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The therapeutic potential of RNA regulation in neurological disorders

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Antisense oligonucleotide, ASO, genetics, gene regulation, miRNA, RNA therapeutics, short interfering RNA, siRNA, brain, precision medicine

Abstract

Introduction: Gene regulation is the term used to describe the mechanisms by which a cell increases or decreases the amount of a gene product (RNA or protein). In complex organs such as the brain, gene regulation is of the utmost importance; aberrations in the regulation of specific genes can lead to neurological disorders. Understanding these mechanisms can create new strategies for targeting these disorders and progress is being made. Two drugs that function at the RNA level (Nusinersen and eteplirsen) have now been approved by the FDA for the treatment of spinomuscular atrophy and Duchenne muscular dystrophy respectively; several other compounds for neurological disease are currently being investigated in preclinical studies and clinical trials.

Areas covered: We highlight how gene regulation at the level of RNA molecules can be used as a therapeutic strategy to treat neurological disorders. We provide examples of how such an approach is being studied or used and discuss the current hurdles.

Expert opinion: Targeting gene expression at the RNA level is a promising strategy to treat genetic neurological disorders. Safe administration, long-term efficacy and potential side effects, however, still need careful evaluation before RNA therapeutics can be applied on a larger scale.

Article Highlights

1. Gene regulation at the level of RNA molecules is a feasible entry point to treat genetic neurological disorders.
2. RNA therapeutics (antisense oligonucleotides (ASO) and short interfering RNA (siRNA)) are an emerging class of therapeutics. So far, two neurological compounds are FDA approved and several others are in (pre-)clinical trials.
3. RNA therapeutics exhibit their effects through cleavage and degradation of mRNAs, altering splicing or targeting regulatory non-coding genes.
4. The CRISPR/Cas tools hold promise to become the future gene therapy, not only through changing the DNA sequence, but also by altering expression levels and correcting transcripts at the RNA level.
5. Some pharmacological obstacles still exist before RNA therapeutics can be used in a routine basis. These obstacles include safe delivery, stability, immunogenicity and off-target effects.

1. Introduction

Every organism needs tight regulation of cellular processes, which have to adapt adequately to changes in developmental stage and environment. Virtually all cells in an organism contain the same genotype. During development, cells differentiate and form a multitude of diverse cell types, with distinct morphology, function and protein content. Gene regulation is the term used to describe mechanisms by which a cell increases or decreases the amount of a certain gene product (RNA or protein) under influence of intrinsic or environmental changes. Several mechanisms, on DNA, RNA and protein level, are known to regulate gene expression, such as epigenetic mechanisms, (post-) transcriptional regulation, translational regulation and post-translational modifications.

Gene expression levels often have strict limits within which the cell can function properly. For certain genes, the dosage is particularly important. Gene dosage is the number of copies of a particular gene that are present in the genome and is often correlated with the amount of gene product (RNA or protein). Normally every individual has two copies of a particular gene (one from the paternal chromosome and one from the maternal). Genomic duplications or deletions containing dosage-sensitive genes can have serious phenotypical consequences. Pathogenic genetic variants can also interfere with this equilibrium. Genetic variants can be classified by the effect of the variant on the function of its gene product. Loss of function (LOF) variants result in the gene product having less or no function. Depending on the function of the gene product, having one normal operating allele is still sufficient for regular cell function. In this situation both alleles must be mutated before it leads to a (recessive) disease. In other cases, 50% of gene product is not enough for the cell to function normally, and one mutated allele leads to a (dominant) disease. This mechanism is called haploinsufficiency. Gain of function (GOF) variants on the other hand result in either an enhanced activity of the normal gene product (GOF), or in the creation of an abnormal toxic function (toxic GOF). Genes that encode for proteins being part of a larger multimeric complex, can harbor variants with yet another mutation mechanism, known as a dominant negative effect. When a gene encoding one subunit of the complex is mutated but still is incorporated in the complex, this can interfere negatively with the function of the entire complex and lead to a greater than 50% reduction of functional complex.

Presumed pathogenic missense variants are not always straightforward to predict whether they cause a LOF, a dominant negative effect, or a toxic GOF. Functional studies to determine the consequence of missense variants on protein function, therefor, are of critical importance to ascertain therapeutic options.

In this review, we focus on targeting gene regulation at the RNA level as a treatment strategy. We concentrate on rare neurological disorders, because of the huge unmet need for better treatments for many of these diseases [1]. Although these are all rare disorders (with a prevalence of less than 5/10 000), together they are fairly common [1] and provoke high direct and indirect costs on society [2]. We provide examples of how gene regulation at the level of RNA molecules is used as a therapeutic strategy to treat neurological disorders in animal models and even humans, illustrating how this can be extrapolated and adapted to other fields. Although we are aware that gene regulation at the RNA level is only one aspect of gene regulation, the therapeutic potential of other levels of gene regulation is beyond the scope of this review. We will discuss first the different types of RNA therapeutics and their current pharmaceutical obstacles. Then, we review several proof-of-concept studies to show their potential (see table 2).

2. RNA therapeutics

RNA therapeutics are an emerging class of therapeutics with a common fundamental principle: an oligonucleotide binds a target RNA through complementary Watson-Crick base pairing. Different approaches have been developed with the aim of modulating the amount of messenger RNA (mRNA). One can downregulate mRNA directly, modify the mRNA sequence via alternative splicing or influence the mRNA catabolism to increase the amount of available mRNA molecules. Different types of RNA therapeutics are listed in table 1.

Antisense oligonucleotides (ASOs) are 15-25 nucleotides long, synthetic single stranded (ss) oligodeoxynucleotides designed to bind complementary RNA targets. The ASO/RNA hybrids are recognized and degraded by RNAse H, resulting in reduced mRNA and consequently protein levels [3]. Later generation ASOs, with modified backbones and altered pharmacological properties, were developed to sterically block splicing at the pre-mRNA level resulting in alternative splicing, or block translation factors, causing the impairment of protein translation (Figure 1) [4].

