sugar component have improved stability, binding strength and specificity which has made antisense oligonucleotides suitable for therapeutic application <sup>2</sup>. For several neurodegenerative disorders, antisense oligonucleotide therapy has now moved from the preclinical to the clinical stage, facilitated by the remarkable widespread distribution and cellular uptake of antisense oligonucleotides once delivered into the brain <sup>3,4</sup>.

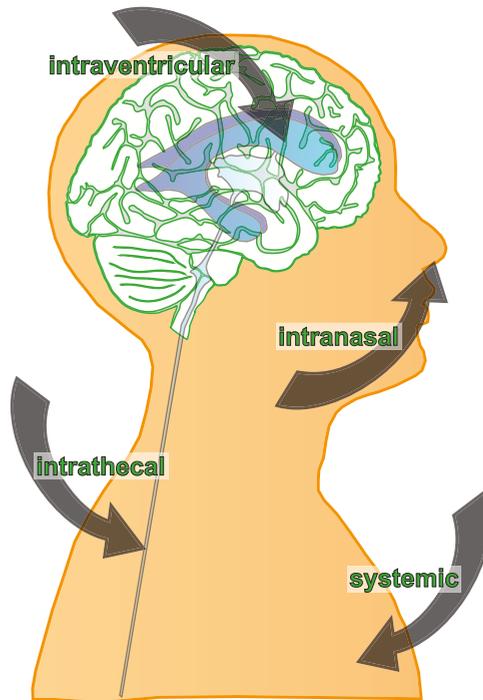
There are many other types of nucleic acid molecules that can interfere at the RNA level using the RNA-induced silencing complex (reviewed in <sup>5</sup>) that promote selective degradation of homologous cellular mRNAs, but these will not be discussed. In this review we will outline the key characteristics of antisense oligonucleotides that make them very suitable for treating neurological disorders. We will discuss the delivery of antisense oligonucleotides to the nervous system and the available chemical modifications of antisense oligonucleotides that have been applied to neurodegenerative disorders. Finally different functional mechanisms to alter RNA function in the nervous system will be discussed, as well as several neurodegenerative disorders where these different antisense oligonucleotide mechanisms are being applied.

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## DELIVERY TO THE NERVOUS SYSTEM

In drug discovery, the aim is to find a substance which is potent, selective, and preferably bioavailable that needs to reach its target at sufficient concentrations <sup>6</sup>. For drugs to reach the nervous system they first have to cross the vascular barrier, which is made up of the blood brain barrier (BBB) or the blood-spinal cord barrier (BSCB). These vascular barriers prevent most molecules from entering the nervous system from the blood circulation. Despite this gate-controlling system, essential nutrients, such as glucose, are permitted to pass <sup>7</sup>. The vascular barriers of the nervous system are comprised of a monolayer of endothelial cells forming tight junctions through interactions of cell adhesion molecules <sup>8</sup>. Other structural components are astrocytes that surround the endothelial cells with their processes, pericytes located between the endothelial cells and astrocytes, macrophages, and finally the basement membrane. The endothelial cells of the BBB are characterized by only few fenestrae and pinocytotic vesicles,

limiting transport to and from the brain. The BBB thus also largely separates the peripheral immune system from the brain. Although the BSCB is largely made up of the same components as the BBB, there are some functional and morphological differences <sup>9</sup>. For instance in the BSCB, the equivalent of the BBB endothelial cells are the choroid plexus epithelial cells <sup>10</sup> and the permeability of the two barriers are different probably due to differences in tight junction protein expression <sup>11</sup>. In neurodegenerative diseases, disruption of the vascular barrier is common <sup>8, 12, 13</sup> and it was shown in animal models that a compromised BBB barrier in itself can lead to neurodegeneration <sup>12</sup>. However, for most diseases of the nervous system that could be treated with antisense oligonucleotides, the antisense oligonucleotides will not be able to cross the vascular barrier when delivered systemically. Several methods have been used to deliver antisense oligonucleotides to the nervous system (see Fig. 1). Antisense oligonucleotide modifications will largely determine the most efficient route of delivery. In this chapter the different mechanisms for antisense oligonucleotides to enter the nervous system will be outlined. We will next review the different types of antisense oligonucleotide modifications used for neurodegenerative disorders in chapter 3.



**Figure 1.** Routes for delivery of antisense oligonucleotides to the central nervous system. Next to systemic delivery, antisense oligonucleotides can be directly delivered into the cerebrospinal fluid through intracerebroventricular or intrathecal infusion using an implanted reservoir that is connected to the ventricles within the brain or spinal cord via an outlet catheter. An alternative less invasive route of delivery of antisense oligonucleotides could be conceivable via intranasal administration.

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## PERIPHERAL DELIVERY

Efforts are ongoing to deliver antisense oligonucleotides to the nervous system via the systemic route. In general, when a drug is administered systemically, a fraction will be bound to proteins (e.g. serum albumin, lipoprotein etc.) and a fraction will be unbound. The bound fraction is the pharmacologically relevant fraction, since it is available to cross the vascular barrier<sup>8</sup> depending on its physicochemical properties.

The first mechanism by which antisense oligonucleotides could cross the vascular barrier is simple diffusion. Small lipophilic substances which have a hydrogen bond are more likely to pass the vascular barrier<sup>14</sup> than compounds without hydrogen bonds and only molecules with a molecular weight smaller than 400 Dalton are able to cross the vascular barrier<sup>15</sup>. Antisense oligonucleotides generally have a molecular weight of approximately 6,000 to 10,000 Dalton and thus are too large to cross the vascular barrier by simple diffusion and reach an effective concentration in the nervous system. Indeed, early studies showed only limited brain uptake of peripherally administered radioactive antisense oligonucleotides with less than 1% of the injected oligonucleotide measured in brain<sup>16-19</sup>. The mechanism by which this antisense oligonucleotide crossed the BBB was named the saturable oligonucleotide transport system<sup>17</sup>.

Another mechanism to cross the vascular barrier is via receptor-mediated endocytosis, which allows macromolecules, such as transferrin, insulin, leptin, and insulin-like growth factor 1, to enter the nervous system<sup>20</sup>. Studies by Lee and associates used biotinylated antisense oligonucleotides captured with a streptavidin conjugated radioactive labelled monoclonal antibody to the mouse transferrin receptor to quantify gene expression *in vivo*<sup>21</sup>. This radioactive labelled antisense oligonucleotide conjugate reached the brain through the endogenous transferrin transport pathway (receptor-mediated endocytosis) in a transgenic mouse model<sup>21</sup>. This same transferrin transport pathway was used to transport nanoparticles carrying antisense oligonucleotides targeting aquaporin 4 into the brain parenchyma<sup>22</sup>.

More recent research has focussed on cell-penetrating peptide (CPP)-based delivery systems<sup>23</sup>. These have shown strong transmembrane capacity and great potential for therapeutic approaches for neurodegenerative disorders<sup>24</sup>. CPPs can be up to 30 amino acids in length and can carry a wide variety of cargos. Different CPPs use distinct cellular translocation pathways, which depend on cell types and cargos<sup>25</sup>. Systemically delivered antisense oligonucleotides tagged with arginine-rich CPPs were able to cross the BBB and were widely distributed throughout the brain of wild-type mice<sup>26</sup>. To note, not all types of antisense oligonucleotide modifications are suitable for coupling with CPPs. Although two promising CPP antisense oligonucleotides were abandoned as therapeutic agents since repetitive intravenous (IV) bolus injections of CPP antisense oligonucleotides caused lethargy and weight loss in rats<sup>27</sup> and tubular degeneration in the kidneys of monkeys<sup>28</sup>, CPP systems are still a promising delivery system.

