Therapeutic RNAi-based gene therapy for neurodegenerative disorders : slowing down the ticking clock

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Chapter 1

General introduction

Concept of gene therapy

Gene therapy is an emerging therapeutic tool to deliver functional genetic material to cells in order to correct a defective gene. By delivering a copy of a therapeutic gene to affected cells, the messenger RNA (mRNA) and/or proteins will be continuously synthesized within the cell, utilizing the cell's own transcriptional and translational machinery[1]. The main advantage of this technology is that it offers a potentially life-long therapeutic effect without the need for repeated administration. Gene therapy can be used to correct defective genes by introducing a functional copy of a defective gene, by silencing a mutant allele using RNA interference (RNAi), by introducing a disease-modifying gene, or by using gene-editing technology.[2–4]

Gene therapy vectors can be either viral or non-viral. Different physical and chemical systems can be applied to deliver therapeutic genes to cells without the need of a viral vector. Non-viral vectors have no size limitation for the therapeutic gene, generally have a low immunogenicity risk and can be produced at relatively low costs.[5] However, because high therapeutic doses are required when using non-viral technologies, and the resulting gene expression is generally transient, most gene therapies now rely on viral vectors. Numerous viral vector types have been tested in clinic, including vaccinia, measles, vesicular stomatitis virus (VSV), polio, reovirus, adenovirus, lentivirus, γ-retrovirus, herpes simplex virus (HSV) and adeno-associated virus (AAV).[6] Vaccinia, measles, VSV, polio, reovirus, adenovirus and HSV vectors are currently mainly used in either vaccines or cancer therapeutics while lenti- and γ-retroviral vectors are predominantly used for transduction of transplantable cells.[7] For *in vivo* gene delivery, AAV is currently the preferred vector. AAV belongs to the Parvoviridae family and is preferred for gene therapy because it is non-replicating (AAV requires a helper virus for replication), has a low immunogenicity profile and is not known to cause disease.[8,9] In the absence of a helper virus, AAV may stably integrate into the host genome, but at relatively low frequency. The genome of wildtype AAV is about 4.7 kb and is flanked between two inverted terminal repeats (ITRs) (Figure 1a).[10] The open reading frame between the ITRs contains a replication (Rep) gene and a capsid (Cap) gene. The ITRs are cis-acting elements and are required for genome replication, integration, and packaging into the capsid. The Rep gene encodes 4 proteins (Rep78, Rep68, Rep52, and Rep40) that have important roles in replication and encapsidation of the viral DNA.[11] The Cap gene encodes three capsid proteins (VP1, VP2, and VP3) and an assembly activating protein that promotes capsid formation.[12] There is a growing number of naturally occurring and engineered AAV serotypes with different viral capsids that have altered tissue tropism, transduction rate, or other features such as the ability to cross the blood-brain barrier[13,14] Recombinant AAV (rAAV) can be produced by replacing the rep and cap genes with an expression cassette containing a therapeutic gene of interest (Figure 1b). Formation of wild type AAV is prevented by expressing the rep and cap genes on a separate plasmid (AAV packaging plasmid or AAV helper plasmid). The ITRs are the minimal region required to be retained in rAAV to allow

packaging of its genome. rAAV might still integrate randomly in the human genome at very low frequencies but most of its genome is maintained as episomal circular structures known as concatamers. rAAVs have been widely used in over 200 human clinical studies and have demonstrated to be safe.[15] AAV-based gene therapies are highly attractive for the treatment of neurogenerative diseases due to the neuronal tropism and good safety profile demonstrated in clinical studies. In addition, a single administration results in long-term, potentially life-long gene expression, which is a main advantage when *in vivo* AAV administration require invasive procedures.

Neurodegenerative diseases

Neurodegenerative diseases are a heterogeneous group of multi-system disorders affecting the central nervous system, ultimately leading to neurodegeneration[16]. Examples of the most common neurodegenerative diseases are amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), spinocerebellar ataxias (SCAs), Huntington's disease (HD), Alzheimer's disease (AD) and Parkinson's disease (PD).[17] The prevalence of these age-dependent disorders is increasing, partly due to the aging population, placing a major economic burden on health care services. Some neurogenerative diseases are caused by genetic mutations and/or cellular and circuit dysregulation. In some cases, different neurodegenerative diseases are linked to the same polymorphisms or mutations, thereby sharing similar pathological mechanisms. It seems that certain environmental or lifestyle factors combined with genetic factors increase the risk for certain neurodegenerative disease but aging is considered to be the most important risk factor for the sporadic cases.[18] Although each neurodegenerative disease have a different pathophysiology, they all lead to damage to the nervous system due to features such as cell death, impaired/failure of axonal regeneration, demyelination and/or neuronal structural/functional deficits.[19] These pathological features occur in different combinations and their causes can be either genetic or unknown.[19] Common underlying causes leading to these conditions can be due to abnormal accumulation of proteins such as amyloid in AD, misfolded proteins (typical for PolyQ diseases), aggregation of proteins such as Tau (AD and traumatic brain injuries), synuclein (PD) or TDP-43 (ALS), RNA toxicity or translational products from repeats expansion within genes.[19,20] Each of these features has unique mechanisms of toxicity which in large part are currently not well understood. Some mechanisms of pathogenesis in neurodegenerative diseases share key characteristics with prions suggesting that progression of certain neurodegenerative diseases may share similar mechanisms involved in prion diseases.[21] For example, misfolded protein aggregates (such as Tau, α-synuclein, amyloid-ß, huntingtin) can spread via cell to cell interaction and invade healthy tissues. The misfolded proteins can induce secondary misfolding of other unrelated aggregation -prone proteins, impairing the entire proteostatic network.[22] Ultimately, they lead to a decline or even complete loss of sensory, motor, and cognitive functions. Symptoms commonly associated with neurodegenerative

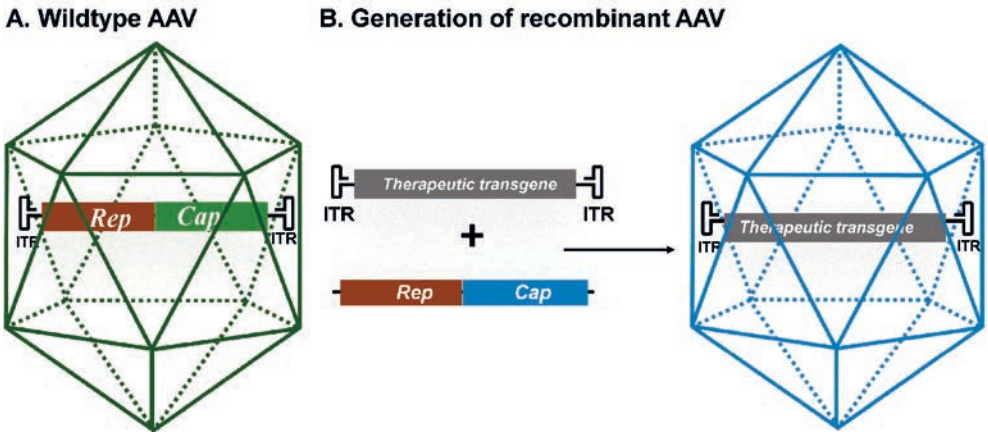

Figure 1. a) Schematic of wild type AAV. Its genome consists of the viral rep and cap genes flanked by two inverted terminal repeats (ITRs). b) Schematic of recombinant AAV (rAAV) generated by replacing the viral genes for a therapeutic gene, flanked by the ITRs. The rep and cap genes are expressed from a different plasmid or viral vector. The rAAV is generated by co-transfecting cells with the transgene cassette flanked by AAV ITRs, the rep and cap genes of a specific AAV serotype, and the adenovirus helper plasmid.

diseases include cognitive impairment, memory loss, apathy, anxiety, muscle weakness, paralyses, difficulties with speech or breath and death.

One great mystery for most neurodegenerative diseases is the onset of clinically manifest symptoms, as pathological alterations usually occur long before symptoms start to develop. Progressive accumulation of neuronal cell damage and the effects of aging are two common explanations and more recently, the role of neuroplasticity in the development and progression of neurodegeneration has also been implicated.[23] The adult brain generally shows less neuroplasticity in response to insults than the developing nervous system.[23] However early-life events and insults such as perinatal infections, an unstructured/abusive environment, social isolation, stress, poor nutrition, exposure to chemicals or metals could possibly interfere with the neuroplastic development in children and adolescents.[24–26] These may place an additional burden on the plastic capacity of the developing neuronal system leading to the disturbance of the structural brain self-organization. A second challenge later in life could trigger the final onset of neurodegeneration and this may be a critical factor determining the onset and course of neurodegeneration.[23]

A major clinical challenge is early diagnosis of neurodegenerative diseases and due to overlapping symptoms, discrimination between the different diseases is difficult. Moreover, early symptoms are often dismissed or interpreted as normal consequences of aging. Apart from diagnostic delay, additional challenges for new therapeutic approaches reaching the stage of clinical development are the lack of druggable targets, the limited choice of delivery methods, and a lack of reliable biomarkers and clinical parameters that

predict therapeutic efficacy or the rate of disease progression. To date neurodegenerative diseases cannot be cured, and only palliative treatments are available. Because these diseases are devastating for patients and their families, and cause a vast burden on society, there is an enormous need to better understand their causes, pathology and clinical progression and to develop early detection methods and new therapeutic interventions. The current thesis presents the development of RNAi-based gene therapies for ALS and SCA3 and discusses the currently available options for delivery to the target cells. Additionally, the preclinical validation of an AAV-based mifepristone-inducible GeneSwitch system is reported as one of the first steps in the development of a small molecule-regulated gene therapy approach.

Amyotrophic lateral sclerosis

ALS (or Lou Gehrig's disease) is the most common adult onset motor neuron disease affecting the upper- and the lower motor neurons in the brain and spinal cord, but other neuroanatomical regions may also be affected.[27,28] The upper motor neurons are found in the motor cortex of the brain, while the lower motor neurons are located along the brainstem, spinal cord and extend to the muscles.[29] Degeneration of the upper motor neurons causes symptoms such as spasticity and hyperreflexia while the loss of lower motor neurons results in progressive muscle weakness, cramps, fasciculations, muscle wasting and paralysis.[29,30] The prevalence of ALS is currently estimated at 5 in 100.000 but the estimated lifetime risk to develop the disease is about 1:400-800.[31,32] The discordance between the low prevalence but high lifetime risk is explained by the fact that ALS patients have a very limited life span with a median survival ranging from two to five years from symptom onset.[28] The understanding of the genetic causes of ALS is continually expanding, but our knowledge of other risk factors such as environmental factors, lifestyle or aging remains poor. Only ten percent of ALS cases are familial, and the causal genetic mutations are usually inherited in a mendelian autosomal dominant manner. Thus, most ALS cases are assumed to occur sporadically, and the main causes are still unknown.