Short interfering RNA (siRNA), on the other hand, are short double stranded (ds) molecules with a chemical composition similar to RNA, interacting with the RNA induced silencing complex (RISC) to downregulate their target mRNA (Figure 1). A siRNA is fully complementary to one target mRNA that gets cleaved and degraded when bound to the siRNA/RISC. siRNAs can be introduced in cells either as (i) long dsRNA molecules, as (ii) synthetic short siRNA molecules or as (iii) short hairpin RNA (shRNA) molecules. shRNA are stem-loop RNAs generally delivered through viral vectors and expressed in the nucleus. Once expressed, they get transported towards the cytoplasm through

exportin 5 (XPO5) and processed by DICER1 to cleave off the loop sequence, before they are loaded into the RISC.

A clear comparison between ASOs and siRNAs is reviewed in [3, 4, 5].

MicroRNAs (miRNAs) are small non-coding RNA molecules that post-transcriptionally regulate gene expression. They are transcribed as a few hundred nucleotides long primary transcripts (pri-miRNA) that needs to undergo several processing steps (Figure 2). Once the pri-miRNAs are transcribed by RNA polymerase II, they fold into one or more hairpin structures. In a first processing step, the microprocessor complex cleaves the hairpins out of the pri-miRNA, releasing the precursor miRNA (pre-miRNA). Then, the pre-miRNA is exported to the cytoplasm via XPO5. In the cytoplasm, the pre-miRNA undergoes a second cleaving step, when DICER1 cleaves the loop of the hairpin structure. One of the remaining mature miRNA arms (either the 5p or 3p arm) is loaded into the RISC, while usually the opposite arm is quickly degraded [6]. Mature miRNAs generally bind the 3' UTR of their target mRNA. MiRNAs are different from siRNAs in a sense that siRNAs need perfect complementarity to downregulate one single target, while miRNAs do not need perfect complementarity and rather repress the translation of many different target genes.

Perfect complementarity between the miRNA and its target mRNA normally results in mRNA degradation. Yet, in animals, imperfect complementarity is more frequently seen, resulting in translational repression [7]. MiRNAs are expressed in a spatio-temporal specific manner [8]. One miRNA usually regulates several (sometimes up to a few hundreds) of target mRNAs and inversely, one mRNA can be regulated by several miRNAs [9]. Targeting of a specific miRNA will thus affect several downstream transcripts, which should be carefully considered from a therapeutic point of view. Using miRNAs to target monogenetic disorders might therefore be less promising. Targeting complex disorders using miRNAs on the other hand, can be promising when a miRNA is identified that regulates several key proteins involved in the pathogenesis of the disorder.

When ASOs target microRNAs, they are often referred to as antagomiRs, because they reduce the level and functioning of the targeted miRNA. MiRNA mimics to increase the level of active miRNAs, are often referred to as agomiRs.

Other non-coding RNA (ncRNA) can have a role in gene regulation as well. They can be arbitrarily subdivided by their length. Long ncRNA (lncRNA) are by definition at least 200 nucleotides long, in comparison to miRNA or siRNA that are generally 20-25 nucleotides long. Targeting regulatory lncRNA can be a promising approach to modulate the mRNA levels of the genes they regulate (see 4.3.).

3. Pharmacological obstacles related to RNA therapeutics

Although many interesting exploratory and pre-clinical experiments are currently ongoing for the use of gene regulating molecules in neurological disorders, a long trajectory still lies ahead before many of these treatments will reach the patient. After initial discovery of a targetable entry point, the precise mechanism of the disorder has to be well characterized and validated. Furthermore, several hurdles still need to be overcome before RNA therapeutics can be used in the clinic on a routine basis.

3.1. Delivery

A major hurdle is the efficient delivery of large, usually negatively charged, molecules to their tissue of interest . Several barriers need to be overcome before the therapeutics can reach their tissue of interest. For CNS disorders, compounds have to exit the bloodstream and cross the blood-brain barrier (BBB), consisting of tight junctions between the epithelial cells, and astrocytic end-feets that surround the blood vessels. Inside the brain, they need to be taken up by the correct cells through endocytosis, after which endosomal escape is necessary to release the compound into the cell. For PNS disorders, compounds need to cross a blood-nerve barrier (BNB) as well. Tight junctions between endothelial cells and between pericytes in endoneurial vasculature isolate the endoneurium from the blood [10]. Compound exchange between the bloodstream and nerve cells occurs mainly through endoneurial capillaries, while perineurial passage constitutes a minor route [10]. Afterwards, the compound needs to be taken up by the neurons after which endosomal escape is again needed to release the compound into the cell.

Several delivery strategies have been developed, including different chemical modifications and conjugations, or delivery via nanoparticles, viral vectors or via administration of naked RNA (excellently reviewed in [11], [5] and [4]).

Viral vectors make use of the efficient methods of certain viruses to enter cells and deliver their own genetic material. They are especially useful for delivery of siRNAs, that can be cloned into a vector and will be expressed from it when the vector enters the nucleus. This approach is less useful for ASOs as they are single stranded molecules. For delivery to the nervous system, adeno-associated viral (AAV) vectors or lentiviral (LV) vectors are commonly used. Both are non-pathogenic, trigger minimal immune responses and cannot replicate in the host [12]. AAVs remain in the cells as nuclear episomes, while LVs integrate their DNA into the host genome [13]. Both vectors can target dividing

and non-dividing cells, which makes them highly suitable for post-mitotic neuronal cells [14]. Viral vectors with specific capsid serotypes can be chosen to specifically transduce tissues of interest. Depending on the capsid serotype, AAVs can preferentially transduce neurons, astrocytes or glial cells (nicely reviewed in [14, 15]). AAV9 for example is often used to target the brain [16], while AAV2 has been used to transduce Schwann cells and peripheral nerve segments [17]. Incorporating targeting ligands into the capsid can furthermore mediate ligand specific receptor binding [18, 19]. Caution should be taken however because many humans have pre-existing antibodies against certain AAV serotypes which might trigger a neutralizing response against the vector [20]. The rather small packaging capacity can be a limiting factor, i.e. max 4.8 kilobases (kb) for AAV and 7-9 kb for LV [14], although due to the short sequence length of siRNAs, this is usually not a problem.