Although not tested thus far, antisense oligonucleotides encapsulated in exosomes would theoretically also be able to cross the vascular barrier after IV injection. Exosomes are a well-studied class of extracellular vesicles known to mediate communication between cells through transfer of proteins and nucleic acids<sup>29,30</sup>. IV injection of exosomes transduced with short viral

peptides derived from rabies virus glycoprotein (RVG) resulted in crossing of the BBB and siRNA delivery to the brain<sup>31</sup>. These IV injected RVG-targeted exosomes bound to neurons, microglia and oligodendrocytes, resulting in a specific gene knockdown in the brain<sup>29</sup>.

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## DIRECT DELIVERY TO THE NERVOUS SYSTEM

Besides mechanisms to cross the vascular barriers, there are also techniques that bypass them through direct infusion into the cerebrospinal fluid (CSF).

Antisense oligonucleotides can be infused intracerebroventricularly (ICV) after which the ASOs would have to pass the ependymal cell layer that lines the ventricular system to enter the parenchyma. Intrathecal (IT) delivery means delivery of the ASOs into the subarachnoid space of the spinal cord. From here it will have to pass the pia mater to enter the parenchyma. ASOs can be delivered ICT or IT through an outlet catheter that is connected to an implanted reservoir. Drugs can be injected into the reservoir and delivered directly to the CSF.

This route of delivery has several advantages over peripheral administration. It results in immediate high drug concentrations in the CSF meaning that a smaller dose can be used, potentially minimizing toxicity. Also, because there is free exchange between the CSF and brain parenchyma, and the BBB prevents transport of the antisense oligonucleotides into the peripheral circulation, direct delivery into the nervous system can relatively rapidly result in therapeutic drug concentrations. This infusion technique was used in antisense oligonucleotide applications in rodent models of neurodegenerative disorders as well as non-human primates<sup>32</sup>. Moreover, IT drug therapy has been applied to a wide variety of neurological conditions<sup>33,34</sup>. To date there have been two phase I clinical trials completed using IT infusion of antisense oligonucleotides in amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) patients<sup>35,36</sup> without any major adverse side effects<sup>35</sup>.

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## INTRANASAL DELIVERY TO THE BRAIN

Although to date there have been very few studies showing delivery of antisense oligonucleotides to the brain via intranasal administration, this is a very promising alternative route of delivery. After intranasal administration, molecules can be transported along the olfactory and trigeminal nerve pathway and the rostral migratory stream<sup>37</sup>. It is less invasive compared to the above mentioned antisense oligonucleotide delivery methods and already was used successfully in clinical trials showing improved cognition after intranasal insulin application in Alzheimer disease patients<sup>38</sup>. Also intranasal delivery of small interfering RNA (siRNA) was shown to be very efficient. CPP conjugated to glycol-polycaprolactone copolymers were able to target siRNAs to the brain via the intranasal route<sup>39</sup>. In rats, intranasal delivery of antisense oligonucleotides was found to lead to good distribution throughout the brain, and was able to slow down intracerebral tumour growth<sup>40</sup>. These studies hence indicate that intranasal delivery may become a viable option for antisense oligonucleotide delivery in the future.

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## CHEMICAL MODIFICATIONS

Initially, antisense oligonucleotides were used in the form of synthetic unmodified DNA<sup>41</sup>. Though successful, these types of oligonucleotides proved very susceptible to degradation by endo- and exonucleases. It quickly became apparent that if antisense oligonucleotides were to be used for clinical applications, their pharmacological profile would have to be enhanced. Oligonucleotides have since benefited from technical advances in chemical modifications leading to significantly improved characteristics. Antisense oligonucleotides are available with a range of different modifications on the phosphate backbone and ribose sugar group in the case of RNA (see Table 1). The modifications used in studies of neurodegenerative disorders and their mode of actions will be discussed in the paragraphs below.

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## BACKBONE MODIFICATIONS: COUNTERACTING NUCLEASES

One of the main factors impeding antisense oligonucleotide efficacy is their rapid degradation by endo- and exonucleases. A 3' to 5' exonuclease is able to degrade unprotected antisense oligonucleotides within 30 minutes in serum<sup>42</sup>, while intracellular exo- and endonucleases can lead to degradation in an even shorter timespan<sup>43</sup>. Brain associated  $\alpha$ -exonuclease is likely responsible for degradation of antisense oligonucleotides in CSF<sup>4</sup>. One of the first successful modifications providing a good degree of protection from these nucleases is the phosphorothioate (PS) backbone. Termed the first generation of oligonucleotide modifications, the PS backbone is accomplished by replacement of one of the non-bridging oxygen atoms in the backbone with a sulphur atom<sup>44</sup>. Oligonucleotides with this modification are more stable with reported half-lives of 9 hours in human serum and 19 hours in rat CSF<sup>45</sup>. The negative charge of PS DNA furthermore allows for good uptake into various cell types *in vitro*<sup>46</sup> and into various brain cell types after microinjection into brain<sup>47</sup>. Another important characteristic of the PS backbone is its ability to activate ribonuclease H (RNase H), allowing for use in applications where target RNA downregulation is desired<sup>48</sup>. RNase H recognizes an RNA-DNA heteroduplex, and then cleaves the RNA strand, resulting in a 5-phosphate on the product and release of the intact DNA strand<sup>49, 50</sup>. A less favourable characteristic of the PS backbone is that it destabilize duplexes, leading to a decrease in melting temperature<sup>51</sup>. Cytotoxic effects of PS at high concentrations have been reported (reviewed in<sup>52</sup>), and are thought to be related to protein binding<sup>53, 54</sup> or complement activation<sup>55</sup>. PS modified antisense oligonucleotides have undergone extensive pharmacokinetic testing aimed at peripheral administration. In this context, it was found that PS oligonucleotides bind serum proteins, leading to reduced renal clearance and an increased circulation time<sup>56, 57</sup>. These favourable pharmacokinetic properties in the periphery are contrasted by the poor ability of PS oligonucleotides to cross the BBB<sup>58</sup>.

**Table 1.** Chemical modifications of antisense oligonucleotides.

	Modification	Main features
Phosphate linkage	phosphodiester	Naturally occurring Inexpensive
	phosphoramidate	High affinity High nuclease resistance Does not support RNase H
	phosphorothioate (PS)	Improved nuclease resistance Improved binding to plasma proteins (preventing kidney clearance)
Sugar modification	LNA	Strong binding affinity Increased nuclease resistance Does not support RNase H
	2OMe	Improved binding affinity Improved nuclease resistance Inhibits immune stimulation of PS backbone Does not support RNase H
	MOE	Improved binding affinity Improved nuclease resistance Inhibits immune stimulation of PS backbone Does not support RNase H
	2'-Fluoro	Improved binding affinity Does not support RNase H cleavage
	cEt	Strong binding affinity Improved nuclease resistance Does not support RNase H
	tc-DNA	Improved nuclease resistance Improved binding affinity Does not support RNase H
	Non-ribose modifications	PMO
	PNA	Uncharged High binding affinity Low toxicity High nuclease resistance

Sugar modifications are typically used together with the PS backbone modification. 2OMe: 2'-O-methyl, cEt: S-constrained-ethyl, LNA: Locked nucleic acid, MOE: 2'-O-methoxy-ethyl, PMO: phosphorodiamidate morpholino oligomer, PNA: peptide nucleic acid, Tc-DNA: Tricyclo-DNA