Two drugs have been approved by the Food and Drug Administration for the treatment of ALS, but the efficacy of both drugs is modest. Riluzole, first approved in 1995, is a glutamate receptor antagonist which may increase survival by 2 to 3 months. More than two decades later in 2017, Edaravone, a free radical scavenger was approved but its efficacy is still unclear, and at best the drug has a moderate effect on disease progression.[33] More than 30 genes have been linked to ALS and mutations in chromosome 9 open reading frame 72 *(C9orf72),* superoxide dismutase1 (SOD1), transactive response DNA-binding protein 43 (TDP-43), or fused in sarcoma (FUS) are responsible for most of the familial ALS cases. A hexanucleotide expansion consisting of GGGGCC (G_4C_2) nucleotides in the first intron of the *C9orf72* gene is the most frequent genetic cause of ALS and is found in about 40-50% of familial ALS cases and 5-10% of sporadic ALS cases.[34] The G_4C_2 repeat

is transcribed bidirectionally and affected patients usually carry more than 30 G_4C_2 copies. Interestingly, the same mutation also causes FTD, the second most common form of dementia after AD. *C9orf72* is responsible for 25% of familial FTD cases and 5-7% of sporadic FTD cases. ALS and FTD are considered overlapping diseases as about 15% of ALS patients develop FTD and up to 50% of ALS patients show some degree of functional loss in the frontal lobe of the brain.[32] The function of the *C9orf72* encoded protein is poorly understood, but it may be a regulator of the autophagy-lysosome pathway during nutrient stress responses. [35] There are at least three proposed pathogenic mechanisms in *C9orf72* related ALS and/or FTD patients; 1) RNA-mediated toxicity, 2) RAN translation and 3) haploinsufficiency. It is also possible that a combination of the three mechanisms contributes to the disease pathogenesis (Figure 2).[36,37]

RNA-mediated toxicity

RNA-mediated toxicity was first described in myotonic dystrophy type 1 (DM1), which is caused by a CTG repeat expansion in the 3'UTR of the myotonic dystrophy protein kinase gene.[38,39] It was shown that RNA consisting of CUG repeats folds into stable structures that colocalize with RNA-binding proteins and sequester their function. For example, an important protein that is sequestered by CUG-containing RNA foci is the splicing factor muscleblind-like 1 (MBNL1).[40] The sequestration of MBNL1 leads to its inactivation which subsequently cause mis-splicing of several pre-mRNAs, such as muscle-specific chloride ion channel and insulin receptor.[41,42] RNA foci are observed in several other neurodegenerative diseases caused by repeat expansions including ALS and SCA3.[43] In *C9orf72*-related ALS and FTD, accumulation of sense and antisense G_4C_2-containing RNA foci is detected in several brain-, spinal cord tissues, lymphocytes and fibroblasts. RNA foci are also detected in patient-derived Induced-pluripotent stem cell (iPSC) -neurons. Although RNA foci mainly accumulate in the nucleus of cells they are also observed at lower concentrations in the cytoplasm. Several RNA binding proteins interact with G_4C_2 RNA repeats, such as ADARB2, hnRNPA1, hnRNPA1B2, Pur-α, FUS, Nucleolin and TDP-43.[36,44,45] However the contribution of these proteins to the neurodegeneration is only partially understood.

RAN translation

Another proposed mechanism of toxicity in *C9orf72* related ALS and/or FTD is by repeat-associated non-ATG (RAN) translation.[36,46,47] It has been shown that the repeat-containing transcripts can be translated into dipeptide repeat proteins (DPRs) even in the absence of an ATG start codon and even though the mutation is in a non-coding region of *C9orf72*. These DPR proteins are toxic and they can form aggregates that accumulate in the brain and spinal cord of patients.[48,49] Six DPR proteins can be produced via unconventional translation in all reading frames. Glycine-alanine (GA) and glycine-arginine (GR) are

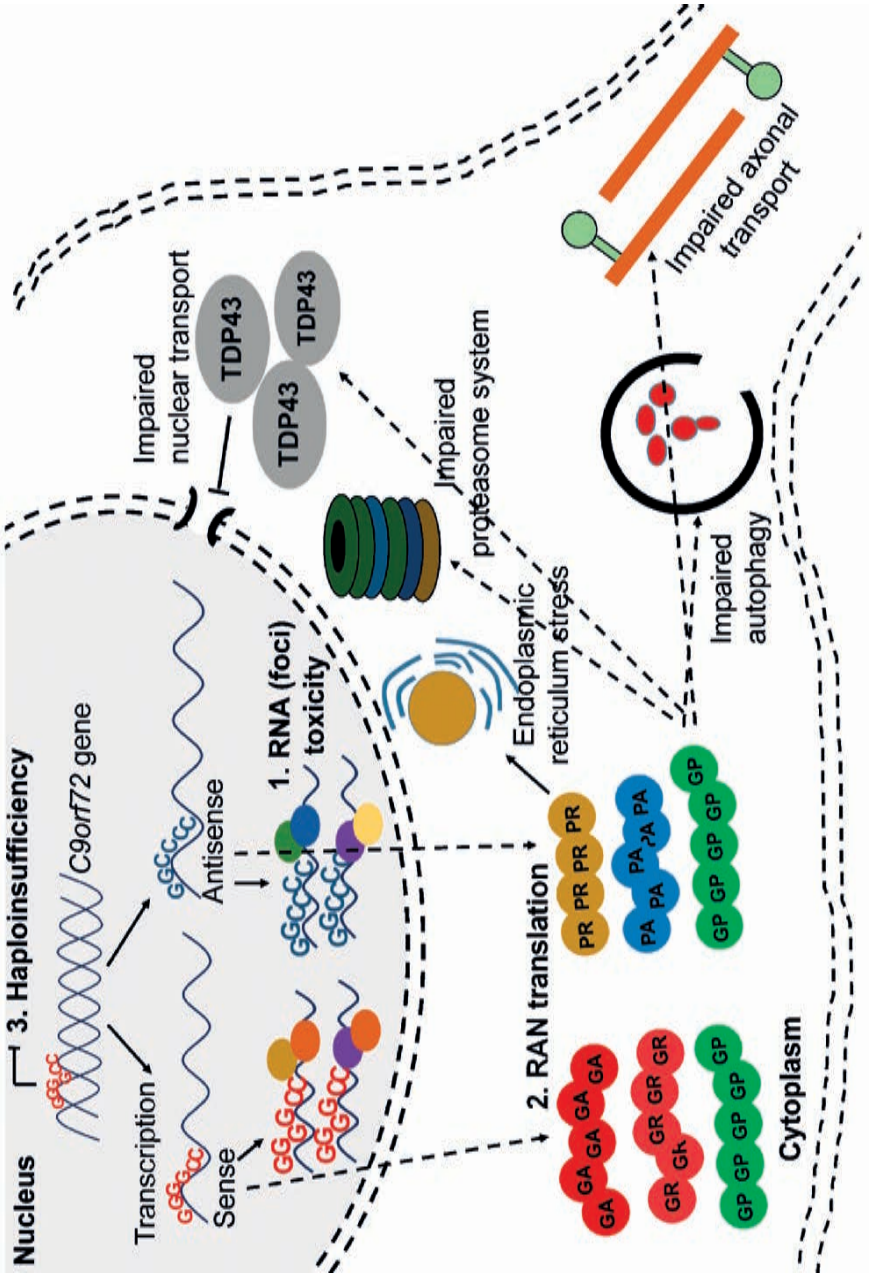

Figure 2. Mechanisms of toxicity associated with *C9orf72* G_4C_2 repeat. a) RNA-mediated toxicity. Repeat-containing sense and antisense RNA transcripts accumulate and sequester RNA binding proteins (1) b) RAN translation. The sense and antisense repeat-containing transcripts undergo RAN translation into five, potentially toxic DPRs (2) b) Haploinsufficiency. Hypermethylation of the expansion leads to reduced transcription of *C9orf72* (3).

produced from the sense repeat-containing transcripts, while proline-arginine (PR) and proline-alanine (PA) are produced from the antisense repeat-containing transcripts. Glycine-proline (GP) is produced from both sense and antisense repeat-containing transcripts.[50] Ample evidence indicates that DPR proteins are toxic and cause neurodegeneration. For example, neurotoxicity, proteasome activity and endoplasmic reticulum stress was observed in primary neurons expressing GA proteins.[51] Addition of recombinant GR and PR proteins to Hela cells and human astrocytes was toxic and caused alterations in RNA processing.[50] Expressing GR and PR proteins in Drosophila caused toxicity and early lethality.[52] Several recent publications demonstrated that DPR proteins can lead to impairment of nuclear transport, causing accumulation of several RNA-binding proteins including TDP-43 in the cytoplasm.[53–55] Cytoplasmic TDP-43 aggregation is observed in ~97% of cases of ALS, including those associated with *C9orf72* mutations. TDP-43 protein is predominantly found in the nucleus but can shuttle between the nucleus and cytoplasm to regulate processes such as RNA processing, transcription, pre-mRNA splicing, transport and stabilization of mRNA.[56–58] In patients, TDP-43 accumulates as cytosolic inclusions with a C-terminal fragment of 25 or 35 kDa. The aggregated TDP-43 is also post-translationally modified, being heavily ubiquitinated, and phosphorylated at the C-terminal region.[56] Notably, mutations in TARDBP, the gene that encodes the TDP-43 protein, have also been linked to ALS, but it remains unclear whether it is depletion of nuclear TDP-43, gain of a toxic function of cytoplasmic TDP-43, or a combination of these processes that are neurotoxic. Recently it was demonstrated that expressing PR in mice also resulted in neurodegeneration and premature death.[59] PR proteins localized to the heterochromatin caused abnormal histone H3 methylation and aberrations in nuclear lamins and heterochromatin protein 1α (HP1α). This resulted in down-regulation of numerous differentially expressed genes and upregulation of many repetitive elements which was accompanied by the accumulation of double-stranded RNA, ultimately leading to neuronal death. Thus, as RNA foci and DPRs both contribute to the pathogenesis of ALS, their inhibition could potentially reduce the disease burden in patients.

Haploinsufficiency

The thought that haploinsufficiency may contribute to *C9orf72*-related ALS originates from the finding that reduced *C9orf72* RNA and protein levels have been reported in brain and spinal cord tissues and in iPSC-neurons derived from ALS patients. Several mechanisms may lead to reduced levels of *C9orf72*, including abortive transcription caused by G-quadruplexes and R-loop structures of the repeat-containing transcripts. In addition, the *C9orf72* locus is hypermethylated in several mutation carriers, which can lead to epigenetic silencing and similar mechanisms of gene silencing are observed in other repeat expansion diseases such as Friedrich ataxia and fragile X mental retardation syndrome. However, haploinsufficiency alone does not explain the observed ALS pathology in patients. Several loss-of-function mutations in *C9orf72* have been identified

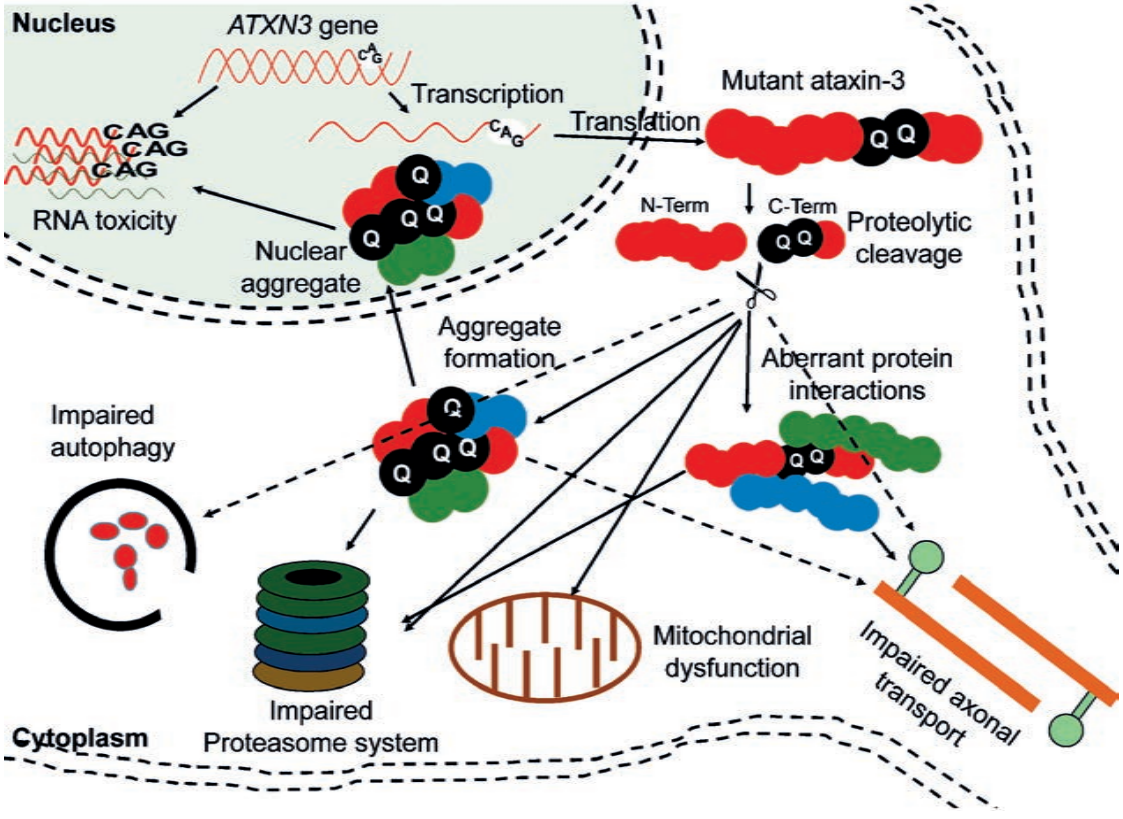

Figure 3. Ataxin-3 mediated mechanisms of toxicity. 1) RNA toxicity. RNA containing the CUG repeats can sequester function of transcription factor 2) Mutant ataxin-3 toxicity. The CAG repeat-containing *ATXN3* transcript is translated into a protein with a polyQ expansion. Proteolytic cleavage of mutant ataxin-3 can generate C-terminal protein fragments containing the polyQ repeat. The mutant ataxin-3 and C-terminal protein fragments can both cause several cellular disturbances such as transcriptional deregulation, impaired autophagy, mitochondrial dysfunction, proteasomal impairment, compromised axonal transport and DNA damage.

and seemed to be non-pathogenic.[60] Furthermore, complete *C9orf72* knockout mice showed no neurodegeneration but rather splenomegaly, enlarged cervical lymph nodes, and autoimmune related premature death.[61,62] Interestingly, this phenotype was rescued in mice hemizygous for *C9orf72,* suggesting that a partial knockdown of the gene would be well tolerated.[62] A recent publication suggest a cooperative pathogenesis between gain- and loss-of function mechanisms.[37] Reduced *C9orf72* protein in cultured motor neurons caused accumulation of glutamate receptors and excitotoxicity in response to glutamate. In addition, these motor neurons showed impaired clearance of DPRs and were hypersensitive for these proteins.