Next to viral vectors, several non-viral approaches have been developed. Nanoparticles consist of a group of diverse structures with a similar nanometric size. They protect the compound by encapsulating it and aid in facilitating cellular uptake [21]. The ideal nanoparticle for biological use is polar at the surface, to give high aqueous solubility and to prevent aggregation. Additionally, it should be biodegradable with a limited life span, so that it is only present as long as therapeutically needed [22]. Many types of nanoparticles exist and include cationic polymeric, solid-lipid, nano-emulsions and liposomes [23]. Cationic polyethylenimine (PEI)-DNA complexes are the golden standard for mammalian cell transfections [24, 25]. Nanoparticles can cross the blood brain barrier either by (i) direct penetration also known as absorptive transcytosis, by (ii) simple diffusion or by (iii) receptor-mediated endocytosis [26]. When nanoparticles are taken up by the cell, they are often encapsulated in endosomes, after which endosomal escape is a major limiting factor. Positively charged nanoparticles are more efficiently able to escape from endosomes in neurons and are thus more frequently used [26].

Another approach is bypassing the BBB by injecting the compound directly into the cerebrospinal fluid (CSF) [4, 27], as is done for nusinersen (see 4.2.1.1.). Intranasal delivery has been reported as a valid alternative in animal studies [28, 29, 30], to provide a non-invasive method to cross the BBB [31].

3.2. Stability and immunogenicity

When cells encounter ssDNA such as ASOs or small dsRNA such as siRNA, they may recognize it as a viral by-product, resulting in an immune response [32] and quick degradation of the compound by nucleases [33]. The fragility of RNA therapeutics is illustrated by the fact that the half-life of naked siRNAs in nuclease-rich serum can be as short as several minutes [33]. Backbone modifications have

been proposed to enhance stability by minimizing DNAse or RNAse degradation. Some of the most frequently used backbone modifications include adjustments at the 2' position of the ribose sugar, such as 2'-O-methyl (2'-O-Me), 2'-O-methoxy-ethyl (2'-O-MOE), or locked nucleic acids (LNA) ASOs. They have a higher affinity to target RNA [34], higher resistance to nuclease degradation [35] and reduced immunostimulatory activity [36]. Another frequently used modified ASO is the phosphorodiamidate morpholino oligomer (PMO). Here, the deoxyribose is replaced by a morpholino ring, and the charged phosphodiester inter-subunit linker is replaced by an uncharged phosphorodiamidate linker. PMOs are very resistant to nuclease and protease degradation and are mostly used for splicing modulation and translation inhibition [37]. Backbone modifications of siRNAs or agomirs can limit the efficiency of RISC loading, . Nevertheless, when properly positioned within the compounds, these modifications have minor effects on their effectiveness. Modifications of the 2' position of the ribose sugar, for example, that does not contain bulky groups such as O-MOE, can still result in potent siRNAs [38, 39]. Furthermore, stabilizing the 5' end of the RNA molecule was shown to enhance the potency of siRNAs [38, 39]

3.3. Saturation of endogenous RNAi pathway

Delivery of shRNAs has been shown to overload the XPO5 transporter, leading to a saturation of the endogenous miRNA or RNAi pathway and to profound toxicity [40, 41]. Optimizing the shRNA dose and sequence will thus be of the utmost importance when using shRNAs in human. Using promotors with only limited expression capacity, or co-expressing XPO5 have been shown to reduce these toxic effects [40, 42].

3.4. Off-target effects

Another limitation is that RNA therapeutics can potentially induce off-target side-effects. Especially downregulation of genes with a partial sequence complementarity, in a manner similar to miRNA targeting, has been noted [43]. Several solutions have been proposed, including carefully designing the compound so that it has high complementarity with the target site only, using modifications of the second nucleotide of the oligonucleotide, or using a combination of several siRNAs also referred to as siPools [5]. The siPools are enzymatic generated complex pools, containing up to 60 accurately defined siRNAs targeting the same mRNA. The low concentration of each individual siRNA dilutes sequence-specific off-target effects below detection limits, while the combination maintains a potent on target silencing [44].

Last but not least, also unanticipated toxicity from gene of interest knock down, caused by non-redundant essential functions of the target gene should be rigorously checked. For patients harboring missense variants, the pathomechanism, e.g. LOF, dominant negative effect or toxic GOF, should be known upfront to make sure that altering RNA levels would not have detrimental effects.

4. Proof-of-concept studies

4.1. Targeting mRNA levels to downregulate gene expression or activity

Some neurological disorders result from the overexpression of one specific gene, either by duplications in the genome or by variants causing an increased activity of gene product. A logical approach for this group of disorders is to normalize expression or activity levels of the culprit gene.

4.1.1. Duplications

Charcot-Marie-Tooth disease, type 1A (CMT1A) is the most common heritable peripheral neuropathy. It results from a duplication on chromosome 17, encompassing the dosage-sensitive *peripheral myelin protein 22* (*PMP22*) gene. Weekly subcutaneous injections of an ASO targeting PMP22 resulted in the reduced expression of PMP22 in both a mouse and rat model of CMT1A. This reduction slowed the disease progression in these models and even improved the CMT1A associated phenotypes as shown using the grip strength test and the rotarod test [45]. Additional experiments to study safety, the optimal dose, frequency and administration route, as well as the pharmacokinetics and –dynamics of the ASOs are still needed before they can be tested in human.

4.1.2. Mutations causing increased activity of gene product

4.1.2.1. Mutations causing increased normal function

Centronuclear myopathies (CNMs) are non-dystrophic muscle diseases characterized by muscle weakness and hypotrophic fibers with centralized nuclei [46]. The most severe form (X-linked CNM) is caused by LOF mutations in myotubularin 1 (MTM1), while the autosomal dominant form is caused by mutations in dynamin 2 (DNM2). DNM2 overactivity is believed to be the disease mechanism in both forms. It was shown that down regulating the expression of Dnm2 in Mtm1 KO mice prevents myopathy development [47] and systemic injection of Dnm2 ASOs into affected mice resulted in the reversal of muscle pathology [47]. Furthermore, a single injection of an AAV vector expressing a shRNA targeting Dnm2 resulted in a long-term reduction of Dnm2 protein, and restored muscle force

and mass in the same mouse model. It brought histology markers back to normal and prevented molecular defects linked to the disease [48].