Main disadvantages	Application	Frequency of use	Refs
Rapidly degraded by nucleases	Steric hindrance	-	[44,105-107]
Quickly cleared by kidney	RNase H cleavage		
Quickly cleared by kidney	Steric hindrance	+	[37]
Can cause immune response/ cytotoxicity at high concentrations	Steric hindrance	+ + + + +	[108-110]
Slightly reduced binding affinity compared to phosphodiester	RNase H cleavage		
Higher toxicity than other modifications	Steric hindrance	+ + +	[74,111,112]
Higher risk of a-specific binding			
Higher propensity for self-annealing			
Lower affinity than most other modifications	Steric hindrance	+ + + +	[113,114]
Lower affinity than most other modifications	Steric hindrance	+ + + +	[28,33,115]
Little improvement for nuclease resistance	Steric hindrance	+	[116]
	Steric hindrance	+ +	[117-119]
Little research data available	Steric hindrance	+	[78,79]
Rapid clearance	Steric hindrance	+ + +	[98,120,121]
Poor uptake in cell nucleus			
Poor pharmacokinetic properties			
Rapid clearance	Steric hindrance	+ +	[92,112,122,123]
Poor uptake/ pharmacokinetic properties			

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## SUGAR GROUP MODIFICATIONS: IMPROVING AFFINITY AND REDUCING TOXICITY

In addition to the PS backbone, RNA oligonucleotides can be further modified at the 2' position of the ribose sugar. These types of modifications are termed the second generation oligonucleotide modification, and, in combination with the PS backbone, have been of great importance for advancing oligonucleotide safety and pharmacologic properties. In this class of modifications, the 2'-O-methyl (2OMe) and 2'-O-methoxy-ethyl (MOE) have proven most successful thus far. These modifications increase hybridization affinity to their target RNA compared to unmodified phosphorothioates<sup>59-61</sup>, as well as increased resistance toward nuclease degradation<sup>61, 62</sup>. An additional key advantage of the 2' modifications is their ability to reduce sequence independent toxicity arising from the PS backbone<sup>63</sup>, which also holds true in the CNS<sup>64</sup>. An important trait of 2'-modifications is their inability to recruit RNase H<sup>65</sup> and oligonucleotides that are fully modified in this fashion thus cannot induce RNase H-mediated target RNA downregulation.

For 2'-O-modified-PS antisense oligonucleotides only very mild toxicity has been reported, which did not interfere with their desired effects after ICV delivery in rodent brain<sup>66</sup>, or in cultured neuronal cells<sup>67</sup>. Although it has been shown that PS antisense oligonucleotides can have an immunostimulatory effect via toll-like receptors, appropriate 2'-O modifications, such as 2OMe and MOE, can suppress these effects<sup>68-70</sup>. It is important to mention that possible toxic and immunostimulatory effects of 2OMe-PS antisense oligonucleotides may also be due to dosage, concentration, or duration of treatment<sup>71</sup>.

Locked nucleic acids (LNA) are a 2'-modification where the 4'-carbon has been tethered to the 2'-hydroxyl group. LNAs provide resistance to nucleases<sup>72</sup> and show much improved hybridization compared to the other 2'-modifications<sup>73,74</sup>. LNAs are also unable to induce RNase H-mediated target RNA downregulation<sup>75, 76</sup>. Though LNAs provide a better RNA binding affinity than most other 2'-modifications, there appear to be more severe toxicological problems with these oligonucleotides in systemic treatment<sup>77</sup>. Whether this also holds true for the CNS will remain to be determined, though one study reports that LNAs are tolerated in rat brain<sup>78</sup>. Additionally, the high affinity of LNAs can lead to a reduction in target specificity<sup>74,79</sup>. For these reasons, a chimera design where LNA modified nucleosides are interspersed by unmodified or differently 2'-O-modified nucleosides can be implemented<sup>73</sup>. LNAs can be further modified by addition of oligospermine nucleobases. The resulting antisense oligonucleotide-oligospermine conjugates are known as zip nucleic acids (ZNA)<sup>80</sup>. These conjugates lack the polyanionic nature and electrostatic repulsion of the negatively charged phosphate backbones, leading to increased binding affinities and improved cellular uptake. ZNA conjugates were found to reduce huntingtin expression in Huntington disease (HD) patient-derived cells<sup>81</sup>. Although ZNA conjugates are primarily used as probes for real-time PCR, they possess some therapeutically interesting properties.

Another conformationally constrained oligonucleotide modification available is tricyclo-DNA (tc-DNA). This modification is aimed at lessening the flexibility around the C3'-C4' and

C4'-C5' bonds by addition of an ethylene bridge fused with a cyclopropane unit. The result in a more stable duplex formation<sup>82</sup> that is not compatible with RNase H. Tc-DNA has been reported to be stable in serum and resulted in more potent splicing correction compared to a 2OMe-PS oligonucleotide when tested in cells<sup>83</sup>. To date, there have been very few studies performed making use of tc-DNA, but there has been one report stating splicing modulation in the brain occurred after peripheral administration of tc-DNA in a Duchenne muscular dystrophy mouse model<sup>84</sup>. Interestingly, dystrophin restoration in the mouse brain resulted in complete reversal of its behavioral phenotype<sup>84</sup>.

Other sugar modifications less frequently used in CNS studies are the 2'-fluoro and S-constrained-ethyl (cEt) oligonucleotides. The 2'-fluoro, akin to OMe and MOE modifications, replaces the 2'-hydroxy with a fluoro group and provides a higher affinity than most other 2'-modifications<sup>65</sup>, in addition to providing resistance to nucleases<sup>85</sup>. The cEt modified antisense oligonucleotides provide similar binding affinity as LNA, yet appear to have a more favourable toxicity profile<sup>86</sup>, and have recently shown good promise in a humanized mouse model for HD<sup>87</sup>.

Taken together, the second generation of oligonucleotide modifications have provided a good degree of improvement on the PS backbone, with enhancements in nuclease resistance, binding affinity and reduction of PS-induced toxicity. However, in light of the fact that most CNS research utilising oligonucleotides aims at downregulation of target RNA, the fact that these modifications are not compatible with RNase H cleavage is an important consideration. For this reason, a RNase H compatible gapmer strategy was conceived of (described in chapter 4), in which a PS oligonucleotide is not modified with 2'O-modifications over its entire length. Importantly, a MOE-PS gapmer oligonucleotide was the first to make it to clinical trials for a neurodegenerative disorder, and has shown favourable tolerability<sup>35</sup>.

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## OTHER OLIGONUCLEOTIDE MODIFICATIONS

Besides the PS backbone and several ribose sugar modifications described above, efforts have been made with newer modifications that combine backbone, ribose and nucleosides modifications.

Peptide nucleic acids (PNA) are generated by replacement of the entire sugar phosphate backbone with polyamide linkages<sup>88</sup>, but are still able to hybridize through Watson-Crick binding<sup>89</sup>. PNAs are uncharged, and provide a high resistance to nuclease and protease degradation<sup>90</sup>. Another important feature of PNAs is their high binding affinity for RNA<sup>91</sup>. In light of their inability to activate RNase H, PNAs are mostly implemented in splicing modulation approaches or translation inhibition. A clear shortcoming of PNAs is their poor cellular uptake<sup>92</sup> and water insolubility<sup>93</sup>. However, both uptake and water solubility can be improved using peptide conjugates<sup>94,95</sup>. Uptake in neuronal cells of unmodified PNA *in vivo* has been reported<sup>96</sup> but the use of PNA antisense oligonucleotides in neurodegenerative disorders

remains somewhat limited. When administered peripherally, PNAs are rapidly cleared<sup>97</sup> and these poor pharmacokinetic properties likely in part explain their limited *in vivo* use thus far.