As most evidence points towards a toxic gain of function resulting from this mutation, an RNAi-based gene therapy would be an attractive therapeutic approach to silence the mutated gene.

Spinocerebellar ataxia type 3

The spinocerebellar ataxias (SCAs) are a large group of neurodegenerative diseases that are characterized by progressive ataxia due to degeneration of the cerebellum and often adjacent regions. SCAs are inherited in an autosomal-dominant manner and have a prevalence of about 1-3 in 100.000, although this highly vary between geography and ethnicity.[63,64] More than 40 different types of SCA have been identified and the most common ones (SCA1, SCA2, SCA3, SCA6, SCA7 and SCA17) are caused by a CAG nucleotide repeat expansion encoding polyglutamine (polyQ).[65] SCA3 (also known as Machado-Joseph disease or MJD) is the second most common PolyQ disorder after HD and the most common among the SCAs. SCA3 patients experience progressive ataxia, affecting balance, gait and speech and frequently symptoms are pyramidal signs, progressive external ophthalmoplegia, dysarthria, dysphagia, rigidity, distal muscle atrophies and double vision.[66] The pathogenic CAG repeat is in the penultimate exon of *ATXN3* gene on chromosome 14q32.1 and the disease severity is related to the number of CAG repeats:[67] Up to 44 CAG repeats are considered normal, between 45 to 51 repeats are associated with intermediate or low penetrance of the disease, while SCA3 patients usually have more than 51 repeats. The survival rate is variable and usually between 10 to 21 years after symptom onset.[68] Similar to ALS, there is no cure for SCA3 and current treatments are based on antispasmodic drugs to help reduce spasticity, speech therapy and physiotherapy. Several molecules such as sulfamethoxazole-trimethoprim, varenicline and lithium carbonate have been tested in clinical trials, but all failed to show clinical improvement.

The *ATXN3* gene encodes a 42-kDa protein called ataxin-3 which consists of a N-terminal catalytic Josephin domain and two to three (dependent on the splice variant) C-terminal ubiquitin (Ub)-interacting motifs flanking the polyQ tract.[65,66,69] The ataxin-3 protein is widely expressed in different cell types of peripheral and neuronal tissues and present in both nucleus and cytoplasm.[70] Ataxin-3 is believed to interact with up to 100

proteins involved in ubiquitin-dependent pathways and quality control. The protein has a role in various ubiquitin-dependent pathways that maintain protein homeostasis. By partnering with ubiquitin ligases, ataxin-3 may be able to regulate, or edit, the lengths and linkage types of ubiquitin chains on proteins and in this manner either rescue proteins from being degraded, or stimulate protein breakdown. [71,72] Additional roles in endoplasmic reticulum-associated degradation, aggresome (aggregates of misfolded proteins) production and DNA repair have also been implicated.[73–77]

Mechanisms of toxicity

The mutant ataxin-3 protein is neurotoxic but the exact pathways leading to neurodegeneration are not completely understood. Two attractive hypotheses postulate a gain of toxicity and RNA-mediated toxicity (Figure 3). The expanded CAG repeat in the *ATXN3* gene leads to formation of an ataxin-3 protein with an expanded polyQ tract at its C-terminal region with toxic gain of function properties. In addition, the mutated protein causes aggregate formation in neurons which is typical for polyQ diseases. Aggregates are found in different types of neurons in the brain stem (ventral pons), substantia nigra, globus pallidus, dorsal medulla and dentate nucleus.[66,69] Although the aggregates are mainly detected in the nucleus, they are also found at low levels in cytoplasm of neurons in affected areas and in axons within fiber tracts known to undergo neurodegeneration in the disease.[78] The aggregates consist of different types of proteins such as ataxin-3 (wildtype and mutant), heat-shock proteins, transcription factors and other polyQ disease-associated proteins. It is believed that sequestering of these functional proteins contributes to cellular dysfunction and neurodegeneration. Furthermore, proteolytic cleavage of the mutant ataxin-3 may lead to generation of shorter soluble PolyQ fragments that are also toxic. Another hypothesis is that the polyQ expansion induces conformational changes in ataxin-3 and alters its function in multiple ubiquitin-dependent pathways that can lead to altered binding properties, loss of protein function, disorganized subcellular localization, aggregation, and altered proteolytic cleavage.[66,79]

In addition to the mechanisms described above, which are based on protein toxicity, RNA toxicity could also contribute to the disease. As mentioned before, RNA containing CUG repeats can sequester various transcription factors and undergo RAN translation. A crucial evidence for RNA toxicity was observed in nematodes. CAG repeats cloned into the 3' UTR of a marker protein showed severe toxicity in a length-dependent manner in Caenorhabditis elegans (C. elegans). The CUG RNAs formed RNA foci and colocalized with C. elegans muscleblind protein. The highest CAG repeats were embryonically lethal while the shorter CAG repeats were tolerated.[80,81] Since the mutated *ATXN3* gene produces a protein with a toxic gain of function, it is an ideal target for an RNAi-based gene therapy approach.

In vivo and *in vitro* models for ALS and SCA3

Model systems that recapitulate the different aspects of the diseases are essential to understand the pathology, and to predict clinical efficacy during early development of new therapeutic approaches. Development of animal models for neurodegenerative diseases is a major challenge as the diseases have complex pathologies and symptoms can take decades to unfold. As a result, most of the currently available animal models for neurodegenerative diseases are not exact phenotypes of the human diseases and therefore lack the ability to forecast clinical success of new therapeutic approaches. Moreover, studies performed *in vitro* and *in vivo* on cells and small animals are difficult to translate to larger animals, because factors such as delivery route, dosing, distribution and toxicity vary between the different model systems.[82]

Animal models for *C9orf72* ALS/FTD

Various mouse models have been generated for *C9orf72* ALS/FTD that recapitulate distinct disease-related pathological, functional, and behavioral phenotypes. An overview of the currently available models is shown in Table 1. The first mouse model for *C9orf72* ALS was created in 2015 by somatic transduction of the C57BL/6J mouse CNS using an AAV carrying 66 G_4C_2 repeat.[83] Although this model did not develop the severe ALS/FTD

Table 1. Overview of the currently available mouse models for *C9orf72* related ALS and/or FTD.

ALS mouse model	Mutation	phenotype
AAV-$G_4C_2$66 (Chew et al. 2015)	$(G_4C_2)_{66}$ + 119 bp 5′ + 100 bp 3′ region (expressed by CBA promotor)	Mild: Histopathological features, Anxiety, decreased sociability, reduced motor function, weight loss, loss of NeuN positive neurons in cortex, motor cortex, Purkinje cells.[83]
BAC-C9-500/300 (Peters et al. 2015)	Human *C9orf72* (exon 1-6) + ~500 G_4C_2 repeats + 141 Kb 5′ region	Only histopathological features (no TDP-43 pathology), no behavioural.[86]
Tg(*C9orf72*_3) Line 112 (BAC-C9-(100-1000)) (O'Rourke et al. 2015)	Human *C9orf72* (1-11) + mix of 100-1000 G_4C_2 repeats + 110 Kb 5′ + 20 Kb 3′ region	Only histopathological features (no TDP-43 pathology), no behavioural.[84]
BAC-C9-450 (Jiang et al. 2016)	Human *C9orf72* (exon 1-5) + ~450 G_4C_2 repeats + 140 Kb 5′region	Mild: Histopathological features, spatial learning deficit, anxiety, ~ 10% loss of hippocampal neurons.[62]
BAC-C9-500 (Liu et al. 2016)	Human *C9orf72* (1-11) + ~500 G_4C_2 repeats + 52 Kb 5′ + 19 Kb 3′ region	Severe: Histopathological features, impaired motor function, reduced grip strength, hindlimb paralysis, decreased survival, loss of Purkinje cells, interneurons, upper and lower motor neurons.[85]

symptoms, some important features were observed that correlate with the pathology in patients. For example, sense RNA foci and DPRs produced from RAN translation were observed and TDP-43 inclusions were found in ~7-8% of neurons in the cortex and hippocampus.[83] In addition, mild neurodegeneration but no motor neuron loss was observed.[83] Four other transgenic mouse models were created expressing either full length or truncated human *C9orf72* with the G_4C_2 expansion. The *C9orf72* Exon 1–6 BAC (G_4C_2)500 SJL/B6 mice expressed sense and antisense RNA foci and poly GP proteins but no motor and/or behavioral impairment was observed.[47] The Tg(*C9orf72*_3) line 112 mouse was created containing the full length human *C9orf72* with multiple G_4C_2-repeat sizes ranging from 100 to 1000 repeats.[84] This model showed sense and antisense RNA foci and mild accumulation of poly GP but despite some evidence for nuclear stress there was no neuronal loss observed. Similarly, the BAC-C9-450 model also produced sense and antisense RNA foci in some brain regions and in the spinal cord as well as DPR proteins with an age dependent increase of poly(GA).[62] Despite a partial learning deficit and increased anxiety, no other motor or behavioral changes were observed. In contrast, the FVB/NJ-Tg(*C9orf72*)500Lpwr/J mouse model expressing the full length mutated human *C9orf72* gene including the 52 Kb 5'upstream region and the 19 Kb 3' downstream region of the gene did show progressive neurodegeneration and decreased survival which is also seen in ALS patients.[85] Sense RNA foci were observed in almost all NeuN positive neurons in the cortex, hippocampus and cerebellum. Antisense RNA foci were predominantly found in the motor cortex, hippocampus, cerebellar Purkinje layer and interneurons in the lateral and posterior horn of the spinal cord. DPR proteins in this mouse model increased with age throughout the brain, and nuclear and cytoplasmic TDP-43 aggregates were mainly observed in degenerating brain regions. The severe phenotype was exclusively observed in about one third of the females, while most of the males and females developed a mild phenotype. Nevertheless, the observations made in this model strongly support the involvement of the G_4C_2 repeat-mediated gain of toxicity in the disease pathology.

Despite the variable penetrance, these models could still be valuable tools for research as they all displayed some specific features such as RNA foci and DPR proteins that are key characteristics of *C9orf72* related ALS/FTD patients.

Animal models for SCA3

More than ten transgenic models for SCA3 have been published, all with variable differences in pathology and phenotype and most of them showing a mild form of neurodegeneration.[87] A summary of the most commonly used mouse models and their key characteristics is shown in Table 2. Three models showed a severe phenotype and will be discussed here in more detail. The first transgenic mouse model for SCA3 was created more than two decades ago expressing either full length or truncated ataxin-3 with 79 CAG repeat (Q79). Surprisingly, a severe ataxic phenotype was observed only in

Table 2. Overview of the currently available mouse models for SCA3.