4.1.2.2. Mutations causing novel gain-of-function

Hereditary amyloidosis is a heterogenous group of autosomal dominant inherited diseases characterized by the deposit of insoluble protein fibrils in the extracellular matrix [49]. Patients typically present with polyneuropathy, carpal tunnel syndrome, autonomic insufficiency, cardiomyopathy and gastrointestinal features [49]. Mutations in transthyretin (*TTR*), a tetrameric protein, are the most frequent cause of hereditary amyloidosis. Mutations in the *TTR* gene misfold the monomer subunits, inhibiting the tetramer formation and causing aggregation into TTR amyloid fibrils (ATTR) [50]. Treatment options focus on decreasing the amount of circulating amyloidogenic protein. Patisiran is a TTR mRNA specific siRNA formulated in lipid nanoparticles (LNP) [51]. In a phase II clinical trial, two intravenous infusions at different doses were administered in a three weeks interval. All doses were generally well tolerated and the highest dose of 0.3 mg/kg was shown effective in reducing serum TTR protein levels by 80%. A phase III clinical trial was recently finished. The study showed improvement with patisiran relative to placebo in the primary endpoint of modified Neuropathy Impairment Score and additional secondary endpoints including sensory, motor, and autonomic neuropathy symptoms at 18 months (http://investors.alnylam.com/news-releases/news-release-details/alnylam-and-sanofi-present-positive-complete-results-apollo). A new drug application for patisiran was filed to the FDA end 2017.

Another example is an ASO targeting SOD1, currently under study in a phase I/II clinical trial. Mutations in *SOD1* are a frequent cause of familial amyotrophic lateral sclerosis (ALS), an adult onset neurodegenerative disease characterized with loss of both upper and lower motor neuron pathways. The exact pathomechanism is still unclear, but toxic GOF is believed to be the underlying cause [52]. Surprisingly, complete absence of SOD1 did not result in an overt disease or in reduced lifespan in a mouse model [53], so that downregulating both wild type (wt) and mutant alleles seems to be safe. An ASO targeting SOD1 reduced SOD1 mRNA and protein levels in an ALS rat model overexpressing the human mutant $SOD1^{G93A}$. Starting ASO treatment approximately 30 days prior to disease onset slowed the disease progression and resulted in extended survival, although it did not change the disease onset or early disease stages [54]. The same ASO was delivered by intrathecal infusion in a first phase I clinical trial, showing no dose limiting toxic effects and no overall safety or tolerability concerns [55]. A larger phase I/II clinical trial is ongoing.

4.1.3. Allele-specific silencing

For many dose sensitive genes, non-specifically altering both wt and mutant levels might have detrimental effects. By specifically targeting variants associated with the disease, allele-specific silencing has the ability to reduce only the expression levels of the mutant allele, while leaving the expression levels of the wild type allele intact. This creates the possibility to not only target gain-of-function, but also dominant negatively acting variants. It should however be taken in mind that a crucial requisite for allele-specific silencing is that haploinsufficiency of the investigated gene is tolerated, as after silencing of the mutant allele, only 50% of the wt allele will remain.

For disorders with a larger number of different pathogenic variants, a disadvantage of allele-specific variant targeting is the need to develop many variant-specific molecules for a limited number of patients. One way to circumvent this problem, is to target a common single nucleotide polymorphism (SNP), present on the mutant allele in a heterozygous state. The latter has the advantage that only a few RNA therapeutics need to be developed to treat a large percentage of the patient population.

Huntington is an autosomal dominant progressive neurodegenerative disorder, caused by a toxic GOF cytosine-adenine-guanine (CAG) repeat expansion in the coding region of the *Huntingtin* (*HTT*) gene [56]. This repeat expansion results in the formation of short polyglutamine-containing peptides and in the misfolding of the HTT protein. Although some studies in Huntington mouse models show that reducing both wt and mutant *HTT* levels is well tolerated [57], the safest approach is to only silence the mutant allele. Skotte and colleagues [58] designed ASOs targeting SNPs associated with the mutant *HTT* allele. They showed an impressive specificity and high potency for their most promising ASOs in primary neurons derived from a humanized Huntington mouse model (containing the SNPs heterozygous on the mutant allele). A phase I clinical trial was recently started to test the safety and tolerability of two ASOs targeting the *HTT* SNPs rs362307 and rs362331. At least one of both SNPs is heterozygous in 65.1% of the Huntington patient population [59].

4.2. Modifying RNA molecules: targeting splicing

In eukaryotic systems, a gene is transcribed as a primary transcript (pre-mRNA) that has to undergo several processing steps in order to generate the mature mRNA. An important step is RNA splicing. The spliceosome removes introns and joins two neighboring exons together. Via alternative splicing, different transcripts can be generated from the same gene. These alternative splicing events can be

spatial and temporal specific and can be influenced by environmental factors [60]. Disturbance of alternative splicing has repeatedly been associated with disease [61].

4.2.1. Create full-length transcript

Interference with alternative splicing can also be a potent strategy for treatment. An excellent example is spinomuscular atrophy (SMA), which is caused by recessive LOF mutations in *SMN1*. SMA is a hereditary disease that causes weakness and muscle wasting due to the loss of lower motor neurons [62]. A duplicate gene (*SMN2*) is a modifier of the disease, and having more copies of *SMN2* results in a milder phenotype [63]. *SMN2* is almost identical to *SMN1* but produces less functional protein compared to *SMN1*, because of an alternative splicing event that removes exon 7 [64]. This results in 80-90% truncated non-functional protein, and only 10-20% full-length protein. End 2016, the FDA approved nusinersen, the first therapy for SMA [27]. Nusinersen is a small 2'-O-(2-methoxyethyl) modified ASO that binds the pre-mRNA of *SMN2*. It targets an intronic splicing silencer (ISS) in intron 7 of the SMN2 pre-mRNA, and displaces heterogenous nuclear ribonucleoproteins (hnRNPs) from this ISS. It thus facilitates accurate splicing of *SMN2* transcripts [65, 66, 67], and promotes inclusion of exon 7, generating more functional protein and so compensating for the *SMN1* loss.