Phosphorodiamidate morpholino oligomers (PMO) have a morpholine ring instead of the ribose ring, and have phosphoroamidate intersubunit linkages. Similar to PNAs, PMO backbones are neutrally charged and are not compatible with RNase H<sup>98</sup>. PMOs are highly resistant to nuclease and protease degradation<sup>99</sup>. In a phase 1 clinical trial using IV administration, PMOs were well tolerated, but were shown to suffer from the same pharmacokinetic shortcomings as PNAs since they are rapidly cleared from the blood<sup>100</sup>. Peptides can be conjugated to the PMO for improved cellular uptake and pharmacokinetics<sup>101</sup>. Also, bare PMO chemistry has been successfully used to modify splicing when administered directly in mouse brain<sup>102</sup>, though toxicity at higher doses may occur<sup>103</sup>. Another interesting conjugated PMO is the vivo-morpholino, which features a terminal octaguanidinium dendrimer aimed at improving cell permeability and thus tissue uptake when administered peripherally<sup>104</sup>. Unfortunately, there have been reports of severe toxicity following IV administration of vivo-morpholino in mice<sup>105</sup>. Importantly, the increased toxicity of vivo-morpholino compared to bare PMOs also appears to hold true in the brain<sup>103,106</sup>. Crossing of the BBB does not appear to occur efficiently for vivo-morpholinos<sup>107</sup>, and, though microinjection in rat brain was effective, target protein downregulation lasted for only 14 days<sup>108</sup>.

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## FUNCTIONAL MECHANISMS

Depending on the chemistry and target site, antisense oligonucleotides can be used in many different ways to modulate gene expression. Below we will discuss the major functional mechanisms that can be used in neurodegenerative disorders (for schematic representation see Fig. 2) where either the RNA is broken down or is altered with the use of antisense oligonucleotides.

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## RNASE H-MEDIATED DEGRADATION

As described in the previous paragraph, 2'-modifications enhance safety and pharmacologic properties of antisense oligonucleotides. However, RNase H requires a free 2'-oxygen and oligonucleotides that are fully 2'-modified cannot induce RNase H-mediated target RNA downregulation<sup>65</sup>. To induce gene knockdown through RNase H, an alternative strategy using gapmers has been developed (Fig. 2A and B). A gapmer antisense oligonucleotide consists of a central DNA region with flanking 2'-modified nucleosides. Because the active site of an exonuclease only binds to two or three residues at the 3'- or 5'-end, a short stretch of 2'-modified RNA nucleosides at both ends is sufficient for the protection against exonucleases<sup>109</sup>. The gapmer molecule thus benefits from nuclease resistance and improved uptake from the wings, whilst activating RNase H owing to the gap region<sup>65</sup>.

If the target protein has important cellular functions general downregulation would be detrimental. In such case specific lowering of the mutation-containing protein is desired.

Selective RNase H-mediated degradation can be achieved using antisense oligonucleotide gapmers targeting (1) specific point mutations <sup>110</sup>, (2) structural differences between wild-type and mutant mRNA <sup>111-113</sup>, or (3) a single nucleotide polymorphism (SNP) that is unique to the mutant RNA <sup>114</sup> (Fig. 2B).

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## STERIC HINDRANCE

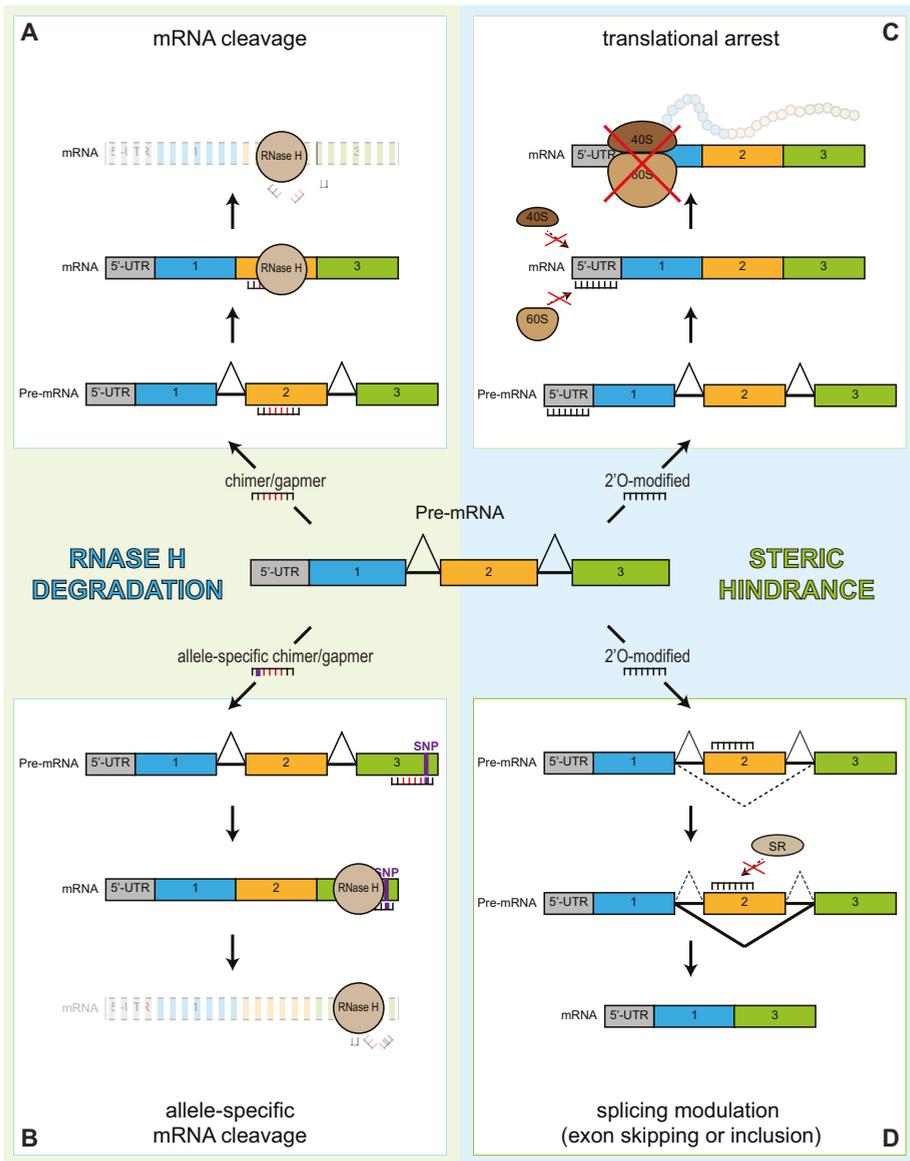
Next to RNase H-mediated breakdown of mRNA, protein levels can also be reduced by preventing translation (Fig. 2C). Here, all nucleotides of the antisense oligonucleotide have sugar modifications rendering them RNase H resistant. Suppressing RNA translation to reduce protein levels could be achieved by antisense oligonucleotides targeting the RNA translation start site or sterically blocking the binding of RNA binding protein complexes, such as ribosomal subunits <sup>115</sup> (Fig. 2C).

Other antisense oligonucleotide applications that do not induce the lowering of transcript levels are gaining more interest. The best-known application is the manipulation of splicing <sup>116</sup> (Fig. 2D). Most human genes express more than one mRNA through alternative splicing <sup>117</sup> and this is an important mechanism for gene regulation. In brain there is a very high level of alternative splicing <sup>118</sup> and disruption of normal splicing patterns can cause or modify human disease. Antisense oligonucleotides can be used to interfere with this naturally occurring regulatory mechanism by targeting splice sites or exonic/intronic inclusion signals that will result in skipping or inclusion of the targeted exon and altering the RNA and protein sequence (Fig. 2D). For neurodegenerative disorders this can have multiple applications, e.g. switching from a harmful isoform to a less harmful isoform <sup>119</sup>, skipping an aberrantly included exon to restore the normal transcript <sup>120</sup>, removing disease-causing mutations from genes <sup>119</sup>, or restoring the reading-frame by removing an exon with a mutation <sup>121</sup>.