SCA3 mouse model	Mutation	phenotype
Q79 (Ikeda et al. 1998)	L7 promotor + Human Ataxin 3 +79 CAG L7 promotor + truncated Human Ataxin 3 +79 CAG	Severe when 79 CAG is expressed by itself: severe ataxia, gait disturbances, motor deficits.[88]
MJD84.2 (Cemal et al. 2002)	Human ataxin-3 (YAC) + 84 CAG + 35 Kb 5′ + 150 kb 3′	Intermediate: gait abnormalities, hypoactivity, Limb clasping, atrophy of the cerebellar Purkinje and molecular cell layers.[95]
Homozygous Q71C (Goti et al. 2004)	Mouse prion promotor + human ataxin-3 + 71 CAG	Severe: progressive postural instability, gait and limb ataxia, weight loss, premature death, neuronal intranuclear inclusions, decreased TH-positive neurons in the substantia nigra. [92]
70.61 (Bichelmeier et al. 2007)	Mouse prion promotor + Ataxin-3 + 70 or 184 CAG	Severe: intranuclear inclusions in cortex and cerebellum, Shrinkage of ~ 50-80% of Purkinje cells, premature death[96]
Ataxin-3-Q79HA (Chou et al. 2008)	Mouse prion promotor + Ataxin-3 + 79 CAG	Intermediate: neuronal dysfunction, ataxia, downregulation and upregulation of several genes.[97]
PrP/MJD77-het/hom (Boy et al. 2009)	Ataxin-3 + 77 CAG	Mild: Cerebellar dysfunction, reduced anxiety, hyperactivity, impaired rotarod performance, weight loss.[98]
HDProm-MJD148 (Boy et al. 2010)	ataxin-3 +148 CAG (expressed by Huntingtin promotor)	Mild: Late onset symptoms, declined motor coordination after one year.[99]
Hemi-CMVMJD94 (Silva-Fernandes et al. 2010)	ataxin-3 + 94 CAG (expressed by CMV promotor)	Mild: represents early disease symptoms, neuronal atrophy and astrogliosis in several brain regions.[100]
Lentiviral Atx3-72Q (Nóbrega et al. 2013)	Ataxin-3 + CAG (expressed by PGK promotor)	Mild: reduced motor coordination, wide-based ataxic gait, and hyperactivity. accumulation of intranuclear inclusions, neurodegeneration.[101]
Humanized SCA3 knockin (Ki91) (Switonski et al. 2015)	Ataxin-3+91 CAG	Mild: deficits in coordination, transcriptional changes in the brain, amyloid depositions, mild degeneration of Purkinje cells in older mice, increased GFAP-positive glia in cerebellar white matter.[102]

the truncated model, with degeneration of the cerebellum and loss of Purkinje cells.[88] Due to this observation, it was strongly suggested that the C-terminal fragment of ataxin-3 containing the expanded polyQ could be more toxic by itself than when expressed as part of full length ataxin-3. Consistently, putative cleavage fragments of expanded ataxin-3 were identified in cell models, in post-mortem brains tissues of a SCA3 mouse model, in a drosophila model and in patients.[89-93] It is now well-accepted that the gain of toxicity is directly caused by the CAG repeat and that the affected neurons express a protease that cleaves the mutant ataxin-3 protein, releasing short soluble polyQ fragments that are highly neurotoxic and more prone to form aggregates.[80,81] Besides proteolytic cleavage of the C-terminal of the mutant ataxin-3 protein, mis-splicing could also play a role. A crucial evidence of mis-splicing leading to production of toxic short polyQ proteins was observed in HD. It was shown that exon 1 of the huntingtin, gene which contains the CAG repeat does not always splice to exon 2 but generates small polyadenylated HTTexon1 mRNA that encodes a small, highly pathogenic exon 1-polyQ HTT protein (also known as "exon-1 protein").[94]

The two other SCA3 mouse models (Q71C and 70.61) with a severe phenotype both express mutant ataxin-3 under control of the prion protein promotor. Within 1 to 8 months, both models showed early onset and a rapidly progressive type of SCA3 with several abnormalities such as progressive neurodegeneration, weight loss, behavioral problems, neuronal inclusions and premature death. Taken together, all these models could be useful to further study the disease mechanism in SCA3 and serve as tools for preclinical studies targeting mutant ataxin-3. It should be noted that the models with a severe phenotype all use non-native promoters to drive expression of mutant ataxin-3, lack regulatory flanking sequences, and often contain an excessive number of transgene copies. Thus, these models do not exactly mimic the expression pattern of mutant ataxin-3 in patients.

In vitro models for ALS and SCA3

Induced-pluripotent stem cell (iPSC) technology is revolutionizing the study of genetic diseases and is now pivotal for the development of targeted therapies. The use of iPSC technology was made possible when Professor Shinya Yamanaka showed that expression of four transcription factors (Myc, Oct3/4, Sox2 and Klf4) in fibroblasts reprograms the cells into pluripotent stem cells with the capability for indefinite self-renewal. For this discovery he was awarded the Noble prize in 2012.[103] It was subsequently demonstrated that iPSCs can be generated from different human somatic cell types that can be easily obtained such as fibroblasts, white blood cells, renal epithelial cells, keratinocytes etc. What made this technology widely accepted was the fact that it bypasses the ethical concerns associated with the use of embryonic stem cells and depending on the type of study, it may even replace animal models. iPSC lines can be generated from patients and differentiated back toward the disease-specific cells. For ALS, iPSC- derived motor neurons

have been of particular interest to study toxicity caused by the G_4C_2 repeat and to test therapeutic compounds. Cortical neurons can also be generated from iPSCs representing the cells primarily affected in FTD. For SCA3, it is currently still challenging to model cerebellar diseases with iPSC technology and although generation of cerebellar neurons such as Purkinje cells from iPSCs has been reported, this seems difficult to reproduce.[104] Hence, modeling cerebellar neurons with iPSC technology is less commonly reported as compared to other neuronal cell types in the frontal brain, midbrain and spinal cord. iPSC technology makes it possible to generate cell models for an individual or a specific group of patients which is a big advantage when compared to the different types of animal models, that always display species-related differences. Human-derived iPSCs with a wide variety of genetic backgrounds can be differentiated into different types of cells. This makes iPSC technology a very useful tool to study disease mechanisms, to use in early drug discovery (e.g screening of new therapeutic compounds), for toxicology testing and for prediction of off-target effects of gene modulating compounds (e.g. RNAi) in humans.

A limitation of iPSC systems is that *in vitro* two-dimension (2D) monocultures fail to represent physiological cellular functions and signaling pathways due to the lack of cell-cell and cell-matrix interactions. Recently, it was discovered that iPSCs can also be cultured in three dimensions (3D) to generate organoids that could represent different types of tissues, including different brain regions.[105,106] These 3D brain organoids allow cell-cell interactions and complex cyto-architectures to be modeled and studied in greater detail and in more physiological contexts. It is currently difficult to control the cell type organization and cell-cell or cell-matrix interactions and most organoid only represent single or partial components of a tissue. Efforts are being made to make organoids with multiple cell types in a more controlled fashion which may be promising in the future to replace animal models. While the use of animal models may be significantly reduced with the current advances on iPSC technology, at present, drug development and study of disease mechanisms still rely on a combination of different in vitro and *in vivo* model systems.

RNAi-based therapeutic strategies for neurogenerative diseases

As discussed, several neurodegenerative diseases are caused by mutations that lead to gain of toxic functions, and gene silencing technologies are attractive to lower the expression of disease-causing genes. A specific technology that can be applied for gene silencing is RNA interference (RNAi).

The discovery of RNAi dates almost three decades (1990) back, when Napoli and Jorgensen reported that injection of an extra chimeric copy of chalcone synthase, a gene responsible for the purple pigment anthocyanin in petunias, unexpectedly resulted in white petunias.[107] Thus, instead of complementing each other and producing extra purple flowers, the two copies of chalcone synthase seemed to interact with each other and

turned themselves off. The mechanism underlying this observation remained unclear until 1998, when Andrew Fire and Craig C. Mello provided an explanation of the RNAi mechanism. They discovered that a small non-coding RNA, miRNA (lin-4), binds to the 3'-UTR of the lin-14 mRNA in C. elegans, silencing its expression. For this work they were awarded the Nobel prize in Physiology or Medicine in 2006.[108] It was subsequently demonstrated that miRNA-mediated silencing also occurs in mammalian cells and this mechanism became a popular new tool to study gene function.[109]

It is now known that RNAi is a naturally occurring process in eukaryotic cells where double stranded RNA molecules can knock down or suppress the expression of genes that contain a homologous RNAi target sequence. RNAi plays an important physiological role in gene regulation and also has a function in the innate immune response of cells by providing protection against foreign nucleic acids from pathogens such as viruses and bacteria.[110] One of the first discovered mediators of RNAi is the RNAi-induced silencing complex (RISC), which has a nuclease activity that can cleave mRNA and knock down its expression. RNAi can be triggered by both endogenous and exogeneous double stranded RNAs.[111–113] Three types of small non-coding RNAs use the RNAi pathway; microRNA (miRNAs), small interfering RNA (siRNA) and piwi-interacting RNAs (piRNAs). miRNAs and siRNAs have a role as negative regulators of gene expression, while the piRNAs defend organisms against transposable elements.[114] miRNAs and siRNA are both widely being used as therapeutics in clinical trials and each system has its own merits. The focus of this thesis will be on miRNAs

miRNAs and their processing

miRNAs are small non-coding RNAs that are thought to regulate about 30% of genes in humans. More than 2000 miRNAs (www.mirbase.org) have been identified in humans and these are known to regulate several important cellular processes. Dysregulation of miRNAs is associated with several types of metabolic and CNS diseases, and with cancer. Apart from regulating gene expression, miRNAs can act as signaling molecules for intercellular communication. For example, it has been shown that miRNAs can be packaged into exosomes or microvesicles that following secretion, can be endocytosed by secondary cells, and re-establish their function.[115]

The biogenesis of a miRNA is tightly regulated in humans and involves 4 key enzymes; Drosha, exportin 5, Dicer and argonaute (AGO) proteins (Figure 4). The precursor of a miRNA is naturally encoded in the genome and is transcribed by RNA polymerase II or III into a long primary miRNA (pri-miRNA) with a cap and poly-A tail. The pri-miRNA transcript folds into a complex hairpin structure consisting of a double-stranded stem of about 30 base pairs, a terminal loop and two flanking unstructured single-stranded tails. The pri-miRNA is further processed in the cell nucleus by a ribonuclease called Drosha, resulting in a short 70-nt stem-loop structured precursor miRNA (pre-miRNA). The pre-miRNA is then transported to the cytoplasm by exportin 5 and is cleaved into

a short double stranded miRNA by a RNAse III enzyme called Dicer. The double stranded miRNA is recognized by AGO proteins and is loaded on RISC, usually preserving the guide strand and degrading the passenger strand. The RISC-AGO complex guides the miRNA guide strand to its target mRNA and induce its degradation and/or inhibit its translation. The first seven nucleotides near the 5'end of the guide strand form the seed sequence and are critical for the target recognition. It is also worth to mention that not all miRNAs are processed as described above and miRNA processing in plants differs at several points from the above described pathway. Furthermore, several mammalian miRNAs can bypass Drosha and currently one miRNA is known to bypass Dicer processing.[116] This Dicer-independent miRNA is microRNA-451a (miR-451a or miR-451), which has a role in the regulation of erythroid development.[117] Its recognition by Dicer seems to be perturbed due to its unusually short base paired stem and as a result, an active strand derived from the stem is produced, consisting of the single-stranded loop and part of the complementary stem region (figure 4). The ability to design artificial miRNAs that specifically silence disease-related genes is the basis for therapeutics such as miRNA mimics, anti-miRs and artificial miRNAs. miRNA mimics are double stranded miRNAs made synthetically to match a corresponding miRNA, aiming to compensate the loss of miRNAs that are downregulated in diseases. Anti-miRs are artificially made single stranded miRNA to bind target miRNAs and inhibit their function. Artificial miRNAs are made by replacing the guide strand sequence of an endogenous miRNA precursor with the sequence of an mRNA of interest, enabling silencing of genes that are upregulated in diseases (figure 5). Recently, the miQURE™ Gene Silencing platform was introduced by uniQure. miQURE uses the advantages of the non-canonical processing of miR-451 to silence genes involved in diseases. miR-451 produces no passenger strand as the pre-miR-451 escapes Dicer cleavage and only a 5' arm strand is active on the targets. Thus, there is no miRNA duplex formation that requires strand separation and selection for the RISC. Because of this efficient processing, off-target effects due to a passenger strand activity can be neglected. This technology was first applied in uniQure's Huntington's disease program and is now being investigated for application in other neurodegenerative diseases such as ALS and SCA3 and for liver indications.