4.2.2. Create functional truncated protein

A similar approach is being investigated for Duchenne muscular dystrophy (DMD), an X-linked disease caused by LOF mutations in the Dystrophin gene. DMD is characterized by progressive proximal muscular dystrophy with characteristic pseudohypertrophy of the calves, and onset usually before the age of 3. Patients become wheelchair-bound by age 12 and develop a severe cardiomyopathy by their twenties [68]. Internal out-of-frame deletions make up about two-third of the cases. In-frame deletions on the other hand usually cause the milder Becker muscular dystrophy (BMD). The most frequent cause of DMD (~ 16%) is a deletion of exon 50, resulting in a frameshift and the occurrence of a premature stop codon. However, deletion of both exon 50 and 51 maintains the reading frame and will cause a milder phenotype. Van Deutekom et al. (2007) injected ASOs targeting exon 51 in the muscle of four patients with DMD diagnosed with a deletion of exon 50. This induced skipping of exon 51 and restoration of the open reading frame, resulting in a local increase of Dystrophin expression [69]. Based on this research, eteplirsen was developed [70], and granted accelerated approval by the FDA in 2016 based on surrogate end-point results showing it was able to

increase dystrophin levels in patients. However, the conflicting results of the first clinical pilot study asked for a still ongoing larger confirmatory clinical trial to evaluate its efficacy and safety. Similar strategies to restore the open reading frame by skipping other Dystrophin exons are also subject of further investigation [71].

4.2.3. Modulate alternative splicing

Epilepsy is a disorder that causes people to have recurrent seizures, which are the clinical manifestations of abnormal neuronal hyperexcitability. It is a heterogenic disorder in terms of age-of-onset, clinical outcome and etiology. Epilepsy can be caused by structural brain abnormalities, metabolic disorders or genetic aberrations. One of the many mechanisms that provoke epilepsy is an increase in voltage-gated persistent sodium currents ($I_{Na}P$) that contributes to neuronal hyperexcitability. To date however, no anti-epileptic drugs can specifically target the $I_{Na}P$ currents without targeting the transient voltage-gated sodium currents ($I_{Na}T$) as well, the latter being critical for normal action potential firing. $I_{Na}P$ amplitudes can however be regulated by alternative splicing. In *SCN1A* for example, the gene encoding the alpha subunit of the neuronal voltage-gated sodium channel also known as $Na_v1.1$, two mutually exclusive exon 5 are present: exon 5N is thought to be mainly expressed in neonates and exon 5A in adults. In HEK293T cells, the *SCN1A-5N* transcript produces channels exhibiting more rapid inactivation and reduced $I_{Na}P$ compared to *SCN1A-5A* transcripts [72]. In Drosophila only one Na_V channel is present, encoded by the *para* gene. A similar exon pair is present in *para*: exon 25 consists of a mutually exclusive exon K or exon L. Channels including exon K exhibit reduced $I_{Na}P$ compared to channels including exon L, without changing the $I_{Na}T$ [73]. Furthermore, increased inclusion of exon L is seen in bang-sensitive mutant flies that have lower seizure thresholds. Lin et al. identified 95 Drosophila genes that, upon knock down, altered splicing of *para* and promoted the inclusion of exon K. Knockdown of these genes, furthermore, reduced seizure duration in epilepsy fly models. The authors then identified five small compounds that were known to inhibit the proteins encoded by five selected genes of interest from their list, and proved that they had a similar anticonvulsant effect [74]. This study shows how alternative splicing can influence excitability of neurons, and how this knowledge can be used to identify potential new anti-epileptic drugs. It is an incentive to investigate whether manipulation of alternative splicing of *SCN1A* is also anticonvulsant in humans. Interestingly, a common genetic variant (rs3812718) disrupting the consensus splice donor sequence of exon 5N blocks the incorporation of exon 5N in mature *SCN1A* mRNA transcripts. Epileptic patients carrying the other haplotype (with higher exon 5N inclusion) required lower doses of the sodium channel blockers phenytoin and carbamazepine for

treatment [75, 76]. In line with this observation, proteins including exon 5N were more sensitive to the *in vitro* application of the anti-epileptic drugs and sodium channel blockers phenytoin and lamotrigine [77].

4.3 Influence catabolism of RNA molecules

4.3.1. Targeting natural antisense transcripts

Endogenous antisense non-coding transcripts can regulate their sense coding genes. One type of long non-coding RNA (lncRNA) called 'natural antisense transcript' (NAT), downregulates the expression of the gene from the opposite strand. NATs are often multi-exonic and are known to function as fine modulators of on-going transcription, affecting a single gene or a small subset of related genes [78]. The exact mechanism of this regulation is still not completely clear, although antisense RNA-induced chromatin remodeling seems to be a feasible and dynamic mode of action [79].

Inhibiting this inhibitory effect of the NAT can thus upregulate the expression of their target coding genes [78], which represents an appealing therapeutic strategy for diseases caused by haploinsufficiency of a specific gene [80].

Dravet syndrome, for example, is a severe early-onset epilepsy syndrome characterized by fever-sensitive seizures starting during the first year of life, followed by developmental delay [81]). The mutational mechanism causing Dravet syndrome is *SCN1A* haploinsufficiency*. SCN1A* has a NAT (termed *SCN1ANAT)* that controls its expression. *SCN1ANAT* can be targeted by an antagoNAT, an oligonucleotide-based compound. Intrathecal injection of this compound was shown to induce a 10-30 % increase of Scn1a mRNA levels in the brain of a Dravet mouse model and was associated with a decrease in average seizure frequency and duration. Interestingly, a 25% increase in brain Scn1a levels was sufficient to elicit a 70% reduction in seizure frequency. Intrathecal injection of the antagoNAT in African green monkey revealed upregulation of SCN1A mRNA in brain but not in peripheral tissues. Furthermore, no antagoNAT-associated histopathology was identified, indicating that intrathecal antagoNAT administration is largely safe [80].

The advantage of therapeutics targeting NATs is their high specificity, since NATs are limited to regulate only specific genes. A disadvantage, however, is that it is currently not clear how many genes are regulated by NATs in human, as the early predictions that NATs commonly regulate their partner coding gene [82] have not been confirmed recently.

4.3.2. Targeting microRNAs

In contrast to the *cis*-acting NATs described above, miRNAs are seen as *trans*-acting antisense transcripts. MiRNAs regulate several target mRNAs, so targeting a miRNA can be especially useful for complex disorders where many genes are deregulated.