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## ANTISENSE OLIGONUCLEOTIDE APPROACHES FOR NEURODEGENERATIVE DISORDERS

For non-neurodegenerative disorders several antisense oligonucleotides therapies are under development with antisense-mediated exon skipping for Duchenne muscular dystrophy the closest to clinical application <sup>122, 123</sup>. Furthermore, one of the first repeat expansion diseases where antisense oligonucleotide mediated RNaseH dependent degradation of mutant RNA was developed for is Myotonic Dystrophy type 1 <sup>124</sup>. However, reaching sufficient concentrations of antisense oligonucleotides in the organ of interest and establishing sufficient high cellular uptake is a major issue. Here neurodegenerative disorders have the advantage. After reaching the nervous system, most antisense oligonucleotides are readily taken up by neurons and glia <sup>3,4</sup>. It has been suggested that uptake occurs through nucleic acid channels <sup>125</sup> but the exact mechanism of cellular uptake is still poorly understood <sup>126-128</sup>. Once the antisense oligonucleotide has been delivered into the nervous system, the vascular barriers will prevent it from entering the periphery and there will be no rapid excretion or break down by the kidney and liver,



**Figure 2. Functional mechanisms of antisense oligonucleotide for central nervous system disorders.** Depending on the chemistry and target site, antisense oligonucleotides can be used in different ways to modulate gene expression: **A.** Antisense oligonucleotide gapmers induce RNase H-mediated breakdown of target RNA. **B.** RNase H-mediated breakdown by antisense oligonucleotide gapmers targeting specific point mutations, structural differences between wild-type and mutant mRNA or a single nucleotide polymorphism (SNP) that is unique to the mutant RNA. **C.** Suppressing RNA translation by antisense oligonucleotides targeting the RNA translation start site or sterically blocking the binding of RNA binding protein complexes, such as ribosomal subunits. **D.** Antisense oligonucleotides binding to targeting splice sites or exonic/intronic inclusion signals that will result in skipping or inclusion of the targeted exon. SR, pre-mRNA splicing machinery.

which means that it is easier to reach clinically effective concentrations. In rodents, it was shown that the tissue half-life of 2OMe-PS antisense oligonucleotides in peripheral tissue was around 10–65 days after IV injection<sup>129,130</sup>. ICV infused MOE-PS antisense oligonucleotides can have a much longer tissue half-life of 71–206 days in the brain and 145–191 days in the spinal cord<sup>131</sup>. This striking difference in tissue half-life between neuronal and peripheral tissue could be explained by lower intrinsic nuclease activity in neuronal tissue compared to peripheral tissues<sup>4</sup>, or by differences in endosome handling between neuronal and peripheral cell types<sup>131</sup>. Although antisense oligonucleotides have a long half-life they are eventually degraded, offering the possibility to discontinue treatment<sup>132</sup>. For clinical trials with long term administration it would be ideal to have the option to terminate the treatment effect if unwanted side effects occur. A so called antidote, or decoy, was recently applied in SMA mice initially treated with MOE-PS antisense oligonucleotides<sup>131</sup>. Administration of a fully complementary oligonucleotide three weeks after ICV injection of a therapeutic MOE-PS antisense oligonucleotide was reported to reverse the antisense oligonucleotide-mediated survival motor neuron 2 (SMN2) exon 7 inclusion in SMA mice<sup>131</sup>.

For many neurodegenerative disorders it is known that certain proteins are specifically up regulated, either as a primary or secondary event in the disease process. In other disorders, proteins are not expressed due to mutations in a gene, or are aberrantly spliced and toxic protein isoforms are expressed. Mutant proteins can also aggregate, which can contribute to disease pathology. This paragraph will outline several neurodegenerative disorders where the use of antisense oligonucleotides is a promising therapeutic strategy. Examples are given where antisense oligonucleotide treatment resulted in therapeutic benefit in animal models and/or clinical trials (Table 2).

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## REDUCING PROTEIN EXPRESSION

Next to blocking translation, antisense oligonucleotides can also modulate splicing to introduce an out-of-frame deletion on transcript level, resulting in nonsense-mediated decay and knock down of target protein expression<sup>133</sup>. This approach is an alternative approach to RNase H-mediated degradation<sup>134</sup>. Alzheimer disease is the most common form of dementia where cleavage of amyloid precursor protein (APP) at the  $\beta$ -secretase and  $\gamma$ -secretase site causes elevated levels of  $\beta$ -amyloid peptide (A $\beta$ ). This is considered a key event in the pathogenesis of Alzheimer disease<sup>135</sup>. One of the first approaches to down regulate APP translation made use of PS antisense DNA oligonucleotides. After tail vein injections of PS antisense oligonucleotide there was a reduction in APP and modest behavioural improvement in a transgenic Alzheimer disease mouse model<sup>19</sup>. Whether these PS antisense DNA oligonucleotides consisted of modified 2' nucleosides or what the mechanism of action was has not been described. Next to A $\beta$  plaque formation, another prominent feature of Alzheimer disease is aggregation of microtubule associated protein tau to form intracellular neurofibrillary tangles and glial tangles<sup>136</sup>. For Alzheimer disease and other tauopathies, antisense oligonucleotides have been applied to reduce expression of the tau protein<sup>133</sup>. Tau is encoded by the *MAPT* gene and several

Table 2. Antisense oligonucleotide approaches for neurodegenerative disorders.

Disease	Target gene	Oligonucleotide chemistry	Mechanism	Organism
SMA	SMN2	MOE-PS	Splicing modulation	SMA patients
	SMN2	MOE-PS, PMO and 2'OMe-PS	Splicing modulation	SMA mice and NH primates
	SMN2	PMO	Splicing modulation	SMA mouse
	SMN2	PMO and vivo-PMO	Splicing modulation	SMA mouse
	SMN2	PMO	Splicing modulation	SMA mouse
	SMN2	2OMe-PS, bifunctional	Splicing modulation + recruitment splicing factors	SMA mouse
	SMN2	MOE-PS	Splicing modulation	SMA mouse
ALS	SOD1	MOE-PS gapmer	RNase-H mediated degradation	ALS patients
	AChE	Oligodeoxynucleotide, 3' 3'-nucleosides 2'Ome modified	RNase-H mediated degradation	SOD1 mouse
	C9ORF72	MOE-PS gapmer and 2OMe-PS	RNase-H mediated degradation or steric hindrance of GGGGCC repeat	iPSC differentiated neurons
	C9ORF72	MOE-PS gapmer	RNase-H mediated degradation	Mouse
	SOD1	MOE-PS gapmer	RNase-H mediated degradation	SOD1 rats
	SOD1	MOE-PS gapmer	RNase-H mediated degradation	SOD1 rats and rhesus monkey
	GluR3	PNA	Not described	SOD1 mouse
	p75NTR	PNA	Translational arrest	SOD1 mouse
HD	Mutant HTT	MOE-PS and cEt-PS gapmers	RNase-H mediated degradation	neuronal culture
	Mutant HTT	MOE-PS and cEt-PS gapmers	RNase-H mediated degradation	HD mouse