Clinical applications for Therapeutic RNAi

Several RNAi-targeted therapeutics have reached clinical development. For example, MRX34 (synthetic miR-34), a miRNA developed by Mirna Therapeutics was the first to enter a clinical trial to treat different types of cancer.[118,119] Its mode of action was to decrease the expression of collagen and other proteins that are involved in fibrous scar formation. However, clinical development was terminated in 2016 due to several immune-related severe adverse events. An example of a successful anti-miR clinical trial is Miravirsen, an Locked Nucleic Acid (LNA) anti-miR-122 oligo developed by Santaris Pharma to treat Hepatitis C.[120] The phase II trials demonstrated reduced Hepatitis C viral load, even at low

therapeutic concentrations and no adverse events was observed.[121–123] Another phase I/II clinical trial to treat patients with Hepatitis C infection was based on shRNAs and was conducted by Benitec Biopharma. Their lead product was TT-034 which is based on intravenous AAV8 delivery of three independent shRNAs targeting three well-conserved

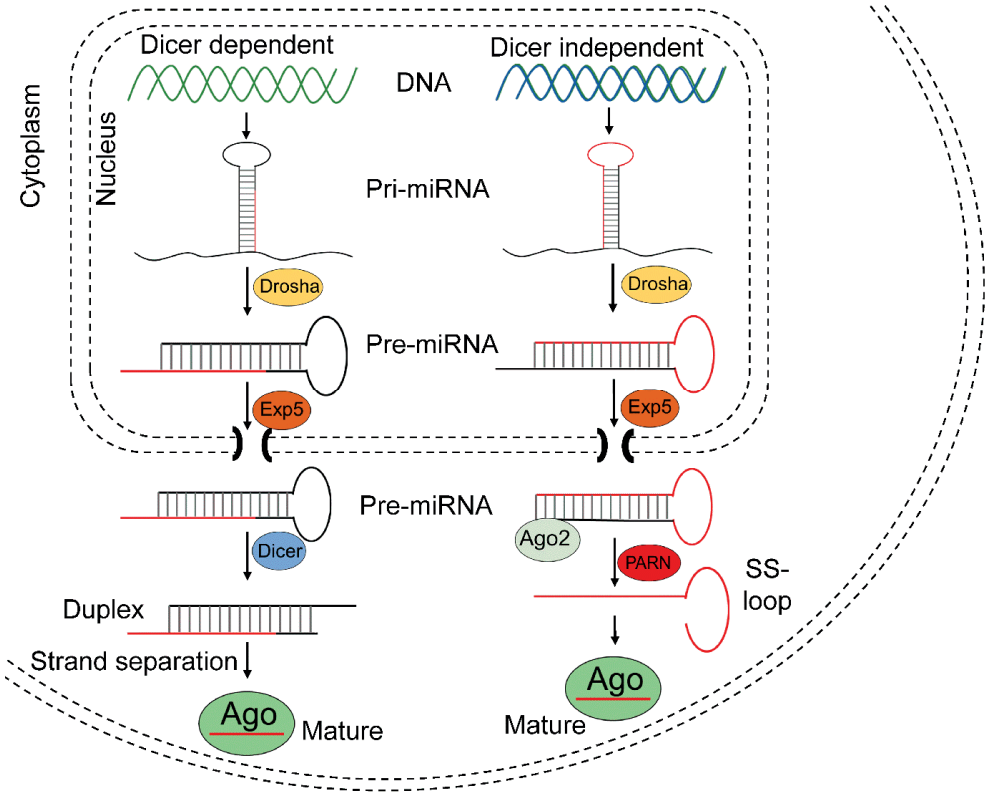

Figure 4. Schematic of miRNA processing pathway. Most miRNAs are processed in the Dicer dependent (canonical) pathway shown in the left part of the figure. After being transcribed, the pri-miRNA transcripts are cropped by Drosha in the nucleus, resulting in a 60–70 nt pre-miRNA. The pre-miRNA is exported to the cytoplasm by Exportin 5 and the hairpin is then diced by Dicer into ~22-nt miRNA duplex, after which it is separated into a guide and passenger strand. The guide strand is usually loaded into Ago proteins to form RISC. There are four Ago proteins and all are capable to repress translation or promote mRNA degradation. Ago2 is the only Ago protein with a slicer activity which plays a critical role in Dicer independent processing of miR451 (right part of the figure). miR451 is also processed in the nucleus by Drosha, but results in a unusually short, 41–42-nt pre-miRNA which is not recognized by Dicer and do not form a miRNA duplex. However, the further processing of miR-451 requires the slicer activity of Ago2. Ago2 cleaves the 3' arm of pre-miRNA by its slicer activity and yields a 30-nt intermediate whose 3' end is further trimmed by PARN to generate a mature, ~23-nt miRNA. This mature miRNA is loaded into Ago proteins to form the RISC.

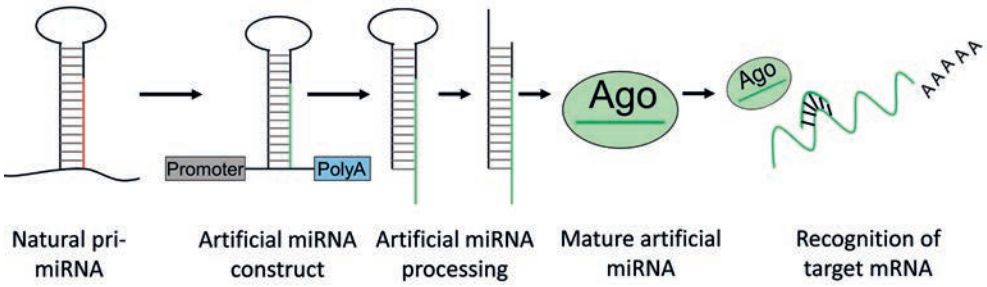

Figure 5. Artificial miRNA design. Artificial miRNAs can be made by replacing the mature miRNA sequence of a natural pri-miRNA for a complementary sequence of a target mRNA of interest. The artificial pri-miRNA sequence can be cloned in an expression construct. Upon transfection with the artificial miRNA construct, the artificial miRNA is processed in the cell into a mature artificial miRNA which can bind and knockdown expression of a mRNA of interest.

sequences located in the 5' UTR and NS5B regions of the HCV genome.[124] Overall, TT-034 was well tolerated and safe in patients. Furthermore, liver biopsies revealed sustained transduction of hepatocytes and expression of the three anti-HCV shRNAs. However, Benitec Biopharma decided to discontinue this program due to decrease in commercial opportunities for TT-034 following the introduction of highly effective viral eradication strategies based on (combinations of) small molecules. Compared to miRNAs, siRNAs have been more widely tested in clinical trials for treatment of different types of diseases such as cancer, viral infections, age-related macular degeneration, diabetic macular, hypercholesterolemia and ocular hypertension.[125] DNA constructs encoding therapeutic RNAi following delivery by lentiviral vectors have also been clinically tested. Benitec, Inc in collaboration with the City of Hope National Medical Center conducted a trial to treat HIV-1 infection in AIDS/lymphoma patients.[126] This was executed by genetically modifying hematopoietic stem cells (hSC) *ex vivo* by transduction with a lentiviral vector expressing three small RNAs targeting HIV: An shRNA targeting an exon in HIV-1 tat/rev , a RNA decoy for the HIV TAT-reactive element, and a ribozyme targeting the host cell CCR5 chemokine receptor. The transduced hSC were then infused into patients whose bone marrow has been ablated to treat their AIDS related lymphoma. Sustained expression of the anti-tat/rev shRNA and ribozyme was observed for up to 24 months post-infusion.[123] Although this study demonstrated feasibility and safety of this approach, it failed to demonstrate clinical benefit because an insufficient number of hSCs was transduced.

In the neurodegenerative field, uniQure recently obtained Food and Drug Administration (FDA) clearance for AMT-130 and this will be the first AAV-miRNA-based gene therapy for a neurodegenerative disease to start a Phase I/II clinical trial. AMT-130 is based on a miRNA designed to target both wild-type and mutant Huntingtin allele (AAV5-miHTT).[127,128] Significant lowering of human mutant huntingtin mRNA and protein was achieved in the brain of a transgenic HD (tgHD) minipig model after a single

administration into the brain. [128] uniQure plans to start the first PhaseI/II clinical trial in early HD patients in the second half of 2019. A similar approach is being pursued by Voyager Therapeutics and promising results have been obtained with their lead candidate VY-HTT01 in preclinical models. miRNA mediated silencing is thought to be an attractive therapeutic modality in many other neurodegenerative diseases, including the SCAs, ALS, FTD, synucleopathies including Parkinson's disease, tauopathies and AD.[129–132] miRNAs may cause toxicity related to (passenger strand) off-target toxicity. Hence, during development these effects should be closely investigated and minimized, by selecting scaffolds with little to no passenger strand activity and by predicting the chances of binding to off-target genes using computer-based bioinformatic tools. Another important limitation of miRNA-based therapeutics is the required efficacy in the nucleus of cells, because active mature miRNAs resulting from canonical Dicer processing in the cytoplasm are thought not to re-enter the nucleus. However, as many of these hurdles are being tackled, more miRNA therapeutics are expected to reach the clinic.

Common alternative silencing strategies

Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are short, synthetic, single-stranded nucleic acids of about 20-25 bases long that bind cellular RNA and reduce, restore, or modify protein expression via several distinct mechanisms. The silencing pathway of ASOs differs from siRNA and miRNAs and there is no known cellular mechanism to facilitate strand recognition. ASOs classically bind to complementary mRNA through Watson Crick base-pairing leading to endonuclease-mediated transcriptional knockdown.[133] Once bound to the mRNA, ASOs can form an RNA–DNA hybrid that becomes a substrate for the enzyme RNase H, which hydrolyzes the mRNA resulting in its degradation.[134] The cleaved mRNA products are then processed by the normal cellular degradation pathways in the nucleus and cytoplasm.[135] ASOs can be engineered to have enhanced pharmacological properties by introducing backbone modifications. For example, modified ASOs can knockdown gene expression by sterically blocking splicing factors and altering pre-mRNA splicing, or by preventing ribosome recruitment to block mRNA translation.[136] Interestingly, ASOs can destabilize splicing sites and displace or recruit splicing factors when designed

Table 3. clinical trials using ASOs for ALS by Ionis Pharmaceuticals

Disease	Trial code	Vector	Delivery route	Status (completion year)	Sponsor
ALS[137,138]	NCT01041222	ASO (SOD1)	Intrathecal	Phase I (2012)	Ionis Pharmaceuticals
ALS	NCT02623699	ASO (SOD1)	Intrathecal	Phase I/II (2019)	Biogen & Ionis Pharmaceuticals
ALS	NCT03626012	*ASO (C9orf72)*	Intrathecal	Phase I (2022)	Biogen & Ionis

to bind within intron–exon junctions. This makes it possible to exclude ("skip") or include exons of interest, which can be beneficial in genetic diseases.[136] Through this mechanism normal gene function can be restored by re-establishing a normal reading frame following a pathogenic frame shift, or by excluding mutated segments of DNA.[133] ASOs have been used in preclinical studies since the 1970s and several programs have made it into clinical development. A phase I ALS trial using intrathecal administration of ASOs targeting SOD1 was completed in 2012 by Ionis Pharmaceuticals (Table 3).[137,138] Unfortunately this study failed to show a reduction of SOD1 protein which was explained by the low target tissue ASO concentration but the treatment proved to be safe, and a follow up phaseI/II trial study is ongoing (BIIB067; IONIS-SOD1). Ionis Pharmaceuticals also initiated a phase I clinical trial in 2019 with ASOs targeting *C9orf72*-ALS patients (BIIB078). BIIB078 targets specifically the intronic region of *C9orf72* to cause reduction of only the repeat-containing *C9orf72* transcripts and is administered intrathecally to adult patients. Ionis Pharmaceuticals has developed ASOs for many other diseases and have ongoing clinical trials for HD, DM1 and SMA. Furthermore, several preclinical studies have shown efficacy of ASOs in *C9orf72*-related ALS and SCA3 in vitro and in vivo but these programs have not yet reached clinical development. A clinical study (NCT03508947) with ASOs that has raised some concerns regarding toxicity was performed by WAVE life Sciences to treat patients with Duchenne muscular dystrophy (DMD). DMD is a fatal genetic disorder characterized by progressive muscle wasting and is caused by mutations in the DMD gene which encodes for dystrophin, an essential protein to maintain muscle integrity. The ASO was administered intravenously and was designed to skip the mutated exon 51 in the DMD gene to restore normal function of dystophin.[139] Adverse events such as headache, fever, vomiting and tachycardia occurred in the low and mid dose groups and these adverse events were even more severe in the high-dosed groups. WAVE life sciences reported to continue with the lower doses for Phase II/III trial which is expected to be initiated in 2019 but it is unclear if these adverse events will re-occur during re-administration and whether or not the lower doses are therapeutically effective. A major limitation of ASOs for clinical application is that they are degraded by endo- and exonucleases and re-administration is required.[140] This is especially problematic in CNS disorders that require invasive delivery methods. However, the pharmacological profiles of ASOs can be enhanced by introducing chemical modifications and efforts are being made to improve ASOs delivery to for example cross the blood brain barrier (BBB) and to improve target engagement.[141]