Temporal lobe epilepsy (TLE), for example, is the most frequent form of focal epilepsy. It is characterized by focal seizures with often intense psychic or autonomic auras and can have a number of causes, including head injury, stroke, brain infections, and structural lesions in the brain or genetic aberrations. Both in animal models and patient brain samples with temporal lobe epilepsy, an upregulation of mir-134 is seen [28, 83]. In a kainic acid mouse model of TLE, mir-134 antagomir injection in the ventricle one hour after status epilepticus resulted in prolonged spontaneous seizure suppression and reduced seizure activity [28]. After this initial finding, more deregulated miRNAs were identified in mouse models of TLE, and either antagomir, to reduce its expression when upregulated in epileptic mice, or agomir treatments to increase its expression when downregulated in epileptic mice, were proven to reduce seizure severity [29, 30, 84, 85]. More studies should be conducted, to validate these findings and to assess safety before entering clinical trials.

5. Future perspectives

5.1. CRISPR/Cas9 targeted gene editing

Gene editing facilitating site-specific genomic modifications, is one of the most promising therapeutic technologies, although it is currently still in its infancy. One encouraging tool is the clustered regularly interspaced short palindromic repeat (CRISPR)- CRISPR-associated protein (Cas) system, that in the future potentially can be used as a therapy at the RNA level. This system is based on a natural bacterial defense mechanism against viruses. When bacteria recognize viral DNA, they produce two small RNA sequences, CRISPR RNA (crRNA) and trans-acting crRNA (tracrRNA) (in gene editing tools fused into one guide RNA (gRNA)), that form a complex with the nuclease Cas protein. This nuclease subsequently binds the DNA that is recognized by the crRNA and cleaves it. Several variations of the CRISPR-Cas system, such as CRISPR-Cas9 [86] or CRISPR-Cpf1 [87], have been developed to edit genomes. This technique holds the promise to cleave virtually every gene. Cells will try to repair the cut either via non-homologous end joining (NHEJ), or via homology directed repair (HDR) [88]. NHEJ is error prone, usually resulting in indels and LOF variants when present in the open reading frame of a gene. HDR on the other hand is less error prone, and when a repair template is present, it can be used to knock-in a specific sequence at the location of the cut, which theoretically

can be used as precision medicine strategy to correct disease-causing variants. A detailed description is beyond the scope of this project, but an overview can be found in Shin et al., 2018 [89]. In general, there are a myriad of difficulties that still have to be overcome before the CRISPR-Cas system can be used to treat neurological disorders. First of all, delivery to the brain remains a problem, because the gene for the commonly used Cas9 is too large to fit in a AAV vector [90]. Then, the CRISPR-Cas should be able to enter a large (sub)population of neurons separately, to efficiently correct the gene of interest in all of them. Last but not least, the gRNA should be designed specifically enough to cleave only the gene of interest, as to avoid off-target effects that could potentially lead to oncogenesis [90].

5.2. CRISPR/Cas9 targeted gene activation

For many genetic neurological disorders, loss of function mutations are the leading cause of pathology. In a recent paper, Liao and colleagues describe a method to activate gene expression using the CRISPR/Cas9 system, providing a mechanism to compensate the loss of gene function. They fused a co-activation complex with a short single guide RNA, together termed dead single guide RNA or dsgRNA, as it does not cleave the target gene. The dsgRNA will lead the Cas9 protein to the locus of interest, where the co-activation complex recruits the transcriptional machinery to activate the target gene. As a proof-of-concept, they used a dual AAV approach to simultaneously inject an AAV-vector containing a dsgRNA targeting *Follistatin* (*Fst*) and an AAV-vector containing Cas9 in the fore and hind limb muscles of a DMD mouse model. Fst was upregulated in muscle and epigenetic markers showed active transcription at the Fst transcription start site three months after injection. Furthermore, Fst overexpression resulted in an amelioration of the DMD symptoms as shown by the grip strength test [91]. This is a promising strategy to treat disorders caused by loss of function mutations, with the additional advantage that no DNA double-strand breaks need to be induced. Of course safety, efficacy and potential side effects should still be thoroughly examined.

5.3. RNA editing

Recently, a variant of the CRIPSR-Cas system, CRISPR-Cas13, has been developed to perform RNA-specific RNA editing. A catalytic dead Cas protein (dCas13) fused with the adenosine deaminase domain of ADAR2 ($ADAR_{DD}$) can direct adenosine (A) to inosine (I) deaminase activity at a specific position guided by the gRNA. Inosine is functionally similar to guanosine. This system is known as the RNA Editing for Programmable A to I Replacement (REPAIR) system.

An advantage of RNA editing compared to DNA editing is that it does not use the endogenous repair system, making it more efficient in post-mitotic cells. Furthermore, its transient and easily reversible effect makes it a potentially safer therapy compared to DNA editing, and could be preferred for selected pathologies [92]. For now, REPAIR still has low efficiency and a limited number of off-target effects throughout the transcriptome. Moreover, only A>I modifications are possible with this tool at the moment, but continuous progress will enable other RNA changes, and profoundly improve the tools.

6. Conclusion

Targeting gene expression at the RNA level is a promising strategy to treat genetic neurological disorders. RNA therapy has the advantage that it can act on virtually all genes, including conventionally non-druggable targets.

In this review, we provide an overview of how gene regulation at the level of RNA molecules can be used to target neurological disorders. We examine several examples and discuss the remaining hurdles. As the first molecules to treat neurological disorders were recently FDA-approved and several other lines of *in vitro* and *in vivo* proof-of-principle evidence is already available, RNA therapeutics will become more and more prevalent in the near future.

7. Expert opinion

In 2010, a Declaration of Principles on rare neurological disorders of childhood was endorsed by researchers, clinicians, patient advocacy groups and drug companies. This effort aimed to advance awareness and knowledge of rare neurological diseases via international collaboration and to enable better diagnosis and management of patients with these diseases. There are an estimated 5000-8000 rare diseases and about 5-8 % of the European population is thought to be affected by a rare disease, many of which have neurological manifestations [1]. About 80% of rare diseases have a genetic basis. Many of these disorders are considered undruggable because they cannot be targeted with 'traditional' pharmacological compounds.