Administration	Molecular effect	Phenotypic effect	Clinical/ preclinical	Reference
Intrathecal	Not tested. Good CSF distribution, no serious adverse events	NA	Phase 1 clinical	178
ICV in mice, intrathecal in NH primates	Mice: up to 3.5 fold change in transcript ratio Primate: predicted therapeutic dose	NA	Preclinical	131
ICV	8 fold increase SMN2 protein level	Lifespan extended from 13 to 54 days	Preclinical	179
ICV and SC	11 fold increase transcript ratio	Lifespan increased by 60 days	Preclinical	103
ICV	6 fold increase full length SMN2 transcript, and 3-fold increase protein	Lifespan extended from 15 to >100 days	Preclinical	102
ICV	3.5 fold increase SMN protein	Lifespan extended by 8 days	Preclinical	180
ICV and SC	ICV: 83% exon inclusion in brain SC: 47% exon inclusion in brain	ICV: lifespan extended to 16 days ICV+IP: lifespan extended to 173 days	Preclinical	181
Intrathecal	Not tested. No serious adverse events.	NA	Phase 1 clinical	35
IP	Higher motor neuron count	Lifespan extended by 10 days	Preclinical	182
NA	Reduction in RNA foci and RNA binding protein aggregation with both strategies	Mitigation excitotoxicity	Preclinical	141
ICV	Reduction C9ORF72 by 65% in brain and spinal cord.	No behavioural deficits induced	Preclinical	142
ICV	SOD1 protein reduction by ~40%	NA	Preclinical	183
ICV	Rat: 60% downregulation SOD1 protein	Lifespan extended by 10 days	Preclinical	132
IP	No effect seen at protein level in spinal cord	Lifespan extended by 10%	Preclinical	184
IP	Apparent p75NTR downregulation in spinal cord, though not quantified	Lifespan extended by ~10 days	Preclinical	185
bath application	Almost complete downregulation mutant htt protein	NA	Preclinical	156
ICV	90% reduction mutant htt protein	NA	Preclinical	87

Table 2. (continued)

Disease	Target gene	Oligonucleotide chemistry	Mechanism	Organism
	Mutant HTT	MOE-PS and cEt-PS gapmers	RNase-H mediated degradation	HD mouse
	HTT	MOE-PS gapmer	RNase-H mediated degradation	HD mouse
	HTT	MOE-PS gapmer	RNase-H mediated degradation	HD mice, Rhesus monkey
	HTT	MOE-PS and cEt-PS gapmers	RNase-H mediated degradation	HD mice
	HTT	PNA and LNA	Unknown mechanism, CAG repeat targeting	Fibroblasts
AD	HTT	2OMe-PS	Splicing modulation	Mouse
	Mutated APP	MOE gapmer	RNase-H mediated degradation	AD mouse
	GSK-3 $\beta$	PS DNA	RNase-H mediated degradation	AD mouse
Creutzfeldt-Jakob	PrPc	MOE-PS gapmer	RNase-H mediated degradation	Prion infected mouse
SCA3	Ataxin-3	2OMe-PS	Splicing modulation	Mouse
Menkes disease	ATP7A	PMO	Splicing modulation	Zebrafish
FTD	Tau	2OMe-PS and PNA	Splicing modulation	Neuroblastoma cells with tau minigene

2OMe, 2'-O-methyl; AChE, acetylcholinesterase; AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; ATP7A, copper-transporting ATPase 1; cEt, S-constrained-ethyl; CJD, Creutzfeldt-Jakob disease; CSF, cerebrospinal fluid; FTD, frontotemporal dementia; GluR3, glutamate receptor subunit 3; GSK-3 $\beta$ , glycogen synthase kinase 3; HD, Huntington disease; HTT, huntingtin; ICV, Intracerebroventricular; IP, intraperitoneal; iPSC, Induced pluripotent stem cells; LNA, locked nucleic acid; NA, not assessed; NH, non-human; MOE, 2'-O-methoxy-ethyl; p75NTR, p75 neurotrophin receptor; PMO, phosphorodiamidate morpholino oligomer; PNA, peptide nucleic acid; PrP<sup>c</sup>, cellular prion protein; PS, phosphorothioate; SC, subcutaneous; SCA3, spinocerebellar ataxia type 3; SMA, spinal muscular atrophy; SMN2, survival of motor neuron 2; SOD1, superoxide dismutase 1

PMOs were designed to target MAPT transcripts. In human neuroblastoma cell lines a reduction of tau protein levels between 50% and 80% was achieved by exon skipping to induce an out-of-frame deletion in MAPT mRNA<sup>133</sup>. Antisense oligonucleotides resulting in steric blockage of the start codon to block translation initiation were also tested, but were found to be less efficient in reducing MAPT mRNA expression<sup>133</sup>.

An antisense oligonucleotide therapeutic approach that is close to clinical application in a neurodegenerative disorder has been developed for ALS<sup>35</sup>. ALS is a progressive

Administration	Molecular effect	Phenotypic effect	Clinical/ preclinical	Reference
ICV	90% reduction mutant htt protein	NA	Preclinical	158
ICV	50% reduction mutant htt protein	Correction of motor and psychiatric phenotypes	Preclinical	186
ICV in mice, intrathecal in monkey	Mouse: up to 75% reduction htt protein Monkey: 60% reduction HTT RNA	Mouse: motor coordination reverted to normal levels	Preclinical	32
Locally in striatum	50% downregulation mutant htt protein	NA	Preclinical	157
Transfection	Up to ~80% allele specific downregulation mutant htt protein	NA	Preclinical	112
Locally in brain	25% exon skipping HTT mRNA	NA	Preclinical	174
ICV	AChE level almost back to normal	NA	Preclinical	110
ICV	~25% reduction GSK-3 $\beta$	Improved learning and memory	Preclinical	187
ICV	Disease causing prion protein reduced by 90%	Incubation period prolonged by 2 months	Preclinical	152
ICV	40% exon skipping of ataxin-3 mRNA	NA	Preclinical	119
Microinjection	ATP7A protein restored to 35% of wildtype levels	Melanin pigmentation and notochord abnormalities rescued	Preclinical	167
Transfection	Up to 4 fold change in exon inclusion	NA	Preclinical	171

neurodegenerative disorder caused by degeneration of motor neurons in the brain and spinal cord. This eventually leads to muscle weakening, twitching, and an inability to move the arms, legs, and body <sup>137</sup>. Only 10% of ALS cases are familial and about 12% of all familial cases are caused by mutations in the gene that encodes for the enzyme superoxide dismutase 1 (SOD1) rendering the protein toxic and prone to aggregation <sup>138</sup>. The antisense oligonucleotides that have been used in ALS were designed to lower mRNA levels of the SOD1 transcripts. Continuous ventricular infusion of MOE-PS gapmer antisense oligonucleotides reduced levels of mutant

SOD1 in a rodent model of ALS and significantly slowed disease progression<sup>132</sup>. A phase I study to test the safety of this antisense oligonucleotide in subjects with familial ALS with a SOD1 mutation showed no serious adverse side effects after IT injection into the CSF<sup>35</sup>. A more recent development for ALS targets the hexanucleotide repeat expansion (GGGGCC) in the noncoding region of the *C9ORF72* gene. This repeat expansion is the most common cause of familial ALS<sup>138</sup> and although the underlying disease mechanism is not known, the repeat is transcribed and leads to accumulation of repeat-containing RNA foci in patient tissues<sup>139</sup>. This same repeat expansion also causes frontotemporal dementia (FTD). FTD is characterised by degeneration of frontal and temporal lobes, leading to changes in personality, behaviour, and language, resulting in death within 5 to 10 years. FTD and ALS are closely linked and share clinical, pathological, and genetic characteristics<sup>140</sup>. Various MOE-PS gapmer antisense oligonucleotides, targeting exon 2 common to all *C9ORF72* transcripts, and others targeting the region in intron 1 adjacent to the repeat, reduced RNA foci formation in motor neurons differentiated from ALS/FTD patients-derived fibroblasts and induced pluripotent stem cells<sup>141-143</sup>.