Gene Editing (CRISPR)

Gene editing is a relatively novel approach to remove, add or alter DNA in a sequence-specific manner. The pivotal discovery that made gene editing possible was the discovery that the endogenous cellular repair machinery can be triggered by targeted DNA double strand breaks (DSBs) through homology-directed repair (HDR) or nonhomologous end-

joining (NHEJ). DNA DSBs can be introduced at a precise location by using engineered nucleases harboring a sequence-specific DNA-binding domain, fused to a DNA cleavage module. The most popular nucleases are zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the RNA-guided clustered regulatory interspaced short palindromic repeats (CRISPR) and CRISPR-associated system 9 (Cas9) (CRISPR/Cas9). ZFNs are currently the only gene editing technology that have made it to clinical trials. In 2018 Sangamo Therapeutics started a clinical trial using this technology in mucopolysaccharidosis II (MPS II). ZFNs consist of ~30 amino acids which folds into ββα "fingers" structures that recognize and bind a trinucleotide sequence of DNA.[142–144] The nuclease domain is based on a restriction endonuclease (FokI) that can cut DNA when following dimerization.[142] Binding to DNA sequences longer than 3 and up to 18 base pairs (in multiples of 3 base pairs) is possible by arranging a series of linked zinc-fingers. However, this requires recoding of proteins for each new target site which is a very challenging and time-consuming process. Another limitation is the restricted target site selection as zinc-fingers can only target binding sites every ~50-200 base pairs in a random DNA sequence [142] Furthermore, all gene editing technologies including zinc-fingers may have off-target effects, resulting in unwanted induction of DNA mutations or deletions. Like ZFNs, TALENs are based on a DNA-protein association but they use a different DNA binding domain termed (transcription activator-like effectors) TALE repeats.[142] A TALE motif can recognize a single nucleotide and an array of TALEs can recognize and bind to a longer DNA sequence. Unlike ZFNs, the design of TALENs are less complicated and the TALE repeat array can be easily extended to whatever length is desired.[142] Furthermore, as compared to ZFNs, site-specific targeting is easier because multiple TALEN pairs are available for each base pair of a random DNA sequence. Some major limitations for TALENs are off-target effects and compared to ZFNs, they are about 3x larger in size (~3 Kb) which makes delivery by viral vectors more challenging.

CRISPR/Cas9 is the latest of the above-mentioned nuclease systems and is based on a naturally occurring process in the adaptive immune system of the bacteria Streptococcus pyogenes. CRISPR/Cas9 is distinct from ZFN and TALEN endonucleases in that it does not use a protein but an RNA-guided system to perform genome editing. Unlike the other nuclease systems, CRISPR/Cas9 does not require custom design of novel proteins for each DNA target site. Thus, the design of constructs is relatively easy and cost-effective. Bacteria use the RNA-guided Cas proteins to create DSBs in exogenous DNA derived from invading viruses (bacteriophages).It does so by creating CRISPR arrays from exogenous DNA by insertion of these DNA sequences between short palindromic repeats in the CRISPR locus of its own DNA. Upon a repeated viral attack, these "foreign" inserts can be transcribed from the virus specific CRISPR locus into CRISPR RNA fragments that match the invading viral DNA. CRISPR RNAs contain a tail transcribed from the CRISPR locus which facilitates incorporation into complexes to form hairpins-like structures that allow them to dock on a Cas protein. The Cas protein is then guided by the CRISPR RNA to hybridize to its parental exogeneous DNA and induce a DSB.[145] This CRISPR/Cas9

system can be implemented in eukaryotes by designing sequences that target a specific DNA sequence and co-expressing Cas9. The Cas9 protein cuts the DNA at the target site, which is repaired by HDR or NHEJ. Several animal models have been created using the CRISPR/Cas9 system and a rapidly increasing number of preclinical studies show the promises of this technology in combination with gene therapy for the treatment of neurodegenerative diseases. For example, in AD, the defective (amyloid precursor protein) APP gene was successfully edited in human fibroblasts using a CRISPR/cas9 construct and this resulted in reduction of Amyloid beta, a main component of plaques found in brain of AD patients.[146] In ALS, the G_4C_2 repeat in the non-coding region of the *C9orf72* gene was successfully deleted in transfected patient-derived iPSCs and this prevented RNA foci formation as well as the promoter hypermethylation that is typical for ALS.[147] For HD, permanent suppression of mutant Huntingtin and it's aggregates was achieved in the striatum of the HD140Q-knock-in mice by CRISPR/Cas9.[148] For SCA3, CRISPR/Cas9-mediated deletion CAG repeat in *ATXN3* gene was successfully performed in patient-derived iPSCs.[149] Following the CAG deletion, the iPSCs retained pluripotency and neurons differentiated from these cells retained a normal ubiquitin-binding capacity of *ATXN3*.

The CRISPR/Cas9 system has great potential for targeting pathogenic genes, but a major hurdle is the occurrence of off-target effects, Cas9 specificity, and potential mutagenesis. Not unexpectedly, continuous expression of Cas9 proteins at high concentrations has been linked to toxicity.[150] The chances for off-target binding in the host genome using current systems are estimated to be relatively high (≥50%).[151] Another limitation is the delivery method as the CRISPR/Cas9 components are about 8-10 kb long whereas AAV vectors have a packaging capacity limited to 4.8 kb. Thus, the CRISPR/Cas9 system has a huge potential to be developed in therapeutic approaches but major challenges need to be overcome to make this technology suitable to treat human diseases.

AAVs for gene transfer to the nervous system

A major difficulty to treat neurodegenerative diseases remains the method of administration. Due to existence of the BBB, many small molecules and most therapeutic nucleotides or gene therapy vectors that are administered systemically or orally fail to reach the brain or spinal cord at a therapeutically relevant dose. Different alternative routes of administration are being investigated for delivery to the CNS and their application is highly depended on the disease pathology. While some diseases require local targeting or transduction, others require widespread distribution throughout the CNS. Intracerebral administration, directly injected in the parenchyma of the brain can be safely and effectively done using convection-enhanced delivery (CED) in combination with precise positioning using magnetic resonance imaging-based guidance technologies (MRI).[152] CED uses a pressure gradient to generate bulk flow within the brain parenchyma. Due to the low velocity of injection, potential structural damage is minimized, and a uniform distribution can be

obtained.[153,154] The advantage of this injection route is that it bypasses the BBB and high local transduction can be obtained with a relatively low dose of AAV, with limited leakage to periphery organs. Further spread of AAV may occur via anterograde or retrograde transport along axons but this is highly dependent on the AAV serotype.[9,155] A drawback of this delivery route is that the procedure itself is invasive and may lead to complications such as bleeding or leakage of the administered vector into the CSF. In addition, intracerebral administration is only suitable for diseases with pathology limited to specific brain areas. Alternative routes to cover larger areas of the CNS are systemic or intrathecal delivery. Systemic delivery by intravenous injection is a relatively simple procedure, less invasive and avoids costly neurosurgical procedures. However, systemic delivery of AAV gene therapy is currently less suitable for neurodegenerative disorders because the bulk of such vectors is taken up by peripheral organs and can cause systemic immunogenicity. Even though some vectors can cross the BBB, the target tissue concentration after systemic delivery is often not therapeutically relevant. Nevertheless, a successful clinical trial with systemic administration was performed by AveXis to treat children with SMA.[156] The vector used was a self-complementary AAV9 and following systemic administration a therapeutic effect in motor neurons of the spinal cord was observed. It remains questionable whether similar effects can be obtained in adults as the integrity of the BBB is known to be different. Despite the initial promising results, AveXis changed the delivery method in children to an intrathecal administration route in the follow up study. This amendment was based on a study in NHP showing improved transduction of the CNS with up to 10 times lower intrathecal dose as compared to intravenous administration.[157] Intrathecal administration can be done through lumbar puncture, direct administration into the cisterna magna or in the cerebral ventricle.[158,159] Thus, the vector is delivered directly into the CNS bypassing the BBB. This approach is less invasive than intracerebral administration and usually show less leakage to the periphery organs as compared to systemic administration. One limitation of intrathecal administration is dilution of vector and the consequent transduction is usually lower when compared to intracerebral administration. Furthermore, transduction of the deeper brain structures is poor, possibly because the vector needs to pass the ependymal cell layer or the pia mater.[160,161]

Gene therapy clinical trials for neurogenerative diseases

Several gene therapies for different types of neurodegenerative diseases have progressed into clinical development. For *C9orf72* related ALS and SCA3, no gene therapy has been clinically investigated yet. An overview of the currently ongoing clinical trials published on https://clinicaltrials.gov is depicted in table 4 and will be further discussed in this paragraph.

Parkinson's disease

PD is a neurodegenerative disorder caused by loss of dopaminergic neurons in the substantia nigra, leading to bradykinesia, rigidity, tremor, and gait dysfunction.

Expressing neurotrophic factors such as GDNF delivered by AAV was tested in a single clinical trial and AAV-delivered neurturin (NTN) was investigated in three clinical trials for PD. The rationale for delivering these neurotrophic factors was not to target a causative pathological molecular pathway but to provide neurotrophic support to the degenerating neuronal population. Although these studies were well tolerated, their efficacies are unclear. However, these studies were important to demonstrate the feasibility and tolerability of intraparenchymal AAV delivery of gene therapy directly in the human brain.