Understanding the molecular mechanisms of a disease can facilitate the rational development of better treatment. For several neurological disorders, this understanding has been improved recently, and since the development and implementation of RNA therapeutics, controlling gene regulation at the RNA level seems to be a feasible entry point. Nonetheless several challenges remain. An

important hurdle is the safe and efficient delivery across the BBB and into the cells. Several tools exist such as viral vectors and nanoparticles, that are constantly being improved but for now, it is still challenging to target all or even a subset of neuronal cells. Furthermore, off-target effects should always be rigorously screened to reduce potential toxic or carcinogenic effects.

A promising strategy is to use a highly complex pool of well-defined siRNAs that target the same mRNA, but dilute potential off target effects [44]. For this strategy to work in clinical settings, however, current drug approval strategies should be designed differently. For now, each cocktail component should undergo individual toxicological and efficacy testing which makes it expensive for highly complex pools. A related problem is seen when a combination of different ASOs is used to skip more than one exon in DMD [71]. Multi-exon skipping can target a larger fraction of patients and allows choosing the most functional resulting protein: skipping of exon 3-9 or exon 45-55 for example can help about 54% of patients and results in the milder BMD phenotype [93, 94]. Moreover, sometimes more than one exon should be skipped in order to obtain an in-frame protein. Deletions of exon 7, for example, need an additional skipping of both exon 6 and 8 to restore the open reading frame [95]. The combination of both ASOs will have a clinical effect, while the single ASOs would not [71]. Therefore, mixtures of different ASOs or siRNAs should be considered as one drug by the drug regulatory agencies. Otherwise, the high costs and complexity to organize clinical trials for each of the compounds in the pool will delay the development of new drugs.

Two neurological drugs that have effect on the RNA level are already FDA approved, and many more are in the pipeline. Therefore, we believe that in the future several additional RNA therapeutics will become available. Interestingly, the cost to screen antisense oligonucleotides is substantially lower compared to small compounds, which makes it highly amenable for treatment development for genetic disorders with small patient populations [80]. This will simultaneously allow for the expansion of precision medicine but will put pressure on the current system of clinical trials. Although the technology exists to make RNA therapeutics for individuals or small groups of patients at limited costs, it is very labor and cost intensive to get these compounds subsequently FDA approved, which will not be profitable for the companies developing them. We will need new policies that enable to generate meaningful but affordable safety and efficacy data for rare patient populations [96].

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Declaration of interest

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Figures and Table legends

Figure 1: Different modes of action for antisense oligonucleotides (ASO) and short interfering RNA (siRNA). dsRNA: double stranded RNA, RISC: RNA induced silencing complex, shRNA: short hairpin RNA, XPO5: exportin 5.

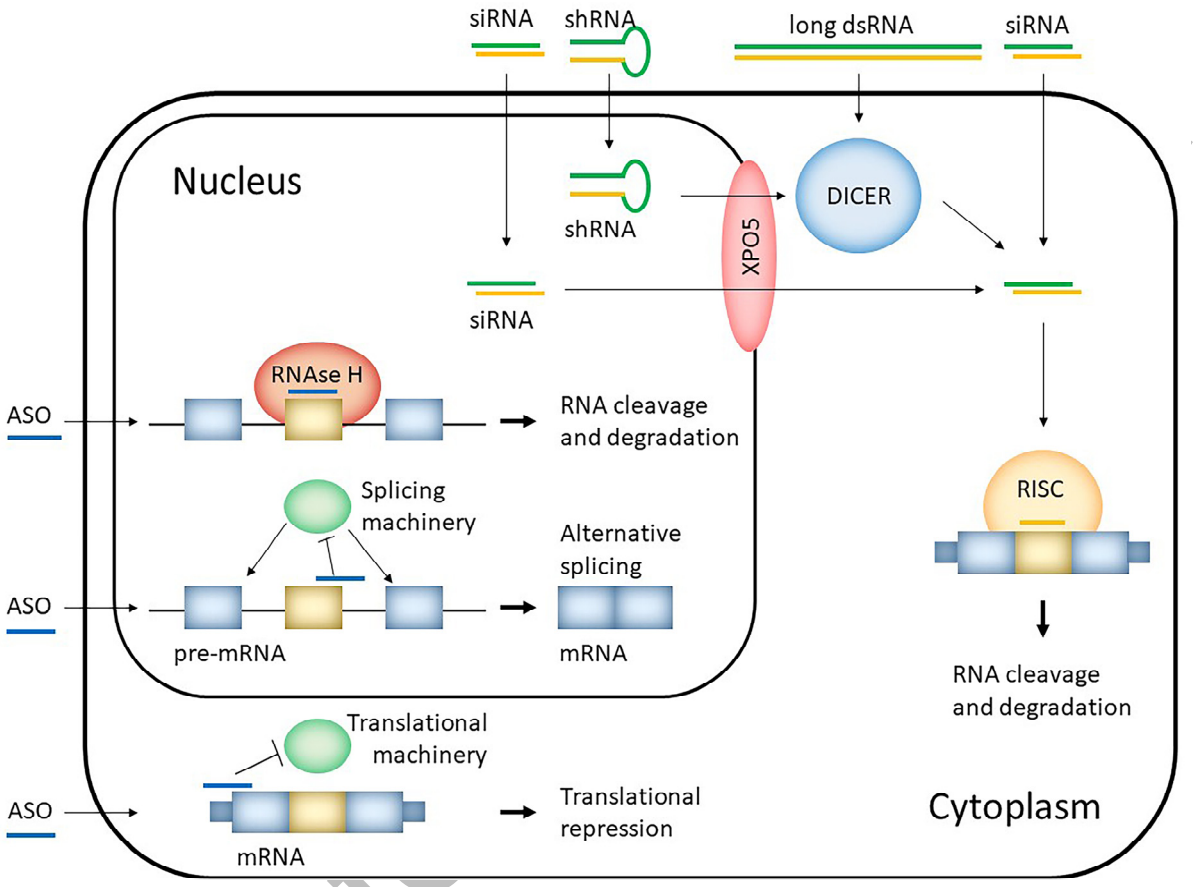

Figure 2: microRNA processing pathway. AntagomiRs reduce the level and functioning of the targeted microRNA. MiRNA mimics or agomiRs increase the level of active microRNAs. Pri-miRNA: primary microRNA, pre-miRNA: precursor microRNA, XPO5: exportin 5

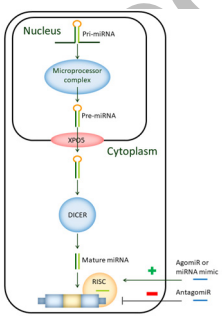

Table 1: RNA therapeutics and their effect on different RNA targets.