For HD, various antisense oligonucleotides with different modifications and backbones have been used to lower overall huntingtin protein levels<sup>144</sup>. HD is one of the nine known polyglutamine (polyQ) disorders, further consisting of the spinocerebellar ataxias (SCAs) 1, 2, 3, 6, 7, and 17), spinal and bulbar muscular atrophy (SBMA) and dentatorubro-pallidoluysian atrophy (DRPLA). PolyQ disorders are caused by a CAG triplet repeat expansion in different genes and result in progressive neurodegeneration with psychiatric, cognitive and motor symptoms<sup>145</sup>. A prominent pathological hallmark of these diseases is the accumulation of aggregated polyQ proteins in the brain<sup>146, 147</sup>. In HD, a generic reduction of huntingtin RNA of up to 75% using MOE-PS gapmer antisense oligonucleotides was found to be well tolerated in rodents and non-human primates<sup>32</sup>. ICV infusion of MOE-PS antisense oligonucleotides in transgenic BACHD mice for two weeks targeting both the human huntingtin transgene and endogenous murine huntingtin resulted in reduced toxicity, extended survival, and significant improved motor performance up to 8 months post treatment<sup>32</sup>. For most of the polyQ disorders, it is known that the wild-type polyQ-containing proteins have important cellular functions, and therefore specific lowering of the mutant polyQ protein levels leaving wild-type levels unchanged, would be favoured over a generic downregulation.

Antisense oligonucleotide-mediated reduction of disease-specific upregulated proteins has been proposed as potential treatment for multiple sclerosis (MS). MS is an autoimmune disease of the CNS where multifocal infiltration of autoreactive T lymphocytes across the BBB takes place. Lymphocytes in MS patients display high levels of  $\alpha$ -4 integrin on their surface<sup>148</sup> and this plays an important role in lymphocyte migration to sites of inflammation<sup>49</sup>. Decreasing leukocyte trafficking into various organs has been successful using monoclonal antibodies against  $\alpha$ -4 integrin<sup>150</sup>. In a commonly used mouse model of MS, the experimental autoimmune encephalomyelitis model, antisense oligonucleotide-induced blocking of  $\alpha$ -4 integrin expression reduced the incidence and severity of paralytic symptoms<sup>151</sup>. The 20-mer antisense oligonucleotides with MOE modifications and a PS backbone were designed to target a sequence just 3' of the translation start site of the murine

$\alpha$ -4 integrin mRNA to block its translation. Subcutaneous daily injections reduced  $\alpha$ -4 integrin surface expression. Although the site of action of this particular antisense oligonucleotide is unknown, it is thought that  $\alpha$ -4 integrin levels are reduced in peripheral lymphoid tissue and this prevents trafficking of activated mononuclear cells into brain and spinal cord <sup>151</sup>.

Reducing protein expression by antisense oligonucleotides was recently also shown as proof-of-principle for patients with Creutzfeldt-Jakob disease (CJD) <sup>152</sup>. CJD is caused by a conformational change of the harmless cellular prion protein (PrP<sup>c</sup>) into an infectious and pathogenic insoluble isoform scrapie PrP (PrP<sup>Sc</sup>) and subsequent deposition of extracellular aggregated prion proteins. The infectious PrP<sup>Sc</sup> has the unique characteristic that it spreads throughout the brain and can be transmitted between people as well as between different species <sup>153</sup>. ICV infusion in PrP<sup>Sc</sup> infected mice of MOE-PS gapmer antisense oligonucleotides for 14 days resulted in reduced PrP<sup>c</sup> as well as reduced disease-causing PrP<sup>Sc</sup> levels <sup>152</sup>. This reduction in disease-causing PrP<sup>Sc</sup> is probably not due to decreased PrP<sup>c</sup>, but due to a, yet unknown, anti-prion action of PS modified antisense oligonucleotides <sup>154, 155</sup>.

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## SPECIFICALLY TARGETING THE MUTANT TRANSCRIPT

If a therapeutic target protein has important cellular functions and general downregulation would be detrimental, a specific lowering of the mutation-containing protein is desired. As described previously, there are several ways to specifically lower mutant transcript and/or mutant protein levels using antisense oligonucleotides.

Targeting the repeat expansion directly has been proposed as potential treatment for ALS/FTD <sup>141, 142</sup>. Binding of antisense oligonucleotides to intronic sequences exclusively linked to the GGGGCC hexanucleotide repeat expansion <sup>142</sup> or to the repeat directly <sup>141</sup> resulted in reduced RNA foci formation in ALS/FTD patient-derived neuronal cells. To achieve GGGGCC repeat specific targeting two mechanisms were proposed: 1) 2OMe-PS antisense oligonucleotides that disrupt the hairpin structure of the expansion and prevents RNA binding proteins to sequester to the GGGGCC repeat, and 2) MOE-PS gapmer antisense oligonucleotides that bind to the repeat and target the mutant C9ORF72 transcripts for RNase H-mediated RNA degradation <sup>141</sup>.

For polyQ disorders the method that has frequently been used is targeting of the common denominator, the expanded CAG repeat. The mechanism behind this selective silencing is either due to structural differences between wild-type and expanded CAG-enclosing mRNA, or because a larger number of CAGs in the expanded repeat provides more binding possibilities for CAG-targeting oligonucleotides. Single stranded PNAs, LNAs, 2OMe-PS, and PMO antisense oligonucleotides targeting CAG repeats have all been used to specifically reduce expanded CAG-containing transcripts *in vitro* in patient-derived fibroblasts <sup>111-113</sup> and *in vivo* in a transgenic and a knock-in HD mouse model <sup>113</sup>. Although results look promising, some of the antisense oligonucleotides described here only show proper allele-specificity at longer CAG repeat lengths that are not very frequent in the patient population. Furthermore, although initial results do not show unwanted downregulation of other CAG-containing transcripts <sup>111</sup>, this needs to be investigated further.

Another way to design a molecule that can distinguish between the wild-type and expanded CAG-containing mRNA is to target a SNP that is located on the mutant transcript <sup>114, 156</sup>. Chimeric MOE-PS DNA and cEt antisense oligonucleotides were shown to selectively reduce mutant huntingtin expression in patient-derived cells <sup>157, 158</sup>. A single ICV injection of chimeric cEt antisense oligonucleotides in a humanized HD mouse model resulted in reduction in mutant huntingtin expression up to 36 weeks post treatment <sup>87</sup>. Although the allele specificity with SNP targeting antisense oligonucleotides is very promising, there are some limitations. The diversity of SNPs within patient populations would make it necessary to develop multiple oligonucleotides. Furthermore, this approach is not applicable for HD patients that do not exhibit heterozygosity for the most frequent SNPs in the coding region of the *HTT* gene.

Another mutant-specific reduction of neurodegenerative disease-causing protein expression makes use of antisense oligonucleotides that target point mutations. For instance, point mutations near the  $\beta$ -secretase site in the human gene for APP lead to a dominantly inherited form of Alzheimer disease <sup>159</sup>. In a transgenic mouse model of Alzheimer disease containing this mutation, translation of the APP mRNA was blocked by MOE-PS gapmer antisense oligonucleotides that bind specifically to the mutated  $\beta$ -secretase site <sup>110</sup>. Repeated injections into the third ventricle (once a week for 4 weeks) reduced the levels of toxic A $\beta$ , indicating that this could be a possible strategy to treat familial Alzheimer disease <sup>110</sup>.