Most gene therapy approaches for PD aim to restore dopamine production in neurons. Deliveryof 1-amino acid decarboxylase (AADC), a key enzyme for dopamine production showed to be more promising than neurotrophic factors. Proof of concept studies in NHP showed an increase in levels of dopamine which was sustained for up 7 years.[154,162] The first AAV delivered AADC study in humans (NCT00229736) was in general well tolerated with some minor side effects.[163,164] A significant improvement of Parkinson's Disease Rating Scale (UPDRS) scores was reported at 6 months post-surgery which was sustained for up to 2 years. These promising results have triggered other AAV-based trials on AADC delivery and several studies are currently ongoing with higher doses and larger cohorts of patients. A clinical trial using a intrastriatal delivered lentivirus (ProSavin) expressing the three key tyrosine hydrolase, AADC, and GTP cyclohydrolase-1, with the aim of providing a continuous source of dopamine in the striatum has also demonstrated to be safe and well tolerated with no surgical complications (CT01856439).[165,166] A significant improvement of motor function was observed, and a long-term analysis is ongoing. Another lentiviral gene therapy that is currently being tested in clinic and very similar to ProSavin also expresses tyrosine hydroxylase, AADC and GTP-cyclohydrolase. While there is no data available yet from this study, the preclinical results in human primary neuronal cultures and NHP showed higher dopamine production compared to ProSavin.[167]

Alzheimer's disease

AD is the most common cause of age-related dementia affecting more than 40% of individuals of 85 years and older. More than 100 therapeutic compounds have been tested to date, but all failed to positively modify the course of the disease. Most gene therapy clinical trials for AD are based on intracerebral delivery of AAV-NGF. NGF encodes nerve growth factor and similar to GDNF and NTN, could provide neurotrophic support to neurons. Preclinical studies have shown that NGF can prevent degeneration of adult cholinergic neurons in the fore brain after injury.[168,169] Although the surgical procedures and the treatments proved to be safe in humans, the efficacy of the studies was inconclusive. A more recent study for AD is based on intracisternal delivery of AAVrh.10hAPOE2 (NCT03634007). It was shown that inherence of an extra allele of *APOE4* gene, a variant

Table I. Gene therapy clinical trials for neurogenerative diseases

Disease	Trial code	Vector
Parkinson[184]	NCT00252850	AAV2- Neurturin
Parkinson[155]	NCT00400634	AAV2-Neurturin
Parkinson[185]	NCT00985517	AAV2-Neurturin
Parkinson[186–188]	NCT01621581	AAV2-GDNF
Parkinson[163]	NCT00229736	AAV-hAADC-2
Parkinson	NCT01973543	AAV2-hAADC
Parkinson	NCT03562494	AAV2-hAADC
Parkinson	NCT02418598	AAV-hAADC-2
Parkinson[166]	NCT01856439	ProSavin (LV-TH-GCH-AADC)
Parkinson	NCT03720418	AXO-Lenti-PD(OXB-102-01)
Parkinson[189,190]	NCT00643890	AAV-GAD
Alzeimer	NCT00087789	AAV2-NGF
Alzeimer[191]	NCT00876863	AAV2-NGF
Alzeimer	NCT03634007	AAVrh.10hAPOE2
Batten[192–194]	NCT03770572	AAV9-CLN3
Batten[195]	NCT00151216	AAV2CUhCLN2
Batten	NCT01161576	AVRh.10CUhCLN2
Batten	NCT02725580	scAAV9.CB.CLN6
Mucopolysaccharidosis Type IIIA[173]	NCT03612869	AAVrh10-h.SGSH
X-Linked Myotubular Myopathy	NCT03199469	AAV8-hMTM1
spinal muscular atrophy Type 1	NCT02122952	AAV9-SMN
spinal muscular atrophy[157,196]	NCT03505099	AAV9-SMN
spinal muscular atrophy[157,196]	NCT03306277	AAV9-SMN
spinal muscular atrophy[156,197]	NCT03461289	AAV9-SMN
spinal muscular atrophy[156,197]	NCT03381729	AAV9-SMN
Pompe disease[198]	NCT03533673	AAV2/8LSPhGAA
Pompe disease	NCT02240407	rAAV9-DES-hGAA
Pompe disease[199]	NCT00976352	rAAV1-CMV-GAA
Canavan disease[200]	NA	AAV2- hASPA
AADC deficiency	NCT01395641	AAV2- hAADC
AADC deficiency	NCT02926066	AAV2- hAADC
AADC deficiency	NCT02852213	AAV2- hAADC
MPS I[201]	NCT02702115	AAV6-ZFN
MPS II[202]	NTC03041324	AAV6-ZFN
MPS IIIA	NCT02716246	AAV9-hSGSH
MPS IIIA[173]	NCT03612869	AAVrh.10- hSGSH
Sanfilippo Disease Type A	NCT02053064	AAVrh10- SGSH
MPSIIIB[174]	NCT03300453	AAV5- hNAGLU
MPSIIIB	NCT03315182	AAV9- hNAGLU
Huntington's disease	NA	AAV5-miHTT

Delivery route	Status (completion year)	Sponsor
Intrastriatal	Phase I (2007)	Ceregene
Putamen	Phase II (2008)	Ceregene
Substantia nigra	Phase II (2018)	Sangamo Therapeutics
Putamen	Phase I (2027)	National Institute of Neurological Disorders and Stroke (NINDS)
Intrastriatal	Phase I (2013)	Genzyme
Intrastriatal	Phase I (2019)	Voyager Therapeutics
Intrastriatal	Phase II (2020)	Voyager Therapeutics
Putamen	Phase II (2022)	Jichi Medical University
Intrastriatal	Phase I/II (2021)	Oxford BioMedica
Intrastriatal	Phase I/II (2020)	Axovant Sciences Ltd
Subthalamic nucleus	Phase II (terminated)	Neurologix, Inc
Basal forebrain	Phase 1 (2010)	Ceregene
Basal forebrain	Phase II (2020)	Sangamo Therapeutics
Intracisternal	Phase I (2020)	Weill Medical College of Cornell University
Intrathecal	Phase II (2022)	Nationwide Children's Hospital & Amicus Therapeutics
Intracranial	Phase I (2019)	Weill Medical College of Cornell University
Intracranial	Phase I (2020)	Weill Medical College of Cornell University
Intrathecal	Phase II (2019)	Nationwide Children's Hospital
Intracerebral	Phase III (2022)	LYSOGENE
Intravenous	Phase II (2025)	Audentes Therapeutics
Intravenous	Phase I (2019)	AveXis, Inc
Intravenous	Phase I (2020)	AveXis, Inc
Intravenous	Phase I (2020)	AveXis, Inc
Intravenous	Phase III (2020)	AveXis, Inc.
Intrathecal	Phase I (2020)	AveXis, Inc.
Intravenous	Phase II (2020)	Actus Therapeutics, Inc
intramuscular	Phase I (2019)	University of Florida
intradiaphragmatic	Phase II (2015)	University of Florida
Intraparenchymal	Phase I (2002)	National Institute of Neurological Disorders and Stroke
Intraparenchymal (Putamen)	Phase I/II (2020)	National Taiwan University Hospital
Intraparenchymal (Putamen)	Phase II (2018)	National Taiwan University Hospital
Intraparenchymal	Phase I (2020)	National Taiwan University Hospital
Intravenous	Phase I (2020)	Sangamo Therapeutics
Intravenous	Phase I (N.A)	Sangamo Therapeutics
Intravenous	Phase I/II (2019)	Abeona Therapeutics
Intracerebral	Phase II/III (2022)	LYSOGENE
Intracerebral	Phase II (2017)	LYSOGENE
Intracerebral	Phase I/II (2019)	UniQure Biopharma B.V.
Intravenous	Phase I/II (2020)	Nationwide Children's Hospital
Intrastriatal	Phase I/II (N.A)	UniQure Biopharma B.V.

of apolipoprotein E (*APOE*) can significantly increase the risk for developing sporadic AD, while *APOE2* homozygotes are protected against the disease. Thus, APOE2 delivery could potentially be beneficial in *APOE4* homozygous patients.[170] Whether this approach indeed modifies the course of the disease is yet to be demonstrated.

Batten disease

Batten disease or neuronal ceroid lipofuscinosis (NCLs) is a rare group of neurodegenerative disorders that can manifest in infants, children and adults. Symptoms include epilepsy, loss of cognitive and motor function, degeneration of the retina leading to blindness, and early death. More than a dozen CLN genes and more than 430 loss of function mutations have been linked to the diseases.[171] AAV-based clinical trials for Batten disease have mainly focused on delivery of AAV-CLN2 and AAV-CLN3. The first study (NCT00151216), using intracranial delivery of AAV2CUhCLN2 showed little therapeutic benefit. A follow up study is still ongoing and used a different vector (AAVRh.10CUhCLN2) but abnormal T2 hyperintensities was observed in first dosed patient and led to a lowering of the dose for the following patient. For CLN3, one study (NCT03770572) is still recruiting and is based on AAV9-CLN3 delivery. For CLN6, an study with intrathecally delivered scAAV9. CB.CLN6 is ongoing. A press release announcement from the preliminary data from the first two dosed patients stated no further progression of the disease (according to the Hamburg motor and language score) during the first two years after dosing.

Mucopolysaccharidoses (MPSs)

Mucopolysaccharidoses (MPSs) are rare metabolic diseases that are caused by impaired degradation of mucopolysaccharides, leading to accumulation of glycosaminoglycans in the lysosome. Symptoms can be detected as early as during the prenatal period but can also occur in adulthood. The symptoms in adults include difficulty of speech, ataxia, weakness, and dyskinesia, while children usually show neurological impairment, developmental delay and premature death.[172] Several gene therapy clinical trials are currently in progress for the treatment of MPS I (Hurler infantile syndrome or Hurler-Scheie and Scheie for the juvenile and adult forms), MPS II (Hunter syndrome), MPS IIIA and MPS IIIB (Sanfilippo syndromes A and B). For MPS I and MPS II, the trials are based on intravenous AAV delivery of ZFN therapeutic agents to insert a functional copy of alpha- L-Iduronidase (IDUA) or iduronate 2-sulfatase (IDS) into the albumin locus of patient hepatocytes. These trials are currently being tested in adult patients aiming to improve the peripheral symptoms of these diseases. For MPS IIIA, the main goal is to deliver a functional copy of the human N-sulfoglucosamine sulfohydrolase (h.SGSH) directly into the brain of young children. Thus far, h.SGSH delivery seems to be safe, but the therapeutic benefit was limited with cognitive improvement observed in only one patient.[173] For the treatment of MPS IIIB, the goal is to deliver N-sulfoglucosamine sulfohydrolase (NAGLU) to restore its expression in patients. Both intracerebral and intravenous delivery of AAV-NAGLU have shown to be

promising in preclinical studies in rodent and dog models but whether this translates to therapeutic benefit in patients is yet to be demonstrated.[174,175]

Pompe disease

Pompe disease is a progressive neuromuscular disorder and is caused by a mutation in the acid alpha-glucosidase (GAA) gene, which encodes an enzyme required to degrade lysosomal glycogen.[176] Accumulation of glycogen in multiple tissues results in cardiac, respiratory, and skeletal muscle dysfunction. Preclinical studies in GAA knockout mouse models showed rescue of glycogen accumulation in muscle and the central nervous system and increased survival upon delivery of GAA by AAV.[177] Delivery of GAA by AAV is currently being tested in three different clinical trials.

Spinal muscular atrophy

SMA is caused by the loss of function of the gene encoding the survival motor neuron 1 (SMN1) protein and is characterized by progressive loss of the lower motor neurons. The most severe form is type 1 SMA which affect infants. The patients usually die before the age of 20 months. Several clinical trials are run by AveXis based on AAV9-SMN delivery. Thus far, remarkable clinical benefit was observed including increased motor functions, head control, sitting, rolling over and speaking. The high dose of intravenous injected AAV9-SMN led to an increase in serum aminotransferase, a sign of liver toxicity, which was managed by oral prednisolone. Intrathecal delivery of AAV9-SMN is also being investigated by AveXis as improved transduction of the CNS was observed in lower dose as compared to intravenous administration in NHP. This program is currently also being investigated in clinic for other types of SMA (NCT03306277, NCT03505099, NCT03461289, and NCT03381729), results of which are pending (Table 1). A Biological License Application was submitted to the US FDA in November 2018 by Novartis, which acquired AveXis. In March 2019, Novartis announced the FDA approval of AAV9-SMN (Zolgensma®) for the treatment of pediatric SMA type 1 patients.

Huntington's disease

HD is the most common autosomal dominant neurodegenerative disorder worldwide.[178,179] The genetic cause is an expansion of 39 CAG triplets or more in first exon of the huntingtin (HTT) gene.[180] The mutated gene produces a mutant HTT protein that contain a long polyglutamine (polyQ) tract and results in a toxic gain of function. The mutant HTT protein causes neuropathology affecting the entire brain, with medium spiny neurons of the striatum being particularly vulnerable at early stages.[181,182] The clinical symptoms include progressive motor, cognitive, and psychiatric disturbances. Currently there is no treatment available that would halt or delay disease progression. Artificial DNA or RNA molecules to achieve lowering of HTT translation as a potential therapy for HD have been

broadly investigated in preclinical studies but few have made it to clinical trials. IONIS was the first to initiate a Phase I/II clinical trial (NCT02519036) in 2015 based on HTT lowering with an intrathecally administered antisense oligonucleotide targeting both wildtype and mutant HTT. This trial was safe and a reduction of HTT protein was detected in the CSF.[183] Roche is continuing this study with a large phase III trial (NCT03761849) that was launch in 2018 and will be conducted at around 80-90 sites in about 15 countries. It remains unknown whether this intrathecally delivered ASO will reach the deeper brain structures to achieve sufficient lowering of the mutant HTT in the striatum. Concerningly, the latest published data using this approach showed a dose- and time-dependent increase of the ventricular volume, which suggests that the treatment cause more CNS atrophy, instead of the desired prevention of CNS atrophy.