Table 2: Overview of proof-of-concept studies relating RNA therapeutics for neurological disorders that are described in this review.

List of abbreviations

2’-O-Me	2’-O-Methyl
2’-O-MOE	2’-O-methoxy-ethyl
A	Adenosine
AAV	Adenovirus-associated-virus
ALS	Amyotrophic lateral sclerosis
ASO	Antisense oligonucleotides
ATTR	TTR amyloid fibrils
BBB	Blood-brain barrier
BMD	Becker muscular dystrophy
BNB	Blood-nerve barrier
CAG	Cytosine-Adenine-Guanine
Cas	CRISPR-associated protein
CMT1A	Charcot-Marie-Tooth disease, type 1A
CNM	Centronuclear myopathies
CRISPR	Clustered regularly interspaced short palindromic repeat
crRNA	CRISPR RNA
CSF	Cerebrospinal fluid
dCas	Dead Cas protein
DMD	Duchenne muscular dystrophy
DNM2	Dynamin 2
ds	Double stranded
dsgRNA	Dead single guide RNA
FDA	Food and Drug administration
Fst	Follistatin
GBM	Glioblastoma
GOF	Gain-of-function
gRNA	Guide RNA
HAT	Histone acetyltransferase
HDR	Homology directed repair
hnRNP	heterogenous nuclear ribonucleoproteins
HSAN1E	Hereditary sensory autonomic neuropathy with dementia and hearing loss
HTT	Huntingtin

I	Inosine
$I_{Na}P$	Voltage-gated persistent sodium currents
I_{NaT}	Transient voltage-gated sodium currents
ISS	Intronic splicing silencer
kb	Kilobases
LNA	Locked nucleic acids
lncRNA	Long non-coding RNA
LNP	Lipid nanoparticles
LOF	Loss-of-function
LV	Lentiviral
miRNA	MicroRNA
mRNA	Messenger RNA
MTM1	Myotubularin 1
NAT	Natural antisense transcript
Na_V	Voltage-gated sodium channel
NHEJ	Non-homologous end joining
PEI	polyethylenimine
PMO	Phosphorodiamidate morpholino oligomer
PMP22	Peripheral myelin protein 22
pre-miRNA	Precursor miRNA
pri-miRNA	Primary transcripts
REPAIR	RNA Editing for Programmable A to I Replacement
RISC	RNA induced silencing complex
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SMA	Spinomuscular atrophy
SMN	Survival of Motor Neuron
SNP	Single nucleotide polymorphism
SOD1	Superoxide dismutase 1
ss	Single stranded
STAGA	Spt-Ada-Gcn5 acetyltransferase
TLE	Temporal lobe epilepsy
tracrRNA	Trans-acting crRNA

TTR	Transthyretin
XPO5	Exportin 5

RNA therapeutics	Molecular target	Mode of action	Therapeutic effect
ASO	mRNA	endonuclease mediated degradation/sterical hindering	protein downregulation/ alternative splicing
siRNA	mRNA	RISC induced knock down	protein downregulation
shRNA	mRNA	RISC induced knock down	protein downregulation
antagoNAT	natural antisense transcripts	reduce NAT levels --> upregulate sense coding gene	protein upregulation
antagomir	miRNA + its targets	reduce miRNA activity	miRNA targets upregulation
agomir	miRNA + its targets	enhance miRNA activity	miRNA targets downregulation

Table 1: RNA therapeutics and their effect on different RNA targets.

Overview of proof-of-concept studies relating RNA therapeutics for neurological disorders that are described in this review.

Disease	**Gene**	**Pathomechanism**	**RNA therapeutics**	**Phase**	**Ref**
Charcot-Marie-Tooth disease, type 1A (CMT1A)	*PMP22*	GOF: duplication	ASO	Pre-clinical	45
Centronuclear myopathies (CNMs)	*MTM1* and *DNM2*	GOF: DNM2 overactivty	shRNA	Pre-clinical	48
Hereditary amyloidosis	*TTR*	Toxic GOF: aggregation into TTR amyloid fibrils	siRNA (Patisiran)	Phase III clinical trail	51
Amyotrophic lateral sclerosis (ALS)	*SOD1*	Toxic GOF	ASO	Phase I/II clinical trial	55
Huntington	*HTT*	toxic GOF: CAG repeat expansion	ASO targeting benign variant on expanded allele	Phase I clinical trial	59
Spinomuscular atrophy (SMA)	*SMN1*	SMN1 LOF	ASO alters splicing of SMN2 to create full length transcript (Nusinersen)	FDA approved	65, 66, 67
Duchenne muscular dystrophy (DMD)	*Dystrophin*	Out-of-frame deletion: LOF, in-frame deletion results in milder Becker muscular dystrophy (BMD)	ASO changes out-of-frame deletion into in-frame deletion (eteplirsen)	FDA approved	71
Dravet syndrome	*SCN1A*	SCN1A LOF	ASO: alternative splicing of a mutual exclusive exon 5	Exploratory	75

Dravet syndrome	*SCN1A*	SCN1A LOF	antagoNAT: ASO binds natural antisense transcript (NAT) that downregulates SCN1A expression, resulting in SCN1A upregulation	Exploratory	81
Temporal lobe epilepsy (TLE)	mir-134	mir-134 upregulation	antagomir: ASO downregulating mir-134 levels was shown to reduce seizure severity	Pre-clinical	28
Duchenne muscular dystrophy (DMD)	*Dystrophin*	Out-of-frame deletion: LOF, in-frame deletion results in milder Becker muscular dystrophy (BMD)	Crispr/Cas targeted gene activation to upregulate Follistatin (Fst)	Exploratory	91