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## RESTORING PROTEIN EXPRESSION – INTERFERING WITH PRE-MRNA SPLICING

The most prominent application of protein modification through antisense oligonucleotides interfering with pre-mRNA splicing has been researched for SMA. SMA is an autosomal recessive neuromuscular disorder caused by dysfunction and loss of motor neurons in the anterior horn of the spinal cord and lower brain stem. The underlying cause of SMA is a homozygous deletion of SMN1. SMN1 depletion is not lethal because of the presence of the almost identical *SMN2* gene. However, the majority of SMN2 mRNA transcripts lack exon 7, due to a silent mutation within this exon. This reduces the inclusion of exon 7 which results in a truncated protein and reduced expression of functional SMN protein <sup>160</sup>. Current therapeutic strategies are aimed at modulating the splicing of SMN2 by blocking exonic splicing silencers (ESS) or intronic splicing silencers (ISS), thereby increasing exon 7 inclusion. Transfecting fibroblasts with an antisense oligonucleotide (termed ISS-N1) blocking an ISS in intron 7 of SMN2 resulted in inclusion of SMN2 exon 7 <sup>120</sup>. Improved efficacy of the antisense oligonucleotide was achieved by incorporation of a uniform MOE chemistry. A single injection of this MOE-PS antisense oligonucleotide into the cerebral ventricles in a severe mouse model of SMA showed increased exon 7 inclusion and SMN protein levels in the spinal cord resulting in increased muscle size and strength <sup>161</sup>. An increased exon 7 inclusion has also been achieved by 2OMe-PS antisense oligonucleotides targeting the 3' splice site region of exon 8 <sup>162</sup>. These 2OMe-PS antisense oligonucleotides were found to result in exon 7 inclusion and elevated SMN protein expression

levels *in vivo*<sup>71, 163</sup>. A phase I clinical trial has been completed for SMA using IT injections of the MOE-PS antisense oligonucleotide targeting exon 7 inclusion<sup>36</sup>. In the high dose treated patients, SMN protein levels in the CSF more than doubled and these children showed increased muscle function scores up to 14 months after the injection<sup>164</sup>, although these results should be interpreted with caution because this was an open label study. Currently a phase 2 trial is ongoing with 6 mg or 12 mg doses of MOE-PS antisense oligonucleotide administered IT on days 1, 15 and 85<sup>165</sup>. Interim results show that the MOE-modified antisense oligonucleotide is also well tolerated after repeated injections<sup>165</sup>.

Restoration of protein expression using antisense oligonucleotides is also applied to ataxia-telangiectasia. The most debilitating feature of ataxia-telangiectasia is the progressive loss of Purkinje cells in the cerebellum and the accompanying progressive ataxia due to mutations in the *ATM* gene (ataxia-telangiectasia mutated). The majority of mutations in the *ATM* gene are splice site substitutions that result in the absence of full-length ATM protein<sup>166</sup>. CPP PMOs targeting prototypic ATM splicing mutations that activated cryptic splicing sites restored ATM protein expression in cells<sup>121</sup>. Whilst the CPP PMO was shown to distribute throughout the mouse brain following repeated IV injection, no phenotypical changes were observed<sup>26</sup>.

In a Menkes disease zebrafish model, correction of the disease phenotype was observed after PMO microinjection<sup>167</sup>. The fatal neurodegenerative disorder Menkes disease is caused by varied mutations in the *ATP7A* gene, resulting in loss-of-function of the transmembrane copper-transporting P-type ATPase<sup>168</sup>. The Menkes disease zebrafish had mutations at the 3' and 5' splice sites of the *ATP7A* orthologue, resulting in activation of cryptic splice sites and loss of the protein's ATPase function<sup>167</sup>. Various PMOs were investigated for their ability to rescue aberrant splicing<sup>167</sup>. However, mutations are distributed throughout the *ATP7A* gene<sup>169</sup> meaning that many different antisense oligonucleotides would have to be developed to treat all patients and the clinical use of antisense oligonucleotides for Menkes disease patients is currently limited.

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## MODIFYING PROTEIN - REMOVING DISEASE-CAUSING MUTATION

Antisense oligonucleotides are also used to remove neurodegenerative disease-causing mutations from genes at the RNA level. Antisense oligonucleotides have been applied to correct the ratio of tau protein isoforms as potential treatment for FTD<sup>170, 171</sup>. Tau interacts with microtubules through its microtubule binding repeat domains encoded by exons 9 to 12<sup>172</sup>. Alternative splicing of exon 10 produces tau isoforms without exon 10 (3R) or with exon 10 (4R). In healthy human brain the ratio of 4R to 3R tau is generally around 1. Due to 5' splice site mutations in FTD patients, the ratio 4R to 3R tau is shifted towards more exon 10-containing 4R<sup>173</sup>, resulting in the formation of intracellular neurofibrillary tangles. Co-transfecting MAPT minigenes with 2OMe-PS<sup>170</sup> or PNA<sup>171</sup> antisense oligonucleotides directed against the 5' splice site of exon 10 prevented exon 10 inclusion and shifted the 4R to 3R tau ratio towards more 3R tau levels.

In polyQ disorders, exon skipping is applied to modify polyQ proteins to prevent their toxic gain-of-function. In SCA3, reduction of polyQ toxicity was proposed by removal of the toxic polyQ repeat from the ataxin-3 protein <sup>119</sup>. By exclusion of exon 9 and the CAG-enclosing exon 10 from the ataxin-3 pre-mRNA using 2OMe-PS antisense oligonucleotides, a modified ataxin-3 protein was formed that lacked the polyQ repeat and retained important wild-type functions <sup>119</sup>. For HD, a more indirect antisense oligonucleotide approach to reduce protein toxicity was proposed <sup>174</sup>. Several studies have implicated the importance of proteolytic cleavage of mutant huntingtin in HD pathogenesis and it is generally accepted that N-terminal huntingtin fragments are more toxic than full-length protein <sup>175-177</sup>. Transfection of 2OMe-PS antisense oligonucleotides resulted in skipping of exon 12 in huntingtin pre-mRNA and the appearance of a shorter huntingtin protein <sup>174</sup>. In the 2OMe-PS antisense oligonucleotide treated fibroblasts, after proteolytic cleavage less 586 amino acid N-terminal huntingtin fragments implicated in HD toxicity was formed <sup>174</sup>. After a single ICV injection of murine 2OMe-PS antisense oligonucleotides, exon skipping of huntingtin and ataxin-3 was shown in the cerebellum <sup>119</sup> and striatum <sup>174</sup> of control mice. The advantage of this exon skipping approach is that there is no reduction in protein levels and the wild-type functions of the proteins likely remain largely unchanged.

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## CONCLUSION

The recent advances towards the clinical application of antisense oligonucleotides for neurodegenerative disorders are encouraging but safe delivery, long term efficacy and side effects of prolonged treatment still need to be assessed. Also, most studies have been performed in small animals and delivering high enough doses of antisense oligonucleotides throughout the much larger human brain will be a challenge. However, the widespread cellular uptake into brain cells is a major advantage over peripheral antisense oligonucleotide applications. The ease of delivery of modified antisense oligonucleotides seems to be linked with a lack of any major adverse side effects, making antisense oligonucleotides suitable candidates as potential treatment for neurodegenerative diseases. Completed clinical trials on antisense oligonucleotide-mediated therapies into the CSF reported thus far have been successful and no major adverse events were reported <sup>35, 164</sup> bringing this application closer to offer relief to many patients and families that so far had to do without effective treatment.

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