UniQure will launch the first AAV-based RNAi therapy for HD in the second half of 2019. The therapeutic candidate is AAV5-miHTT (AMT-130), which is based on an anti-HTT miRNA targeting a region close to the repeat in exon 1 of the HTT gene to silence both wildtype and mutant HTT. AMT-130 will be injected directly in the striatum and should provide a long-lasting production of miHTT with no re-administration required. Due to the close proximity of the miHTT target site to the CAG repeat, it is expected that AMT-130 also targets the short HTTexon1 fragments which are highly toxic.

Controlled gene therapy using inducible systems

One potential issue with current gene therapies is the irreversible state as transgene expression cannot be stopped. Thus, if continued expression of a therapeutic gene causes unwanted effects, these side effects would most likely persist in the patient without the possibility to terminate the treatment or control the dose. For example, the expression of recombinant GDNF showed side effects associated with cerebellar Purkinje cell loss in non-human primate models.[203] In this case, inducible systems that could provide a more controlled expression of the transgene would be highly beneficial and would add considerably to the safety profile of the gene therapy.

Most classical inducible systems are based on two elements: the first is a chimeric transactivator protein capable of modulating gene expression in a drug-dependent (inducer) manner and the second is an inducible promoter which is often a minimal pol II promoter linked to sequences recognized by the chimeric transactivator protein.[204] These two elements can also be combined in a single expression system to express both transgene and the transactivator protein in a single plasmid or expression vector.[205] Additionally, two mechanisms of gene regulation can be distinguished, called On-switch and Off-switch. In the On-switch mechanism, the chimeric transactivator protein binds specifically to its DNA recognition sequence within the inducible promoter in the presence of the inducer, which in consequence activates gene expression. In the Off-switch mechanism, the chimeric protein-repressor is constantly bound to its recognition sequences and dissociates upon induction allowing transcription. An inducible gene

expression system should fulfil several requirements for safe and long-term gene therapy application, including low basal expression in absence of the inducer and a high induction ratio. Another important safety requirement is that the chimeric proteins used in inducible systems, in active or inactive form, do not interfere with endogenous gene expression, because chimeric proteins are formed from potent transcription factors that can lead to unspecific activation of endogenous promoters and thus interfere with cellular processes. The safety aspects should also be determined for all inducible system components within the context of immune system activation.

Regulated gene expression should ideally exhibit a wide dynamic range of induction with dose-response over a broad range of inducer concentrations, as opposed to acting merely as an on-off switch. For therapeutic applications, additional safety measures are required to minimize potential immune responses or other unwanted effects. For example, the regulatory proteins should be built from human proteins and should respond to an inducer that is safe, physiologically inert at the doses used, and with pharmacokinetics that permit a clinically tractable dosing regimen.

Several inducible gene expression systems have been developed including tetracycline, rapamycine, Isopropyl β-D-1-thiogalactopyranoside (IPTG), ecdysone and mifepristone dependent technologies.[206–208] In this paragraph the GeneSwitch and tetracycline-dependent (tet) system are discussed as they are currently the most widely used inducible systems.

GeneSwitch

GeneSwitch is one of the best characterized inducible systems for regulation of gene expression.[207,209] It exploits a chimeric transactivator GeneSwitch protein which consists of a DNA binding domain from the yeast Gal4 protein (Gal4 BD), a truncated ligand binding domain from human progesterone receptor (PR) and a p65 transcriptional activation domain of the human NF-kappa B transcription factor (p65) (Figure 6). The second component is the GeneSwitch inducible promoter, which consists of a minimal pol II promoter with upstream activation sequences (Gal4) that can be recognized by Gal4 BD of the GeneSwitch protein. This hybrid system can be activated by the antiprogestin mifepristone (MFP), a clinically approved synthetic steroid that binds to the PR domain of the GeneSwitch protein. MFP induces GeneSwitch conformational changes and dimerization, resulting in binding of the GeneSwitch dimer to the Gal4 sites placed upstream of a promoter to activate transcription of a transgene. In absence of mifepristone, the transgene will remain silent. The GeneSwitch system is highly attractive for clinical application as it does not contain any bacterial or viral components and is considered to be less immunogenic than most other inducible systems. The classical GeneSwitch system has been optimized to be delivered in a single vector, with low background levels and a high transgene expression after induction. Furthermore, GeneSwitch proved promising in combination with AAV-based gene therapy in preclinical studies for neurodegenerative

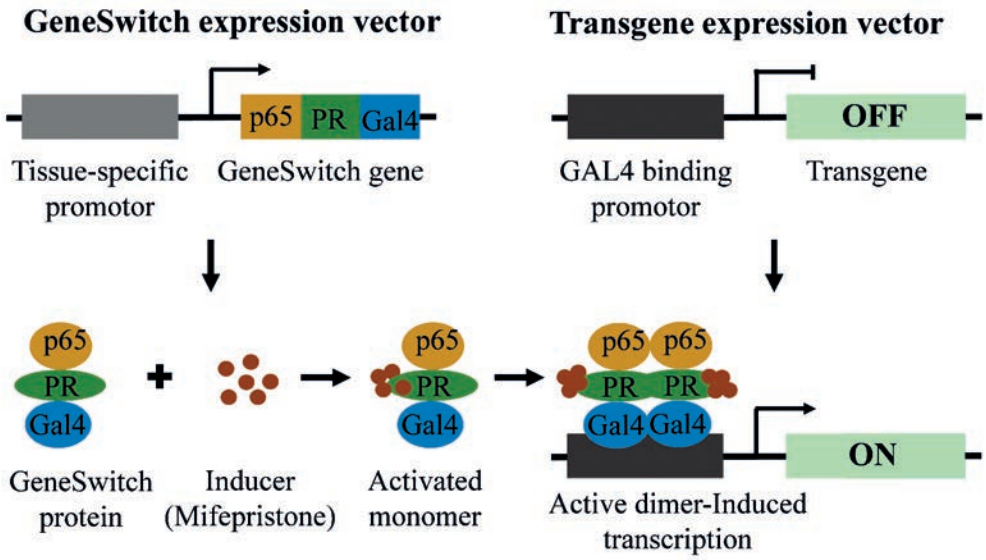

Figure 6. A schematic of the GeneSwitch system and its mechanism of action. The classical GeneSwitch system consists of two expression cassettes on two separate vectors; one containing the GeneSwitch gene and one containing the transgene. The expression of GeneSwitch is driven by a promoter, which can be tissue specific. Expression of the transgene is driven by a minimal pol II promoter with Gal4 binding sites. The GeneSwitch protein comprises yeast Gal4 DNA-binding domain, a human p65 activation domain and a Mifepristone (MFP) controlled domain derived from the human progesterone receptor. The GeneSwitch protein (monomer) can be activated by the steroid inducer MFP. And active dimer is formed that can attach to the Gal4-binding sites in the inducible promoter of the transgene. Hence only in the presence of MFP the transgene is transcribed.

diseases.[205] For example, GDNF expression was successfully induced up to three hundred-fold in rat brains and the expression was reversible with no background expression. It was also possible to re-activate GDNF expression after long-term off-status, and therapeutic benefits of induced GDNF was demonstrated in a PD rat model by restoring motor pathology.[205,210] The major advantage of the GeneSwitch system is that it lacks virally- or bacterially derived components thus limiting a potential immune reaction to GeneSwitch expression. Another advantage of the system is its inducer MFP, which is a marketed drug with well characterized pharmacokinetics and the induction occurs at sub-therapeutic MFP concentrations.[211] One concern is that expression of GeneSwitch for one month resulted in transcriptional changes in the murine liver.[209] Although these alterations were not associated with any biochemical or morphological changes, this aspect needs more attention.

(tet) system

Another inducible system is the tetracycline-dependent (tet) system which is currently the most widely used regulatable system.[212] There are two versions, namely a tetOFF and a tetON system. TetOFF is based on a fusion between the wild-type tet repressor and the activation domain of the HSV VP16 transcription factor.[213] In the tetON system, the tet repressor sequence has been replaced by a mutant which reverses the tet-repressor to a tet-inducer system.[214] Compared to tetOFF, tetON seems more suitable for further development to use in clinic as repeated punctual treatments using an inducible system is more favorable than a repressible system.[214] TetON consists of a transactivator-protein (rtTA) that can be activated by the antibiotic doxycycline and bind to a Tet promoter upstream of a transgene of interest. The TetON system was also successfully used in combination with AAV gene therapy in rodents and showed controlled expression of GDNF in brain of rats.[213] However, some major concerns are the side effects that may be caused by the inducer (an antibiotic) and the immunogenicity caused by the rtTA protein. Long-term exposure to antibiotics is undesirable and the rtTA protein of this system which is derived from bacteria showed to be highly immunogenic in non-human primates.[215,216] Therefore, the GeneSwitch system is currently preferable for further development into an applicable system in humans.

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Scope of this thesis

During the past decades, several discoveries have highlighted the potential of AAV-based gene therapy for the treatment of inherited diseases. By means of gene replacement or RNAi, gene therapy has emerged as an attractive and clinically viable option to treat neurodegenerative diseases. In the current thesis we developed and characterized RNAi-based gene therapy for ALS and SCA3 aiming to silence two single genes that are the root cause of the diseases. These diseases have a very high unmet medical need with a progressive decline in the quality of life and no effective treatments available. Additionally, different delivery methods of AAV-based gene therapy were investigated for the CNS, as well as methods to regulate the transgene expression.

Chapter 1 introduces the concept of gene silencing using miRNAs and provides an overview of the pathogenesis of neurodegenerative diseases that are the subject of this thesis. The challenges for developing novel therapies for neurodegenerative diseases are discussed with an emphasis on ALS and SCA3. Chapter 2 describes the design of a novel miRNA-based gene silencing technology for ALS. miRNAs targeting different regions of *C9orf72* were designed and tested *in vitro* for silencing efficacy. For a long time, the functionality of miRNAs was thought to be restricted to the cytoplasm but some diseases, including *C9orf72*-related ALS require intranuclear silencing of the pathogenic repeat-containing transcripts. We demonstrated that nuclear silencing is possible with our miRNAs. We also report on feasibility of different targeting strategies to silence the sense- and/or antisense transcripts. Targeting exons within the *C9orf72* gene resulted in lowering of the healthy and mutated sense transcript, but not the antisense transcripts. Targeting both sense and antisense transcript can be achieved by expressing two miRNAs in a concatenated fashion in a single expression vector. An approach to target only the mutated transcripts is also possible by developing miRNAs close to the repeat region, but targeting the repeat region directly was not successful as this region consisted solely from GC nucleotides and has a highly complicated tertiary structure. In chapter 3, the therapeutic potential of *C9orf72*-targeting miRNAs was tested on patient-derived iPSC-neurons and in an *C9orf72* ALS mouse model. We confirmed the presence and activity of the therapeutic miRNAs in the nucleus of cells and showed reduction of the repeat-containing transcripts as well as nuclear RNA foci formation in the brain of an ALS mouse model. In chapter 4, the miQURE technology was applied to further develop a miRNA gene therapy for SCA3. Several miRNAs were tested *in vitro*, and three lead candidates were incorporated into AAV5. The therapeutic potential of these constructs was tested in human iPSC-neurons and in an SCA3 knock-in mouse model using different routes of delivery. We demonstrated strong reduction of *ATXN3* mRNA in both model systems. Further support for this approach was provided by the finding that mutant ataxin-3 protein was reduced in the brain stem and cerebellum of treated mice. Targeting the affected brain regions is critical for AAV-based silencing technologies. In chapter 5 we studied the biodistribution of AAV5 in the rat brain using different delivery routes. We

found that each delivery route has its own merits and should be carefully investigated for each type of disease. In many cases it may be useful to be able to regulate the expression of therapeutic constructs in the brain following delivery. In chapter 6, a proof on concept study was performed to investigate the ability to regulate expression of a therapeutic transgene using the mifepristone regulated GeneSwitch system. Using a novel design that can fit both the GeneSwitch system and the transgene in a single vector, we showed that this system is safe and that it is possible to regulate expression of the transgene *in vitro* and *in vivo* using. In chapter 7, we discussed our main findings and compare our therapeutic strategies with others. We further discussed the future perspectives for gene therapies for neurodegenerative diseases. Overall, we provided evidence that our RNAi-technology has the potential to be further developed in effective therapies for a group of severe diseases with no medical cure and a high demand for novel treatments.